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CHAPTER 8

Biopesticides—*Bacillus thuringiensis*

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8.1 Introduction

Bacillus thuringiensis (Bt) is the most successful biopesticide as of today being used actively in agriculture, forestry and the public health sector. The Bt group has four commercial principal subgroups, namely, Bt var. *kurstaki* (lepidoptera); Bt var. *aizawai* (lepidoptera); Bt var. *israelensis* (diptera); Bt var. *san diego* and Bt var. *tenebrionis* (coleopteran). Bt has specific activities against species of the orders Lepidoptera (Moths and Butterflies), Diptera (Flies and Mosquitoes) and Coleoptera (Beetles). Upon sporulation, Bt forms crystals of proteinaceous insecticidal δ-endotoxins which are encoded by *cry* genes. Cry toxins have specific activities against species of different insect orders. Spores and crystalline insecticidal proteins produced by *B. thuringiensis* are used as specific insecticides under trade names of Dipel and Thuricide. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects. Bt-based insecticides are often applied as liquid sprays on crop plants, where the insecticide must be ingested to be effective. It is thought that the solubilized toxins form pores in the midgut epithelium of susceptible larvae. Recent research has suggested that the midgut bacteria of susceptible larvae are required for Bt insecticidal activity (Broderick et al., 2006).

The success of the Bt biopesticides is gauged from different technical drivers: a) fermentation (cheaper raw material and process costs); b) harvesting (ease of product separation and recovery efficiency); c) formulations (product stability and viability); d) registration (reduced mammalian and flora-fauna toxicity except the target pest); and e) field application (synergy with application equipment; field efficacy of formulations and resistance to adverse environmental factors). Meanwhile, production of Bt biopesticides worldwide has undergone tremendous changes in terms of utilization of alternate raw materials to decrease the process burden as well as changes in formulation technologies

(advanced versions like microencapsulations). These trends of Bt production and formulations have been discussed in great details in earlier reviews (Tirado-Montiel et al., 1998; Brar et al., 2006a). Thus, the objective of this chapter is to briefly review the studies carried out in this field over the last decade (as shown in Table 8.1) and then discuss the trends and general patterns in the use of wastewater and wastewater sludge as a raw material for Bt biopesticides production referred to as the Bt-INRS process.

8.2 Fermentation

Bt fermentation of wastewater and/or wastewater sludge has been conventionally carried out by using submerged fermentation (Burges, 1998). Submerged fermentation, despite some benefits (e.g., ease of control of process parameters and potential scale-up), encompasses limitations of high operational costs due to higher agitation and aeration. Despite being a cost intensive process, submerged fermentation is more extensively studied and well documented in the literature.

Figure 8.1 illustrates the biopesticide voyage covering all steps in our laboratory at INRS-ETE which will be discussed in this chapter. The production process involves the following steps: a) sludge fermentation, b) product recovery/harvesting, c) product formulation. Fermentation is the process of deriving energy from the oxidation of organic compounds, such as carbohydrates, using an endogenous electron acceptor, which is usually an organic compound. In this case, fermentation of sludge is carried out to achieve the objectives. However, before the sludge is fermented using *Bacillus thuringiensis* as the microorganism, it needs to be sterilized in order to kill the pre-existing spores and cells of numerous microorganisms which are an inherent part of wastewater sludge. Generally, sterilization refers to a process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses, prions and spore forms etc.) from a biological culture medium. A widely-used method for heat sterilization is the autoclave; in this case, sterilization is performed at 121^{o}C for 30 minutes which can even kill prions (that causes the disease scrapie). Once sterilized, the wastewater sludge needs to be inoculated with the micoorganism (Bt, in this case) seed so that the microorganisms proliferate in the fermenter.

Preliminary experiments on utilization of wastewater sludge as a raw material for Bt biopesticides production were carried out by Tirado-Montiel et al. (2001). The sludge from various wastewater treatment plants, with or without hydrolysis, was tested for Bt growth, sporulation and entomotoxicity production. The entomotoxicity (Tx) level reported was very low to the tune of 3,000–4,000 SBU/µl, (spruce budworm units per micro liters) as the sludge solids concentration was not yet optimized. However, the wastewater and wastewater sludge did show encouraging signs of growth support for production of Bt biopesticides. Spruce budworm units refer to the toxicity of Bt crystal

Table 8.1 *Bacillus thuringiensis* fermentation in wastewater (WW) and wastewater sludge (WWS).

TSS/ SS (g/L)	Reactor Type	Type of WW or WWS	TC_{48h} (CFU/mL)	VS_{48h} (CFU/mL)	Tx (x 10^9 SBU/L)	Industrial Standard Reference	References
-	Batch (shake flask)	Raw Acid hydrolyzed Supernatant (Primary and 2ndary)	1 x 10^6 – 1.5 x 10^7 1.7 x 10^6 – 2 x 10^7 1.3 x 10^6 – 2.4 x 10^6 2 x 10^8 (soya)	1 x 10^6 – 1.3 x 10^7 1.6 x 10^6 – 1 x 10^7 1.2 x 10^5 – 2 x 10^6 1.8 x 10^8 (soya)	1.3–3.3 3.0–4.0 1.0–3.0 3.8 (soya)	48 B (12.6 x 10^9 IU/l)	Tirado-Montiel et al., 2001, 2003
25– 40	Batch (shake flask)	2ndary (raw and pre-treated)	5 x 10^8 – 6 x 10^8 3.9 x 10^8 (soya)	3 x 10^8 – 3.5 x 10^8 3 x 10^8 (soya)	9.0–11.0 4.5 (soya)	48 B (12.6 x 10^9 IU/L)	Sachdeva et al., 1999, 2000
2 – 45	Batch (fermenters)	Mixed sludge	5 x 10^9 (25g/L) 9 x 10^8 (soya)	4.2 x 10^9 (25g/l) 8 x 10^8 (soya)	12.3 (25g/l) 10.5 (soya)	48 B (12.6 x 10^9 IU/L)	Lachhab et al., 2001
25	Batch (shake flasks)	Wastewater sludge	1.2 –6.4 x 10^9	1.5 x10^5 – 3.3 x 10^9	10.6 –14.8	76 B (19.5 x 10^9 IU/L)	Mohammedi et al., 2006
25	Batch (shake flasks)	2ndary and mixed	5.8 x 10^8 6.2 x 10^8 (soya)	5.1 x 10^8 5.2 x 10^8 (soya)	10.5 9.6 (soya)	76 B (19.5 x 10^9 IU/L)	Vidyarthi et al., 2001, 2002
25, 30	Batch (fermenters)	2ndary (raw and hydrolyzed) & addition of external supplements	6.7 x 10^8 (raw) 7.8 x 10^8 (hydrolyzed)	6.5 x 10^8 (raw) 5 x 10^9 (hydrolyzed)	13–18	76 B (19.5 x 10^9 IU/L)	Leblanc, 2003
25	Batch (fermenters)	2ndary (raw and hydrolyzed)	1.8 x 10^8 6.2 x 10^8 (soya)	5.1 x 10^8 5.2 x 10^8 (soya)	10.6 – 16	76 B (19.5 x 10^9 IU/L)	Lamontagne, 2004
25	Batch (bench/ pilot scale fermenters)	2ndary	1.5 x 10^9	5.5 x 10^8	12–14	76 B (19.5 x 10^9 IU/l)	Yezza et al., 2004
25	Fed-batch (fermenters)	2ndary	9 x 10^8	8.6 x 10^8	18	76 B (19.5 x 10^9 IU/L)	Yezza et al., 2005a
25	Batch (fermenters)	Thermal hydrolyzed Oxidative hydrolyzed 2ndary	8.3 x 10^8 6.3 x 10^8	7.9 x 10^8 5.8 x 10^8	17 13	76 B (19.5 x 10^9 IU/L)	Yezza et al., 2005b
25 (WWS)	Batch (fermenters)	Starch industry wastewater 2ndary	1.7 x 10^9 6.7 x 10^8	6.6 x 10^8 5.5 x 10^8	18 13	76 B (19.5 x 10^9 IU/L)	Yezza et al., 2006
25	Batch (fermenters)	Raw Thermal hydrolyzed 2ndary	5.4 x 10^8 3 x 10^8	4 x 10^8 3.9 x 10^8	13 18	76 B (19.5 x 10^9 IU/L)	Brar et al., 2005b
-	Batch (fermenters)	Starch industry wastewater	1.7 x 10^9	8 x 10^8	16	76 B (19.5 x 10^9 IU/L)	Brar et al., 2005c

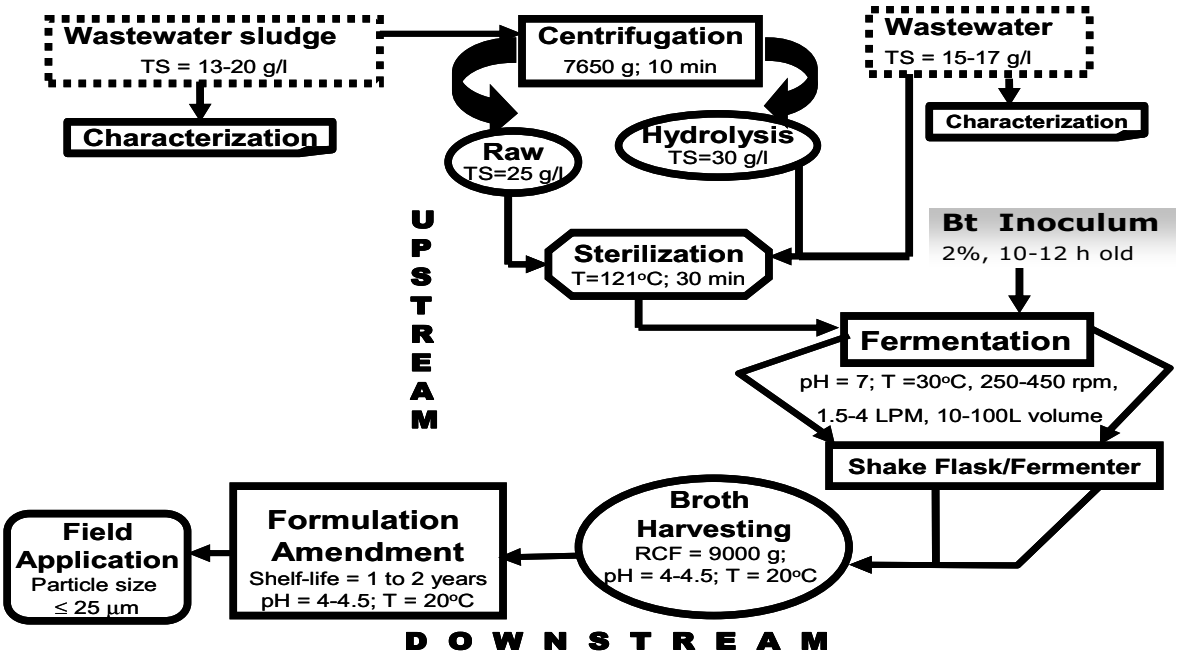

Figure 8.1 *Bacillus thuringiensis* biopesticides production voyage in wastewater and/or wastewater sludge at INRS-ETE (RCF = relative centrifugal force; TS = total solids; T = temperature).

proteins when measured against spruce budworm larvae. Meanwhile, generally, the toxicity is measured against standard insects which are specific to each region and is reported as international units per liter (IU/L). In North America, these insects comprise cabbage looper (*Trichoplusia ni*). The reported low entomotoxicity (Tx) level was attributed to uncontrolled pH conditions, and also the total solid concentration and the process parameters like inoculum were not optimized.

Tirado-Montiel et al. (2001) also noted a low spores concentration of 1.0×10^6–1.3×10^7 CFU/mL (non-hydrolysed sludge), 1.6×10^6–1.8×10^7 (hydrolysed sludge, acidic conditions) and 1.2×10^5–2.0×10^6 CFU/mL (sludge supernatant) (colony forming units /mL) in wastewater sludge when compared to a soya medium (1.5×10^8 CFU/ml). However, this research laid the foundation to the fact that wastewater sludge does contain the necessary nutrients to support the growth, sporulation and crystal formation by Bt. Later, Tirado-Montiel et al. (2003) pursued the Bt growth in wastewater and/or wastewater sludge; they optimized different fermentation process parameters in shake flasks, namely, pH (7 ± 0.1); temperature (30 ± 1^oC) and agitation rate (200–250 rpm). The results showed a tremendous shift from earlier uncontrolled experiments with the spore concentration increasing to 1.2×10^8 CFU/mL and entomotoxicity (Tx) of 7000 SBU/μL. Our experimental results indicate that there is a linear relation established between Tx and the maximum specific growth rate; overall Tx increased with the spore count, but the specific entomotoxicity (spTx) or Tx per 1000 spores decreased with an increase in the spore count as illustrated in Figure 8.2. Nevertheless, the results support Bt growth in wastewater and/or wastewater sludge, which has induced series of experimental strategies and the process being referred to as the Bt-INRS process.

Interestingly, the Bt fermentation also follows diauxic growth. This is in accord with an earlier study where diauxic growth was observed when Bt was grown on mixed substrates (different combinations of complex proteins and simple sugars and vice versa) (Ribbons, 1969). The diauxic growth was probably due to the transient accumulation of acids such as gluconate, 2-keto gluconate, α-ketoglutarate or pyruvate in the culture medium that required a short lag phase before they were completely oxidized (Ribbons, 1969). Thus, the presence of different degrees of biodegradable materials (simple and complex) in sludge could have contributed to diauxic Bt growth in wastewater sludge (Tirado-Montiel et al., 2001). As will be discussed later, the diauxic growth was a common feature observed in all wastewater sludge studies (Vidyarthi et al., 2000; Barnabé et al., 2001; Tyagi et al., 2001; Lacchab et al., 2001; Vidyarthi et al., 2002; Leblanc, 2003; Vidyarthi et al., 2003; Brar et al., 2004 a,b, Lamontagne, 2004; Yezza et al., 2004; Barnabé et al., 2005a, b; Brar et al., 2005 a,b,c; Yezza et al., 2005 a, b, c; Brar et al., 2006 a, b, c; 2008a,b; 2009; Yezza et al., 2006a, b). Thus, wastewater sludge can be a very good source of carbon, nitrogen, phosphorus, and other nutrients for many microbial processes that could add value to sludge by producing certain valuable

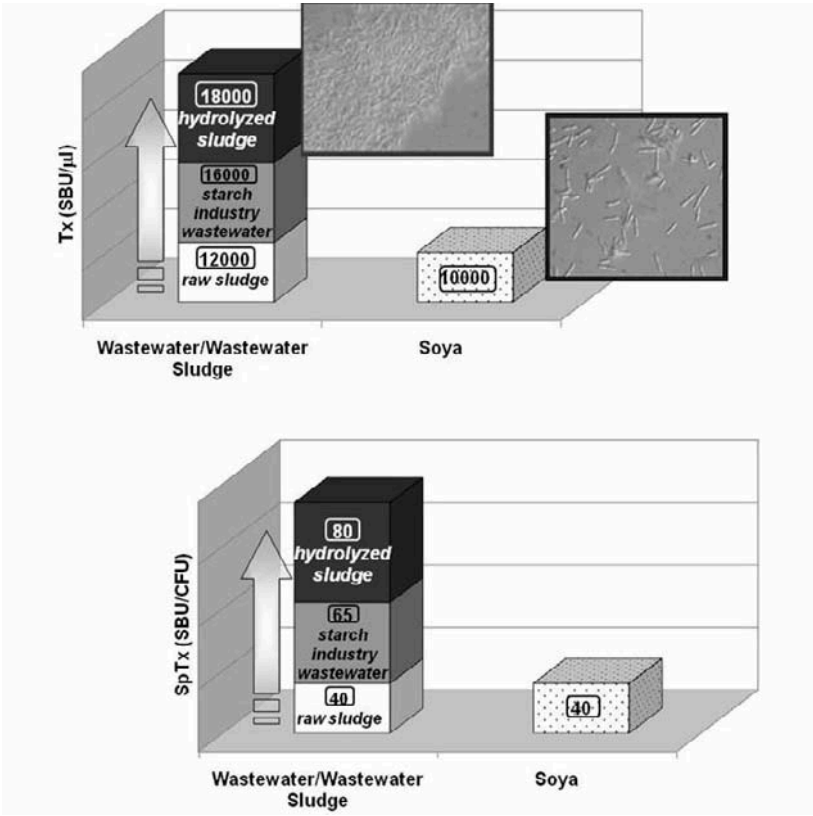

Figure 8.2 Fermentation profile in terms of biopesticidal potential represented with (top) Tx, entomotoxicity (spruce budworm units per µL) or (bottom) SpTx (specific entomotoxicity per 1,000 spores) that are produced by Bt using wastewater and/or wastewater sludge as compared to the synthetic medium (soya). Wastewater used was starch industry wastewater (SIW); and wastewater sludge used was secondary (raw and hydrolyzed) sludge. The photographs inset represent the microscopic view of Bt growth in sludge and soy media at 100 x magnification. Data is derived from 10 years of study at INRS-ETE.

products, such as endotoxins and spores of Bt as well as certain other compounds (vegetative insecticidal proteins–Vips, hemolysins, enterotoxins, chitinases, proteases, phospholipases and others) (Kaur et al., 2001), which contribute towards mortality of insects defined with the term of entomotoxicity (or biopesticidal potential). Currently, the most attractive way to dispose wastewater or municipal sludge is agricultural and/or forestland application (Casado-Vela et al., 2006). Utilization of sewage sludge for Bt production followed by its application to agricultural crops and forests for pest control seems to be fully compatible with current sludge disposal practices. However, only the sludge that follows the regulatory standards (USEPA, 1984) should be used for Bt production and has been discussed further during the course of this section.

Meanwhile, a higher level of entomotoxicity is desired to further reduce the production cost of biopesticides. Higher entomotoxicity depends on the type of raw material, assimilation of nutrients by Bt, production of different synergistic factors in the fermented broth and post-fermentation by adding additives which synergize the crystal proteins. In this context, Vidyarthi et al. (2002) conducted a study to evaluate sludge solids, dewatered sludge and mixed sludge in search of the increased level of entomotoxicity. At the same time, the protein to carbohydrate ratio in the synthetic medium has been reported to have a subtle effect on the spore formation and entomotoxicity yield by Bt (Pearson and Ward, 1988; Morris et al., 1996). However, the specific value of the carbon to nitrogen ratio in the production medium for Bt production was lacking in the existing literature. Therefore, the effect of the C:N ratio in sludge medium on the sporulation and the entomotoxicity yield was also studied (Vidyarthi et al. 2002).

A low C:N ratio in the secondary sludge and a high C:N ratio in the mixed sludge resulted in a higher entomotoxicity. The optimum value of the C:N ratio in combined sludge for Bt production was found to be 7.9–9.9. A higher specific sporulation rate and hence higher entomotoxicity was observed in sludge (10,000–12,000 SBU/μL) than synthetic media (9,500 SBU/μL). The entomotoxicity value increased linearly with the maximum specific growth rate. The specific sporulation rate (0.55 h^{-1}) exhibited an optimum value for maximum entomotoxicity.

8.2.1 Oxygen Transfer

Oxygen mass transfer is important for any aerobic bioprocess. An increase in the broth viscosity can decrease oxygen transfer rates. Hence, it is important to track oxygen uptake rate (OUR), specific oxygen uptake rate (Q_{O2}), and the volumetric oxygen mass transfer coefficient (k_La) as a function of fermentation time and broth viscosity. These parameters are important in order to ensure that sufficient amounts of oxygen are available for the microorganisms, help optimize biopesticide production, and gather relevant oxygen transfer data for scale-up and bioreactor design.

Maintaining the appropriate concentration of dissolved oxygen has been pointed out as an important factor in the fermentation of Bt (Flores et al., 1997). There is sufficient literature available in relation to the effects of oxygen on the biomass concentration and toxin synthesis (Maldonado-Blanco et al., 2003). In order to avoid oxygen limitation, the oxygen supply, or more correctly, the oxygen transfer rate, must be at least equal to the maximum oxygen demand during the exponential growth phase of Bt (Avignone-Rossa and Mignone, 1995).

As the oxygen was supplied to meet the oxygen demand of Bt culture, the spore and toxin yields increased. However, beyond a certain stage of excess oxygen supply, the cell and spore yield decreased and the entomotoxicity remained constant suggesting the possible inhibitory effect of oxygen on metabolic activity of the bacterium, especially on its growth (Avignone-Rossa and Mignone, 1995). Additionally, it is economical to avoid a higher air supply rate.

In fact, studies carried out by Brar et al. (2005a, b) demonstrated that properties like viscosity do play an important role during oxygen transfer in Bt fermentation. There were variations in viscosity during fermentation due to the concerted effects of increases in the viable cell and spores, cell lysis, agitation, aeration and anti-foam addition. The particle size decreased markedly at the end of fermentation. The viscosity showed an initial decrease (0 to 6 h depending on the medium type) followed by a hump and then later a decrease (after 9 or 12 h depending on the fermentation medium). The general profile of viscosity can be better understood from the typical profile given in Figure 8.3.

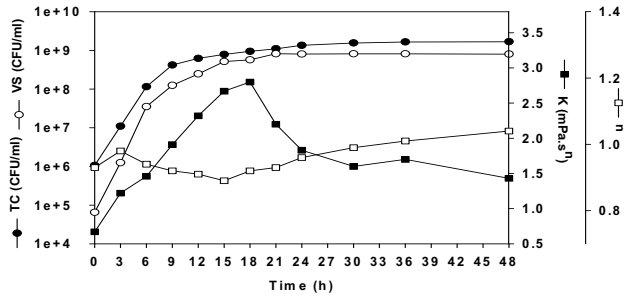

Figure 8.3 Time courses of Bt growth characteristics using wastewater sludge as a medium in relation to rheology. Different abbreviations in the Figure are as follows: TC = total cell count; VS = viable spores; CFU = colony forming units; K = consistency index; and n = flow behaviour index.

8.2.2 Foaming

Increased cell growth, sporulation and δ-endotoxin production by Bt requires higher aeration rates which causes foaming. Foam formation during biopesticides fermentation is a serious disadvantage to the industry. Excessive foaming can cause: (a) fouling of the gas outlet condenser and as a consequence compromising the aseptic operation of the vessel; (b) loss of biopesticide spores and other metabolites that stick to the wall of the vessel after foams are broken; (c) mass transfer problems in the fermenter; and (d) questionable final characteristics of the fermented broth due to the loss of some important biopesticidal components. It is widely accepted that polypeptides are the most foam promoting compounds (Jegou et al., 2000). Since the foam formation influences both the biopesticide quality and productivity, several methods of foam controlling are employed, but none of them is perfect. The use of antifoam is the most common, although it cannot always be applied and in some cases can compromise the quality of the final product. Moreover, it can cause cleaning and hygiene problems, and it makes the biopesticide production more expensive. Thus, it would be desirable to control the foam level in the fermenter in a different way, the most favourable without using additives. Thus, varying aeration and agitation rates can control foaming.

Foam formation in biopesticides production is controlled externally by the addition of polypropylene glycol (PPG) and silicone based anti-foam agents (Holmberg et al., 1980; Pearson and Ward, 1988). These anti-foam agents affect nutrient and oxygen transport across the Bt cell membranes. Furthermore, when alternative medium like sludge is used, the proteins and cell debris present cause foam formation. This foam creates non-homogeneity in the medium due to floatation of physiologically different microorganisms. In this context, Vidyarthi et al. (2000) studied the effect of various anti-foam agents, including PPG, silicone anti-foam, canola, olive, soyabean and peanut oil on wastewater sludge and soya medium fermentation in bench scale fermenters. They observed that the chemical anti-foams decreased the entomotoxicity by 25 to 40% whereas the vegetable oils did not cause any inhibitory effect. In fact, the synergistic effect of vegetable oils increased with the monounsaturated fat content supporting Bt growth.

Foam formation in sludge medium depends on sludge solids differing with the type of sludge (primary, mixed and secondary) and sludge composition which varies over space and time, season, plant location, type of wastewater treated, and treatment type. Thus, foam control plays an important role in wastewater sludge based Bt fermentation which needs to be carried out prudently to enhance the biopesticidal potential and the same is being actually practiced in series of fermentations in our laboratory.

8.2.3 Solids Concentration and Inoculum