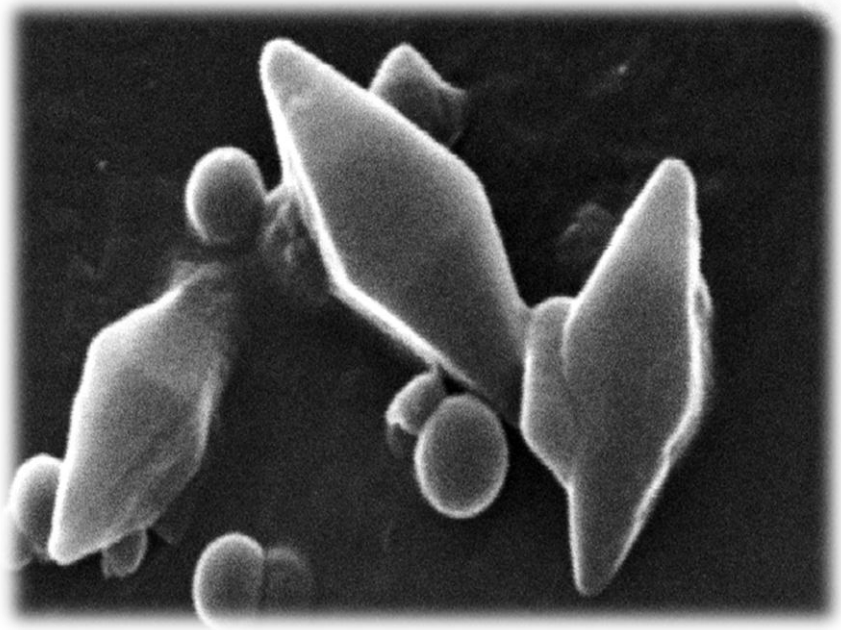
Mário Brum Teixeira



Using sewage sludge from Municipal and Industrial Solid Wastes to produce a *Bacillus thuringiensis* Biopesticide

UNIVERSIDADE DOS AÇORES

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In Biotechnology in Biological Control

Orientador: Doutor Nelson José de Oliveira Simões

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A todos vocês um grande abraço e obrigado.

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ABSTRACT

The objective of this work was to ascertain the sludge with capacity for the development of *Bacillus thuringiensis* (Bt) with the aim of producing a bio-pesticide. In this study, we carried out tests of bacterial growth in municipal and industrial waste-water treatment plant sludge. The used bacterium was a native strain of Bt isolated in São Miguel Island. The total viable cells, maximum specific growth rate, rate of sporulation, and entomotoxicity against the pest *E. kuehniella* were used as a method of comparison and selection of the sludge.

The selection of native Bt started with the isolation of 216 soil samples, taken in different areas and habitats, covering the entire island of São Miguel. From these samples it was possible to isolate and identify 248 Bt corresponding to an index of 0.23. The morphological characterization of crystals revealed the predominance of bipyramidal shape (having activity against Lepidoptera) in respect to other shapes: spherical, amorphous and associated to spore. Toxicity assays against *E. kuehniella* larvae allowed the selection of the native isolate (S170a) that presented the lowest LC50 (9.2 µg/ml). To grow Bt, sludge was submitted to a pre-alkali treatment to reduce viscosity and to elevate nutrient availability. Despite all the sludge have presented nutritional capacity for the development of Bt it was seen that sludge from an ETARI related to the slaughter of animals showed the best values. This sludge presented the highest yield of viable cells (4.76 x 10^8 CFU/ml) that represents an increase of 20% compared with the semisynthetic medium (TSB) with 84 hours of fermentation. Furthermore, Bt produced in this sludge has one of the lowest toxicities (LC50 of 58.30 µg/ml).

Our data clearly show that it is feasible the use of IDWWTP sludge to produce efficiently and at a low cost a bio-pesticide based in local Bt isolates to use in biological control of lepidopteran pests.

RESUMO

O objetivo deste trabalho foi determinar que lamas têm capacidade para o desenvolvimento de *Bacillus thuringiensis* (Bt) com a intenção de produzir um biopesticida.

Neste estudo, realizamos testes de crescimento bacteriano em lamas de estações de tratamento de águas residuais urbanas e industriais. A bactéria utilizada foi uma estirpe nativa de Bt isolada na ilha de São Miguel. Os totais de células viáveis, taxa de crescimento máximo, taxa de esporulação e entomotoxicidade contra a praga *E. kuehniella* foram usados como um método de comparação e selecção das lamas.

A selecção da Bt nativa foi iniciada a partir do isolamento de 216 amostras de solo colhidas em diversas zonas e habitats, cobrindo em grande parte a ilha de São Miguel. A partir destas amostras foi possível identificar 248 isolados de Bt o que faz um índex de 0.23. A caracterização morfológica dos cristais revelou a predominância de cristais bipiramidais (activos contra lepidópteros) sobre as restantes morfologias: esférico, amorfo e associado ao esporo. Baseado em ensaios de toxicidade sobre o insecto *E. kuehniella* seleccionou-se o isolado S170a de Bt nativo de S. Miguel.

A fim de aumentar o crescimento de Bt foram realizados pré- tratamentos alcalinos das lamas para redução de viscosidade deste modo elevando a disponibilidade de nutrientes. Apesar de todas as lamas terem apresentado capacidade nutricional para o desenvolvimento da bactéria verificou-se que as lamas provenientes de uma ETARI relacionada com o abate de animais apresentaram valores distintivos. Estas lamas produziram o maior número de células viáveis (4.76×10^8 CFU/ml), representando um aumento de 20% comparativamente com o meio semi-sintético comercial (TSB) no final de 84 horas decrescimento. Os Bt produzidos nestas lamas apresentaram uma entomotoxicidade com um dos mais baixos valores de LC50 (58.30 µg/ml).

Tendo em conta estes factos aqui apresentados ficou provada a viabilidade de utilização de lamas de ETAR/I para a produção a custos reduzidos de um biopesticida eficiente no controlo biológico de pragas de lepidópteros.

1. INTRODUCTION

The control of insect pests in agriculture and some human vector pathogen is usually controlled and achieved using chemical insecticides. The continually use of these chemical compounds has been responsible for various problems, environmental pollution and the increase of many world diseases, such as cancer and several other immune system disorders (Devine and Furlong, 2007). The use of bacterial insecticides is proposed to be a substitute to chemicals. Bacterium bio-pesticide has a low persistence and impact in the environment and is not pathogenic to humans. The most successful insect pathogen used for insect control is the bacterium *Bacillus thuringiensis* (Bt), which presently represents 2% of the total insecticidals commercialized products. Bt is mostly active in larval stages of insect orders and kills the insect by disruption of the midgut tissues followed by septicemia that is caused not only by Bt spore germination but also by other bacterial species present in the insect intestinal flora (Raymond et al., 2010). When sporulating Bt produces crystal inclusions that are formed by a variety of insecticidals protein Cry or Cyt toxins. These toxins were shown to have highly selective activity killing a narrow range of insects. The Cry and Cyt toxins fit into a class of bacterial toxins known as pore forming toxins (James, 2009). Cry toxins are classified by their amino acid sequence and more than 500 *cry* genes have been categorized in 67 groups from *cry*1 to *cry*67 until today (Crickmore et al., 2010).

Despite commercially use of Bt to control insects in agriculture; its use is restricted due to the high costs of production in bioreactor. The raw materials used for the production of Bt bio-pesticides represent between 35% and 59% of the overall costs of production. Therefore, for commercial purposes, there is an urgent need to improve the efficiency and lower costs of obtaining raw materials available throughout the year for the production of Bt. (Abdel, et al 2001). In recent years, the research focused on the use of sludge from wastewater treatment plants (WWTP) as a single substrate for fermentation of Bt. Research has shown promising results in increased toxicity in relation to commercialized semi-synthetic media (Yezza et al, 2005). The insecticidal activity of Bt is known not only by relying on the activity of the bacterial culture, but also by abiotic factors, such as the composition of the culture mediums. Different carbon

sources and nitrogen and C/N ratio, alter the shape, composition and content of δ-endotoxin of crystal toxin affecting the entomotoxicity (Farrera et al, 1998). Sludge from WWTP can be a good source of carbon, nitrogen, phosphorus and other nutrients for microbial processes and for production of metabolic added value compounds. The use of sludge allows the growth of Bt and the production of protein crystals and spores that act as bio-pesticides. These two components are in insoluble phase (solid phase). Simultaneously, the Bt also produces alkaline proteases, which are in the soluble phase fermentation of sludge. These proteases are abundantly used in the manufacture of detergents, as well as in the tannery industry, with a global market of over one thousand and eight hundred million euros. Thus, the use of WWTP sludge can be an alternative source and profitable relatively to the use of synthetic commercialized media for the production of Bt (Khanh, 2010).

Sludge is an abundant waste in São Miguel Island as a result of municipal and industrial waste-water treatment plants (WWTP). The sludge wastes produce elevated costs for Azorean industries and have high management expenses before disposal in land fields. In this work, we propose to evaluate four different sludge sources to produce a bio-pesticide based in a native isolate of *Bacillus thuringiensis*, creating an added value product from this waste.

2. MATERIAL AND METHODS

2.1 Soil sampling and bacterial isolation

Bacillus thuringiensis strains were isolated according to the methods described by the World Health Organization in 1985 and Travers et al, (1987). A soil sample of about 500 g was obtained from 2–5 cm depth soil by scraping off soil surface material. Soil samples were collected in sterile bags and stored away from sun light at 4°C until processed. The local sampling place was registered using a GPS Garmin e-Trex and the coordinate format registered in Universal Transverse Mercator (UTM). The sample source, habitat and vegetal surrounding area were also registered. To isolate sample bacteria, 4 g of each homogenized soil sample were suspended in 10 ml of peptonized water sterile. The suspension was vortex for 1 minute and heat-shock at 80ºC for 10 minutes to kill all vegetative forms. 1 ml of the heated treated suspension was used for serial dilutions up to 10 -3. A volume of 0.1 ml from the dilutions 10^{-2} and 10^{-3}, was plated in T3 agar (3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.002 g $MnSO_4$, $7H_2O$; 0.02 g $MgSO_4$, $7H_2O$; 1.4 g Na_2HPO_4; 1.2 g NaH_2PO_4, and 15 g of agar per liter) adjusted to pH 6.8 and incubated at 30 ºC for 24 h.

2.2 Identification of B. thuringiensis isolates and preservation in a collection

Five colonies with egg shape morphology resembling Bt were selected from each sample and observed under a phase-contrast microscope to check for the presence of spores and parasporal inclusions. Inclusions morphology was grouped in three types: bipyramidal; spherical or amorphous. (four types: bipyramidal; spherical or amorphous and associated to spore) The bacteria presenting inclusions body and spore were cryopreserved in 20% glycerol and stored at -80ºC. They were also preserved lyophilized in filter paper and conserved at room temperature on sealed glass vials (Krieg, 1981; Bernhard, Jarrett et al. 1997). These isolates belong to the Azorean Entomopathogenic Collection belonging to the Bio-insecticide Research Group.

2.2.1 Preparation of spore - crystals mixtures

From a single spore colony, a first pre-culture was made in 5 ml of T3 at 30ºC overnight and at 250 r.p.m., in a rotary shaker. From these pre-culture 0.2 ml were diluted in 100 ml of T3 and incubated at 30ºC, 250 r.p.m., for 96 hours to ensure complete spore and crystal formation and bacteria autolysis. After sporulation and crystal production, the culture was transferred into centrifuge tubes at 4 °C and centrifuge at 10000 r.p.m. for 10 minutes. The pellet containing the spores and crystals was recovered, re-suspend and washed with a solution of 1 M NaCl + 0,1% Triton X-100 using the vortex. The washed crystals and spores were again centrifuge at 4 °C, 10000 r.p.m., for 10 minutes and the pellet recovered. The same procedure was repeated four times using cold water until most of the cell debris was eliminated. The use of cooled water allowed the elimination of most of the spores, concentrating the crystals in the mixture which was checked under the contrast phase microscope. This crystals spore isolation technic was the same used to obtain crystal for scanning electron microscopy (SEM), for obtaining crystals for SDS-PAGE profiles and for all bioassays.

2.2.2 Identification of parasporal bodies shape by Scanning electron microscopy (SEM)

After sporulation and spore-crystal mixtures preparation, the pellet containing the spores and the crystals was recovered, re-suspend and washed with a solution of 1M NaCl + 0,1% Triton X-100 using a vortex, the washed crystals and spores were again centrifuge at 4°C, 10000 r.p.m., for 10 minutes and the pellet recovered. This pellet was prepared for SEM several dilutions were made to obtain a low concentration of crystals. This was achieved by checking the dilution under the optical microscope until few crystals 3 to 4 remain in the observation zone field. 10 µl of this dilution was dropped in a round glass slide of 1 cm wide, and let to dry at room temperature overnight.

2.2.3 Characterization of crystal protein (SDS–PAGE)

To extract crystal proteins for characterization by the SDS-PAGE, 500 µl of the partially purified crystals were centrifuge at 4ºC, 10000 r.p.m. and the

pellet was suspended in 500 µl of a 50 mM Na_2CO_3 (pH 11.0) + 10 mM dithiothreitol at 4ºC for 24 hours. Microscopic observation was carried out to ensure the solubilization by observing the absence of crystals. Crystal solubilized mixtures were centrifuge at 4 °C and 10000 r.p.m. for 15 min. The supernatant containing the pro toxins was recovered and the pH stabilized to 7 with Tris-HCl 1 M pH 4.75 buffer (Naimov et al. 2008) Protein concentration was determined using Pierce reagent (Pierce BCA Protein assay kit, Cat. 23225). Protein composition of spore-crystal mixtures was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) as accordingly to Laemmli (1970). Separation gel was 12%, for 10 ml of total gel volume it was used 4 ml of acrylamide at 30%, 2.5 ml Tris-HCl at 0.375 M pH 8, 0.1 ml of the SDS at 10%, bringing the remaining with H_2O milliQ, finally 75 µl of APS at 10% and 30 µl of TEMED was added for gel polymerization. A running buffer with 33 g/l, Tris, 144g/ l Glycine, and 10 g/l SDS was used. Each isolates spore crystal solubilized proteins were diluted (1:2) in bromophenol blue loading buffer (2% of 0.5%of blue bromophenol; 20% of SDS at 10%; 25% of glycerol; 12.5% of 0.5M Tris-HCl, pH 6.8 with 5% of β-mercaptoethanol) and pre-treated at 100 ºC for 5 minutes. 10 µg of protein from spore crystals solubilized mixture was applied per gel wells. Electrophoresis was run at 100 V until complete migration of the front line. The staining solution consisted in Coomassie Blue Colloidal this solution consisted of 98 ml H_2O of a solution A, which contained ammonia sulphate 9.8 g (10% w/v), 1.96 g of ortho-phosphoric acid (2% w/v), and a solution B with 2 ml of H_2O with 0.1 g Coomassie Blue G250 (5% w/v). To fix the proteins it was added to staining mixture 25 µl of methanol.

2.3 Bioassays to select Bt isolates active against Lepidoptera.

To screen for the most toxic Bt isolate against Lepidoptera insects, *E. kuehniella* was used. We screened the 238 isolates against third-instar larvae using the technique of incorporating spore-crystal complex in the larvae diet. The spore crystals mixtures were added with the flour usually used to feed the insect. The spore-crystal suspensions consisting of 100µg of toxin in 600 µl of distilled water, were mixed in 1 g diet (corn flour), and placed in a Petri dish. Assays were performed using 15 larvae per dish

with three replicates. The strain *B. thuringiensis* subsp. *Kurstaki* (HD-1) and the solubilization buffer and water were used as positive and negative controls. Larvae mortality was recorded 96 hours post treatments. The isolates with high toxicity (> 75%) were selected for a further characterization of the toxicity, LC50 (concentration that kills 50% of exposed insects) values were determined for the 4 most active isolates. Five dilutions of toxin (10, 25, 50, 100, 150 µg) in 600µl were used for each isolate, and 45 third-instar larvae were tested in each dilution. Mortality was record 96 hours later. All toxicity tests were carried out at 25 ºC, 70 ± 10 % HR with 14: 10 h, L: D photoperiod. The LC50 calculation was calculated using SPSS Probit analyzer. Bioassays with Bt produced in sewage sludge were also conducted as above.

2.4 Waste water Sludge Physical analyses and Alkaline hydrolysis Pre-treatment

For the Bt growth evaluation in the waste-water sludge, four different sources were used from Urban and industries waste-water plants. One sludge source was from an urban waste-water treatment plant (UWWTP) that collected water from the public sewage water grid. Two other sludge sample source came from dairy related industrial factories. One of these industries waste-waste treatment plants (IDWWTP) were dedicated to dairy product transformation in butter and powder milk (IDWWTP-B), the second dairy industry was related to milk and cheese production (IDWWTP-M). The last industrial waste-waste treatment plant sludge came from a slaughterhouse (ISWWTP).The samples were collected in polypropylene containers and carried to the laboratory where they were sterilized (121 °C for 30 min) to eliminate pathogens and prevent the sludge deterioration during storage at 4 °C until used. This sludge was submitted to its physical constitution to determine the total solids concentration. Total solids are a measure of the suspended and dissolved solids in water matter suspended or dissolved in water or wastewater is considered as solids. The measure units are given in mg per liter and its calculation formula (total solids = [(TSA – TSB)] X 1000 / sample mL), were TSA is equal to the weight of dried residue + dish in milligrams and TSB to the weight of dish in milligrams. For this procedure, a 2.5 g of sludge sample were mixed with 50 ml of miliQ

water and stirring overnight, after the mixture was set to dry until no water remains at 105 ºC. Wet and dry weights were taken (Table 1) and total solid calculation was made for each sludge (APHA, 1989). The final total solids concentration of production sludge was set to 26 g/l as referred in Lachhab, (2001) just before pretreatments start.

Table 1- Wet and dry weights for total solids calculation in the sludge from different source

Sludge source	Sludge (g/50ml)	Dish weight (mg)	Dish weight +dry sample (mg)	Volume sample (ml)
ISWWTP	2,5g	64400	64900	52
IDWWTP-M	2,5g	67000	67600	52
IDWWTP-B	2,5g	67000	67300	53
UWWTP	2,5g	64400	64800	53

The alkaline pretreatments of sludge was done as in Chang, (2007) this was achieved adding 5 g of NaOH to one liter of the sludge mix (26 g/l) and adjusting the pH to 10. With continually slow stirring at room temperature for 24 hours and the final pH was set to 7. This sludge medium was distributed by the Erlenmeyer flask before autoclaving.

2.5 Bt crystals production in Sewage sludge

For pre-culture inoculum a 100 ml Erlenmeyer flask with 20 ml of a dilution (1:2) of TSB and pretreated sludge was used. After pretreatment, the sludge pretreated production medium (100 ml) was introduced into a 500 ml capacity Erlenmeyer flask. Tryptic soy broth (TSB) 3 and 0.3 g yeast extract were added to another 500 ml capacity Erlenmeyer flask containing 100 ml of demineralized water. The Erlenmeyer flasks were autoclaved at 121 °C for 30 min. A colony of Bt was added to 100 ml inoculum flask, Bt colonies were picked from culture grown on standard agar plates. The flasks were then incubated in a rotary shaking incubator at 30 °C with an agitation speed of 250 rpm until the culture reached exponential phase (about 10 h). The inoculum (pre-culture), thus obtained was transferred to the production sludge medium 500 ml flask and incubated in a shaking incubator at 30ºC for the period of 84 hours. Samples drawn in were drawn every four-hour interval and diluted with physiological solution (0.85% NaCl) and applied to standard solid agar medium

on plates. The plates were then incubated in an incubator at 30°C for 24 h the colonies, which appeared on the plates corrected for dilution factors, were counted as total viable cell count. Between 30 and 300 colonies were counted per plate. To determine the viable spores count, the dilutions of different samples were treated at 80 °C for 10 min followed by application to standard solid agar plates and incubated at 31 °C for 24 h. The colonies counted on plates were corrected for dilution factor and registered as spore viable count (Lachhab, 2001).

3. RESULTS

3.1 Sampling and isolation

In São Miguel Island, a total of 216 soil samples were collected from which 1066 sporulating colonies were obtained (Table 2). These colonies were checked for the presence of parasporal inclusion bodies, which is the determinant morphological characteristic in the identification of Bt isolates. B. thuringiensis was identified in 50.9% of soil collected samples. The 248 B. thuringiensis colonies founded represented a Bt index of 0.23.

Table 2- Number of samples and number of colonies with crystal

Nº. of samples examined	Nº. of colonies analyzed	No. of crystaliferous colonies	Samples with Bt recovery (%)	*Bt Index
216	1066	248	50,9	0,23

*Bt index represent the ratio between the number of colonies with parasporal inclusion bodies and the number of total sporulating colonies.

3.2 Morphological characterization of Bacillus thuringiensis

All the bacteria identified has Bt are rod-shaped and present a terminal spore. In all the isolates the sporulation process was followed by the formation of crystals as expected. Crystals characterization was performed by phase contrast microcopy and by scanning electron microscopy. Figure 1 showed the 4 distinct morphological crystal groups surveyed in S. Miguel: bipyramidal, spherical, amorphous, and associated to the spore. The most frequent crystals have bipyramidal shape, with 72.2%, spherical shape with 19%, amorphous with 7.7% and associated to spore with 1.2% of occurrence (Table 3).

Table 3- Morphological groups of crystals and the occurrence of crystal shape

Crystal shape	Nº of isolates	Occurrence (%)
Bipyramidal	179	72.2
Spherical	47	19.0
Amorphous	19	7.7
Associated to spore	3	1.2
TOTAL	248	100

The bipyramidal crystals have a size of about 1.8 µm wide. Worth being noted that about 90% of the isolates that presents bipyramidal crystals produced one or more secondary inclusions the spherical crystals were 0.8 µm diameter. A few isolates were found to present secondary round inclusions. The inclusions with amorphous shapes were from many sizes. Most of these isolates produced more than one amorphous inclusion per cell. The parasporal bodies associated to spore had less than 1.0 µm. These parasporal bodies showed two distinct morphologies, rod-shape and irregular shapes (Figure 1).

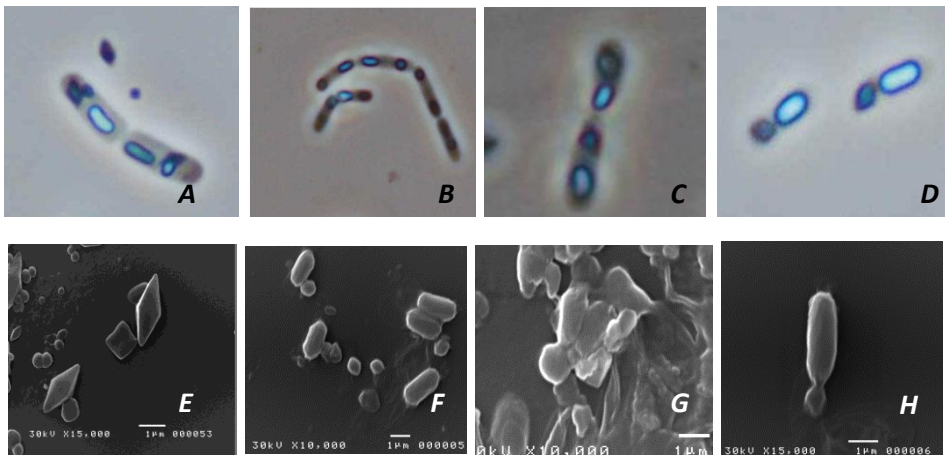

Figure 1- Crystals Shape under phase contrast microcopy and Electron Microscope. A,E) Bipyramidal; B,F) Spherical; C,G) Amorphous; D,H) Associated to spore crystals.(irregular).

3.3 Distribution of Bt in São Miguel Island

The distribution of Bt was not related with the microbial load of the sampling zone. In the volcanic complex of Sete Cidades the microbial load was

very low and the presence of Bt almost inexistent as for these areas. We had to double the initial weigh of soil before dilutions to achieve five colonies per soil sample whereas in the central part of the Island (Lagoa and Ribeira Grande) where a high microbial load was registered the presence of Bt was equally not observed (Figure 2).

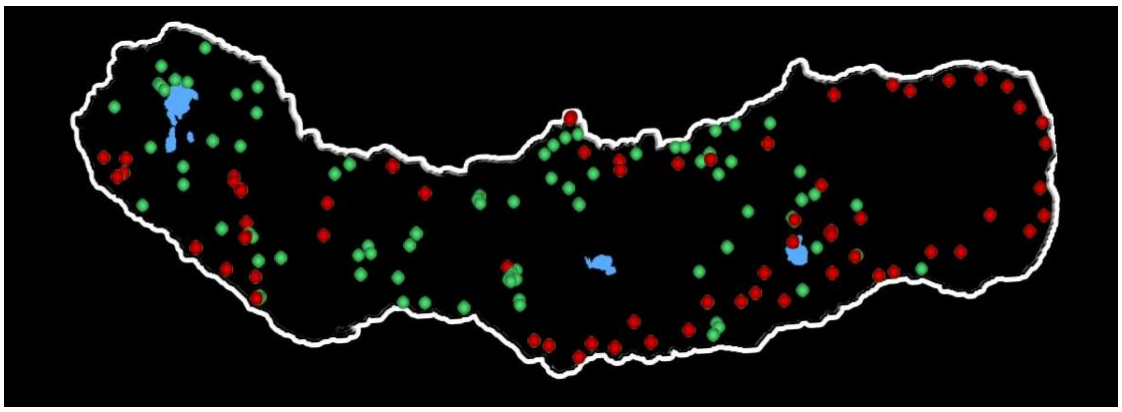

Figure 2 - Geographic distribution of soil samples in São Miguel Island (green-soil samples; Red-positive Bt soil samples)

3.4 Distribution of Bt by habitat

The distribution of Bt was not related with the microbial load of the sampling zone. In the volcanic complex of Sete Cidades the microbial load was very low and the presence of Bt almost inexistent as for these areas. We had to double the initial weigh of soil before dilutions to achieve five colonies per soil sample whereas in the central part of the Island (Lagoa and Ribeira Grande) where a high microbial load was registered the presence of Bt was equally not observed (Figure2).

Table 4 shows the distribution of the 248 soil samples positive to Bt in S. Miguel. It can be seen that samples were distributed by 10 different habitats considering the soil or water source and the surrounding vegetation. In semi-intensive pastures, the samples were collected in a wasteland or abandoned fields. The intensive pastures were relative to intensive cattle farming fields for dairy or beef production. In the cultivated fields of corn crop rotation between corn and beet. In cultivated field horticulture, we could find several different crops like cabbage, tomatoes, beans, pumpkins, watermelons and lettuce. In soil samples collected in the habitat woodland, we can find cryptomeria trees,

moss and brambles. In orchards soil samples, the surrounding vegetation was manly exotic flowers and trees, in the habitat hedge rows, there were *Hedychium gardnerianum*, *Pittosporum undulatum* and *Hydrangea macrophylla*. The water ponds and manure are relative to small stagnated water zones or cattle fountain and cow cattle manure, respectively. The last habitat corresponds to hot spring water and sediments that were collected in high volcanic activity zones most of them with temperatures above 50ºC. Orchards are the habitat where the Bt recovery samples, was higher with 83.3%, followed by hedge rows with, 67.6% and intensive pastures with 65.5%.

Table 4- Distribution of Bt by habitat in the Island of São Miguel.

Habitat	Nº of total samples	Nº of total sporulation colonies analyzed	No. of colonies with crystal	*Samples with Bt recovery (%)
Herbaceous cover				
Semi-intensive pastures	59	327	26	27,1
Intensive pastures	55	255	91	65,5
Cultivated Fields-Corn	8	36	9	50,0
Cultivated Fields-Horticulture	6	29	3	50,0
Woody plant cover				
woodland	16	67	19	56,3
Orchards	6	33	13	83,3
Hedge Rows	34	157	45	67,6
Particular habitats				
Waterponds	9	51	6	33,3
Manure	9	46	16	55,6
Hot spring water and soil	12	61	16	33,3
TOTAL	216	1066	248	50,9

*(the ratio between number of positive Bt soil samples in a particular habitat/number of total soil samples from that habitat)

The Bt index in different habitats is shown in Figure 5. The major Bt index was found in the orchards with 0.39, followed by the intensive pastures with 0.36 and by manure with 0.35.

3.5 Occurrence of Bt crystal shape by habitat

The occurrence in each habitat of particular crystal shape is shown in Figure 6. Bipyramidal crystals are predominant in the intensive pastures and hedge rows with 37% and 16%, respectively. Furthermore, the spherical crystals are predominant in these habitats with 38% and 23% respectively. Regarding the amorphous crystals, they are predominant in semi-intensive-pastures with 32% followed by intensive pastures and hedge rows, both with 26%. The associated to spore crystals only occurred in intensive pastures 67% and in semi-intensive pastures 33%. In addition, it was analyzed the occurrence of crystal morphology per habitat (Figure 7). In an herbaceous cover, the most predominant crystal shape was the bipyramidal crystals except in corn fields, where the spherical crystals assume the leadership with 56% against and the bipyramidal crystals have only 44%. Concerning horticulture fields only bipyramidal and amorphous crystals were found. In the other three habitats groups, the bipyramidal crystals are the predominant.

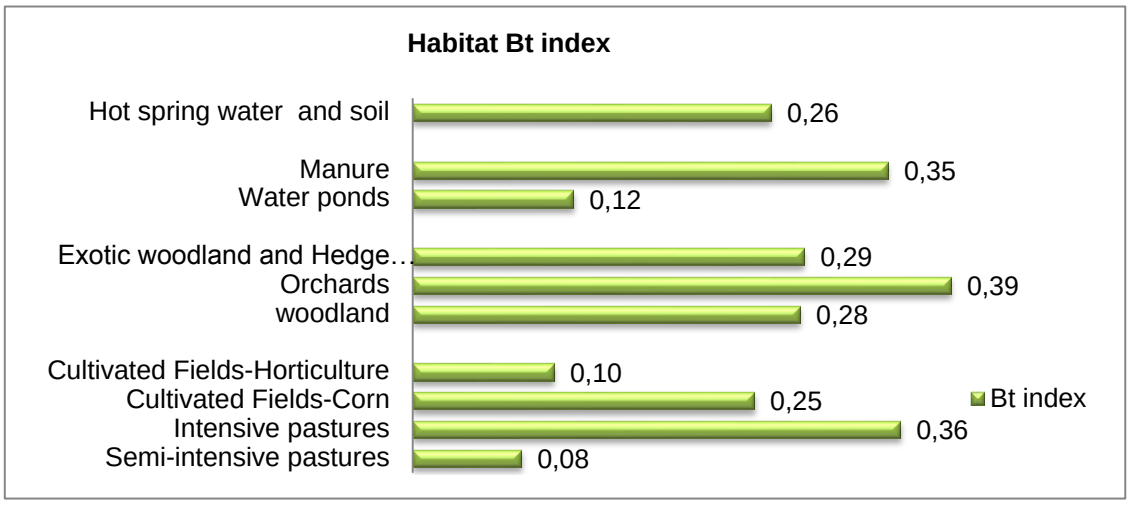

Figure 5 - Bt distribution index by Habitat number of colonies with crystal/ number of total colonies analyzed (number of colonies with parasporal inclusion body in a particular habitat/total of sporulating colonies analyzed in that habitat)

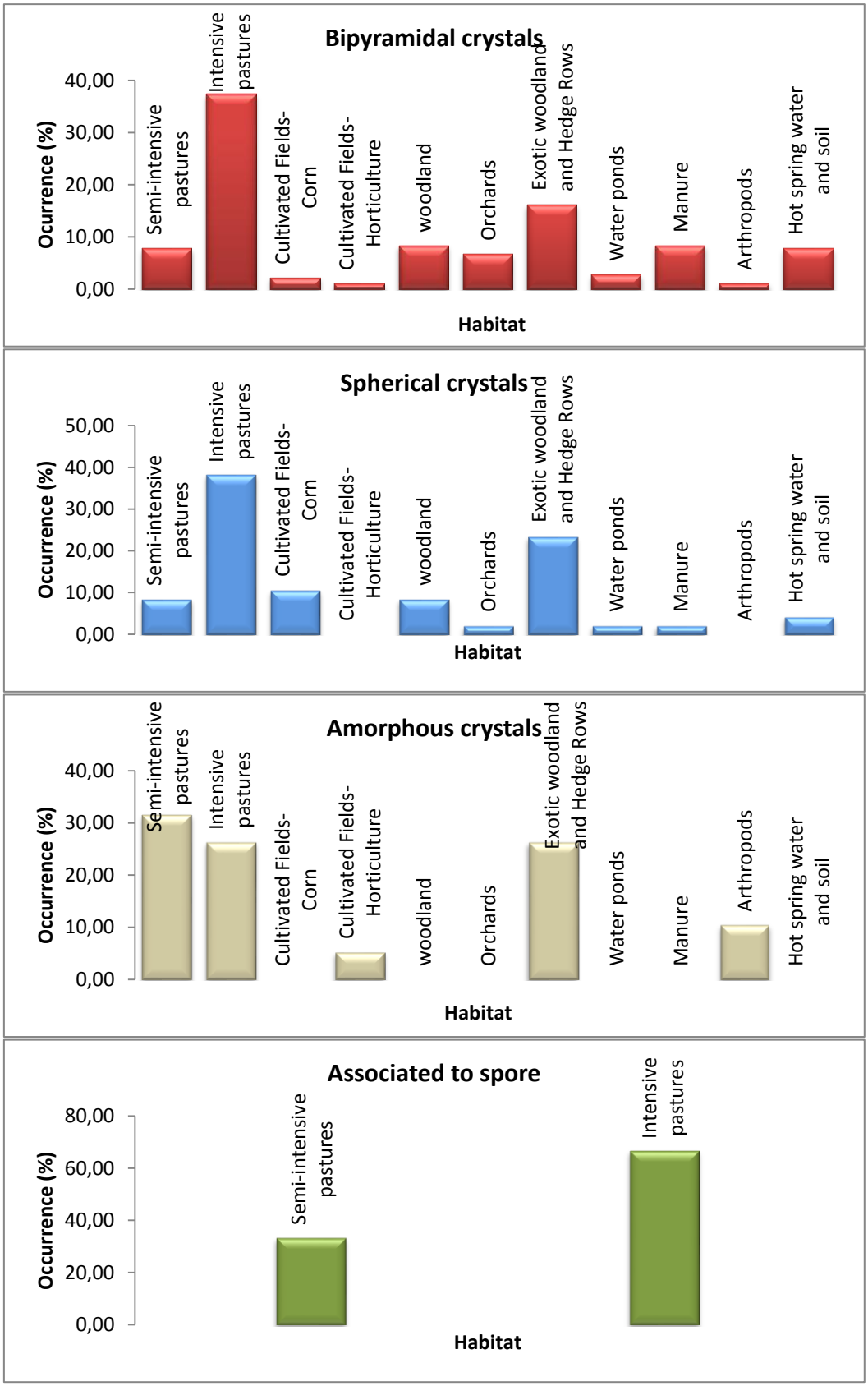

Figure 6 - Occurrence percentage of Bt crystal shape by habitat in the Island of São Miguel (the ratio between number of a particular crystal shape in that habitat and the total number of that particular crystal shape)

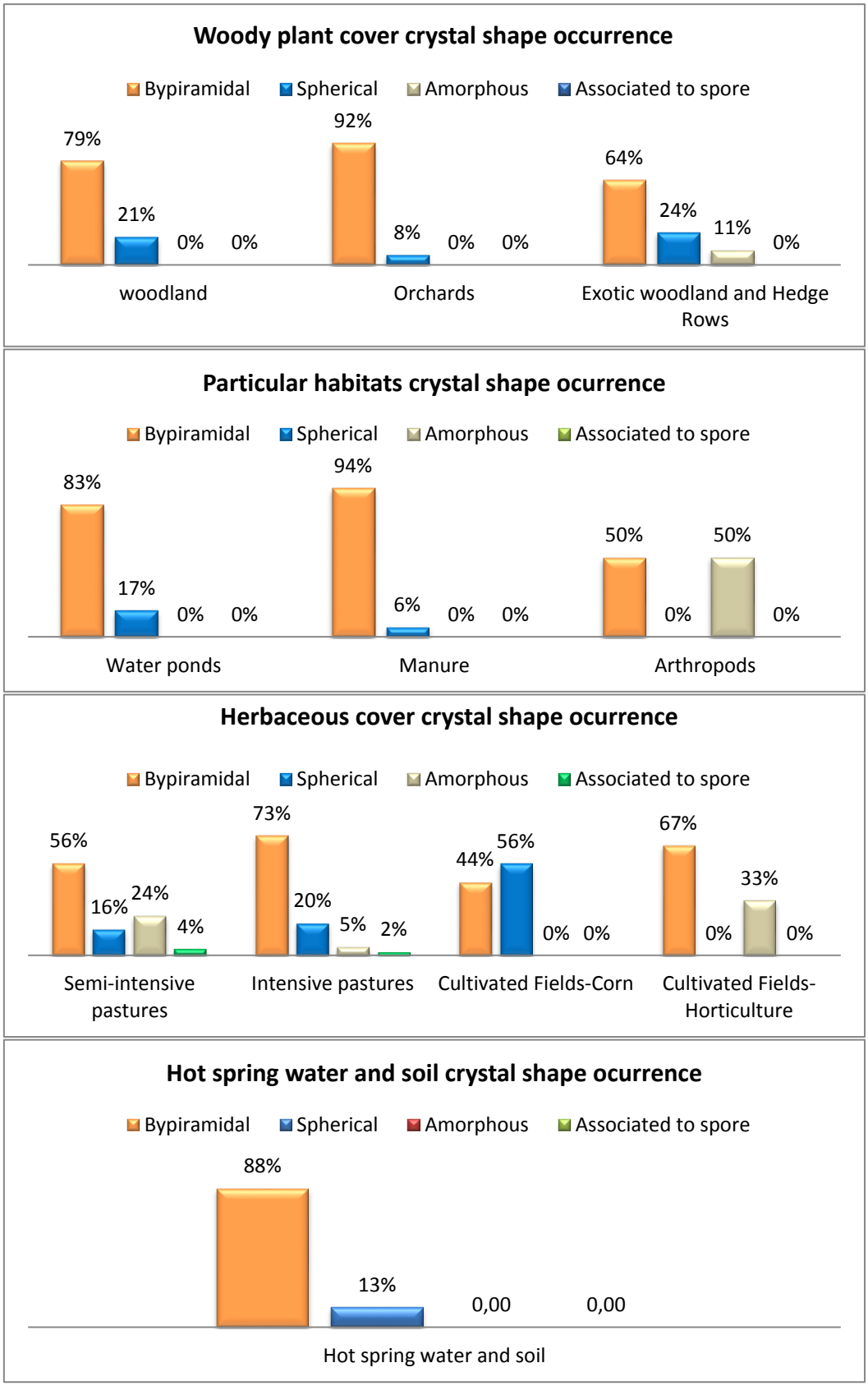

Figure 7 - Crystals morphology index by Habitats (the ratio between the number of particular crystal morphology in that habitat and the total of crystals found in that habitat)

3.6. The distribution of crystal morphology by altitude

Figure 8 allows concluding that in São Miguel, the Bt crystal shapes are differentially distributed by altitude. The bipyramidal, spherical and amorphous crystals are predominant in low altitude (0 to 300 meters), whereas associated to spore crystals are more frequent in intermediate altitudes (301 to 600 meters). In the zone between 0 to 300 meters, which is considerate with predominance in agricultural fields the most frequent crystal shape is bipyramidal like it in the altitudes from 301 to 600 considered with predominance of pastures and above 600 m, the zone of wild woodland (Figure 9).

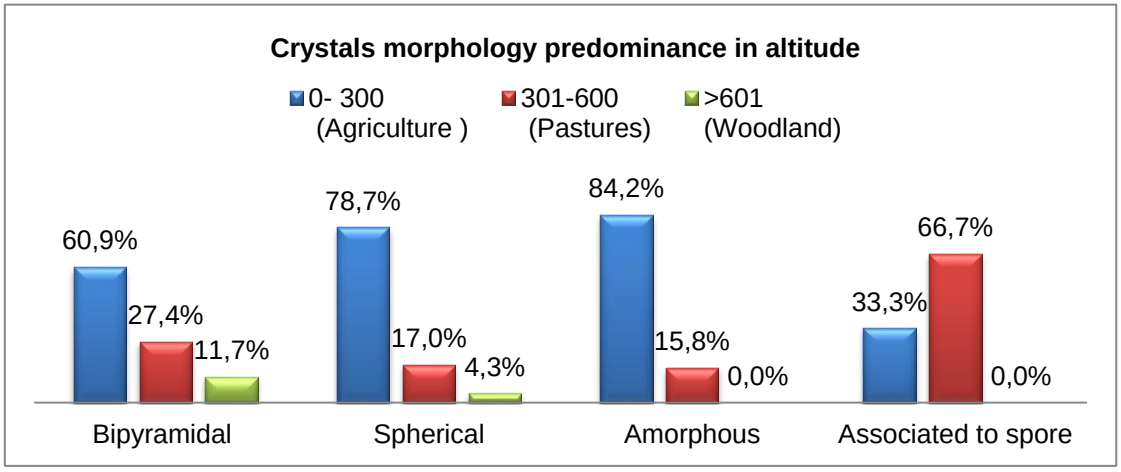

Figura 8 - Crystals morphology distribution by altitude (the ratio between the number of a particular crystal shape by altitude interval and the total number of that particular crystal shape).

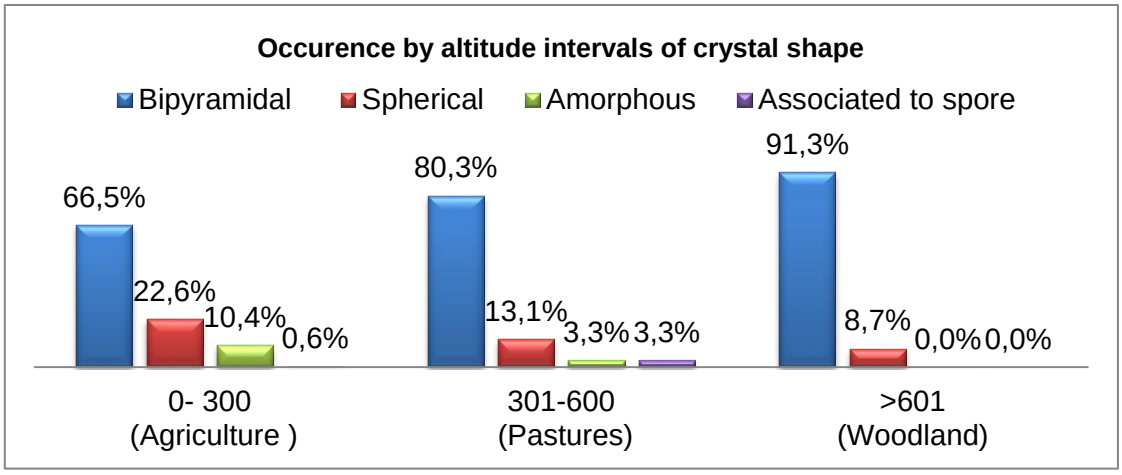

Figure 9 - Occurence by altitude intervals of crystal shape (The ratio of the total number of a particular crystal shape by the sum of all the crystals in that altitude interval)

3.7. Selection of Bt *isolates active against Lepidoptera*

The selection of a native Bt isolates to use as a biopesticide was performed based in the mortality efficiency against E. kuehniella. In the initial screening 238 Bt isolates were tested against third instar larvae. Figure 10 shows that 56 isolates caused more than 75% mortality. This mortality started between 24 and 48 hours after the application of the crystals and spore mix and reach the peak at 72 hours. All the 56 isolates had crystals with bipyramidal shape.

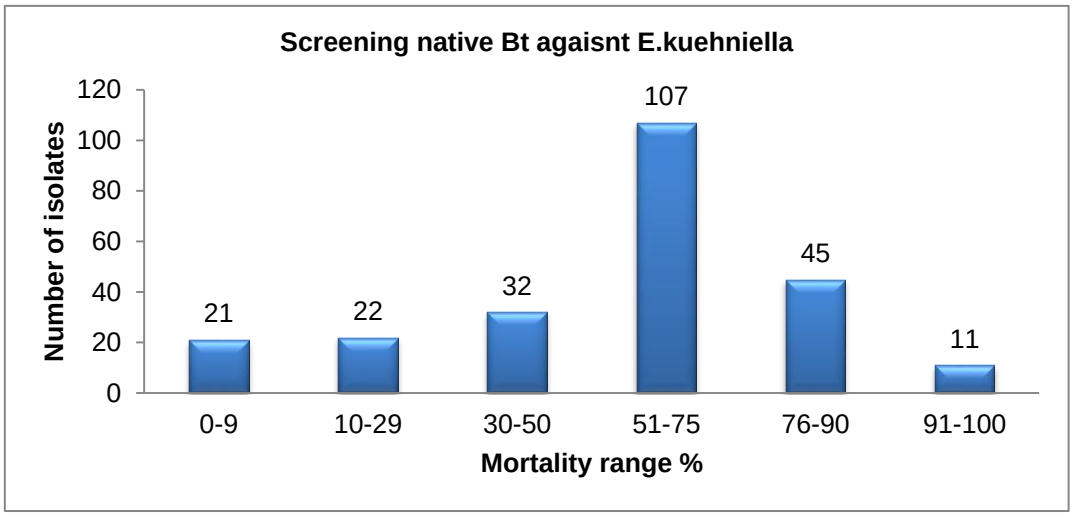

Figure 10 - Screening and mortality range of the 238 bipyramidal isolates against *E.kuehniella*

To gain a better understanding in the virulence of these isolates, they were submitted to an evaluation of LD50 against the same insect. It is well known that the virulence of Bt isolates is due to the proteins constituting the crystal. Therefore, to exclude redundancy in this assay, we determine the protein profile in each of the 56 isolates by SDS-PAGE (Figure 11).

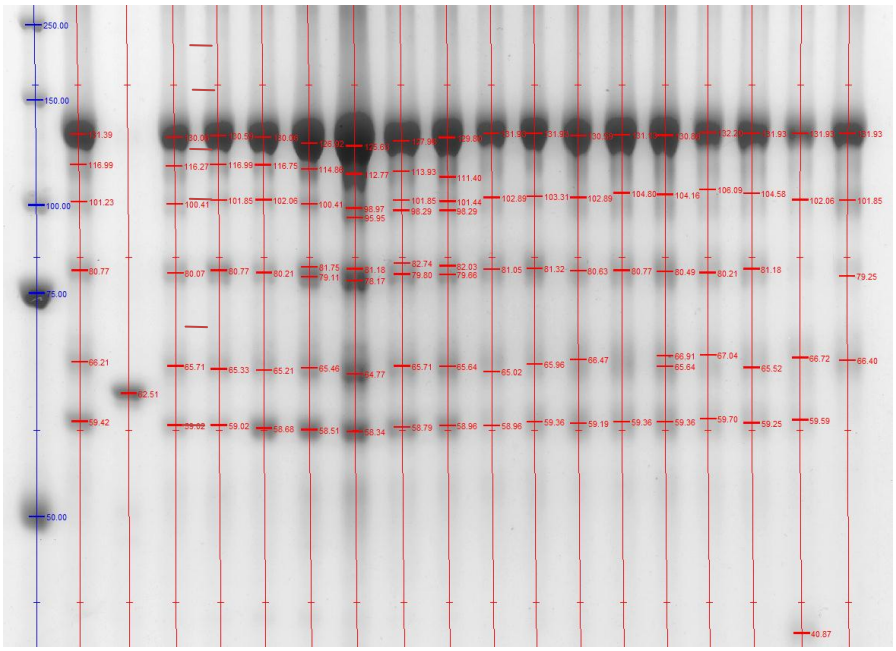

Figure 11 - SDS-PAGE protein crystal profiles

Protein profiles of the 56 isolates revealed three different patterns the group 1 including 36 isolates had two major polypeptides with molecular weights of 130 kDa and 65 kDa. These two dominant proteins usually correspond to Cry1 and Cry2 pro toxins, which are known to be active in Lepidoptera. Another protein profile was formed with 17 isolates that was named group 2. Two major polypeptides, with 110 kDa and with 55 kDa were detected. The last protein profile grouped 2 isolates that showed three major bands, 130 kDa, 65 kDa and 40 kDa. In the three protein profile groups, it can be found several minor polypeptides as shown in Table 5.

Table 5- Molecular weight of protein crystals

Isolate	SDS-PAGE groups	Protein molecular weight (KDa) selection base	Number of isolates
S170a	1	130;100;90;72;65;27;26;20	36
S69c	2	110;77;75;70;62;40;20	17
S170c	3	130;100;90;70;65;60;50;45;40	2

Figure 12 presented the distribution of the proteins by size of 56 isolates that had mortality above 75%. These isolates had crystals proteins with molecular mass from 20 to 130 kDa and all the isolates produced more than

one band. We can also conclude that 100% of the isolates presented one polypeptide in the interval of 100 to 140 kDa. 98% of the isolates presented one or more polypeptides in the interval of 50 to 80 kDa. In the interval of 21 to 30 kDa 66% of the isolates presented several polypeptide bands and in the interval 17 to 20 kDa only 30% of the isolates presented one or more polypeptides. We can see the occurrence of the band sizes intervals in the 56 isolates that had mortality above 75%. This group of isolates produced proteins with molecular mass from 20 to 130 kDa and all the isolates produced more than one band. From the SDS-PAGE profile, we can see that 100% of the isolates presented one polypeptide in the interval of 100 to 140 kDa. 98% of the isolates presented one or more polypeptides in the interval of 50 to 80 kDa. From the interval of 21 to 30 kDa 66% of the isolates presented several polypeptide bands and from the interval 17 to 20 only 30% of the isolates presented one or more polypeptides.

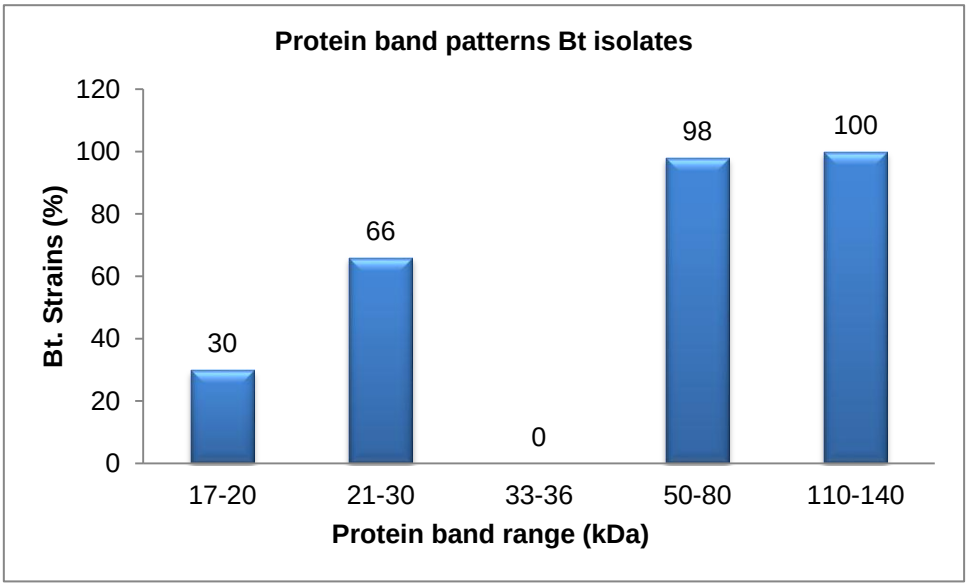

Figure 12 - Protein band pattern in the isolates with mortality above 75% in *E.kuehniella*

In Figure 13, we can see the geographic distribution of the 56 isolates with mortality above 75% in Ephestia by their SDS-PAGE groups. Worth being noted that the isolates belonging to group 1 were collected all over the island with predominance in the east part. The group 2 isolates are mostly from the southern central part of the island and from the east end of the island. The

group 3 was collected only in the east end of the island in a restricted zone as can be seen in the map.

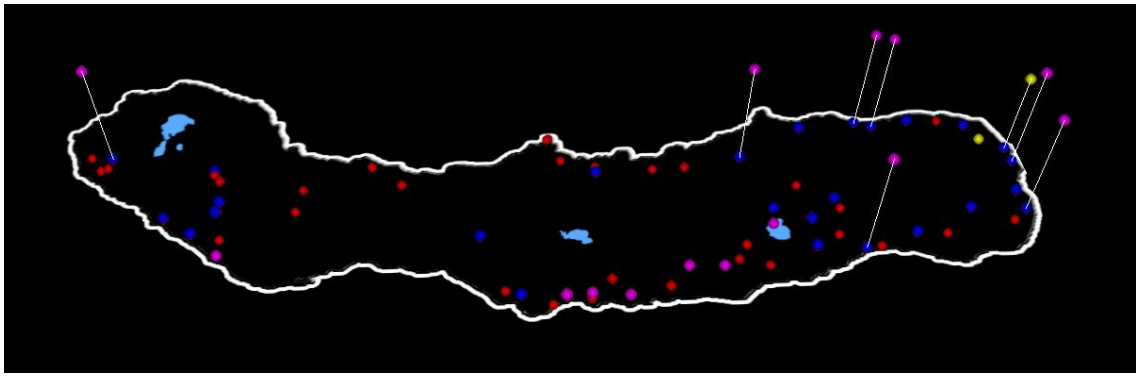

Figure 13 - SDS-PAGE profiles groups geographic distribution of the 56 isolates with mortality above 75% (Red dots – positive Bt samples; blue dots- group1; Pink Dots-group 2; yellow dots-group 3).

3.8 Selection of the Bt isolate with the lowest LC50

For the growth test in the sludge subtracts it was selected the native Bt isolate with the lowest LC50 against E. kuehniella, to ensure the efficiency of the bio-pesticide produced. The *B. thuringiensis kurstaki* was used as a reference to compare the mortality rates. We compare the LC50 of one isolate of each SDS-PAGE group. The isolate belonging to group 1 had the lowest LC50 (9.2 µg/ml) among the native isolates. Therefore, isolate S170a was selected to grow in the sludge.

3.9 Evaluation of Bt growth in sewage sludge

Having in consideration that the sludge nature is certainly an influence factor for the growth of microorganisms it was selected four different sources of sludge to growth the native Bt S170a. Most important parameter in sewage sludge that influences growth and entomotoxicity of Bt is the total solids concentration (Lachhab, 2001; Chang, 2007; Yezza, 2005). It is postulated that this value is optimal at around 26 g per liter. As we can see in Table 7 the source of sludge with the highest determined total solids concentration was IDWWTP-M with 12 g per liter and the lowest the UWWTP sludge with 8 g per liter. Therefore, the total solid concentration in all the sludge was set to 26 g.

After the total solid set, and the alkaline pretreatment finished the Bt growth was initiated, and the growth curves registered. Entomotoxicity was tested at the end of Bt growth.

Table 7- Total solid calculation results for secondary waste water sludge from the four WWTP

Sludge source	Determined Total solids (g/l)	Ideal Total solid (g/l)*
ISWWTP	10	26
IDWWTP-M	12	26
IDWWTP-B	6	26
UWWTP	8	26

Analyzing the growth curves in Figure 14 we can see similar growth patterns between the different sludge subtracts for the 84 hours. This growth pattern differed from the TSB medium, except for the first eight hours in which all growth curves were very identic. After the initial eight hours, we can notice differences in total viable cell counts between the five subtracts.

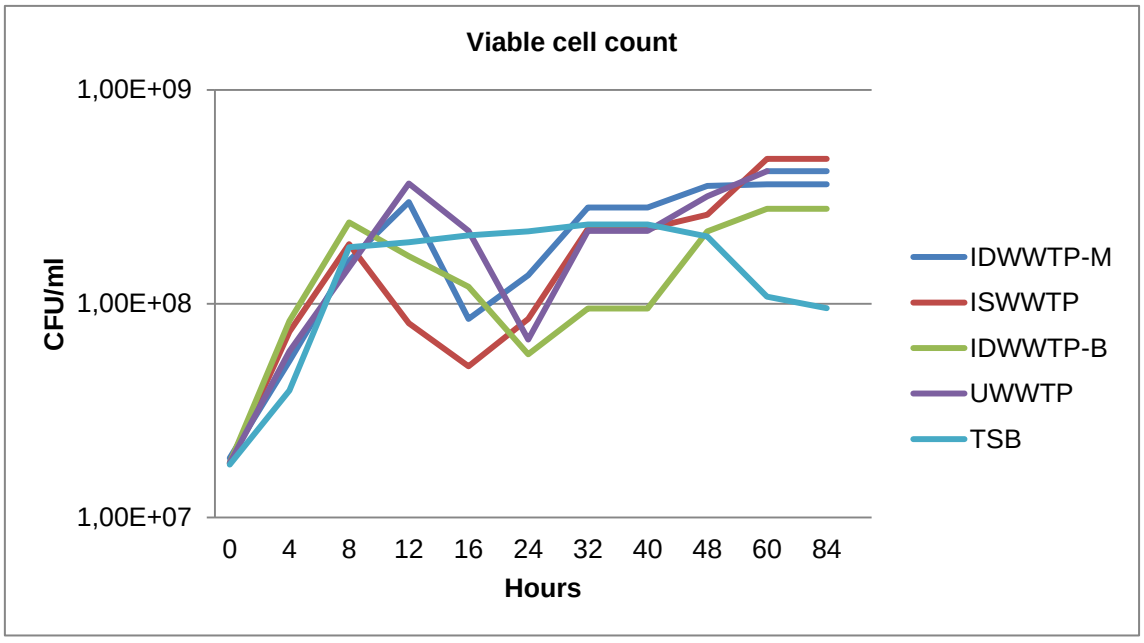

Figure 14 - Growth curves of viable cell count for the 5 different substrates used for Bt growth.

The final of fermentation (84 hours), the substrate with the highest viable cell count was the sludge from the ISWWTP with 4.76 x 10^8 CFU/ml. In second place was the sludge from UWWTP with 4.17 x 10^8 CFU/ml. The third subtract was from the IDWWTP-M with 3.62 x10^8 CFU/ml the fourth was the IDWWTP-B with the value of 2.78 x 10^8 CFU/ml. The lowest viable cell count after the 84 hour was the TSB medium with 2.78 x10^8 CFU/ml (Table 8).

Table 8- Total initial and final cells viable counts and maximum specific growth rate.

Sludge source	Initial total Viable cell count (CFU/ml)	Final total Viable cell count (CFU/ml)	Maximum specific growth rate (μ h^{-1})*
ISWWTP	1.80x10^7	4.76x10^8	0.2946
UWWTP	1.80x10^7	4.17x10^8	0.2508
IDWWTP-B	1.80x10^7	2.78x10^8	0.3238
IDWWTP-M	1.90x10^7	3.62x10^8	0.2297
TSB	1.77x10^7	9.53x10^7	0.2927

* The maximum specific grow rate was calculated to the end of exponential grow phase

3.10 Evaluation of Bt sporulation

Figure 15 and Table 9 shows the data scored in spore count in the five different substrates used. As it can be seen the beginning of the sporulation was different among them. In the industrial medium TSB and in the sludge from IDWWTP-M, the sporulation started at 12 hours, this was a difference of four hours relative to another sludge. The sludge achieving the highest number of spores was ISWWTP with 2.16x10^8 CFU/ml.

Table 9- Total viable cell count and maximum specific growth rate.

Sludge source	Final Spore count (CFU/ml)	Sporulating rate (%)
ISWWTP	2.16x10^8	45
UWWTP	1.64x10^8	39
IDWWTP-B	1.29x10^8	47
IDWWTP-M	1.39x10^8	38
TSB	6.23x10^7	65

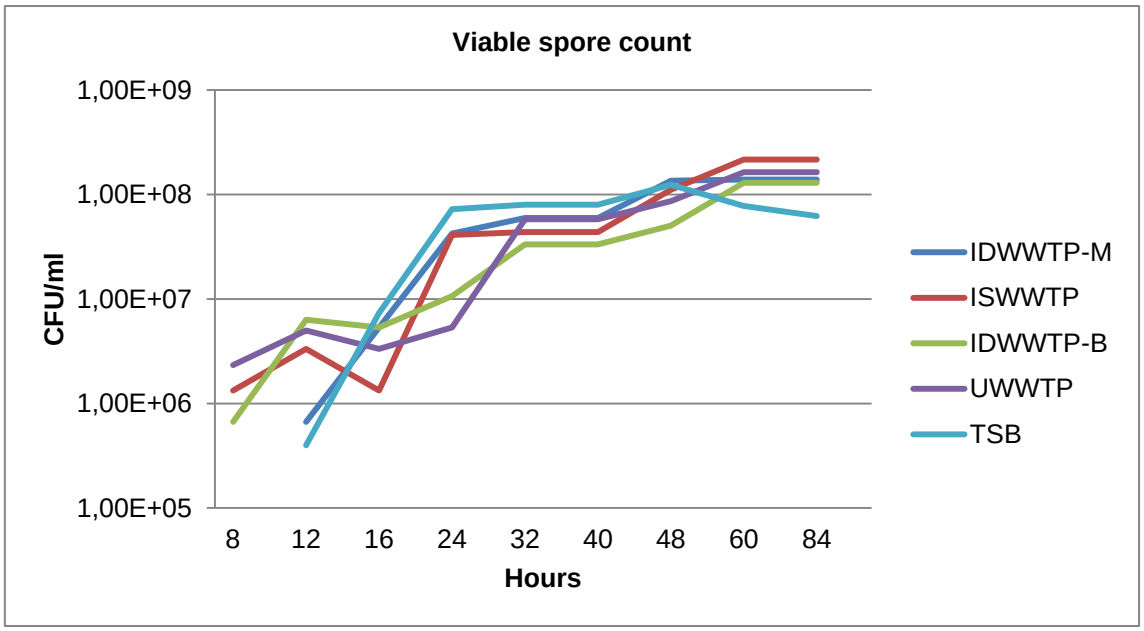

Figure 15- Sporulating curves for the 5 different substrates used for Bt growth.

3.11 Evaluation of Bt toxicity

Bioactivity of Bt S170a grown in the different sewage sludge was evaluated by determining the LC50 at 72 hours (Table 10). The lowest LC50 was achieved in Bt produced in TSB (LC50 = 48.46 µg/ml) however, this value was not significantly different from those of the Bt grown in ISWWTP (LC50 = 58.30 µg/ml). The highest LC50 was observed in Bt grown in IDWWTP-B (LC50 = 181.72)

Table 10 - Estimated LC_{50} (96 hours) concentrations of isolate S170A grown in sludge, against larvae of *Ephestia kuehniella*.

Sludge source	N^{β}	$LC_{50\ (95\%\ CL)}{}^{\alpha}$	Slope ± SE	X^2
ISWWTP	30	58.30 [a] (31 - 94,25)	1.25 ± 0.40	0.511
UWWTP	30	69.43 [ac] (34,26 - 157,55)	0.90 ± 0.23	1.629
IDWWTP-B	30	181.72 [bc] (97,34 - 31798,88)	0.96 ± 0.41	0.007
IDWWTP-M	30	117.92 [ac] (73,97 - 507,89)	1.15 ± 0.40	0.908
TSB	30	48.46[-]	2.8 ± 0.82	-

$^{\beta}$ Number of larvae test excluding controls

$^{\alpha}$ LC_{50}= Lethal Concentration, 50% values and 95% confidence limits (CL) expressed in number of µg/ml of protein required to kill the larvae. LC values within a column followed by the same letter are not significantly different based on non-overlapping 95% CL.

4. DISCUSSION

The aim of this work was to produce a bio-pesticide based in a native isolate of *B. thuringiensis* using waste water sludge. Moreover, this work allowed us to isolate Bt and to characterize 238 Bt isolates that was preserved in Azorean *B. thuringiensis* collection. This investigation shows for the first time, the morphological diversity, geographic distribution, and predominant habitats of Bt in São Miguel Island. This data can be useful in pest management for bio-control as the bacteria can be found associated to specific habitats were some insect hosts are usually present (Hernandez and Ferre, 2009).

Two hundred and sixteen soil samples were collected from wide-range vegetation coverage, covering intensive pastures, semi-intensive pastures, corn fields, horticulture fields, woodlands, orchards, hedge rows, water ponds, manure and hot spring soils and waters. Also the altitude of sampling places was highly variable ranging from 44 to 769 meters above sea level. Bt strains were found in 110 of the 216 soil samples (Table 2). This result gives us a Bt recovery percentage of 50.9, suggesting a wide dispersal of Bt in the Island. After colonies isolation from soil samples, a total of 1066 sporulating colonies were obtained. The observation of these colonies allowed us to select 248 colonies with crystal inclusions, giving a Bt index of 0.23, which indicates the Bt presence relative to other spore-forming bacteria in Azorean soils. This index is 3 fold higher than the Bt soil sampling performed in Canaries Islands (Quesada et al., 2004). Regarding the habitats where the Bt recovery was the highest, we can conclude that the highest prevalence of Bt was in orchards and intensive pastures.This finding, suggest that in these habitats, Bt founds an ideal environment for proliferation, which could be due to a high host population density.

The identification of Bt isolates was fundamentally based in the position of endospore and in the presence of crystal inclusions, by contrast phase microscopy. By M.O., we identified four different crystal morphology shapes that were confirmed by scan electron microscopy. The most common crystal morphology found was bipyramidal representing 72.2% of the crystals in the Island. These bipyramidal crystals are geographic distributed all over the Island,

mainly in intensive pastures and hedge rows. Knowing that this crystal shape usually enclosure Cry proteins with specificity to Lepidoptera insects (Frankenhuyzen, 2009), we can attribute the high density of bipyramidal crystals to the presence of several Lepidoptera potential hosts like. One of the most frequent Lepidoptera is *Mythimna unipuncta* that usually reaches high densities, particularly in pastures, becoming a pest with elevated economic impact in the Azores (Carneiro, 1979). This conclusion is supported by our finding that showed 86% of bipyramidal crystals found in the intensive pastures and hedge rows caused above 50% mortality in *E. kuehniella*. Furthermore, we observed that 60.9% of bipyramidal crystals were found between 0 and 300 meters of altitude, certainly the most favorable areas for insect development due to temperature.

The second and third most frequent crystals in the Island are spherical crystals and amorphous with 19 and 7.7 % of occurrence, respectively. With less representation are the associated to spore crystals with only 1.2%. The spherical isolates were distributed mostly by intensive pastures and hedge rows and had no expression in the samples collected in horticulture cultivated fields. We can hypothesize that this distribution is also related with host densities. In fact, it is known that spherical crystals usually have insecticidal proteins against Diptera and most of the Diptera in Azores must be associated to cattle, which is reared in pastures. Therefore, we speculate that the prevalence of this crystal shape in pastures is due to the presence of susceptible hosts. Amorphous crystals seem to have a preference for abandoned fields as they are predominant in semi-intensive pastures and hedge rows. Crystals associated to spore have very particular habitats as they are restricted to semi-intensive and intensive pastures only.

Checking the prevalence of the crystal shape by habitats we saw that bipyramidal crystals were predominant in almost all habitats, including water ponds, where the spherical were expected to be predominant (Frankenhuyzen, 2009). In water ponds, habitat's bipyramidal crystals occurrence was 6 fold higher than the spherical shape (Figure 7). Spherical isolates were predominant over bipyramidal in corn cultivated fields suggesting the presence of a Diptera in this particular habitat as a suitable host despite it has not been identified so far.

Relatively, to altitudes the spherical and amorphous crystals are predominant below 300 meters. Only the associated to spore crystals are preferentially found in middle altitudes from 301 to 600 meters (Figure 8).

All the isolates collected in São Miguel were submitted to a screening against *E. kuehniella* with the aim of selecting the isolate for growth in the sludge. 56 isolates killed above 75% of insects in 96 hours, all of them with bipyramidal crystals. Eleven of this isolates had a mortality activity above 91% that was higher than the mean mortality (77%) of *B.thuringiensis Kurstaki*.

Using SDS-PAGE to avoid redundancy as suggested by Vidal (2009), we grouped this 56 isolates in three protein groups. All groups had bands between 110 and 140 kDa, which are associated to Cry 1 (Valicente et al. 2010) and a band between 50 and 80 kDa that is usually encoded by *cry*2 (Valicente et al. 2010). The differences in the molecular weight of these major proteins allowed us to form group 1 and group 2 profiles. A third major band of 40 kDa allowed us to form group 3. These molecular weights differences indicate variations in the respective encoding *cry* genes that were confirmed later by the identification of different subfamilies of *cry1* and *cry2* genes (personal communication Ferreira, 2011).

Based in the lowest LC50 against *E. kuehniella* we selected the isolate S170A, which belong to the protein group 1 profile and to the group of isolates that killed more than 91% in *E. Kuenhiella* on the initial screening.

Many parameters can influence the Bt growth in sludge subtracts. Variables like physical and chemical constitution, total solids concentration, available oxygen, initial volume inoculum, or temperature are common to limit bacterial growth. In this batch fermentations ideal parameters for Bt growth referred in previous publications were used (Lachhab, 2001; Chang, 2007; Yezza, 2005; Zhuang, 2011). Some of these parameters we maintained equal between production sludge mediums, so the only aspect changing was the nutritional and chemical characteristics of the different sludge. All the sludges were submitted to an alkaline pretreatment. The pretreatments greatly enhance the availability of nutrients and hence increasing Bt growth and entomotoxicity by 40% relatively to non-pretreated sludge (Chang, 2007). Taking that in consideration, all the four sludges were submitted to a pretreatment. Other

maintained parameter was the total solids concentration sated at 26 g/l. The solid concentration is highly related to oxygen availability in the sludge culture medium, the most relevant parameters in Bt growth (Lachhab, 2001), Low concentration of the total solids would elevate the oxygen presence in the medium but lower its nutritional values, essential for Bt metabolism growth. The upper values of total solids should increase the nutritional value lowering the oxygen. Low concentration of oxygen will result in low cell concentration, sporulation and entomotoxicity. The inoculum type medium used and the percentage of inoculum are also very important. It was used for the inoculum a TSB and sludge medium mix with 10 hours of incubation. The use of this type of inoculum mix allowed us to eliminate the lag phase in production medium culture. The use of relatively small or large amounts of initial inoculum results in low entomotoxicity. A high concentration of an initial cell culture medium can result in a rapid consumption of oxygen and nutrients, thus allowing the low growth and cell concentration (Lachhab, 2001).

The incubation temperature and initial pH in the production culture mediums was set equal in all cases. The base for the selection of one of this tested sludge for a bio-pesticide production was first its capability of producing a high entomotoxicity related to the capability of producing an elevated number of viable cells, suggesting which sludge has more nutritional values for Bt growth. After the 84 hours of fermentation, we concluded that all the four different sludge sources used in this work were suitable or had enough minimal nutritional value for Bt growing (Figure 14). Beside the best specific growth rate and sporulation rate using IDWWTP-B sludge the LC50 of produced Bt was the highest (Table11) , thus excluding it in this work as an suitable sludge for a bio-pesticide production, although taking this data in consideration the future optimization of this sludge characteristic for optimal LC50 achievement is a possibility.

Table 11 – Resume of the 5 tested medium for their specific growth rate sporulation rate and their LC50

Sludge source	Initial total Viable cell count (CFU/ml)	Final total Viable cell count (CFU/ml)	Maximum specific growth rate(μh^{-1})*	Final Spore count (CFU/ml)	LC50% (µg/ml)$^{+}$	Sporulating rate (%)
ISWWTP	$1.80x10^{7}$	$4.76x10^{8}$	0.2946	$2.16x10^{8}$	58.30	45
UWWTP	$1.80x10^{7}$	$4.17x10^{8}$	0.2508	$1.64x10^{8}$	69.43	39
IDWWTP-B	$1.80x10^{7}$	$2.78x10^{8}$	0.3238	$1.29x10^{8}$	181.72	47
IDWWTP-M	$1.90x10^{7}$	$3.62x10^{8}$	0,2297	$1.39x10^{8}$	117.92	38
TSB	$1.77x10^{7}$	$9.53x10^{7}$	0,2927	$6.23x10^{7}$	48.46	65

*The maximum specific grow rate was calculated to the end of exponential grow phase

$^{+}$At the end of 84 Hours fermentation

The sludge that was able to produce Bt with the lowest LC50 (58.30 µg/ml) was ISWWTP. LC50 in this sludge compared with that in TSB did not show significant differences. This sludge allowed the production of the most elevated viable cell count (4.76×10^{8} CFU/ml), which represents an increment of 20% relatively to the commercial medium (TSB). In this sludge Bt has a specific growth rate of 0.2946 that is very close to the one observed in TSB (0.2927). These factors were enough for supporting the use of this sludge for a possible scale up using a fermenter.

The sporulation rate in the ISWWTP sludge was considerable lower than in TSB. This could be due to the presence of some heavy metals or reduced oxygen concentration during the fermentation process (Zhuang, 2011). Both limitations can be overcome. The low-oxygen concentration in a fermenter can be overcome by controlling aeration. On the other hand, heavy metals concentration can be reduced by using iron and sulfur-oxidizing bacteria, once these bacteria are capable of reducing heavy metals like Cr, Cu and other heavy metals that usually affect Bt sporulation rates (Chan, et al. 2003).

The physical and chemical analyses to the sludge after fermentation with Bt is an important parameter to conclude if they meet the agricultural limit values for use as and fertilizer in forest and agricultural soils. Although this analysis was not performed in this work it should be done in a future work. If this

parameters meet the environmental safety parameters then we can use the results of this fermentation as a soil fertilizer.

This work involved two components: the selection of a native Bt with high activity against Lepidoptera and the production of the native isolate in amounts and with activity that supports its use as a bio-insecticide. Resulting from a wide sampling in S. Miguel we selected an isolate that has toxicity comparable to commercial Bt. Using four different sludge we selected one in which the native isolate could grow, to form spores and Cry toxins in a similar rate to the synthetic growth medium. Moreover, this work gave insights in the local diversity of Bt and in the possibility to profit on local wastes to control many important insect pests in Azores, namely the armyworm, *Mythimna unipuncta*.

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