Journal Pre-proof

# Biochemical limitations of *Bacillus thuringiensis* based biopesticides production in a wheat bran culture medium

Rita Barssoum, Gabrielle Al Kassis, Rayan Nasseredine, Jihane Saad, Meriem El Ghoul, Joanna Abboud, Nancy Fayad, Stéphanie Dupoiron, Julien Cescut, César Arturo Aceves-Lara, Luc Fillaudeau, Mireille Kallassy Awad

PII: S0923-2508(23)00018-9

DOI: https://doi.org/10.1016/j.resmic.2023.104043

Reference: RESMIC 104043

To appear in: *Research in Microbiology*

Received Date: 24 October 2022

Revised Date: 30 January 2023

Accepted Date: 31 January 2023

Please cite this article as: R. Barssoum, G. Al Kassis, R. Nasseredine, J. Saad, M. El Ghoul, J. Abboud, N. Fayad, S. Dupoiron, J. Cescut, .C.A. Aceves-Lara, L. Fillaudeau, M.K. Awad, Biochemical limitations of *Bacillus thuringiensis* based biopesticides production in a wheat bran culture medium, *Research in Microbiologoy*, https://doi.org/10.1016/j.resmic.2023.104043.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2023 Published by Elsevier Masson SAS on behalf of Institut Pasteur.

1 # Biochemical limitations of *Bacillus thuringiensis* based
2 biopesticides production in a wheat bran culture medium

3

4 Rita Barssoum[a,b], Gabrielle Al Kassis[a], Rayan Nasseredine[a], Jihane Saad[a,c], Meriem El Ghoul[d],
5 Joanna Abboud[a,c], Nancy Fayad[a,e], Stéphanie Dupoiron[c], Julien Cescut[c], César Arturo Aceves-
6 Lara[b], Luc Fillaudeau[b], Mireille Kallassy Awad[a*]

7 [a]Saint-Joseph University of Beirut, UR- EGP, Functional Genomic and Proteomic Laboratory,
8 Faculty of Sciences, Mar Roukos- Dekwaneh, B.P. 17-5208, Mar Mikhael, Beirut 1104 2020,
9 Lebanon.

10 [b]Toulouse Biotechnology Institute, Bio & Chemical Engineering, Université de Toulouse- (CNRS
11 UMR5504- INRAE UMR792, INSA), 135 avenue de Rangueil, 31077 Toulouse cedex 04, France.

12 [c]Toulouse White Biotechnology (INRAE UMS1337, CNRS UMS3582, INSA), INSA-Toulouse, 135
13 avenue de Rangueil 31077, Toulouse cedex 04, France.

14 [d]Pharmacological laboratory Médis, route de Tunis Km 7-BP 206, Nabeul 8000, Tunisia.

15 [e]Multi-Omics Laboratory, School of Pharmacy, Lebanese American University, P.O. Box 36,
16 Byblos 1401, Lebanon

17 Contact e-mails:

18 Rita.barssoum@net.usj.edu.lb, kassisgabrielle@gmail.com, Rayan.nassereddine@net.usj.edu.lb,
19 Jihane.saad@net.usj.edu.lb, merighoul@gmail.com, joanna.abboud@inrae.fr, nancy.fayad@net.usj.edu.lb,
20 stephanie.dupoiron@inrae.fr, julien.cescut@inrae.fr, aceves@insa-toulouse.fr, lfillaud@insa-toulouse.fr,
21 mireille.kallassy@usj.edu.lb*

22

Journal Pre-proof

23 Abstract

24 *Bacillus thuringiensis*, a gram-positive sporulating bacteria found in the environment,
25 produces, during its sporulation phase, crystals responsible for its insecticidal activity,
26 constituted of an assembly of pore-forming δ-endotoxins. This has led to its use as a
27 biopesticide, an eco-friendly alternative to harmful chemical pesticides. To minimize
28 production cost, one endemic *Bacillus thuringiensis* sv. *kurstaki* (*Btk*) strain Lip, isolated from
29 Lebanese soil, was cultivated in a wheat bran (WB) based medium (IPM-4-Citrus project EC n°
30 734921). With the aim of studying the biochemical limitations of *Btk* biopesticide production
31 in a wheat bran based medium, the WB was sieved into different granulometries, heat treated,
32 inoculated with *Btk* Lip at flask scale, then filtered and separated into an insoluble and a
33 permeate fractions. Several biochemical analyses, *ie.* bio performances, starch, elemental
34 composition, total nitrogen and ashes, were then conducted on both fractions before and
35 after culture. **On a morphological level, two populations were distinguished, the fine starch**
36 **granules and the coarse lignocellulosic particles. The biochemical analyses showed that both**
37 **the raw and sieved WB have a similar proteins content (0.115 g/gdm WB), water content**
38 **(0.116 g/gdm WB) and elemental composition (carbon: 45 %, oxygen: 37 %, nitrogen: 3 %,**
39 **hydrogen: 6 %, ashes: 5 %). The starch content was 17 %, 14 % and 34 % and the fermentable**
40 **fraction was estimated to 32.1 %, 36.1 % and 51.1 % respectively for classes 2, 3 and 4. Both**
41 **the elemental composition and Kjeldahl analyses showed that the nitrogen is the limiting**
42 **nutrient of the culture.**

43 Keywords: *Bacillus thuringiensis*, wheat bran, elemental composition, fermentable fraction,
44 biochemical limitations.

45

Journal Pre-proof

Journal Pre-proof


46 # 1 Introduction

47 Insects, be it plant pests or vectors of human diseases, continue to pose a worldwide problem
48 in agriculture and public health [1]. To get rid of these insects, farmers have traditionally
49 resorted to the excessive use of chemical pesticides, harmful to nature and human health [2].
50 This has led the scientific community to look for less harmful alternatives, hence the
51 introduction and industrialization of "biopesticides", mainly those produced by
52 microorganisms [3]. *Bacillus thuringiensis* (*Bt*) is a facultative anaerobic gram-positive
53 sporulating bacteria, that is among the most used in the production of biopesticides.
54 Moreover, *Bt* has been a genetic source for the production of transgenic plants, able to secrete
55 insecticidal molecules on their own [4]. It usually inhabits different environments such as soil,
56 settled dust or water [5]. *Bt* has been shown to be toxic to various phytopathogen insects
57 including lepidopterans, coleopterans, dipterans, or nematodes, but is considered safe for
58 mammals [6]. *Bt* based products provide effective and eco-friendly pest control, be it disease
59 vectors or phytopathogens problematic in agriculture and forestry. *Bt* life cycle has two main
60 phases: (i) the exponential phase where the bacteria multiplies and produces vegetative
61 biomass and (ii) the sporulating phase where *Bt* begins to sporulate [7] and produces crystals.
62 The latter are a combination of Cry toxins, or $\delta$-endotoxins (https://www.bpprc.org/ ). These
63 toxins are solubilized in the insect gut by the alkaline pH and activated by proteolysis. They
64 will then interact with gut receptors to create pores in the intestinal cell membranes leading
65 to the death of insect larvae [8]. **In addition, the *Bacillus cereus* group to which *Bacillus***
66 ***thuringiensis* belongs, have a large number of transcriptional regulators, responsible for the**
67 **activation of the biofilm formation [9].** *Bacillus thuringiensis* sv. *kurstaki* (*Btk*) is well known
68 for its activity against lepidopteran larvae. Most of the biopesticides distributed in the world
69 are mainly based on *Btk* HD1 [10]. Two endemic strains, *Btk* Lip [11] and Blb1 [12], isolated
70 from Lebanese and Tunisian soils respectively, have exhibited a higher efficiency than HD1
71 against the lepidopteran larvae *Ephestia kuehniella*.

72 For its growth and production, in the laboratory and on industrial scales, *Bt*, similarly to other
73 bacteria, needs a source of sugar. Several sugars, including glucose, fructose, starch, maltose,
74 and ribose, can be fermented by this bacterium [13]. Some new *Bt* strains have also been
75 shown to ferment xylan and cellulose [14]. Glucose, in a limited quantity [15], is the
76 bacterium's most utilized carbon source and the most effective for sporulation and δ-

77 endotoxin synthesis [16]. Organic nitrogen is also a key element for bacterial growth. Some
78 amino acids (leucine, valine, arginine) are crucial for biomass production and sporulation.
79 However, other amino acids like cysteine have an opposite effect [17]. Minerals, like
80 potassium ($K_2HPO_4$, 50-100 mM), are also required for δ-endotoxins synthesis [18]. The
81 Anderson medium (yeast extract, bactopeptone, glucose and $(NH_4)_2SO_2$) yielded $7x10^{11}$
82 spores/mL compared to other media used in *Bt* culture [18,.19]. Özkan et al., 2003 found that
83 NSYM (nutrient yeast salt medium) based on peptone, glucose, meat extract and yeast extract
84 produced $3x10^{10}$ cfu/mL [21]. Because they contain yeast or animal extracts, all of these
85 synthetic media were classified as complex media. Since the cost of production is a key step
86 in biopesticide production at large scale, several other complex media with reduced cost have
87 been proposed for *Btk* culture [22]**. Wheat bran (WB), a low- cost by-product (192 USD/
88 tonnage) of cereal grain milling is estimated to be 650 million tons per year in the world**
89 [23]**, contains all the elements needed for the bacterial growth (sugar, proteins and
90 minerals). As previously demonstrated, WB medium presented a higher yield for *Btk* crystals
91 production compared to semi-synthetic medium [22, 23, 24]. Following an experimental
92 design, a response surface methodology was employed to define the operating parameters
93 for optimal *Bt* growth.** The wheat bran was sieved in order to understand the effect of the
94 granulometry on biopesticide production.[27] In this context, IPM-4-Citrus project (MSCA
95 RISE, n° 734921, April 2017-December 2022, www.ipm-4-citrus.insa-toulouse.fr) developed
96 simultaneously research and transfer activities about two new biopesticides based on the δ-
97 endotoxins produced by *Btk* Blb1 and Lip, active against citrus pests *Phyllocnitis citrella* and
98 *Prays citri*. The project englobing 11 partners from 6 countries, aims to understand and raise
99 awareness among stakeholders about the health risks related to citrus pests and to develop
100 an alternative integrated pest management (IPM) approach based on biological control. These
101 actions and scientific locks include bioproduction, formulation and bioactivity, and transfer to
102 the market. To this end, the two selected strains of *Btk*, Blb1 and Lip and the one reference
103 HD1, were cultivated in a wheat bran based medium (WB).

104 The aim of this study is to define the bacteria's nutritional requirements during the culture
105 and to determine the biochemical limitations of the production process, such as the
106 fermentable fraction and the limiting nutrient. To this end, the WB was sieved into three
107 particle sizes (classes). Each class was suspended in water, sterilized by autoclaving and

Journal Pre-proof



108 inoculated by *Btk* Lip at flask scale. The suspensions were then filtered and separated into two
109 fractions: the insoluble fraction containing the remaining WB and the permeate fraction
110 containing the vegetative cells, the spores and the δ-endotoxins. Several chemical and
111 biochemical analyses were conducted on wheat bran, on the strains and on both fractions.

# 112 2 Materials and Methods

## 113 2.1 Strains and substrate

114 Three strains of *Bacillus thuringiensis sv. kurstaki (Btk)* were selected for this study:

115 1. *Btk* HD1, the industrial reference strain [28]
116 2. *Btk* Blb1, isolated from a Tunisian soil sample [12]
117 3. *Btk* Lip, isolated from a Lebanese soil sample [11]

118 Wheat bran (WB) as an industrial by-product is the main component of the culture medium
119 to produce the *Btk* spores/crystals mixture. **The natural** WB was bought from Nehmet rabna-
120 Grocery store (Mansourieh, Lebanon, 14/04/2021). This by-product was sieved and
121 characterized by several physico-chemical analyses: dry matter, water content, minerals,
122 protein, total sugar, nitrogen and elemental composition.

## 123 2.2 Sample preparation

### 124 2.2.1 Sieving

125 Wheat bran (Nehmet Rabna, Mansourieh, Lebanon, 14/04/2021) was sieved in order to study
126 its physicochemical characteristics. Batches (70 g) were sieved on a vibrating sifter (ELE
127 international, UK, SN: 80-0352, max frequency: 60 Hz, width: 380 mm, height: 1085 mm during
128 20 minutes at 5 Hz. Three square mesh sieves (250 µm, 500 µm, 850 µm) generated 4 fractions
129 (class 1 to 4). Material balances were established for 2 batches (200 g) and the biochemical
130 composition are reported in table 1 with an average water content of 0.12 g/grams dry matter
131 (gdm) and a density of 1.13 grams humid matter (ghm)/mL. Class 1 (>850µm) represents less
132 than 1% w/w and is not of industrial interest considering the separation and formulation steps
133 downstream of bioproduction. Classes 2, 3 and 4 were adopted for further biochemical and
134 physical analyses.

Journal Pre-proof

135 2.2.2 Flask cultures

136 *Btk* strains were initially isolated on petri dishes containing T3 solid medium [29]. The dishes
137 were incubated for 12 h at 30 °C. Transfer to liquid medium was ensured by inoculation of 6
138 mL Luria Broth (LB) medium [30]. Each suspension was incubated for 12 h at 30 °C with shaking
139 at 250 rpm. Cell growth was measured by optical density (OD) at 600 nm. **For the liquid**
140 **fermentation,** 3.68 ghm of WB from classes 2, 3 and 4 were suspended in a total volume of
141 50 mL in **500 ml flasks**, equivalent to a concentration of [WB]=73.6 ghm/L**. According to our**
142 **previous study** [27]**, this concentration was the optimal concentration for *Btk* growth.**
143 **Moreover, on a physical level, the selected concentration does not represent any physical**
144 **limitations because the interaction between the WB particles in this suspension is**
145 **moderated. However, choosing a higher concentration means that the WB particles will**
146 **have a stronger interaction with each other and this limited the oxygen transference to the**
147 **culture which induces problems during the fermentation.**

148 Then, the suspensions were sterilized by autoclaving at 121 °C for 20 min. **It is of note that**
149 **the autoclaving may generate some compounds that can inhibit the bacterial growth** [31]**.**

150 Afterwards, the media were inoculated at an equivalent initial OD= 0.15 (600 nm) and
151 incubated for 48 h at 30 °C with shaking at 250 **revolutions** per minute (rpm) and **an initial**
152 **pH= 6.2.**

153 2.2.3 Solid/liquid separation

154 Flask contents (with or without culture) were filtered on a Whatman paper (#28413902, cut
155 off: 13 µm), generating insoluble (substrate) and permeate (cells, spores, δ-endotoxins)
156 fractions. This was only done for classes 2 and 3, not 4, because the fine particles of the latest
157 clogged the filter. All chemical analyses were realized with both fractions for classes 2 and 3,
158 and on the whole suspension for class 4, in order to establish the mass and elemental
159 balances.

160 2.3 Physico-chemical analysis of the substrate

161 2.3.1 Morpho-granulometry of WB particles

162 The morphological analysis of the raw and sieved WB was performed ex-situ using a
163 morphogranulometer (Mastersizer G3S, Malvern Instruments Ltd. SN: MAL1033756,
164 Morphologi v7.21 software). This optical device includes a lens system (magnification: from

Journal Pre-proof

Journal Pre-proof



×1 to ×50, min/max size: 0.5/3000 µm) and a camera (Nikon CFI60) with a resolution close to 0.06 µm/pixel. The analyses were conducted in dry mode by dispersing WB (0.25 mg) through a specific unit (DSU) under a pressure of 4 bar for 10 ms. Image acquisition and analysis were performed according to a standard operating procedure (SOP) that defined the type of light source (diascopic light, bright mode), illumination parameters (light intensity: 80 % ± 0.2), magnification (×2.5) and particle detection threshold (thresholding = 140) and scanned area (40x40 mm). The number (raw data) and volume (conversion under assumption of a spherical model) distributions associated with each parameter (particle size, morphometry) were generated.

#### 2.3.2 Relative (x) and absolute (n) humidity of WB

In order to determine the dry WB moisture content, empty crucibles were dried in an oven (Thermo Fischer Scientific, ref: 0562202010) for 2 h at 105 °C and weighted. WB (3.68 ghm) was dried in the oven (24 h, 105 °C) then the total mass (crucible + WB) was measured. Absolute (g water/ gdm) and relative humidity (g water/ ghm) were then calculated:

$$\boldsymbol{humidity} = \frac{M_{water}}{Dry\ or\ humid\ matter\ (WB)} \qquad \textbf{Eq. 1}$$

#### 2.3.3 Dry matter analysis

WB (3.68 ghm) was suspended in water (50 mL) then autoclaved (**20 min,** 121 °C, saturated steam sterilization). Insoluble and permeate fractions of sterilized suspensions were separated by filtration. The filter papers were first dried in an oven (2 h, 105 °C) and weighted before filtration and then they were put back in the oven for 24 h. The total filtrate was collected in 50 mL flasks. Empty crucibles were put in an oven for 2 h at 105 °C, followed by at least 1 h in the desiccator (SICO) until reaching a constant weight. Ten mL of permeate placed in crucibles were dried in an oven for 24 h at 105 °C. The dry matters of permeate (gdm sol WB/ g) and insoluble (gdm ins WB/g) fractions were measured and calculated [32].

#### 2.3.4 Ashes

Half a gram of insoluble fraction or 7.5 mL of the permeate were placed with the dried crucibles in the oven (600 °C, 2 h). The ashes concentration for both insoluble (g ash/gdm ins) and permeate (g ash/gdm sol) fractions was estimated. These analyses could help understand the mineral composition of insoluble and soluble fractions of the WB based medium.

Journal Pre-proof

195 2.3.5 Water retention capacity (WRC)

196 Empty Falcon tubes (50 mL) were first weighted, before adding 1.84 g of WB and suspending
197 it in 25 mL of water. The suspensions were agitated for 60 min and sterilized in the autoclave
198 (121 °C, 1 h) then vortexed (30 s) and centrifuged (3000 rpm, 15 min, 16 °C). The supernatant
199 was eliminated and the pellet was weighted. The water retention capacity (% w/w) was then
200 calculated [33].

201 2.4 Bioproduction analysis

202 2.4.1 Cells and Spores counting

203 First, dilutions **rate** of the permeate of a 48 h culture (30 °C) were realized (dilution rate: $10^{-1}$
204 to $10^{-9}$). Then, petri dishes containing solid T3 medium were inoculated with 10 µL of diluted
205 culture at $10^{-9}$, in triplicate, and then incubated at 30 °C for 12 h for counting of vegetative
206 cells and living spores. As described for total biomass, spore concentrations alone were
207 counted by inoculating diluted culture on T3 medium, after actively killing vegetative cells by
208 heat treatment (80 °C, 10 minutes). Spores and cfu concentrations were then calculated
209 considering the average count of the triplicate (A), the dilution factor ($F_d$) and the inoculum
210 volume (v):

211 $$[\boldsymbol{Total\ flora}]\ \boldsymbol{or}\ [\boldsymbol{Spores}] = \frac{A*Fd}{V} \quad \left(\frac{CFU\ or\ Spores}{mL}\right)$$ **Eq. 2**

212 2.4.2 Protein dosage (Bradford)

213 Protein concentration was estimated using the Bradford reagent [34]. After 48 h of culture, 1
214 mL of spores/crystals mixture were centrifuged (13000 rpm, 16 °C, 5 min). The supernatant
215 was thrown out and the pellet was washed twice with NaCl 1M/Triton 0.01 M and four times
216 with 1 mL of cold autoclaved water. The crystals in the pellet were solubilized with 50 mM of
217 NaOH by incubating at 30 °C for 2 h in a rotary shaker (250 rpm). **The samples were
218 centrifugated and 10 µl of the supernatant were mixed with 200 µl of Bradford reagent, and
219 790 µl of water [31].** The corresponding tubes were incubated in the dark for 12 min. The
220 optical density was then measured at λ = 595 nm, and the protein concentration in the
221 samples was calculated based on a standard calibration curve established using BSA
222 standards.

Journal Pre-proof

223 ## 2.5 Biochemical analysis

224 ### 2.5.1 Determination of starch content

225 Starch is an important source of glucose for the bacterial culture. To determine the starch
226 content in WB, the colorimetric method [30, 31] was used. Iodine solution was prepared by
227 crushing 22 mg of Iodine and mixing it with 10 mL of water. KI (44 mg) was added and the total
228 volume was adjusted to 50 mL and homogenized. The standard range was prepared by using
229 starch solutions (Commercial ref: S58150-3J - batch number: 19005-25-81) at concentrations
230 variating between 0.05 and 3 g/L. Calibration curve was constructed by adding 0.2 mL of a
231 standard solution with 1 mL of I2/KI. The solution was left to react for 2 min at room
232 temperature and the optical density was measured at 540 nm (spectrophotometer: Perkin
233 Elmer, ENSPIRE, SN 23000751). Starch was quantified by determining the dilute solutions
234 absorbance using the Beer-Lambert law considering that ε is the slope of the standard curve:

235 $$\boldsymbol{Abs_{\lambda} = \varepsilon_{\lambda} \times l \times c = 0.825 \times l \times c} \qquad \textbf{Eq. 3}$$
236

237 ### 2.5.2 Elemental composition: CHONS

238 The elemental composition analysis [37] was conducted **at the beginning and the end of the**
239 **culture** using the Flash Smart 2000 (Thermo Fischer Scientific, ref: 20140222). The analysis is
240 based on flash combustion (1800 °C) under an inert atmosphere (Helium) followed by gas
241 chromatography. Analysis of CHONS were done through two distinct analysers. The machine
242 is constituted of a quartz tube (Thermo Fischer Scientific, ref: 46802015) and two gas
243 chromatography columns (ref: 26007900 for CHNS, ref: 26008215 for O) and a thermal
244 conductivity detector with increased sensibility. The experiment was done under the following
245 conditions: helium flow rate: 950 mL/min, oven temperature: 950 °C and pressure below 5
246 kPa.

247 Sample preparation included a preliminary step to remove residual water. It is required to
248 ensure that hydrogen and oxygen only come from the substrate or cell activity. For the
249 insoluble fraction, frozen solid samples were dried in an oven (24 h, 70 °C, 300 mbar) whereas
250 for the permeate, liquid samples were thawed then lyophilized (Freeze drier: Epsilon 2-4 LSC
251 plus, ref: TWB /MO/PS/2, 19/10/2017) for 48 h under a pressure of 10 mbar.

Journal Pre-proof

Journal Pre-proof


252 Dried samples were weighted on a precision balance (METTLER TOLEDO, Model XP6/52, SNR
253 B444194554, precision: 0.1 µg, maximum capacity: 2.1 g) in Sn capsules (Thermo Fischer
254 scientific, ref: 24006400) for C, H, N and S analysis and Ag (thermo Fischer scientific, ref:
255 24005400) for oxygen analysis. The masses (sample, capsule) ranged between 2 and 3 mg. The
256 capsules were rolled into balls with pliers, then placed on a carousel for analysis. All samples
257 were analysed in duplicate.

258 Calibrating the device with a standard is a critical step. The standard, bbot (ref: PJ. 33835210),
259 has a known mass elemental composition: C (72.53 %), H (6.09 %), O (7.43 %) N (6.51%) and S
260 (7.44 %). The standard range consisted of 5 samples of different masses between 2 mg and 4
261 mg. The calibrating curves were established. The equations, precision and correlation
262 coefficient are shown in the supplementary material.

263 All analyses with an area below or above the limits of detection (lods) will be considered
264 outside of the calibration range. Nevertheless, since linear regression passes through the
265 ordinate at the origin, the weak values will be interpreted (supplementary material).

266 Statistical analysis (mean and standard deviation) of CHONS analysis was systematically
267 reported. The statistical inference to compare samples is carried out by a Student test (t-test).
268 Assuming a Normal distribution and considering that the variables are quantitative and
269 discrete and that samples are independent, the variable t allowed to compare 2 samples for a
270 population size $n < 30$. The analysis of variance and mean is performed using the t-test
271 function (Microsoft Office Excel, 2019), the samples were compared item by item with a p-
272 value $< 0.05$.

273 2.5.3 Nitrogen (Kjeldhal): Ntot, Nmin, Norg, Eq. protein

274 The Kjeldahl method [38] was applied to quantify total, mineral and organic nitrogen.
275 Insoluble and permeate fractions for sterilized WB before and after culture were analysed.
276 The evolution of nitrogen during culture can be interpreted to evaluate cell growth,
277 sporulation, δ- endotoxins production and nitrogen consumption.

278 Kjeldahl mineral nitrogen (KMN) was quantified by adding 0.5 g of the insoluble fraction (dried
279 WB) or the permeate (7.5 mL) with 10 mL of sulfuric acid (Sigma-Aldrich, ref: 1506921000,
280 molarity: 98 %). The mixture was heated to 350 °C in a heating block. The tube was placed in
281 the Kjeldahl machine (Kjeltec$^{TM}$, 8400). 25 mL of boric acid (Sigma-Aldrich, ref: B6768,

molarity: 4 %) were added in an Erlenmeyer with: 40 mL NaOH (Sigma-Aldrich, ref: 1587931000) and 40 mL distilled water for 4 min. The Tashiro indicator was added to the Erlenmeyer content and green colour was obtained. A titration with sulfuric acid solution (0.05 N) was done to obtain the pink colour. Each mole of ammonium corresponds to 2 moles of sulfuric acid. The number of moles of nitrogen is equal to the ammonium one. To deduce the mass of nitrogen, the number of moles was converted to mass by considering the nitrogen molar mass (14 g/mol) hence the factor 0.014 was deduced. The mineral nitrogen concentration was calculated as follows:

$$KMN = \frac{v_{H2SO4} * N * 0.014007}{m\ sample} \left(\frac{g\ Nmin}{g\ dm}\right) \quad \textbf{Eq. 4}$$

Kjeldahl total nitrogen (KTN) was quantified by adding 0.5 g of the insoluble fraction (WB) or 7.5 mL of the permeate in Kjeldahl tubes with 10 mL of sulfuric acid (98%), 5 g of Kjeldahl catalyst and 3 glass beads. The tubes were incubated in the mineralizer for 2 h, then each sample was passed throughout the Kjeldahl machine (same conditions of the mineral nitrogen). The total nitrogen concentration was calculated as follows:

$$KTN = \frac{V_{H2SO4} * N * 0.014007}{m\ sample} \left(\frac{g\ Ntot}{g\ dm}\right) \quad \textbf{Eq. 5}$$

The concentration of organic nitrogen was then determined:

$$[Norg] = [KTN] - [KMN] \quad \left(\frac{g\ Norg}{g\ DM}\right) \quad \textbf{Eq. 6}$$

The proteins amount was deduced by considering a vegetative factor F = 6.25 g Protein Bovine Serum Albumin (BSA)/ g nitrogen:

$$[proteins] = [Norg] * F \quad \left(\frac{g\ proteins}{g\ DM}\right) \quad \textbf{Eq. 7}$$

The analysis of variance and mean is performed using the T-test function (Microsoft Office Excel, 2019), the samples were compared item by item with a p-value < 0.05.

# 3 Results

## 3.1 Substrate characterization

### 3.1.1 Sieving and granulometry

Firstly, to characterize the WB itself and the different classes, the raw material was sieved. Four classes were generated: class 1 >850 µm, class 2 500-850 µm, class 3 250-500 µm, class 4 <250 µm. The average post-sieving material balance of the substrate indicates that class 3 is dominant (60.5 % w/w) followed by classes 2 (19.9 % w/w) and 4 (19 % w/w). The particle size

Journal Pre-proof

Journal Pre-proof


311 analysis of the dry substrate allowed the establishment of distribution functions (in number
312 and volume) of the particles as a function of the equivalent circle diameter ($d_{CE}$). Comparative
313 analysis of distribution functions combines the use of graphical representations (such as
314 dendrograms) and descriptive statistics (e.g., mean, median, mode and standard deviation).
315 The assessment of normality can be based on the comparison between the sample moments
316 and the theoretical moments of the normal distribution (method of "moments"). The
317 similarities between the distribution functions can be examined, by applying the Student's t-
318 test and determining the overlap coefficient [35, 36].

319 Two populations, "fines" and "coarse" are present. Morphological analysis of the particles
320 indicates that the "fines" have an ovoid shape (aspect ratio= 0.735, circularity = 0.849) with a
321 smooth surface and that the granules can be aggregated. The "coarse" ones have a rough
322 surface (aspect ratio = 0.700, circularity = 0.783) and a rather angular polyhedral geometry
323 (with 3, 4 or 5 sides) in coherence with the milling process and the extraction of the pericarp
324 of the wheat grain by mechanical attrition following a wetting operation. Considering the
325 deviation of these morphologies from the sphere model, the transition from a number
326 distribution to a volume distribution can be considered. The number, $En(d_{CE})$ and volume,
327 $Ev(d_{CE})$ distributions highlight the ratio of "fines" (5-50 µm) and "coarse" (150-1000 µm)
328 population of granules. Figure 1 illustrates the bimodal nature of each class. The volume
329 distribution and the circle diameter equivalent to 90 % of the population, (Dv0.9),
330 demonstrate that the sieving effect on coarse particles agrees with the maximum size of a
331 square mesh (1200, 700 and 350 µm). Nevertheless, the volume distributions show a wide
332 range (Dv0.1 to Dv0.9) and a low overlap rate (obtained by calculating the integral of the
333 distribution function) of 25.68 % (between classes 2 and 3) and 29.71 % (between classes 3
334 and 4). At this stage a secondary population between 5 and 50 µm seems to be present but
335 negligible in volume.

336 3.1.2 Dry matter, water content (n, x) and biochemical composition of WB substrates

337 To explore the impact of granulometry on the biochemical composition of WB classes, water,
338 starch, proteins, ashes contents and elemental composition were studied for both raw and
339 sieved WB. All data was summarized in table 1.

340 No difference was observed in the water content neither in the raw WB nor in that sieved to
341 classes (0.116 g water/gdm). Similarly, the ash content varies slightly from 3.23 % to 4.43 %

342 (p>0.05). The starch content of WB varies inversely to the particle size: 0.173 g/gdm (class 2),
343 0.144 g/gdm (class 3) and 0.347 g/gdm (class 4). The Kjeldahl analysis demonstrated that the
344 total nitrogen is equal to 0.025, 0.027 and 0.03 g/gdm WB respectively for classes 2, 3 and 4.
345 On the other side, the organic nitrogen was calculated to be: 0.018, 0.020 and 0.025 g/gdm
346 WB for classes 2, 3 and 4. Protein content was then deduced from organic nitrogen content
347 (vegetative factor: 6.25) and was estimated to be 0.127, 0.145 and 0.155 g/gdm WB for classes
348 2, 3 and 4. However, these differences were not significative. The mean elemental
349 composition of the substrate is 44.37 % (±0.37), 6.59 % (±0.17), 36.6 % (±0.42) and 2.53 %
350 (±0.2) for carbon, hydrogen, oxygen, and nitrogen, respectively. Sulfur remains an
351 unquantifiable trace element with this method of analysis (limit of quantification loq < 0,5 %
352 w/w) regardless of its class. The differences in elemental composition between the different
353 particle sizes are not significant (Student test, $p > 0.05$). Based on the mean value of elemental
354 composition, the mass and molar formula of WB were calculated to be: $CH_{0.14}O_{0.71}N_{0.05}ash_{0.1}$
355 and $CH_{1.74}O_{0.53}N_{0.04}$ respectively.

356 In addition, a physical property, the water retention capacity, (WRC) was included in this
357 study. The variation of the WRC decreases with the decrease of the particle size: 500% w/w
358 for class 2, 400 % w/w for class 3 and 250 % w/w for class 4.

### 359 3.2 Bioproduction and fermentable fraction.

360 Following, to better understand the evolution of the WB medium composition after Lip
361 culture, we studied subsequently the elemental composition of the *Btk* strains.

#### 362 3.2.1 Comparison between strains and WB elemental composition:

363 The elemental composition of the strains (Lip, Blb1 and HD1) following culture in LB was
364 characterized and was shown to be identical as represented in table 3. Based on the mean
365 value of the elemental composition, the strains mass and molar formula were calculated to
366 be: $CH_{0.14}O_{0.59}N_{0.24}ash_{0.15}$ and $CH_{1.72}O_{0.44}N_{0.21}$ respectively. The elemental composition of the
367 strains was then compared to literature as shown in table 3. *Btk* strains elemental composition
368 come closest to *Bacillus cereus*. Hereafter, a comparison was established between the mean
369 elemental composition of the strains and the WB one. As highlighted in Fig.2, the strains are
370 less rich in oxygen, but richer in nitrogen compared to the WB ($p < 0.05$). This difference is also
371 confirmed by comparing the strain and the WB molar formula. **The observed difference**
372 **between the nitrogen composition of our *Btk* strains and that of WB cannot be definitively**

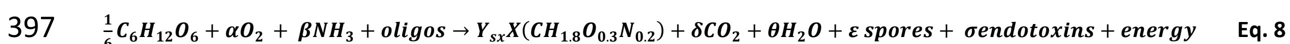
**explained. Nonetheless, a speculation could be made about this difference: it is due to bacteria being quite rich in proteins, be it metabolic proteins, enzymes, structural proteins, surface receptors or others, hence their richness in nitrogen, a major component of amino acids.**

### 3.2.2 Cell and spore counting and δ-endotoxin production

To determine the fermentable fraction, it is important to assess the productivity of *Btk* Lip (δ-endotoxins concentration), and the distribution of vegetative cells and living spores in the permeate. After 48 hours of culture produced in various classes of WB media, the concentration of spores and δ-endotoxins was examined. No significant difference of spore's concentration was observed among classes. As for the δ-endotoxins yield, a small difference was noted with class 2 and 3, compared to class 4. However, protein quantification was not significantly different (table 2).

### 3.2.3 Mass balance and fermentable fractions

To estimate the fermentable proportion across classes, the mass balance between insoluble and permeate fractions was established (Fig. 3). Our findings revealed that, after culture, the mass balance of the insoluble fraction decreased from 0.85 g/gdm WB to 0.63 g/gdm WB. However, the mass balance in the permeate increased from 0.15 g/gdm WB to 0.25 g/gdm WB for both classes 2 and 3. Due to a filter clogging induced by fine particles with WB class 4, the separation was not possible and the mass balance analysis for this class corresponded to both insoluble and permeate fractions. Assuming the stoichiometry of the bioreaction that describes the growth and the sporulation, and with a hypothesis that the production yield $Y_{s/x}$= 0.5, the fermentable fraction was calculated to be 32.3, 36.1 and 51.1 % for classes 2, 3 and 4 respectively which is far from WB's starch content of each class (17.3 % (class 2), 14.4 % (class 3) and 34.7 % (class 4)) (table 1).



$$\frac{1}{6}C_6H_{12}O_6 + \alpha O_2 + \beta NH_3 + oligos \rightarrow Y_{sx}X(CH_{1.8}O_{0.3}N_{0.2}) + \delta CO_2 + \theta H_2O + \varepsilon\, spores + \sigma endotoxins + energy \quad \textbf{Eq. 8}$$

### 3.2.4 Elemental composition (CHONS)

Knowing the elemental composition of the strains and the WB, and having determined the fermentable fraction among WB classes, the next step was to know how the WB elemental composition would evolve and what would be the limiting nutrient. Our results demonstrated that in the insoluble and permeate fraction, and for both classes 2 and 3, the carbon, hydrogen, ashes and oxygen contents did not represent any significant difference ($p > 0.05$)

Journal Pre-proof

404 before and after culture. However, for class2, nitrogen significantly, decreased ($p< 0.05$) from
405 2.67 % to 1.49 % in the insoluble fraction and increased ($p< 0.05$) from 1.44 % to 5.58 % in
406 permeate one. Similar pattern was observed for class 3. Minerals also increased in permeate,
407 approximatively from 5 % to 10 % in both classes 2 and 3 (Fig. 4).

408 3.2.5 Nitrogen and Proteins (Kjeldahl method)

409 Since nitrogen concentrations in the insoluble and the permeate fractions significantly
410 changed after culture, a more thorough analysis of the nitrogen was conducted. Hence, the
411 nitrogen concentration was examined using the Kjeldahl method in order to understand the
412 evolution of organic, mineral, and total nitrogen in both fractions. For classes 2 and 3, after
413 culture, the total nitrogen significantly decreased from 0.03 g/gdm WB to 0.02 g/gdm WB ($p<$
414 $0.05$) in the insoluble fraction, along with the significative decrease ($p< 0.05$) of the organic
415 nitrogen (0.025 g/gdm WB to 0.02 g/gdm WB). The mineral nitrogen remained stable at 0.01
416 g/gdm WB (Fig. 5A). In the permeate fraction, for classes 2 and 3, the total nitrogen increased
417 significantly from 0.025 to 0.075 g/gdm WB ($p< 0.05$) along with the increase ($p< 0.05$) of the
418 organic nitrogen from 0.015 g/gdm WB to 0.035 g/gdm WB for class 2 and 0.05 g/gdm WB for
419 class 3. Even though the mineral nitrogen increased from 0.01 g/gdm WB to 0.025 g/gdm WB
420 in the permeate (Fig. 5B) but this increase was not significant ($p> 0.05$). Thus, the ratio mineral
421 nitrogen/organic nitrogen decreases after culture in the permeate fraction and increases in
422 the insoluble fraction. Considering class 4, and due to its tiny particles size, there was no
423 separation into fractions. However, the total nitrogen in this suspension increased significantly
424 ($p< 0.05$) from 0.03 to 0.05 g/gdm WB along with the organic nitrogen (0.025 to 0.04 g/gdm
425 WB). These results are consistent with the permeate results for classes 2 and 3, thus we
426 showed them as a permeate in Fig.5B.

427 4 Discussion

428 Producing low cost biopesticides is one of the main objectives of IPM-4-Citrus project. Present
429 work explores more precisely: how WB media satisfies the nutritional requirements of the
430 strains, the evolution of the biochemical composition during the culture and the identification
431 of the limiting nutrient. Several scientific and technical bottlenecks were cleared up by: (i) size
432 distribution of WB substrate after sieving and establishing the mass balance, (ii) quantifying
433 the fermentable fraction, (iii) determining the biochemical composition of both the WB and

Journal Pre-proof

Journal Pre-proof


434 the strains and (iv) identifying the limiting nutritional factors by an analysis of elemental
435 composition.

436 The granulometry of the WB particles affect the downstream process (DSP) during the
437 clarification of the fermentation broth, the solid/liquid separation and the formulation
438 process (spray drying). Moreover, after formulation, remaining coarse particles may plug the
439 nozzles during field treatment and spreading. Therefore, a subsequent morpho-granulometry
440 analysis was used to identify the WB particle range composing each class. On a morphological
441 level, the spherical shape of the fine particles may be attributed to starch granules. On one
442 hand, the number distribution functions confirm that the fine population is dominant. This
443 population is concentrated between 5 and 50 µm particle size, with characteristic values that
444 are more or less identical for the three classes. On another hand volume distribution did
445 confirm the effect of sieving on the coarse particles. However, nor number, nor volume
446 distribution were enough to estimate the relative proportion of 'fines' and 'coarse' particle
447 populations. This is due to the fact that starch granules remain attached to lignocellulosic
448 fraction. Therefore, the biochemical studies were carried out in order to analyse the WB starch
449 content, and the results showed that it was 0.173, 0.144, and 0.347 g/gdm WB for classes 2,
450 3, and 4, respectively. This result confirm that the dominant proportion of starch is found in
451 class 4. Wet processing and heating (sterilization) of the culture media prior to inoculation
452 improved the accessibility of the starch to the microorganism by inducing swelling and starch
453 release into the suspended wheat bran [41].

454 Hereafter, an enlarged chemical analysis of raw and sieved WB was conducted. Our results
455 were consistent with those previously reported in the literature [36, 37, 38, 39, 40, 41, 42]
456 proving that the starch content in WB varies between 0.13 and 0.40 g/gdm WB among classes.
457 The protein (around 0.15 g proteins/gdm WB) and the water content (between 0.015 and
458 0.019 g water/gdm WB) and the elemental composition of the WB were shown to be
459 independent from the particle size. Taking into consideration all the obtained results regarding
460 the chemical analysis of raw and sieved WB samples, the total mass balance is supposed to be
461 100 %. However, it was found to be slightly inferior (95.54 % for raw WB, 95.58 % for class 2,
462 94 % for class 3, and 95.52 % for class 4), which was caused by the experimental error and the
463 fact that the trace elements (phosphor, zinc, calcium, etc.) were not quantified.

Journal Pre-proof


464 Moreover, our result showed that the water retention capacity (WRC, explained by the ability
465 of a matrix to trap water), was proportional to the particle size. The class 2 (highest
466 granulometry) has the biggest capacity to retain water [49]. This is due to its richness in
467 lignocellulosic matrix and its low starch content. Additionally, WRC decreases in class 4
468 prepared suspension. This can be explained by the fact that starch granules aggregate and
469 prevent the matrix from retaining water [50].

470 After confirming the productivity (spores and δ-endotoxins concentration of *Btk*-Lip) in the
471 different classes of WB-based media, we focused on fermentable fraction estimation across
472 classes by evaluation the mass balance in insoluble and permeate fractions relative to WB dry
473 matter content. Following culture, the mass balance in the insoluble fraction decreased for
474 classes 2 and 3. However, it increased in the permeate. In addition, in total (insoluble+
475 permeate), a decrease in the mass balance can be noticed. Based on the general fermentation
476 reaction, this decrease is equal to the half of the fermentable fraction since the other half is
477 lost as carbon dioxide. Knowing that glucose is the preferred source of carbon for *Btk* growth
478 [19], we wondered whether the fermentable fraction is equal to starch content. Based on our
479 results, starch concentration was much lower than the calculated fermentable fraction (starch
480 concentration in WB equal approximatively 50% of the fermentable fraction). Based on this
481 information, we deduced that *Btk* have used an alternative source of carbon to grow. This
482 source is most probably the lignocellulosic matrix of the WB, **since *Btk* genetically is capable**
483 **to novel cellulases which could liberate glucose from lignocellulosic matrix.**

484 In order to better understand and evaluate more deeply, the nutrient requirements of
485 bacteria growing in a wheat bran-based medium, the elemental composition analyses were
486 conducted first on the WB, then on the strain and lastly on the medium after *Btk* culture for
487 48 hours [51]. Our results show that, independently from the strain origin, the elemental
488 composition of all *Btk* strains is similar and consistent with what has been described in the
489 literature particularly the one of *Bacillus cereus* [45, 46, 47]. Furthermore, the comparison
490 between the strains and the WB elemental composition affirms that, the strains are
491 particularly richer in nitrogen than the WB. Since no supplements were added to the medium,
492 the WB is the only nitrogen source during *Btk* growth and sporulation. Consequently, this
493 information was essential for identifying a component to track the change in the chemical
494 composition of the WB during the fermentation process. Moreover, to determine the culture

495 limiting nutrient, we asked how the elemental composition of the WB would evolve across
496 classes in both insoluble and permeate fraction. Five elements were analyzed: carbon, oxygen,
497 hydrogen, nitrogen and minerals. After culture, in the insoluble fraction, only nitrogen
498 decreased significantly. However, in the permeate fraction, nitrogen increased significantly.
499 In addition, we can observe in the insoluble fraction, that only 30 % of the available nitrogen
500 was consumed which affirms that the residual nitrogen is inaccessible for the bacteria from a
501 physical standpoint. These results confirm that nitrogen is the culture limiting nutrient that
502 induces the spores/crystals formation.

503 Afterwards, since the nitrogen could emanate from both mineral or organic components of
504 WB, we wanted to assess which type of nitrogen *Btk* has consumed. To this end, we analyzed
505 the variation of the total, the mineral and the organic nitrogen in both insoluble and permeate
506 fractions. Our results did show that in the insoluble fraction, the total nitrogen decreased
507 significantly along with the decrease of the organic nitrogen. However, in the permeate
508 fractions, the total and the organic nitrogen increased significantly. The mineral nitrogen, on
509 the other hand, did not represent any significant changes. Since the organic nitrogen is in
510 principle found in the proteins, we can deduce, that *Btk* has partially consumed the proteins
511 of the WB. This information affirms that sugar is not the only ingredient required for *Btk*
512 growth and sporulation. Furthermore, as previously described, the strain is richer in nitrogen
513 compared to the WB. This can explain the increase of the organic nitrogen in the permeate
514 that is probably due to the production of the biomass. These results obtained by Kjeldahl are
515 aligned with those obtained by elemental composition technique.

516 Based on all these results, we can conclude that the WB stands as an interesting by-product
517 for biopesticide production since its composition meets the full nutritional requirements of
518 *Btk* strains with nitrogen being the limiting factor. However, in order to optimize
519 bioproduction on a large scale, the analysis should be conducted on a bioreactor level.
520 Moreover, to improve the appropriate downstream process for biopesticides formulation and
521 application, the physical limitations of such a medium should be examined.

522 **Conflict of interest**

523 The authors declared no conflict of interest.

Journal Pre-proof

524 # 5 Funding

525 EU Project IPM-4-CITRUS - Horizon 2020 – https://www.ipm-4-citrus.insa-toulouse.fr Grant
526 n°734921 (April 2017- January 2023). Agence Universitaire de la Francophonie AUF- Bureau
527 du Moyen Orient, BMO (November 2021) and INSA funds (Septembre 2022) (Mobility of
528 Mireille KALLASSY from Lebanon to INSA Toulouse)

529

530 # 6 Acknowledgements

531 A special thanks to the organizing committee of the 10th international conference on *Bacillus*
532 *cereus*, *Bacillus anthracis* and *Bacillus thuringiensis* (Paris, April 2022).

533

Journal Pre-proof

Journal Pre-proof


534 # 7 References

535 1. Spence N, Hill L, Morris J. **How the global threat of pests and diseases impacts plants,**
536 **people, and the planet**. *Plants People planet.* 2020;2(1):5–13.,
537 https://nph.onlinelibrary.wiley.com/doi/full/10.1002/ppp3.10088

538 2. Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L. **Chemical**
539 **Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. Front**
540 **Public Health**. *Journal of Frontiers in Public Health*,
541 https://doi.org/10.3389/fpubh.2016.00148

542 3. Samada LH, Tambunan USF. **Biopesticides as Promising Alternatives to Chemical**
543 **Pesticides: A Review of Their Current and Future Status**. *OnLine Journal of Biological*
544 *Sciences*, *20*(2), 66-76. https://doi.org/10.3844/ojbsci.2020.66.76.

545 4. Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P. ***Bacillus thuringiensis*: a**
546 **century of research, development and commercial applications.** *Plant Biotechnology*.
547 2011;9(3):283–300, https://doi.org/10.1111/j.1467-7652.2011.00595.

548 5. Palma L, Muñoz D, Berry C, Murillo J, Caballero P. ***Bacillus thuringiensis* Toxins: An**
549 **Overview of Their Biocidal Activity.** *Toxins,*2014 Dec;6(12):3296–325,
550 https://doi.org/10.3390/toxins6123296.

551 6. Siegel JP. **The Mammalian Safety of *Bacillus thuringiensis*- Based Insecticides**. J*ournal*
552 *of Invertebrate Pathology*, 2001 Jan 1;77(1):13–21, https://doi.org/10.1006/jipa.2000.5000.

553 7. Lambert B, Peferoen M. **Insecticidal Promise of *Bacillus thuringiensis*.** *BioScience.*
554 *1992;42(2):112–22*, https://doi.org/10.1128/Fmmbr.62.3.775-806.1998.

555 8. Bravo A, Gill SS, Soberón M. **Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins**
556 **and their potential for insect control.** Toxicon. 2007 Mar 15;49(4):423–35, https://doi.org/
557 10.1016/j.toxicon.2006.11.022.

558 9. Verplaetse E, Slamti L, Gohar M, Lereclus D. **Cell Differentiation in a *Bacillus***
559 ***thuringiensis* Population during Planktonic Growth, Biofilm Formation, and Host Infection**.
560 *mBio*, 2015 Apr 28;6(3): e00138-15, https://doi.org/10.1128/mBio.00138-15.

561 10. Rowe GE, Margaritis A. **Bioprocess design and economic analysis for the commercial**
562 **production of environmentally friendly bioinsecticides from *Bacillus thuringiensis HD-1***
563 ***kurstaki***. *Biotechnol Bioeng*. 2004;86(4):377–88, https://doi.org/10.1002/bit.20146.

564 11. El Khoury M, Azzouz H, Chavanieu A, Abdelmalak N, Chopineau J, Awad MK. **Isolation**
565 **and characterization of a new *Bacillus thuringiensis* strain Lip harboring a new cry1Aa gene**
566 **highly toxic to *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae.** *Arch Microbiol*. 2014 Jun
567 1;196(6):435–4, https://doi.org/10.1007/s00203-014-0981-3

568 12. Saadaoui I, Rouis S, Jaoua S. **A new Tunisian strain of *Bacillus thuringiensis kurstaki***
569 **having high insecticidal activity and δ-endotoxin yield.** *Arch Microbiol*. 2009 Feb
570 12;191(4):341, https://doi.org/10.1007/s00203-009-0458.

Journal Pre-proof

Journal Pre-proof


571 13. Setlow P, Kornberg A. **Biochemical studies of bacterial sporulation and germination:**
572 **energy metabolism in early stages of germination of *Bacillus megaterium* spores.** J Biol
573 Chem. 1970 Jul 25;245(14):3637–44, https://doi.org/10.1128/jb.98.1.75-81.1969.

574 14. Agustini L, Efiyanti L, Faulina SA, Santoso E. **Isolation and Characterization of Cellulase-**
575 **and Xylanase-Producing Microbes Isolated from Tropical Forests in Java and Sumatra,**
576 *scientific scolar*, 2012, Corpus ID: 52205648.

577 15. Ireton K, Jin S, Grossman AD, Sonenshein AL. **Krebs cycle function is required for**
578 **activation of the Spo0A transcription factor in *Bacillus subtilis*.** Proc Natl Acad Sci. 1995 Mar
579 28;92(7):2845–9, https://doi.org/10.1073/pnas.92.7.2845.

580 16. Mazmira, M M, Ramlah, A SA, Rosfarizan, M, et al. **Effect of saccharides on growth,**
581 **sporulation rate and d-endotoxin synthesis of *Bacillus thuringiensis***. Afr J Biotechnol. 2012
582 May 17;11(40):9654–63, https://doi.org/10.5897/AJB11.1391.

583 17. El-bendary M. ***Bacillus thuringiensis* and *Bacillus sphaericus* biopesticides production**.
584 J *Basic Microbiol*. 2006 Apr 1; 46:158–70, https://doi.org/10.1002/jobm.200510585.

585 18. Özkan M, Dilek FB, Yetis Ü, Özcengiz G. **Nutritional and cultural parameters**
586 **influencing antidipteran delta-endotoxin production.** *Res Microbiol*. 2003 Jan 1;154(1):49–
587 53, https://doi.org/10.1016/S0923-2508(02)00006-2.

588 19. Amin G, Alotaibi S, Narmen YA, Saleh WD. **Bioinsecticide production by the bacterium**
589 ***Bacillus thuringiensis*. Pattern of cell growth, toxin production and by-product synthesis.**
590 *Arch Agron Soil Sci.* 2008 Aug 1;54(4):387–94, https://doi.org/10.1080/03650340802158201.

591 20. Rahbani J, Salameh D, Awad MK, Brandam C, Lteif R. **Evaluation of a cereal milling by-**
592 **product for the low-cost production of *Bacillus thuringiensis kurstaki* in submerged**
593 **fermentation,** *European Journal of Biotechnology and Bioscience,* Vol 1, Issue 6, 2014, ISSN
594 2321-9122, Hal ID: hal-02042841, https://hal.archives-ouvertes.fr/hal-02042841.

595 21. Özkan M, Dilek FB, Yetis Ü, Özcengiz G. **Nutritional and cultural parameters**
596 **influencing antidipteran delta-endotoxin production.** *Res Microbiol*. 2003 Jan 1;154(1):49–
597 53, https://doi.org/ 10.1016/s0923-2508(02)00006-2.

598 22. Jallouli W, Driss F, Fillaudeau L, Rouis S. **Review on biopesticide production by *Bacillus***
599 ***thuringiensis subsp. kurstaki* since 1990: Focus on bioprocess parameters.** *Process Biochem*.
600 2020 Nov 1; 98:224–32, https://doi.org/10.1016/j.procbio.2020.07.023.

601 23 Prückler M, Siebenhandl-Ehn S, Apprich S, Höltinger S, Haas C, Schmid E, et al. **Wheat**
602 **bran-based biorefinery 1: Composition of wheat bran and strategies of functionalization.**
603 *LWT - Food Sci Technol. 2014 May 1;56(2):211–21,* https://doi.org/10.1016/j.lwt.2013.12.004.

604 24. Devi PSV, Ravinder T, Jaidev C. **Cost-effective production of *Bacillus thuringiensis* by**
605 **solid-state fermentation.** *J Invertebr Pathol*. 2005 Feb 1;88(2):163–8,
606 https://doi.org/10.1016/j.jip.2005.01.013.

Journal Pre-proof

Journal Pre-proof


607 25. Mounsef JR, Salameh D, Lteif R. **Evaluation of a cereal milling by-product for the low**
608 **cost production of *Bacillus thuringiensis kurstaki* in submerged fermentation,** *European*
609 *Journal of Biotechnology and Bioscience,* Vol 1, Issue 6, 2014, . ISSN 2321-9122, Hal ID: hal-
610 02042841, https://hal.archives-ouvertes.fr/hal-02042841.

611 26. Sarrafzadeh MH, Belloy L, Esteban G, Navarro JM, Ghommidh C. **Dielectric monitoring**
612 **of growth and sporulation of *Bacillus thuringiensis.*** *Biotechnol Lett*. 2005 Apr 1;27(7):511–7,
613 https://doi.org/10.1007/s10529-005-2543-x.

614 27. Fayad N., Abboud J., Idriss F, Louka N., Kallassy M. **Optimization of Culture Conditions**
615 **and Wheat Bran Class Selection in the Production of *Bacillus thuringiensis*-Based**
616 **Biopesticides.** *Fermentation 2022, 8(12), 666*; https://doi.org/10.3390/fermentation8120666

617 28. Wiwat C, Thaithanun S, Pantuwatana S, Bhumiratana A. **Toxicity of Chitinase-**
618 **Producing *Bacillus thuringiensis ssp. kurstaki* HD-1 (G) toward *Plutella xylostella*.** *J Invertebr*
619 *Pathol.* 2000 Nov 1;76(4):270–7, https://doi.org/10.1006/jipa.2000.4976.

620 29. Morris ON, Converse V, Kanagaratnam P, Coté JC. **Isolation, characterization, and**
621 **culture of *Bacillus thuringiensis* from soil and dust from grain storage bins and their toxicity**
622 **for *mamestra configurata* (lepidoptera: noctuidae)**. *Can Entomol*. 1998 Aug;130(4):515–37,
623 https://doi.org/10.10802F03235408.2011.599134.

624 30. Li T, Deng X, Wang J, Chen Y, He L, Sun Y, et al. **Biodegradation of nitrobenzene in a**
625 **lysogeny broth medium by a novel halophilic bacterium *Bacillus licheniformis***. *Mar Pollut*
626 *Bull*. 2014 Dec 15 ;89(1):384–9, https://doi.org/10.1016/j.marpolbul.2014.09.028.

627 31. Le T, Anne-Archard D, Coma V, Cameleyre X, Lombard E, To KA, et al. **Using in-situ**
628 **viscosimetry and morphogranulometry to explore hydrolysis mechanisms of filter paper and**
629 **pretreated sugarcane bagasse under semi-dilute suspensions.** *Biochem Eng J. 2017; 127:9–*
630 *20.*, http://doi.org/10.1016/j.bej.2017.07.006

631 32. Hemery Y, Rouau X, Dragan C, Bilici M, Beleca R, Dascalescu L. **Electrostatic properties**
632 **of wheat bran and its constitutive layers: Influence of particle size, composition, and**
633 **moisture content.** *J Food Eng*. 2009 Jul 1;93(1):114–24,
634 https://doi.org/10.1016/j.jfoodeng.2009.01.003.

635 33. Traynham TL, Myers DJ, Carriquiry AL, Johnson LA. **Evaluation of Water-Holding**
636 **Capacity for Wheat–Soy Flour Blends.** *J Am Oil Chem Soc*. 2007;84(2):151,
637 https://doi.org/10.1007/s11746-006-1018-0.

638 34. Jones CG, Daniel Hare J, Compton SJ. **Measuring plant protein with the Bradford**
639 **assay: 1. Evaluation and standard method**. *J Chem Ecol*. 1989 Mar ;15(3):979–92,
640 https://doi.org/ 10.1007/BF01015193.

641 35. Thivend P, Mercier C, Guilbot A. **Dosage de l'amidon dans les milieux complexes.** *Ann*
642 *Biol Anim Biochim Biophys*. 1965;5(4):513–26, hal-00896310, HAL Id: hal-00896310
643 https://hal.archives-ouvertes.fr/hal-00896310.

644 36. Xie X (Sherry), Cui SW, Li W, Tsao R. **Isolation and characterization of wheat bran**
645 **starch.** *Food Res Int.* 2008 Nov 1;41(9):882–7, https://doi.org/10.1016/j.foodres.2008.07.016.

646 37. Singhal A, Konttinen J, Joronen T. **Effect of different washing parameters on the fuel**
647 **properties and elemental composition of wheat straw in water-washing pre-treatment. Part**
648 **1: Effect of washing duration and biomass size**. *Fuel.* 2021 May 15;292:120206,
649 https://doi.org/10.1016/j.fuel.2021.120206.

650 38. Sáez-Plaza P, Navas M, Wybraniec S, Michałowski T, Garcia Asuero A. **An Overview of**
651 **the Kjeldahl Method of Nitrogen Determination. Part II. Sample Preparation, Working Scale,**
652 **Instrumental Finish, and Quality Control.** *Crit Rev Anal Chem*. 2013 Oct 2;43,
653 https://doi.org/10.1080/10408347.2012.751787.

654 39. Clemons TE, Bradley EL. **A nonparametric measure of the overlapping coefficient.**
655 *Comput Stat Data Anal.* 2000 Jul 28;34(1):51–61, https://doi.org/10.1016/S0167-
656 9473(99)00074-2.

657 40. Inman HF, Bradley EL. **The overlapping coefficient as a measure of agreement**
658 **between probability distributions and point estimation of the overlap of two normal**
659 **densities.** *Commun Stat - Theory Methods*. 1989 Jan 1;18(10):3851–74,
660 https://doi.org/10.1080/03610928908830127.

661 41. Cordeiro MJM, Veloso CM, Santos LS, Bonomo RCF, Caliari M, Fontan R da CI. T**he**
662 **Impact of Heat-Moisture Treatment on the Properties of *Musa paradisiaca* L. Starch and**
663 **Optimization of Process Variables.** *Food Technol Biotechnol.* 2018 Dec;56(4):506–15,
664 https://doi.org/10.17113/ftb.56.04.18.5490.

665 42. Hell J, Prückler M, Danner L, Henniges U, Apprich S, Rosenau T, et al. **A comparison**
666 **between near-infrared (NIR) and mid-infrared (ATR-FTIR) spectroscopy for the multivariate**
667 **determination of compositional properties in wheat bran samples.** *Food Control*. 2016 Feb
668 1;60:365–9, http://dx.doi.org/10.1016/j.foodcont.2015.08.003.

669 43. Onipe OO, Jideani AIO, Beswa D. **Composition and functionality of wheat bran and its**
670 **application in some cereal food products.** *Int J Food Sci Technol.* 2015;50(12):2509–18,
671 https://doi.org/10.1111/ijfs.12935.

672 44. Apprich S, Tirpanalan Ö, Hell J, Reisinger M, Böhmdorfer S, Siebenhandl-Ehn S, et al.
673 **Wheat bran-based biorefinery 2: Valorization of products**. LWT - *Food Sci Technol.* 2014 May
674 1;56(2):222–31, https://doi.org/10.1016/j.lwt.2013.12.004.

675 45. Gominho-Rosa M do C, Rodrigues APO, Mattioni B, de Francisco A, Moraes G,
676 Fracalossi DM. **Comparison between the omnivorous jundiá catfish (*Rhamdia quelen*) and**
677 **Nile tilapia (*Oreochromis niloticus*) on the utilization of dietary starch sources: Digestibility,**
678 **enzyme activity and starch microstructure**. *Aquaculture*. 2015 Jan 1;435:92–9,
679 http://dx.doi.org/10.1016/j.aquaculture.2014.09.035.

Journal Pre-proof

680 46. Kaur A, Yadav MP, Singh B, Bhinder S, Simon S, Singh N. **Isolation and characterization**
681 **of arabinoxylans from wheat bran and study of their contribution to wheat flour dough**
682 **rheology**. *Carbohydr Polym*. 2019 Oct 1; 221:166–73,
683 https://doi.org/10.1016/j.carbpol.2019.06.002.

684 47. Naidu DS, Hlangothi SP, John MJ. **Bio-based products from xylan: A review.** *Carbohydr*
685 *Polym.* 2018 Jan 1; 179:28–41, https://doi.org/10.1016/j.carbpol.2017.09.064.

686 48. Reisinger M, Tirpanalan Ö, Prückler M, Huber F, Kneifel W, Novalin S. **Wheat bran**
687 **biorefinery – A detailed investigation on hydrothermal and enzymatic treatment.** *Bioresour*
688 *Technol.* 2013 Sep 1; 144:179–85, https://doi.org/10.1016/j.biortech.2013.06.088.

689 49. Holloway WD, Greig RI. **Water Holding Capacity of Hemicelluloses from Fruits,**
690 **Vegetables and Wheat Bran.** *J Food Sci*. 1984;49(6):1632–3, https://doi.org/10.1111/j.1365-
691 2621.1984.tb12867.x

692 50. Qazi IM, Kumar Rakshit S, Tran T. **Effect of physico-chemical properties of tropical**
693 **starches and hydrocolloids on rice gels texture and noodles water retention ability.** *Starch -*
694 *Stärke.* 2011;63(9):558–69, https://doi.org/10.1002/star.201000140.

695 51. Shevkani K, Singh N, Bajaj R, Kaur A. **Wheat starch production, structure, functionality**
696 **and applications—a review.** *Int J Food Sci Technol.* 2017;52(1):38–58,
697 https://doi.org/10.1111/ijfs.13266.

698 52. Fagerbakke K, Heldal M, Norland S. **Content of carbon, nitrogen, oxygen, sulfur and**
699 **phosphorus in native aquatic and cultured bacteria**. *Aquat Microb Ecol.* 1996; 10:15–27,
700 https://doi.org/10.3354/AME010015.

701 53. Lawford H, Rousseau J. **Studies on Nutrient Requirements and Cost-Effective**
702 **Supplements for Ethanol Production by Recombinant *E. coli.*** *Appl Biochem Biotechnol.* 1996
703 Mar 1;57–58:307–26, https://doi.org/10.1007/BF02941710.

704 54. Popovic M. **Thermodynamic properties of microorganisms: determination and**
705 **analysis of enthalpy, entropy, and Gibbs free energy of biomass, cells and colonies of 32**
706 **microorganism species.** *Heliyon.* 2019 Jun 1;5(6):e01950,
707 https://doi.org/10.1016/j.heliyon.2019.e01950.

708

709 # 8 Legends for tables

710

711 Table 1: Wheat bran characterization per class

712 Table 2: *Btk* Lip Spore counting and protein content with classes 2, 3 and 4

713 Table 3: Elemental composition (% g/gdm), molar composition (g/c_mol) of biomass and molar mass of biomass (g/c-mol)
714 obtained from experimental data (*Btk*) and literature

715

716

Journal Pre-proof

717

Journal Pre-proof

718 Table 1: Wheat bran characterization per class

| | | **Granulometry (µm)** | | | | **Mass balance (mean value)** | **Water content** | **WRC** | **Starch** | **Total nitrogen** | **Organic nitrogen** | **Protein KJ** | **Elemental composition %** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Class | Size (µm) | $Dv_{0.1}$ | $Dv_{0.5}$ | $Dv_{0.9}$ | D(4,3) | [%w/w] | [g water /gdm] | % g/gdm | [g/gdm] | [g/gdm WB] | [g/gdm WB] | [g/gdm] | C | H | O | N | Ash |
| Raw | | 270.6 | 557.4 | 969.5 | 598.5 | 100 | 0.119 ±0.07 | | 0.207 ±0.008 | | | | 44.79 ±0.44 | 6.74 ±0.20 | 36.53 ±0.37 | 2.50 ±0.24 | 4.43 ±0.12 |
| 2 | 500-850 | 687.8 | 852.1 | 1055.8 | 865.4 | 19.80 | 0.115 ±0.04 | 500 | 0.173 ±0.35 | 0.025 ±0.01 | 0.018 ±0.008 | 0.127 ±0.012 | 44.21 ±0.19 | 6.50 ±0.12 | 35.62 ±0.45 | 2.60 ±0.23 | 3.70 ±0.12 |
| 3 | 250-500 | 325.1 | 531.3 | 761.5 | 531.1 | 51.41 | 0.116 ±0.04 | 400 | 0.144 ±1.35 | 0.027 ±0.01 | 0.02 ±0.01 | 0.145 ±0.014 | 45.34 ±0.08 | 6.46 ±0.04 | 37.89 ±0.44 | 2.48 ±0.04 | 4.28 ±0.12 |
| 4 | <250 | 166.0 | 269.8 | 391.1 | 275.3 | 28.20 | 0.116 ±0.04 | 250 | 0.347 ±0.47 | 0.03 ±0.02 | 0.025 ±0.01 | 0.155 ±0.016 | 42.40 ±0.04 | 6.61 ±0.12 | 34.60 ±0.37 | 2.61 ±0.24 | 3.23 ±0.12 |

719

Journal Pre-proof

Journal Pre-proof


720 Table 2: *Btk* Lip Spore counting and protein content with classes 2, 3 and 4

| Class | Spore [cfu/mL] | Endotoxin [g eq. BSA/L] |
|---|---|---|
| 2 | 3.50E+09 ± 2.36E+08 | 0.549 ± 0.117 |
| 3 | 4.12E+09 ± 1.63E+09 | 0.547 ± 0.075 |
| 4 | 3.50E+09 ± 1.04E+09 | 0.432 ± 0.062 |

721

722 Table 3: Elemental composition (% g/gdm), molar composition (g/C_mol) of biomass and
723 molar mass of biomass (g/C_mol) obtained from experimental data (*Btk*) and literature

724 *na =not analysed

| Strain | Elements (% w/w) | | | | | Molar formula | Molar Mass |
|---|---|---|---|---|---|---|---|
| | Ashes | C | H | O | N | (C_mol) | (g/C_mol) |
| ***Btk* Blb1*** | 7 | 43.37 | 6.22 | 24.84 | 10.48 | $CH_{1.72}O_{0.45}N_{0.21}$ | 25.98 |
| ***Btk* HD1** | 6.7 | 45.7 ±0.067 | 6.61 ±0.090 | 26.89 ±0.870 | 10.51 ±0.011 | $CH_{1.7}O_{0.42}N_{0.2}$ | 25.42 |
| ***Btk* Lip** | na | 44.65 ±0.300 | 6.42 ±0.072 | 26.56 ±0.680 | 11.23 ±0.068 | $CH_{1.73}O_{0.43}N_{0.19}$ | 25.51 |
| ***Btk* - mean** | 6.80 | 44.82 ±0.330 | 6.46 ±0.117 | 26.35 ±0.775 | 10.79 ±0.039 | $CH_{1.72}O_{0.44}N_{0.21}$ | 25.52 |
| ***Lactobacillus helveticus*** *(Fagerbakke et al, 1996)* | 9.03 | 47.54 | 6.25 | 24.39 | 12.79 | $CH_{1.58}O_{0.39}N_{0.23}$ | 25.26 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***Flavobacterium Dehydrogenans*** *(Lawford Rousseau et al, 1996)* | 13.5 | 45.16 | 6.15 | 24.29 | 10.87 | $CH_{1.63}O_{0.40}N_{0.21}$ | 26.60 |
| ***Escherichia coli*** *(Popovic et al, 2019)* | 11.3 | 47.83 | 6.95 | 21.65 | 12.3 | $CH_{1.74}O_{0.34}N_{0.22}$ | 25.11 |
| ***Bacillus cereus*** *(Popovic et al, 2019)* | 9.98 | 46.05 | 5.73 | 26.26 | 11.98 | $CH_{1.49}O_{0.43}N_{0.22}$ | 26.08 |

725 na: not analysed

726 *Blb1 elemental composition analysis was conducted once due to a lack of material

727

# 9 Supplementary material

728 Precision and calibration coefficients for CHONS analysis

| Element | Element mass (for 2 mg BBOT) | Equation (m = a x signal) | Precision (%) | $R^2$ | LOD min (timexΔθ) | LOD max (timexΔθ) |
|---|---|---|---|---|---|---|
| C | 1,45 | a = $323x10^7$ | 2,190 | 0,997 | $7x\ 10^6$ | $17x10^6$ |
| H | 0,12 | a=$890x10^7$ | 2,240 | 0,999 | $19x10^6$ | $48x10^6$ |
| N | 0,14 | a=$132x10^7$ | 2,030 | 0,999 | $26x10^4$ | $70x10^4$ |
| S | 0,15 | a= $116x10^7$ | 10,530 | 0,918 | $19x10^4$ | $63x10^7$ |
| O | 0,15 | a= $231x10^7$ | 3,300 | 0,986 | $47x10^7$ | $10x10^6$ |

729

Journal Pre-proof

Journal Pre-proof

Figure legends

Fig 1: Cumulative distribution function in number and volume for WB particles (classes 2, 3 and 4) including a microscopic image of WB class2 (Diascopic illumination, Intensity 80%, surface length: 10x10 $mm^2$, BF, magnification x2.5).

Fig 2: Comparison between the strain and substrate elemental composition

Fig 3: Evolution of the WB mass balance in both insoluble and permeate fractions regarding granulometry

Fig 4: Evolution of WB elemental composition for (A) class 2 and (B) class 3 in both insoluble and permeate fraction

Fig 5: Evolution of total, organic and mineral nitrogen in both insoluble (A) and permeate (B) fractions among all WB classes 2, 3 and 4.

Journal Pre-proof

Table 1: Wheat bran characterization per class

| | | **Granulometry (µm)** | | | | **Mass balance (mean value)** | **Water content** | **WRC** | **Starch** | **Total nitrogen** | **Organic nitrogen** | **Protein KJ** | **Elemental composition %** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Class | Size (µm) | $Dv_{0.1}$ | $Dv_{0.5}$ | $Dv_{0.9}$ | D(4,3) | [%w/w] | [g water /gdm] | % g/gdm | [g/gdm] | [g/gdm WB] | [g/gdm WB] | [g/gdm] | C | H | O | N | Ash |
| Raw | | 270.6 | 557.4 | 969.5 | 598.5 | 100 | 0.119 ±0.07 | | 0.207 ±0.008 | | | | 44.79 ±0.44 | 6.74 ±0.20 | 36.53 ±0.37 | 2.50 ±0.24 | 4.43 ±0.12 |
| 2 | 500-850 | 687.8 | 852.1 | 1055.8 | 865.4 | 19.80 | 0.115 ±0.04 | 500 | 0.173 ±0.35 | 0.025 ±0.01 | 0.018 ±0.008 | 0.127 ±0.012 | 44.21 ±0.19 | 6.50 ±0.12 | 35.62 ±0.45 | 2.60 ±0.23 | 3.70 ±0.12 |
| 3 | 250-500 | 325.1 | 531.3 | 761.5 | 531.1 | 51.41 | 0.116 ±0.04 | 400 | 0.144 ±1.35 | 0.027 ±0.01 | 0.02 ±0.01 | 0.145 ±0.014 | 45.34 ±0.08 | 6.46 ±0.04 | 37.89 ±0.44 | 2.48 ±0.04 | 4.28 ±0.12 |
| 4 | <250 | 166.0 | 269.8 | 391.1 | 275.3 | 28.20 | 0.116 ±0.04 | 250 | 0.347 ±0.47 | 0.03 ±0.02 | 0.025 ±0.01 | 0.155 ±0.016 | 42.40 ±0.04 | 6.61 ±0.12 | 34.60 ±0.37 | 2.61 ±0.24 | 3.23 ±0.12 |

Journal Pre-proof

Table 2: *Btk* Lip Spore counting and protein content with classes 2, 3 and 4

| Class | Spore [cfu/mL] | Endotoxin [g eq. BSA/L] |
|---|---|---|
| 2 | 3.50E+09 ± 2.36E+08 | 0.549 ± 0.117 |
| 3 | 4.12E+09 ± 1.63E+09 | 0.547 ± 0.075 |
| 4 | 3.50E+09 ± 1.04E+09 | 0.432 ± 0.062 |

Table 3: Elemental composition (% g/gdm), molar composition (g/C_mol) of biomass and molar mass of biomass (g/C-mol) obtained from experimental data (*Btk*) and literature

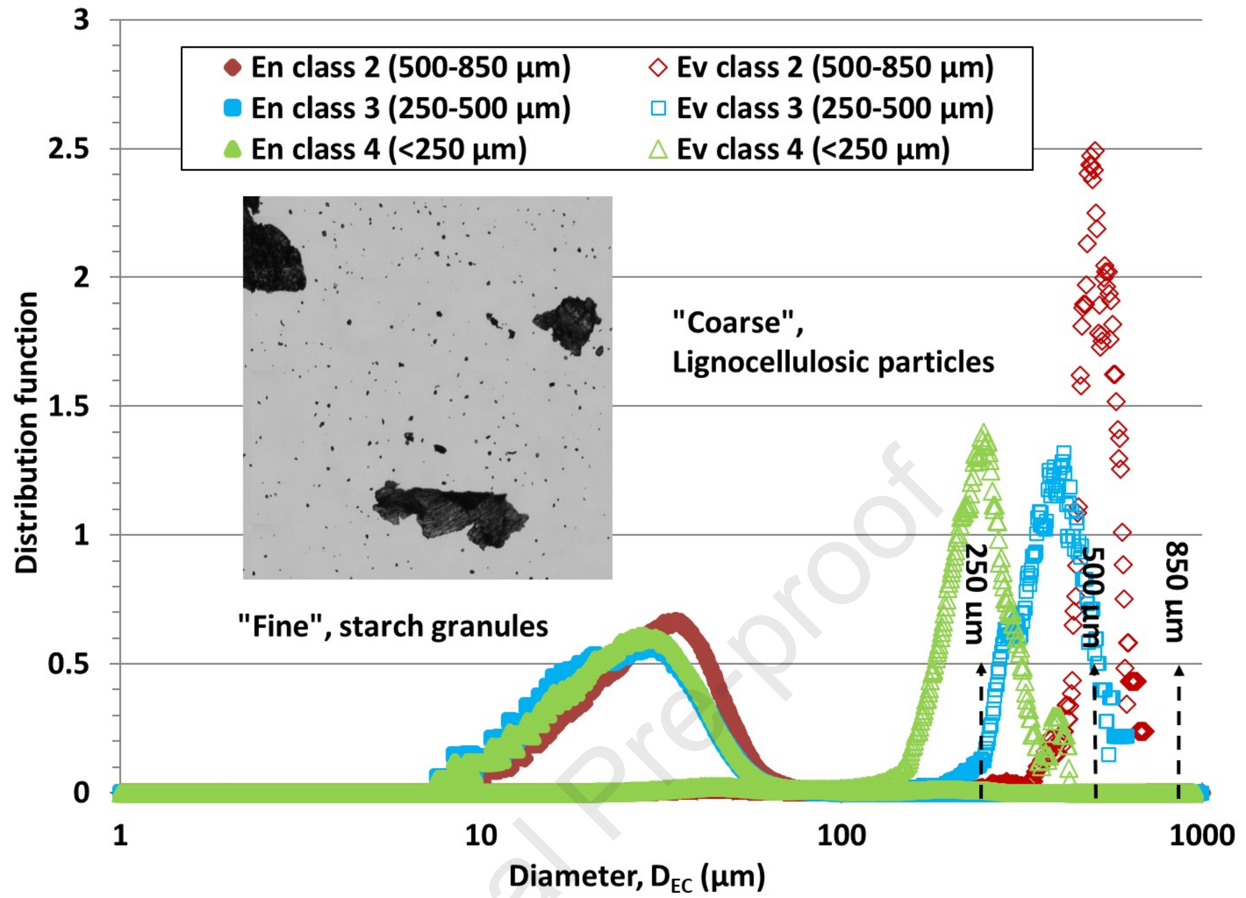
na: not analysed

| **Strain** | **Elements (% w/w)** | | | | | **Molar formula** | **Molar Mass** |
|---|---|---|---|---|---|---|---|
| | **Ashes** | **C** | **H** | **O** | **N** | **(C_mol)** | **(g/C_mol)** |
| ***Btk* Blb1*** | 7 | 43.37 | 6.22 | 24.84 | 10.48 | $CH_{1.72}O_{0.45}N_{0.21}$ | 25.98 |
| ***Btk* HD1** | 6.7 | 45.7 ±0.067 | 6.61 ±0.090 | 26.89 ±0.870 | 10.51 ±0.011 | $CH_{1.7}O_{0.42}N_{0.2}$ | 25.42 |
| ***Btk* Lip** | na | 44.65 ±0.300 | 6.42 ±0.072 | 26.56 ±0.680 | 11.23 ±0.068 | $CH_{1.73}O_{0.43}N_{0.19}$ | 25.51 |
| ***Btk* - mean** | 6.80 | 44.82 ±0.330 | 6.46 ±0.117 | 26.35 ±0.775 | 10.79 ±0.039 | $CH_{1.72}O_{0.44}N_{0.21}$ | 25.52 |
| ***Lactobacillus helveticus*** *(Fagerbakke et al, 1996)* | 9.03 | 47.54 | 6.25 | 24.39 | 12.79 | $CH_{1.58}O_{0.39}N_{0.23}$ | 25.26 |

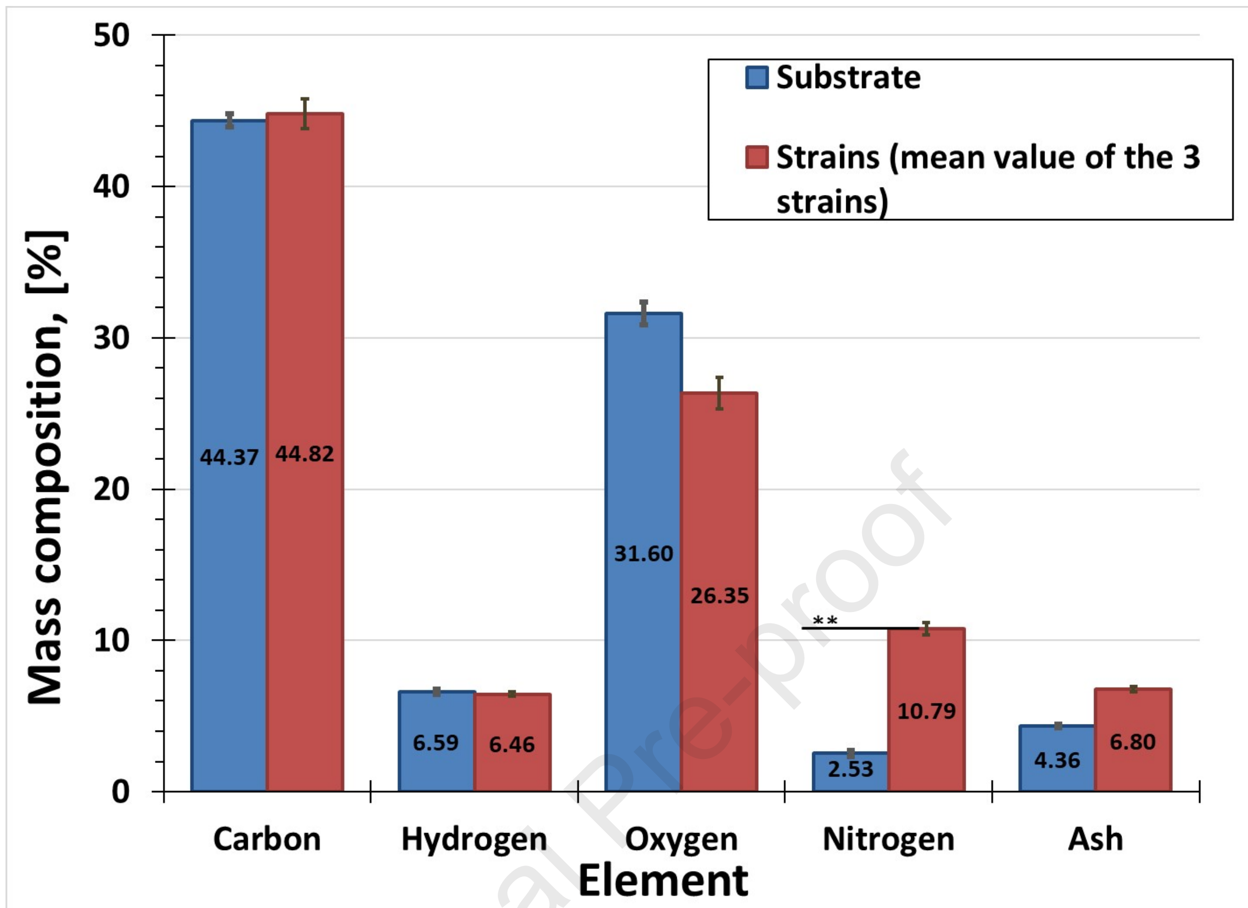
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ***Flavobacterium Dehydrogenans*** *(Lawford Rousseau et al, 1996)* | 13.5 | 45.16 | 6.15 | 24.29 | 10.87 | $CH_{1.63}O_{0.40}N_{0.21}$ | 26.60 |
| ***Escherichia coli*** *(Popovic et al, 2019)* | 11.3 | 47.83 | 6.95 | 21.65 | 12.3 | $CH_{1.74}O_{0.34}N_{0.22}$ | 25.11 |
| ***Bacillus cereus*** *(Popovic et al, 2019)* | 9.98 | 46.05 | 5.73 | 26.26 | 11.98 | $CH_{1.49}O_{0.43}N_{0.22}$ | 26.08 |

*Blb1 elemental composition analysis was conducted once due to a lack of material

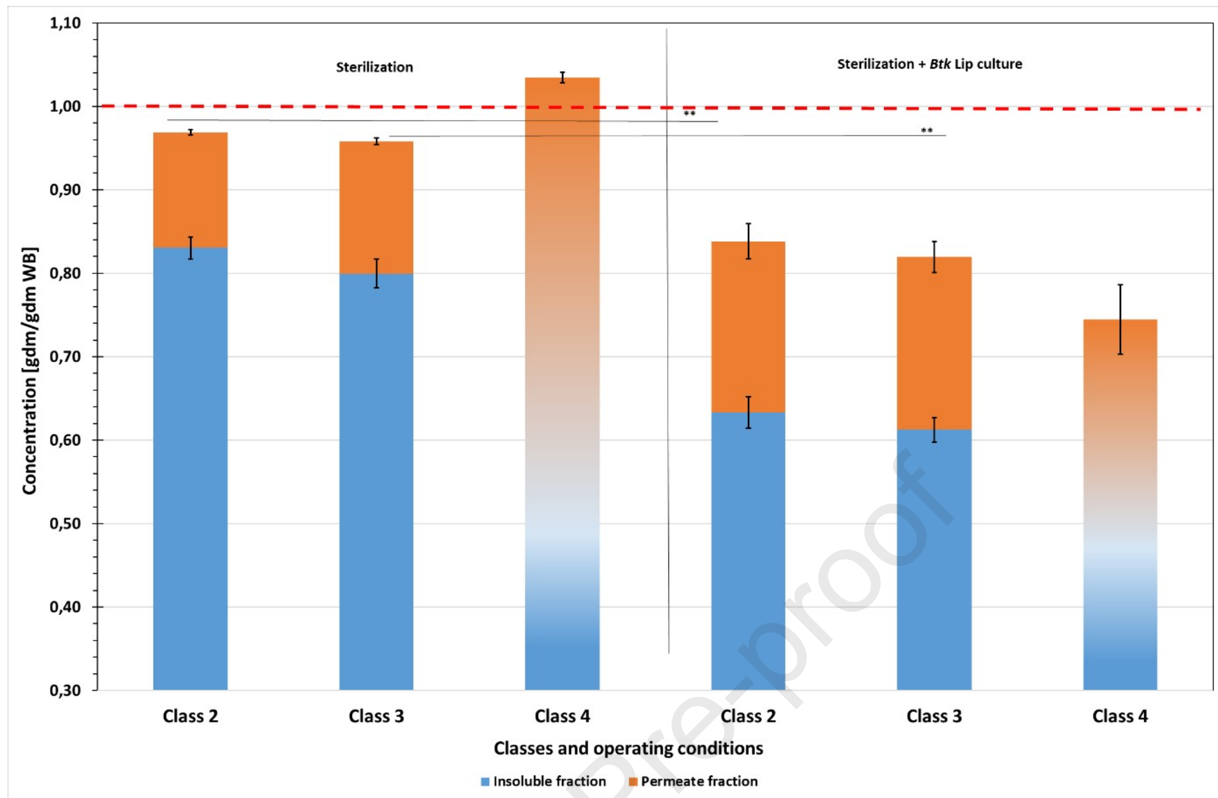
Journal Pre-proof



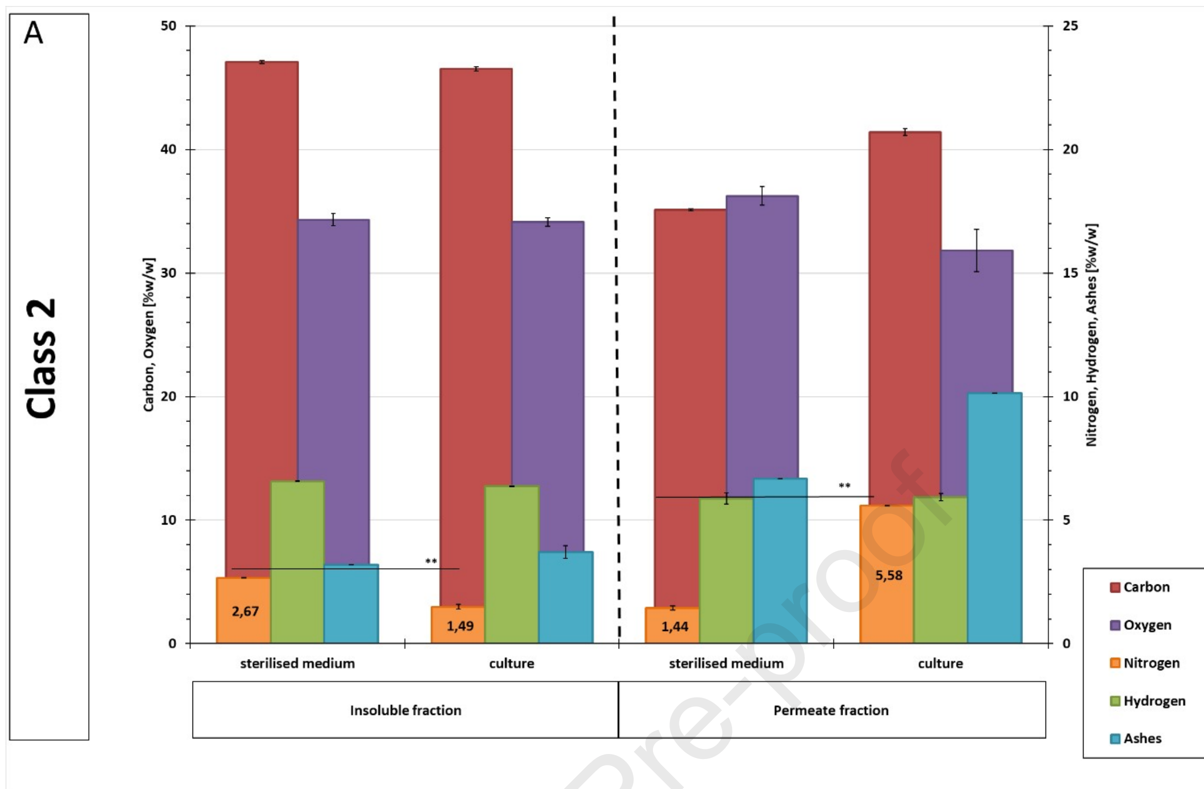

Journal Pre-proof



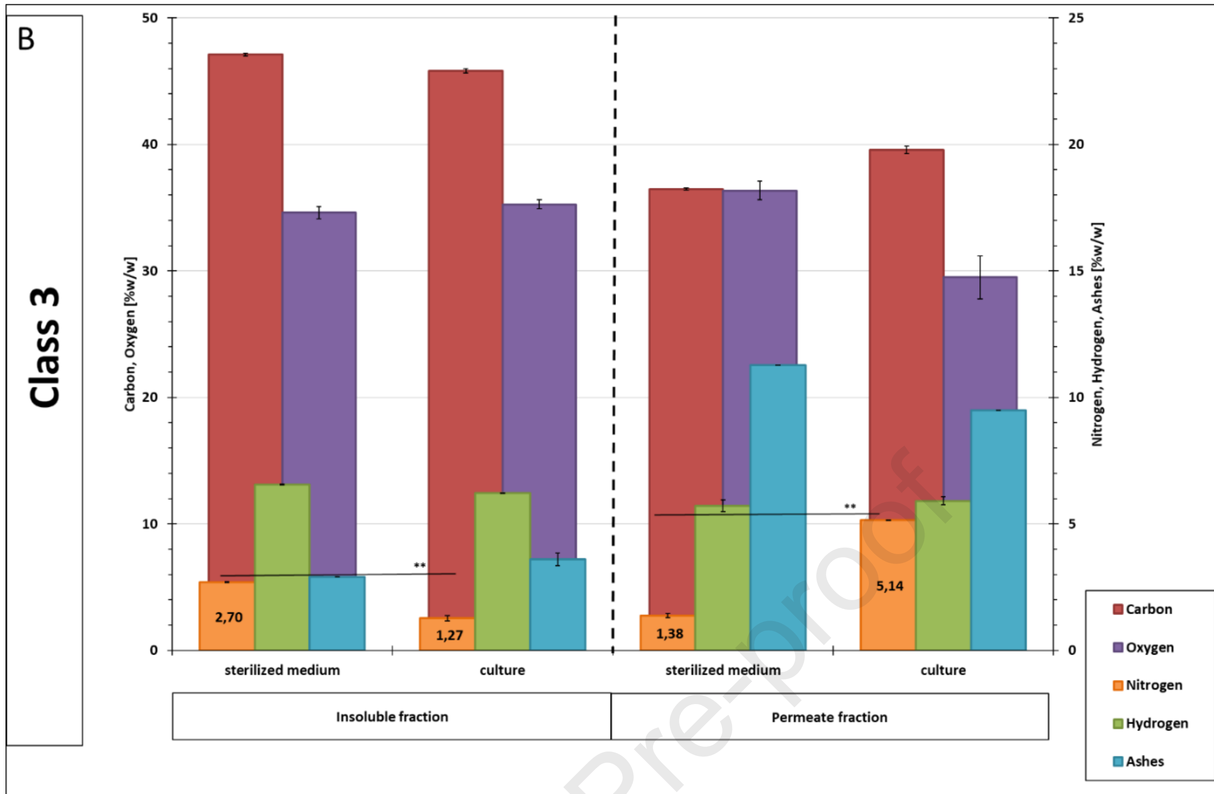

Journal Pre-proof



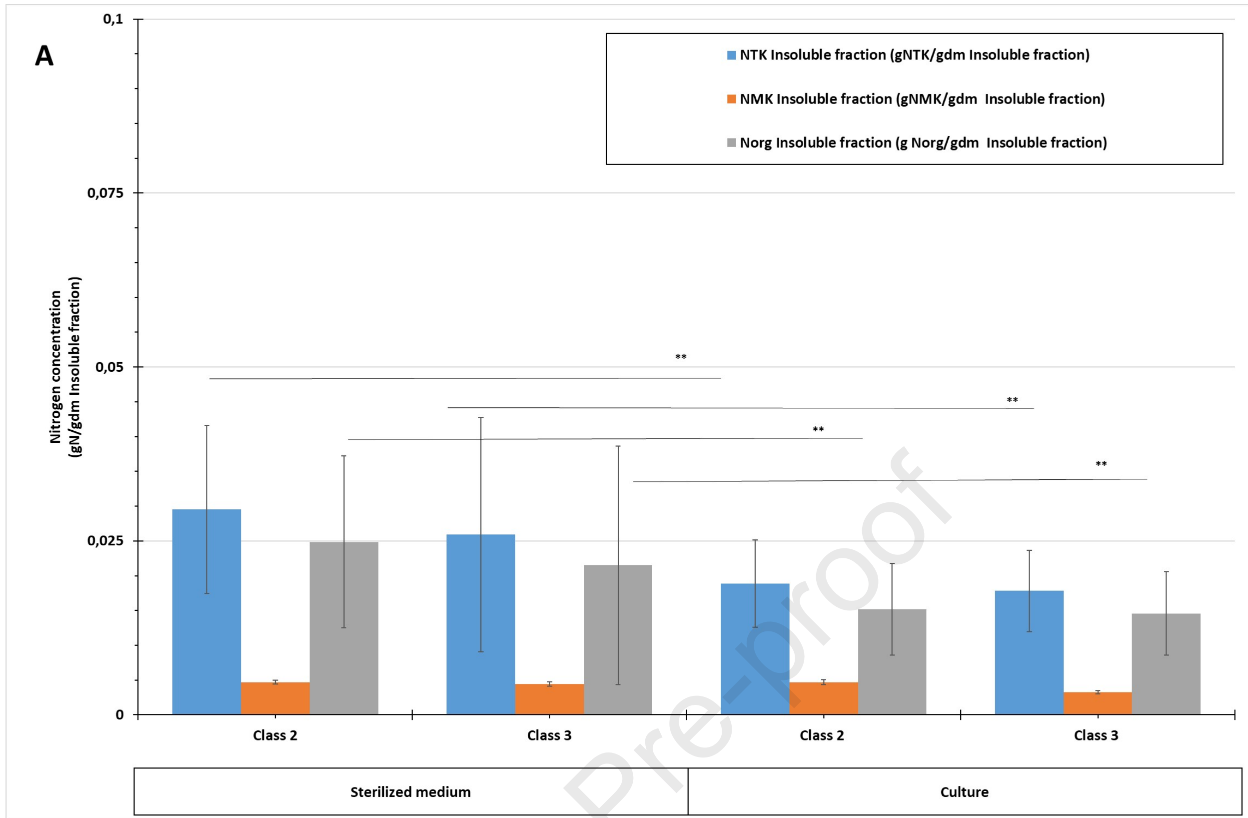

Journal Pre-proof



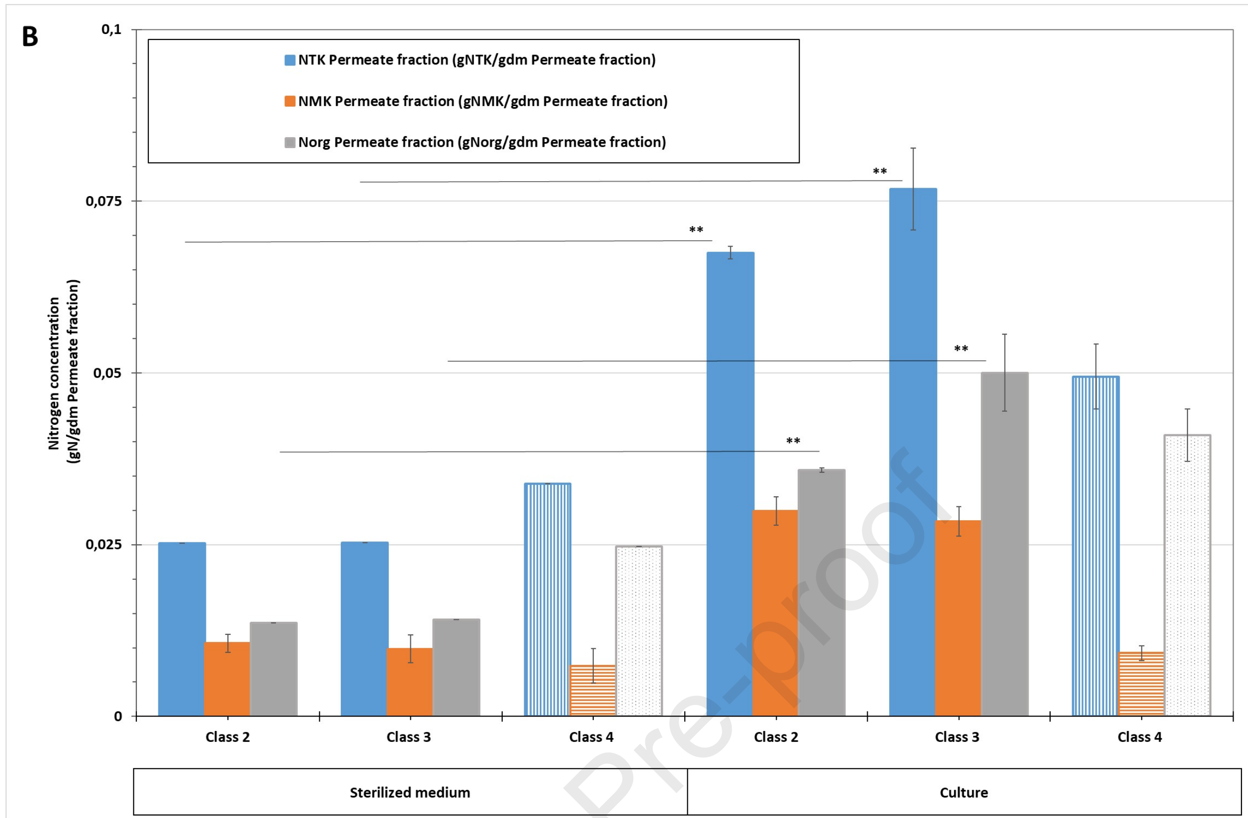

Journal Pre-proof




Journal Pre-proof




Journal Pre-proof




Journal Pre-proof