2.0 LITERATURE REVIEW

2.1 *Bacillus thuringiensis* - the bacterium

Bacillus thuringiensis is a rod-shaped, aerobic, spore forming bacterium characterized by the production of one or more proteinaeous parasporal crystals during the sporulation cycle as indicated in Figure 1 (Bechtel and Bulla, 1976). These parasporal crystals which are composed of proteins are designated the delta endotoxin which are responsible for the pathogenicity of *B. thuringiensis* to insects. Various species of *B. thuringiensis* differ in the degree of activity towards insects which include the Lepidoptera (Dulmage, 1981), Diptera (Desai and Shethna, 1991), Orthoptera, Hymenoptera, and Coleoptera (Angus, 1971), Acari, Phthiraptera (Drummond and Pinnock, 1994). *Bacillus thuringiensis* can be isolated readily from insects, stored product material sericulture environment and phylloplane. It is also a ubiquitous soil bacterium (Chilcott and Wigley, 1994).

Bacillus thuringiensis shows several interesting features. The pathogenicity of the bacterium is activated at the time of sporulation when the crystals produced contain a stomach poison to susceptible insects. The bacterial cells autolyse to release a mixture of spores and crystals. The mixture is then ingested by insect larvae feeding on contaminated vegetation which causes gut paralysis leading to lethal septicemia (Deacon, 1983).

In addition to the delta endotoxin, most strains of *B. thuringiensis* also produce a range of other toxic metabolites, including a toxin termed exotoxin or thuringiensin (Deacon, 1983). The delta endotoxin and the exotoxin are the best known toxins, but there are other toxins, as yet unidentified, responsible for the pathogenicity to some hosts eg. lice (Drummond and Pinnock, 1994).

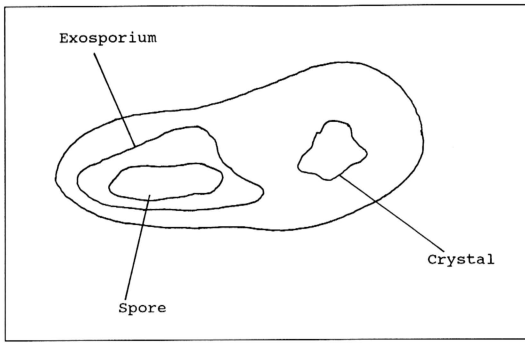

Fig. 1 ***Bacillus thuringiensis*** (Bechtel *et al.*, 1976)

2.2 *Bacillus thuringiensis* - A brief history of its development

The bacterium was first recorded in 1901 as the cause of the 'sotto' disease of silkworms in Japan (Deacon, 1983). The existence of a parasporal body or 'Restkorper' in sporulated cells of *B. thuringiensis* was noted by Berliner (1915) and Mattes (1927) although the observation attracted little attention then.

In 1953, Hannay observed the extra-sporal inclusion and characterized its nature and significance. With the realization of the parasporal body to a toxic proteinaeous crystal, it became the centre of considerable attention (Norris, 1971).

In 1955 Hannay and Fitz-James showed that the crystal was composed of protein subunits. Angus (1956) demonstrated that it caused most of the symptoms of toxicosis observed in silkworm larvae. Heimpel (1967) termed the crystal protein a delta endotoxin and ever since it has been studied extensively all over the world.

2.3 Biosynthesis of the spore and crystal

The life cycle of *B. thuringiensis* is characterized by two distinct steps : vegetative growth and sporulation. As the growth ceases due to a limitation of an essential nutrient or the exhaustion of a carbon source, sporulation begins. It is an ordered sequence of events which results in the construction of a complex extracellular structure, the bacterial endospore (Figure 2). Post-exponential *B. thuringiensis* cells produce both endospore and a variety of ultracellular inclusions. The latter are comprised of protoxins, each being specific for the larvae of certain species (Aronson, 1993).

The development process comprises the following : (a) axial formation (localization of genetic material along the longitudinal axis), (b) forespore development, which involves invagination of the cytoplasm membrane and engulfment of genetic material, (c) cortex synthesis and spore coat deposition, and (d) dehydration of spore protoplast with a concomitant accumulation of dipicolinic acid and calcium. The last stage after the completion of sporulation involves total refractility of the spore which results in the release of the mature spore from the sporangium (Bulla, 1975).

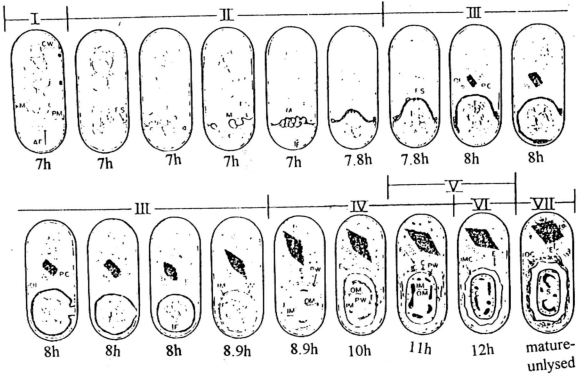

Fig. 2: Diagram of sporulation in *B. thuringiensis*. M, mesosome; CW, cell wall; PM, plasma membrane; AF, axial filament; OM, outer membrane; PW, primordial cell wall; E, exosporium; LC, lamella spore coat; OC, outer spore coat; C, cortex; IM, incorporated mother cell cytoplasm; S, mature spore in a unlysed sporangium. (Source: Fast, 1981)

There are various theories regarding the formation of the crystal. Somerville and Pockett (1975) suggested that the crystal is a major component of the spore coat while Heimpel and Angus (1960) reported that the crystal could be the undesirable substance produced as a metabolic by-product of sporulation which is removed from the soluble environment by crystallization.

Synthesis of the crystal toxin in *B. thuringiensis* is evidently associated with sporulation as crystal toxin is not found in vegetative cells and does not appear in sporulating cells until about 3 hours after its onset (Andrews *et al.*, 1985). It is synthesized within the sporulating cell during stages II and III (Fig. 2). Presumably, subunits are synthesized and progressively assembled to produce a crystal structure that becomes refractile to light. The perasporal crystal is enclosed within the spore's outermost envolope (exosporium), as determined by transmission electron microscopy (Lopezmeza and Ibara, 1996).

2.4 The crystal protein of *Bacillus thuringiensis*

There is a considerable variation in the shape and size of the parasporal inclusion produced by different varieties of *B. thuringiensis*. Some crystals are regular, others less so. Murty *et al.* (1994) reported that *B. thuringiensis* subsp. *kurstaki*, a toxic strain to lepidopteran insects that produces small bipyramidal crystals, and *B thuringiensis* subsp. *yunnanensis*, a nontoxic strain produces usually large bipyramidal and spindle-shaped crystal. Cidaria *et al.* (1991) have also reported rare flat, square crystals. The most common form is best described as diamond shaped, that is octahedral which appears tetragonal.

Scherrer *et al.* (1973) reported that the shape and size of the crystal can be influenced by the media composition, while Heimpel (1967) related the shape of the crystal to the degree of toxicity, resulting in thromboid, truncated and wedge shaped forms. Data from electron microscopic and X-ray powder diffraction studies indicate that the readily visible subunit comprising the crystal is rod or dumb-bell shaped with dimensions of the order of 5 x 15 nm (Norris, 1971) and has a molecular weight of 230 000 daltons (Fast, 1981). The mature crystal may account for some 30% of the total dry weight of the sporulated cell (Norris, 1971).

The crystal is a glycoprotein where carbohydrate constitutes about 5.6% of the molecule and the balance is protein (Bulla *et al.*, 1977). The carbohydrate consists of glucose (3.8%) and mannose (1.8%) (Bulla *et al.*, 1979). Table 1 lists the amino acid composition of the crystal.

2.5 Biochemistry of the crystal

The crystal is heat-labile, insoluble in water and organic solvents but is readily soluble under alkaline reducing conditions (Deacon, 1983). The solubility of the crystal cannot be attributed to the number of disulphide bonds in the protein, as believed earlier because the cystine content of the protein is low (Cooksey, 1971). Studies have shown the presence of significant amounts of silicone in the crystal and it forms a lattice upon which the protein molecules are assembled. This silicaneous framework offers an attractive explanation for its lack of solubility.

Table 1: Amino acid analyses of *B. thuringiensis* crystals: grams of amino acid residues/100g

Reference	Somerville *et al.* (1968)	Angus (1956)	Lecadet (1965a)		Holmes & Monro (1965)	Spencer (1968)			Cookey (unpublished)			
Esterase type[a]	*alesti*	*sotto*	*berliner*	*anduze*	*berliner*	*entomocidus*	*alesti*	*galleriae*	*kenya*	*kenya*	*berliner*	*berliner*
Crystals or Protein	+	+	+	+	+	+	+	+	+	+	+	+
Aspartic acid	10.7	9.6	12.6	11.0	11.9	14.0	13.0	15.5	12.4	13.2	12.7	11.2
Threonine	4.8	4.5	5.8	5.1	5.2	6.8	7.0	7.0	6.3	6.4	6.3	5.6
Serine	4.8	4.8	4.4	5.6	5.4	6.0	6.5	6.2	5.3	5.9	5.0	4.2
Glutamic acid	11.6	11.8	12.0	14.3	12.5	16.0	17.0	14.5	13.7	14.1	13.0	13.2
Proline	3.5	7.5	4.4	4.9	4.0	4.4	4.3	4.7	3.4	3.9	3.8	3.4
Glycine	3.3	3.2	4.3	4.5	3.0	4.8	4.8	4.2	3.6	4.5	3.4	4.0
Alanine	2.9	2.8	3.8	4.1	3.1	4.8	5.0	5.0	4.0	5.0	3.9	4.7
Cystine	1.2	1.2	1.4	1.3	0.3	1.4	1.9	1.5	2.0	N.D.	N.D.	N.D.
Valine	6.0	5.3	5.0	5.1	5.7	7.8	7.4	6.4	5.8	6.8	6.0	6.1
Methionine	0.9	1.3	1.5	1.7	1.6	0.7	0.2	0.8	0.4	2.0	1.1	1.8
Iso-leucine	5.1	11.2[b]	4.4	5.1	6.3	6.6	6.4	6.3	5.6	6.7	5.6	5.4
Leucine	7.1		7.7	7.9	8.8	10.5	9.5	9.3	8.3	10.0	8.1	8.3
Tyrosine	5.9	6.8	6.6	5.9	7.0	6.5	6.7	6.7	5.8	6.6	6.9	5.7
Phenylalanine	5.7	8.6	6.8	5.2	8.1	6.7	6.8	6.6	5.9	7.0	5.6	5.8
Lysine	3.1	3.6	3.4	4.0	4.0	4.4	5.4	4.6	4.6	5.8	4.9	7.2
Histidine	2.2	2.7	2.7	3.3	2.5	2.8	2.8	2.8	2.8	2.4	2.5	2.2
Arginine	7.5	9.6	7.8	7.5	8.5	9.0	10.4	9.9	8.6	7.5	7.6	5.2
Tryptophan	1.9	2.6	2.1	1.8	5.2	2.3	2.3	1.9	N.D.	N.D.	N.D.	N.D.
Ammonia	1.9	N.R.	N.R.	N.R.	2.0	N.R.	N.R.	N.R.	1.9	2.3	1.6	1.4
Total recovered	97.1	96.6	98.3	105.1	105.5	117.4	113.9	90.1	100.4	98.0	110.1	95.4

a Norris (1964)

b Angus (1956) used a paper chromatographic method incapable of resolving iso-leucine and leucine

N.D. not determine; N.R. not reported [Source: Cooksey, 1971]

The crystals are composed of several proteins (Tyrell *et al.*, 1981). One of the problems associated with assaying isolated crystal proteins is that the crystals, when dissolved, show little or no toxicity *in vivo*. In the intact crystal structure the S-S bonds are not accesible to reducing agents without a preceeding loosening of non-covalent intermolecular forces, probably hydrogen bonds.

Huber *et al.* (1981), reported that urea and guanidine not only break inter- but also intramolecular hydrogen bonds. This explains the inactivation of the toxin when these solvents are used, whereas the use of moderate alkaline pH does not affect the activity.

It is established that the crystal is composed of individual subunits (Norris, 1971). These subunits are, in turn, composed of one or more proteins which themselves are probably built from polypeptide chains covalently linked by disulphide bridges. Andrews (1985) demonstrated that the initial crystal protein produced by *B. thuringiensis* has a molecular weight of 135 000 daltons which is then converted to the activated toxin (molecular weight, 68 000) by proteolytic cleavage during its synthesis. This observation strongly disproves the earlier hyphothesis that the 135 000 molecular weight molecule is a dimer.

Herbert *et al.* (1971) separated the crystal protein of *B. thuringiensis* var. *tolworthy* into two polypeptide chains with molecular weights of 55 000 and 120 000; they appeared in the ratio of 1:2, and the component with the molecular weight of 55 000 accounted for most of the toxicity of the dissolved crystal. Yamamoto *et al.* (1983) concluded that the crystals of *B. thuringiensis*

var. *israelensis* are composed of proteins with molecular weights of 28 000, 42 000, 67 000 and 120 000. Nicolas et al. (1993) observed that 42kDa protein inclusions of *Bacillus sphaericus* was toxic to *Culex pipiems* larvae and the 51kDa protein inclusions were not. Similar proteins may also be produced by *B. thuringiensis* which is toxic to Dipteran. Orduz *et al.* (1994) have shown that *B. thuringiensis* serovar medellin shows polypeptides at 100kDa, multiple bands at 80, 75, 70, 67 and 65 kDa, and two duplets at 40-41 and 28-30kDa when analysed by SDS-PAGE method.

Tyrell (1981) established that *B. thuringiensis* toxic to lepidoptera produce crystals that are structurally, biochemically and immunologically different from those that are toxic to dipteran. Further the specificity of *B. thuringiensis* may be due to a toxin with a unique amino acid sequence, different carbohydrate content, or both.

2.6 Mode of action

A common feature of all insects susceptible to the proendotoxin of *B. thuringiensis* is an extremely alkaline midgut pH, often exceeding a value of 10 (Cooksey, 1971) . Under these conditions the crystal dissolves and the toxin is released by proteolytic enzymes. There is evidence that the enzymes of different insects release different polypeptides from the crystals, thus contributing to further specificity (Deacon, 1983).

This was further established by Haider *et al.* (1985) with *B. thuringiensis* var. *colmeri*. After the activation of the crystal with *Aedes aegypti* gut extract the preparation was found to be toxic to all the mosquito cell lines but only

one lepidopteran line, whereas an activated preparation produced by treatment with *Pieris brassicae* gut enzymes was toxic only to lepidopteran cell lines.

The site of action of crystal protein is definitely the midgut area and the effect of the toxin is to damage the midgut epithelium (Angus, 1971). Following investigation of spores and inclusions, toxicity results in the spores gaining access to haemolymph, a source of nutrients suitable for gemination and growth (Aronson, 1993). Thomas and Ellar (1983) indicated that the toxin binds readily to multilammelar liposomes containing phosphatidyl choline, sphingo-myelin, or phosphatidyl ethanolamine provided that the lipids contain unsaturated acyl residues. Knowles *et al.* (1984) later identified these lipids as plasma membrane receptors for *B. thuringiensis* var. *israelensis*.

English and Slatin (1992) suggested that these toxins bind to the epithelium of the insect midgut and open the cation-selective channels. Delta-endotoxin form cation-selective channels in planar lipid bilayer, suggesting that these channels are responsible for toxicity. All susceptible lepidoptera suffer from gut paralysis shortly after feeding on sporulated cultures or on crystals (Heimpel and Angus, 1960; Cooksey, 1971; Bulla, 1980; Couch and Ross, 1980).

Histological studies, *in vivo* have shown that the larval midgut epithelial cells swell and lyse causing severe disruption of the gut wall (Heimpel and Angus, 1960). Cells of the gut epithelium slough off into the lumen and expose areas of the basement membrane which are attacked by vegetative cells.

The toxin acts as a non-competitive inhibitor of the potassium ion and amino acid co-transporters of the larval midgut of lepidoptera (Giordana *et al.*, 1993). Increase in haemolymph K^+ concentration begins after the metabolism of gut epithelial cells has been effectively broken down and general leakage of ions from gut to haemolymph had begun. This is followed by an inhibition of glucose and leucine uptake into the gut epithelial cells.

The resulting changes in composition and pH of the haemolymph can cause general paralysis, as observed in silkworms as early as 90 minutes after treatment. Often changes in gut and haemolymph permit vegetative propogation of normal gut microbial flora or of introduced organisms, resulting in septicaemia which contribute to or cause the death of larvae (Fast, 1981).

2.7 Metabolism of *Bacillus thuringiensis*

Much research has been done on the metabolism of *Bacillus subtilis, Bacillus cereus, Bacillus larvae*, and other spore forming bacilli (Gollakota and Halvorson, 1960; Hanson, *et al.*, 1963; Baillie and Norris, 1963; Hanson and Cox, 1967; Warren, 1968; Tochikubo, 1971; Diesterhaft and Freese, 1973). The biochemical events related to spore and crystal formation of *B. thuringiensis* do not differ much from other *Bacillus*.

Generally, *B. thuringiensis* catabolizes carbohydrates by a concurrent operation of the Embden-Meyerhof-Parnas and pentose-phosphate pathways (Wang and Krackov, 1962; Julian and Bulla, 1971; Bulla *et al.*, 1980; Bulla and Hoch, 1985). The overall metabolic pattern during growth and sporulation

as described by Yousten and Rogoff (1969) for *B. thuringiensis* is very similar to that of *B. cereus* (Hanson *et al.*, 1963). Studies conducted by Benoit *et al.* (1990) on *B. thuringiensis* HD-1 suggest that lactate, pyruvate, acetate, acetoin, and poly-beta-hydroxybutyrate (PHB) are produced via the 2,3-butanediol cycle.

For both *B. cereus* and *B. subtilis*, the Embden-Meyerhof-Parnas pathway is the primary mechanism for glucose assimilation, whereas the pentose-phosphate pathway aids formation of biosynthetic intermediates rather than functioning as a respiratory pathway (Wang and Krackov, 1962).

Julian and Bulla (1971) have shown that the method of glucose metabolism in *B. larvae* differs significantly from that of other spore forming bacilli. The authors observed that glucose was assimilated predominantly by an oxidative mechanism rather than by the Embden-Meyerhof-Parnas scheme and glucose-grown cells contain enzymes peculiar to the Entner- Doudoroff pathway.

At the end of the exponential growth, when glucose is exhausted from the growth medium, the cells undergo a sequential development that results in spore formation. During the transition from vegetative growth to sporulation, certain metabolic changes occur. End-products of glucose catabolism that accumulate during growth are oxidised via the tricarboxylic acid cycle (Julian and Bulla, 1971). Apparently this cycle provides the energy necessary for biosynthesis and sporulation (Bulla *et al.*, 1971).

Yousten and Hanson (1972) provided the first evidence that the tricarboxylic acid cycle may not be an absolute requirement for sporulation

while Nickerson *et al.* (1974) later reported a similar observation. Studies by Aronson *et al.* (1975) have revealed the presence of an ancillary pathway in *B. thuringiensis* which allows for glutamate and alpha-ketoglutarate catabolism via the gamma-aminobutyric acid pathway (Fig. 3). The enzyme involved in this pathway are listed in Table 2. Similar findings were reported by Bulla *et al.* (1980) which show that *B. thuringiensis* possesses a modified tricarboxylic acid cycle in which alpha-ketoglutarate dehydrogenase is absent. The author further showed that alpha-ketoglutarate is converted to glutamate and then to succinate via the gamma-aminobutyric acid. Figure 3 summarises the metabolic pathway utilized by *B. thuringiensis* during growth and sporulation.

Table 2: Enzymes which are involved during growth and sporulation of *B. thuringiensis* as depicted in Figure 3

a	Fructose diphosphate aldolase
b	Glyceraldehyde phosphate dehydrogenase
c	Aconitate hydrase
d	Isocitrate dehydrogenase
e	Fumarate hydrase
f	Malate dehydrogenase
g	Malate dehydrogenase (decarboxylating)
h	Isocitrate lyase
i	Malate synthase
j	Glutamate dehydrogenase
k	Alanine dehydrogenase
l	L-Aspartate aminotransferase
m	L-Alanine aminotransferase
n	Glutamate decarboxylase
o	Aminobutyrate aminotransferase
p	Succinate-semialdehyde dehydrogenase

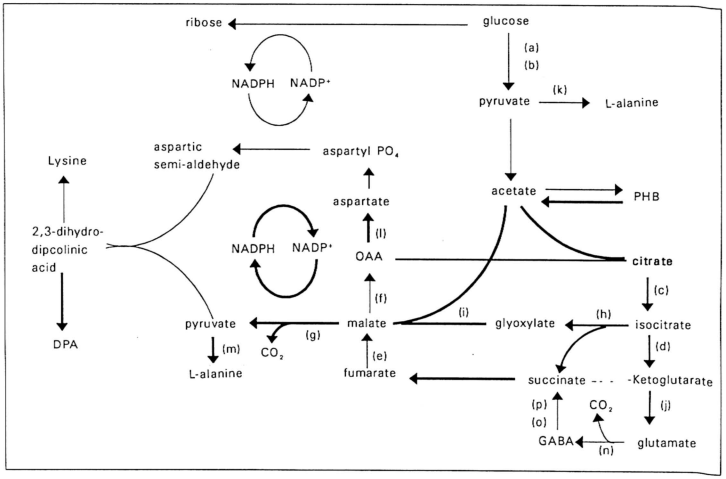

Fig. 3: Summary of some metabolic pathways utilized by *B. thuringiensis* during sporulation (heavy arrows). Those pathways thought to be important during growth or during both growth and sporulation are shown by light arrows. Letters in parentheses refer to enzymes tabulated in Table 2. (Source: Aroson, 1975)

Bulla *et al.* (1971) observed that four and five-carbon compounds, such as alpha-ketoglutarate and glutamate are required for the continued operation of the tricarboxylic acid cycle. A mechanism to form four-carbon compounds becomes necessary if they are not supplied exogenously. Presumably, this mechanism is the glyoxylate cycle.

Ohne (1975) found that the addition of glucose leads to the repression of all the tricarboxylic acid cycle enzymes. The synthesis of the tricarboxylic acid cycle enzymes (Table 3) is also regulated by specific induction and repression mechanisms, acting on individual enzymes. These mechanisms are outlined in Figure 4.

Bulla and Hoch (1985) observed that sporulation was associated with the accumulation of dipicolinic acid (DPA). Young forespores accumulate DPA, as well as calcium by facilitated diffusion from the mother cell cytoplasm, and a chelate of calcium dipicolinate is formed as the forespore matures.

Another aspect of sporulation is the synthesis of a unique peptidoglycan that consists of three repeating subunits : (1) a muramic lactam with no amino acids attached, (2) an alanine subunit with only an L-alanyl residue, and (3) a tetrapeptide subunit having the sequence L-ala-D-glu-meso-diaminopimelic acid-D-ala. These components occur in a general ratio of 3.5:1:2, respectively.

Table 3 : Enzymes of citric acid cycle as depicted in Figure 4

1	Pyruvate dehydrogenase
2	Citrate synthase
3	Aconitase
4	Isocitrate dehydrogenase
5	α-Ketoglutarate dehydrogenase
6	Succinyl CoA synthatase
7	Succinate dehydrogenase
8	Fumarase
9	Malate dehydrogenase
10	Malic enzyme
11	Pyruvate carboxylase

Hanson *et al.* (1963) found that cells of *B. cereus* also accumulated poly-beta-hydroxybutyric acid (PHB) during the initial stages of sporulation. PHB was also observed in *B. thuringiensis* during the stationary phase by Benoit *et al.* (1990). The authors further suggested that PHB probably provided energy for spore formation.

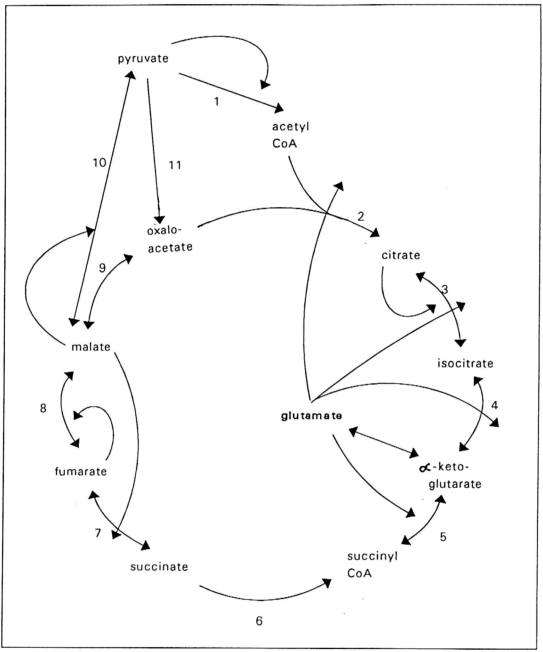

Fig. 4: Mechanisms regulating the synthesis of citric acid cycle enzyme and some metabolically related enzymes in *B. subtilis*. The reactions shown are catalyzed by the enzymes shown on Table 3. ($\underline{\downarrow}$) indicates induction, ($\underline{\uparrow}$) indicates repression. (Source: Ohne, 1975)

2.8 Enzymes of *Bacillus thuringiensis* in relation to spore and crystal formation

The protein content of the spore is different from that of the vegetative cells. During sporulation the complement of protein changes in both the mother cell and the developing spore. Setlow (1981) has delineated three groups or classes of proteins associated with sporulation : group 1 proteins, which occur in both the mother cell and forespore at similar levels, group 2 proteins, which are present in the mother cell but not in the forespore, and group 3 proteins, which are associated with the forespore but not with the mother cell.

Group 1 proteins include glycoprotein enzymes, a few enzymes involved in terminal electron transport, enzymes for vegetative cell wall biosynthesis, and some enzymes responsible for amino acid catabolism. Group 2 proteins include most of the tricarboxylic acid cycle enzymes, alanine dehydrogenase, several enzymes specific for biosynthesis of cortex, DPA biosynthetic pathway enzymes and some nonspecific endoproteases. Among the group 3 proteins are glucose dehydrogenase, enzymes for cortex processing and salvaging of by-products of cortex synthesis, aspartase, low molecular-weight proteins associated with the spore interior, and a variety of proteases.

The transition from the vegetative to the sporulation cycle in a sporeformer is marked by a change in the enzymatic machinery of the cell. The tricarboxylic acid cycle enzymes can be completely repressed during growth of all bacilli and they appear under all conditions conducive to good sporulation.

Hanson *et al.* (1963) found that cells of *B. cereus* harvested during vegetative growth lacked a functional tricarboxylic acid cycle, and the enzymes required for the completion of this cycle were synthesized during the transition from growth to sporulation. Similar findings were reported by Bulla *et al.* (1971) for *B. thuringiensis, B. alvei, B. lentimorbus* and *B. popilliae*.

The regulation of alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase, and malic enzymes has been studied in *B. subtilis* by Ohne (1975). The levels of these enzymes increase rapidly during late exponential phase and are maximal 1 to 2 hours after the onset of sporulation.

In extracts of *B. subtilis*, carbon dioxide fixation occurs primarily through the constitutive enzyme pyruvate carboxylase, which is strongly activated by acetyl-CoA (Diesterhaft, 1973). This enzyme was found to be necessary for growth on glucose but was not required for sporulation.

In contrast, aconitase and isocitrate dehydrogenase were repressed during exponential growth of *B. subtilis* (Fortnagel, 1970). Both enzymes were derepressed when growth ceased, and the cells entered the developmental stage. Warren (1968) also reported similar observations on the activity of aconitase. Unlike aconitase, the activity of malate dehydrogenase remained at a highly constant level throughout sporulation. The activity of fumarase was reported to be highest, 1-5 hours after the end of the exponential growth.

One of the earliest enzymes produced during sporulation is the exoprotease. The importance of this enzyme in sporulation is shown by the fact that mutants that had lost the ability to synthesize it lack the ability to form spores (Mandelstam and Waites, 1968).

Reviews on the study of proteases produced by Bacillus species can be found in Bernlohr (1964), Millet (1970), Prestidge (1971), Li (1975), Epremyan *et al.* (1981), Egorov and Loriya (1983) and Andrews (1985). A diverse group of proteases are produced by bacilli. Several reports suggest that the crystal-associated proteases are serine, metal, and sulfhydryl classes (Bulla *et al.*, 1977; Chestukhina *et al.*, 1978; Chestukhina *et al.*, 1980). Despite the lack of a proven role for the proteases of *B. thuringiensis*, their synthesis is interesting as they are produced very early in the sporulation cycle, and they interfere with biochemical studies of sporulating bacteria.

Alkaline phosphatase appears during sporulation (Warren, 1968). The activity of this enzyme increases fivefold at the end of the exponential growth. It is not certain whether the alkaline phosphatase synthesized after the logarithmic growth has ceased is identical to the vegetative enzyme synthesized during conditions of phosphate depletion (Hanson *et al.* 1970).

Kingan and Ensign (1968) isolated and characterized three autolytic enzymes associated with sporulation. These enzymes were found to be N-acetylmuramidase, N-acetylmuramyl-L-alanine amidase and endopeptidase. Other enzymes that appear during sporulation include those required for the synthesis of critical peptidoglycan and dipicolinate (Hanson *et al.*, 1970).

2.9 Production of *Bacillus thuringiensis* delta-endotoxin

The technology used in the production of microbial insecticides varies from that of a cottage to one that equals the sophistication as in the pharmaceutical industry. The preparations of *B. thuringiensis* used in the early

studies were grown under laboratory conditions on simple nutrient media. At the end of the growth period, the culture was simply scraped from the surface of the medium, suspended in water and used in experiments (Angus, 1971). This method only sufficed for simple, nonqualitative laboratory tests. Thus, other methods of growing *B. thuringiensis* on a large scale had to be developed in order to produce the insecticide in bulk.

In commercial production a suitable strain of *B. thuringiensis* is cultured either on a solid substrate (semi-solid fermentation) or in aerated broth cultures (submerged fermentation). The choice of the strain is dictated by several considerations : growth habits, bacteriophage sensitivity, host spectrum, toxicity of crystal protein and exotoxin produced, yield, and characteristics of spores produced (Angus, 1971).

In the semi-solid fermentation, the organism is grown in a liquid nutrient which is adsorbed on the surface of small particles of a carrier. This enables a high ratio of surface area to volume and furnishes, in a relatively small space, a large liquid-gas interface (Dulmage and Rhodes, 1971). The common carrier used in these fermentations is wheat bran (Angus, 1971; Dulmage and Rhodes, 1971; Bulla *et al.*, 1979; Dulmage, 1981) although ground corn, peanut meal, oat or rice hulls, cottonseed meal and alfalfa meal have been used (Dulmage and Rhodes, 1971).

Bran has a peculiar property of holding water on its surface without being wet (Dulmage, 1981) and is also an excellent source of starch and protein (Dulmage and Rhodes, 1971). Dulmage (1981) further found that if, instead of water, a nutrient solution is applied to bran, each bran particle becomes an individual miniature fermenter.

In submerged fermentation, microbes are grown in a liquid medium in large tanks sealed from the environment. The liquid inside is kept agitated and aerated by the passage of sterile, filtered air (Dulmage, 1981). Pilot plant studies and large scale production use deep-tank fermentors. These range in size from 5 litres in a laboratory model up to 200 000 litres in production vessels (Dulmage and Rhodes, 1971).

Semi-solid fermentation has more advantages compared to submerged fermentation, whereby all soluble exotoxins produced are preserved whereas in the broth it is largely discarded in the supernatant fluid (Angus, 1971). Recovery of product from submerged fermentation too, is often difficult since there is only a small amount of the desired material in a large amount of fermentation liquour (Dulmage and Rhodes, 1971).

Submerged fermentation is expensive whereby the cost is largely determined by the energy requirements to run the fermenter (steam for sterilization, chilled water for temperature control, compressors to supply air). However, submerged fermentation offers an over-riding advantage in that various process parameters can be controlled during the process. Yields are also usually higher. Thus, submerged fermentation is preferred to semi-solid fermentation.

There are other less popular methods for the production of microbial insecticides. A control agent may be produced from insects collected from the field. Field collected larvae are infected by injecting with *B. popillae* or *B. lentimorbus*. The resultant spore-laden cadavers are formulated as a wettable powder for protecting lawns, field crops and market garden (Stockdale, 1985).

One of the keys to successful commercialization of the product would be the development of a suitable and economical fermentation medium. Most media employed now use natural products as a source of carbon, nitrogen and trace minerals. The list of nitrogen sources used include : fishmeal, cottonseed flour, corn steep liquor, soybeans, autolysed yeast and casein. Carbohydrate sources include : hydrolysed corn products, starch and dextrose.

Preparations of *B. thuringiensis* are available as wettable powders, dusts, and water-dispersible emulsions and the viability of the spores in the mixture are preserved (Angus, 1971).

2.10 Formulation and field trial

Bacillus thuringiensis is used against a wide range of lepidopterous defoliaters in cotton, soybean, forest, grape, tobacco, fruits, nuts and vegetables. The organism is capable of controlling 92 species of insects worldwide (Couch and Ross, 1980). Thus, extensive field tests are required to determine the efficacy of *B. thuringiensis*. Formulation has a critical effect on the efficacy of microbial insecticides. A pesticide formulation is defined as the resultant composition when the candidate is mixed with anything, including water. Therefore, any combination of an active biocide with another material is technically a formulation. Angus and Luthy (1971) mentioned that the development of a formulated microbial insecticide closely paralled that of chemical insecticides. This is true because both chemicals and insect pathogens must be formulated to facilitate mixing and application.

Basic formulations of insecticides derived from *B. thuringiensis* comprise : (1) liquids (aqueous suspensions, or emulsifiable suspension), (2) wettable powders, (3) dusts, (4) baites, (5) granules (Angus and Luthy, 1971; Angus,1971; Couch and Ignoffo, 1981). In the commercial development of a basic formulation of an entomopathogen, research mainly concerns the maintainance of the pathogen's viability and virulence during the production process and developing a product which preserves or enhances its properties. Like many other products offered for field use, preparations of *B. thuringiensis* usually include additives such as wettable agents, stickers, emulsifiers, and anticaking agents (Angus, 1971).

Biological control agents have been inadequately studied as components of integrated pest management systems. An integrated pest management system does not involve the use of biological control only, nor, indeed, the use of any other single method of pest control. Rather, an integrated pest management system entails simultaneous or sequential use of several methods of control. Biological control is of particular interest as a component of integrated pest management systems because its suppressive effect depends on the density of the pest population.

In many instances, there is a lag period between the buildup of the pest population and the buildup of the biological control agent. Indeed, the buildup of the pest population is frequently so explosive that the crop is badly damaged or lost before indigenous biological agents can overcome the pest population. In such instances it is desirable to supplement the use of biological control agents with other methods of control (Klassen, 1971).

Bacillus thuringiensis is compatible with a wide range of fungicides, insecticides, herbicides, and acaricides. Table 4 indicates the types of chemical pesticides which are compatible with *B. thuringiensis*. In addition to being compatible with chemical pesticides the combination of *B. thuringiensis* and chemicals may increase the effectiveness of one or both (Falcon, 1971). Furthermore, this may be accomplished by using lower dosages of each with a resultant synergistic effect.

The efficacy of microbial control is affected, as are other forms of control to a certain extent, by a multiplicity of factors. Prominent among these are the habitat in which the control is to be attempted and the degree of control required, the age and the habits of the target, as well as the weather. In the field the factor most likely to inactivate a microbial insecticide is sunlight. Consequently sunlight protectants such as dyes, carbon black or titanium dioxide are added (Stockdale, 1985).

The application of *B. thuringiensis* is continuously extended as growers are educated to the unique properties of this bacterium. Also with the advent of new strains, particularly those which destroy insects affecting man and animals, entirely new areas of growth and penetration are expected (Couch and Ross, 1980).

2.11 Safety

The application of entomopathogens in biological control is not new. According to Martignoni (1964) the production of these pathogens started soon after 1880 and continued over the years. Obviously if a product is intended for use on food and forage crops or is to be used freely in the forests, it is hji-

Table 4: Compatibility between chemical pesticides and *Bacillus thuringiensis*

Pesticide	Pathogen	Reference	Compatibility
Insecticides			
DDT			
Dieldrin	Bt	Heimpel (18967)	Field
Malathion	Bt	Commercial literature	Rec
Methyl trithion	Bt	Commercial literature	Rec
Parathion	Bt	Commercial literature	Rec
Demeton	Bt	Commercial literature	Rec
Fungicides			
Daconit	Bt	Commercial literature	Rec
Difolatan	Bt	Commercial literature	Rec
Dichlone	Bt	Heimpel (1967)	Field
Dimethoate	Bt	Commercial literature	Rec
Thiocarbamates	Bt	Heimpel *et al.* (1967)	Field
Acaricides			
Aramite	Bt	Commercial literature	Rec
Difocol	Bt	Heimpel (1967)	Field
Tetradifon	Bt	Commerical literature	Rec
Miscellaneous			
Maleic hydrazide	Bt	Commercial literature	Rec

Field: Good results obtained in field trials implying that, under the conditions stated, the additive did not significantly reduce effectiveness of *Bacillus thiringiensis*

Rec: Use of additive noted or recommended without supporting evidence about effect on pathogen; presumably harmless.

(Source: Angus and Luthy, 1971)

essential that its use does not generate a hazard. In 1960, United States Food and Drug Administration granted full exemption from tolerence for *B. thuringiensis* on food and forage crops (Bulla *et al.*, 1979). This implies that there is no specific residue limit on the crops for which the product is registered for use. It can be used up to the day of harvest if the user so desires. The information and data sheets issued by manufacturers of *B. thuringiensis* indicate that no special precautions need to be taken when using such products; it is suggested that unnecessary contact with spray materials (whatever their nature) should be avoided (Angus, 1971).

Occasionally, it has been suggested that there may be a danger of mutation or genetic exchange through transduction or transformation that would allow the development of human pathogenicity to *B. thuringiensis*. This fear is usually associated with the fact that *B. thuringiensis* has some similarities to the human and animal pathogen, *B. anthracis* (Somerville and Jones, 1972). However, there has been no evidence of acute or chronic toxicity in rats, guinea pigs, dogs, mules, swine, or other mammalian test animals (Couch and Ross, 1980). *Bacillus thuringiensis* as a commercial formulation is self-limiting in the environment. This is due to its failure to compete effectively with other soil bacteria, adverse growing conditions on the surface of plants, and its susceptibility to weathering and solar radiation.

The precaution necessary in applying chemicals relates directly to residual problems and this points to one of the desirable attributes of bacterial insecticides which are highly specific to many noxious insects but are not harmful to their parasites (Heimpel and Angus, 1960). However, the

the chemicals. However, broad field evaluation of resistance to entomopathogenic microorganisms will have to await wide-scale testing and the commercial utilization of insect pathogens.

2.13 Application of *Bacillus thuringiensis*

The main advantages of *B. thuringiensis* as a biological control agent is that it has a broad spectrum of activity against larvae of lepidoptera (about 300 in all) and this order includes some of the most serious crop pests (Heimpel and Angus, 1960; Deacon, 1983). A broad spectrum of activity is desirable for the commercial producer of a pesticide because it represents a large potential market. *Bacillus thuringiensis* has no effect on vertebrates, including man (Couch and Ross, 1980; Heimpel, 1971; Hall, 1964), and it has a generally negligible effect on insects that form part of the natural enemy of pests (Deacon, 1983). The ease of production enables the product to be competitive in price with conventional chemical pesticides (Hall, 1964; Deacon, 1983). Another obvious advantage is the apparent delay whereby a susceptible host develops resistance (Hall, 1964; Burges, 1961).

The spores and to a lesser extend, the crystals are inactivated by prolonged exposure to ultraviolet radiation (Stockdale, 1985). The spores and crystals remain on the plant surface and therefore are effective only against surface-feeding insects or the surface-feeding stages of boring and burrowing insects. They also show a general lack of persistance in the environment, at least at levels that can achieve lasting control of a pest problem (Deacon, 1983).

2.14 Recent developments in the application of *Bacillus thuringiensis*

Current research into biopesticides aims to lower production costs and improve pest control efficacy. Moreover, the target range of biopesticides is being broadened. The host range for *B. thuringiensis* products is broadened by introducing genes coding for several different toxins into one strain. Whereas, the persistance of the toxins is improved by transferring the toxic genes to other microbes, such as *Pseudomonas fluorescens* (Commandeur and Komen, 1992). Several firms are exploring the possibility of propagating genetically engineered plants carrying the toxins of *B. thuringiensis* (transgenic plants). Hofte and Whiteley (1989) reported that transgenic tobacco plants tested in field trials were fully protected against damage by *Monduca sexta* and *Heliothis virescens*. Similar approaches are now being used for other commercial crops with important lepidopteran or coleopteran pests and for which a transformation system is available.

Research on the genetics and molecular biology of *B. thuringiensis* during the past 10-12 years has led to a reasonably clear understanding of the genetic organization and diversity of the insecticidal crystal protein genes responsible for the bioactivity of *B. thuringiensis* based products, recombinant DNA technology has been utilized to transfer the *B. thuringiensis* delta endotoxin gene to microbes and plants (Gelernter, 1992). Current opportunities for enhancing insect resistance include insertion of the toxin gene from the bacterium *B. thuringiensis* and transfer proteinase inhibitor genes from other plant species. Work is under way in a number of laboratories throughout the world to insert *B. thuringiensis* toxin genes into forest tree species (Strauss *et al.*, 1991).

Another area of interest to researchers is the mass prodution of *B. thuringiensis* endotoxin by fermentation using agrowaste as substrate. Lee and Seleena (1991) reported that grated coconut waste, fishmeal and rice bran could be used as substrate for producing *B. thuringiensis* endotoxin. Desai and Shetna (1991) reported similar findings by using defatted groundnut (*Arachis hypogaea*) cake as the first nitrogen source and gram flour (*Cicar arietinum*), soybean (*Glycine max*) or defatted milk powder as the second nitrogen source.