3.0 MATERIALS AND METHODS

3.1 Microorganisms

The microorganisms studied were Florbac, IPT Bt 6, IPT Bt 15, IPT Bt 16, IMR Bt 8, and IMR Bt 16. Florbac (Zeenax (M) Ltd.), is a commercial preparation of *B. thuringiensis* var *aizawai*. IPT Bt 6, IPT Bt 15, and IPT Bt 16 were obtained from the culture collection (Singh, R. 1994), Institute of Advanced Studies, University of Malaya. IMR Bt 8 and IMR Bt 16 were obtained from the Institute of Medical Research, Kuala Lumpur. The cultures were maintained in the laboratory on Nutrient agar slants at 4°C

3.2 Growth studies

For the growth studies a liquid medium (designated G-medium) composed of 4.0g yeast extract; 4.0g protease peptone; 5.6g NaCl; 5.6g K_2HPO_4; 1.2g KH_2PO_4; 0.3g $MgSO_4$; 0.02g $MnSO_4$; and 0.02g $FeSO_4$; per litre of distilled water was used. Glucose 0.5% (w/v) was autoclaved separately and added aseptically to the medium. 500 ml flasks each containing 100ml of the medium were inoculated with 1ml of the respective cultures and incubated in a rotary incubator at 200 rpm and 35°C.

The inoculum was prepared by making individual suspensions of the different strains of *B. thuringiensis* maintained on agar slants with sterile distilled water. The inoculum was standardized by measuring the absorbance (optical density) at 600nm using a Shimadzu UV 120 spectrophotometer. 1.0ml of inoculum with an optical density within 0.5 - 0.6 was used to

inoculate each flask. The biomass was determined by measuring the optical density at 600nm whereas pH was determined by a pH meter.

Samples each containing 20ml were taken at 5 stages representative of the standard growth curve : lag phase (2 1/2 hours), exponential phase (5 hours), early stationary phase (8 hours), early sporulation phase (16 hours) and late sporulation phase (20 hours). These samples were used to determine pH, biomass, reducing sugar, and for the enzymatic studies. 1 unit of activity is the amount of enzyme converting 1μmole of substrate (or producing 1 μmole of product), per minute at X°C under the text conditions.

3.3 Analysis

Reducing sugar and protein

Reducing sugar in the sample broth was analyzed by the Dinitrosalicylic acid (DNS) method (Miller, 1959). Protein was analyzed by Lowry method (Peterson, 1977) using the Sigma Protein Assay Kit.

3.4 Enzyme Assay

Exoenzymes

The assay for exoenzymes was conducted on the sample supernatants obtained at the various growth phases after the broth was centrifuged at 4000 rpm for 10 minutes.

3.4.1 Protease

Protease was assayed by using 1.0ml of suitably diluted enzyme (broth supernatant) preparations were equilibrated at 25°C. To this, 1.0ml of azoca-

sein solution, also equilibrated at 25°C was added, and the resulting reaction mixture was incubated at 25°C for 15 minutes. The reaction was stopped by the addition of 8ml 5% (w/v) trichloroacetic acid and the precipitated protein was filtered by using Whatman No. 54 filter paper. 5ml samples of the supernatant were taken and 0.5ml 10N-NaOH was added to develop the colour of the 'protease-solubilized' material. Absorbance (OD) was measured at 440nm. Protease activity was determined against a standard curve prepared with Azocasein at concentrations of 1000 to 5000 μg/ml.

3.3.2 Alkaline phosphatase

Alkaline phosphatase was assayed by the method of Malamy and Horecker (1966). The assay is based on the formation of *p*-nitrophenol by the hydrolysis of *p*-nitrophenylphosphate. To 1.0ml of the buffered *p*-nitrophenylphosphate solution, 1.0ml of the enzyme solution (supernatant) was added. Absorption was read at 420nm. Alkaline phosphatase activity was determined against a standard curve prepared with buffered p-nitrophenol at concentrations of 10 to 50μg/ml.

3.5 Endoenzymes

The culture broth was centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. The pellets were washed repeatedly in distilled water. The cells were disrupted by sonication in a glass cell with a Braun Sonicator Model 2000. To minimize heating the cells were immersed in ice, and sonication was accomplished at 0.3 repeating duty cycles for 15 minutes

with a 30 second delay between each of 3 minutes. The cell debris were removed by centrifugation at 4000 rpm for 10 minutes and the supernatant was used for the assay of endoenzymes.

3.5.1 Isocitrate dehydrogenase

Isocitrate dehydrogenase was assayed by the method of Cleland, Thompson and Barden (1969). The following mixture was employed : 1.0ml of a solution containing 1mM EDTA, 0.3mM DTT, and 100mM Tris-HCl buffer, pH 7.4; 20mM $MnSO_4$, 0.2ml; 1.5mM NADP, 0.2ml; 80mM threo-D L -isocitrate, 0.25ml; enzyme solution, 0.1ml; and water to a total volume of 3.0ml. The reaction was carried out at 25°C in a 3ml silica cuvette with a 1cm light path. Absorbance was read at 340nm. The increase was measured at intervals of 15 second for 2 minutes. Isocitrate dehydrogenase activity was determined against a standard curve prepared with 0 to 1.0ml of 0.3mM NADPH.

At the end of this research study, isocitrate dehydrogenase was assayed again on Florbac and IPT-Bt6 to reconfirm its high activity after 8 hours of growth.

3.5.2 Citrate synthase

Citrate synthase was assayed by the method of Parvin (1969). CoASH liberated in this reaction reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form a mercaptide ion which absorbs ultraviolet light at 412nm. Tris-HCl, 0.2ml; DTNB, 0.1ml; oxaloacetate, 0.1ml; acetyl-CoA, 0.1ml; and water to 1.0ml was added to a spectrophotometer cuvette of 1cm light path. The

reaction was started at room temperature (25-28°C) by the addition of 0.4ml of the enzyme, and the increase in absorbance was measured at intervals of 15 or 30 seconds at 412nm for 1 minute. Citrate synthase activity was determined against a standard curve prepared with 0 to 1.0ml of 0.25 mM CoASH.

3.5.3 Pyruvate carboxylase

Pyruvate carboxylase was assayed by the method of Seubert and Weicker (1969). The assay consists of coupling the carboxylation of pyruvate with the reduction of the oxaloacetate formed to malate using reduced nicotinamide adenine dinucleotide (NADH) and malate dehydrogenase. The reaction mixture contained : buffer, 1.1ml; magnesium chloride, 0.15ml; potassium bicarbonate, 0.2ml; pyruvate, 0.02ml; NADH, 0.05ml; ATP, 0.02ml; serum albumin, 0.02ml; and malate dehydrogenase, 0.005ml.

The reaction was initiated by the addition of 0.4ml of the enzyme preparation. The oxidation of NADH was followed spectrophotometrically at 366nm. The decrease in absorbance was measured at intervals of 60 seconds for 3 minutes. The activity of pyruvate carboxylase was determined against a standard curve prepared with 0 to 50ul of 10mM NADH.

3.6 Production of the spore-crystal complex for purification

The production of the spore-crystal complex was carried out in a B Braun Biolab fermenter, of 1.5 litre vessel capacity containing 900ml of G-medium (Plate 1).

Plate 1: B. Braun Biolab Fermentor

The production of spore-crystal complex was carried out at pH 7.0 and 35°C. The impeller speed was 200 rpm.

3.6.1 Inoculum

The culture were streaked on Nutrient agar slants in universal bottles. The agar slants were incubated at 35°C for 24 hours. A convenient volume of distilled water was pipetted into each of the universal bottles in order to obtain suspensions which gave absorbance of 0.5 to 0.6 at 600nm. 1ml of this suspension was added to each of 100ml of G-medium in shake flasks. The shake flasks were incubated at 35°C for 24 hours in an orbital incubator at 200rpm. The contents of these flasks were used as inocula for fermentation.

3.6.2 Growth in a 1.5 litre fermenter

The inoculum consisted of 100ml culture. 0.5% (w/v) of glucose which was autoclaved separately was added aseptically to the G-medium in the fermenter vessel. The impeller speed was maintained at 200 rpm, while the air flow rate was kept at 1200cm/minute, pH and temperature were maintained at 7.0 and 35°C. pH was controlled by the addition of 1.0M NaOH and 1.0M HCl, and 0.1% antifoam A was used for foam control. The fermentation was carried out for 7 days.

3.6.3 Preparation of the spore-crystal complex

The culture was collected after 7 days of fermentation, dispensed into 250ml centrifuge tubes and centrifuged at 4000 rpm at 5°C for 10 minutes. The supernatant was discarded and the pellet which was washed repeatedly with distilled water was pooled and used for the purification of the crystals.

3.7 Purification of crystals from the spore-crystal complex

Two methods were used for the purification of crystals from the spores. The method which resulted in increased purity was used subsequently.

3.7.1 First method

This method described by Goodman *et al.*, (1967) involved a two-phase separation. Sodium dextran sulfate 500, 334ml of a 20% (w/v) solution ; polyethylene glycol 6000, 234ml of a 20% (w/v) solution; sodium chloride, 100ml of a 3M solution; and the spore-crystal suspension was added together. The final volume was brought to 1 litre with distilled water.

Fresh polyethylene glycol which constituted the upper phase was prepared by adding the following components to sufficient distilled water to make 6 litres : sodium dextran sulfate 500, 1.8g; polyethylene glycol 6000, 421.8g; and sodium chloride, 105.0g. Solvent constituting 250ml of the lower phase was placed in a separatory funnel. Approximately 250ml of fresh upper phase was added to this, mixed and the liqiud phase were allowed to separate. The upper phase, which constituted the spores and cell debris was carefully aspirated off without disturbing the interface. Fresh upper phase (250ml) was added and the process was repeated until few or no spores were observed microscopically in the upper phase.

The viscous crystal-rich lower phase was diluted with an equal volume of distilled water and centrifuged at 12000g for 30 minutes at 5°C. The supernatant was discarded and the sediment was washed by resuspending in

distilled water and centrifuged at 200g for 20 minutes at 5°C. The pellet was washed repeatedly and freeze-dried.

3.7.2 Second method

This method described by Pendleton and Morrison (1966) involved phase separation in which an aqueous phase consisting of a suspension of spores and crystals was emulsified with an organic solvent. To eliminate the spores, a convenient volume of the suspension was shaken vigorously by hand for 5 - 15 seconds and allowed to stand. The froth which appeared on the surface was held back on filtration with Whatman No. 1 filter paper or mechanically removed. This was repeated several times. The spore-crystal suspension was further purified by adding 30ml of 1% (w/v) sodium sulfate and 35ml of carbon tetrachloride to 35ml of the suspension. This was mixed at 7000 -8000 rpm for 2.5 minutes in a homogenizer (Ultra-Turrax T25).

The mixture was then allowed to stand for 15 minutes while the organic phase settled. The aqueous phase, which contained the crystals was aspirated. This was then centrifuged at 4000 rpm for 10 minutes and the supernatant was discarded. The remaining pellet was washed repeatedly with distilled water and freeze-dried. This was used for electrophoretic studies.

3.7.2.1 Dorner method for staining endospores

The purified crystals still contained some vegetative cells and endospores. To differentiate the endospore from the crystals, the Dorner method (Doetsch, 1981) was employed. An aqueous suspension of bacterial was mixed in a test tube with an equal volume of carbolfuchsin. The tube was immersed in a boiling water bath for 10 min. Then a loopful of 7% (w/v)

aqucous nigrosin is mixed on a glass slide with one loopful of the boiled carbolfuchsin - organism suspension and air-dried in a thin film. Vegetative cells are colourless, the endospores are red, and the background is black.

3.8 Molecular weight determination by Dodecyl Sulfate - Polyacrylamide gel electrophoresis (SDS - PAGE)

The procedure for the determination of molecular weight by SDS - PAGE was a modified method by Laemmli (1970) and Weber and Osborn (1969).

3.8.1 Preparation of stock solutions

All solutions were kept at 4°C unless otherwise stated.

1. Solution A

45% (w/v) acrylamide was added to 1.2% (w/v) N,N'-methylene-bis-acrylamide. This solution was deionized with amberlite MB-1, filtered and stored in a dark bottle.

2. Solution B

To 100ml of 0.5M Tris-HCl (pH 8.8) was added 0.13% (v/v) tetramethyl-etyhlenediamine (TEMED).

3. Solution C

12% (w/v) sodium dodecyl sulfate (SDS) was prepared and the solution was kept at room temperature (28 $\pm$ 2°C).

4. Solution D

10% (w/v) ammonium persulfate was freshly prepared just before use.

5. Solution E

0.65M Tris-HCl was prepared and pH adjusted to 6.8.

6. Running buffer (5X)

To 100ml of 125mM Trizma base was added 100ml of 660mM glycine and 100ml of 1% (w/v) SDS. The solution was adjusted to a pH of 8.6 and kept at room temperature.

3.8.2 Preparation of gel

1. Separating gel

A 10% acrylamide gel was prepared by adding the following solutions to sufficient distilled water to make 60ml : solution A, 13.3ml; solution B, 15.0ml; solution C, 0.5ml; solution D, 0.5ml. The solution was immediately poured into the casting apparatus and allowed to polymerize.

2. Stacking gel

A 4% stacking gel was prepared by adding the following stock solutions as follows to 18.67ml of distilled water : solution A, 2.64ml; solution C, 0.20ml; solution D, 0.20ml; TEMED, 24μl. The solution was poured onto the top of the separating gel and allowed to polymerize.

3.8.3 Solubilization of the crystal protein

The crystals of *B. thuringiensis* were dissolved under both reducing and non-reducing conditions. The sample buffer for the reducing agent contained 1% (w/v) SDS, 3μl of tracking dye (0.05% Bromophenol blue in water), 10%

(v/v) glycerol and 5% (w/v) dithiothreitol (DTT). The sample buffer for the non-reducing agent was prepared by dissolving 1% (w/v) SDS in 5mM Tris-HCl (pH 6.5). To this 3μl of tracking dye (0.05% Bromophenol blue in water) and 10% (v/v) glycerol were added.

The crystal protein 1mg of which was added to 1ml of sample buffer, and this was dissolved by incubating for 5 minutes. The following proteins served as standards : phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, aprotinin, triosephosphate isomerase, lactic dehydrogenase, fumarase, pyruvate kinase, fructose-6-phosphate kinase, ß-galactosidase and α_2-macroglobin.

3.8.4 Staining and destaining

The gels were placed in small containers filled with the staining solution prepared by dissolving 1.25g of Coomassie brilliant blue R250 in a mixture of 454ml of 50% methanol and 46ml of glacial acetic acid. Staining was carried out at room temperature for 1 hour. The gels were then removed from the staining solution, rinsed with distilled water, and placed in destaining solution (75ml of acetic acid, 50ml of methanol, and 875ml of water) overnight. The mobility of the protein and the marker dye were recorded. Mobility was calculated as :

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

3.9 Production of spore-crystal complex for bioassay

Production of spore-crystal complex for the bioassay was performed in a 10L Biostat E fermenter (Plates 2 and 3) consisting of 9 litres of G-medium. Glucose 0.5% (w/v) was autoclaved separately and added to the fermentor vessel. This was transferred aseptically into the vessel. The seed medium was prepared by inoculating a suspension of *B. thuringiensis* taken from slant agar in 250ml of G-medium in four 1L shake flasks. These flasks were incubated in an orbital shaker at 200rpm and 35°C overnight. The fermentation was carried out at 35°C with an agitation of 250rpm and dissolved oxygen saturation of 30%. pH was adjusted to neutral pH with 3M NaOH and 3M HCl. The fermented broth was dispensed into centrifuge tubes after 7 days of fermentation and centrifuged at 4000rpm for 10 minutes in order to harvest the spore-crystal complex which was then freeze dried into a powder. This was used for bioassay studies.

3.10 Preliminary evaluation of IPT Bt 6, Florbac and Avermectin against *Plutella xylostella*

The spore-crystal complex of IPT Bt 6, Florbac and the chemical, Avermectin were evaluated in the laboratory against *Plutella xylostella*. The efficacy of these was compared individually and in combinations. The bioassay was carried out at MARDI in Cameron Highlands.

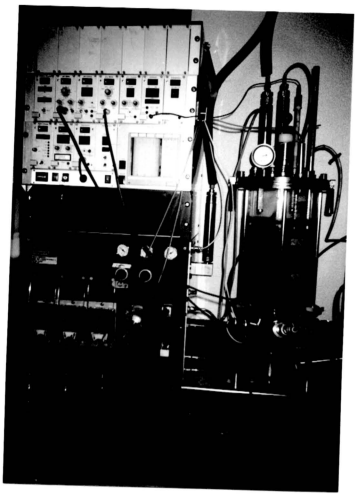

Plate 2: Biostate E (10 L)

Fermentation was carried out at 35°C with an agitation of 250 rpm and dissolved oxygen saturation of 30%

Plate 3: Biostate E

The 1 L vessel of Biostat E fermentor equiped with the pH probe, dissolved oxygen probe and foam sensor.

3.10.1 Target organisms

Larvae of *P. xylostella* were collected from vegetable farms in Kampung Raja and Melson Valley in Cameron Highlands (Plate 4). These were propagated in the laboratory. The larvae used in the bioassay consisted of F1 and F2 off-spring of those collected from the farms. The larvae were fed with untreated cabbage leaves grown in glass houses (Plate 5).

3.10.2 Preparation of test solution for the bioassay

For the bioassay of *P. xylostella* from Kampung Raja, two concentrations each of Florbac and IPT Bt 6 were used. The spore-crystal suspension was prepared by dissolving 0.2g and 0.05g of the powdered extract in 200ml distilled water containing Extravon (Ciba Geigy (M) Ltd.) as a wetting agent at a rate of 0.5ml/L. This produced solutions with concentrations of 1g/L and 0.25g/L, respectively. Agrimec 1.8EC (Hoechst (M) Ltd.), a chemical insecticide with Avermectin B1 as the active ingredient was used as control. This was used at a concentration of 0.1ml/L. Various combinations of the treatments were used in the bioassay.

A lower concentration of the crystals was used for the Melson Valley strains which were more susceptible than the Kampung Raja variety due to less intensive spraying of chemicals in the former. Florbac and IPT Bt 6 were used at concentrations of 0.03g/l and 0.1g/l, respectively. These doses were the LD_{50} values for *P. xylostella* (Singh, R. 1994). The various combinations tested are indicated in Tables 5 and 6.

Plate 4: Collecting larvae of *P. xylostella* from a vegetable farm in Kampung Raja (Healthy larvae were brought to the laboratory for propagation)

Plate 5: Isolated glass house

Larvae fed with untreated cabbage leaves, propagated in isolated glass house kept at room temperature.

Table 5 : Treatment regime for the bioassay of *P. xylostella* strains from Kampung Raja.

No.	Treatments	Concentration
1	IPT Bt 6	0.25g/l
2	IPT Bt 6	1.0g/L
3	Florbac	0.25g/L
4	Florbac	1.0g/L
5	Agrimec	0.1ml/L
6	IPT Bt 6 + Florbac	0.25g/L + 0.25g/L
7	IPT Bt 6 + Agrimec	0.25g/L + 0.1ml/L
8	Florbac + Agrimec	0.25g/L + 0.1ml/L
9	Control	untreated

Table 6: Treatment regime for the bioassay of *P. xylostella* strains from Melson Valley

No.	Treatments	Concentration
1	IPT Bt 6	0.11g/L
2	Florbac	0.03g/L
3	IPT Bt 6 + Florbac	0.11g/L + 0/03g/L
4	IPT Bt 6 + Agrimec	0.11g/L + 0.1ml/L
5	Florbac + Agrimec	0.03g/l + 0.1ml/L
6	Control	untreated

3.10.3 Leaf-dip method for the bioassay of larvae of *P. xylostella*

The leaf-dip method (Tabasnik and Cushing, 1987) was used for the bioassay of *P. xylostella*. They consisted of leaf discs (5cm diameter) cut from the centre of the middle leaves of cabbage plants using a metal hole punch. Each leaf disc was immersed in the test solution and allowed to dry on a corrugated sheet of aluminium foil for 1-2 hours at room temperature with the underside of the leaf uppermost. Control leaf discs were not treated. These discs were then placed in pairs in disposable plastic cups (6.5cm x 6.0cm x 4.5cm) (Plate 10), containing a single, moistened, Whatman No. 1 filter paper (2cm diameter). Five early 3rd instar larvae were placed in each cup. Larval mortality was assesed on the 2nd day up to the 8th day, after which pupation usually occurred among the survivors in all treatments. After 3 days, the treated leaf discs were replaced by fresh, untreated cabbage leaves in order to prevent deaths due to starvation.