REVIEW

Bacillus thuringiensis Growth and Toxicity

Basic and Applied Considerations

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Abstract

Despite the known importance of the composition of culture media and culture conditions on *Bacillus thuringiensis* growth and toxicity, very few reviews are concerned with this subject. This article reviews some aspects of the microbiology of *Bacillus thuringiensis*, and how toxicity is affected by the composition of growth media and bioreactor operation.

Index Entries: *Bacillus thuringiensis*; toxic proteins; medium composition.

1. Introduction

Since its discovery by Ishiwata in 1902, *Bacillus thuringiensis* (BT) has raised the attention of investigators researching in many different areas of microbiology, entomology, and biochemistry, because of its ability to synthesize a toxic protein, specific against several insects. Early studies were focused on the control of some agricultural pests and field applications, with variable success. Soon after the suggestion that the parasporal crystal was the toxic agent *(1,2)*, studies on the purification and characterization of the toxic component were performed *(3)*. This led to several groups working on the biochemistry and characterization of the parasporal crystal *(4,5)*, together with research on the physiology and biochemistry of BT *(6–8)*. In the last 10 years developments in molecular biology and genetic engineering techniques have led to an increased understanding of the molecular biology of crystal formation *(9)*. Simultaneously, exhaustive studies have been made regarding the mode of action of BT δ-endotoxin *(10–12)*.

Obviously, a great number of reviews and books dealing with different aspects of BT have been published. Among them, it is worthwhile to mention the reviews by Rogoff and Yousten *(13)* and Bulla et al. *(14)*, related to general aspects of BT; Lüthy et al. *(15)*, with a complete analysis of BT metabolism; Aronson et al. *(16)*, dealing with BT and other insect pathogens; Rowe and Margaritis *(17)*, covering almost all subjects related to biotechnology of BT (including economic aspects); Whiteley and Schnepf *(9)* and Höfte and Whiteley *(18)*, two excellent reviews on the molecular biology of BT crystal toxin; Priest *(19)*, a short review on mosquito control; and Gill et al. *(20)*, a thorough analysis of BT toxin mode of action. At least three books have been published, dealing with different aspects of BT *(21–23)*. However, despite the known importance of the composition of culture media and culture conditions on BT growth and toxicity, very few reviews are concerned with these subjects *(24,25)*. The purpose of this work is to review some aspects of the microbiology of BT, and how toxicity is affected by the composition of growth media and bioreactor operation.

2. Biology of *Bacillus thuringiensis*

2.1. Microbiology and Biochemistry

BT is a large Gram-positive, spore-forming bacteria that produces, concomitantly with sporu-

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lation, a proteinaceous parasporal crystal known as δ-endotoxin.

2.1.1. Carbon Metabolism

This microbe is a chemoheterotroph, and aerobically oxidizes carbohydrates to organic acids, which are further oxidized to carbon dioxide. During vegetative growth, sugars are mainly metabolized through the Embden-Meyerhof-Parnas pathway yielding pyruvic and acetic acids, which are further oxidized via the Tricarboxylic Acids cycle enzymes *(15)*. These enzymes are fully derepressed when sporulation begins. As BT shows no α-ketoglutarate dehydrogenase activity, it has been concluded that an incomplete TCA cycle is present in this microorganism *(7,26)*. The latter demonstrated the presence of the γ-aminobutyric acid pathway, which transforms glutamate to succinate, thus circumventing the α-ketoglutarate dehydrogenase step.

The rate of catabolism of pyruvic and acetic acids is lower if amino acids are present in the medium *(27)* because these compounds may act as carbon and nitrogen sources. In the case of glutamate, it is converted to γ-aminobutyric acid, which is metabolized via succinate.

Another important feature of BT is the strong exoprotease production observed when the acids begin to be metabolized. These exoproteases are different from those produced during vegetative growth. They are neither repressed by ammonia nor stimulated by glutamate *(28)*. In addition, they have different substrate specificities *(29)*. The appearance of exoproteases is an early event recognized to be associated with the initiation of sporulation.

2.1.2. Nitrogen Metabolism

Because of the synthesis of both sporulation specific enzymes and crystal proteins, BT must mobilize, during the sporulation phase, most of the nitrogen assimilated during vegetative growth. More than 50% of the amino acids that are present in the pool of free amino acids are alanine and glutamate. The transfer of nitrogen from glutamate to other metabolites is catalyzed mainly by glutamate oxaloacetate transaminase and, to a lesser extent, by glutamate pyruvate transaminase and glutamate dehydrogenase. The pathways for ammonia assimilation are catabolized by alanine dehydrogenase and glutamate dehydrogenase *(26,30)*. Although glutamate synthase and glutamine synthetase have been found in *Bacillus megaterium (31,32), Bacillus licheniformis (33),* and *Bacillus subtilis (34),* as far as the authors are aware, no report about the presence of this system in BT has been made. Meers et al. *(35)* found that the expression of these enzymes was very high for *B. megaterium* (among other bacteria) when the growth was ammonium limited. As the majority of the work on BT has been performed in batch cultures (i.e., excess of all nutrients), it should be very interesting to discover whether these enzymes are present when BT is cultivated under NH_4+ limitation.

2.2. Genetics

2.2.1. Toxin Genes of Bacillus thuringiensis

It has been demonstrated that crystal protein genes of BT are present on one or more plasmids *(36,37)*. Studies related to gene expression have demonstrated that these genes are transcribed by a sporulation-specific RNA polymerase *(38),* and that the control of gene expression is exerted at the transcriptional level *(39)*.

The genes encoding for crystal proteins have been classified by Höfte and Whiteley *(18)* into four classes and several subclasses (Table 1). The major gene classes are *Cry*I, *Cry*II, *Cry*III, *Cry*IV, and *Cyt*A. All genes belonging to class I encode for polypeptides with molecular weights of ca 130 kDa, which are specific for Lepidoptera larvae. Genes from Lepidoptera- and Diptera-specific *Cry*II group and from *Cry*III group (Coleoptera-specific) encode for polypeptides of about 70 kDa. The genes from group IV (present in BT var *israelensis*, Diptera-specific) are quite different from the others: *Cry*IVA encodes for a 134-kDa protein; *Cry*IVB, for a protein of 128 kDa; *Cry*IVC encodes for a protein of 78 kDa; and the product of gene *Cry*IVD is a polypeptide of 72 kDa. BT var *israelensis* presents also a Cyt gene, which codifies for a 27-kDa polypeptide, with activity against invertebrate and vertebrate cells. A closely related gene is found in BT var *morri-*

Table 1
Classification of Crystal Protein Genes Present in Different Varieties of *Bacillus thuringiensis*

Class	Subclass	MW, kDa	Specificity toward	Variety	Reference
*Cry*I	IA(a)	130–140	Lepidoptera	*kurstaki HD1, sotto, aizawai*	*41*
	IA(b)			*berliner, aizawai, kurstaki HD1*	*42*
	IA(c)			*kurstaki HD73*	*43*
	IB			*thuringiensis HD2*	*44*
	IC			*entomocidus, aizawai*	*45*
	ID			*aizawai*	*18*
	IE				
*Cry*II	IIA	63	Lepidoptera diptera	*kurstaki* HD263	*46*
	IIB			*kurstaki* HD1	*47*
	IIC	61			*48*
*Cry*III	IIIA	72	Coleoptera	*san diego, tenebrionis*	
	IIIB			*tolworthi*	*50*
*Cry*IV	IVA	135	Diptera	*israelensis*	*51*
	IVB	128		*israelensis*	*52*
	IVC	78		*israelensis*	*53*
	IVD	72		*israelensis*	*46*
*Cry*V	VA(a)	?	Nematodes	?	*40*
	VA(b)				
	VB				
	VC				
*Cry*VI	VIA	?	Nematodes	?	*40*
	VIB				
Cyt	*Cyt*A	27	Diptera/cytolytic	*israelensis, morrisoni*	54

[a]Table adapted from Höfte and Whiteley *(18)*.

soni. Recently two new Cry genes, *Cry*V and *Cry*VI, have been reported *(40)*.

At least 20 *Cry*I sequences have been reported, from which six different genes were classified. All of them encode for proteins of molecular masses from 130–140 kDa, which form characteristic bipyramidal-shaped inclusions within the sporangium. These proteins are actually protoxins that are proteolytically activated in the larval midgut *(55,56)*.

Genes belonging to *Cry*II group encode for 65-kDa proteins, present in 15 strains of BT vars *kurstaki*, *thuringiensis*, *tolworthii*, and *kenyae* *(57,58)*. There are two genes, *Cry*IIA and *Cry*IIB, that present a very high (87%) amino acid homology *(47)*. However, they differ in their insecticidal activity: *Cry*IIA is Lepidoptera- and Diptera-specific, whereas *Cry*IIB is only Lepidoptera specific.

Genes belonging to the *Cry*III group (*Cry*IIIA and *Cry*IIIB) encode for proteins with activity against Coleoptera. There are three BT varieties possessing gene IIIA: *tenebrionis (59)*, *san diego (49)*, and EG2158 *(46)*. The crystal protein gene is the same in all the three varieties, and encodes for a 72-kDa polypeptide, which is proteolytically converted into a 66-kDa polypeptide. Crystal gene

*Cry*IIIB has been reported to be present in var *tolworthi (50).*

BT var *israelensis* genes belong to classes *Cry*IV and *Cyt*A. There are four different *Cry*IV genes: *Cry*IVA, *Cry*IVB, *Cry*IVC, and *Cry*IVD, encoding for proteins with predicted molecular weights of 135, 127, 78, and 72 kDa, respectively, that are assembled in the crystal complex together with the 27-kDa protein encoded by *Cyt*A gene. Studies on the products of the cloned genes have proved that the single components are not as toxic as the whole crystal; this is explained by a possible synergism among the components of BT var *israelensis* crystals *(60–62).*

The structure of *Cry*IVA and *Cry*IVB genes is very similar to the structure of *Cry*I genes, and their product (a 135-kDa protein) is proteolytically converted into a smaller fragment of about 70 kDa *(52,53). Cry*IVC, encoding for a 78-kDa polypeptide, presents a region specifying the active toxin that is highly divergent to the regions of *Cry*IVA and *Cry*IVB. *Cry*IVD encodes for a major component of BT var *israelensis* crystal, with a predicted mol wt of 72 kDa, which is converted into an active fragment of ca 30 kDa *(63). Cyt*A, the other gene present in BT var *israelensis*, has no homology with Cry genes, and its product (a 27-kDa protein) presents different properties: it is cytolytic for several insect and mammalian cells, neurotoxic, and hemolytic *(64–66).* Despite these unique features, insecticidal activity of *cyt*A in vivo remains unclear, and it has been demonstrated recently that this polypeptide is not essential for mosquiticidal activity *(67).*

The *Cry*V and *Cry*VI genes recently reported encode for nematode-active toxins *(40).* These proteins are not so closely related to the proteins encoded by the other genes, and, although these authors divided the new classes into several subclasses, further research seems to be necessary for their complete characterization.

2.2.2. Cloning of the Crystal Protein Genes

The first report on the molecular cloning of a crystal protein gene was made in 1981 *(68),* and it was followed by the cloning of the crystal protein gene(s) of different varieties of BT *(69–72).* Those experiments allowed the study of the molecular biology of BT parasporal crystal formation, reviewed by Whiteley and Schnepf *(9).* Several authors have proposed that all genes probably present a common evolutionary origin.

One of the goals of genetic analysis of toxin genes is the identification of the regions involved in toxicity and specificity. To date, DNA sequences of toxin genes of several BT varieties have been reported *(9,54,58,73).* From these, together with deletion analysis and amino acid sequencing, it has been established that the toxic domain is located in the *N*-terminal half of the protoxin. The C-terminal half is highly conserved for *Cry*I genes, suggesting a structural function for crystal formation *(20)* rather than for toxicity or specificity.

As was mentioned by Spear *(74),* genetic engineering of bacterial insecticides can have three different aims: first, to enhance the yield or potency of bacterial toxins; second, to change the host organism that carries or expresses the genes; and third, to change the spectrum of toxicity in order to alter or increase the insecticidal specificity.

Changes in yield of bacterial toxins seem to be difficult to achieve; more than 30% of the total protein of the sporangium corresponds to δ-endotoxin, so it is unlikely to be increased. An alternative to the increase in δ-endotoxin yields may be the reduction in the fermentation cycle of BT (that is, the same amount of toxin in less fermentation time, or, in other words, higher δ-endotoxin productivity). This approach is supported by the results of Shivakumar et al. *(73),* which demonstrated that a δ-endotoxin gene from BT can direct the synthesis of crystals in *Bacillus subtilis* without a requirement for a stationary phase of growth, and also by the results of Mettus and Macaluso *(75),* which reported the expression of BT var *kurstaki* toxin genes during vegetative growth of a recombinant strain of *Bacillus megaterium.* Further work employing this approach may lead to the successful expression of δ-endotoxin genes during vegetative growth, which would permit the use of continuous fermentation processes for BT δ-endotoxin production.

The change of the host organism carrying the protein gene has been attempted by different

groups; apart from *Escherichia coli* and *B. subtilis*, toxin genes of BT have been cloned in *Pseudomonas fluorescens (40),* in the water occurring bacteria of the genus *Caulobacter (76),* and in various species of cyanobacteria *(77),* with variable success. One of the recombinant products has received approbation by the Environmental Protection Agency (EPA), to be employed in the United States *(40).* However, some questions remain unanswered regarding the advantages of the use of recombinant bacteria to be released into the environment. Potency, persistence, and plasmid stability should be taken into account, as well as potential pathogenicity of the recombinant microorganism, so careful studies must be carried out before the release of microorganisms carrying BT toxin genes. The lack of information about the employment of engineered microorganisms implies a drawback that reinforces arguments against its use *(78).*

The third approach is the improvement of bacterial insecticides through protein engineering. Studies on toxin structures involved in specificity and toxicity must be accompanied by research on insect receptors. Single changes in the primary structure of δ-endotoxin severely affect the toxicity and the expression of the toxin *(51,79).*

2.3. The Toxin

2.3.1. Structure and Biochemistry

As has already been mentioned, the special feature of BT is the toxin synthesized during sporulation. Since it was first described by Angus *(2),* innumerable reports on δ-endotoxin biochemistry and structure have been made. BT synthesizes at least seven different toxins, from which only δ-endotoxin and β-exotoxin *(thuringiensin)* present toxicity against insects. β-Exotoxin is known to be teratogenic and mutagenic, so its use has been banned in several countries. The US EPA requires BT-based products to be free of the β-exotoxin *(80).*

There is a high amino acid homology between *Cry*I proteins. Three of them (*Cry*IA[a], *Cry*IA[b], and *Cry*IA[c]) share more than 80% of the amino acid sequence. The other *Cry*I proteins do not present such high similarities between them. It is interesting to note that the C-terminal moiety is highly conserved for all *Cry*I proteins, and that almost all cysteine residues are located in this part of the toxin *(81).*

Special attention must be paid to the composition of BT crystal proteins. As has been mentioned, changes in the primary structure dramatically affect not only the expression of the toxin but its toxicity as well *(51).* Several reports on the biochemistry of crystal proteins of BT have been published *(55,82–85).* From these, a series of biochemical structural features can be extracted: Parasporal crystals from different varieties of BT present very similar amino acid composition, except for BT var *israelensis*, which shows significant differences in aspartate, cysteine, leucine and phenylalanine contents.

Early studies demonstrated that BT var *kurstaki* parasporal crystal presents variable amounts of carbohydrates (mainly glucose and mannose), ranging from 0.1–12% in the different reports *(83,84,86)* and it was suggested that those carbohydrates were not covalently bound to the proteins.

Studies on BT var *israelensis* parasporal crystal also diverge; whereas some authors reported a carbohydrate content of 6% *(87),* others found an undefined amount of different hexoses (glucose, mannose, fucose, rhamnose, xylose, galactosamine) *(55).* More recently *(85),* it could be demonstrated that BT var *israelensis* crystal proteins present covalently bounded carbohydrates, including neutral sugars, hexosamines, and *N*-acetylated hexosamines. In an excellent report *(88),* it was recently demonstrated that sugars covalently attached to BT HD-1 protein crystals are the product of nonenzymatic glycosylation, thus explaining the variable results found in the literature. The presence of amino sugars in the BT var *israelensis* protein crystals suggests that a lectinlike receptor could be present in the larval mosquito gut *(89).* The recognition of the amino sugars portion by the specific receptor would be responsible for the high specificity of BT var *israelensis* δ-endotoxin toward Diptera, but not toward other insects or animals.

The covalent modification of lysine side chains (guanidination, carbamylation, dansylation, or succinylation) leads to a loss of toxicity that

reaches, in some cases, more than 50% of the original toxicity *(90)*. This can be related to the proteolytic activation of the δ-endotoxin inside the larval midgut *(see the following)*.

Another important feature of the BT protein crystal lies on the presence of disulfide bonds. These are chiefly of importance in the stabilization of tertiary structure, and they play a main role in the solubility of the crystal proteins *(16)*. In special, the solubilization of BT var *israelensis* *Cry*IVA and *Cry*IVB proteins requires disulfide reductive conditions *(81)*, and the 27-kDa protein *Cyt*A is found as an oligomer (trimer or tetramer) because of the existence of disulfide bonds. As has been mentioned, the amino acid composition of different BT crystals shows an average cysteine composition of 1.6% (1.1–1.8%), except for BT var *israelensis* where the percentage of cysteine is 2.1% *(55)*. This fact must be considered when studying stability of BT crystals in alkaline media; BT var *thuringiensis* presents crystals with cysteine groups exclusively as disulfide bonds *(91)*, whereas analysis of BT var *israelensis* crystals shows a rather complex crystal structure *(81,90)*, in which disulfide bonds seem to show no participation in toxicity, in contrast with other BT protein crystals *(92)*.

These structural features allow the use of solubility as a tool for toxin separation. At alkaline pH (9.0–9.5), *Cry*I toxins are solubilized, while *Cry*II toxins remain insoluble unless the pH is raised to 12. *Cry*IVA, *Cry*IVB, and *Cyt*A are soluble at pH 9.5, but *Cry*IVD is solubilized at pHs of about 12. *Cry*IA(c) is soluble at pH values higher than 9.5 or lower than 3.5 *(81,93,94)*.

Studies on the three-dimensional structure of the *Cry*IA(c) protein show that it consists of a 20% α-helix, 35% β-pleated sheet, and 45% unordered *(95,96)*. A study at 2.5 Å resolution *(97)* shows that the δ-endotoxin of BT var *tenebrionis* presents three domains, which may explain, at least partially, the basis for insecticidal function. According to Gill et al. *(20)*, the α-helix domain is probably crucial for the toxicity, whereas the β-sheet domains are related to the toxin specificity toward the target insect.

2.3.2. Mode of Action

An important point is the study of how δ-endotoxin interacts with insect cell membranes. The mode of action greatly depends on toxin structure and also on the existence of δ-endotoxin specific receptors.

Primary structure varies with the target insect (Lepidoptera, Diptera, Coleoptera), and a certain degree of amino acid homology among different toxins has been found. Tertiary structure also varies with the BT variety producing the δ-endotoxin. For instance, BT var *kurstaki* inclusion body is composed of three *Cry*I and two *Cry*II polypeptides *(18)*, which are held together by means of different kinds of chemical and physicochemical interactions, such as hydrogen bonds, disulfide bonds, and hydrophobic interactions *(98)*. However, parasporal bodies of var *israelensis* present only structural disulfide bonds *(90)*.

The 130-kDa proteins (which are actually protoxins) require processing to become active. This activation is achieved by alkaline pH and proteases, which are present in the larval midgut and play an important role on both solubilization and toxicity *(94)*. The products (active toxins) have mol wt of about 60–70 kDa, which are protease resistant and derive from the *N*-terminal half of the unprocessed protein. This model accounts for *Cry*IA(c) protein, and may be generalized for other 130-kDa proteins. Size and stability of the protease resistant core vary with BT variety, and for var *israelensis* the less stable core is found *(16,63,99)*.

The action of Cry toxins occurs primarily in the midgut epithelium; these toxins require a specific receptor, which is believed to be a glycosylated protein *(11,89,100)*. Probably, Cry toxins bind to the receptor via a membrane binding domain. A conformational change allows the toxin to be inserted into the cell membrane. Once within the membrane, toxin molecules may oligomerize, thus forming a pore that provokes an osmotic imbalance in the epithelial cells *(20)*. The reader is referred to the latter for a complete interpretation of BT toxin mode of action.

*Cyt*A toxins of the mosquiticidal BT var *israelensis* show a different mode of action: Ini-

tially, *Cyt*A binds to unsaturated phospholipids *(10,101)*, and aggregates of about 300–400 kDa are formed, leading to pore formation and cytolysis *(102)*.

3. Effects of Medium Composition and Culture Conditions on the Toxicity of *Bacillus thuringiensis*

There are diverse reports regarding variations in toxicity as influenced by culture conditions and media composition. Good growth and sporulation do not always imply high toxicity.

Dulmage *(103)* was among the first authors to correlate medium composition with toxicity. In his paper, Dulmage stated principles that are sometimes forgotten:

1. It is impossible to predict activity from spore count;
2. The insecticidal activity varies with the strain and with culture conditions; and
3. It is necessary to specify the variant used and the conditions of δ-endotoxin production when reporting toxicity of cultures of BT.

3.1. Medium Composition

The nutritional requirements of different varieties of BT are known to be variable *(25)*. A good culture medium for one variety may be useless for another one, which implies the impossibility of defining a specific fermentation medium for BT. Early studies recognized the difficulty of obtaining a chemically defined medium for BT cultures *(104,105)*. However, Nickerson and Bulla *(106)* reported a defined medium, composed of glucose and salts, and supplemented by either aspartate, glutamate, or citrate, in which growth, sporulation, and crystal formation were observed for 18 strains of BT. Singer and Rogoff *(6)* and Rajalakshmi and Shethna *(107)* found that some amino acids do not support growth of BT var *thuringiensis*, and that cysteine alone was enough to support growth and crystal formation *(107)*, but concentrations higher than 0.25% totally inhibited spore and crystal formation *(108)*. These results could not be extended to BT var *israelensis*. Experiments performed in minimal media supplemented with diverse amino acids and vitamins showed low or no growth at all (Avignone-Rossa and Mignone, unpublished results). Moreover, it was found that δ-endotoxin protein production was enhanced when yeast extract-containing media were supplemented with ammonium sulfate *(109)*. It is evident that complex media give the microbe all the nutrients needed for growth, sporulation, and toxin production, and that the presence of amino acids is not always enough to obtain good yields.

Media for industrial production are based on complex carbon and nitrogen sources *(110)*. Carbon sources have the most variable origins: starches, molasses, dextrose, flours, and glycerol, among others, have been reported. However, best results are achieved when simple carbon sources are employed. Carbohydrate concentration influences the production of δ-endotoxin, and attention must be paid in this respect. Scherrer et al. *(111)* found that crystal size, protein content, and toxicity varied with glucose concentration, being the highest at concentrations of about 6–8 g/L glucose, which they determined to be the optimal concentration. Pearson and Ward *(112,113)* reported results for BT var *israelensis* cultures with different carbon and nitrogen sources, from which a range varying from 3400–5800 ITU per 10^8 spores could be derived. Glucose, molasses, and starch were tested alone or in combinations, and no media showed differences from the range mentioned. Results obtained with BT var *galleriae* cultured at high substrate concentrations show that glucose concentrations higher than 50 g/L result in great decreases of sporulation and cell count, whereas a high yeast extract concentration (20 g/L) combined with glucose concentrations from 30–100 g/L suppressed spore formation *(114)*. Unpublished results from Mignone and Avignone-Rossa show a similar trend for BT var *israelensis*. Lower values of μ_{max}, deformation of cells, and filamentous growth of the bacilli, could be observed. Crystal formation and lysis were suppressed.

With regard to the nitrogen source, Mummigatti and Raghunathan *(115)*, working with BT var *thuringiensis*, observed that the specific toxicity of the product obtained with sugar cane

molasses as the carbon source was 2.5 times higher than that obtained with soluble starch, when greengram (*Vigna radiata*) powder was used as the nitrogen source. However, high toxicity was achieved, irrespective of the carbon source, when the nitrogen source was soybean powder. These results are probably masked by the several combinations of carbon and nitrogen sources that were employed. The same problem could be inferred from other reports in which very complex media are used for BT cultures; most of them were composed of agroindustrial byproducts *(116,117)* or inexpensive locally available ingredients *(118,119)*.

Reports on the effect of nutrient limitations on toxicity are scarce. Sakharova et al. *(120)* studied the effect of different nutrient limitations on batch growth of BT var *galleriae* in a semisynthetic medium. The higher crystal titer was obtained in a phosphate limited medium, but, on a per crystal basis, the toxicity was independent of the kind of limitation. This kind of approach should be useful for elucidating how different nutrient limitations affect toxicity.

Another interesting approach for medium design is that of Goldberg et al. *(121)*, which employed the continuous culture system combined with the pulse and shift technique to improve growth and toxin production of BT var *berliner*. The optimized medium yielded 400 times more spores than the original one, whereas the toxicity on a per spore basis remained the same.

In BT var *israelensis* cultures, it could be observed that the replacement of glucose by glycerol yielded higher toxin concentrations *(122, 123)*. Similar observations were not reported for other varieties of BT. As pointed out by Battacharya et al. *(88)*, nonenzymatic glycosylation is likely responsible for sugars detected in BT crystals. So, it is probable that protein glycosylation is mainly affected by fermentation conditions. This fact would explain differences in results reported by different laboratories; several factors must be taken into account, such as differences in growth medium, residual nutrient levels, pH, and so on. This nonenzymatic glycosylation may now explain some features of BT fermentation processes, such as batch-to-batch variations, or product variations despite equal starting material. These authors also proposed an explanation as to how changes in toxicity occur. Based on the fact that glycosylation occurs preferentially on lysine side chains at alkaline pHs, they proposed three possible mechanisms:

1. Glycosylation inside the alkaline insect midguts, depending on the availability of sugars;
2. Protoxin activation by trypsin-like proteases, which can be altered by glycosylation of lysine or arginine residues of the toxin; and
3. Lysine residues could be necessary for toxicity themselves, and glycosylation can dramatically alter this toxicity.

As was mentioned by Pfannenstiel et al. *(90)*, covalent modification of lysine side chains led to a loss of toxicity. Obviously, further research is needed to find an explanation for this phenomena.

The presence of diverse ions in the culture media is necessary to obtain high toxicity and abundant growth. It has been demonstrated that potassium stimulates δ-endotoxin production *(124)*, and replacement by sodium dramatically affects δ-endotoxin production *(125,126)*. Arcas et al. *(127)* indicate that the addition of Ca, Mn, and Mg to yeast extract containing media substantially increases yields, suggesting that the concentrations of those ions found in that complex component are not enough to obtain high yields.

Another important point to be considered in relation to growth and δ-endotoxin production is oxygen supply. BT is aerobic, and oxygen supply is known to be an important constraint for BT cultures. As is known, the oxygen consumption rate (rO_2) is related to growth rate by the equation

$$rO_2 = \mu . X / Y_{x/o}$$

where μ is the specific growth rate, X is the biomass concentration, and $Y_{x/o}$ is the cell yield based on oxygen (grams of produced biomass per gram of consumed oxygen). According to this equation, the higher the growth rate, the higher the oxygen demand. Thus, in order to avoid oxygen limitation, the oxygen supply, or, more correctly, the oxygen transfer rate, at least must be equal to the maximum oxygen demand. Shake-flasks (250

rpm, air to medium ratio: 10:1) have a maximum transfer rate value of about 35 mmol O_2/L/h *(128)*. Values of μ reported for BT vary from 1.5/h, for var *galleriae (129)*, to 0.7/h for vars *kurstaki* and *israelensis (130,131)*, and cell yields based on oxygen can be calculated to be of about 2 g/g *(132)*. So, taking conservative values for those parameters (that is to assume a cell concentration of about 3 g/L, growing at μ = 0.5/h, and presenting a yield value $Y_{x/o}$ = 2 g/g), the value of rO_2 will be equal to 28 mmol O_2/L/h. As cell yields based on carbohydrates are of about 0.5–0.6 g/g, depending on the nitrogen source, carbohydrate concentration in shake-flask cultures should not exceed 6 g/L, approximately, in order to avoid oxygen limitation. Therefore, results obtained in these cultures should be analyzed carefully.

Not only growth is affected by oxygen supply. Toxin synthesis, as well as sporulation, is known to be highly dependent on oxygen. Dulmage et al. *(25)* showed that higher toxicity is found in culture systems presenting an efficient aeration. Foda et al. *(124)* studied the effect of different aeration rates in shake-flask cultures of BT var *entomocidus*, varying the air-to-medium ratio from 9:1–19:1. Viable count and sporulation titers were higher and the toxin potency showed a sixfold increase under the 19:1 (air to medium ratio) conditions.

In a patent issued to Zamola and Kajfez in 1977 *(25)*, interrupted aeration is reported to be an efficient method for BT production. Periods of 12–14 h of aerated fermentation are followed by 3–6 h periods without aeration in which lysis is prevented. Further aeration is allowed, and sporulation begins. Nonaerated phase suppresses sporulation and lysis, but cell growth continues. Toxin yields are claimed to be enhanced. However, results obtained in our laboratory demonstrate that, at least for BT var *israelensis*, spore count and δ-endotoxin production are lower when cultures are under oxygen limitation, and interruption of aeration in nonlimited cultures gives δ-endotoxin yields similar to those of oxygen limited cultures, although spore counts are similar to those found in nonlimited cultures *(131)*.

More recently, Bravo et al. *(133)* reported high toxin production by a mutant strain of BT var *sotto*, presenting higher respiratory capacity than the wild-type strain. In this mutant, cytochrome aa_3 expression is not repressed during the sporulation phase, which leads to enhanced oxygen consumption. According to the authors, this fact would be responsible for the higher toxin production.

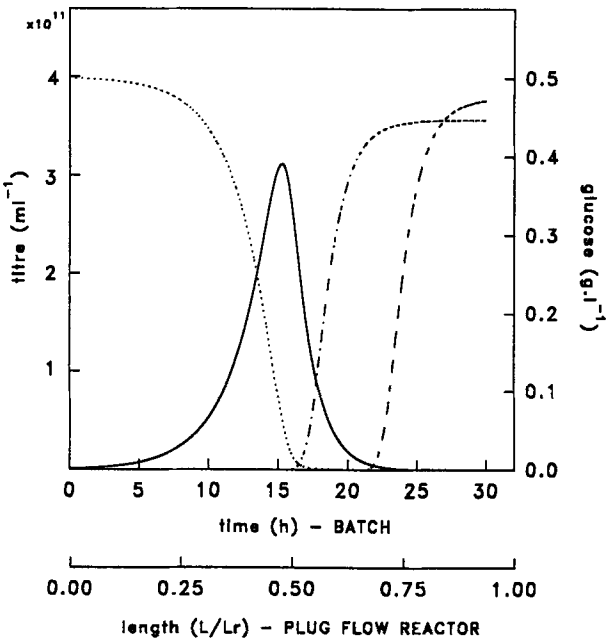

Fig. 1. Batch culture of BT var. *galleriae* (adapted from ref. *139*). —, vegetative growth; ·····, glucose consumption; —·—·, δ-endotoxin formation; – —, sporulation; Lr, length of the tubular reactor (*see text* for explanation).

Oxygen effects on sporulation and toxicity are far from being completely understood, and systematic research seems necessary.

3.2. Bioreactor Configurations for BT Production

Batch cultures are usually employed for BT production. The events that can be differentiated during a typical batch culture (cell growth, sporulation, and glucose consumption) are schematically shown in Fig. 1. Batch growth of BT can be regarded as a two-stage process. In the first one, vegetative growth takes place and nutrients are consumed, whereas in the second stage accumulation of cells carrying spores and crystals can be observed.

During vegetative growth, the carbon source is oxidized, and, owing to organic acids accumulation (*see* Section 2.1.1.), pH drops dramatically. This can be deleterious for both growth and δ-endotoxin production. In order to avoid this effect, media must be buffered, or pH controlled, especially those with high carbon source contents *(124)*. Values of maximum specific growth rate (μ_{max}) have been reported to be of about 1.3/h for BT var *galleriae* and var *thuringiensis (129,134)*, 0.83/h for var *berliner (121)*, and 0.7/h for var *kurstaki* and var *israelensis (127,131)*.

Once the sporulation is initiated, the cell is commited to complete the sequence of events leading to maturation of a spore. The time required for this process is known as maturation time, t_M *(135)*. At the end of the process, cellular lysis is complete and spore–crystal complexes are released into the medium. Mature spores account for at least 15% of the vegetative cell mass, whereas crystals represent 17–20% of the same *(75,136)*. During spore and crystal formation, amino acids derived from proteolysis are primarily metabolized, and some evidence of the incorporation of amino acids from the medium to crystal protein has been reported *(137)*. The reader is referred to the reviews of Lüthy et al. *(15)* and Rowe and Margaritis *(17)* for a detailed description of the biochemical events occurring in batch cultures.

Obviously, this growth pattern is not found in other culture systems. Freiman and Chupin *(138)* reported results from one- and two-stages continuous cultures of BT var *galleriae*. In one-stage cultures, with dilution rates from 0.16–0.71/h, only with the lower dilution rate spores and crystals are obtained, although in a low concentration (3% of the total population). These results are not surprising, because the fraction of spores that remains in a continuous culture and become mature is equal to $exp(-D.t_M)$, D being the dilution rate *(135)*. Thus, the lower the dilution rate, the higher the spore titer within the reactor. Analysis of the two stages of configuration showed a higher degree of spore maturation in the second stage. There were no spores in the first stage ($D = 0.28$/h), whereas in the second stage ($D = 0.08$/h) spores titers reached 5–10% of the total population. So, in order to attain higher spore concentrations, the second reactor was operated as a maturation tank. In this case, the second stage reactor was filled with the medium outcoming from the first stage reactor. Afterward, the flow was interrupted, and the second reactor was operated as a batch system, to ensure total spore maturation. With this procedure, spores and crystals account for 80–100% of the population.

Another design of a two-stages continuous culture system is that employed by Kang et al. *(140)* for the cultivation of BT var *kurstaki*. In this case the first stage is a total cell retention culture system *(see the following)* from which part of the culture is fed to the second stage; this consists of a continuous reactor with a low dilution rate. Spore production reached values as high as 70%, but it seems that the system is far from reaching the stationary state.

Based on the necessity of a maturation time to reach a high level of sporulation and toxin synthesis, Dreier et al. *(141)* and Moser *(142)* proposed a continuous system composed of a continuous bioreactor as the first stage; and an aerated tubular reactor, with plug flow behavior, as the second stage. Plug flow reactors (PFR) can be regarded as batch reactors, but the temporal events occurring in the latter occur along the longitudinal axis of the PFR (Fig. 1). It was found *(141)* that at the beginning of the tubular reactor the cells showed few inclusion bodies, and at the end many bipyramidal shaped bodies (crystals) could be seen. The residence time was 19 h. Thus, the first part of the reactor can be employed for vegetative growth, whereas in the second part spore formation can be achieved. Residence times within the reactor can be optimized to obtain a complete spore maturation.

A major drawback presented by continuous cultures is the spontaneous mutation of cells to either asporogenous or acrystalliferous variants. The selection of oligosporogenous mutants of *B. cereus* in continuous cultures was reported *(143)*. After 8 d growing at $D = 0.29$/h, the oligosporogenous strain comprised 50% of the population within the reactor. Sachidanandham and Jayaraman *(144)* reported the formation of asporogenic crys-

talliferous variants in continuous cultures of BT var *galleriae*. The crystals so obtained were amorphous and difficult to solubilize, and the toxicity toward *Bombyx mori* was lower than the toxicity of the wild-type strain.

Another interesting culture system is fed-batch culture (FBC), and some reports on the subject have been published. Arcas et al. *(130)* reported higher spore counts (1.2×10^{10} CFU/mL) in FBC of BT var *kurstaki* than those reached in batch cultures (0.74×10^{10} CFU/mL). The toxicity on a per spore basis was the same as that found in batch cultures. Kang et al. *(145)* attained similar results (namely, 1.2×10^{10} CFU/mL) with the same variety, by employing intermittently fed batch cultures. However, no report about toxicity was made. In an additional report *(140)*, a total cell retention culture system for BT cultivation was employed. This system presents a ceramic membrane filter incorporated inside a bioreactor, able to exclude particles greater than 0.2 μm. In this case, culture volume is constant, and biomass is retained within the reactor. Once the maximum biomass is reached, the feed is stopped and the cells are allowed to complete sporulation. At a dilution rate of 0.1/h, with a feeding glucose concentration of 50 g/L, 1.6×10^{10} CFU/mL were obtained, but the system failed when the glucose concentration in the feed was 100 g/L. This can be attributed to the growth inhibition caused by the high nutrient accumulation within the reactor.

Avignone-Rossa and Mignone *(146)* reported linearly fed-batch cultures of BT var *israelensis*, where the concentration of nutrients entering the reactor showed a linear profile. Regardless of the growth limiting substrate (cultures were either glucose- or ammonium-limited), the spore counts reached were not significantly different from those of batch cultures, but the toxicity against *Culex pipiens* larvae was one order of magnitude lower. These results reinforce the idea that performing bioassays in order to assess toxicity is unavoidable when culturing BT, either for industrial processes or δ-endotoxin composition studies.

Fed-batch cultures of recombinant microorganisms were also reported *(147,148)*. A strain of *E. coli* harboring the 135-kDa protein gene *Cry*I(a) from BT var *aizawai* was cultivated in intermittent fed-batch cultures *(147)*. Accumulation of acetate, which inhibits growth and toxin production, was avoided by cross flow filtration. Under these conditions, final cell and toxin concentrations were of 125 and 6.6 g/L, respectively. Cayuela et al. *(148)* carried out a fed-batch culture of a recombinant *B. subtilis* strain harboring several copies of the *Cry*I protein gene from BT var *kurstaki*. By controlling glucose concentration at low levels (2 g/L) they obtained a cell concentration and a toxin titer of 104 g/L and 3.49×10^5 ITU/mL; when glucose was controlled at a higher level, these yields were lower. This could be related to the conclusions raised by Battacharya et al. *(88)*, discussed in Section 2.3.1.

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