Evaluation of a cereal milling by-product for the low cost production of *Bacillus thuringiensis kurstaki* in submerged fermentation

Jihane Rahbani Mounsef, Dominique Salameh, Mireille kallassy Awad, Cedric Brandam, Roger Lteif

Jihane Rahbani Mounsef
Université de Toulouse ; INPT, UPS; Laboratoire de Génie Chimique; 4, Allée Emile Monso, F-31030 Toulouse, France.

CNRS ; Laboratoire de Génie Chimique ; F-31030 Toulouse, France

Université Saint Joseph de Beyrouth B.P. 11-514 Riad El Solh Beyrouth 1107 2050 Liban

Dominique Salameh
Université Saint Joseph de Beyrouth B.P. 11-514 Riad El Solh Beyrouth 1107 2050 Liban

Mireille kallassy Awad
Université Saint Joseph de Beyrouth B.P. 11-514 Riad El Solh Beyrouth 1107 2050 Liban

Cedric Brandam
Université de Toulouse ; INPT, UPS; Laboratoire de Génie Chimique; 4, Allée Emile Monso, F-31030 Toulouse, France.

CNRS ; Laboratoire de Génie Chimique ; F-31030 Toulouse, France

Roger Lteif
Université Saint Joseph de Beyrouth B.P. 11-514 Riad El Solh Beyrouth 1107 2050 Liban

Correspondence:
Jihane Rahbani Mounsef Université de Toulouse ; INPT, UPS; Laboratoire de Génie Chimique; 4, Allée Emile Monso, F-31030 Toulouse, France.

CNRS ; Laboratoire de Génie Chimique ; F-31030 Toulouse, France

Université Saint Joseph de Beyrouth B.P. 11-514 Riad El Solh Beyrouth 1107 2050 Liban

ABSTRACT
With the idea of finding a cheap medium for the mass production of *Bacillus thuringiensis* (*Bt*), a cereal milling by product (CMB) was evaluated and was shown efficient to be used as a source of carbohydrates, proteins and minerals for the production of δ-endotoxins in submerged fermentation of a new strain of *Bacillus thuringiensis kurstaki* "Lip". The results obtained in 1000 mL shake flasks experiments showed that the toxin proteins concentration produced in the 6% (w/v) CMB medium was 2.4 fold and 1.54 fold greater than the values reached in the Anderson medium and in a standard semi synthetic medium respectively, while there was no significant difference between the cell, spore and crystal counts or between the protein profiles of the "Lip" spore/crystal complex obtained in the three mediums. Considering all three factors, relative yield, productivity and cost, the use of the CMB mono-component medium was proved much more economical for an industrial production of "Lip" than the references mediums. Moreover, the suitability of the medium for large scale production of *Bt* based bio insecticide was evidenced in a 5 L lab bioreactor.

Keywords: *Bacillus thuringiensis*, Cereal milling byproduct, δ-endotoxins, Industrial production, Productivity

1. Introduction

Bacillus thuringiensis (*Bt*) based bio insecticides are the most broadly used microbial biological agents in the control of harmful insects [1, 2]. They represent natural, biodegradable and safe pest control means. *Bacillus thuringiensis* is a ubiquitous, gram positive, spore forming bacterium. Its insecticidal activity resides in the production of a proteinaceous parasporal crystal called δ-endotoxin, which is formed during sporulation. Despite the increasing need for environment friendly, chemical residue free and safe control agents such as *Bt* based biopesticides, the biopesticides market was valued in 2011 at $1.3 billion for $37.5 billion for the global pesticide market [3]. The use of bio-insecticides remains very limited compared to chemical insecticides for two main reasons: the production cost and the low stability of the product over time forcing applications more frequent. *Bacillus thuringiensis* requires, for its growth, sporulation and crystal synthesis, a carbon and energy source like glucose, nitrogen sources such as peptone or yeast extract and ammonium sulfate, and mineral salts [4, 5]. Metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} were found essential for *Bt* growth and potassium ions were showed necessary for the production of *Bt* crystals [6, 7]. In lab experiments, synthetic mediums with all these elements are generally used. However, at industrial scale, the synthetic and expensive substrates are usually replaced by agro-industrial by-products to reduce the cost of mass production of *Bt*. This approach allows also to process waste into valuable products and help dispose of these byproducts in an ecologically sound manner. Different agro-industrial residues and byproducts were used in *Bacillus thuringiensis* production. Soybean meal, corn steep liquor, groundnut seed meal extract, fish meal, gruel fish, cheese whey, *Bombyx mori* pupae, powders of edible leguminous seeds and fodder yeast were used as sources of proteins [8, 9, 10, 11, 12, 13, 14, 15] but carbohydrates (glucose, starch or molasses) and/or mineral sources were added. Cereal milling by products are available in many countries at low prices. They are used as sources of proteins, minerals, energy, carbohydrates, fat and fibers for commercial livestock feed [16].

In this work, we studied the efficiency of using a cereal milling byproduct (CMB) as a complete substrate for *Bt* production which could substantially reduce the culture medium cost. The cereal milling by-product (CMB) mono-component medium was investigated and compared to synthetic mediums in terms of δ-endotoxin yield and productivity. Production of the bio-insecticide in lab-bioreactor in controlled conditions was equally performed to give basic elements for extrapolation in industrial conditions.

2. Materials and Methods:

2.1 Microorganism

A bacterial strain *Bacillus thuringiensis* variety *kurstaki*, registered as Lep^{MK} 101, designated by 'Lip', isolated from the Lebanese soil and genetically characterized [17], was used. The strain was provided by the biotechnology laboratory of Saint Joseph University, Campus of Science and Technology, Lebanon.

2.2 Media composition

A "CMB" based medium

It contained a cereal milling byproduct (particles size < 200 mesh) as a mono component medium in distilled water. In the results section we indicate the ratio of the CMB (w/v) used in each experiment. The CMB was provided by *arcenciel* NGO; a sustainable agricultural lead developer in Lebanon.
Two mediums of literature were used as references:

2.2.1 A complete rich medium proposed by Anderson [18]

The medium contained: Glucose 9.77 $g.L^{-1}$; Yeast extract 4.62 $g.L^{-1}$; Bacto peptone 4.62 $g.L^{-1}$; $(NH_4)_2SO_4$ 1 $g.L^{-1}$; KH_2PO_4 3.4 $g.L^{-1}$; K_2HPO_4 4.15 $g.L^{-1}$; $MgSO_4.7H2O$ 0.3 $g.L^{-1}$; $CaCl_2$. 2 H_2O 0.106 $g.L^{-1}$; Fe-Citrate 0.075 $g.L^{-1}$; $ZnSO4.7H_2O$ 0.0075 $g.L^{-1}$; $CuSO_4$ 0.0045 $g.L^{-1}$; $MnSO_4.7H_2O$ 0.05 $g.L^{-1}$.

2.2.2 A semi synthetic medium [19]

The medium contained: soybean flour 15.0 $g.L^{-1}$; glucose 5.0 $g.L^{-1}$; starch 5.0 $g.L^{-1}$; K_2HPO_4 1.0 $g.L^{-1}$; KH_2PO_4 1.0 $g.L^{-1}$; $MgSO_4.7H_2O$ 0.3 $g.L^{-1}$; $FeSO_4.7H_2O$ 0.02 $g.L^{-1}$; $ZnSO_4.7H_2O$ 0.02 $g.L^{-1}$; $CaCO_3$ 1.0 $g.L^{-1}$.

2.3 Preparation of seed culture

The first seed culture was prepared by inoculation of a 500 mL Erlenmeyer flask containing 100 mL Luria Bertani broth by a loopful of bacteria from the slant. The flask was incubated on a rotary shaker at 30 °C and 340 rpm for 8 h. At this time 2% (v/v) of the first passage seed was used to inoculate a 500 mL flask containing 98 mL of tryptose phosphate broth, which was incubated at the same conditions as the first passage seed for 12 h.

2.4 Fermentation procedure

2.4.1 Shake flasks experiments

2% (v/v) of the second-passage seed was used to inoculate 1000 ml flasks containing 50 mL of the Anderson medium, semi synthetic medium or CMB based medium. These flasks were incubated for 48 h at 30 °C in a rotary shaker set at 340 rpm.

2.4.2 Bioreactor experiments

The bioreactor used in this study was a 5 L Sartorius BIOSTAT B fermenter. The volume of the culture medium was 2.9 L and the bioreactor was inoculated by 100 mL of a second stage seed. Foaming was controlled by adding a natural foaming agent. The air flow was adjusted to 0.033 L/L of medium/min and the stirrer speed to 500 rpm. The temperature was set at 30 °C and the pH was monitored but not controlled.

2.5 Analytical methods

2.5.1Cell and crystal count

Cell and crystal counts were microscopically determined with a Petroff Hausser chamber (cell depth: 0.02 mm, ruling pattern: Improved Neubauer, 1/400 square mm) using high power, oil immersion objective of an Olympus microscope. Serial dilutions with thorough sonication were used to avoid cells or spores crystals clumping. The result is expressed in number of cells or number of crystals per ml.

2.5.2 Viable spores count

Samples taken from each culture were heat treated at 80 °C for 15 min, serially diluted with physiological solution (0.9% NaCl), and plated onto T3 plates. Plates were then incubated at 30 °C for 24 h and the developed *B. thuringiensis* colonies were counted and expressed in CFU (colony forming units) per mL. The samples were plated in duplicate.

2.5.3 Estimation of toxin proteins

A 1 mL sample of lysed cell suspension was centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the pellet was washed two times with NaCl 0.14 M - 0.01% Triton X-100 solution and four times with cold water. The crystal protein in the pellet was dissolved with 0.05 N NaOH for three hours with stirring. The suspension was centrifuged at 13000 rpm for 5 min and the pellet, containing spores and cell debris was discarded. The concentration of the crystal protein in the supernatant was determined by the Bradford method [20] using the bovine serum albumin as a standard. Some of the supernatant was kept for SDS-PAGE analysis.

2.6 SDS-PAGE

Samples of the supernatants, obtained after alkaline dissolution of the crystal protein from the different culture mediums, were mixed to the Laemmli buffer (18.7% (v/v) Tris HCl 1 M, 30% (v/v) SDS 2 $g.L^{-1}$, 30% (v/v) glycerol, 15% (v/v) ß-mercaptoethanol, 5% (v/v) bromophenol blue) at a ratio of 5:1 and then boiled for 5 min and chilled in ice bath. Toxin proteins were separated by electrophoresis on 10% sodium dodecyl sulphate- polyacrylamide gel (SDS-PAGE) [21]. The proteins profiles were visualized by staining in 0.1% Coomassie blue/ 7.5% (v/v) ethanol/5% glacial acetic acid and then destaining in 7.5% ethanol/ 5% acetic acid.

2.7 Sugars determination

2.7.1Carbohydrates extraction and hydrolysis

In order to extract and hydrolyze the CMB carbohydrates into simple sugars 1.5 ml of 72% H_2SO_4 was added to 0.122 g of CMB or to 5 mL of the culture medium and incubated for 60 Minutes at 30 °C. After dilution with distilled water (42 ml), samples were autoclaved at 120 °C for 20 minutes.

2.7.2 HPLC analysis

The filtered liquid obtained after acid hydrolysis was analyzed on an HPLC column (Bio-Rad Aminex HPX-87H). For HPLC analysis, 20 µl samples were injected at a temperature of 63 °C and flow rate of 0.6 ml/min (eluent 4 mM H_2SO_4). Glucose, xylose and arabinose were detected by a refractive index (RI)

detector. The calibration concentration was in the range between 0.1 to 0.8 $g.L^{-1}$ for each sugar. The linearity r2 for all sugars was greater than 0.99.

2.8 Statistical analysis of results

All the results related to determination of cells and crystals counts, CFU counts and toxin proteins concentration were the average of two replicates of two separate experiments for each cultural condition. Mean values were compared by a student's t test at the 0.05 level.

3. Results and discussions

3.1 Formulation of a CMB based medium

The Anderson medium was used in literature for the production of *Bacillus thuringiensis kurstaki* [18, 22, 23]. It has all the nutrients required for *Bt* growth and sporulation and for δ-endotoxin production. A series of experiments was carried out in order to study the influence of substituting each of nutrients sources in this medium by 3% (w/v) cereal milling byproduct (CMB), on the toxin protein concentration obtained at the end of fermentation typified by 90% free spores (table 1).

When the carbon and nitrogen sources in the Anderson medium were replaced by CMB 3% (w/v), the toxin proteins concentration achieved, increased from 1 to 1.3 $g.L^{-1}$ (medium 2 compared to medium 1). No significant difference was observed between the mean values of the toxin proteins concentrations obtained in mediums 3, 4, 5 and 6 compared to that obtained in medium 2 by a student's t test at the 0.05 level. These results show that the CMB at a ratio of 3% (w/v) can replace the sources of carbohydrates, proteins, minerals and oligoelements of the Anderson medium and the toxin proteins production is improved.

Table 1: Effect of substituting the nutrients of Anderson medium by CMB 3% (w/v) on toxin proteins production

Culture medium	Toxin proteins concentration ($g.L^{-1}$)
Medium 1 : Anderson, 1990	1 ± 0.04
Medium 2 : Anderson medium where glucose, yeast extract, bactopeptone and $(NH4)_2SO_4$ (carbohydrate and nitrogen sources) were substituted by CMB 3% (w/v)	1.3 ± 0.04
Medium 3 : Medium 2 without minerals: ($MgSO_4.7H2O$; $ZnSO_4$. $7H_2O$ $CuSO_4$; $MnSO_4.7H_2O$)	1.27 ± 0.02
Medium 4: Medium 3 without iron, citrate and calcium ($CaCl_2$. $2\ H_2O$; Fe-Citrate)	1.22 ± 0.04
Medium 5: Medium 3 without phosphate and potassium (KH_2PO_4; K_2HPO_4)	1.26 ± 0.02
Medium 6: Only CMB 3% (w/v)	1.25 ± 0.03

3.2 Determination of the optimal amount of CMB

With the idea of enhancing the effectiveness of the CMB medium, different ratios of the CMB were tested in shake flasks experiments. As it is shown in figure (1 a), a proportional increase of the protein concentration with the CMB ratio in the culture medium was observed from 1 to 6% (w/v) CMB. The crystal count, determined by microscopic observation and the toxin proteins concentration were perfectly correlated in this range of CMB w/v ratio. A further increase in the CMB ratio led to a proportional increase in the crystal count but not in the toxin proteins concentration (figure 1 b). The toxin yield, defined as the ratio between the toxin proteins concentration achieved (mg/L) and the CMB ratio in the culture medium, remained constant at 40 mg/g for 3, 4.5 and 6% CMB but decreased to 29.44 mg/g when using 9% CMB (figure 1 a).

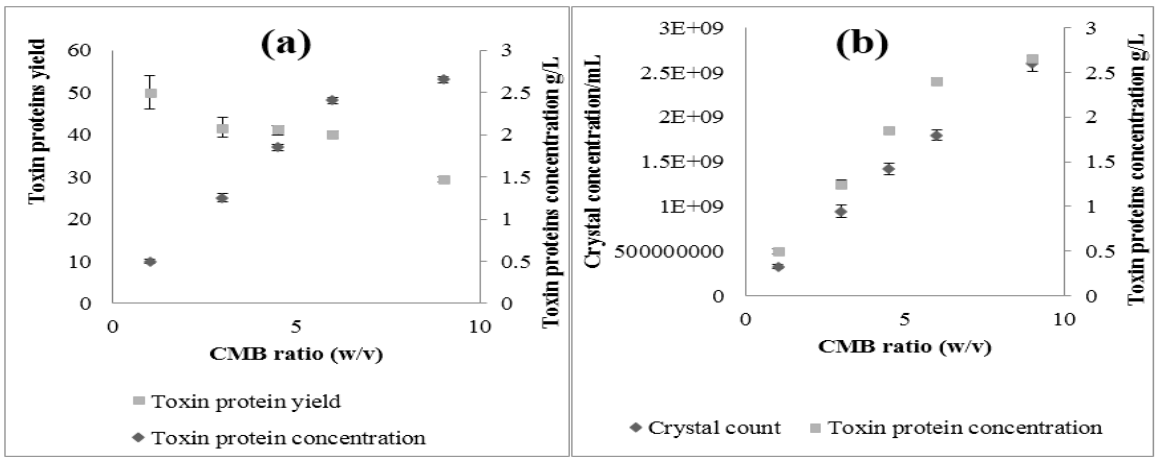

Fig 1: (a) Variation of toxin proteins concentration and toxin proteins yield with CMB ratio; (b) Variation of crystal count and toxin proteins concentration with CMB ratio

3.3 Testing the CMB liquid extract for the production of "Lip"

In order to test the suitability of the CMB liquid extract for the "Lip" production, the 6% CMB medium was autoclaved for 20 minutes at 120 ºC then filtered through a strainer. The filtrate was used as a culture medium for the local strain of *Btk* in shake flasks experiments. The toxin concentration obtained was only 0.6 $g.L^{-1}$ for 2.4 $g.L^{-1}$ when the liquid-solid medium was used.

3.4 Analysis of the CMB sugars consumption by "Lip"

The CMB contains essential carbohydrates in the form of polymers: cellulose, hemicellulose and starch. Samples of solid CMB recuperated from the 6% CMB culture medium at the end of the fermentation and samples of fresh CMB were submitted to acid hydrolysis in order to extract and hydrolyze the carbohydrates. The obtained sugars were then analyzed by HPLC. In the same way the concentrations of sugars remaining in the liquid phase of the culture medium were determined. The mass of the recuperated CMB was found to be 1/3 of the initial mass. All these data were used to calculate the consumed mass of each of the sugars during *Bt* fermentation in the 50 mL CMB 6% culture medium in shake flasks experiments.

The obtained results, grouped in table 2, show that 0.972 g of glucose was consumed during the fermentation, which represents 66.48% of the mass of glucose in the fresh CMB. All xylose and arabinose measured in the fresh CMB were found at the end of fermentation in liquid or solid phase of the culture medium. Thus, the bacteria did not use the pentose (xylose and arabinose) that derive from the hydrolysis of the CMB hemicellulose.

Table 2: Sugar consumption during *Bt* fermentation in the 50 mL CMB 6% culture medium in shake flasks experiments.

	Fresh CMB (a)	**Used CMB (b)**	**Liquid medium at T_f (c)**	**Sugars consumed (a-(b+c))**
Glucose mass (g)	1.462 ± 0.047	0.221 ± 0.019	0.269 ± 0.025	0.972 ± 0.002
Xylose mass (g)	0.503 ± 0.035	0.295 ± 0.016	0.205 ± 0.019	0.003 ± 0.0008
Arabinose mass (g)	0.287 ± 0.029	0.178 ± 0.023	0.105 ± 0.005	0.004 ± 0.0002

T_f: the time corresponding to the end of the fermentation

3.5 Comparison of the CMB medium, to the Anderson medium and to the standard semi-synthetic medium for the production of "Lip"

Cultures in shake flasks were performed to compare the formulated mono component 6% CMB medium to the Anderson, medium and to the semi synthetic medium in terms of cells, spores and crystals counts. The obtained toxin proteins and the consumed glucose concentrations as well as the relative yield, the toxin proteins yield based on glucose, the productivity and the cost of preparing 1 L of each of the three mediums were also compared (table 3).

Table 3: Comparison of the CMB medium, to the Anderson medium and to the semi-synthetic medium for the production of "Lip"

	Anderson, 1990	**Standard semi synthetic**	**6% CMB medium**
Cells ($x10^9$ cell.mL^{-1})	2.05$^{(a)}$	1.98$^{(a)}$	1.96$^{(a)}$
Spores ($x10^9$ CFU.mL^{-1})	1.7 $^{(a)}$	1.68 $^{(a)}$	1.66 $^{(a)}$
Crystals ($x10^9$crystal.mL^{-1})	1.79 $^{(a)}$	1.78 $^{(a)}$	1.80 $^{(a)}$
Toxin proteins $g.L^{-1}$	1 $^{(a)}$	1.56 $^{(b)}$	2.4 $^{(c)}$
Relative yield			
(mg of toxin proteins /10^9 CFU)	589.35 $^{(a)}$	932.56 $^{(b)}$	1447.42 $^{(c)}$
Consumed glucose $g.L^{-1}$ mg of toxin proteins/g of	9.91 $^{(b)}$	8.95 $^{(a)}$	19.44 $^{(c)}$
consumed glucose	100.77 $^{(a)}$	174.46 $^{(c)}$	123.50 $^{(b)}$
mg of toxin proteins/L/h	30.27 $^{(a)}$	43.35 $^{(b)}$	50.03 $^{(c)}$
Cost of 1 L of each medium	1.8 $	3.9 $	0.006 $

Values in the same row that are preceded by the same upper case letter are not significantly different

Similar growth was observed in the three mediums. Spores and crystals counts were also of the same order in the three mediums. Spore counts were slightly lower than the crystal counts. This could be attributed to the difference in the count methods used. The value of toxin proteins concentration obtained in the 6% (w/v) CMB medium was 2.4 fold and 1.54 fold greater than the values reached in the Anderson medium and in the semi synthetic medium respectively. The proteins profiles of the "Lip" spore/crystal complex obtained from different culture media, determined by gel electrophoresis,

were similar as it is shown in figure 2, 130 and 65 kDa bands were mainly obtained. The greatest yield of toxin proteins based on glucose was obtained in the semi synthetic medium. Higher productivity and higher relative yield were achieved in the CMB medium.

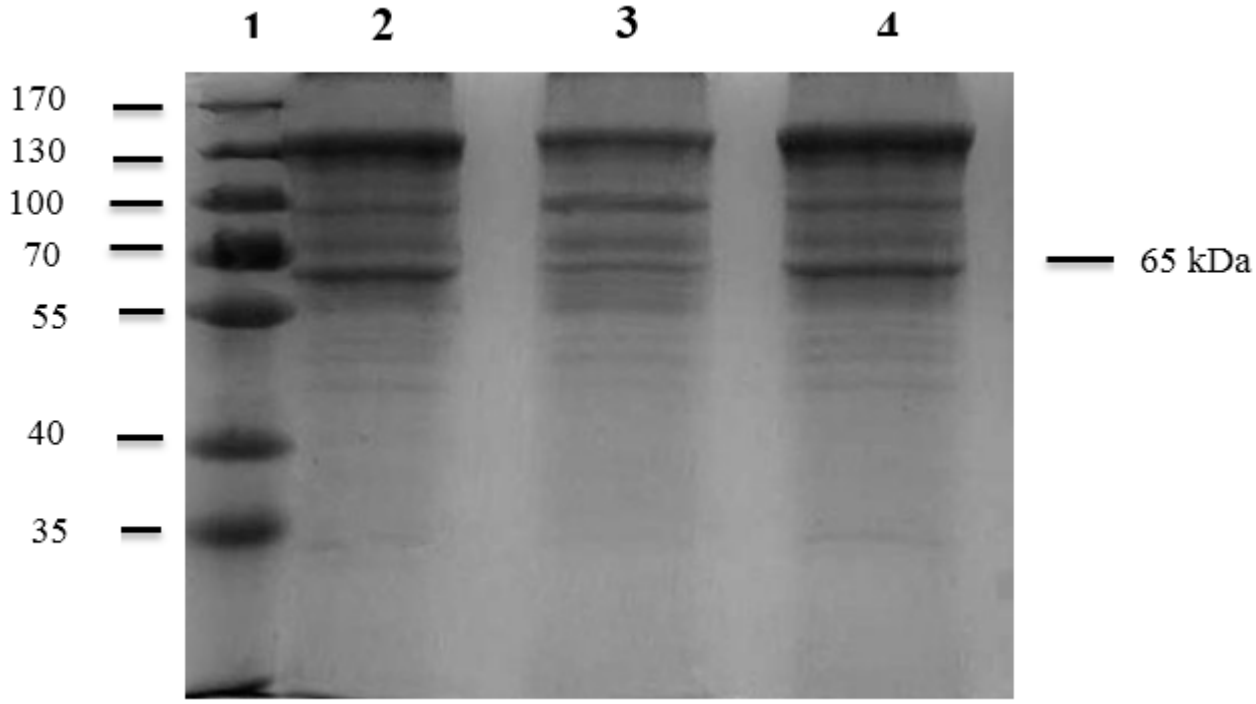

Fig 2: Comparative analysis by SDS PAGE of the protein profiles of the "Lip" spore/crystal complex obtained from different culture media: lane 1: protein marker; 2: standard semi synthetic medium; lane 3: Anderson, 1990 medium; lane 4: CMB medium

3.6 Bioreactor experiment

The strain of *Bacillus thuringiensis* was cultured in a 5 L bioreactor in the CMB 6% medium. The aeration conditions (flow rate 0.033L/L/min; stirrer speed 500 rpm) were selected so as to simulate the limited aeration conditions in the shake flasks experiments. A toxin proteins concentration of 2.19 g.L^{-1} was obtained in 48 hours. The cell and the free crystal count variation throughout the fermentation are presented in figure 3. A slight variation of pH is noticed during the fermentation. The initial pH value was 6.22. The minimum and maximum values reached by the pH during the fermentation were respectively 5.63 and 6.63.

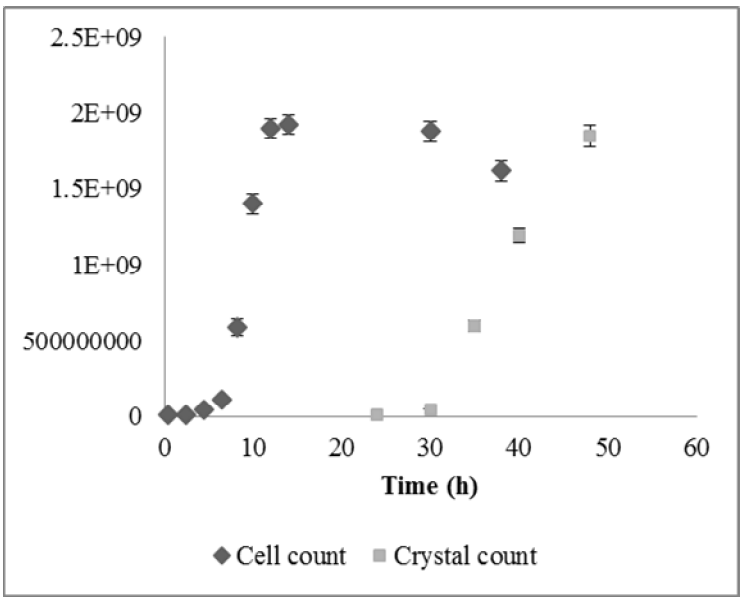

Fig 3: Cell and crystal count variation throughout the fermentation of "Lip" in a 5 L bioreactor

4. Discussions

In the present study the CMB was proved efficient to be used as source of carbon, nitrogen and minerals for the δ-endotoxin production in a submerged fermentation of a *Bacillus thuringinesis kurstaki*. The optimal CMB ratio (w/v) in the shake flasks cultures was 6%. Although we could predict the δ-endotoxin concentration achieved based on a crystal count in the CMB medium at different ratios of the substrate up to 6%, the prediction was not possible when the CMB ratio in the culture medium increased to 9%. The ratio, between the toxin proteins concentration and the crystals concentration, decreased revealing that the crystalline inclusions synthetized in the 9% CMB medium had a lower content of proteins. The low toxin yield obtained at 9% (w/v) CMB may be due to poorer oxygen availability in the thick culture medium or to a carbon catabolic repression of the δ-endotoxin synthesis as a result of growing cells at a high concentration of substrate. The suitability of the CMB 6% liquid extract for the production of "Lip" was tested. The low concentration of toxin produced suggests that the nutrients needed for the δ-endotoxin production by the bacteria, are not all extracted from the cereal milling byproduct and dissolved in the culture medium after autoclave. And that an enzymatic activity of the bacteria is needed to break down the present nutrient sources in the CMB to be available. Analysis of CMB sugars consumption by "Lip" showed that the bacteria used only the glucose. The amylase activity of *Bt* was proved by many authors [24, 25, 26]. Further study is needed to determine the capacity of the local strain of *Bt* for cellulose degradation and xylan hydrolysis. Therefore, we suggest that the consumed glucose derives mainly from the CMB starch hydrolysis by *Bt* amylases. The 6% (w/v) CMB medium was compared to the Anderson and to standard semi-synthetic mediums. It is reported that the size, shape and toxin content of the crystals can be affected by the composition of the medium [27, 28]. Different sources of proteins and carbohydrates at different concentrations were used in the three compared mediums which may explain the difference in the δ-endotoxin concentration in the synthetized crystals. Keshavarsil *et al.* [29] studied the relationship between δ-endotoxin production and the biochemical constituents, including carbohydrate and protein, of the culture media and found that the optimal media, in terms of protein composition, contained high levels of glutamic acid, the most abundant amino acid constituent of the crystal protein [30]. On the other hand, more glucose was consumed in the CMB medium, while the cell concentrations value obtained in the three mediums were similar. The nature and characteristics of the carbon/energy and nitrogen sources have a predominant role to play in the metabolism of microorganisms [31]. The glucose in the Anderson and semi synthetic mediums may be used by the local strain of *Bt* as energy source only and the carbon source could be the yeast extract in the Anderson medium and the soybean flour in the semi synthetic medium while the greater consumption of glucose in the CMB medium could be explained by the use of glucose (deriving from starch) as carbon and energy source. Another explanation could be that part of the glucose in the CMB medium was used for the formation of other metabolic products. Anyway, the toxin proteins yield based on consumed glucose, achieved in the CMB medium was not low in comparison with those obtained in the Anderson and the standard semi synthetic medium. Since the toxin proteins play the ultimate role in bio-control [32] high protein productivity is the goal for optimization of *Bt* fermentation. The present work showed that, in comparison with the references mediums, the 6% CMB medium gave the highest toxin proteins productivity. On the other hand, as the CMB is an agro industrial byproduct, the cost of the preparation of 1 L of the 6% CMB mono component medium is negligible, while the preparation of the Anderson medium and the semi-synthetic medium involves 1.8 $ and 3.9 $ respectively. Considering all three factors, relative yield, productivity and cost, the use of the CMB mono-component medium is much more economical for the large scale industrial production of the local strain of *Bacillus thuringiensis kurstaki* (*Btk*) "Lip" than the references mediums. Considerable savings could be achieved using this mono component medium where there is no need to add carbohydrates or salts and *Btk* could then be cheaply produced. In a 5 L laboratory bioreactor experiment, the CMB mono component medium was evidenced suitable for a large scale production of 'Lip' in submerged fermentation. The slight variation of pH throughout the fermentation reveals the balance between the carbon and nitrogen sources in the CMB medium and show that fermentations could be performed with no need to control the pH. Optimization of operating parameters (e.g. agitation, aeration) in bioreactor fermentation deserves further investigations to produce the toxin at industrial scale.

5. References

1. Lord JC. From Metchnikoff to Monsanto and beyond: The path of microbial control. Journal of Invertebrate Pathology 2005; 89:19-29.
2. Brar SK, Tyagi VRD, Valéro JR. Recent advances in downstream processes and formulations of Bacillus thuringiensis based biopectides. Process Biochemestry 2006; 41:323-342.
3. Kumar S, Biopesticides: A Need for Food and Environmental Safety. J Biofertil Biopestici, 2012, 3:4.
4. Dulmage HT. Production of the spore-d-endotoxin complex variants of *Bacillus thuringiensis* in two fermentation media. Journal of Invertebrate Pathology 1970; 16:385-389.
5. Nickerson KW, Bulla LA. Physiology of Spore-Forming Bacteria Associated With Insects, Minimal Nutrition Requirements for Growth, Sporulation and Parasporal Crystal formation of *Bacillus thuringiensis*. Appl. Microbiol 1974; 28:124-128.
6. Wakisaka Y, Masaki E, Nishimoto Y. Formation of Crystalline 8-Endotoxin or Poly-3-Hydroxybutyric Acid Granules by Asporogenous Mutants of *Bacillus thuringiensis*. Applied and Environmental Microbiology 1982; 43(6):1473-1480.
7. Foda MS, Salama HS, Selim M. Factors affecting growth physiology of *Bacillus thuringiensis.* Appl Microbiol Biotechnol 1985; 22:50-52.
8. Salama HS, F oda MS, Selim MH El –Sharaby A. Utilization of fodder yeast and agro-industrial by-products in production of spores and biologically active endotoxins from *Bacillus thuringiensis*. Zentralblatt fur Mikrobiologie 1983; 83:553-563.
9. Arcas J, Yantorno O, Arraraas E, Ertola R. A new medium for growth and d-endotoxin production by

Bacillus thuringiensis var. *kurstaki*. Biotechnology Letters 1984; 6:495-500.
10. Mummigatti SG, Raghunathan AN. Influence of media composition on the production of delta-endotoxin by *Bacillus thuringiensis* var. *thuringiensis* J Invertebr Pathol 1990; 55:147-151.
11. Zouari N, Dhouib A, Ellouz R, Jaoua S. Nutritional requirements of a *Bacillus thuringiensis* subsp. *Kurstaki* strain and use of gruel hydrolysate for the formulation of a new medium for delta-endotoxin production. Appl Biochem Biotechnol 1998; 69:41-52.
12. Vora D, Shethna YI. Enhanced growth, sporulation and toxin production by *Bacillus thuringiensis* subsp. *Kurstaki* in soil seed meal extract media containing cysteine World J Microb Biot 1999; 15:747-749.
13. Kumar A, Sra K, Sangodkar UMX, Sharma VP. Advances in the bio-control of mosquito vectors utilizing *Bacillus sphaericus* and *Bacillus thuringiensis* var *israelensis*. Proc Natl Acad Sci India LXX 2000, 1-20.
14. Dregval OA, Cherevach NV, Vinnikov AI. Influence of composition of the nutrient medium on growth and development of entomopathogenic bacteria *Bacillus thuringiensis*. Mikrobiol Z 2002; 64:44-48.
15. Luna CL, Silva GR, Rios E.M. *Bacillus thuringiensis* var. *israelensis* production involving re-use of the supernatant. Biotechnol Lett 2004; 26:143-145.
16. Klopfenstein TJ. By-products feeds: plant origin. In encyclopedia of animal science edited by Wilson G pond 2004, 84-186.
17. El Khoury M, Azzouz H, Chavanieu A, Abdelmalak N, Chopineau J, Awad kM. Isolation and characterization of a new *Bacillus thuringiensis* strain Lip harboring a new cry 1 Aa gene highly toxic to *Ephestia kuehniella* (Lepidoptera: *Pyralidae*) larvae. Archives of Microbiology 2014, DOI 10.1007/s00203-014-0981-3.
18. Anderson T. Effects of carbon: nitrogen ratio and oxygen on the growth kinetics of *Bacillus thuringiensis* and yield of bioinsecticidal crystal protein, M.Sc Thesis, The University of Western Ontario, London, Canada, 1990.
19. Montiel MLT, Tyagi RD, Valero JR. Wastewater treatment sludge as a raw material for the production of Bacillus thuringiensis based biopesticides. Water Res. 2001; 35:3807-16.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72:248-254.
21. Lammeli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-685.
22. Rivera D, Margariris A, De Lasa H. A sporulation kinetic model for batch growth of *Bacillus thuringiensis*. The Canadian Journal of Chemical Engineering 1999; 77:903-910.
23. Amin G, Alotaibi S, Narmen A. Youssef and Saleh W.D. Bioinsecticide production by the bacterium *Bacillus thuringiensis* (Pattern of cell growth, toxin production and by-product synthesis). Journal of Basic and Applied Sciences 2008; 4(1):27-31.
24. Baumann I, Okamoto1 K, Unterman MB, Lynch JM, Baumann P. Phenotypic Characterization of *Bacillus thuringiensis* and *Bacillus cereus*. Journal of Invertebrate Pathology 1984; 44:329-341.
25. Kuppusamy M, Balaraman K. Extra cellular hydrolytic enzyme secretion in *Bacillus thuringiensis* H14 & *B. sphaericus* and their significance in media design. Indian J Med Res 1990; 91:149-50.
26. Avignone-Rossa C, Mignone CF. *Bacillus thuringiensis* growth and Toxicity. Basic and Applied Considerations, Molecular Biotechnology 1995; 4 (1):55-71.
27. Scherrer P, Luethy P, Trumpi B. Production of d -endotoxin by *Bacillus thuringiensis* as a function of glucose concentrations. Applied Microbiology 1973; 25:644-646.
28. Faloci MM, Yantorno OM, Marino HA, Arcas JA, Ertola RJ. Effect of the media composition on the growth parameters and biological properties of *Bacillus thuringiensis* var. kurstaki d -endotoxin. World Journal of Microbiology and Biotechnology 1993; 6:32-38.
29. Keshavarzi1 M, Salimi H, Mirzanamadi F. Biochemical and Physical Requirements of *Bacillus thuringiensis* subsp. *kurstaki* For High Biomass Yield Production J Agric Sci Technol 2005; 7:41-47.
30. Fast PG. The Crystal Toxin of *Bacillus thuringiensis*. In: Microbial Control of Insects and Mites. (Eds.): Burges H. D. and Hussey H. Academic Press, London, New York, 1981, 223-244
31. Anderson RKI, Jayaraman K. Influence of Carbon and Nitrogen Sources on the growth and sporulation of *Bacillus thuringiensis* var *Galleriae* for Biopesticide Production Chem Biochem Eng Q 2003; 17(3):225-231.
32. Yang XM, Wang SS. Development of *Bacillus thuringiensis* fermentation and process control from a practical perspective. Biotechnol Appl Biochem 1998; 28:95-98.