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1 **Effects of temperature, pH and water activity**
2 **on the growth and the sporulation abilities of *Bacillus subtilis* BSB1**

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13 **Keywords**

14 Growth kinetics, sporulation kinetics, cardinal model, growth boundaries, spores

15

16 **Abstract**

17 Spore-forming bacteria are implicated in cases of food spoilage or food poisoning. In their
18 sporulated form, they are resistant to physical and chemical treatments applied in the food
19 industry and can persist throughout the food chain. The sporulation leads to an increase in
20 the concentration of resistant forms in final products or food processing equipment. In
21 order to identify sporulation environments in the food industry, it is necessary to be able to
22 predict bacterial sporulation according to environmental factors. As sporulation occurs
23 after bacterial growth, a kinetic model of growth-sporulation was used to describe the
24 evolution of vegetative cells and spores through time. The effects of temperature, pH and
25 water activity on the growth and the sporulation abilities of *Bacillus subtilis* BSB1 were
26 modelled. The values of the growth boundaries were used as inputs to predict these
27 effects. The good description of the sporulation kinetics by growth parameters suggests
28 that the impact of the studied environmental factors is the same on both physiological
29 process. Suboptimal conditions for growth delay the appearance of the first spores, and
30 spores appear more synchronously in suboptimal conditions for growth. The developed
31 model was also applicable to describe the growth and sporulation curves in changing
32 temperature and pH conditions over time.

33

34 **1. Introduction**

35 Spore-forming bacteria have been responsible for 30 to 40% of foodborne illness cases
36 in France over recent years (Institut de Veille Sanitaire, 2015, 2014, 2013). They can produce
37 toxins that make food unfit for human consumption but also spoilage enzymes (Pavic et al.,
38 2005; Sorokulova et al., 2003). They are natural contaminants of raw materials in their
39 sporulated form. Throughout the food chain, from farm to fork, contaminating spores can
40 encounter environmental conditions that enable their germination leading to vegetative cell
41 outgrowth. Following their emergence, the vegetative cells can grow until they reach high
42 concentrations when they can form biofilms or they can differentiate into resistant spores. The
43 sporulation conditions have a strong impact on the production of spores and their resistance
44 properties. These newly-formed spores will be sources of re-contamination of food products,
45 end products and food processing equipment lines, and can be resistant to subsequent
46 treatments. Many studies aim at gaining a better understanding of (i) ecological niches of
47 spore-formers (Heyndrickx, 2011; Miller et al., 2015; Postollec et al., 2012) , (ii) the abilities
48 of vegetative cells to grow in foods according to environmental factors (Doyle and Buchanan,
49 2012; Pinon et al., 2004; Sant'Ana, 2017) and (iii) the abilities of spores to resist chemical
50 and physical treatments (Baril et al., 2012b; Mah et al., 2008; Mtimet et al., 2015; Nguyen Thi
51 Minh et al., 2011; Peña et al., 2009). However, few studies have identified the sporulation
52 niches and the steps of food processes that would be favorable to bacterial sporulation in
53 foods and on food processing equipment lines.

54 Predictive microbiology provides useful tools for predicting bacterial behavior
55 according to environmental factors, such as bacterial growth according to the environmental
56 factors (Augustin et al., 2000; Pinon et al., 2004; Rosso et al., 1995), or the resistance of
57 spores according to physical and chemical treatments (Baril et al., 2011; Gaillard et al., 1998;
58 Mafart et al., 2010). As far as sporulation is concerned, primary models are sparsely

59 developed (Baril et al., 2012a; Das and Sen, 2011). Some studies have investigated the effects
60 of temperature, pH or water activity on sporulation (Baril et al., 2012a; Lundgren, 1967;
61 Monteiro et al., 2005; Mtimet et al., 2015) but, to our knowledge, no secondary model has
62 been developed or suggested to quantify and predict these effects.

63 The sporulation and growth are two closely linked bacterial processes as sporulation
64 starts with DNA replication as does bacterial division, so the two processes share some
65 molecular **processes** (Narula et al., 2016b). The objective of this work was to (i) assess the
66 effects of environmental factors on the growth and the sporulation of *Bacillus subtilis* BSB1
67 in static conditions of environmental factors and (ii) to suggest a secondary model to describe
68 these effects. The environmental factors studied were those which could have a significant
69 impact on the sporulation behavior in the food industry environment (Gauvry et al., 2017)
70 such as temperature, pH and water activity. As the environmental conditions are mainly
71 dynamic, the model prediction was challenged for dynamic profiles of each factors.

72

73 **2. Materials and methods**

74 **2.1. Biological material**

75 The strain used in this study was the bacterial model *Bacillus subtilis* BSB1 which is a
76 *trp+* derivative of *B. subtilis* 168 (Buescher et al., 2012; Nicolas et al., 2012) kindly provided
77 by the Micalis institute, at the National Institute for Agricultural Research (INRA) center in
78 Jouy-en-Josas, France. This strain has been extensively studied, **much** knowledge on its
79 metabolism is available in the literature and many tools or methods for molecular
80 investigation are available. The strain was isolated on Luria Bertani (LB) plates (Difco™,
81 Becton, Dickinson and Company) and incubated overnight at 37°C. A colony was re-
82 suspended in LB broth, under 100 rpm agitation at 37°C until it reached an absorbance $A_{600\text{nm}}$
83 of 1.0. From this pre-culture, a 100-fold dilution was performed in 100 mL of LB broth in a
84 flask, in the same culturing conditions for 3 h. A second dilution was then performed in the
85 same conditions. When the $A_{600\text{nm}}$ reached 1.0, glycerol was added to the culture at a final
86 concentration of 25% w/w in cryovials. Conservation was performed at -80°C and the stocks
87 were renewed every month to avoid cellular death overtime in the cryovials.

88 **2.2. Assessment of the impact of temperature, pH and water activity on growth**

89 **2.2.1. Assessment of growth boundaries**

90 Growth or No Growth experiments (G/NG) were performed to evaluate the growth
91 boundaries for temperature, pH and a_w . Different pH values were tested between pH 4.50 to
92 5.00 (6 values) and between pH 8.90 and 9.30 (5 values), with a step of 0.10. The
93 temperatures tested were 5°C, 10°C, 13°C and 15°C for the lower limits and between 54°C
94 and 60°C with a step of 2°C for the upper limits. The values of a_w tested were comprised
95 between 0.910 and 0.940 with a step of 0.05. For one environmental factor tested, the other
96 factors were set to the standard values of 37°C, pH 7.0 or maximum a_w (0.996).

97 From a cryovial, a convenient dilution was performed in Tryptone Salt Broth (TS, Biokar
98 Diagnostics, Allonne, France) and 1 mL of this dilution was inoculated in a 250 mL flask
99 containing 100 mL Luria Bertani (LB) broth, in order to obtain an initial concentration of 6
100 \log_{10} (CFU/mL). Cultures were performed under an agitation of 100 rpm at the wanted
101 conditions of temperature, pH and a_w . The pH was adjusted by adding 2 M sodium hydroxide
102 or 2 M hydrochloric acid sterilized on 0.22 μm filters. The water activity of the medium was
103 adjusted with sodium chloride. Total cell counts were performed for each culture after 3, 7, 14
104 and 21 days by pouring 1 mL of adequate dilutions into TS broth in Nutrient Agar (NA,
105 Biokar Diagnostics, Allonne, France), with incubation of the plates for 24 h at 37°C and
106 counting of the colonies. The growth was observed if a significant increase of the cell
107 concentration was observed compared to the inoculum concentration.

108 *2.2.2. Effects of temperature, pH and a_w on growth*

109 The effects of pH, temperature and water activity on the bacterial growth were evaluated
110 with three independent experimental designs (Table 1), from pH 4.90 to 9.00, from 15°C to
111 54°C and from a_w 0.996 to 0.940 respectively. Here again, for one environmental factor
112 tested, the other factors were set to the standard values of 37°C, pH 7.0 or maximum a_w
113 (0.996) in order to test the effect of each environmental factor individually. Bacterial cultures
114 were performed in 100 mL of LB in 250 mL shaking flasks under an agitation of 100 rpm.
115 The inoculation was performed following the procedure described above, but targeting an
116 initial concentration of 3 \log_{10} (CFU/mL). The growth kinetics were monitored by total cell
117 counts over time. The initial pH was adjusted by adding sodium hydroxide 2 M or
118 hydrochloric acid 2 M sterilized on 0.22 μm filters. The initial water activity of the medium
119 was adjusted with sodium chloride. No significant variation of pH or a_w was noted before the
120 end of the exponential phase.

121 **2.3. Assessment of the impact of pH, water activity and temperature on growth**
122 **and sporulation abilities**

123 **2.3.1. Evaluation of sporulation boundaries**

124 Sporulation abilities were assessed with Sporulation or No-Sporulation experiments
125 (S/NS) at 5 °C, 9 °C, 13 °C and 15 °C for the lower limits and between 48 °C to 60 °C with a
126 step of 2 °C for the upper limits. The values of a_w tested were comprised between 0.910 and
127 0.945 with a step of 0.05. The effects of pH could not be evaluated in these conditions
128 because the pH could not be maintained at a constant value throughout the culture in the
129 shaking flask. **Indeed, as a function of temperature and water activity of the medium, the pH**
130 **of the culture decreases by more than 0.5 unit in the end of the exponential phase due to the**
131 **bacterial growth and increases until 8.0 at the beginning and during sporulation.** The
132 conditions of incubation were the same as for the evaluation of the growth boundaries, with
133 an inoculation concentration **close** to 6 log₁₀ (CFU/mL). The spore count was performed after
134 3, 7, 14 and 28 days of incubation. To do so, the total culture was treated for 10 minutes at
135 80°C by the capillary method (Baril, 2011) in order to inactivate the vegetative cells. With
136 this method, the detection limit of the concentration of spores was 10 CFU/mL. Sporulation is
137 observed if colonies are visible on plate after incubation.

138 **2.3.2. Effects of static temperature, pH and a_w on growth and sporulation**

139 The effects of pH (5.0; 5.2; 7.0; 8.0; 8.8), temperature (20°C; 25°C; 30°C; 37°C;
140 45°C; 49°C) and a_w (0.945; 0.960; 0.975; 0.985; 0.996) were evaluated on the growth and the
141 sporulation of *B. subtilis* BSB1 in batch cultures. Only one environmental factor at a time was
142 changed between experiments, the two other environmental factors were set again to the
143 standard values (37°C, pH 7.0 or a_w 0.996). For each condition tested, the bacterial cultures
144 were performed in triplicate. *B. subtilis* BSB1 was cultivated in batches, in an Applikon
145 bioreactor (Applikon Biotechnology, Netherlands) in 2 L Brain Heart Infusion (BHI,

146 BIOKAR Diagnostics, Beauvais, France) supplemented with sporulation salts (Hageman et
147 al., 1984), with an agitation of 250 rpm and an aeration of 1.5 L/min. The initial inoculum of
148 10^3 CFU/ml was made from an adequate dilution of a cryovial in TS broth. The water activity
149 of the medium was adjusted with sodium chloride. The pH was maintained at a constant value
150 across the culture, by adding sodium hydroxide 2 M or hydrochloric acid 2 M sterilized on
151 0.22 μ m filters. At each measurement time, 5 mL were sampled, and the total cell counts and
152 spore counts were performed as previously described.

153 ***2.3.3. Effects of dynamic conditions of pH and temperature on sporulation***

154 In order to test the effects of dynamic temperature during incubation, *B. subtilis* BSB1
155 was cultivated in 250 mL flasks containing 100 mL of LB broth, under an agitation of 100
156 rpm. Two profiles were tested with a temperature shift from temperature allowing sporulation
157 to adverse conditions. The first temperature profile was the succession of three periods: an
158 incubation at 37°C during 16 h, then at 10°C for 24 h and at 37°C until the end of the
159 experiment. The growth and sporulation kinetics observed in these conditions were compared
160 the kinetics obtained at the static temperature of 37°C. The second temperature profiles was
161 the succession of three periods: an incubation at 43°C during 24 h, then at 25°C during 144 h,
162 and at 50°C until the end of the experiment was compared to a constant temperature profile at
163 43°C.

164 The effects of pH variations during bacterial culture were tested on cultures of *B.*
165 *subtilis* BSB1 in batches (Applikon Biotechnology, Netherlands), in 2 L of BHI with an
166 agitation of 250 rpm and an aeration of 1.5 L/min. The dynamic profile of pH were as two
167 steps, the first shift at a pH close to the minimum pH of growth and the second below this
168 value. The pH profile was submitted at constant temperature of 37°C with three phases: pH
169 7.0 during 23 h, pH 5.0 during 40 h and pH 4.5 until the end of the experiment. The growth
170 and sporulation kinetics obtained were compared to the kinetics obtained at a static pH of 7.0.

171 **2.4. Primary and secondary modelling**

172 **2.4.1. Kinetic model of growth and sporulation**

173 The growth parameters were evaluated for the different conditions tested (Table 1).
174 The data of the natural logarithm for the total cell concentration were plotted against time and
175 the logistic model of growth (Rosso et al., 1995) was used to fit experimental data with Eq.
176 (1).

$$177 \ln(N(t_i)) = \begin{cases} \ln(N_0), & t_i < \lambda \\ \ln\left(\frac{N_{max}}{\left(1 + \frac{N_{max}}{N_0}\right) \times \exp(-\mu_{max} \times (t_i - \lambda))}\right), & t_i \geq \lambda \end{cases} \quad (1)$$

178 with $N(t_i)$, the concentration of total cells at a given time of the incubation t_i , N_0 the initial
179 concentration of vegetative cells and N_{max} the maximum total cell concentration (CFU/mL), λ
180 the lag before growth (h) and μ_{max} the maximum vegetative specific growth rate (h^{-1}).

181 The sporulation kinetics in batch cultures were described and fitted with the primary
182 model of Gauvry *et al.* (2019) given by Eq. (2):

$$183 S(t_i) = \begin{cases} 0, & t_i < t_f \\ S(t_{i-1}) + [N(t_i - t_f) - S(t_{i-1})] \times P(t_i - t_f), & t_i \geq t_f \end{cases} \quad (2)$$

184 with $N_{t_i-t_f}$ the concentration of total cells at time $t_i - t_f$ (CFU/mL) given in Eq. (1), $S_{t_{i-1}}$,
185 the concentration of spores at time t_{i-1} (CFU/mL), t_{i-1} is the previous analyzed sampling
186 point, then $t_i - t_{i-1}$ is the interval of time used for the computation (fixed at one hour in this
187 study). t_f is the time needed for the spore formation, i.e. the time from the sporulation
188 initiation to the appearance of spores (heat resistant cell, as defined above). P is the
189 probability to form a spore over time which was described with a Gaussian law:

$$190 P(t_i) = P_{max} \times \left[\frac{1}{\sigma \times \sqrt{2\pi}} \times \exp\left(-0.5 \times \left(\frac{t_i - t_{max}}{\sigma \times \sqrt{2}}\right)^2\right) \right] \quad (3)$$

191 where P_{max} (unitless), t_{max} (h) and σ (h). P_{max} is used to weight the maximum proportion of
 192 cells which are able to sporulate at the time t_{max} (h). Consequently the probability of
 193 sporulating at time t_{max} can be easily calculated as follows:

$$194 \quad P(t_{max}) = P_{max} \times \frac{1}{\sigma \times \sqrt{2\pi}} \quad (4)$$

195 P_{max} is obtained at the time t_{max} which mainly has an impact on the time at which the first cells
 196 initiate sporulation and subsequently, on the time until the first spores appear. The parameter
 197 σ is the scattering of the probability curve which accounts for the dispersion of the time of
 198 sporulation initiation. This **parameter influences** the shape of the sporulation curve i.e. the
 199 appearance of spores over time.

200 **2.4.2. Describing the impact of temperature, pH and a_w on growth and** 201 **sporulation**

202 The effects of temperature, pH and water activity on bacterial growth were described with
 203 a model inspired by the cardinal model of Rosso et al. (1995) and suggested by Gauvry
 204 (2017). This model is detailed by the equations Eq. (5) and Eq. (6):

$$205 \quad Y_{max}(T^{\circ}C, pH, a_w) = Y_{opt} \times CM_2(T^{\circ}C) \times CM_{0.1}(pH) \times CM_1(a_w) \quad (5)$$

$$206 \quad CM_n(X) =$$

$$207 \quad \begin{cases} \frac{(X-X_{max}) \times (X-X_{min})^n}{(X_{opt}-X_{min})^{n-1} \times [(X_{opt}-X_{min}) \times (X-X_{opt}) - (X_{opt}-X_{max}) \times (X_{opt}+X_{min}-nX)]}, & X_{min} < X < X_{max} \\ 0, & X \leq X_{min} \text{ ou } X \geq X_{max} \end{cases} \quad (6)$$

208 Where Y is the growth or the sporulation parameters (e.g. μ_{max} , $1/\lambda$, $-(\log_{10}(P_{max}))$, t_{max} or σ),
 209 and X is the environmental factor (pH, temperature or water activity). X_{max} , X_{min} and X_{opt} were
 210 the maximum, minimum and optimum values of the environmental factors at which the strain
 211 was able to grow or to sporulate. a_w opt and a_w max were set up equal to 0.996 and 1.000
 212 respectively. Moreover, n is a shape parameter. The values of the shape parameter used to

213 describe the effects of temperature, pH and water activity on the growth and sporulation
214 parameters were 2, 0.1 and 1 respectively. The lower is the n value, more abrupt is the change
215 in the response (Y_{max}) when the factor is close to the boundary.

216 The cardinal values of growth were estimated based on the combined data of μ_{max} of
217 the three factorial designs, obtained in LB in Erlenmeyer flasks. The effects of temperature,
218 pH and water activity on the lag before growth λ were described with the cardinal values as
219 inputs and the optimal value of $1/\lambda$ was estimated. For prediction in dynamic conditions, it is
220 important to notice that the shift of temperature or pH occurs after a time of incubation longer
221 than the lag time.

222 The optimal values of the sporulation parameters were estimated in a two-step
223 procedure. The growth and sporulation kinetics obtained in favorable conditions for growth
224 and sporulation (45°C, pH 7.0, a_w 0.996) were fitted by the primary model given by Eq. (1)
225 and Eq. (2) in order to estimate the growth and sporulation parameters in this particular
226 condition. The values of these parameters ($Y_{max}(45^\circ C, pH\ 7.0, a_w\ 0.996)$) were used to calculate
227 the optimum values Y_{opt} with Eq. (5). The effects $CM_n(X)$ were computed with Eq. (6) using
228 the cardinal values of growth as inputs.

229 As detailed above, the growth parameters were estimated from growth kinetics and the
230 optimal values of the sporulation parameters from one growth and sporulation kinetic (45°C,
231 pH 7.0, a_w 0.996). The other growth and sporulation kinetics in static or dynamic conditions
232 were predicted and compared to the observations. The growth and the sporulation parameters
233 were estimated by minimizing the Sum of Square Error (SSE) in Eq. (6) between the
234 experimental values and the values calculated by the kinetic models (functions `nlinfit` or
235 `fmincon`, MATLAB and Statistics Toolbox Release 2013a, The MathWorks, Inc., Natick,
236 Massachusetts, United States). The standard deviations of the estimates were calculated from

237 the confidence intervals at 95% (function nlparci). Triplicates were performed for each
238 condition tested for the growth and sporulation kinetics in batch cultures. As there was little
239 biological and experimental variability, estimations of the growth and sporulation parameters
240 were made on the global data of the triplicates.

$$241 \quad SSE = \sum (y_i - \hat{y}_i)^2 \quad (7)$$

242 with y_i , experimental data of the concentration of total cells or spores ln (CFU/mL) and
243 \hat{y}_i , corresponding concentrations estimated with the model.

244 From the fitted or predicted kinetics of growth and sporulation with Eq. (1) and Eq.
245 (2), criteria of interest to characterize the efficiency of sporulation could be calculated in order
246 to compare these results to previous works (Baril et al., 2012a; Carvalho et al., 2010;
247 Monteiro et al., 2014). These criteria are the maximum concentration of spores S_{max}
248 (CFU/mL) and the time to see the first spore t_{1s} (h) *i.e.* the time at which the concentration
249 reaches one spore per mL (1 CFU/mL).

250 The goodness of fit of the model was assessed with the Root Mean Square Error
251 (RMSE). The relative error (RE) was used to assess the mean error between (i) observed and
252 predicted values of $1/\lambda$ and (ii) between estimated and simulated values of the time to see the
253 first spores (t_{1s}) and the maximum concentration of spores S_{max} (ln (CFU/mL)) (Ross, 1996).
254 The formulas were detailed below:

$$255 \quad RMSE = \sqrt{\frac{SSE}{n-p}} \quad (8)$$

256 with SSE calculated in Eq. (7), n the number of experimental data of $1/\lambda$ (h^{-1}), t_{1s} (h) or S_{max}
257 (ln (CFU/mL)), and p the number of parameters of the model.

258 $RE (\%) = 100 \times \frac{|y_i - \hat{y}_i|}{y_i} \quad (9)$

259 with y_i the experimental data of $1/\lambda$ or the estimated values of the sporulation parameters
260 ($-\log_{10}(P_{max})$), t_{max} , σ , and \hat{y}_i the corresponding simulated values computed with the cardinal
261 model.

262

263 **3. Results**

264 **3.1. Characterization of the growth of *B. subtilis* BSB1 according to temperature,** 265 **pH and water activity**

266 Growth/No Growth experiments allowed to show that *Bacillus subtilis* BSB1 could grow
267 between 13°C and 54°C, between pH 4.9 and pH 9.1, and at water activities superior to 0.935
268 (filled black circles in Fig. 1). The cardinal values of growth were estimated for temperature,
269 pH and a_w based on the maximum growth rates μ_{max} (Fig. 1 and Table 1). *B. subtilis* BSB1 had
270 an optimal growth rate μ_{opt} of 4.04 h⁻¹ in optimal conditions i.e. at 46.9 °C, pH 6.8 and
271 maximum a_w 0.996. It had a minimum latency of 1.22 h for the conditions of inoculation used
272 (inoculum concentration, medium agitation and aeration). The ranges of temperature, pH
273 and water activity for which the strain was able to grow were from 5.5°C to 55.7°C, from pH
274 4.8 to 9.2 and beyond a_w 0.929. These estimated cardinal values of growth were within the
275 range defined by the Growth/No Growth boundaries, except for the minimum temperature of
276 growth estimated at 5.5°C whereas the growth was observed at 13°C and no growth was
277 observed at 10°C. But the uncertainty is large on this estimated value as its confidence
278 interval range (95%) is from -2.3°C to 13.1°C.

279 The impacts of the three factors (T, pH and a_w) on the lag before growth could be correctly
280 described by the model with a mean relative error of 27% for the 47 experiments. The
281 maximum concentration of total cells N_{max} could be considered as a constant with a mean
282 value of $\ln(N_{max})$ of 20.76 (+/- 4.36%) i.e. 1.04×10^9 CFU/mL.

3.2. Characterization of the sporulation of *B. subtilis* BSB1 according to temperature, pH and water activity

3.2.1. Typical impacts of environmental factors on sporulation kinetics

In order to show how the sporulation kinetics could be affected by the environmental factors, three characteristic curves of growth and sporulation obtained in batch culture in Brain Heart Infusion (BHI) are presented in Fig. 2. The environmental factors have an impact on the time to obtain the first spore t_{1s} (Fig. 3d, i and n), the sporulation rate and/or the maximum concentration of spores S_{max} (Fig. 3e, j and o). The differences of the shapes of the sporulation curves between conditions tested (Fig. 2) could be explained with the probability to sporulate over time (Fig. 2 d, e and f) and its three parameters (P_{max} , t_{max} and σ , see Equation 3). For example, the spores appeared **synchronously** at 25°C, pH 7.0 and a_w 0.996 (Fig. 2b) or at 37°C, pH 7.0 and a_w 0.945 (Fig. 2c) than at 49°C, pH 7.0 and a_w 0.996 (Fig. 2a). In the first conditions, the probability scatterings were lower with values of σ of 4.8 h (Fig. 2e, incubation at 25°C, pH 7.0, a_w 0.996) and 3.7 h (Fig. 2f, incubation at 37°C, pH 7.0, a_w 0.945). At 49°C, pH 7.0 and a_w 0.996 (Fig. 2a), the cells sporulated less synchronously as the sporulation probability over time was much more scattered ($\sigma= 25.1$ h) and led to a more progressive appearance of spores over time.

The combination of the three parameters (P_{max} , t_{max} and σ) explained the differences of the maximum concentration of spores S_{max} between conditions. At 37°C, pH 7.0 and a_w 0.945 (Fig. 2c), the spore yield was 3500 times lower than at 25°C, pH 7.0 and a_w 0.996 (Fig. 2b). We could have expected that it was the **result** of a very low maximum probability of forming a spore (P_{max}). However, P_{max} was 2.5-fold higher for an incubation at 37°C, pH 7.0 and a_w 0.945 than at 25°C, pH 7.0 and a_w 0.996 (Fig. 2e and f). On one hand, at 37°C, pH 7.0 and a_w 0.945, the maximum sporulation probability was reached much sooner during the incubation ($t_{max} = 13.4$ h), when the concentration of total cells was only $1.2 \cdot 10^4$ CFU/mL. On the other

308 hand, at 25°C, pH 7.0 and a_w 0.996, the maximum sporulation probability was reached at 48.2
309 h of incubation when the bacterial concentration was 8.0×10^9 CFU/mL. Consequently,
310 despite the fact that the maximum proportion of cells was lower, there is a higher amount of
311 vegetative cells which have initiated sporulation, explaining the higher level of spore
312 concentration at this temperature.

313 **3.2.2. Effects of environmental factors on sporulation kinetics**

314 In the Sporulation/No Sporulation experiments, the boundaries of sporulation were
315 comprised between 10°C and 15°C for the lower bounds, between 50°C and 52°C for the
316 upper bounds, and the minimum a_w for sporulation was between 0.935 and 0.940. However,
317 estimations of the sporulation boundaries directly depend on the spore detection limit (10
318 CFU/mL), inherent to the experimental procedure. Consequently, the sporulation boundaries
319 could be underestimated with this method.

320 We defined the optimal conditions for sporulation as the conditions in which the
321 spores appeared the soonest and in which the highest amount of spores was obtained. The
322 spores appeared sooner in the most optimal conditions for sporulation and this time increased
323 when the culture conditions approached the sporulation boundaries (Fig. 3d, 3i and 3n). For
324 example, while the first spores appeared at 14 h of culture at the three temperatures 37°C,
325 45°C and 49°C, they appeared at 29 h at 20°C and at 51 h at 25°C. An exception was
326 observed at a_w 0.945 (Fig. 3n) for which the first spores appeared at 10 hours of culture. The
327 spores appeared more synchronously in suboptimal conditions while they appeared more
328 gradually over time in more optimal sporulation conditions (Fig. 2). The maximum
329 concentration (S_{max}) of spores was not strongly affected by the temperatures and the pH
330 values tested (Fig. 3e and j) with a mean value of S_{max} of $7.4 \log_{10}$ (CFU/mL). However, the
331 maximum concentration of spores was more strongly affected as the water activity decreased

332 (Fig. 3o) with 3.0×10^4 CFU/mL obtained at a_w 0.945 while the maximum concentration of
333 spores obtained was 6.54×10^8 CFU/mL at 37 °C, pH 7.0 and a_w 0.996.

334 In optimal conditions for growth (i.e. at 45°C, pH 7.0, a_w 0.996), the probability of
335 forming a spore was $7.32 \times 10^{-5} \text{ h}^{-1}$, the time t_{max} at which cells had the highest probability of
336 sporulation was 109.8 h and the optimal probability scattering σ was 21.7 h (see the table S1
337 in the supplementary data). Interestingly, when the environmental conditions were closer to
338 the boundaries of the growth region, the maximum sporulation probability increased from
339 $7.32 \times 10^{-5} \text{ h}^{-1}$ in optimal conditions to $1.10 \times 10^{-2} \text{ h}^{-1}$ at 25°C or $3.38 \times 10^{-2} \text{ h}^{-1}$ at a_w 0.945.
340 The time t_{max} at which the cells had the maximum sporulation probability decreased close to
341 the growth boundaries until values of 35.1 h at pH 8.8 or 13.2 h at a_w 0.945 for example.
342 Lastly, the probability scattering dropped as the temperature, pH or water activity became
343 more and more drastic for growth and sporulation. For example, the values decreased from
344 22.1 h in optimal conditions to 3.7 h at a_w 0.945, 5.7 h at pH 8.8 or 7.4 h at 20°C. Our
345 observations indicated that when the conditions are adverse to the growth (i.e. close to the
346 growth boundaries), the cells sporulated more synchronously and in higher proportions.
347 However vegetative cells sporulated sooner, when the concentration of vegetative cells was
348 still low, ultimately leading to fewer amounts of sporulating cells.

349 The optimal conditions for growth (45°C, pH 7.0, a_w 0.996) were used to calculate the
350 optimal values of the probability parameters that would be obtained in optimal conditions for
351 growth i.e. at 46.9°C, pH 6.8, a_w 0.996. The values of P_{max} , t_{max} and σ in these optimal
352 conditions for growth were 3.98×10^{-3} , 111.7 h and 22.1 h respectively. Then, these optimal
353 values of the sporulation parameters and the growth boundaries were used as inputs of the
354 cardinal model to describe the effects of the static profiles of temperature, pH and water
355 activity on the sporulation parameters, i.e. to calculate the time to see the first spore and the
356 maximum concentration of spores (Fig. 3). The simulated kinetics of growth and sporulation

357 gave a good prediction of the time to see the first spores with a mean relative error of 23.9 %.

358 The maximum concentration of spores (\log_{10} (CFU/mL)) could be predicted with a mean

359 relative error of 17.8%.

360

361 ***3.2.3. Effects of environmental shifts during bacterial culture on sporulation kinetics***

362 For the tested conditions, the growth kinetics were similar ($p < 5\%$) between static
363 conditions and dynamic conditions of pH or temperature because the shifts were applied after
364 the stationary phase was reached (see Fig. 4). The maximum concentrations of total cells were
365 2.9×10^8 CFU/mL in flasks and 2.2×10^9 CFU/mL in bioreactors.

366 In order to simulate the sporulation kinetics in dynamic conditions with the growth and
367 sporulation model (Eq. 1 and Eq. 2), some hypothesis were made. For the first temperature profile
368 (Fig. 4a), the sporulation curve started at 37°C as in static conditions. At the time shift (16 h), we
369 hypothesized that no more cell could initiate the sporulation and that the vegetative cells which
370 were already committed to sporulation were blocked in the sporulation process by the low
371 temperature of 10°C. Consequently, no more spore could be produced and no spore appeared at
372 10°C from 16 h to 40 h of culture. When the temperature was restored at 37°C, the vegetative
373 cells which were already committed to sporulation before the switch at 10°C finished the
374 sporulation process, leading to an increase of spores concentration observed from 40 h to 47 h of
375 incubation. At the same time, when the temperature shifted from 10 °C to 37 °C, we hypothesized
376 that the ability to sporulate was restored at the level reached before the shift. The probability to
377 sporulate was fixed equal to the quartile of cells having initiated the sporulation previously
378 reached at 16h of incubation (shift from 37°C to 10°C).

379 For the second dynamic temperature profile (Fig. 4b) and for the dynamic pH profile (Fig.
380 4c), the first spores appeared identically to static conditions (43°C, pH 7.0, a_w 0.996 or 37°C, pH
381 7.0, a_w 0.996). When the environmental shift was applied, the vegetative cells already committed
382 to sporulation could finish the sporulation process as the new environmental conditions were still
383 favourable for sporulation (25°C, pH 7.0, a_w 0.996 or 37°C, pH 5.0, a_w 0.996; Fig. 4). The
384 sporulation kinetics continued and followed trends computed. The previous hypothesis were used
385 to compute the apparition of the spores until the end of the incubation: at the shift, the ability to
386 sporulate restarted from as if there were 1 cell/mL, the probability to sporulate was equal to the

387 quartile of cells having initiated the sporulation previously reached, and this probability evolved
388 as expected in static environmental conditions. The use of the growth and sporulation model led
389 to good descriptions of the sporulation curves in the three dynamic profiles tested with RMSE
390 values associated to the \log_{10} spores concentrations of 0.438 (Fig. 4a), 0.314 (Fig. 4b), and
391 0.243 (Fig. 4c).

392 **4. Discussion**

393 *B. subtilis* is able to grow between 5.5°C and 55.7°C, between pH 4.8 and pH 9.2 and
394 beyond a_w 0.929. These values were consistent with the growth boundaries evaluated with the
395 Growth/No Growth experiments and are consistent with previous observations (Holtmann and
396 Bremer, 2004; Pandey et al., 2013; Pant et al., 2015; Tapia et al., 2007). As the environmental
397 conditions were increasingly drastic for growth, the latency before growth increased and the
398 growth rate decreased. The experiments were performed with bacterial cells which were in the
399 same physiological states and the size of the inoculum was high enough ($3.0 \log_{10}$ (CFU/mL))
400 to neglect the heterogeneity of behavior between cells. Consequently, a linear relationship
401 could be observed between the growth and the inverse of the latency before growth as
402 previously observed elsewhere (Munoz-Cuevas et al., 2010; Robinson, 1998).

403 Predictive microbiology has proven its efficiency to predict the bacterial growth rate
404 according to environmental factors by using a cardinal model. This model uses the cardinal
405 values of growth i.e. the minimum, optimum and maximum values of environmental factors
406 for which bacterial growth is possible. As a relationship could be established between the
407 growth rate and the latency before growth, the cardinal model could be used to predict both
408 these growth parameters. On the contrary, the environmental condition tested did not have a
409 significant impact on the maximum concentration of total cells. Consequently, the maximum
410 bacterial concentration could be considered as a constant of 1.04×10^9 UFC/mL in the culture
411 conditions tested. This also means that provided that vegetative cells are able to grow, they

412 will theoretically meet the conditions of starvation and quorum sensing that trigger
413 sporulation (Grossman and Losick, 1988). Close to the optimal conditions for growth, the first
414 spores appeared the soonest and the highest concentrations of spores were obtained (Fig. 3).
415 These criteria are commonly used to define the efficiency of sporulation (Baril et al., 2012a;
416 Carvalho et al., 2010; Monteiro et al., 2005) and are of interest for food applications.
417 Consequently, we defined the optimal conditions for sporulation as being identical to the
418 optimal conditions for growth. The maximum concentration of spores was not strongly
419 affected by suboptimal temperature and pH conditions. These results are consistent with
420 previous results on *B. subtilis* (Monteiro 2005) and were also observed for other species such
421 as *Bacillus weihenstephanensis* KBAB4 (Baril et al., 2012a). On the contrary, water activity
422 had a strong effect on the maximum concentration of spores as also observed for *B.*
423 *weihenstephanensis* KBAB4. In our study, this could be related to the rapid mortality
424 observed after the stationary phase at low water activity (results not shown). Indeed,
425 sporulation is a last resort differentiation option for survival but, before committing to
426 sporulation, a checkpoint is activated to assess whether the cell will succeed or fail to
427 complete the sporulation process (Lemon et al., 2000; Stephens, 1998; Veening et al., 2009).
428 In suboptimal conditions, the spores of *B. subtilis* BSB1 appeared later as observed for *B.*
429 *weihenstephanensis* KBAB4 and *Bacillus licheniformis* AD978 but they appeared more
430 synchronously contrarily to what is commonly observed in the literature (Baril et al., 2012a).

431 The effects of environmental factors on the sporulation kinetics can be explained by
432 their effects on the sporulation probability over time. In suboptimal conditions of growth, the
433 maximum probability to sporulate (P_{max}) increases, the time (t_{max}) and the probability
434 scattering (σ) decrease. These results mean that vegetative cells sporulate more synchronously
435 and in higher proportions in suboptimal conditions for sporulation. In suboptimal conditions,
436 the phosphorylated Spo0A level **increases** to high level in stressed cells allowing for a more

437 efficient initiation of the sporulation process (González-Pastor, 2011; Fujita *et al.*, 2005). This
438 process is not efficient if the vegetative cells sporulate very early (lower t_{max}), before high
439 concentration or before the maximum concentration of vegetative cells is reached. Indeed,
440 although a high proportion of cells sporulate (high P_{max}) in the same range of time (lower σ),
441 this gives a small amount (or concentration) of spores as depicted at low water activity (see
442 Fig 2f, incubation at 37°C, pH 7.0, a_w 0.945). Compared to these observations made in static
443 environmental conditions, shifts from favorable to unfavorable conditions led to delay and to
444 slow down, even to stop the spores formation. But if the population of vegetative cells is
445 placed again in favorable conditions, the sporulation process restarts. This could be explained
446 by the fact that low temperatures and low pH lead to the inhibition of sporulation genes
447 expression and slow down enzymatic reactions (Budde *et al.*, 2006; Cosby and Zuber, 1997;
448 Movahedi and Waites, 2002).

449 Sporulation and growth are strongly linked. Indeed, a linear relationship exists between
450 the maximum growth rate and the sporulation rate (Baril, 2011; Dawes and Mandelstam,
451 1970), because these two bacterial processes share common molecular machineries (Mendez
452 *et al.*, 2004; Narula *et al.*, 2016a; Reder *et al.*, 2012). Moreover, the sporulation boundaries
453 were consistent with the growth boundaries and the optimal conditions for sporulation (in
454 which the spores appear rapidly and in high concentrations) matched with the optimal
455 condition for growth. Lastly, the temperature, pH and water activity affected both the growth
456 (Fig. 1) and sporulation (Fig. 3) parameters in the same way. These observations led to
457 suggest that the sporulation parameters could be described with the same model as for growth,
458 i.e. the cardinal model with the growth boundaries as inputs. **These results suggest a**
459 **correlation observed between the maximum growth rate (μ_{max}) and the sporulation parameters**
460 **which impact highly the efficiency of the sporulation process (probability scattering (σ), the**

461 maximum proportion of cells which are able to sporulate (P_{max}) or the time t_{max} at which this
462 probalibility is observed).

463 The only information needed to predict sporulation in any environmental conditions was
464 the optimal values of the sporulation parameters in optimal conditions i.e. at 46.9°C, pH 6.8,
465 a_w 0.996. To calculate these optimal values, only one set of experimental values for the
466 growth and sporulation kinetics in favorable conditions for sporulation is required as a set of
467 calibration kinetics and then, by using with Eq. (5) and Eq. (6). Thanks to these optimal
468 parameter values and the cardinal values of growth, the used model described efficiently the
469 growth and sporulation kinetics obtained in the 13 other conditions tested for the batch. With
470 this model, the data of interest for food applications such as the time until the first spores
471 appear and the maximum concentration of spores could be satisfactorily and safely predicted
472 by computing with mean relative errors of 23.9% and 17.8% respectively. Further
473 investigation is needed for good prediction close to the boundary, as the interaction between
474 factors is neglected. In these areas, growth might be overestimated, which could lead to an
475 overestimation of spore formation.

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479

480 6. References

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659

660 Table 1. Growth boundaries and estimations of the cardinal values of *B. subtilis* BSB1
 661 estimated based on the maximum growth rate.

Parameter	Lower boundaries	Estimates (\pmSD)	Upper boundaries
T_{min} ($^{\circ}$ C)	10.0 ^{ng}	5.5 (\pm 3.9)	13.0 ^g
T_{opt} ($^{\circ}$ C)	-	46.9 (\pm 3.6)	-
T_{max} ($^{\circ}$ C)	54.0 ^g	55.7 (\pm 2.9)	56.0 ^{ng}
pH_{min}	4.8 ^{ng}	4.82 (\pm 0.77)	4.9 ^g
pH_{opt}	-	6.79 (\pm 1.37)	-
pH_{max}	9.1 ^g	9.15 (\pm 0.45)	9.2 ^{ng}
$a_w min$	0.925 ^{ng}	0.929 (\pm 0.026)	0.930 ^g
μ_{opt} (h^{-1})	-	4.04 (\pm 1.00)	-
$1/\lambda_{opt}$ (h^{-1})	-	0.82 (\pm 0.25)	-

662 g: growth was observed at the corresponding temperature, pH or a_w with the growth-no
 663 growth experiments.

664 ng: no growth was observed at the corresponding temperature, pH or a_w with the growth-no
 665 growth experiments.

666 - : not applicable.

667

668

669

670 Fig. 1. Effect of temperature, pH and a_w on the growth rate (μ_{max}) and the lag (λ) before
671 growth of *Bacillus subtilis* BSB1. The experimental data (\circ) of μ_{max} (a, c and e) and $1/\lambda$ (b, d
672 and f) were plotted against temperature (a and b), pH (c and d) or a_w (e and f). The conditions
673 in which no growth was observed are indicated by bold empty circles on the horizontal axis (
674 \bullet) and the conditions in which growth was observed in the Growth/No Growth experiments
675 are indicated by black filled circles (\bullet). The cardinal values of growth were estimated based
676 on growth rates observations (μ_{max}) with the cardinal model (solid lines). These estimated
677 cardinal values were used as inputs to estimate ($1/\lambda_{opt}$) and then describe the effects of
678 environmental factors on ($1/\lambda$)

679

680 Fig. 2. Growth and sporulation kinetics of *B. subtilis* BSB1 obtained in BHI, in batch cultures
681 at 49°C, pH 7.0 and a_w 0.996 (a and d), at 25°C, pH 7.0 and a_w 0.996 (b and c) and at 37°C,
682 pH 7.0 and a_w 0.945 (c and f). The experimental data of total cells (\circ) and heat resistant spores
683 (\bullet) were fitted with the growth-sporulation model (solid lines). The probability of vegetative
684 cell to commit in sporulation over time (h^{-1}) is represented in addition to the growth and
685 sporulation kinetics (d, e and f).

686

687 Fig. 3. Prediction of the effects of temperature (a to e), pH (f to j), and water activity (k to o)
688 on the sporulation parameters of *B. subtilis* BSB1. The effects of each environmental factor
689 were assessed on the probability to form a spore ($-\log_{10}(P^*_{max})$ see a, f and k), the time to
690 reach the maximum sporulation probability (t_{max} see b, g and l), the probability scattering (σ
691 see c, h and m), the inverse of the calculated time to see the first spore ($1/t_{1s}$ see d, i and n)

692 and the calculated maximum concentration of spores reached \log_{10} (S_{\max} see e, j and o). The
693 cardinal values of growth (Tab. 1) were used as inputs for the cardinal model (solid lines).

694

695 Fig. 4. Growth and sporulation kinetics of *B. subtilis* BSB1 in dynamic conditions of
696 temperature (a and b) and pH (c) compared to kinetics obtained static conditions. The
697 experimental data of total cells (\bullet) and of heat resistant spores obtained in static conditions
698 (\bullet) were fitted with the growth-sporulation model in Eq. (1) and Eq. (2) (solid lines). The
699 experimental data of heat-resistant spores in dynamic conditions (\square) were fitted with Eq. (1)
700 and Eq. (2) following materials and methods (dashed lines). Time shifts are indicated with
701 vertical dotted lines. (a) The incubation was performed in static conditions (37°C, pH 7.0 and
702 a_w 0.996) or 37°C during 16 h, then at 10°C for 24 h and at 37°C until the end of the
703 experiment. (b) The incubation was performed in static conditions (43°C, pH= 7.0 and a_w
704 0.996) or at 43°C during 24 h, then at 25°C during 144 h, and at 50°C until the end of the
705 experiment. (c) The incubation was performed in static conditions (37°C, pH 7.0 and a_w
706 0.996) or at pH 7.0 during 23 h, pH 5.0 during 40 h and pH 4.5 until the end of the
707 experiment.

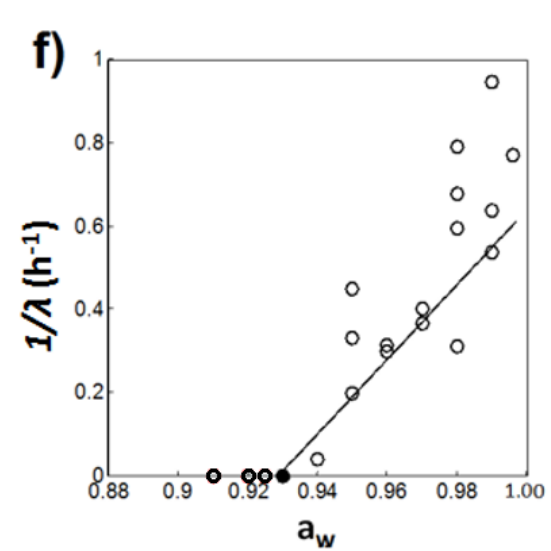
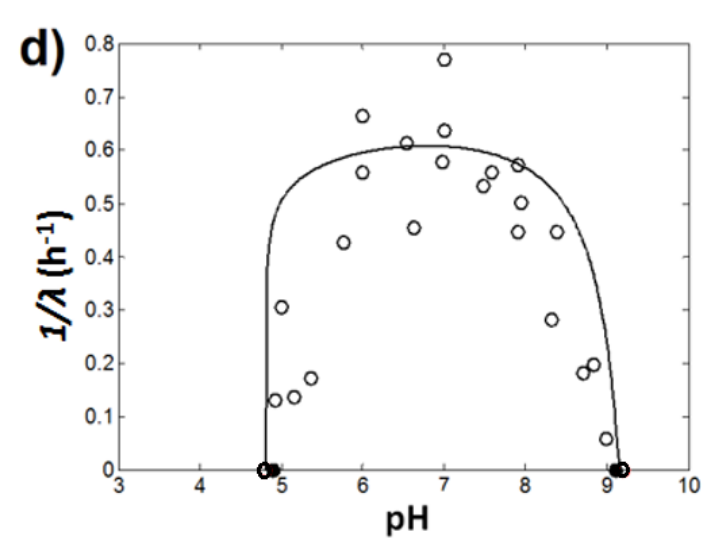
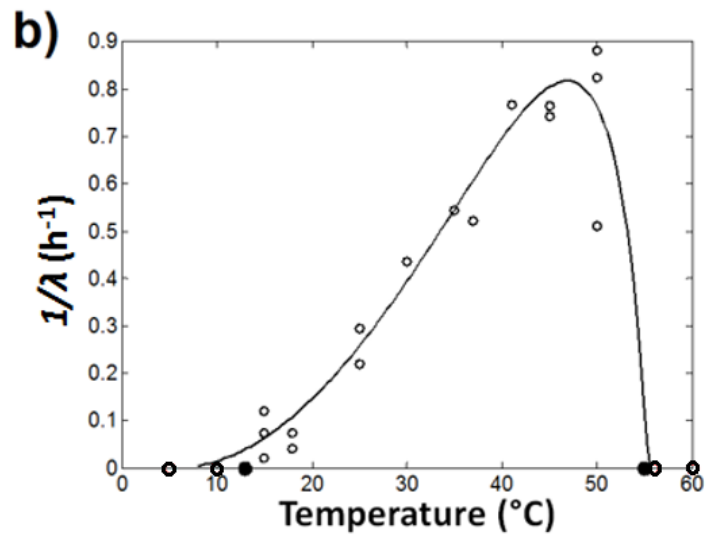
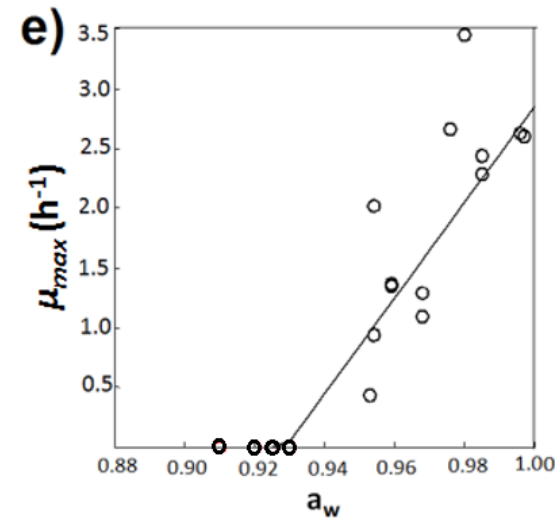
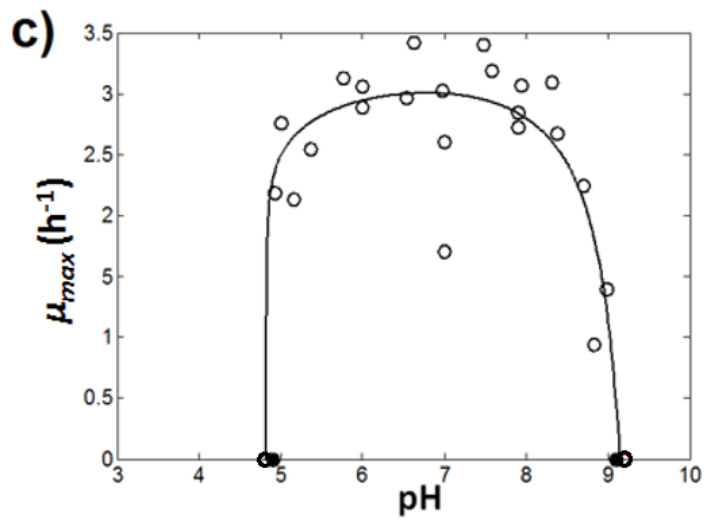
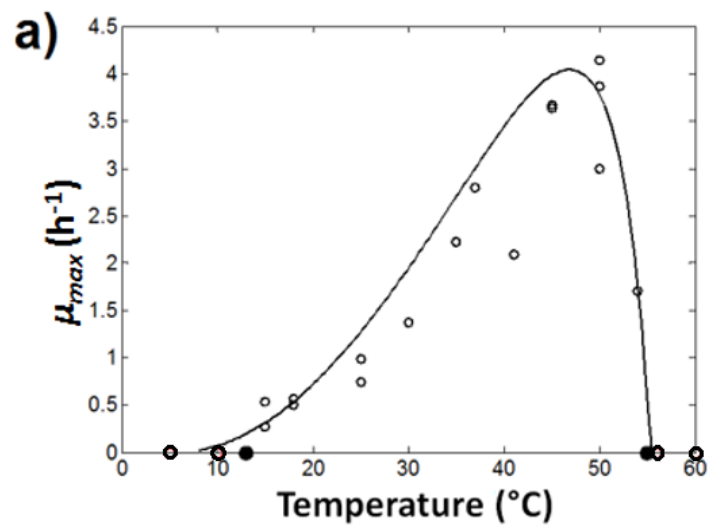
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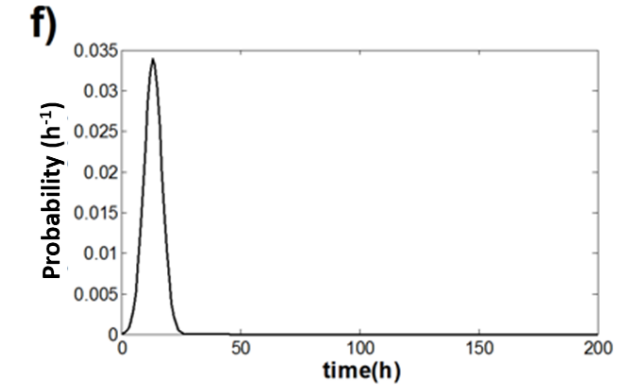
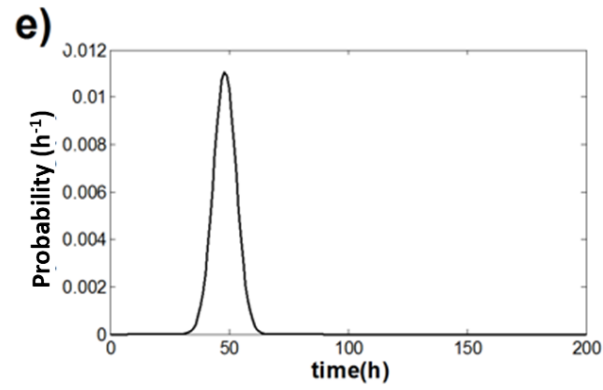
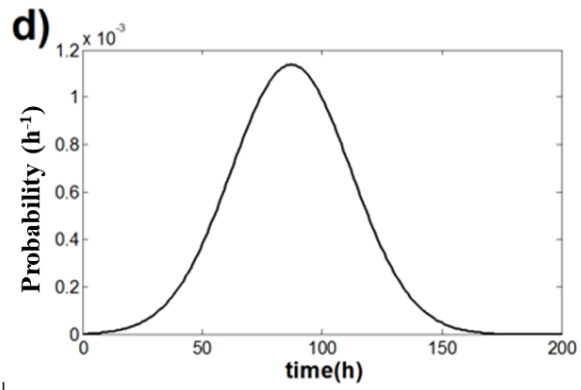
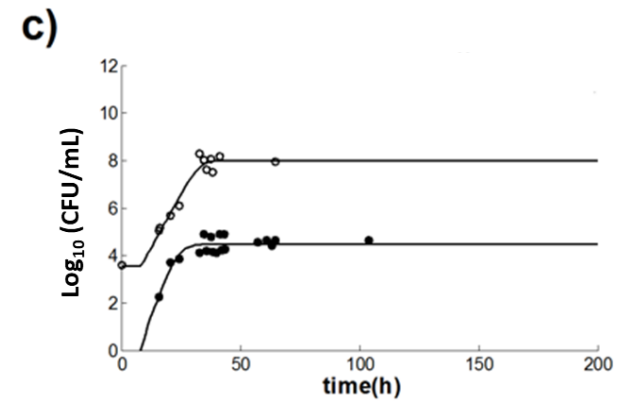
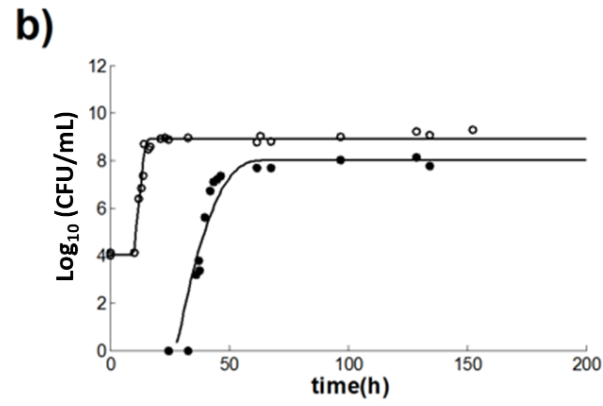
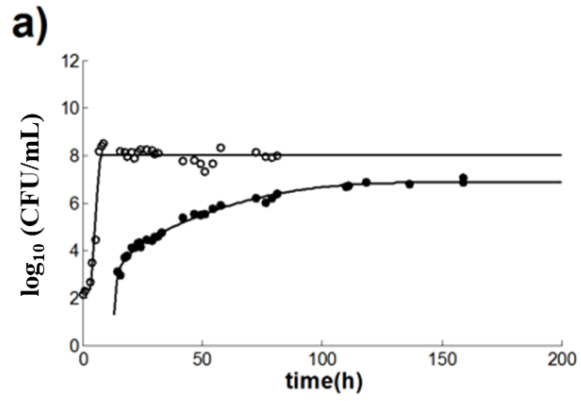
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710 Fig. S1. Growth, sporulation and probability kinetics of *B. subtilis* BSB1 at various
711 temperature, pH and water activity.

712 Table S1. Growth, sporulation parameters estimated for kinetics obtained at static conditions
713 of temperature, pH and water activity and with *B. subtilis* BSB1.

714





Time (h)

