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

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Abstract

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Biological material

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

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

2.2.1. Assessment of growth boundaries

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

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

2.2.2. Effects of temperature, pH and a_w on growth

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

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

2.3.1. Evaluation of sporulation boundaries

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

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

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

2.4. Primary and secondary modelling

2.4.1. Kinetic model of growth and sporulation

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. Results

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

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

The impacts of the three factors (T, pH and a_w) on the lag before growth could be correctly described by the model with a mean relative error of 27% for the 47 experiments. The maximum concentration of total cells N_{max} could be considered as a constant with a mean 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 synchroneously 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 (σ= 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 10^4 CFU/mL. On the other

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

3.2.2. Effects of environmental factors on sporulation kinetics

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

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

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

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

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

gave a good prediction of the time to see the first spores with a mean relative error of 23.9 %. The maximum concentration of spores (log_{10} (CFU/mL)) could be predicted with a mean relative error of 17.8%.

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 to10°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

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

4. Discussion

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

Predictive microbiology has proven its efficiency to predict the bacterial growth rate according to environmental factors by using a cardinal model. This model uses the cardinal values of growth i.e. the minimum, optimum and maximum values of environmental factors for which bacterial growth is possible. As a relationship could be established between the growth rate and the latency before growth, the cardinal model could be used to predict both these growth parameters. On the contrary, the environmental condition tested did not have a significant impact on the maximum concentration of total cells. Consequently, the maximum bacterial concentration could be considered as a constant of 1.04×10^9 UFC/mL in the culture 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

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

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

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659

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

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

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

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

- : not applicable.

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 (○) 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 ⭘) and the conditions in which growth was observed in the Growth/No Growth experiments
675 are indicated by black filled circles (●). 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 (o) and heat resistant spores
683 (●) 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 (⭘) and of heat resistant spores obtained in static conditions
698 (●) 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 (□) 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.

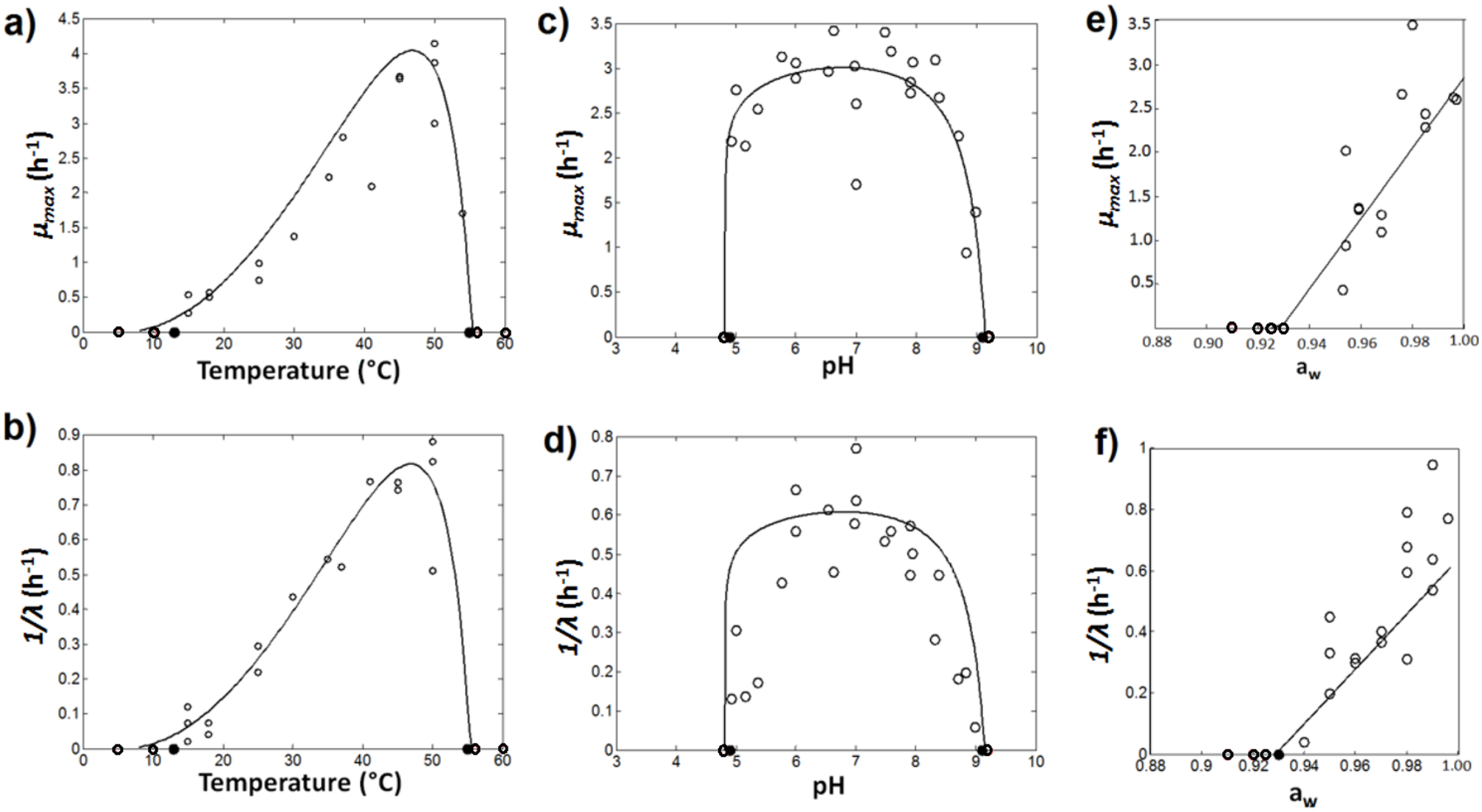
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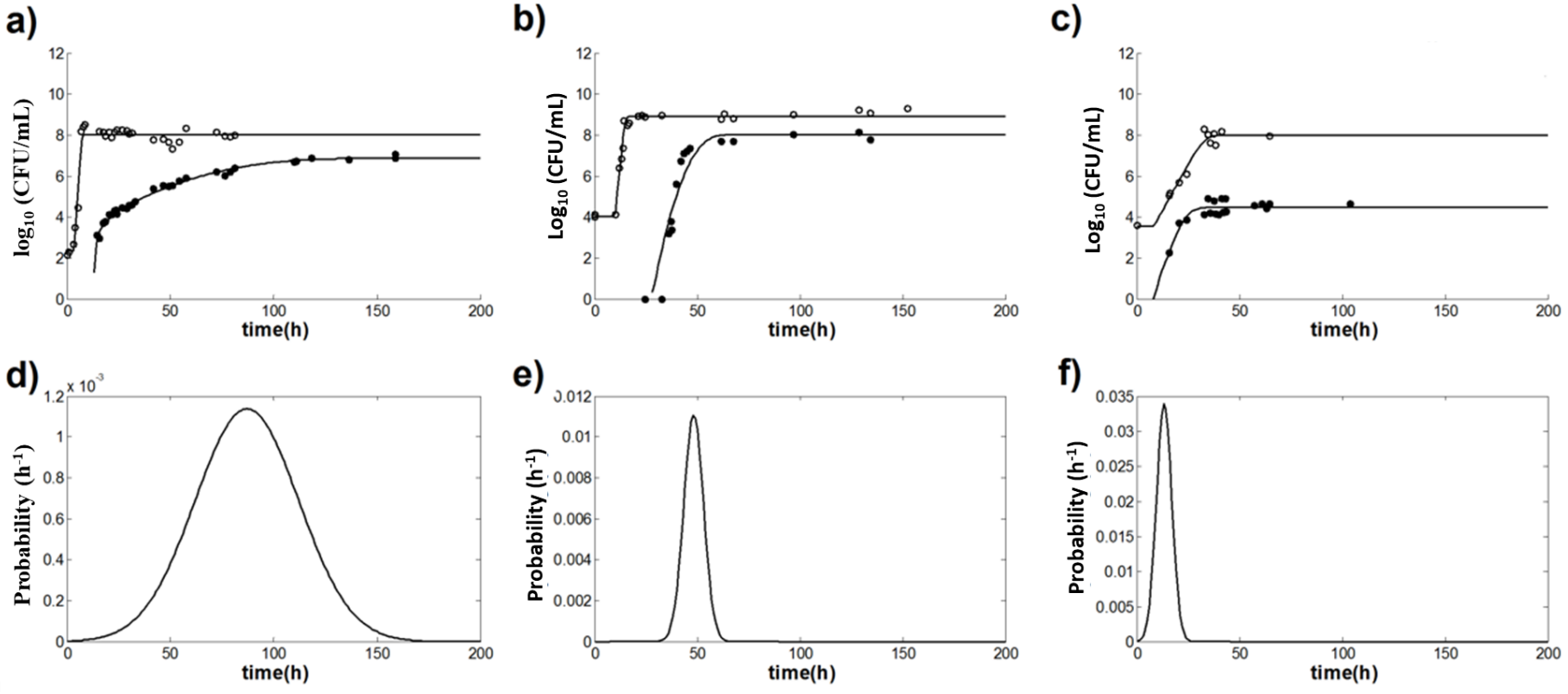
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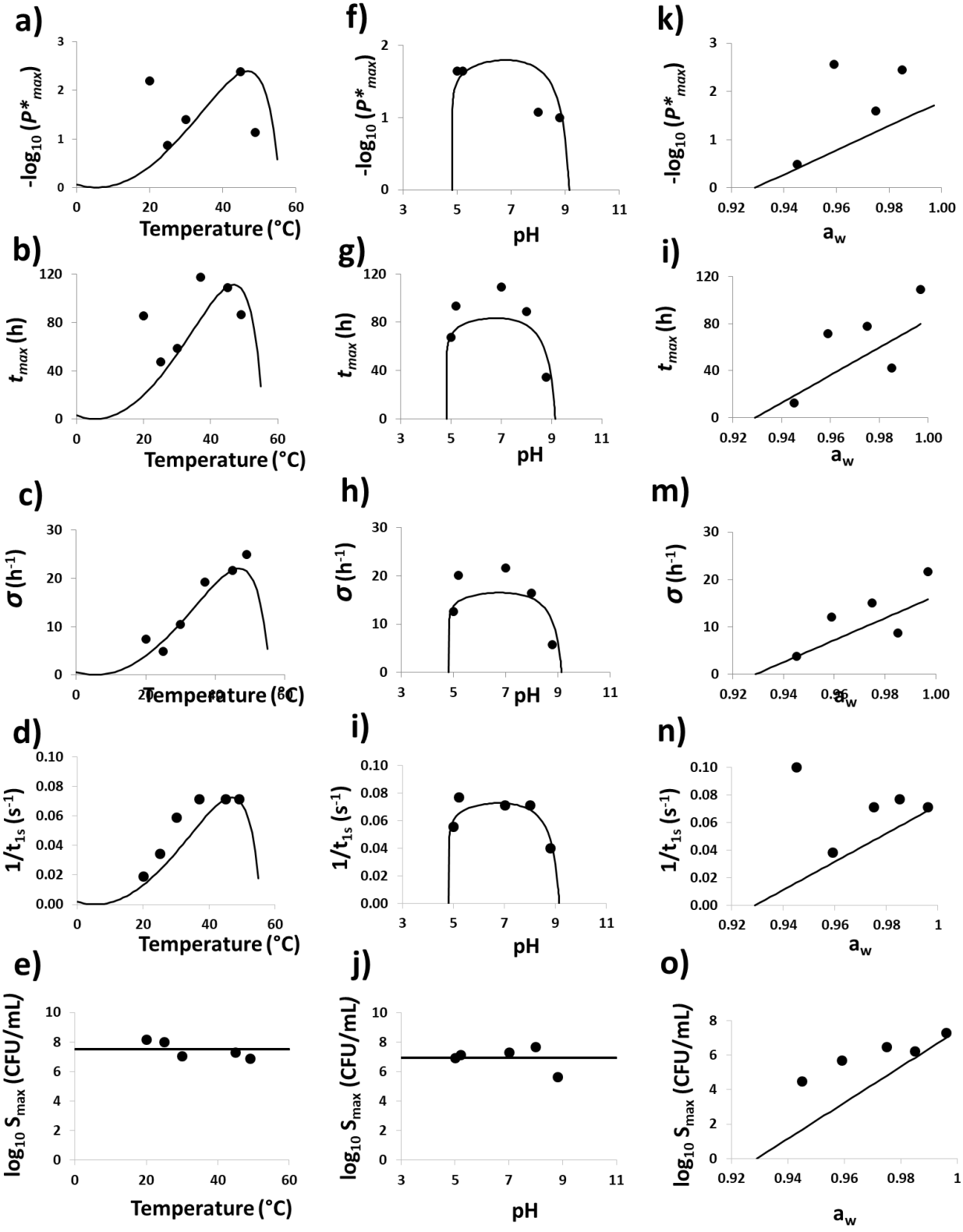
710 Fig. S1. Growth, sporulation and probability kinetics of *B. subtilis* BSB1 at various
711 temperature, pH and water activity.

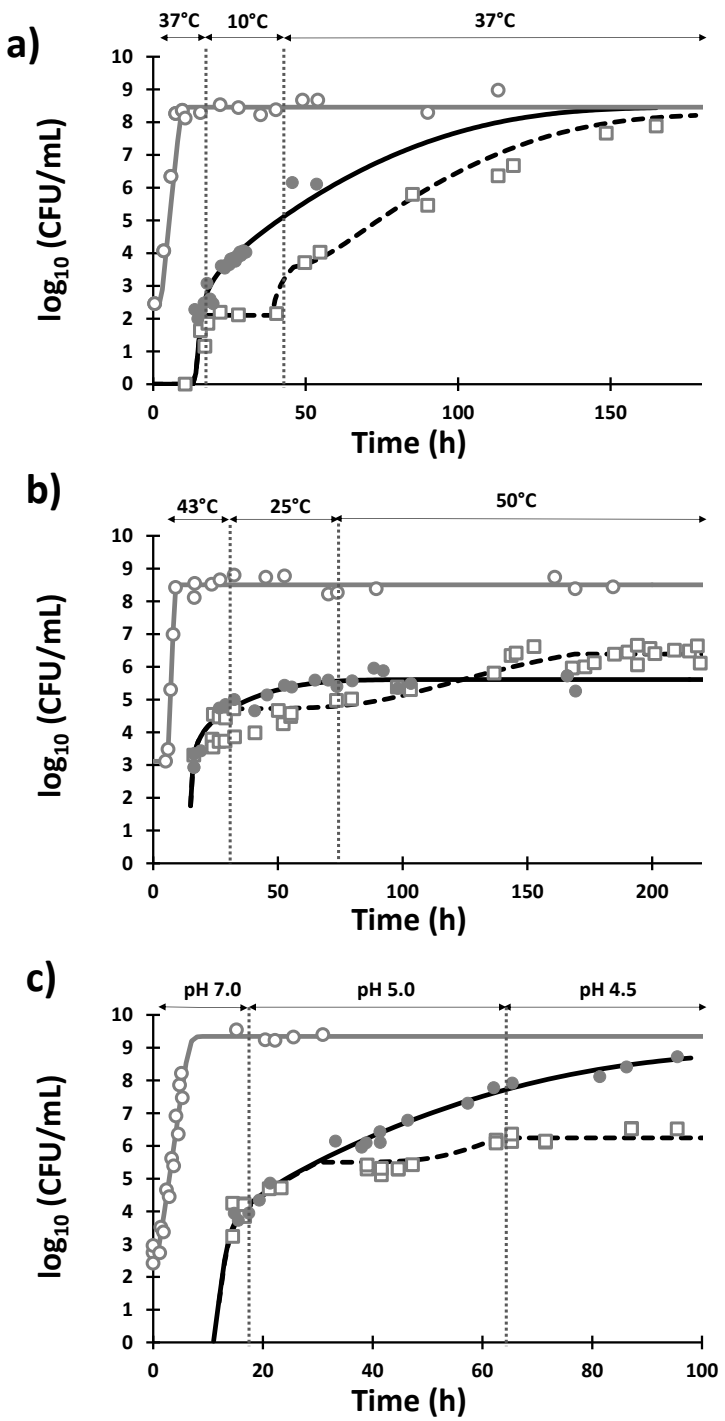
712 Table S1. Growth, sporulation parameters estimated for kinetics obtained at static conditions
713 of temperature, pH and water activity and with *B. subtilis* BSB1.

714


a)
μ_{max} (h^{-1})
Temperature (°C)
b)
1/λ (h^{-1})
Temperature (°C)
c)
μ_{max} (h^{-1})
pH
d)
1/λ (h^{-1})
pH
e)
μ_{max} (h^{-1})
a_w
f)
1/λ (h^{-1})
a_w


a)
b)
c)
d)
e)
f)
log10 (CFU/mL)
Log10 (CFU/mL)
Probability (h-1)
x 10-3
time(h)
Time (h)


a)
$-\log_{10}(P^*_{max})$
Temperature (°C)
f)
$-\log_{10}(P^*_{max})$
pH
k)
$-\log_{10}(P^*_{max})$
a_w
b)
t_{max} (h)
Temperature (°C)
g)
t_{max} (h)
pH
i)
t_{max} (h)
a_w
c)
σ (h^{-1})
Temperature (°C)
h)
σ (h^{-1})
pH
m)
σ (h^{-1})
a_w
d)
$1/t_{1s}$ (s^{-1})
Temperature (°C)
i)
$1/t_{1s}$ (s^{-1})
pH
n)
$1/t_{1s}$ (s^{-1})
a_w
e)
$\log_{10} S_{max}$ (CFU/mL)
Temperature (°C)
j)
$\log_{10} S_{max}$ (CFU/mL)
pH
o)
$\log_{10} S_{max}$ (CFU/mL)
a_w


a)
37°C
10°C
37°C
log10 (CFU/mL)
Time (h)
b)
43°C
25°C
50°C
log10 (CFU/mL)
Time (h)
c)
pH 7.0
pH 5.0
pH 4.5
log10 (CFU/mL)
Time (h)