

IPM-4-CITRUS



# Monitoring of *Bacillus thuringiensis* (Bt) growth and sporulation: exploration and comparison of on-line and off-line measurements.

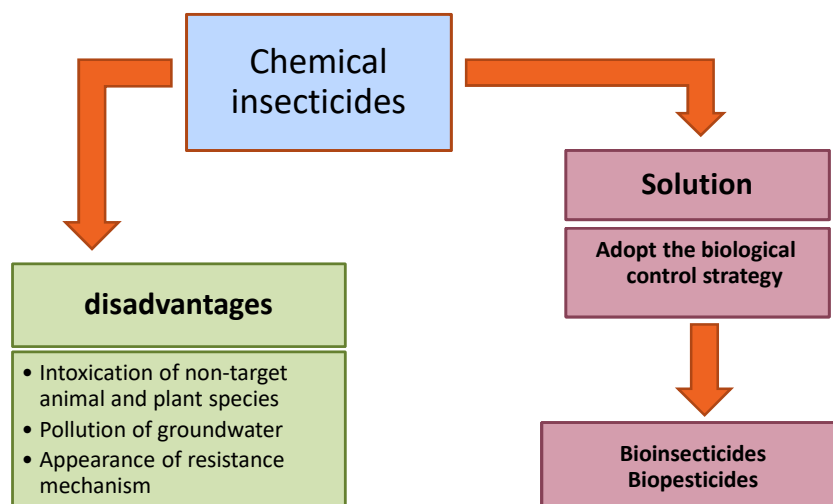
Presented by: **Nouha ABDELMALEK**

MCB2 – 16 to 20 March 2019 – Yessmine Hammamet, Tunisia

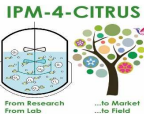


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# Biopesticides market



- The global biopesticides market was ca. \$3.5B in 2016 & is projected to reach \$8.8B by 2022
- **Biopesticides today hold only 5% of the total crop protection market**, representing significant room for growth
- **The biopesticide market is growing at a CAGR of ~17% vs. ~2.0% in the chemical pesticide market**


**Estimated Global Biopesticide Market<sup>1</sup>**



Year	Market Value (\$B)
2016	\$3.4B
2017E	
2018E	
2019E	
2020E	
2021E	
2022E	\$8.8B

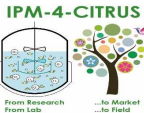
CAGR = 17.4%




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3



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# Bioinsecticides




Biological pesticides that are derived from natural materials as animals, plants, bacteria, and certain minerals.

➤ Advantages

- ✓ Specific
- ✓ SAFE for human and environment

***Bacillus thuringiensis***



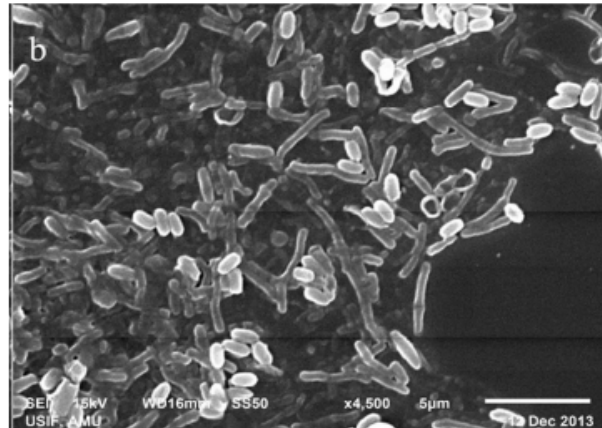
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4

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## *Bacillus thuringiensis*

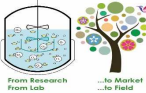


Scanning electron micrographs of *Bt* cells (Lone and al., 2017).

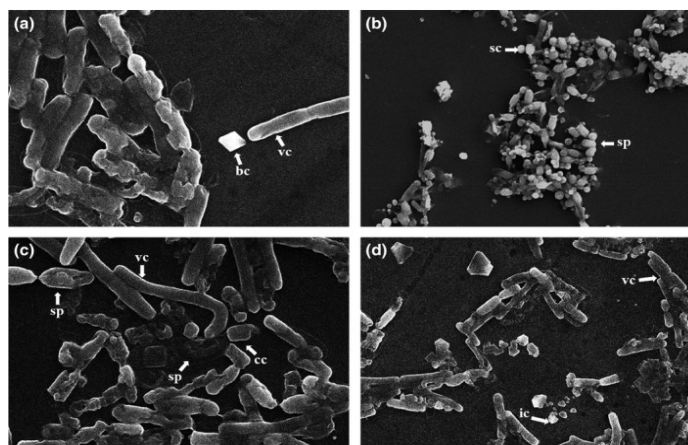


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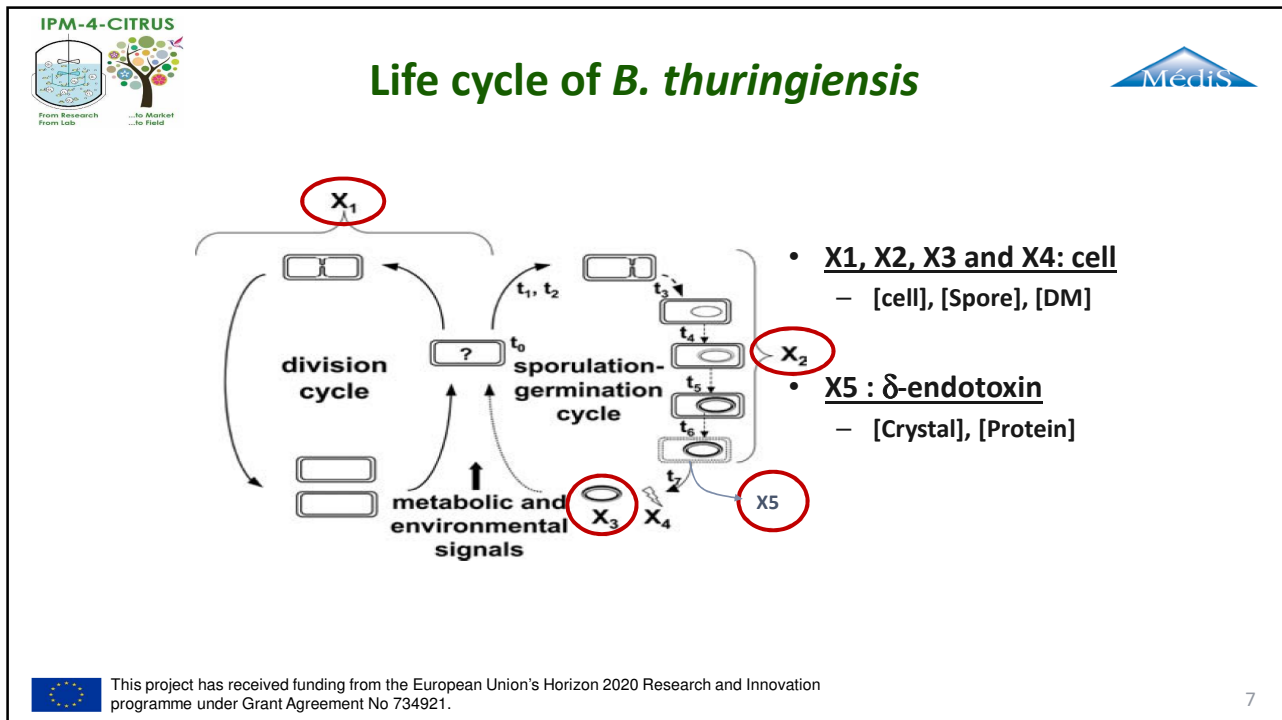
## *Bacillus thuringiensis* delta-endotoxins



Scanning electron micrographs of *Bt* cells showing presence of crystals, spores, and vegetative cells. (a) bipyramidal crystal (bc), (b) spherical crystal (sc), (c) cuboidal crystal (cc), (d) irregular crystal (ic) (Lone and al., 2017).



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## IPM-4-Citrus aims...

<http://www.ipm-4-citrus.insa-toulouse.fr/>

**IPM-4-CITRUS** aims to strengthen collaborations between academic and non-academic partners based in 3 European Member States (France, Germany and Italy), 2 Associated Countries (Turkey and Tunisia) and 1 Third Country (Lebanon), to develop two new bio-pesticides active against citrus pests and scale them up from lab to market.

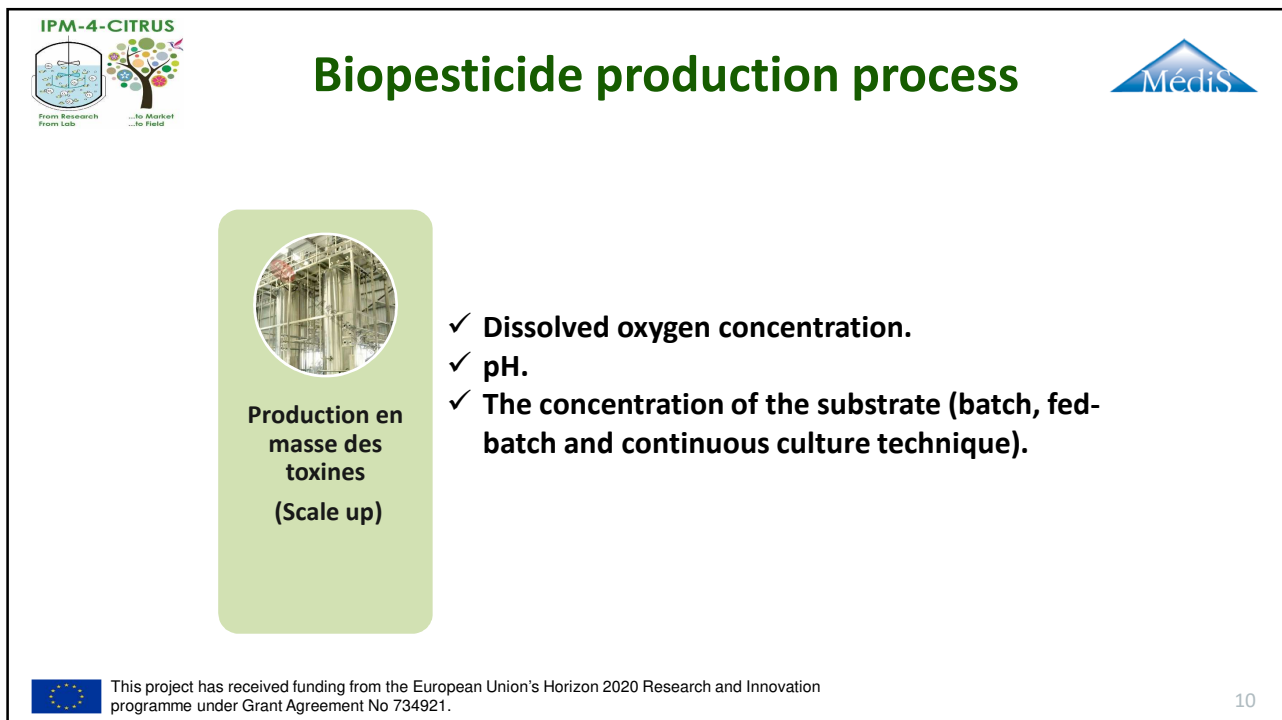
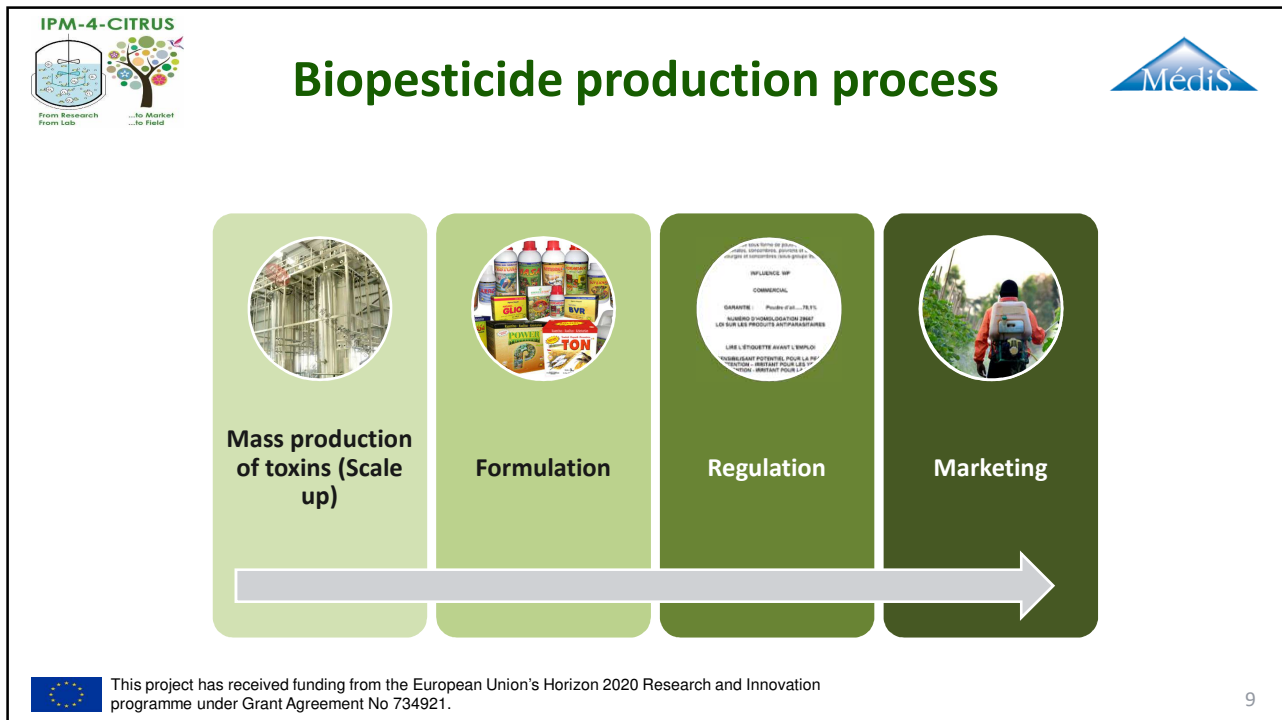
Bacillus thuringiensis

The project's research and innovation activities are based on a multidisciplinary approach, which aims at understanding and sensitising stakeholders about the health risk factors related to citrus pests and developing an alternative **Integrated Pest Management (IPM)** approach based on biological control. In conjunction with validation through field tests, the project will pave the way for future commercial exploitation of these new biopesticide products by drawing up a feasibility study for future spin-off activities and/or new production lines in partner SMEs.

Staff secondments and inter-sector and international mobilities between complementary partners will represent a unique opportunity to optimise bioproduction processes and obtain high added-value bioproducts, while building up the partners' skills and reinforcing the training of early-stage researchers through knowledge sharing and networking. The project will also adopt a concrete RRI approach by favouring public engagement and informal education through the different outreach activities aimed at a variety of target groups.

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8



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# Objective

**IDENTIFYING THE VARIOUS PHYSIOLOGICAL STATES OF *BACILLUS THURINGIENSIS* DURING CULTURE, USING COMBINATION OF ONLINE AND OFFLINE MEASUREMENTS.**



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11

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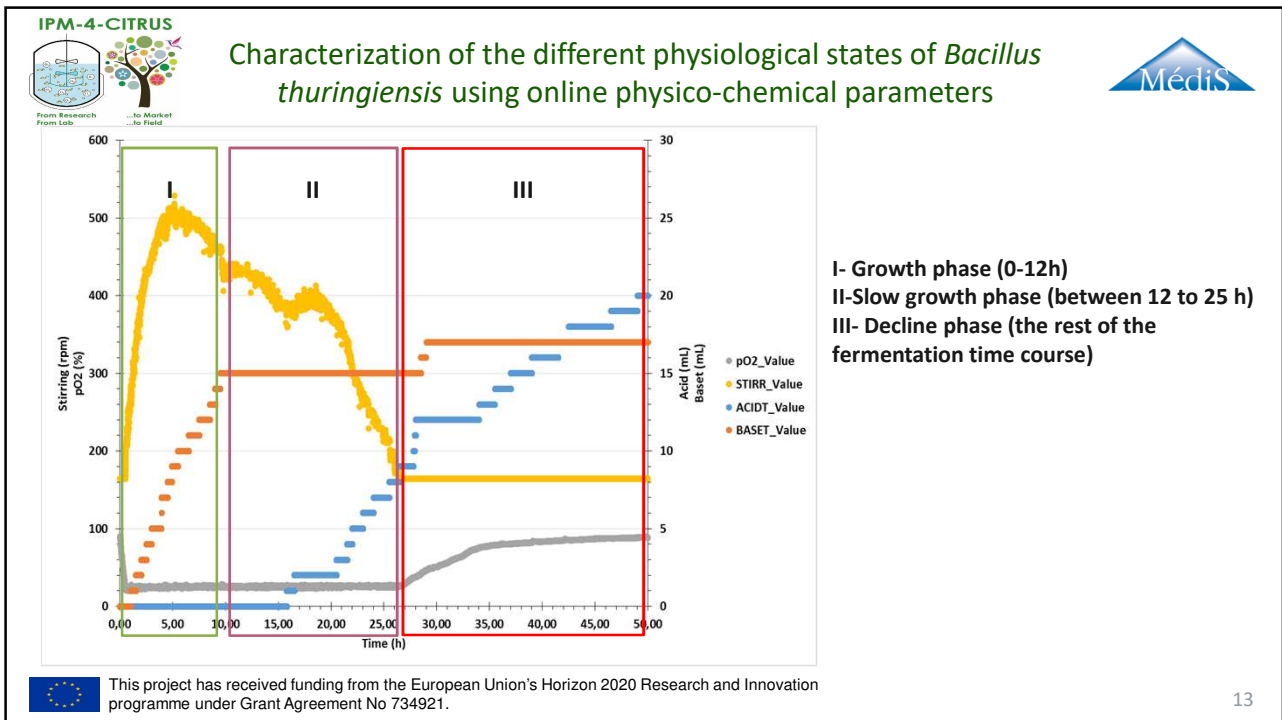
## Culture conditions in the semi-synthetic medium



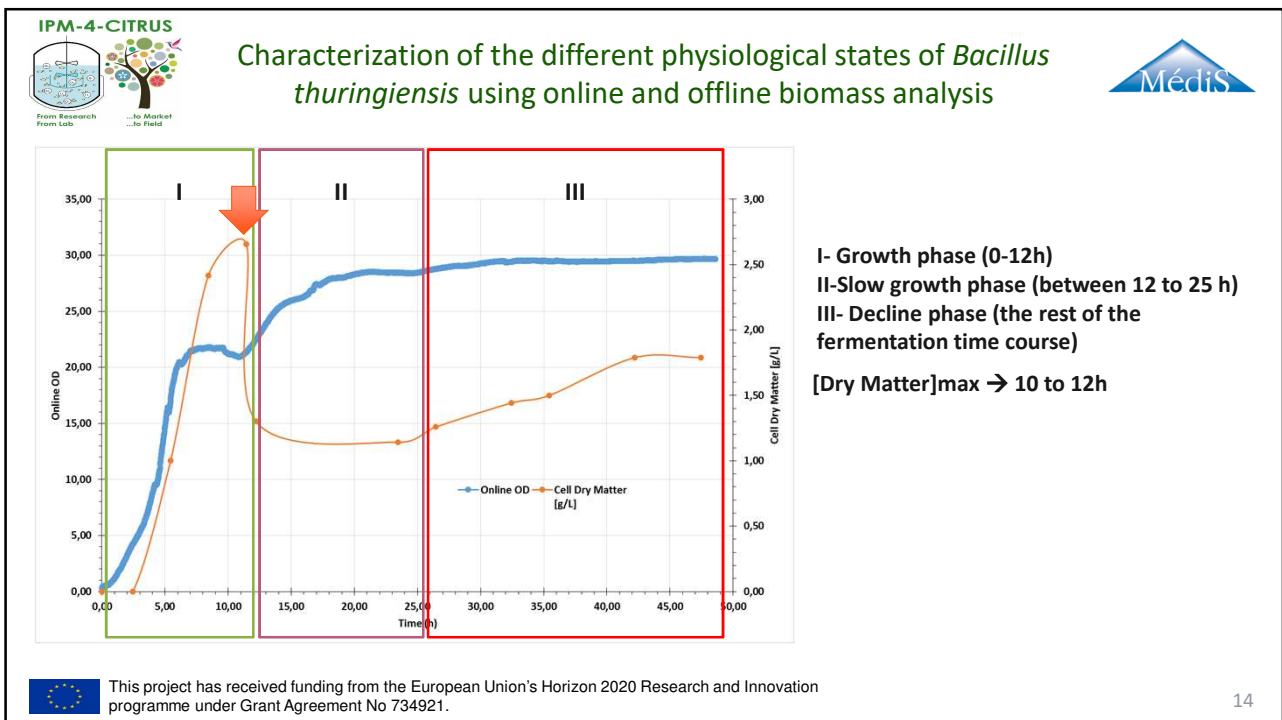
	BLB1	HD1
pH	6,8	6,8
T°C	30	30
pO <sub>2</sub> (%)	50	50
String (rpm)	Cascade	Cascade
Gazflow (L/min)	The aeration rate is fixed at 0,5	The aeration rate is fixed at 0,5
Initial OD	0,15±0,05	0,15±0,05
Fermentation duration (h)	48	48



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13




14


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**Refractility evolution during fermentation of *Bacillus thuringiensis* using phase contrast microscopy**

Médis



**1- Vegetative cells at the beginning of the fermentation (till 12h)**

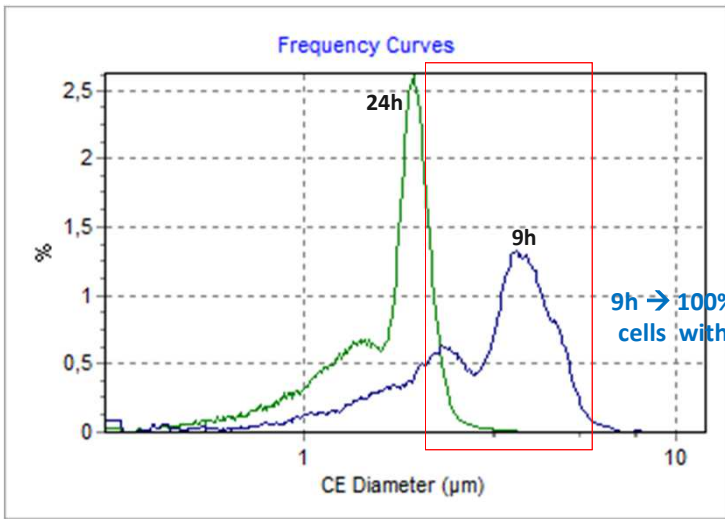
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15

**IPM-4-CITRUS**  
From Research From Lab ...to Market ...to Field

**Characterization of the size distribution of the different evaluative phases of *Bacillus thuringiensis* using Morphogranulometry**

Médis




**Frequency Curves**

24h

9h

9h → 100% of the population are formed of Vegetative cells with a mean diameters of 3,14µm and bacterial chains of around 6 to 8 bacteria.

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16


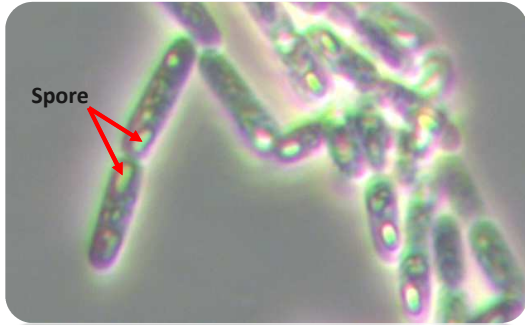


**IPM-4-CITRUS**  
From Research From Lab ...to Market ...to Field

**Refractility evolution during fermentation of *Bacillus thuringiensis* using phase contrast microscopy**

MédiS

**2- At about 12h of fermentation, small and slightly refractile white body (spore) located at one end pole of the cells appears .**

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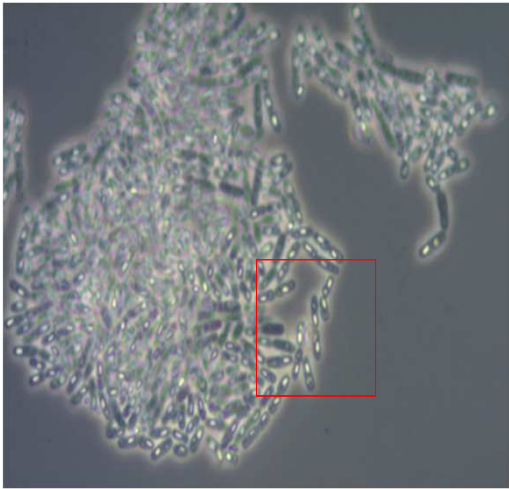
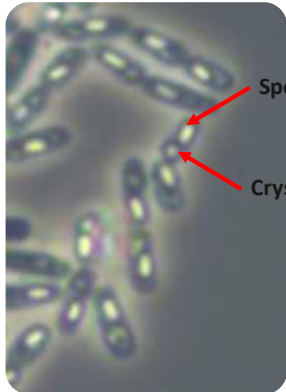
17

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From Research From Lab ...to Market ...to Field

**Refractility evolution during fermentation of *Bacillus thuringiensis* using phase contrast microscopy**


MédiS

**3- The refractile unlysed sporulated cells and their linked crystals (between 12 till 18h)**





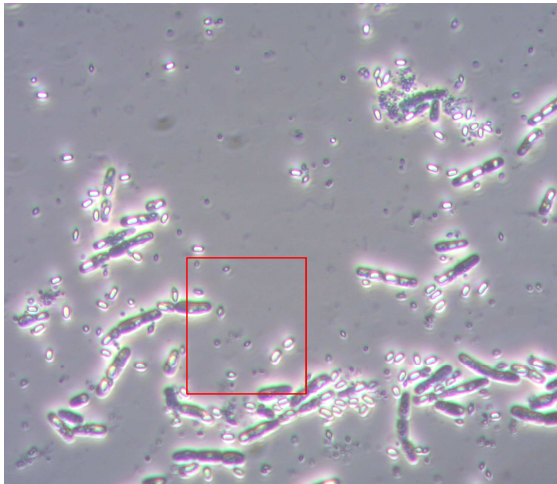
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18

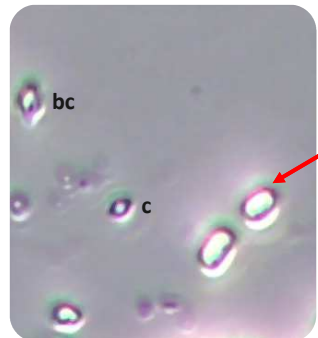


### Refractility evolution during fermentation of *Bacillus thuringiensis* using phase contrast microscopy







**4- Fully mature spores, and their crystal, free from the mother cell (from 18h till the end of fermentation).**




*bc: bipyramidal crystal; c: cubic crystal*

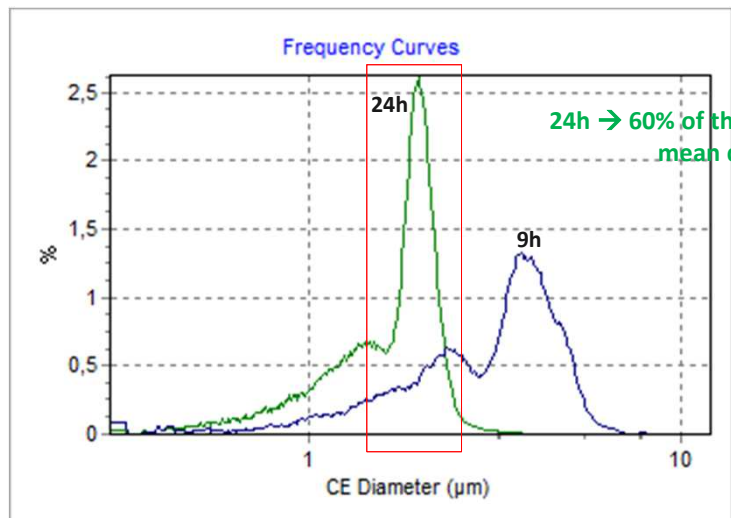


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### Characterization of the size distribution of the different evaluative phases of *Bacillus thuringiensis* using Morphogranulometry

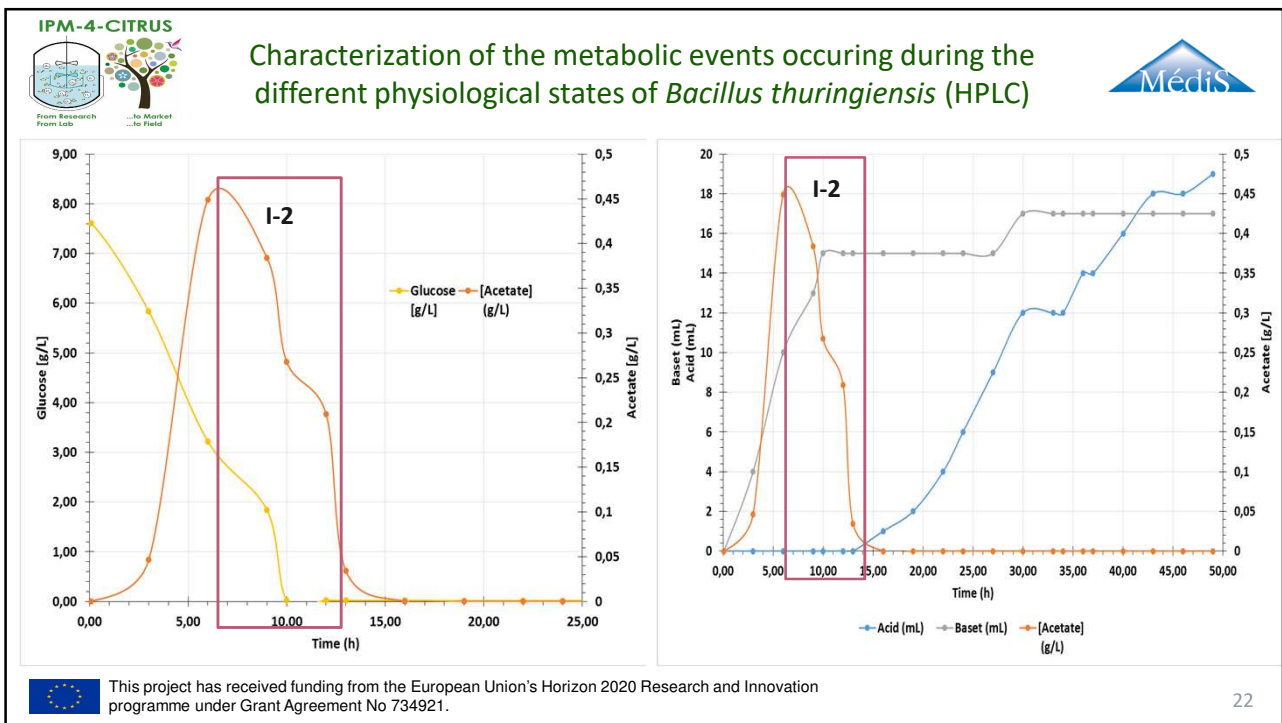
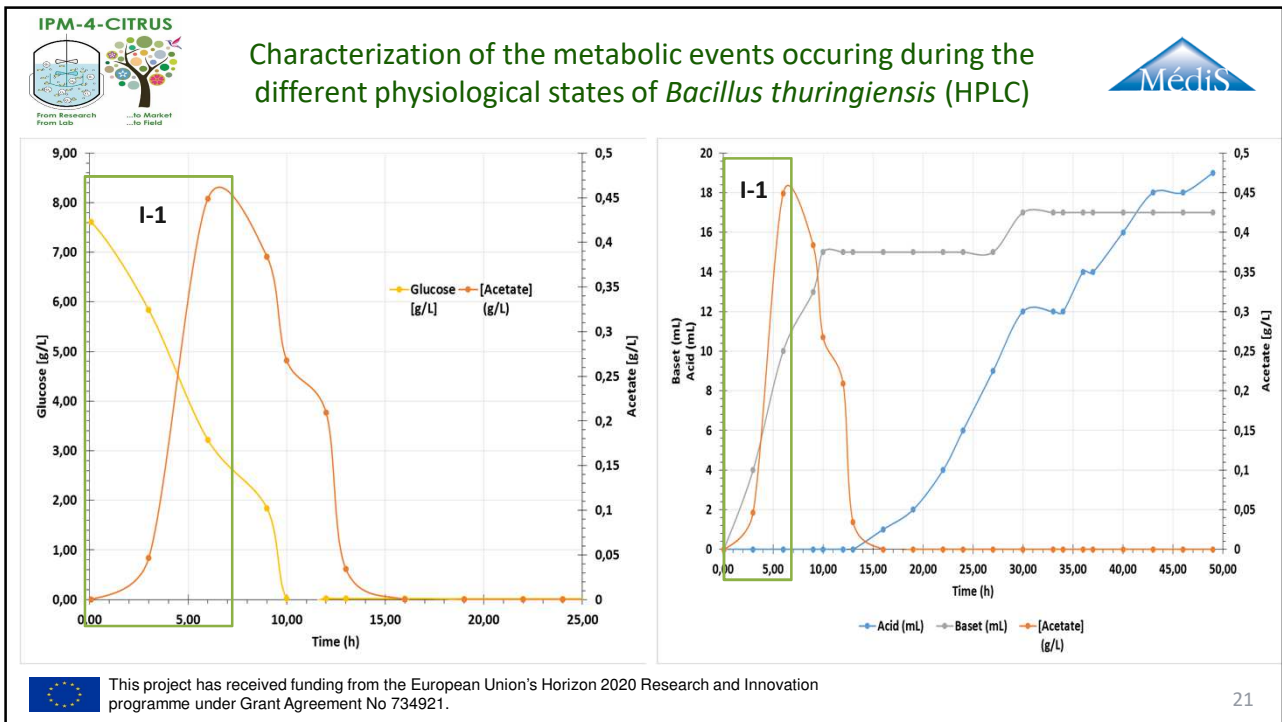


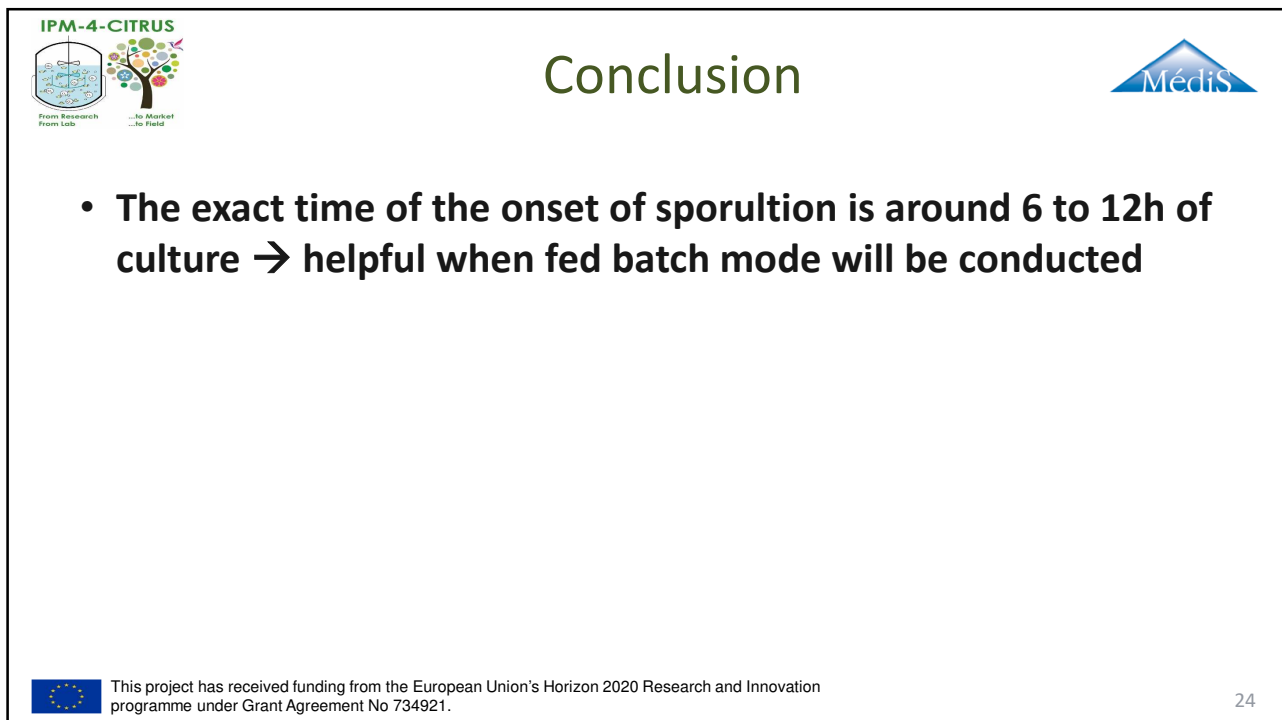
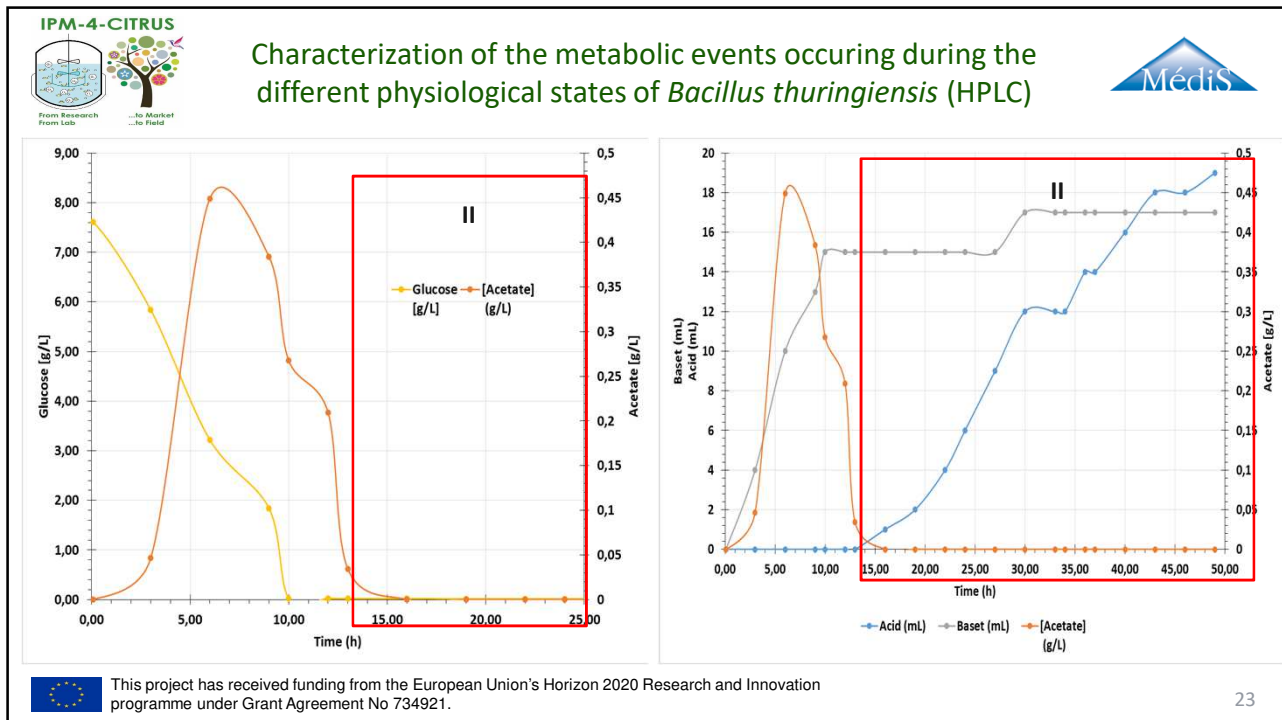


**24h → 60% of the population are Spores with a mean diameters of 1,68µm.**




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**Culture conditions in the semi-synthetic medium**




	BLB1	BLB1
pH	6,8	6,8
T°C	30	30
pO <sub>2</sub> (%)	50	25
String (rpm)	Cascade	Cascade
Gazflow (L/min)	The aeration rate is fixed at 0,5	0 at the beginning of the fermentation then fixed at 0,3 at the moment of the regulation
Initial OD	0,15±0,05	0,15±0,05
Fermentation duration (h)	48	48


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**Biokenetic parameters of BLB1**



pO <sub>2</sub> (%)	50	25
tX <sub>m</sub> (h)	12	10
t <sub>p</sub> (h)	36	36
t <sub>f</sub> (h)	48	48
X <sub>m</sub> (g/L)	2,66	5,62
DSX <sub>m</sub> (g/L)	5,38	6,07
YX/S	0,5	0,93
P (g/L)	824,85	835,04
P <sub>x</sub> (g/L.h)	0,22	0,57
Spores (UFC/mL)	4,41E+09	1,20E+09
μ <sub>m</sub> (h <sup>-1</sup> )	0,27	0,57
YX/P	3,22	6,73

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26

## IPM-4-CITRUS



## Conclusion



→ Three distinct phases:

- The exponential phase (0 to 12h) : 100% vegetative cells.
- The transition (slow growth from 12h to 25h) : cells containing spores and crystals.
- Sporulation and cellular lysis (the rest of fermentation).

→ pH regulation and acetic acid production were found to be a main indicator to detect the exact sporulation phase.

→ A better biomass concentration and global yield was obtained using pO<sub>2</sub>sat=25%.



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27

## IPM-4-CITRUS



## Perspective

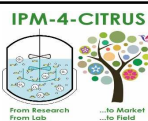


- Future cultures will initiate fed-batch mode considering the critical time of sporulation, in order to increase the biomass and consequently  $\delta$ -endotoxins production.
- The overarching aim will be to transpose biopesticide production with semi-synthetic medium to wheat bran industrial substrate.



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28



# THANK YOU!

## DO YOU HAVE ANY QUESTIONS?

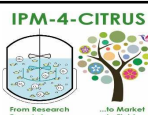


<http://www.ipm-4-citrus.insa-toulouse.fr/>



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29



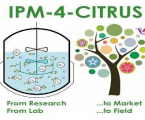
### Tableau récapitulatif des paramètres de suivi de croissance hors ligne



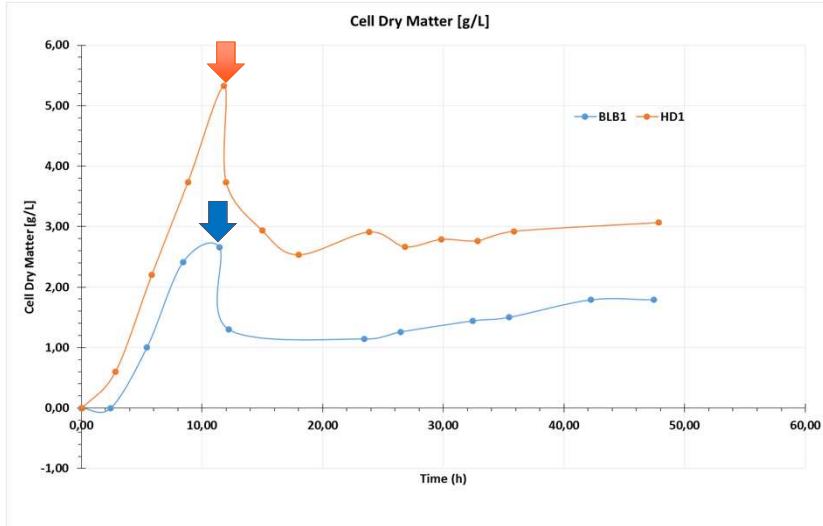
	BLB1	HD1
tXm (h)	12	12
tf (h)	48	48
Xm (g/L)	2,66	5,32
DSXm(g/L)	5,38	7,36
YX/S	0,5	0,72
P (g/L)	824,85	943,46
Px (g/L.h)	0,22	0,44
Spores (UFC/mL)	4,41E+09	5,83E+08
$\mu$ m(h-1)	0,27	0,47
YX/P	3,22	5,6



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## Biokinetic parameters comparison between HD1 and BLB1

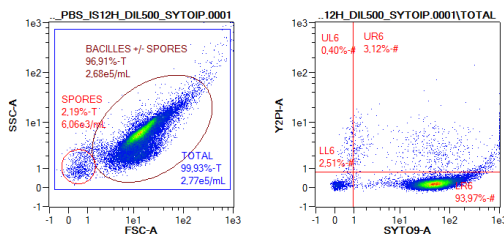


→ [CDM]<sub>max</sub>BLB1 = 2,66 g/L → μ<sub>max</sub> = 0,27h<sup>-1</sup>

→ [CDM]<sub>max</sub>HD1 = 5,32 g/L → μ<sub>max</sub> = 0,47h<sup>-1</sup>

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## ECHANTILLON DU 26-09-18 TEMPS = 9H DE CULTURE



File BT2018-09-26\_CELLULES\_9H\_PBS\_IS12H\_DIL500\_SYTOIP.0001.mqd 2018-Sep-26 17:03

SID CELLULES\_9H\_PBS\_IS12H Descr. DIL500\_SYTOIF

Name	% #	FSC-A Median	SSC-A Median	Y2PI-A Median	SYTO9-A Median
BACILLES +/- SPORES	96.91	14.15	5.52	0.18	50.58
SPORES	2.19	0.60	1.01	0.12	0.20
TOTAL	99.93	13.96	5.45	0.18	49.95
LL6	2.51	0.69	1.18	0.12	0.19
UL6	0.40	1.45	1.82	2.92	0.67
UR6	3.12	29.38	10.34	2.40	67.84
LR6	93.97	14.12	5.50	0.18	50.68

ECHANTILLON SANS TRAITEMENT = CELLULES

- REPRISE EN PBS → PAS DE LYSÉ CELLULAIRE, MORPHOLOGIE OK POUR ANALYSE EN CYTOMETRIE
- MARQUAGE AU SYTO9 ET IODURE DE PROPIDIUM

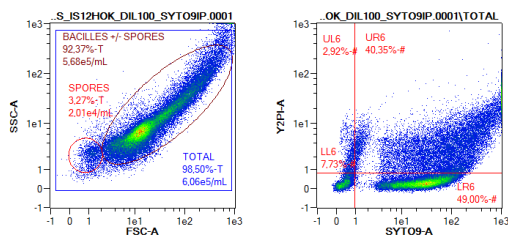
ANALYSE DES POPULATIONS :

- SYTO9+/IP- → CELLULES VIVANTES
- SYTO9-/IP- → SPORES, très peu présentes

APPARITION DE CHAINETTES = Médiane et CV augmentés sur FSC et SSC



## ECHANTILLON DU 26-09-18 TEMPS = 12H DE CULTURE



File BT2018-09-26\_CELULES\_12H\_PBS\_IS12HOK\_DIL100\_SYTO9IP.0001.mqd 2018-Sep-26 12:01  
SID CELLULES\_12H\_PBS\_IS12HOK Descr. DIL100\_S1

Name	%-#	FSC-A Median	SSC-A Median	Y2-PI-A Median	SYTO9-A Median
BACILLES +/- SPORES	92.37	16.91	7.90	0.56	33.35
SPORES	3.27	1.24	2.33	0.15	0.23
TOTAL	98.50	16.13	7.60	0.53	31.41
LL6	7.73	2.83	3.19	0.22	0.38
UL6	2.92	4.04	3.29	1.82	0.68
LR6	40.35	70.72	24.13	3.71	101.22
LR6	45.00	13.80	6.53	0.25	26.99

### ECHANTILLON SANS TRAITEMENT = CELLULES

REPRISE EN PBS → PAS DE LYSE  
CELLULAIRE, MORPHOLOGIE OK  
POUR ANALYSE EN CYTOMETRIE  
MARQUAGE AU SYTO9 ET IODURE  
DE PROPIDIUM

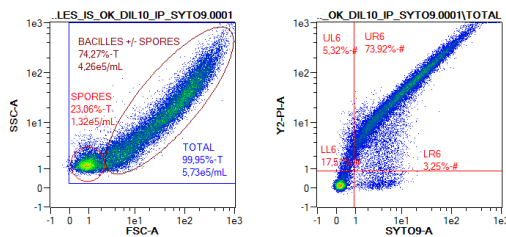
#### ANALYSE DES POPULATIONS :

- SYTO9+/IP- → CELLULES VIVANTES
- SYTO9-/IP- → SPORES, très peu présentes
- SYTO9+/IP+ → CELLULES LYSEES

CHAINETTES +++ = Médiane et CV  
augmentés sur FSC et SSC  
APPARTION DE LA LYSE CELLULAIRE  
PROPORTION DES SPORES SANS  
TRAITEMENT AUGMENTEE

33

## ECHANTILLON DU 11-09-18 TEMPS = ?? H DE CULTURE



File BT2018-09-11\_CELULES\_IS\_OK\_DIL10\_IP\_SYTO9.0001.mqd 2018-Sep-11 16:45  
SID CELLULES\_IS\_OK Descr. DIL10\_IP\_SYTO9

Name	%-#	FSC-A Median	SSC-A Median	Y2-PI-A Median	SYTO9-A Median
BACILLES +/- SPORES	74.27	38.83	13.13	30.45	8.70
SPORES	23.06	0.97	1.30	0.20	0.27
TOTAL	99.95	15.21	5.86	13.62	4.53
LL6	17.51	0.87	1.33	0.15	0.20
UL6	5.32	2.53	2.08	2.12	0.64
LR6	73.92	40.18	13.45	31.32	8.88
LR6	3.25	1.74	1.30	0.27	3.49

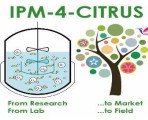
### ECHANTILLON SANS TRAITEMENT = CELLULES

- REPRISE EN EAU → LYSE  
CELLULAIRE, MAUVAISE  
MORPHOLOGIE POUR ANALYSE EN  
CYTOMETRIE
- MARQUAGE AU SYTO9 ET IODURE  
DE PROPIDIUM

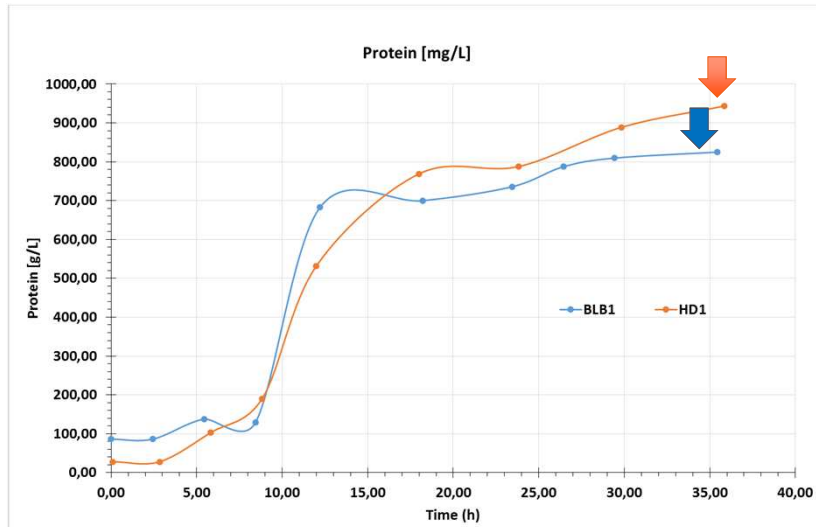
#### DEUX POPULATIONS SE DISTINGUENT:

- SYTO9+/IP+ → CELLULES LYSEES ?
- SYTO9-/IP- → SPORES RESISTANTES  
EXPULSEES PAR LA LYSE CELLULAIRE

34



## Biokinetic parameters comparison between HD1 and BLB1



→ [Prot]maxBLB1 = 824,85 mg/L

→ [Prot]maxHD1 = 943,46 mg/L



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35