

IPM-4-CITRUS



IPM-4-CITRUS
H2020-MSCA-RISE-2016
Project no. 734921
April 2017-Jan 2023

IDB 2022 – 20th December 2022
Satellite session IPM-4-Citrus

WP2	Proof of concept, from <i>B. thuringiensis kurstaki</i> cultivation to δ-endotoxin production (bioperformances, bioprocess and scale-up)
WP2-1	Physical limitations induced by a wheat bran-based medium for the production of biopesticides by <i>Bacillus thuringiensis</i> serovar <i>kurstaki</i>. Rita BARSSOUM, Karim Mohamed CHALBI, Rayan NASSERDINNE, Julien CESCUT, Mireille KALLASSY, César ACEVES-LARA, Luc FILLAUDEAU
WP2-2	Modelling and Dynamic Optimization of protein and spore production by <i>Bacillus thuringiensis</i>. Tatiana Segura MONROY, Nouha ABDELMALEK, Souad ROUIS, Mireille KALLASSY, Joanna ABDOUD, Julien CESCUT, Nadia BEN SAÏD, Luc FILLAUDEAU, César Arturo ACEVES-LARA
WP2-3	In-depth genomic analysis of <i>Bacillus thuringiensis</i> serovar <i>kurstaki</i> strains: Genome dynamics in play. Nancy FAYAD, Rita BARSSOUM, Nathalie MARSAUD, Julien CESCUT, Luc FILLAUDEAU, Mireille KALLASSY
WP3	Optimisation of the new biocontrol activity (formulation, efficacy at lab and field scales, risk assessment and ecotoxicology)
WP3-1	Study of ecotoxicological effects and efficacy tests of biopesticides based on <i>Bacillus thuringiensis</i> “Lip” and “BLB1” against <i>Phyllocnistis citrella</i> and <i>Prays citri</i>. Rayan NASSEREDDINE, Zhanerke AMANGELDI, Gül AYYILDIZ, Fatma IDRIS5, Souad ROUIS, Mireille KALLASY AWAD, Asime Filiz CALISKAN KEÇE, Stephan DIETRICH.
WP3-2	Novel biopesticides <i>Bacillus thuringiensis kurstaki</i> : Toxicity Studies on Non-Target aquatic Organisms. Rim EL JENI, Sayda DHAOUADI, Hazar KRAÏEM, Gül AYYILDIZ, Zakaria BENLASFAR, Zeynep YURTKURAN CETEREZ and Balkiss BOUHAOUALA-ZAHAR
WP3-3	Toxicity assays on Rabbit, Rat and Guinea pig: Biosafety assessment of new BLB1 and LIP <i>Btk</i>-based formulated products. Balkiss BOUHAOUALA-ZAHAR, Gül AYYILDIZ, Hazar KRAÏEM, Rim EL JENI, Sayda. DHAOUADI, Zeynep Yurtkuran-CETERE & Zakaria BENLASFAR
WP4	Transfer & economic maturation (benchmarking, business plan)
WP4-1	Economic maturation & business plan development for new <i>Bacillus thuringiensis</i> based biopesticide. Souad ROUIS, Nadia BEN SAID, Tayssir HAMMAR, Sara FALVO



This project has been funded by the European Union’s Horizon 2020 Research and Innovation Programme under Grant Agreement No 734921

Physical limitations induced by a wheat bran-based medium for the production of biopesticides by *Bacillus thuringiensis serovar kurstaki* (WP2-1)

Rita BARSSOUM¹⁻²⁻³, Karim Mohamed CHALBI², Rayan NASSERDINNE¹, Julien CESCUT³, Mireille KALLASSY¹, César ACEVES-LARA², Luc FILLAUDEAU²

¹Faculté des Sciences, Université Saint-Joseph de Beyrouth, Liban

²Toulouse Biotechnology Institute-Bio & Chemical Engineering, INSA Toulouse, France

³Toulouse White Biotechnology, INSA Toulouse, France

E-mail (corresponding author): rita.barssoum@net.usj.edu.lb

Background and aim: Biopesticides are an alternative to chemical pesticides that respond to the development of the bio-economy and are respectful to humans and the environment. *Bacillus thuringiensis* is a gram-positive, spore-forming bacterium that forms the basis of most biopesticides. This insecticidal activity is mainly due to the production, during the sporulation phase, of δ -endotoxins constituted by an assembly of crystalline proteins called Cry. The European project IPM-4-Citrus (MSCA RISE, No. 734921, 2017-2023) aims to optimise this bioproduction during the culture of 3 strains of *Bacillus thuringiensis serovar kurstaki* (Lip, BLB1 and HD1) in a wheat bran based complex medium. The biochemical composition and nutritional limitations induced by the medium were identified. This study aims to explore the evolution of the physical properties of wheat bran suspension (morpho-granulometry, solid-liquid separation by settling and rheological behaviour) as a function of grain size and concentration.

Methods: Morphological characterisation was carried out *ex-situ* using a morphogranulometer (Mastersizer G3S, Malvern Instrument). Solid-liquid separation by decantation (Falcon 50mL) was used to determine the settling velocity and the volume fraction of supernatant. The water retention capacity (WRC) is also measured. Rotational viscometry was achieved with a rheometer (Mars III, Thermo Scientific) equipped with an helical ribbon ensuring the homogeneity of the wheat bran suspensions (0-150 ghm/L).

Results: The solid-liquid separation mechanisms determines the initial operating conditions (suspension homogeneity, pumping rate) and the downstream processing. The suspension properties result from their technological pathway (with/without heat treatment and after culture) and their composition (starch and lignocellulosic particles). The main trends observed are: (i) an increase of WRC regardless of the granulometry, (ii) an increase in the pellet volume fraction with the technological pathway and (iii) a decrease in the limiting fall velocities. The apparent viscosity of the initial suspensions (after heat treatment) is reported as a function of shear rate, particle size and concentration. The suspensions evolve from a Newtonian behaviour (diluted regime, [WB]<<1 gmh/L) to a non-Newtonian behaviour (shear-thinning) in a semi-diluted ([WB] > 10 gmh/L) then concentrated ([WB] > 80-150 gmh/L) regime. A model of the rheological behaviour considering the equilibrium between the "fines" and the "coarse" can thus be proposed.

Keywords: biopesticides, *Bacillus thuringiensis*, wheat bran, rheometry, morpho-granulometry, decantation.

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921

Modeling and Dynamic Optimization of protein and spore production by *Bacillus thuringiensis* (WP2-2)

Tatiana Segura MONROY¹, Nouha ABDELMALEK², Souad ROUIS³, Mireille KALLASSY⁴, Joanna ABDOUD⁵, Julien CESCUT⁵, Nadia BEN SAÏD², Luc FILLAUDEAU¹, César Arturo ACEVES-LARA¹

¹ Université de Toulouse; UPS, INSA, INP, TBI F31077; the INRA, UMR792, Toulouse Biotechnology Institute, Bio & Chemical Engineering; and the CNRS, UMR5504, 135 avenue de Rangueil, Toulouse, France F-31077 (e-mail: [aceves*, luc.fillaudeau]@insa-toulouse.fr and seguramo@etud.insa-toulouse.fr) - ² Laboratoires pharmaceutiques MédiS, Nabeul, Tunisia. nouha_abdelmalek@yahoo.com and nadia.bensaid@labomedis.com - ³ Centre of Biotechnology of Sfax, B.P 1177 Sfax 3018, Tunisia; e-mail: souad.rouis@cbs.rnrt.tn - ⁴ Faculty of Sciences Saint-Joseph University, Riad El Solh, Beirut, Lebanon; e-mail mireille.kallassy@usj.edu.lb - ⁵ Toulouse White Biotechnology (UMS INRAE/INSA/CNRS), 135 Avenue de Rangueil 31077 Toulouse CEDEX 04; email [Julien.Cescut, joanna.abboud-1]@inrae.fr.*

Background and aim: Dynamic optimization of proteins and spore production by *B. thuringiensis* needs to use robust models coupled to control strategies. In this work, two models were proposed to describe proteins and spores production using *B. thuringiensis*. Models were calibrated and the parameters obtained were in the literature range. The best model was selected to implement two control strategies, for a Fed-batch culture and a Sequential Batch culture.

Methods: Two dynamic model were used to optimize proteins and spores production using three *B. thuringiensis* strains.

Results: The experimental simulations developed in the present study, on *B. thuringiensis* culture made it possible to analyze their behavior and to adjust two models to the experimental data sets. The calibration of both models allows calculate the kinetic parameters of the culture and the experimental data presented a good fit. Additionally, although the BLB strain showed the highest maximum specific growth rate (μ_{max}), the HD strain presented the highest yield Biomass/substrate yield coefficient values (Y1). The LIP strain presented the lowest values for this yield. As for the production of proteins, which are mainly used for insecticidal effects, the BLB strain presented the highest concentration and the proteins/substrate yield coefficient (Y2) was the highest of all the strains, while the LIP strain showed the lowest values for this yield.

In order to study the performance of the control strategies, three different cases were considered, batch without optimization and with initial Substrate equal to 25g/L, fed-batch strategy and sequential batch. It was found that the optimization of proteins and spores productions depicted an increasing profile for glucose feeding until the upper limit of volume was achieved. Fed-batch strategy had the best proteins and spores' productivity with high biomass productivity.

Conclusion: Two models were proposed in this work. One model was chosen to compare two approaches of dynamical optimization. The results demonstrated that the model based on control strategy allowed maximizing proteins and spores productivity. An experimental validation of the control law will be performed to prove the correct proteins and spore productivity.

Keywords: Model optimization, Proteins and Spores optimization, Fed-batch fermentation, Sequential batch fermentation.

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921.

In-depth genomic analysis of *Bacillus thuringiensis* serovar *kurstaki* strains: Genome dynamics in play (WP2-3)

Nancy FAYAD^{1,2*}, Rita BARSSOUM^{1,3}, Nathalie MARSAUD³, Julien CESCUT⁴,
Luc FILLAUDEAU³, Mireille KALLASSY^{1*}

¹Faculty of Sciences, Saint-Joseph University of Beirut, Lebanon

²Multi-Omics Laboratory, School of Pharmacy, Lebanese American University, Lebanon

³Toulouse Biotechnology Institute-Bio & Chemical Engineering, INSA Toulouse, France

⁴Toulouse White Biotechnology (UMS INRAE/INSA/CNRS), Toulouse, France

Author emails: nancy.fayad@net.usj.edu.lb; rita.barssoum@net.usj.edu.lb; nmarsaud@insa-toulouse.fr; julien.cescut@inrae.fr; luc.fillaudeau@insa-toulouse.fr; mireille.kallassy@usj.edu.lb

* co-corresponding authors: nancy.fayad@net.usj.edu.lb ; mireille.kallassy@usj.edu.lb

Background and aim: Biopesticides are an eco-friendly alternative to harmful chemical pesticides. A major player in the biopesticides market are *Bacillus thuringiensis*-based products. The latter is a Gram-positive sporulating soil bacteria that owes its entomopathogenic properties to a parasporal crystal constituted of an ensemble of pore-forming δ -endotoxins. Genes encoding the latter are carried by toxin-carrying plasmids, a pillar of *Bacillus thuringiensis*' genetic makeup. *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) is known for its activity against lepidopteran larvae. *Btk* Lip and BLB1, isolated from Lebanese and Tunisian soils respectively, exhibited a higher efficiency than the reference HD1 against lepidopteran larvae *Ephestia Kuehniella*. IPM-4-Citrus (MSCA RISE, No. 734921, 2017-2023) is a project aiming to optimize the culture of *Btk* Lip, HD1 and BLB1 on a wheat bran based medium. In this study we aimed to elucidate several in-depth genomic aspects of the three strains in question. This analysis included toxin-coding genes, mobile genetic elements, and potential virulence and human health related factors.

Methods: Lip and BLB1 entire genomes were subject to Pacific Biosciences sequencing approach with Illumina HiSeq polishing. Several bioinformatic analysis then followed including complete genome annotation by RAST and Prokka, the detection of toxin-coding genes and the mining of inter- and intra-cellular mobile genetic elements. A maximum likelihood phylogenetic analysis was also conducted.

Results: Based on the sequencing results, we identified 13 plasmids carried by HD1, 12 plasmids for Lip and 11 plasmids for BLB1. The size of the plasmids ranged between 2 and 400 Kb. Some plasmids (7) were common between the three strains, whereas other were specific for each strain. Despite belonging to the same serovar *kurstaki*, the differences in their plasmid content and detected single nucleotide polymorphisms demonstrate the strains' high genomic variability. Results pertaining to the remaining analyses are on-going. All in all, genomic data is indispensable in improving strains' bioperformances and host selection. Moreover, any microorganism which can be disseminated in the environment should be extremely verified for potential virulence factors, prior to its formulation and use as a biopesticide.

Keywords: *Bacillus thuringiensis*, *cry* genes, plasmids, mobile genetic elements, phylogeny

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921.

Study of ecotoxicological effects and efficacy tests of biopesticides based on *Bacillus thuringiensis* “Lip” and “BLB1” against *Phyllocnistis citrella* and *Prays citri* (WP3-1)

Rayan NASSEREDDINE¹, Zhanerke AMANGELDI², Gül AYYILDIZ³, Fatma IDRIS⁵, Souad ROUIS⁵, Mireille KALLASY AWAD¹, Asime Filiz CALISKAN KEÇE², Stephan DIETRICH⁴.

¹Laboratory of Biotechnology Faculty of Sciences Saint-Joseph University, Beirut, Lebanon - ²Çukurova University, Adana, Türkiye – ³Biyans, Biological Products R&D LTD. Co., Ankara, Türkiye – ⁴Julius Kühn-Institut- Federal Research Centre for Cultivated Plants Institut, Dossenheim, Germany, ⁵Laboratory of Biopesticides, Centre of Biotechnology of Sfax, University of Sfax, P.O. Box. “1177”, 3018, Sfax, Tunisia.

E-mail: rayan.nassereddine@net.usj.edu.lb; gul@biyans.com; amangeldi-zhanerke@mail.ru; fatma_driss@yahoo.fr; souad.rouis@cbs.rnrt.tn; mireille.kallassy@usj.edu.lb; filizcaliskan@gmail.com; Dietrich.Stephan@julius-kuehn.de

Background and aims: This study shows the results obtained in the use of an alternative biopesticide based on *Bacillus thuringiensis* tested at lab scale in the department of plant protection, Çukurova university, and in the citrus fields of Mersin targeting two main Lepidoptera: *Phyllocnistis citrella* and *Prays citri*, with the objective of discovering an alternative to chemical pesticides that lead to severe ecological issues.

Methods: *Bacillus thuringiensis* strain "Lip" was identified from Lebanese soil, and *Bacillus thuringiensis* strain "BLB1" was recovered from Tunisian soil. The primary goal of our research is to evaluate the efficiency of our two designed products against the citrus flower moth (*Prays citri*) and the citrus leaf miner (*Phyllocnistis citrella*), as well as to estimate the lethal dosage concentration (LD50) and verify the spore persistency through the assay.

Results: At the field scale, the results showed that the efficacy of the two applied products on both insects reached 70% within 3 days of spraying and increased to between 80-85% by 14 days. At the laboratory scale, the fatal quantity was found to be between 50 and 70 ug/ml. The spores persistence tests verified our findings. The amount of spores dropped with time, indicating that the insects digested them.

Conclusion: Overall, our findings indicate that in order to improve and confirm the efficacy of the products, it is required to first determine the lethal dosage concentration at lab scale before evaluating it at field scale.

Keywords: *Bacillus thuringiensis*, *Prays citri*, *Phyllocnistis citrella*, Ecotoxicological test, Lethal concentration, Citrus field.

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921.

Novel biopesticides *Bacillus thuringiensis* kurstaki Toxicity Studies on Non-Target aquatic Organisms (WP3-2)

Rim EL JENI¹, Sayda DHAOUADI¹, Hazar KRAÏEM¹, Gül AYYILDIZ², Zakaria BENLASFAR¹, Zeynep YURTKURAN CETEREZ² and Balkiss BOUHOUALA-ZAHAR^{1,3}.

¹Laboratoire des Venins et Biomolécules Thérapeutiques, Institut Pasteur de Tunis, 13 Place Pasteur, BP74, 1002 Tunis Belvédère, Université Tunis-El Manar. ²Biyans Biyolojik Urunler Ar-Ge Dan. Tic. Ltd. Beycanoglu Is Merkezi no:104/20 Balgat Cankaya Ankara. ³Faculté de Médecine de Tunis, Université Tunis-El Manar.

E-mail: eljeni.rime@gmail.com , Sayda.Dhaouadi@hotmail.fr

Background and aim: Citrus fruits are among some of the most popular agricultural products contributing in the economic and social progress of nations. Integrated Pest Management (IPM) approach based on controlling citrus insect pests via the use of biopesticides could largely boost yield production. In this optic, newly *Bacillus thuringiensis* (Bt) formulations-based bio pesticides (BLB1 and LIP), active against citrus pests were identified and in order to scale them up from lab to market, eco-toxicological risk assessment has to be monitored. Therefore, the acute toxicity of these new formulations on non-target living aquatic organism freshwater microalgae (*Pseudokirchneriella subcapitata*) as well as the crustaceans water flea *Daphnia magna* Straus, was assessed.

Methods: Toxicity assays were conducted in accordance with the ISO and OECD guidelines. The effects of various BLB1 and LIP concentrations on the sensitivity response of *Daphnia magna* and on the *microalgae* growth, were evaluated after different exposure point times. The assays were realized in comparative manner with Bt-standard formulation (Delfin WG).

Results: As results, realized investigations based on experiments endorsed by regulatory guidelines led us to define the impacts of the new formulations BLB1 and Lip on the environment, accurately aquatic microorganisms. The test provides the fact that Btk Lip and Btk BLB1 are far less toxic than the reference product Delfin.

Conclusion: The generated results reveal the no harmful effects of the new Btk- BLB1 and Lip formulations on aquatic non-target organisms. These results prove their possible commercial exploitation counteracting the health risk factors related to citrus pests treatment by chemical pesticides.

Keywords: *Bacillus thuringiensis*, BLB1, LIP, risk assessment, non-target organisms.

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921.

Toxicity assays on Rabbit, Rat and Guinea pig: Biosafety assessment of new BLB1 and LIP Btk-based formulated products (WP3-3)

Balkiss BOUHAOUALA-ZAHAR^{1,2}, Gül AYYILDIZ³, Hazar KRAÏEM¹, Rim EL JENI¹, Sayda. DHAOUADI¹, Zeynep Yurtkuran-CETERE³ & Zakaria BENLASFAR¹

¹Laboratory of Biomolecules, Venoms and Theranostic Applications (LR200IPT01), Institut Pasteur de Tunis, 13 Place Pasteur, BP74, 1002 Tunis Belvédère, University Tunis-El Manar.

²Medical School of Tunis, University Tunis-El Manar.

³Biyans Biyolojik Urunler Ar-Ge Dan. Tic. Ltd. Beycanoglu Is Merkezi no:104/20 Balgat Cankaya Ankara.

E-mail: balkiss.bouhaouala@fmt.utm.tn

Background and aim: Two new *Bacillus thuringiensis*-based biopests, namely *Bt kurstaki* BLB1 and LIP, efficiently targeting lepidopteron pests, were recently formulated as dried products. To assess their biosafety, toxicity assays were carried out on lab animals (i.e. rabbits, rats and guinea pigs), according to the European ISO and OECD guidelines. The ultimate dual objective was to evaluate the risk for workers/exposed populations and the impact on Health.

Methods: According to a well-established Action Plan (AP), all the experiments were conducted in comparative manners with similar reference products already on the market and a blank formulation of additives without active ingredient, in respect to animal welfares. The eye irritation/corrosion tests were carried out on cohorts of New Zealand rabbits (2-3 kg/rabbit) for acute (one instillation) and sub-acute (single day instillation, 5 days) assays, and the maximum mean total scores (MMTS) were calculated, respectively. The dermal (skin) irritation assays were carried out on three rabbits/product (single exposure) and the Primary Irritation Index (PII) determined. The acute (one gavage and 14 days observations) and subacute (daily gavage during 28 days) oral toxicity assays were carried out on rats (200-250g/rat) in collaboration with Kobay lab animal facilities. The sensitization test was performed on guinea pigs by intradermal administration.

Results: First, all the protocols received an early approval from the Ethical Committee of Institut Pasteur Tunis. The effects of BLB1 and LIP Bt-based formulations were compared to the equivalent DELFIN reference product. As results, no mortality and abnormal clinical findings were observed following external and gavage toxicity tests. No significant weight loss and reduction in feed and water consumption were observed. Barely the skin sensitization test has to be redone because of few mortality observed in negative control group. Collected data will be shown in details. Biochemical parameters and histological observations will be illustrated to support the conclusion.

Conclusion: The first evaluation of the new Btk-based biopests risk on human is promising. The active ingredients as well as the formulated products are non-irritating eye/skin and non-toxic on rat by gavage.

Keywords: *Bacillus thuringiensis*, formulation, toxicity, irritation, mortality

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921.

Economic maturation & business plan development for new *Bacillus thuringiensis* based biopesticide (WP4-1)

Souad ROUIS¹, [Nadia BEN SAID](mailto:Nadia.bensaid@labomedis.com)², Tayssir HAMMAR², Sara FALVO³

¹Centre de Biotechnology de Sfax, Sfax, Tunisia, ²MEDIS, Nabeul, Tunisia; ³BioIndustryPark, Italy,

Souad.rouis@cbs.rnrt.tn, Nadia.bensaid@labomedis.com, Tayssir.hammar@labomedis.com,
Falvo@bioindustryPark.it,

Background and aim: Within the IPM-4-Citrus project, different results are acquired and can be exploitable from scientific and commercial point of view. The exploitation of the project results is at the heart of its implementation. Therefore, different activities are devoted for the economic maturation and business plan development for future spin-off and/or for the creation of new production lines. The aim of this work, is to summarize different exploitation activities developed with the help of the EC Booster experts in order to bring the results closer to the market.

Methods: Key Exploitable Results (KER) are defined. Based on these KERs, Business Plan Canvas (BPC) is developed. The methodology used for BPC development includes: market analysis, legal, regulatory and Intellectual Propriety Rights aspects, clear action plan and roles, milestones, financial costs and revenues, and the impact of the project in three years.

Results: KERs defined are new innovative, cheaper, user friendly biopesticide and simplified production process. Despite the strict regulatory aspects for product homologation, the market analysis shows that biopesticide market is strongly emerging and that 90% of this growing market is based on *Bacillus thuringiensis* strains which increase the competitiveness. To overcome competitiveness, IPM-4-Citrus product will be 25% less expensive than commercial products and will target 100% of local market and 10% of regional market. Intellectual property rights of the products formulations and process will be protected via patents. The exploitation roadmaps main actions are: finalization of the construction and installation of the biopesticide unit, technology transfer and homologation application. Revenues are estimated to increase during the first three years. Also the project is expected to increase biological crops and to save public health and environment from chemicals effects.

Conclusion: In summary, KERs generated during the IPM-4-Citrus project are value added results that makes the economic maturation possible and the commercialization of the new biopesticides cost effective despite the high competitiveness and all regulatory aspects.

Keywords: Integrated Pest Management, Biopesticides, *Bacillus thuringiensis*, Key Exploitable Results, Exploitation, Market, Economic maturation, Business Plan

Funding: IPM-4-Citrus project, H2020-RISE-MSCA, EU project n°734921.