3. MATERIALS AND METHODS

3.1. Source of organism

3.1.1. Bacterium

The standard strain of *Bacillus thuringiensis* subspecies *kurstaki* (*Btk*) was procured from the Institute of Microbial Technology, Chandigarh (Strain designation: BA 83B and the MTCC number: 868), and maintained in Luria-Bertani (LB) medium. Figure 1 shows the single sporulated *Btk* cell.

3.1.1.1. Subculturing

Frozen stocks on agar slants were activated periodically (fortnightly) and maintained on LB-agar slants. For long-term storage, glycerol stocks were made and preserved at -72 °C (Operon, Korea).

3.1.2. Mite

The mite, *Aceria guerreronis* [Vernacular (Malayalam): mandari] was collected locally from the vicinity of Calicut University Campus [8°29’N, 45-50 M, Mean Sea Level (MSL)] from the tender coconut buttons of the infested coconut palms, which were identified by Dr. B. Ramani, Professor, Department of Zoology, University of Calicut. Figure 2 shows a single mite.

3.2. Chemicals

Analytical- and bacteriological-grade chemicals from Chromous (India), Genei (India), Himedia (India), Merck India Ltd., Qualigens (India) and Sigma-Aldrich (USA) were used throughout the study.
3.3. Plastic/ Glasswares

Different glasswares made by Borosil, and Riviera were used for the whole study. Measuring jars (10, 100, 200, 500 and 1000 mL), pipettes (1.0, 5.0, 10.0 mL), beakers (10 mL, 50 mL, 100 mL, 250 mL, 500 mL, 1000 mL), conical flasks (100 mL, 250 mL, 500 mL), Petri- plates (100 x 15 mm) were used for the whole study. Micropipettes (0.5 – 10.0 μL, 20 – 200 μL, 200 – 1000 μL, 1 – 5 mL) from Accupipete, Biosystems and Microlit were used.

3.4. Cultivation of Btk

For the production of extracellular amylase, endospore and δ-endotoxin, Btk was cultivated in various media combinations as described in the succeeding sessions. LB was the basic medium used throughout the study. During the study, it was supplemented with soluble/raw starch, as written in the respective sessions. Prior to inoculation, all the media were autoclaved at 15 psi, 121 °C.

Table 7: Composition of LB medium.

<table>
<thead>
<tr>
<th>LB medium</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>~1.0 L</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

3.4.1. Submerged Fermentation (smF)

3.4.1.1. SmF in LB medium without starch

LB medium without starch was used to monitor the extracellular amylase production by SmF. Samples were withdrawn for assays at 3 h intervals.
3.4.1.2. SmF with soluble starch

For SmF, LB medium was supplemented with 1% (w/v) (Himedia). No starch was added in the control.

3.4.1.3. SmF with natural raw supplements

The LB medium was prepared supplemented with 1% (w/v) various raw starch supplements viz. Banana powder (BP), Bengal gram powder (BgP), Jack seed powder (JP), Potato powder (PP), Tapioca powder (TP) and Taro powder (TaP). Of the raw starch powders, BP was purchased locally (Banatone), JP, PP, TP and TaP were home-made and BgP powdered locally in a mill. No starch was added in the control. Source of raw starch was maintained constant throughout the experiments.

3.4.2. Semisolid- or Solid-State Fermentation (SSF)

3.4.2.1. SSF with soluble starch

The LB medium prepared supplemented with different concentrations of 5%, 10%, 15%, 20% and 25% (all w/v) soluble starch (Himedia). No starch was added in the control.

3.4.2.2. SSF with natural raw supplements

The LB medium was prepared supplemented with different concentrations (5%, 10%, 20%, 30%, 40%, 50%, 60%, 80% and 100%, all w/v) of natural raw starch powders such as BP, BgP, JP, PP, TP, TaP. No starch was added in the control.

3.4.3. Seed culture

The frozen stock was streaked with a sterile toothpick on the LB-agar medium in the petri-plate. For the preparation of overnight seed culture (12 h), single colony from LB-agar plate was inoculated with a sterile toothpick in the liquid medium.
3.4.4. Inoculum

50 µL seed culture was used to inoculate 10 mL sterilised production medium, which was equivalent to $6.2 \times 10^5$ cfu (colony forming units) per 1mL medium. Cfu was calculated by serial dilution, and the colonies were counted by Magnus Compound Microscope (India).

3.4.5. Incubation

For SmF, the medium was incubated at 37 °C with constant shaking (140 rpm) in an environmental shaker (Orbitek, India). For SSF, the samples were incubated in an oven (Technicho, India) at 37 °C.

3.5. Crude extracellular α-amylase harvest

3.5.1. SmF

3.5.1.1. SmF in LB medium

The LB medium was centrifuged at 800g for 10 min at 4 °C in a refrigerated centrifuge (Plastocrafts/Remi, India). 0.5 mL samples were withdrawn for assay at 3 h intervals. The supernatant was used for enzyme assay.

3.5.1.2. SmF in LB medium supplemented with 1 % (w/v) soluble/raw starch

0.5 mL samples were withdrawn for assay at 6 h intervals. The broth was centrifuged at 800g 10 min at 4 °C in a refrigerated centrifuge. The supernatant was used for enzyme assay.

3.5.2. SSF

0.5 g samples were withdrawn for assay at 6 h intervals. The fermented matter was weighed and mixed with 5.0 mL double distilled water (ddH$_2$O) and centrifuged at 800g 10 min at 4 °C in a refrigerated centrifuge. The supernatant was used for extracellular amylase assay.
3.6. α-Amylase assays

α-Amylase was assayed by employing the 3,5-dinitrosalicylic acid (DNS) method of Bernfeld [207], modified by Ezeji and Bahl [158].

3.6.1. DNS Reagents

DNS reagent contained DNS (1%), potassium sodium tartrate (Rochelle salt, (1 M) and NaOH (0.4 M) and ddH₂O.

3.6.1.1. Procedure for 100 mL Reagent

Dissolve by stirring (Remi, India) at room temperature (RT) 1g DNS in 50 mL ddH₂O, then added 20 mL 2 M NaOH and 28.2 g Rochelle salt, finally made up to 100 mL by ddH₂O. The reagent was stored at RT.

3.6.2. Other solutions

- **Buffer:** 0.02 M sodium phosphate buffer (pH, 6.9) with 0.006 M sodium chloride.
- **Starch solution:** 1.0% starch solution was prepared fresh by dissolving 1.0 g soluble starch in 100 mL 0.02 M sodium phosphate buffer (pH, 6.9).
- **Maltose stock solution:** Dissolved 50 μg maltose in 50 mL ddH₂O in a standard flask and stored at 4 °C.

3.6.3. α-Amylase activity assay procedure for SmF (unless otherwise specified)

- Pipette out 0.5 mL enzyme solution (prepared as explained in 3.5.1.) and incubate tubes at 25 °C for 3 min.
- Add 0.5 mL starch solution and incubate for 5 min (RT).
- Stop the reaction by adding 1 mL DNS reagent.
- Heat the solution in a boiling water bath for 5 min.
- Cool it in running tap water.
- Make up the volume to 10.0 mL by the addition of ddH₂O.
➢ Read the absorbance at 540 nm using UV-Vis Spectrophotometer (Schimadzu UV 1601, Japan).
➢ Blank is prepared without enzyme.
➢ Prepare a standard graph with 0 - 100 μg maltose.

3.6.3.1. Calculation of enzyme activity Extinction coefficient (Σ)

For stock solution, 2g D-glucose was weighed into 1 L capacity volumetric flask and made upto 1 L with ddH2O. 5, 15, 25, 50, 75 and 100 mL of stock solutions were pipetted into 100 mL volumetric flasks and made upto 100 mL with ddH2O to give 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0 mg/mL glucose respectively. 2 mL 3,5, DNS reagent, 1mL of each of the different glucose concentrations and 1mL of 0.1 M acetate buffer, pH 5.5 were added into each of the reaction tubes. The mixtures were boiled in water bath for 5 min. and their extinction measured using Spectrophotometer at 540 nm. These extinctions were plotted against their concentrations and thus the glucose standard curve or regression equation was established.

\[ Y = 1.1576X - 0.0429 \quad (R^2 = 0.999) \] …………equation 1

X = 0.18 mg = 1 μM glucose

Substitute 1 μM glucose in equation 1,

Therefore \( Y = 0.165 \text{ cm}^2/\text{μM glucose} = \text{Extinction coefficient (Σ)} \)

3.6.3.2. Calculation of α-amylase activity in SmF

One unit (U/mL) of α-amylase activity is defined as: the amount of protein (α-amylase) required to liberate 1 μmol (0.18 mg equivalence) of reducing sugar (D-glucose) from starch/min, under the assay conditions [158].

\[
\text{Formula: } \alpha\text{-amylase activity (U/mL)} = \frac{\Delta E \times V_f}{\Delta t \times \sum V_s \times d}
\]

\( \Delta E = \text{Absorbance at 540 nm} \)

\( V_f = \text{Final volume including DNS} \)

\( V_s = \text{Volume (mL) of } \alpha\text{-amylase used} \)
Δt = Time of hydrolysis  
Σ = Extinction coefficient  
d = Diameter of cuvette (1 cm for standard cuvette)

3.6.3.3. α-Amylase activity assay procedure for SSF (unless otherwise specified):

- Pipette out 0.5 mL enzyme solution (prepared as explained in 3.5.2.) and incubate tubes at 25 °C for 3 min.
- Add 0.5 mL starch solution and incubate for 5 min (RT).
- Stop the reaction by adding 1 mL DNS reagent.
- Heat the solution in a boiling water bath for 5 min.
- Cool it in running tap water.
- Make up the volume to 10.0 mL by the addition of ddH₂O.
- Read the absorbance at 540 nm using UV-Vis Spectrophotometer.
- Blank is prepared without enzyme.
- Prepare a standard graph with 0 -100 μg maltose.

3.6.3.4. Calculation of α-amylase activity in SSF

One unit per gram dry substrate (U/gds) of α-amylase activity is defined as: the amount of protein (α-amylase) in one gram dry substrate (gds) required to liberate 1 μmol (0.18 mg equivalence) of reducing sugar (D-glucose) from starch/min, under the assay conditions [140]; [158].

Therefore, α-amylase activity is calculated as follows:

\[
\text{Formula: } \alpha\text{-amylase activity (U/gds)} = \frac{\Delta E \times V_f \times V_s}{\Delta t \times \Sigma \times gds \times d}
\]

\( \Delta E \) = Absorbance at 540 nm  
\( V_f \) = Final volume including DNS  
\( V_s \) = Volume (mL) of α-amylase used  
\( \Delta t \) = Time of hydrolysis  
\( \Sigma \) = Extinction coefficient  
gds = dry weight of the substrate in gram

d = Diameter of cuvette (1 cm for standard cuvette, the one used)
3.7. Protein estimation

Protein content was estimated using Lowry’s method with bovine serum albumin (BSA) as the standard [208].

3.7.1. Reagents

- Reagent A: 2% Na₂CO₃ in 0.1 N NaOH
- Reagent B: 500 mg CuSO₄ in 1% Rochelle salt solution
- Reagent C (alkaline copper solution): 50 mL of Reagent A + 1 mL of Reagent B
- Folins-phenol reagent: Commercial Folins-phenol reagent was used after dilution in a 1:1 ratio with ddH₂O.

3.7.2. Procedure

- Pipette out 0.5 mL of the enzyme in test tube and make up to 1 mL with 0.1 N NaOH. Add 5.0 mL of alkaline copper reagent. Mix well and allow standing for 10 min.
- Add 0.5 mL of Folin’s reagent, mix well and incubate at room temperature for 30 min.
- Read the absorbance at 670 nm using Spectrophotometer.
- Calculations were done using the graph generated from the standard graph of BSA.

3.7.3. Standard BSA graph

- 1 mg/mL stock solution is prepared with BSA
- Pipette out different aliquots of stock solution (0.05, 0.10, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 µL) in test tubes and make up to 1 mL with 0.1 N NaOH. Add 5.0 mL of alkaline copper reagent. Mix well and allow standing for 10 min.
- Add 0.5 mL of Folin’s reagent, mix well and incubate at room temperature for 30 min.
- Read the absorbance at 670 nm using Spectrophotometer.
- The values were plotted against concentration vs optical density at 670 nm.
3.8. Enzyme purification and characterisation

Amylase was purified by the method of Ezeji and Bahl [158]. The strategy included fractionation by ammonium sulphate followed by dialysis and gel permeation chromatography. The purity was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [209].

3.8.1. Ammonium sulphate fractionation

Finely powdered ammonium sulphate was added slowly into the crude enzyme preparation until it reached 80% saturation (0-20%, 20-40%, 40-60% and 60-80%). A magnetic stirrer was used for the continuous stirring and the procedure was carried out at 4 °C in an ice bath. The precipitated protein was removed by centrifugation at 2400g for 10 min at 4 °C. The pellet was resuspended in a minimum volume of 0.02 M sodium phosphate buffer (pH 6.9).

3.8.2. Dialysis

The precipitate obtained after ammonium sulphate fractionation was dialysed against sterile ddH₂O for 24 h at 4 °C with continuous stirring and occasional changes of the buffer. Cellulose membrane dialysis tubes were used for dialysis. The amylase activity and protein content of the dialysate were determined as described under 3.6.3 and 3.7.2, respectively.

3.8.3. Gel permeation chromatography

Gel permeation chromatography was done in a column (50 x 3 cm) packed with sephadex G 100 (Sigma Aldrich, USA) using a peristaltic pump (Riviera, India) in a cold room. The dialysate obtained by 40-60% ammonium sulphate fraction was used for gel permeation chromatography. It included the following steps.

- 2.5 g of sephadex G 100 was suspended in ddH₂O and kept for 72 h at 20 °C (as directed by the manufacturer).
- Swollen sephadex was poured into the chromatography column (50 x 3 cm) and allowed to settle under gravity while maintaining a slow flow rate through the column. Care was taken to avoid trapping of air bubbles in the
column. The column was stabilised and equilibrated by passing about 1 L of buffer (0.02 M sodium phosphate buffer, pH 6.9). This step was repeated twice.

- The concentrated dialysate (5 mL) was loaded on the top of the column. The enzyme was eluted using phosphate buffer (0.02 M, pH 6.9) in a sequential manner. Flow rate was adjusted to about 12 mL/h and fractions of 2.0 mL/10 min were collected.
- The absorbance of each fraction was read at 280 nm using a Spectrophotometer.
- The fractions with major peaks were subjected to amylase enzyme activity and protein content estimation.
- Those fractions with maximum amylase enzyme activity were subjected to SDS – PAGE [209].

The gel permeation chromatography (BioRad Biologic LP, Italy, 50 x 1.5 cm) was also done in National Institute for Interdisciplinary Science and Technology, Papanamcode, Thiruvananthapuram.

### 3.8.4. Electrophoresis

The purified enzyme was subjected to SDS–PAGE to confirm the purity and to determine the approximate molecular weight (MW) of the purified protein. SDS-PAGE was conducted using a vertical mini gel (8 x 7 cm) slab with notched glass plate system (BioTech, India). Gels of 1.5 mm thickness were prepared for the whole study.

#### 3.8.4.1. Stock solutions

- Acrylamide-bisacrylamide (30:0.8%) was prepared by dissolving 30 g of acrylamide and 0.8 g of bisacrylamide in a total volume of 100 mL of ddH$_2$O. The solution was filtered through Whatman No. 1 filter paper and stored at 4 °C in a dark bottle.
1.5 M Tris buffer, pH 8.8 – 23.6 g tris was dissolved in 40 mL of ddH₂O and titrated to a pH of 8.8 with 6.0 N NaOH and made upto a final volume of 100 mL. Filter through Whatman No.1 filter paper and stored in a refrigerator.

1.0 M Tris buffer, pH 6.8 – 15.7 g tris was dissolved in 48 mL of 1.0 N NaOH and made upto a final volume of 100 mL with ddH₂O. Filter through Whatman No.1 filter paper and stored in a refrigerator.

10% SDS

10% Ammonium persulphate (make fresh)

TEMED (N,N,N',N'-tetra methyl ethylene diamine) was used as such which was stored in a dark bottle at 4 °C (make fresh).

Electrophoresis buffer - 12 g tris, 57.6 g glycine and 2.0 g SDS was dissolved in 500 mL of ddH₂O and made upto a final volume of 1L and stored in a refrigerator.

Sample buffer.

**Table 8: Ingredients required for sample buffer**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6 M Tris buffer, pH 6.8</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>0.05 mL</td>
</tr>
<tr>
<td>10 mM bromophenol blue</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>20% Glycerol</td>
<td>0.4 mL</td>
</tr>
</tbody>
</table>

Make upto 10.0 mL with ddH₂O and store in a refrigerator.

**3.8.4.2. Stacking gel composition**

**Table 9: Ingredients required for stacking gel**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>0.38 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.03 mL</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.03 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.003 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>2.1 mL</td>
</tr>
</tbody>
</table>
3.8.4.3. Composition of 12% gel

Table 10: Ingredients required for 12% gel

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>3.3 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004 mL</td>
</tr>
</tbody>
</table>

3.8.4.4. Sample preparation

Enzyme solution and sample buffer were mixed in the ratio of 3:1, respectively. The contents were mixed well in a clean eppendorf tube and heated in a boiling water bath for 3 min. For MW determination, wide range markers (Chromous, Bangalore) were used. The MW markers in the ladder were: lysozyme (14 kDa), lactoglobulin (18 kDa), BamHI (25 kDa), carbonic anhydrase (30 kDa), amylase (51 kDa), bovine serum albumin (66 kDa), Phosphotylase (97 kDa) and β-galactosidase (110 kDa). Electrophoresis was performed using a constant voltage (30 V) till the sample dye reached the bottom of resolving gel.

3.8.4.5. Protein staining

Gel was stained using 0.1% coomassie brilliant blue (CBB) G-250 in 50% methanol and 10% glacial acetic acid. It was stirred at room temperature for 60 min and filtered through Whatmann No. 1 filter paper. The solution was stable indefinitely at RT.

3.8.4.6. Destaining

The destaining solvent system contained 10% glacial acetic acid: 45% methanol: 45% ddH₂O.
3.8.4.7. Visualisation

The SDS-PAGE gels were visualised through Gel-documentation system (BioRad, Italy) and also by Canon digital camera (EOS 450D, Japan).

3.8.5. Characterisation of α-amylase

The 40–60% ammonium sulphate fraction was used for the characterisation studies. The purified enzyme was characterised and its various properties were studied. Effects of pH, temperature, substrate concentration, chelating agents and different metal ions on enzyme activity were the factors studied.

3.8.5.1. Effect of pH on enzyme activity

Optimum pH on the enzyme activity was studied by performing the assay at different pH using acetate (pH 4.0 – 5.0) and phosphate (pH 6.0 – 8.2) buffers.

3.8.5.2. Effect of temperature on enzyme activity

Optimum temperature needed for enzyme activity was estimated by incubating the reaction mixture for 5 minutes at different temperatures (30°C – 100°C) at pH 6.0.

3.8.5.3. Effect of different metal salts on enzyme activity

Effect of different metal ions on amylase activity was determined by incubating the reaction mixture with different metal salts, *i.e.*, Ag^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Mo^{2+}, Na^{2+}, SO_{4}^{2-}, SO_{3}^{-} and Zn^{2+} to a final concentration of 0.5 μM, 1.0 μM, 2.0 μM, 3.0 μM, 4.0 μM and 5 μM at pH 6.0 and temperature 60 °C.

3.8.5.4. Effect of complex compounds on α-amylase activity

The role of complex compounds like ethylene diamine tetra acetic acid (EDTA), β - mercaptoethanol, SDS and thiourea were tested. Cysteine was used for the effect of enzyme towards activity.
3.8.5.5. Effect of substrate concentration

The enzyme was treated with soluble starch with concentrations of 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% and 4%. The reaction mixture was incubated with 5 min time intervals ranging from 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min and 60 min at pH 6.0, temperature 60 °C and the metal Ca$^{2+}$.

3.8.5.6. Calculation of $K_{m}$ and $V_{max}$

The $K_{m}$ and $V_{max}$ values were calculated using the effect of soluble starch on enzyme activity using the software, Hyper 32.

3.9. Endospore production

3.9.1. Endospore production by SmF

Spore production also done with various raw starches as supplements. The medium prepared with JP, PP and TP at concentration of 1% w/v and 6 h time interval ranging from 6h to 72h.

3.9.2. Endospore production by SSF

Spore production also done with various raw starches as substrates. The medium prepared with JP, PP and TP at different concentrations of w/v (1%, 5%, 10%, 20% 30%, 40%, 50%, 60%, 80% and 100%) and 6 h time interval ranging from 6h to 72h. The supplements were mixed in a vortex mixture and incubated at 37 °C in an incubator.

3.9.3. Preparation of working spore solution

Culture medium was centrifuged at 8900g for 10 min. Pellets collected and resuspended in 4 °C sterile ddH$_2$O and made upto 10 mL. The resulting solution was centrifuged again at 8900g for 10 min. The pellet taken and the centrifugation procedure repeated 3 times more. Then the pellet collected and stored at 4 °C for overnight for bursting the vegetative cells. Spore suspension was centrifuged and the pellet taken and resuspended in 4 °C ddH$_2$O and centrifuged at 8900g for 10 min.
Final pellet diluted to 50% concentration with ddH₂O to make the working spore solution.

3.9.4. **Slide preparation**

10 μL working spore solution was applied on to a cleaned glass slide with ethanol. Using pipette tip, the sample was smeared into a circle about 1.5 cm in diameter. The slide was placed on a 42 °C metal heating block to dry for ~ 3 min. Slide was then washed for 1 min in Coplin jar with 50 mL ice cold 100% absolute ethanol. The slide was removed from the ethanol and allowed to dry on a metal heating block (42 °C) to dry for approximately 3 min.

3.9.5. **Staining**

3.9.5.1. **Spore staining by malachite green**

Malachite-green staining technique was used as demonstrated by Sherman and Cuppuccino [210]. Bacterial smears were prepared in the usual manner using 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. Counter stain with safranin for 1.0 min. Wash with running tap water and air-dried. The slides were observed under the binocular microscope (100 X). The photographs were taken by Image Analyser (Nikon Eclipse E 400, Towa Opticals, Japan) fitted with Nikon digital camera (DXM 1200F, Japan).

3.9.5.2. **Spore staining by Acridine orange**

Acridine orange staining technique was used as demonstrated by Schichnes et al., [211]. 10 μL of 0.1 μg/mL acridine orange staining solution was applied to the area of spore smear and applied a cover-slip. Slides were observed using an Olympus fluorescent microscope equipped with a BP 480/20 excitation filter fitted with Nikon digital camera (Japan).
3.9.5.3. Measurement of endospores

The average length and breadth of the endospores were measured using a microscope attached with oculometer.

3.9.5.4. Visualisation of endospores

The spores stained by malachite-green were visualised by Image Analyser (Nikon Eclipse E 400, Towa Opticals, Japan) fitted with Nikon digital camera (DXM 1200F, Japan). The spores stained by acridine orange were visualised by Olympus fluorescent microscope equipped with a BP 480/20 excitation filter fitted with Nikon digital camera (Japan). The fluorescent photographs were taken from St. Bazalios College Changanassery, Kottayam.

3.10. Production of Btk-toxin (δ-endotoxin/ Crystal protein)

3.10.1. Production of Crystal protein in SSF

Crystal production was done with various raw starches as supplements. The LB medium was prepared supplemented with JP, PP and TP at 10% (w/v) concentration and 6 h time interval ranging from 48h to 72h. The media were mixed in a vortex mixture and incubated at 37 °C.

3.10.1.1. Maximising Btk-toxin production

For maximisation of crystal production, the fermented matter at 12 h was centrifuged at 800g for 10 min in a refrigerated centrifuge. This step was to remove free water in the medium. The pellets were collected and incubated further at 37 °C.

3.10.2 Slide preparation

The technique employed for the preparation of slides for crystals was the same in 3.9.4.

3.10.3. Purification of crystal protein

The sporulated culture were harvested by centrifugation and crystals purified on a step-wise sucrose gradient [19]. The band containing crystals was washed 3
times in 50 mM tris – HCl, pH 7.5, resuspended in 3 mL of ddH$_2$O containing 1.0 mM Phenyl Methane Sulfonyl Flouride (PMSF).

3.10.4. Crystal staining with safranin

Crystals were stained with malachite-green and safranin. The slides were observed and the photographs were taken by Image Analyser.

3.10.5. Scanning Electron Microscopy (SEM)

3.10.5.1. Procedure

Purified crystals were treated with Sorensen phosphate buffer pH 7.2 and stored at 4 °C overnight. Fixed in gluteraldehyde phosphate buffer for 10 min. Washed with phosphate buffer 15 min.

Dehydration

- 30% ethanol: 15 min 2 changes
- 50% ethanol: 15 min 2 changes
- 70% ethanol: 15 min 2 changes
- 90% ethanol: 30 min 2 changes
- 100% ethanol: 30 min 2 changes

Purified crystals were dried on a metal support, at RT, and covered with gold for 60 sec. at 40 mA. Samples were observed and photographed by scanning electron microscope (SEM) (JEOL, JWS 3000). The SEM photomicrographs were taken from Sree Chitra Tirunal Institute for Medical Science and Technology, Poojappura, Thiruvananthapuram.

3.10.5.2. Protein Estimation

Protein estimation of the purified crystals was done by Lowry’s method [208] as described under 3.7.
3.10.5.3. SDS-PAGE

The purified crystals were analysed on 12% SDS – PAGE under standard conditions as described under 3.8.4.

3.11. Efficacy studies of Btk-toxin against A. guerreronis (mandari)

3.11.1. Selection of palms for collecting coconut buttons

Coconut palms of about 10 M height growing near Calicut University Campus (Calicut University Botanical Garden, Villunnial and Kakkanchery) at 8°29’N and 45-50 M MSL were selected for the present study. Collections were made at about 10 AM in summer (April-May) days with an average day temperature ~33 °C. It is during this time maximum infestation is seen in Kerala.

3.11.2. Collection of coconut buttons

The coconut buttons of about 30 days old were collected every day and used as fresh for culturing the mite A. guerreronis as described under 3.1.2. Hundreds of coconut buttons were cultured repeatedly every week and standardised the culture conditions prior to toxicity assay.

3.11.3. Culturing of A. guerreronis

Commercially available borosil glass rods of 5 cm diameter were bored and cut as rings with internal diameter of 2.5, 3, 3.5 and 4 cm with a height of 1.5 – 2.5 cm and thickness of 0.5 – 1 cm. Cover glasses of 1 mm thickness were suitably cut and used as lids for these culture rings. Coconut buttons (developing small nuts) of about 1 month old were selected from non-infested healthy palms and the appropriate glass ring was fixed with the help of paraffin wax to each button in such a way that the ring touched the boundary between meristematic yellow (the exposed region after the removal of the perianth or cap) and non meristematic green regions. The area (meristematic region within glass ring) of the coconut buttons was calculated from the diameter using vernier calipers.
Diameter = MSR + VSR x LC

MSR = Mean Scale Reading

VSR = Vernier Scale Reading

\[ LC \ (\text{Least count}) = \frac{\text{Value of the smallest division on the main scale}}{\text{Total number of divisions on the vernier scale}} \]

Area = \( \pi r^2 \)

\( \pi = 3.14 \)

Using a sterile brush, about 45 mites/cm\(^2\) were carefully introduced in the meristematic region separated by the glass ring. After introducing the mites, the mouth (rim) was lined with a drop of water so that the cover glass remains intact at the mouth of the ring. This arrangement considerably helped in observing the behavior of individual stages of the mite through the cover glass and their manipulation according to the need. A plastic tray (8.5 cm diameter and 3.5 cm height) was used for rearing \( A. \) guerreronis. The arrangement was placed in the centre of the plastic tray. The tray was then filled with ddH\(_2\)O up to the outer boundary of the glass ring only, so as to prevent the movement of the mites away from the meristematic zone and also to maintain proper humidity. Maximum aseptic conditions were maintained throughout the experiments. Cultures were maintained at 37 °C in an incubator.

**3.11.4. Bioassay for \( A. \) guerreronis**

For toxicity assay (bioassay), the standardised \( A. \) guerreronis cultures were used. 48 h raw pellet of PP (10% w/v) supplemented medium as described in **3.10.1.1.** was used as the crude Btk-toxin/\( \delta \)-endotoxin for the bioassay. This crude Btk-toxin (including the remnants of PP starch supplement, endospores, bacterial debris) was dried in an oven (37 °C for 24 h) and then made into fine powder using mortar and pestle. 1.25, 1.88, 2.5, 3.13, or 3.73 \( \mu g/cm^2 \) crude powder was applied in the culture set-up containing standardised healthy mites. The control mites were fed with the powder prepared as above, which was not inoculated with Btk.
3.11.5. Monitoring the growth and mortality of mites

The life cycle of the mite and mortality rates were observed through a Magnus compound microscope. The photographs were taken using a digital camera attached to the microscope (Webcam companion 2.0 MEM 1300, Japan).

3.12. Statistics

All experiments described in this thesis were performed at least in triplicate. SigmaPlot 11.0 (Systat Software Inc) was used for making calculations and drawings. The $K_m$ and $V_{max}$ values of amylase were calculated using the software Hyper 32.