Process Biochemistry 98 (2020) 224–232

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Review

# Review on biopesticide production by *Bacillus thuringiensis* subsp. *kurstaki* since 1990: Focus on bioprocess parameters

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ARTICLE INFO

*Keywords:*
*Bacillus thuringiensis*
*kurstaki*
Bioprocess
Bioinsecticide
δ-endotoxins

ABSTRACT

Irrespective use of chemical pesticides has led, over the last decades, to several problems such as soil, water and food sources pollution, and generation of a selective pressure causing the emergence of pest resistance. Consequently, researchers have been focusing more on the use of biological control as an alternative strategy. *Bacillus thuringiensis* (*Bt*) is one of the most widely studied bacteria in industrial biotechnology and commercialized as an environmentally sustainable biopesticide. Therefore, a huge interest has been allocated for research on this bacterium and several scientific studies have been published on the issue. In this review, we tried to evaluate the scientific production over the last thirty years, for the first time, in terms of number and geographical origin, focusing particularly on *B. thuringiensis kurstaki* (*Btk*). It is worth emphasizing that the *Btk* process engineering involving factors affecting growth, sporulation and toxin formation yields by *Bt* has not been fully investigated in previous reviews. To this end, the second section of this review provided an updated survey about these conditions, such as nutritional requirements, culture media and fermentation technologies. Relevant information was collected in comparative tables that could be very useful for the scientific community interested in *Btk*-based biopesticides.

## 1. Introduction

Since the 1960s, pest management in industrialized countries has been based on the intensive use of synthetic chemical pesticides. Undeniably, these pesticides have contributed to increasing crop yields by nearly 70 % in Europe and 100 % in the USA [1]. However, the use of synthetic pesticides has significantly become debatable due to a number of interacting factors, especially the fact that all major insect pests are developing resistance to the various classes of chemical insecticides used against them worldwide. Over 500 species of arthropod pests have resistance to one or more insecticides [2], while herbicide-resistant weeds count about 200 species [3]. Biopesticides are used as an integral part of Integrated Pest Management (IPM) and they are classified into three groups according to their origin [microbial, plant (biochemical) or animal (semiochemical)], and can be used both in conventional and in organic farming [4].

Most commercial biopesticides are of microbial origin and are primarily based on the *Bacillus thuringiensis* (*Bt*) microorganism [5]. *Bt*-based biopesticides are of overarching importance and represent almost 90 % of the world's biopesticide market. While the *Bt* products are widely available in North America and represent 55 % of the bioinsecticide market, they are less popular in Europe representing only 8% of the same market. The low level of *Bt* products in the European Union (EU) is mainly due to the greater complexity of EU-based biopesticide regulations [6]. Nevertheless, market share growth of biopesticides is predicted to outpace that of chemical ones, with an annual growth rate of 15 % [7]. In fact, they are expected to increase from about 2% of the global pesticide market in 2003 to about 8% (estimated to exceed 82 billion USD) in 2020. *Bt* spores and crystals have been commercialized to control a range of different insect orders during their larval stages, such as Diptera, Coleoptera, Hymenoptera, Mallophaga among others [8]. The major *Bt*-based bioinsecticides targets are herbivorous lepidopteran larvae like cabbageworms, cabbage loopers, hornworms, European corn borers, cutworms, some armyworms, diamondback moths, tent caterpillars and Indianmeal moth larvae in stored grain. This might explain the fact of relying on *kurstaki* serotype strains, especially that of *Btk* HD1 [9,10]. *Bt* is known to be safe for vertebrates and a good number of reviews has concluded that it is one of the safest

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https://doi.org/10.1016/j.procbio.2020.07.023
Received 9 March 2020; Received in revised form 7 July 2020; Accepted 29 July 2020
Available online 11 August 2020
1359-5113/ 

products available in terms of impacts on non-target insects [11,12]. Consequently, the use of this bacterium is an important environmentally friendly part of pest management tool kit.

*Bt* is a ubiquitous Gram-positive bacterium. It can be found in a great diversity of ecosystems including soil, water, dust from grain storage, dead insects, leaves from deciduous trees and diverse conifers [13]. It is a spore forming bacterium producing crystalline inclusions consisting of one or more insecticidal proteins known as δ-endotoxins and commonly called Cry proteins [14]. When δ-endotoxins (or Cry toxins) are ingested by a susceptible insect, the crystal proteins are solubilized by the extreme alkaline pH of the insect midgut and proteolytically activated by midgut proteases. Then, the activated toxins bind to specific receptors located in the insect cell membrane leading to the destruction of the epithelial cells lining the insect gut. It is generally believed that these toxins act by creating pores in the cell membrane [15]. Although the bacterium contributes to the death of the insect, the δ-endotoxins are capable of killing some species on their own if produced at sufficient high doses [16].

Whether it is through government grants or companies doing research and development or even via non-profit foundations funding, a considerable investment was made in the field of *Bt*-based biopesticides which have gained more and more interest. For instance, the European commission has been funding more than twenty projects dealing with the ability of *Bt* to be used as biopesticides from 1986 to the present date. Among these projects, we can cite the ongoing project coordinated in France, entitled "IPM-4-Citrus, Citrus disease Integrated Pest Management: from Research to Market" (ID: 734921; Period: From 2017 − 04-01 to 2021 − 03-31; Total cost: 801 000 EUR) [17]. IPM-4-CITRUS focuses on two promising and newly identified strains (*Btk* BLB1 and Lip). These strains were shown to be more efficient than the commercial strain (*Btk* HD1) against Lepidopteran pests, both in terms of activity and production yield [18,19]. The ultimate objectives of the project are: optimization of the bioproduction processes, increase of the strains intrinsic toxicity and generation of high added-value bioproducts.

Within the framework of this project, it is expected to define the nutritional requirements for *Bt* cell growth, δ-endotoxin production and entomotoxicity and to improve the productivity beyond the current state. To this end, an exhaustive state of the art reporting the previous conducted research on *Bt* bioinsecticide production with *Btk* HD1, BLB1 and Lip would be very useful. Over the last thirty years, several scientific publications dealing with *Bt* biopesticide have appeared thanks to the important development in the understanding of *Bt* and its mode of action [20]. Reviews reporting this literature focused on gene discovery, toxin mode of action and resistance evolution, with less interest to *Bt* production. In parallel, most of the work conducted by the private companies is either unpublished or patented.

The present review provides the readers an overview on the available publications about *Btk* biopesticide production, during the last thirty years, and addresses this bacterium culture media and culture conditions emphasizing on the fermenter operation modes for biopesticide production.

## 2. Scientific literature related to *Bt* biopesticide production

Bibliographic research using the database in engineering sciences (web of science (WOS); Thomson Reuters) for the timespan 1990–2000 (done the 10th of June 2020), reveals 4998 publications quoting "*B. thuringiensis*" in the title (profile 1). This number includes publications dealing with biopesticide production from different *Bt* subspecies such as subspecies *israelensis* [21], *aizawai* [22], *tenebrionis* [23], etc. As stated above, in this review, we focused on the literature dealing with the subspecies *kurstaki*.

**Table 1**
Number of scientific publications per profile. Timespan: 1990 to 2020.

| Profile | Interrogation field | | Total publications number |
|---|---|---|---|
| | Title | Title/Keywords/ Abstract | |
| **Profile 1** | *B. thuringiensis* | | 4998 |
| **Profile 2** | *B. thuringiensis* | *kurstaki* | 1153 |
| **Profile 3** | *B. thuringiensis* | *kurstaki*/ endotoxin | 398 |
| **Profile 4** | *B. thuringiensis* | *kurstaki*/ sporulation | 73 |
| **Profile 5** | *B. thuringiensis* | *kurstaki*/ fermentation | 62 |
| **Profile 6** | *B. thuringiensis* | *kurstaki*/ kinetics | 9 |
| **Profile 7** | *B. thuringiensis* | *kurstaki*/ bioprocess | 8 |

### 2.1. Quantitative analysis of publication on Btk

The first section of this review highlighted the scientific publications quoting "*B. thuringiensis*" in the title associated with "*kurstaki*" in the title/keywords/abstract concomitant with five topics *viz.*, "endotoxin", "sporulation", "fermentation", "kinetics" and "bioprocess". Thus, profiles from 2 to 7 are proposed (Table 1). The bibliographic research was basically conducted using WOS. The obtained results were enriched by those collected from SpringerLink and ScienceDirect. However, several data are missing either because they are reported in unavailable papers or unpublished. Table 1 states the total number of publications per profile between 1990 and 2020. Further information about the annual and the cumulative publication numbers per profile were given in (Fig. 1). The obtained results showed that the cumulative number of publications about *Btk* associated with the above cited topics increased continuously, since 1990, to reach 1153, 398, 73, 62, 9, and 8 in profiles 2–7, respectively. The corresponding curves have the same shape but with different starting points. Indeed, publications about "endotoxin" (profile 3) and "sporulation" (profile 4) started to appear in 1990 just like those dealing with *Btk* (profile 2). However, publications started to use the terms "fermentation" (profile 5), "kinetics" (profile 6) and "bioprocess" (profile 7) in 1991, 1999 and 2004, respectively (Fig. 1).

Taking these data into account allows us to conclude that the first studies conducted on *Btk* mainlyfocused on the exploration of the bacterium especially its life cycle and the metabolites responsible for its entomopathogenic activity. The number of the corresponding publications peaked around the years 1994 and 2007 for profile 3 and around the years 2007–2008 for profile 4. However, papers related to *Bt* large-scale exploitation began to appear later, in the beginning of the 21st century, with the appearance of publications dealing with "bioprocess" in 2004 (profile 7). Indeed, as reported by Sanchis [24], two main facts have oriented the scientific research in relation to commercial interest in *Bt*: the first is the discovery of HD1 strain which is 2–200 times more toxic against key agricultural pests [25] and designated for the first time as a *kurstaki* variety; the second main fact is when scientists and environmentalists recognized that chemical pesticides were harmful to the environment and could be replaced by *Bt*-based products.

### 2.2. Geographical origin of Btk scientific literature

This review was written in the context of the above cited project involving six countries *viz.*, France, Tunisia, Lebanon, Italy, Turkey and Germany. Thus, it investigated these countries positioning in the scientific production among those interested in research on *Btk* biopesticide. It mainly focused on the scientific production related to the bioprocess parameters revealed by profiles 5–7 dealing with the topics of "fermentation", "kinetics" and "bioprocess", respectively (Table 2). A quick glance at these profiles allows us to deduce that the exploration of the issue is much more concentrated in America (USA, Canada, Mexico and Brazil). Fewer works originated in Asia with a Chinese and Indian predominance. Africa and Europe rank third and fourth, respectively.

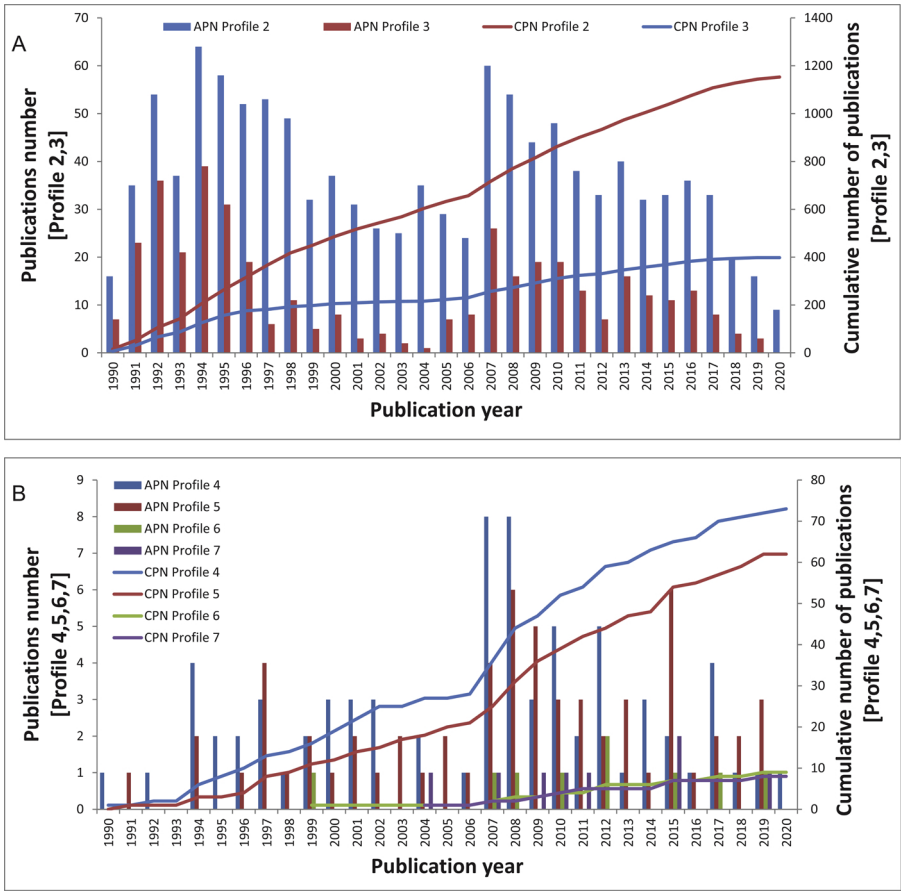


**Fig. 1.** Annual (APN) and cumulative numbers of publication (CPN) for **A**) profiles 2 and 3; **B**) 4, 5, 6 and 7. Profiles are defined as explained in Table 1. Timespan: 1990 to 2020.

Interestingly, the data showed that Tunisia and France play an important role in their respective continents for the publication of such studies (5 for Tunisia and 3 for France). Fewer publications were produced in Italy (2 publications) and Lebanon (1 publication). No scientific production was recorded for Germany and Turkey. These observations may indicate that the *Bt*-based biopesticide industrial production is entailed by the evolution of the geographical distribution of scientific production.

## 3. *Btk* biopesticide production

Unlike profiles 2, 3 and 4 which mostly focused on *Btk* culture media and nutritional requirements, profiles 5–7 dealt with *Btk* bioprocess parameters. Occasionally, the provided information is compared to those of other subspecies allowing the *Btk* study in a broader *Bt* context. In the literature included in profiles 5–7, the bioprocess is investigated from different stand points. For instance, Rowe and Margaritis [26] studied the economic side of the bioprocess, while Mounsef et al. [27] considered the technological feature which is our

**Table 2**
Geographical origin and number of scientific publications of profiles 5, 6 and 7. Timespan: 1990 to 2020.

| Profile 5 Country | Number of publications* | Rate (%) | Profile 6 Country | Number of publications* | Rate (%) | Profile 7 Country | Number of publications* | Rate (%) |
|---|---|---|---|---|---|---|---|---|
| Canada | 22 | 27.2 | Mexico | 4 | 40.0 | Mexico | 3 | 37.5 |
| USA | 13 | 16.0 | Brazil | 2 | 20.0 | Canada | 3 | 37.5 |
| India | 6 | 7.4 | Belgium | 1 | 10.0 | USA | 1 | 12.5 |
| Tunisia | 5 | 7.4 | Italy | 1 | 10.0 | Iran | 1 | 12.5 |
| France | 2 | 6.2 | France | 1 | 10.0 | | | |
| Iran | 2 | 6.2 | Lebanon | 1 | 10.0 | | | |
| Qatar | 2 | 4.9 | | | | | | |
| Egypt | 2 | 2.5 | | | | | | |
| Taiwan | 2 | 2.5 | | | | | | |
| Argentina | 1 | 2.5 | | | | | | |
| Colombia | 1 | 2.5 | | | | | | |
| Syria | 1 | 2.5 | | | | | | |
| Netherlands | 1 | 2.5 | | | | | | |
| South Korea | 1 | 1.2 | | | | | | |
| Italy | 1 | 1.2 | | | | | | |

* All countries are counted for publications issued from more than one country.

main concern in this review. Relevant information about all these issues is detailed below.

### 3.1. Nutritional requirements

A good number of the consulted works focused on factors affecting growth, sporulation and toxin formation. These factors are mainly related to the *Bt* nutritional requirements such as, potassium, metal ions and carbon/nitrogen sources. Indeed, the majority of *Bt* strains are able to ferment a variety of carbohydrates including glucose, fructose, starch, maltose, trehalose and ribose [28]. Recently, newly isolated *Bt* strains have been demonstrated to be able to ferment cellulose and xylan [29]. The most used carbon source to produce *Bt* δ-endotoxins is glucose which was proven to stimulate the growth and trigger δ-endotoxin formation in *Bt* MPK13 [30]. The increase of glucose concentration improves δ-endotoxin production which contributes to the increase of the *Bt* insecticidal activity. Scherrer et al. [31] reported that the optimal glucose concentration for optimal crystal formation is between 6 and 8 g $L^{-1}$. Similar results were obtained by Mazmira et al. [30], confirming the existence of 130 kDa corresponding to Cry protein at 8 g $L^{-1}$ of glucose. At this concentration, crystal reached a length of 2 µm compared to 0.5 µm at 1 g $L^{-1}$ and only 2200 parasporal bodies were enough to stop the uptake of food by *Pieris brassicae* insect larvae compared to 8000 parasporal bodies at 1 g $L^{-1}$ of glucose [31].

When compared to other saccharides used for *Bt* cultivation, glucose at 8 g $L^{-1}$ was demonstrated to enhance the sporulation rate [30]. Indeed, the breakdown of glucose through the Krebs cycle and oxidative phosphorylation produces ATP required for spore germination which is an energy intensive process [28]. However, an excessive glucose concentration in the medium can also inhibit spore formation by the repression of Spo0A activity, which triggers the spore production [32,33]. Moreover, some *cry* genes are sporulation dependent and are controlled by sporulation-specific sigma factors [34]. Thus, the inhibition of *cry* gene expression by high glucose concentration reflects directly the repression of sporulation.

The catabolite repression in *Bt* has been reported by several authors. For instance, for *Bt galleriae*, it has been observed that a glucose concentration of 28 g $L^{-1}$ led to a maximum of biomass production, but increasing its concentration up to 35 g $L^{-1}$ resulted in an heterogeneous population (vegetative cells, sporulated cells and free spores) [35]. For *Bt israelensis* (*Bti*), however, the use of a glucose concentration higher than 75 g $L^{-1}$ was demonstrated to result in growth inhibition as revealed by the reduction of the maximal specific growth rate [36]. Holmberg and Sievanen [37] reported that the high concentration of nutrients inhibited the *Bt* growth and was decisive for sporulation and toxin synthesis. Moreover, Amin et al. [38] found that bacterial spores and crystal protein concentrations were very low when *Btk* is cultured at 50 g $L^{-1}$ or even 90 g $L^{-1}$ of glucose. According to these authors, the carbohydrate concentration should not exceed 23 g $L^{-1}$.

*Bt* was demonstrated to be enabled to assimilate inorganic nitrogen source as a sole nitrogen source in the growth medium. Thus, in order to allow growth of this bacterium, at least one amino acid such as glutamate, aspartate, valine, leucine, serine or threonine could be added in the medium. Other amino acids, like cysteine, however, inhibit *Bt* growth, sporulation and toxin formation [39]. Among the various organic nitrogen sources tested, peptone is as good as some other complex nutrients such as commercial grade food of *Saccharomyces cerevisiae* powder used as the main nitrogen source in glycerol yeast (GY) medium. These organic nitrogen sources gave a similar *Bt* biomass production, productivity and efficiency of the final product to that obtained in LB medium [40].

Balance between carbon and nitrogen sources is needed to avoid lower pH values than 5.6 which could affect cell growth and final spore concentrations [41]. A medium with a C/N ratio of 7.5 results in a larger growth yield coefficient $Y_{X/S}$ (g biomass/g substrate) and increases glucose consumption when compared to C/N ratios of 5 or 11 [42]. Similarly, using a C/N ratio of 7, Farrera et al. [43] demonstrated that the δ-endotoxin production has been improved six times by increasing 2.5 fold the initial concentration of total solid through the addition of glucose and soya bean meal. To control the C/N ratio, Vidyarthia et al. [44] experimented various combinations of primary (carbon-rich) and secondary sludge (nitrogen rich). Their results confirmed the hypothesis of Sachdeva et al. [45] who postulated that the C/N ratio of seven plays an important role in the growth, sporulation and entomotoxic potency of *Bt*.

Among the required ions for *Bt* δ-endotoxin production, potassium is considered essential. Indeed, a concentration of 50–100 mM of $K_2HPO_4$ is required for an effective synthesis of the protoxin Cry4 by *Bti* [46]. Similarly, Banerjee-Bhatnagar [47] reported that potassium is essential for the production of 135-kDa protoxin by *Bt* HD522. Several metal ions such as $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Cu^{2+}$ and $Fe^{2+}$ were shown to be essential for an adequate sporulation and δ-endotoxin formation [39,48]. Özkan et al. [46] stated that $Mn^{2+}$ was the most critical element in the biosynthesis of Cry4Ba and Cry11Aa by *Bti*, when used at a concentration of $10^{-6}$ M, and that $Mg^{2+}$ and $Ca^{2+}$ favor toxin production when provided at concentrations of $8 \times 10^{-3}$ M and $5.5 \times 10^{-4}$ M, respectively. In addition, $Fe^{2+}$ and $Cu^{2+}$ positively influenced δ-endotoxin biosynthesis [49]. Studies related to the investigation of *Bt* nutritional requirements are mandatory for the understanding of its behavior in different fermentation media. Since all the reported studies showed that *Bt* is able to assimilate diverse carbon and organic nitrogen sources and requires a variety of ions for its growth, a broader investigation of its nutritional requirements becomes crucial for a better understanding of its behavior in the different fermentation media.

### 3.2. Culture media

Because of the economic importance of *Bt* as powerful biological control agent against harmful insect pests, special attention was paid to elucidate and optimize its growth conditions that lead to the highest δ-endotoxin yield. Therefore, different synthetic media (Table 3) were used for *Bt* cultivation such as Anderson medium based on glucose, yeast extract, bacto peptone and $(NH_4)_2SO_4$ [42]. Using this medium with various glucose concentrations, Amin et al. [38] showed that at 20 g $L^{-1}$ of glucose, $7.1 \times 10^{11}$ spores $mL^{-1}$ and 3.4 g $L^{-1}$ of crystal protein were obtained. Using nutrient yeast salt medium (NYSM) based on glucose, peptone, beef extract and yeast extract for *Bti* production, allowed a biomass dry weight of 3 g $L^{-1}$ with the lowest spore count of $3 \times 10^{10}$ CFU $mL^{-1}$ [46]. Even lower spore counts of $6 \times 10^9$ and $2.12 \times 10^5$ spores $mL^{-1}$ were obtained by using a clean medium based on 50 g $L^{-1}$ glucose and 2.59 g $L^{-1}$ yeast extract and Luria-Bertani medium, respectively [50,51]. Additionally, Sarrafzadeh et al. [52] proved that by using a medium based on glucose, casein hydrolysate and yeast extract, 90 % of sporulation along with 16.5 optical density were reached at the end of fermentation. Other synthetic media were used for *Bt* production like the one based on glucose, glycerol, yeast extract and ammonium sulphate [53]. For further information, the *Btk* δ-endotoxin production and spore concentration in the last medium were of 531 mg $mL^{-1}$ and $22 \times 10^8$spores $mL^{-1}$, respectively, after 72 h of incubation. Özkan et al., 2003 [46] reported that among the various inorganic nitrogen sources added in Yousten's medium (YSM) the highest yields of Cry11Aa and Cry4Ba proteins of *Bti* HD500 were obtained on $(NH_4)_2HPO_4$. The same authors showed that among the tested carbon sources in YSM medium, inulin, dextrin, maltose, lactose, sucrose, whey and glycerol were all stimulatory, while glucose, starch and molasses were suppressive. Furthermore, Sikdar et al. [49] indicated that for a high δ-endotoxin production yield, mineral salt medium should contains $K_2HPO_4$, $MgSO_4.7H_2O$ and $CaCl_2.2H_20$ at 1, 0.3 and 1 g $L^{-1}$. According to Table 3, all synthetic media reported in literature are actually complex media because they contain all yeast extract, associated with beef extract in one case [54]. Moreover, we

**Table 3**
Synthetic media used for *Bt* cultivation.

| Synthetic medium | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Reference | [50] | [51] | [38] | [49] | [46] | [54] | [53] | [52] |
| **Compound (g $L^{-1}$)** | | | | | | | | |
| Glucose | 12 | | 20 | 10 | 1 | 10 | 15 | 5 |
| Glycerol | | | | | | | 5 | |
| Casein hydrolysate | | | | | | | | 4.5 |
| Beef extract | | | | | | 3 | | |
| Yeast extract | 2.59 | 5 | 4.62 | 2 | 2 | 0.5 | 5 | 0.5 |
| Bactopeptone | | | 4.62 | | | 5 | | |
| Tryptone | | 10 | | | | | | |
| $(NH_4)_2SO_4$ | | | 1 | 2 | 2 | | 5.4 | 6 |
| $KH_2PO_4$ | | | 3.4 | | | | 1 | 1.4 |
| $K_2HPO_4$ | | | 4.15 | 1 | 0.5 | | 1 | 1.4 |
| $CaCl_2.2H_2O$ | | | 0.106 | | 0.08 | 0.102 | | 0.332 |
| NaCl | | 10 | | | | 5 | | |
| $MgSO_4.7H_2O$ | 0.2 | | 0.3 | 0.3 | 0.2 | 0.203 | 0.3 | 0.61 |
| $MnSO_4.7H_2O$ | 0.04 | | 0.05 | 0.015 | 0.05 | 0.01 | 0.01 | 0.006 |
| $FeSO_4.7H_2O$ | 0.00135 | | | 0.01 | | | 0.01 | |
| $C_6H_5FeO_7$ | | | 0.075 | | | | | |
| $ZnSO_4.7H_2O$ | 0.0058 | | 0.0075 | | | | | |
| $CuSO_4$ | 0.0075 | | 0.0045 | 0.001 | | | | |
| $CaCO_3$ | | | | 1 | | | 20 | |
| $CoCl_2.6H_2O$ | 0.03 | | | | | | | |
| KCl | 3 | | | | | | | |

noted that there is no similarity neither in the needed elements for the growth nor in their amount added in the culture medium. Consequently, researches on *Bt* biopesticide production were conducted by different reference media, which hampers the interpretation of the resulting parameters.

Sachdeva et al. [45] reported that the commercial application of *Bt* depends on several parameters such as the raw material cost, strain efficiency, fermentation cycle, fermentation product bioprocessing and its formulation. The raw materials cost is one of the overall *Bt* production main costs. It varies between 30 and 40 % of the total cost depending on the plant production capacity. Therefore, cheap production media based on locally available sources including agro-industrial by-products and wastes (residues) should be developed. In the literature, the most cited are wheat bran [55], fish meal and gruel [56], soybean meal and starch [57], cheese whey, soya bean milk, ground *Bombyx mori* pupae and cane molasses [58], soy flour [54], barley flour [59], bird feathers and deoiled rice bran [60], glycerol from the biodiesel industry [61], brewer's yeast extract [62], molasses with corn steep liquor [22,63] and broiler litter extract [64] (Table 4). Ghribi et al. [57] demonstrated that by using soya bean meal and starch for *Btk* production, δ-endotoxin production and spore count reached 2.71 g $L^{-1}$ and 38 × $10^8$ spores $mL^{-1}$, respectively. A similar δ-endotoxin concentration was obtained by Mounsef et al. [55] using 6% wheat bran (2.4 g $L^{-1}$). However, using a combination of gruel and fish meal, Zouari et al. [56] showed that a highest δ-endotoxin production could be reached with *Btk* strains (3–3.3 g $L^{-1}$), but the lowest production was reached with *Bti* strains (1.24–1.99 g $L^{-1}$). Only 1 g $L^{-1}$ and 0.75 g $L^{-1}$ of δ-endotoxin were obtained by culturing *Btk* in gruel hydrolysate and 2% molasses based medium, respectively [65]. Moreover, Alves et al. [58] showed that, using different combinations of agro-industrial residues and by-products, the spore count varied between 5.5 and 21.6 × $10^8$ spores $mL^{-1}$. A comparative spore count of 15 × $10^8$ spores $mL^{-1}$ was obtained when using brewer's yeast extract as the main nitrogen source [62]. Interestingly, the highest spore count (480 × $10^8$

**Table 4**
Complex media used for *Bt* cultivation.

| Scale | Culture medium | Strain(s) | Spore and δ-endotoxin bioproduction | Reference |
|---|---|---|---|---|
| Erlenmeyer (250 mL) | Gruel 42 g $L^{-1}$, fish meal 20 g $L^{-1}$ | *Btk* & *Bti* | *Btk* HD1: 3060 mg $L^{-1}$ ; 35 × $10^8$ spores $mL^{-1}$<br>*Bti* T14: 1410 mg $L^{-1}$ ; 33 × $10^8$ spores $mL^{-1}$ | [56] |
| Erlenmeyer (250 mL) | Cheese whey 50 %, soya bean milk 10 % and molasses 0.5 % | *Btk* | 21.6 × $10^8$ spores $mL^{-1}$ | [58] |
| Erlenmeyer (250 mL) | Soya bean protein 5%, molasses 0.5 % | *Btk* | 5.5 × $10^8$ spores $mL^{-1}$ | [58] |
| Erlenmeyer (250 mL) | Ground *Bombyx mori* pupae 15 %, molasses 0.5 % | *Btk* | 10.5 × $10^8$ spores $mL^{-1}$ | [58] |
| Erlenmeyer (1 L) | Gruel hydrolysate15 g $L^{-1}$, ammonium sulfate 5.4 g $L^{-1}$, yeast extract 5 g $L^{-1}$ | *Btk* BNS3 | 1 g $L^{-1}$ ; 3.4 × $10^8$ spores $mL^{-1}$ | [65] |
| Erlenmeyer (50 mL) | Hydrolysed sludge of Black Lake | *Btk* HD1 | 0.12 × $10^8$ spores $mL^{-1}$ | [67] |
| Batch (100 L) | Soy flour 2.5 % + 1% solution of ($MgCl_2$ 20.3 g $L^{-1}$; $CaCl_2$ 10.2 g $L^{-1}$ ; $MnCl_2$ 1.0 g $L^{-1}$) | *Bti* | 480 × $10^8$ spores $mL^{-1}$ | [54] |
| Batch (5 L) | Barley flour 2.5 % (m/v) ; soy flour 1%; salt solution ($MgCl_2$ 20.3 g $L^{-1}$ ; $CaCl_2$ 10.2 g $L^{-1}$; $MnCl_2$ 1 g $L^{-1}$) | *Btk* HD1 | 9.58 mg $10^8$ $spores^{-1}$ ; 0.31 × $10^8$spores $mL^{-1}$ | [59] |
| Erlenmeyer (NA) | Bird feathers, deoiled rice bran | *Bti* | 13.4 g $L^{-1}$ | [60] |
| Batch (3 L) | Soya bean meal 25 g $L^{-1}$, starch 30 g $L^{-1}$ | *Btk* | 2711 mg $L^{-1}$ ; 38 × $10^8$ spores $mL^{-1}$ | [57] |
| Erlenmeyer (1 L) | Wheat bran 6% | *Btk* Lip | 1.80 × $10^9$ crystal $mL^{-1}$ and 2.40 g $L^{-1}$ toxin;<br>1.66 × $10^9$ spores $mL^{-1}$ | [55] |
| Batch (20 L) | Brewer's yeast extract 1%; glucose 2.5 %; ammonium sulfate 0.2 % | *Btk* HD1 | 15 × $10^8$ spores $mL^{-1}$ | [40] |
| Batch (2 L) | Molasses 2%, corn steep liquor 3%, sea salt 0.003 % | *Bt* KH4 | 52 × $10^8$ spores $mL^{-1}$ ; 750 mg $L^{-1}$ | [63] |
| Batch (15 L) | Starch industry wastewater | *Btk* HD1 | 1.2 × $10^8$ spores $mL^{-1}$; 1043 mg $L^{-1}$ | [72] |
| Erlenmeyer (500 mL) | Broiler litter extract | *Btk* | 2.46 × $10^8$ spores $mL^{-1}$ | [64] |
| Erlenmeyer (250 mL) | Milky effluent 74 %, beer wastewater 26 % | *Btk* | 2.9 × $10^8$ spores $mL^{-1}$; 4.1 × $10^8$ crystal $mL^{-1}$ | [75] |

NA: Not available.

spores $mL^{-1}$) was generated when using a medium based on soya bean flour with salt solution [54] and the lowest one was reached when using a medium based on barley flour [59]. For a low-cost *Bt* biopesticide production, the most used raw-material is the wastewater sludge [44,66–71]. When used at different preparations, the highest viable cell ($0.14 \times 10^8$ spores $mL^{-1}$) and spore count ($0.12 \times 10^8$ spores $mL^{-1}$) were obtained when *Btk* was grown in hydrolysed Black Lake sludge [67] (Table 4). Lachhab et al. [66] demonstrated also that using the same medium (wastewater sludge) for inoculum preparation, higher sporulation ($17 \times 10^8$ spores $mL^{-1}$) and toxicity (12 300 international units (IU) $mL^{-1}$) values were obtained. They also reported that the optimum sludge solid concentration was 26 g $L^{-1}$, which resulted in an improved potency and high spore count achieving $42 \times 10^8$ spores $mL^{-1}$ and 12 970 IU $mL^{-1}$, respectively. In a later study, Vidyarthia et al. [44] compared the growth and δ-endotoxin production by *Btk* in a tryptic soya yeast extract (TSY) medium, soybean based commercial medium and wastewater sludge medium. They found that the highest toxicity was obtained in a sludge medium and was comparable to that of the concentrated commercial *Bt* formulation available on the market (Foray 48B). Among the used wastewaters for *Bt* cultivation, starch industry wastewater (SIW) is the most used one [72–74]. Compared to wastewater sluge, *Bt* fermented SIW showed a low spore count ($1.2 \times 10^8$ spores $mL^{-1}$) but high entomotoxicity ($18.4 \times 10^9$ spruce budworm units (SBU) $L^{-1}$). Higher spore concentration ($2.9 \times 10^8$ spores $mL^{-1}$) was obtained using a combination of milky effluent and beer wastewater [75]. These studies indicate that the choice of an adequate production medium triggers both spore and δ-endotoxin production ensuring the effectiveness of *Bt* as a biological agent. In all the presented cases, it is not possible to make comparative study to select the appropriate medium for *Bt* cultivation due to the differences in the ways in which biomass, δ-endotoxin and toxicity are measured and expressed.

### 3.3. Culture conditions

Studies conducted by different researchers have shown that process conditions can significantly influence *Bt* crystal-spore complex production. The fermentation parameters that play an important role in the *Bt* production are pH, temperature, dissolved oxygen concentration and inoculum preparation.

Oxygen supply is a decisive parameter in *Bt* fermentation. Oxygen transfer is a function of the volumetric oxygen transfer coefficient (KLa). It is also one of the most important factors for the process scale-up [76]. In practice, the measurement of this coefficient expresses the oxygenation capacity of the medium contained in the bioreactor. It is commonly used to measure the oxygen transfer rate (OTR) and the oxygen consumption rate (OUR) [77]. Several papers have discussed the effect of aeration on growth, sporulation and crystal production for different *Bt* strains [21,27,78,79]. Most *Bt* submerged fermentations were carried out using an aeration rate of one air volume per medium volume per minute. However, other studies used a higher aeration level of 1.4 air volume per medium volume per minute [80]. Sarrafzadeh and Navarro [78] have reported that, using different oxygen transfer rates of 0, 20, 100 and 250 mmol $L^{-1}$ $h^{-1}$ in *Bt* fed-batch cultures, the spore counts were of 100, 93, 84 and 48 %, respectively. So, the highest sporulation rate (100 %) was observed in the absence of oxygen and the mature spores were the only population present under this condition at the end of fermentation. We can even observe that sporulation in a large proportion of cells failed under saturated oxygenation [81]. At 100 mmol $L^{-1}$ $h^{-1}$, cells in different physiological states could be observed. Furthermore, keeping the dissolved oxygen (DO) concentration at 50 % during the vegetative and transition phases then raising the DO to 100 % of saturation throughout sporulation allowed a higher *Bti* toxicity (IPS-82) [21]. However, Ghribi et al. [79] noted a lower spore yield but a higher δ-endotoxin synthesis by a *Btk* strain BNS3 when 60 % and 70 % oxygen saturation were ensured during the first six hours, then decreased to 40 % up to the end of fermentation. Likewise, Bhowmik et al. [82] demonstrated that the fermentation carried out with 30 % DO and an aeration fixed at one vvm allowed an increase of 1.67 fold of δ-endotoxin production by *Btk* strain HD 73 compared to a non-controlled culture. Moreover, in semi-continuous processes combined with batch processes for sporulation, there is an a 53.6 % increase in sporulation of *Bti* IPS 82 under aerated conditions, but toxicity is about four times higher under non aerated conditions [83]. Considering these findings together, it is proved that the optimal conditions for spore and δ-endotoxin yields are not the same, even though sporulation and δ-endotoxin formation occur simultaneously during the fermentation process.

pH and temperature are major factors influencing *Bt* biopesticide production. Indeed, *Bt* growth occurs in the pH range of 5.5–8.5 [28,46,48]. The usual initial pH is 6.8–7.2; decreasing to 5.8 as acetate is released, then increasing to 7.5–8 as it is consumed. Sodium acetate was found to be the best pH control agent for *Bt* entomotoxicity and δ-endotoxin production [72]. Ndao [84] stated that the maximum sporulation and toxicity were obtained in a medium buffered at an initial pH of 7.5 and that the optimal temperature for growth and toxin production is 30 °C. However, Özkan et al. [46] stated that the optimal temperature for toxin production depends on which toxin is produced by the bacterium. They found that Cry4Ba synthesis by *Bti* HD500 was the best when the microorganism was grown at 25 °C, whereas Cry11Aa synthesis was optimal at 30 °C. Culture synchrony is also considered an important parameter during *Bt* biopesticide production since the maximal efficiency of the final product is achieved when fermentation is close to 100 %. In this context, different inoculum preparation strategies such as the use of heat-preteated spores at 60 °C for 15 min [40] and the use of exponential growing cells (cells aged of 6 − 10 h) [53] were applied to generate synchronized *Bt* cultures.

### 3.4. Biokinetics and bioperformances

The production of *Bt* cells and spores depends on the specific growth rate (μ) of the micro-organism, which, in turn, depends on the used strain, the concentration of available nutrients, temperature, pH and dissolved oxygen as well as the metabolic state. Research studies carried out on *Bt* production have demonstrated that the maximum *Bt* growth rate varies from 0.4 to 1.9 $h^{-1}$ [85]. Anderson and Jayaraman [35] have suggested that a high specific growth rate does not necessarily promote sporulation and toxin synthesis. Indeed, a negative correlation between the growth rate and *Bt* production parameters occurred by varying glucose and yeast extract concentrations. In general, *Bt* growth is characterized by an exponential vegetative growth followed by a stationary phase due to substrate depletion. Spores and crystals formation causes a decrease in the growth rate due to the decrease in the energy needed to perform the binary fission, which contributes to the final biomass reduction [86].

The specific sporulation rate is another parameter measured during *Bt* kinetics. This rate varies with the medium composition and culture conditions like pH and dissolved oxygen (DO) concentration, etc. Indeed, an optimization of culture medium composition is critical to control *Bt* sporulation, since high growth-favoring media of vegetative cells may not be adequate for sporulation [35]. Vidyarthi et al., 2002 [44] reported that comparative low specific sporulation rate values (0.05 $h^{-1}$) were obtained in TSY and soya bean based medium. However, the highest value of 0.12 $h^{-1}$ was obtained by using wastewater sludge. This high specific sporulation rate leads to the highest *Btk* entomotoxicity. The same authors reported that a linear relationship exists between the specific sporulation rate and that entomotoxicity. The optimum value of specific sporulation rate was of 0.55 $h^{-1}$.

In *Bt*, sporulation and δ-endotoxins synthesis are greatly dependent on oxygen supply which favored the cellular respiration and metabolism leading to higher viable cell and spore counts and δ-endotoxin concentration [87]. Interestingly, at 12 h of fermentation, the OUR,

which is the third parameter followed during *Bt* fermentation was the highest (0.25 mmol $L^{-1}$ $h^{-1}$) due to the increase in cell concentration (growth) and metabolic activities (enzymatic synthesis) [87]. However, it is worth noting that the OUR peak depends on several parameters such as the microorganism requirements and pH regulation. Commonly, at 12 h *Bt* sporulation is triggered and transition to sporulation phase begins. For this reason, a ten-hour incubation period was chosen by numerous researchers to perform the substrate feeding achieving high levels of biomass, sporulation and toxicity [40,88,89]. Moreover, Rowe at al. [90], demonstrated that the specific OUR of *Btk* HD1 decreased from 8 to 10 mmol $g^{-1}$ $h^{-1}$ at one hour after inoculation to less than 2 mmol $g^{-1}$ $h^{-1}$ by the growth end. Additionally, Mounsef et al. [27] showed that the *Bt* oxygen demand for growth and sporulation is not identical to that for optimal toxin synthesis. Besides, they found that a linear correlation exists between the amount of consumed oxygen and the maximum cell concentration obtained at different cereal milling byproduct (CMB) ratios in the culture medium, with a correlation coefficient of 0.99. In general, OUR increases at the exponential growth phase. Indeed, at this step, a high substrate consumption rate takes place after which it decreases with the cell metabolic activity decline [91].

### 3.5. Culture technologies

For *Bt* practical application as a biological insecticide, high δ-endotoxin production titers are required. For this reason, several works have been carried out on the *Bt* bioinsecticide production using batch fermentation. Boniolo et al. [21] demonstrated that the use of batch fermentation allowed a higher biomass concentration, cell productivity and cell yield. All this closely depended on the amount of 50 % DO applied throughout the fermentation period. In addition, this concentration resulted in a higher spore count and markedly improved the toxic activity of the fermentation broth compared to that achieved at a low DO concentration (5%). Moreover, the application of various DO profiles during batch fermentation proved that the best profile corresponds to 60 % and 70 % of oxygen saturation during the first 6-h fermentation period [79]. Then, 40 % of oxygen saturation should be ensured until the end of fermentation, independently of the carbon source origin. Furthermore, Vu et al. [89] demonstrated an improvement of δ-endotoxin concentration using a batch process performed with SIW. Using the same fermentation technology, Jouzani et al. [63] developed a low-cost bioprocess based on agriculture wastes. The oxygen demand increased with the fermentation time. Besides, the δ-endotoxin production as well as the bacterial growth increased by raising the oxygen concentration up to 70 %.

Fed-batch culture has also been widely used for the production of *Bt* bioinsecticide. Indeed, an extended dynamic model for *Btk* growth and sporulation using an intermittent fed- batch culture each 3.28 h with total cell retention in glucose based medium was proposed by Atehortúa et al. [92]. Using this same strategy, but with two intermittent feeds of SIW (at 10 and 20 h) during the 72-h fermentation period [89], demonstrated that the δ-endotoxin concentration and entomotoxicity were significantly higher than those obtained by applying the batch process. Indeed, δ-endotoxin concentration and entomotoxicity reached 1672.6 mg $L^{-1}$ and 18.59 × $10^6$ SBU $mL^{-1}$, using two intermittent feeds of SIW compared to 511.0 mg $L^{-1}$ and 15.8 × $10^6$ (SBU) $mL^{-1}$, respectively, obtained using the batch fermentation in SIW. However, a fed-batch fermentation carried out with three SIW intermittent feeds at 10, 20 and 34 h of fermentation, resulted in the formation of asporogenous variants which decreased the δ-endotoxin concentration and consequently the entomotoxicity value [89]. Moreover, Rojas et al. [40] demonstrated that a pulse fed-batch process and one-pot combination processes performed at different scales and carried out by the addition of glycerol at 10 h of incubation, significantly increased the biomass production, spore count and toxicity compared to batch fermentation.

Despite the relative improvement of δ-endotoxin production by fed-batch fermentation when compared to batch fermentation, both bioreactor productivity and toxin yield were markedly low due to an incomplete consumption of the added substrate. For this reason, a two-stage exponentially fed-batch fermentation process involving an initial stage for vegetative growth followed by a second stage for sporulation and toxin production was applied. The best condition corresponds to a fermentation supplied with 190 g glucose in 1500 mL. At this condition, up to 20.1 g of bacterial insecticides per litre were recovered from the fermentation broth with glucose to toxin conversion yield of 0.159 g $g^{-1}$ [93].

It is worth noticing that less information was given about the *Btk*-based bioinsecticides production from an economic stand point. Among the few authors dealing with this issue, Rowe and Margaritis [26] compared fermentation broths obtained from different fermentation technologies: i) batch, ii) low density fed-batch (LDFB) and iii) high density fed-batch (HDFB). *Bt*-based bioinsecticides cost was higher for a batch fermentation than for a LDFB. However, the HDFB has relatively little additional cost benefit.

Although different fermentation technologies have been described, probably others are yet to be studied to increase the potential of *Bt* spores-crystal complex which varies according to the medium composition, the strain used and the production conditions.

## 4. Conclusion

Over the last thirty years, the number of scientific publications dealing with *Bt*-based bioinsecticides showed a gradual and steady increase. The highest number of publications in relation with “sporulation” and “fermentation” is recorded by America followed by Asia, Africa and Europe. Fewer publications in relation to “bioprocess” follow the same geographical distribution. This distribution is correlated with the *Bt* biopesticide market which is mostly developed in America. The development of *Bt* biopesticide in the other continents seems to be dependent on many factors such as consumer demand and government policies regarding the use of *Bt* in agriculture. Therefore, it is necessary to strengthen the collaboration between research and industrial institutions and accelerate the practical application of research results to facilitate large-scale industrial development, mainly in developing countries.

Moreover, it is worth noticing that reports providing information about kinetics and fermentation technologies are scare and incomplete. Besides, there are differences in the way of measuring the parameters and expressing the results, which might hamper carrying out comparative studies. Therefore, standardized measurement methods should be established to enhance the whole field.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

This work was conducted under the IPM-4-Citrus project, funded by the European Commission (ID: 734921; Period: From 2017-04-01 to 2021-03-31; https://cordis.europa.eu/project/rcn/207633/factsheet/en). This work was also funded in part by the Tunisian “Ministère de l’Enseignement Supérieur et de la Recherche Scientifique” (Program-contract 2019-2023).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

online version, at doi:https://doi.org/10.1016/j.procbio.2020.07.023.

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