Impact of strain, pH and ethanol concentration on ethanol activation method of *Bacillus* spores using a single spore approach

Type of article: Original research papers

Abstract:

Aims: This work aims to determine the ideal conditions for ethanol activation of spores during their enumeration and compare to thermal activation which is the reference method.
Place and duration: Department of Microbiology of the University of Yaoundé I between May2016 and June 2018.
Methodology: *Bacillus cereus* and *Bacillus subtilis* spores were activated according to an experimental central composite design with three-factor. The factors considered were exposure time, ethanol concentration, pH and activation temperature according to the types of activation. Germination yield was carried out by individually monitoring each spore of a population on solid medium in order to determine the population germination kinetic parameters (time and rate of colony appearance within a population during germination) and germination yields. These parameters were compared with those obtained after thermal activation known as a reference method.
Results: The factors strain and pH, significantly influenced the rate of spore germination within the population after ethanol activation. In the case of thermal activation, the specie and activation temperature were the most influential factors. The best germination yields were obtained for alcoholic activation of spores at 30% ethanol for 60min exposure at pH7, while for thermal activation the best yields varied from one strain to another depending on the activation conditions.
Conclusion: Ethanol activation can be considered as a good substitute of thermal activation during spore enumeration provided activation conditions are well controlled. This is in our opinion the first detailed study comparing ethanol activation to heat activation of *Bacillus* spores. It will impact future revisions of spore enumeration protocols proposed by norms that take into consideration spore activation and reduce bias in spore enumeration.

Keys words : *Bacillus* spore, activation, heat, ethanol, germination, distribution

1-Introduction

Bacteria are considered to be one of the main contaminants in food industries and bacterial spores are known to be one of the most resistant forms of living organisms. Due to their resistance to commonly used treatments in the fight against microorganisms [1; 2], they are able to germinate and produce vegetative cells that cause concerns, ranging from sensory modification of food to food borne illnesses that can be so severe to make them a real public health problem [3,4,5]. The costs endured due to food spoilage and food diseases amount in millions of dollars [6]. Researching spores in food is a real challenge to industries particularly in the context of predicting the microbiological stability of foods. Many works and food microbiology standards recommend thermal activation in the protocols for spores enumeration in food [7]. In general, this method leads to an underestimation of the spore load as a result of the outcome of two main phenomenon: a heat sensitive fraction that is killed during heat activation and a pronounced delay of spores germination over the time dedicated to analysis [8]. Germination delay may be due to heat-induced dormancy and heat induced resistance of spores which are concepts firstly introduced in literature by [9] and [10]. All this can induce an unreliable prediction of the food stability. In this regards, ethanolic activation is proposed as an alternative [11,12, 13]. Indeed, exposure of spores to ethanol improves germination yields with respect to heat exposure and bacterial spore rate of germination [12, 13]. Its relatively low cost and easy implementation makes it an ideal method. There is no evidence of resistance or "alcohol-induced" dormancy up to date. Some species of *Bacillus* spore-formers like *Bacillus coagulans* and *B*acillus *subtilis*, are used as probiotic for human and animal consumption [14, 15, 16, 17]. In this way ethanolic activation could be very important in the acceleration of certain food biotechnological processes using spore-forming bacteria. Moreover, according to [18], spore germination-induction can enhance decontamination

52 effort in biodefence scenario, food manufacturing and hospital environment. This germination-
53 induction may be more enhanced using ethanol activation than thermal activation in our opinion.
54 However, proportions of alcohol conditions for optimal activation of the spores vary depending on the
55 authors, 50% for [11], 60% for [12] and 70% for [19]. In addition to that, little data exists on the ideal
56 conditions for an ethanol activation of spores. The objective of this work is therefore to evaluate the
57 effect of ethanol activation conditions of spores in reference to thermal activation recommended by the
58 Food Microbiology standards.

59

60 **2-MATERIAL AND METHODS**

61 **2-1-Strain and culture media:**

62 *Bacillus cereus* ATC11966 and *Bacillus subtilis* DMS210 were used in this work. Nutrient Broth
63 (Oxoid, Basingstoke, UK) was used for the propagation of the species. Regarding sporulation media,
64 1L of sporulating agar medium was prepared by dissolving, 5 g of peptone, 5 g of sodium chloride, 1 g
65 of meat extract, 2 g of yeast extract, 15 g of agar, 0.5 g of dissodic phosphate, 0,1g of calcium
66 chloride, 0.04g of manganese sulphate and the pH adjusted to 7±0.2 before sterilization at 121°C for
67 15 min [20]. The medium was poured into sterile Petri dishes. Nutrient agar (oxoid, Basingstoke, UK)
68 was used for spore germination.

69 **2-2-Spore production:**

70 Strains previously stored at -80°C were propagated in nutrient broth (oxoid, Basingstoke, UK) at 37°C
71 for 24h three times before been sowed in the sporulating medium. The incubation was done at 37°C
72 for 7 days, spores were harvested and washed as describe by [21]. Spores obtained were suspended
73 in sterile distilled water and stored at -18°C for one month before use and considered as stock
74 solution. These spores were more than 99,99% free of growing or sporulating cells and germinating
75 spores as assessed through An "Ivymen System" optic microscope equipped with a phase-contrast
76 device that was used to observe spores at a x100 objective

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79 **2-3-Spore activation:**

80 A preliminary test performed by counting spores using a Malassez cell on microscope and plating
81 method gave a correlation of 0.99 between the two methods. Microscope count was then use in the
82 rest of the work. Decimal dilutions permitting to have about 10^3 spores/ml in test tubes were
83 performed. Spores contained in the selected dilution were heat activated, modulating pH, time and
84 temperature of exposure, and ethanol activated at different pH, ethanol concentration and time of
85 exposure following an experimental Central Composite Design (CCD) with three factor and five levels
86 (Table 1). For ethanol activation, ethanol and spores suspensions at a final volume of 10ml were
87 mixed in a sterile 250ml beaker at different proportion of ethanol (30 to 70%) adjusted at different pH
88 (5-9) with NaOH or HCl. The mixtures were shaken using an IKA Hs 260 shaker bath at 70tr/min for
89 different durations (40 to 80min) indicated by the CCD plane (Table1) ethanol activation was stopped
90 by immediate dilution as described by[8]. For thermal activation, the selected stock solution dilutions
91 adjusted at different pH (5 to 9) were introduced in a water bath and the tubes maintained for different
92 durations (2-18min) at different temperatures (70-90°C) according to the CCD design (Table1).

93 **Table1: real values of variables tested of CCD design for thermal and alcohol activation of**
94 **spores**

Activation	Variables	Symbol coded	Range and levels of variables				
			2	1	0	-1	-2
Thermal	pH	pH	9	8	7	6	5

	Time (min)	t	18	15	10	5	2
	Température	T	90	85	80	75	70
alcohol	pH	pH	9	8	7	6	5
	Time (min)	t	80	70	60	50	40
	Ethanol (%)	a	70	60	50	40	30

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97 2-4-Spore Germination and Outgrowth Assessment:

98 2-4-1-Theoretical background

99 According to [22] the difference in the time taken by two bacteria with the same growth rate and initial
100 concentration to reach a given load is proportional to the difference between their latent phase times.
101 This reflects in the case of cells from the same strain a difference in their history or the previous
102 environment in which they originate. The study hence verified in a preliminary experiment, that
103 vegetative cells from the same bacterial strain have the same growth rate as their respective spores
104 during their outgrowth (Figure 1). This permitted us to deduce that the difference in time taken by two
105 spore cells to form a visible colony (about 10^6 cells) is proportional to their germination time (Figure 1).
106 During spore counting, the time of appearance of visible colonies on the agar surface is hence
107 proportional to their germination time. Thus the kinetics of single spore appearance within a population
108 can be obtained by the cumulative form of the time to colonies appearance distribution. Thereby the
109 activated spores were counted after their outgrowth and cumulatively expressed in terms of colonies
110 appearing in function of time as earlier demonstrated in our previous work [13]. In this regards the time
111 to first colony appearance is used, in place of the single spore germination time due to the protocol
112 used.

113

114 2-4-2-Protocol

115 Spores inoculated on nutrient agar medium were incubated at 37°C for 24h (time assuring 99.999%
116 of spore germination in nutrient agar obtained after preliminary assessments). The number of colonies
117 from spore outgrowth observed at the end of incubation was considered as the maximum number of
118 spores that could germinate and grow. In order to reduce the probability of more than one spore
119 forming the same colony after outgrowth, an average of 300 spores were inoculated per Petri dish.
120 Colonies were considered as such when the diameter was around 2 mm. Random counting of cells in
121 such colonies through suspension in broth and plating indicated that their cell load was about 6±0.05
122 Log of cells/colony. During incubation, new colonies (2 mm diameter average) were counted every 30
123 minutes between 0 h and 24hours identified with a permanent marker and recorded as number of
124 colonies having the same time to outgrowth.

125 The germination yield (G_{yield}) was calculated by the following relation:

126
$$G_{yield} = \frac{(Number\ of\ spore\ outgrowth\ after\ treatment\ after\ 24h)}{(Initial\ numer\ of\ spores\ inoculated)} * 100 \quad (1)$$

127 The data obtained for each specie was analysed by multiple regression in order to obtain a model for
128 germination as function of factors selected for heat or ethanol activation. The kinetics of colonies
129 appearance as function of time were fitted into the [22] model to estimate the minimum time for spore
130 outgrowth in a population, intended as the time for the detection of the first colony within the
131 population (Plag). As earlier said, the outgrowth rate being the same for all individual spores. Plag is
132 hence proportional to the germination time. The colony appearance rate is considered in this work as
133 an indication of the population germination time homogeneity. The higher the rate the lower the time
134 difference between spores germination time in the population. The same kinetic data was
135 subsequently transformed in order to express it in terms of outgrowth ratio as function of time by using

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136 the following equation:

137 Outgrowth ratio = Nt/N0 (2)

138 where Nt and N0 are the number of outgrowth spores at time t and the number of outgrowth spore
139 after 24h respectively. Outgrowth after 24h was considered as the maximum number of spores
140 available and hence equal to the initial number N0. These transformed data were fitted to the Weibull
141 exponential equation.

142 $$S(t) - Nt/N0 = exp[-b * t^n] \quad (3)$$

143 Where N0 is the maximum number of spores that germinated after 24h, Nt the number of spores that
144 germinated at a time t, "b" and "n" are scale and shape parameters respectively to be estimated and
145 "t" the time of incubation. "b" and "n" parameters estimated were used to generate the time for a single
146 spore outgrowth (tgrowth) distribution using the Weibull pdf function

147 $$PDF = b * n * t^{n-1} exp[-b * t^n] \quad (4)$$

148 The distributions parameters (mode and mean) were calculated as describe by [23]

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150 2-5-Statistical Analysis:

151 All the experiments were conducted in triplicate and data merged together to have a more reliable and
152 consistent counts per reading time. The effects of the variables on spore population outgrowth kinetics
153 parameters (Plag and rate) values and on the distribution parameters (mode and mean) were
154 assessed through an ANCOVA analysis using statistica 12.5 of statsoft. The same software was also
155 used for fitting the experimental data to the Weibull model, the plotting of the curves were performed
156 on an excel spreadsheet.

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159 3-Results:

160 The detail result of the experimental plan (Table 1) regarding outhgrowth kinetics are presented in
161 Table 2. As a general observation, after ethanol activation, *Bacillus cereus* colonies appear quicker
162 and with a higher rate than those of *Bacillus subtilis*. However the best rate is obtained after activation
163 of *Bacillus cereus* (68.5 colonies/min) at 30% of ethanol after 60min exposure at pH7. In the case of
164 *Bacillus subtilis* the best rate (66. 2 colonies/min) is obtained at 30% of alcohol after an exposure time
165 of 80min at pH 9 (Figure 2a). The factor strain (P=0.01) and pH (P=0.00) demonstrated after and
166 ANCOVA analysis to influence the rate of colonies appearance (Fig 2b and fig 2c). Statically pH
167 (P=0.01), activation temperature (P=0.01) and strain (P=0.00) significantly affected rate after thermal
168 activation, while in the case of P.lag only strain (P=0.00) have a significant effect (Figure 2d). It can
169 hence be noted that the rate of colonies appearance (germination) obtained after ethanol activation
170 are higher than those obtained with thermal activation. Globaly, when merging the data of the two
171 activation methods together, strain (P=0.01) and activation method (P=0.00) significantly affected the
172 rate of colony appearance while they affected the time for first colony appearance with P=0.00 and
173 P=0.00 respectively.

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180 **Table 2: Spores population outgrowth parameters (rate, P.lag) of *Bacillus cereus* and *Bacillus*
181 *subtilis* after heat and ethanol activation**

Thermal activation conditions			Rate ** (colonies/min)		P.lag** (min)		Ethanol activation conditions			Rate ** (colonies/min)		P.lag ** (min)	
pH	Time (h)	Heat (°C)	*B.s**	*B.c**	*B.s*	*B.c*	Time (h)	Heat (°C)	ethanol	*B.s*	*B.c*	*B.s*	*B.c*
7	10	90	6.5	0.3	341.8	270.2	9	80	30	66.2	23.8	520.9	498.2
5	10	90	7.5	0.1	362.7	276.3	7	80	50	15.7	17.7	404.1	356.7
9	10	90	5.1	1.9	343.6	242.0	9	80	70	11.0	1.1	396.1	183.7
9	18	90	12.4	0.5	421.1	314.9	8	70	40	12.4	1.8	404.3	191.4
5	2	90	17.7	0.8	397.6	287.4	6	70	40	6.4	1.2	365.9	218.9
6	15	85	11.7	2.0	414.6	394.0	6	70	60	17.5	0.4	464.2	292.7
8	5	85	7.1	0.5	364.7	328.2	8	70	60	18.7	1.4	437.1	306.6
8	15	85	9.0	3.8	354.6	259.3	7	60	30	17.4	68.5	423.9	386.2
6	5	85	11.1	0.3	371.5	226.9	5	60	30	6.8	0.7	421.6	256.7
7	18	80	2.6	1.3	346.6	258.7	9	60	30	5.9	0.6	358.4	210.2
7	2	80	6.5	11.9	352.4	427.4	7	60	50	18.9	23.5	392.3	389.3
7	10	80	14.8	5.2	466.9	326.1	7	60	50	6.7	59.6	331.4	439.4
7	10	80	10.4	2.1	414.0	299.0	7	60	50	7.1	23.9	430.8	409.7
7	10	80	6.2	1.1	421.3	263.0	9	60	50	24.4	5.4	431.8	344.7
9	10	80	5.5	0.6	407.2	274.6	5	60	50	18.4	4.4	449.9	348.5
5	10	80	4.3	0.5	355.0	248.5	7	60	70	16.5	41.1	427.4	450.8
8	15	75	5.5	0.2	347.9	226.8	5	60	70	21.7	0.3	462.5	348.6
6	5	75	7.6	0.1	350.9	269.4	9	60	70	16.4	1.4	433.1	386.4
6	15	75	13.4	1.1	391.2	342.7	6	50	40	7.1	4.3	373.1	415.7
8	5	75	57.0	0.2	413.8	292.6	8	50	40	28.5	2.3	445.6	350.8
7	10	70	6.9	0.8	375.0	263.3	8	50	60	9.2	6.7	409.4	356.3
5	10	70	10.3	1.8	363.9	318.7	6	50	60	7.3	0.2	362.1	296.0
9	10	70	5.2	0.9	336.1	270.5	5	40	30	25.8	6.7	426.1	356.3
9	18	70	5.1	2.9	351.5	343.7	7	40	50	10.3	2.7	367.7	364.1
5	2	70	7.0	2.4	354.6	305.0	5	40	70	12.9	0.2	434.1	296.0

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182 *B.s=*B*acillus *subtilis* and B.c=*B*acillus *cereus*); **Fitting Baranyi and Roberts (1994) model to the data
183 gave and R^2 >0.99 in general and permitted the estimation of Rate and Plag . in general, parameters
184 estimated were all statistically significant with p<0.05 and standard error of estimation lower than 10%
185 in general
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189 In order to best assess spore population behavior during germination and outgrowth, the distribution of
190 the time to single spore colony appearance where obtained through the Weibull PDF function. The
191 detailed data are presented in Table3 and table 4. Globally, the mode and mean time to colony
192 appearance of ethanol activated spores of *Bacillus subtilis* were higher than those of *Bacillus cereus*
193 (Table3 and Figure 3a), indicating that *Bacillus subtilis* spores germinated quicker than *Bacillus cereus*
194 spores. Outgrowth distribution mode after ethanol activation was only influenced by strain (*P*=0.03)
195 while pH (*P*=0.04) affected the mean colony appearance time. Regarding the spores time to colony
196 appearance distribution mode and mean after thermal activation (table4), in absolute terms we can
197 observe a higher value of mode and mean for *Bacillus subtilis* in comparison to *Bacillus cereus* (
198 Figure 3b). The strain was the main factors which affected the mode (*P=0.02*) and mean (*P*=0.01)
199 after thermal activation. It was generally observed that the mode *Bacillus subtilis* after ethanol
200 activation were lower than those obtained after thermal activation (Figure 3c). In the case of *Bacillus*
201 *cereus* spores it was at the contrary thermal activation that induced lower values of the time
202 distribution mode (Figure 3d). The analysis of independent factors (pH) and other factor like strain and
203 activations methods (data not shown) shows that the mode is significantly affected by strain (*P*=0,01)
204 and the activation pH (*P*=0,03). On other hand the distribution mean is only influenced by strain
205 (*P*=0.01).

206 **Table 3: Distribution parameters (mode, mean) for *Bacillus cereus* and *Bacillus subtilis* after**
207 **ethanol activation**

Activation condition			*Bacillus subtilis*			*Bacillus cereus*		
pH	Time (min)	Ethanol (%)	Sample code*	Mode**(h)	Mean**(h)	sample code*	Mode** (h)	Mean** (h)
9	80	30	**S-9-80-30**	7.0	7.4	**C-9-80-30**	7.1	7.2
7	80	50	**S-7-80-50**	5.9	6.1	**C-7-80-50**	5.2	5.4
9	80	70	**S-9-80-70**	6.0	6.3	**C-9-80-70**	4.9	6.7
8	70	40	**S-8-70-40**	7.2	8.5	**C-8-70-40**	4.3	5.0
6	70	40	**S-6-70-40**	6.8	7.4	**C-6-70-40**	5.2	7.7
6	70	60	**S-6-70-60**	6.7	6.9	**C-6-70-60**	8.2	11.8
8	70	60	**S-8-70-60**	6.8	7.3	**C-8-70-60**	5.4	5.8
7	60	30	**S-7-60-30**	7.0	7.7	**C-7-60-30**	5.6	5.8
5	60	30	**S-5-60-30**	6.3	6.6	**C-5-60-30**	6.3	10.7
9	60	30	**S-9-60-30**	6.7	7.5	**C-9-60-30**	5.1	6.7
7	60	50	**S-7-60-50**	6.5	6.9	**C-7-60-50**	5.6	5.7
7	60	50	**S-7-60-50**	6.5	7.4	**C-7-60-50**	6.3	6.5
7	60	50	**S-7-60-50**	6.5	6.9	**C-7-60-50**	5.7	6.2
9	60	50	**S-9-60-50**	6.4	6.5	**C-9-60-50**	6.1	6.8
5	60	50	**S-5-60-50**	6.7	6.8	**C-5-60-50**	5.9	6.9
7	60	70	**S-7-60-70**	6.6	7.0	**C-7-60-70**	5.6	6.2
5	60	70	**S-5-60-70**	6.9	7.3	**C-5-60-70**	7.8	10.9
9	60	70	**S-9-60-70**	6.8	7.4	**C-9-60-70**	7.2	8.7
6	50	40	**S-6-50-40**	6.4	6.8	**C-6-50-40**	6.2	6.4
8	50	40	**S-8-50-40**	6.6	6.8	**C-8-50-40**	6.8	8.5
8	50	60	**S-8-50-60**	6.6	6.9	**C-8-50-60**	4.9	5.5
6	50	60	**S-6-50-60**	6.5	7.2	**C-6-50-60**	6.2	6.7
5	40	30	**S-5-40-30**	6.5	6.9	**C-5-40-30**	8.4	12.3
7	40	50	**S-7-40-50**	6.6	7.2	C-7-40-50	6.2	6.7

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5	40	70	**S-5-40-70**	6.8	7.1	C-5-40-70	8.4	12.3

208 ***sample code**: strain (S=*B*acillus *subtilis*; C=*B*acillus*cereus*)-pH-time-alcohol percentage; ****Fitting of
209 S(t) = exp(-bt^n) to the data gave and R^2 >0.98 in general, parameters estimated were all statistically
210 significant with p<0.05 and standard error of estimation lower than 10% in general
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214 **Table4: Distribution parameters (mode, mean) of *Bacillus cereus* and *Bacillus subtilis* after**
215 **thermal activation**

Activation condition			**Distribution parameters of *Bacillus subtilis***			**Distribution parameters of *Bacillus cereus***		
pH	**Time (min)**	**heat (°C)**	**sample code ***	**Mode** (h)**	**Mean ** (h)**	**Sample code ***	**Mode** (h)**	**Mean** (h)**
7	10	90	S-7-10-90	6.4	7.1	C-7-10-90	7.2	11.8
5	10	90	S-5-10-90	6.1	6.6	C-5-10-90	8.1	12.4
9	10	90	S-9-10-90	6.5	6.9	C-9-10-90	5.7	9.4
9	18	90	S-9-18-90	6.4	6.8	C-9-18-90	5.9	6.8
5	2	90	S-5-2-90	6.0	6.3	C-5-2-90	5.8	7.2
6	15	85	S-6-15-85	6.3	6.7	C-6-15-85	6.8	8.8
8	5	85	S-8-5-85	6.4	6.9	C-8-5-85	7.4	10.6
8	15	85	S-8-15-85	6.3	6.8	C-8-15-85	5.4	6.5
6	5	85	S-6-5-85	6.2	6.7	C-6-5-85	7.4	11.8
7	18	80	S-7-18-80	6.4	6.8	C-7-18-80	5.8	8.2
7	2	80	S-7-2-80	6.9	8.1	C-7-2-80	5.9	6.4
7	10	80	S-7-10-80	7.5	8.3	C-7-10-80	5.9	6.8
7	10	80	S-7-10-80	7.3	8.7	C-7-10-80	7.5	11.5
7	10	80	S-7-10-80	7.4	8.7	C-7-10-80	5.4	6.2
9	10	80	S-9-10-80	7.0	7.8	C-9-10-80	5.7	7.2
5	10	80	S-5-10-80	6.6	7.2	C-5-10-80	5.6	8.1
8	15	75	S-8-15-75	5.9	6.5	C-8-15-75	6.1	11.1
6	5	75	S-6-5-75	6.6	7.4	C-6-5-75	6.5	10.9
6	15	75	S-6-15-75	6.2	6.7	C-6-15-75	5.2	5.5
8	5	75	S-8-5-75	6.0	6.2	C-8-5-75	5.7	6.7
7	10	70	S-7-10-70	6.5	7.1	C-7-10-70	5.4	6.2
5	10	70	S-5-10-70	6.6	7.3	C-5-10-70	5.6	6.2
9	10	70	S-9-10-70	6.5	7.1	C-9-10-70	5.9	8.1
9	18	70	S-9-18-70	6.8	7.7	C-9-18-70	6.6	8.6
5	2	70	S-5-2-70	6.5	7.0	C-5-2-70	5.9	7.1

216 ***sample code**: strain (S=*B*acillus *subtilis*; C=*B*acillus *cereus*)-pH-time-alcohol percentage; ****Fitting of
217 S(t) = exp(-bt^n) to the data gave and R^2 >0.98 parameters estimated were all statistically significant
218 with p<0.05 and standard error of estimation lower than 10% in general
219
220

221 The germination yields of *Bacillus cereus (B. cereus)* and *Bacillus subtilis (B. subtilis)* spores obtained
222 after thermal and ethanol activation are presented in table 5. In the case of ethanol activation, we
223 generally observed for the two strain that the germination yield was increased when the percentage of
224 ethanol exposure decreased. For *Bacillus cereus* the lowest yield (16.3%) is obtained at 70% after
225 60min of activation at pH5, while for *Bacillus subtilis* the lower yield (21.9%) is obtained at 70% after
226 80min of exposure at pH9. On the other hand the best germination yield for the two strains is also
227 obtained at the same percentage of ethanol (30%) after 60min of exposure at pH7. Regarding thermal
228 activation, we can say that germination yield of *Bacillus subtilis* are higher than those of *Bacillus*
229 *cereus* (Table 5). We can also observe that the increase of exposure time generally reduce de
230 germination yields. The best germination yield (80.0%) for *Bacillus cereus* and (87.7%) for *Bacillus*

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231 *subtilis* are obtained when spores are exposed at 80°C for 10 min at pH7. The lower value of yield
232 (8.2%) for *Bacillus cereus* is obtained at temperature of 90°C after 18 min of activation at pH9. For
233 *Bacillus subtilis* (16.1%) is obtained after activation at 80°C, during 18min at pH7.

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238 **Table 5: Experimental conditions for assessing outgrowth colony appearance kinetics and**
239 **germination yield of *Bacillus cereus* and *Bacillus subtilis* spores after ethanol and thermal**
240 **activation. Only germination yields results are presented here.**

Thermal conditions of activation			Germination yield (%)		Ethanol conditions activation			Germination yield (%)	
heat	time	pH	*B.cereus*	*B.subtilis*	alcohol	time	pH	*B.cereus*	*B.subtilis*
90	10	7	26.2	53.2	70	80	9	26.4	21.9
90	10	5	18.6	32.0	70	60	7	30.2	40.5
90	10	9	49.2	37.2	70	60	5	16.3	25.3
90	18	9	8.2	28.1	70	60	9	33.9	27.6
90	2	5	20.7	31.8	70	40	5	38.6	33.6
85	15	6	35.0	25.2	60	70	6	23.4	31.9
85	5	8	34.2	39.8	60	70	8	39.4	41.1
85	15	8	34.4	52.8	60	50	8	28.5	30.9
85	5	6	45.4	48.2	60	50	6	24.0	45.1
80	18	7	48.1	16.1	50	80	7	49.0	40.5
80	2	7	21.9	63.3	50	60	7	66.7	67.0
80	10	7	78.7	70.8	50	60	7	69.8	62.7
80	10	7	80.0	83.0	50	60	7	71.7	63.1
80	10	7	82.0	87.7	50	60	9	58.2	40.2
80	10	9	31.5	35.1	50	60	5	32.2	29.5
80	10	5	27.5	32.9	50	40	7	48.9	52.1
75	15	8	55.8	24.6	40	50	6	26.1	48.2
75	5	6	48.7	64.9	40	50	8	37.9	43.7
75	15	6	39.6	39.4	40	70	8	44.5	47.3
75	5	8	45.7	71.2	40	70	6	30.7	42.3
70	10	7	29.2	42.2	30	80	9	70.8	75.9
70	10	5	28.2	72.9	30	40	5	48.6	51.4
70	10	9	36.7	58.2	30	60	5	35.6	30.0
70	18	9	39.3	19.7	30	60	9	21.3	62.7
70	2	5	40.4	48.8	30	60	7	89.0	90.8

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242 A multiple regression analysis was used to analyze the data of germination yields and polynomials
243 equations were derived. Equations 5 and 6 describe the influence of pH, alcohol concentration (a) and
244 time of exposure (t) on the germination percentage yield (G_{yield}) during ethanol activation of *Bacillus*
245 *subtilis* and *Bacillus cereus* spores respectively. Their response surface representation are presented
246 in fig 3A and 3B respectively

247 $$G_{Yield} = Exp\left(3.077 + 0.014 * a * pH + 0.025 * t * pH - 0.00013t * pH * a - 0.0012t^2 - 0.0006a^2 - 0.13pH^2\right)$$
248 (5)

249 $$G_{Yield} = Exp\left(1.15pH + 0.016t * pH - 0.000041t * pH * a - 0.138t^2 - 0.00087pH^2\right) \quad (6)$$

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Moreover, Equations 7 and 8 describe the influence of pH, temperature of activation (T) and time of activation (t) on the germination percentage yield (G_{yield}) of *Bacillus subtilis* and *Bacillus cereus* respectively

$$G_{Yield} = Exp\left(-767.17 + 777.23\sqrt{pH} - 14.24\sqrt{pH*T} + 3.33\sqrt{t*pH*T} - 15.25t - 140.5pH\right) \quad (7)$$

$$G_{Yield} = Exp\left(-31.36 + 0.9T + 0.030t*pH - 0.01t^2 - 0.006T^2 - 0.091pH^2\right) \quad (8)$$

The variability of data explained by the models were comprised between 54 and 84%. According to the models and response surfaces at fixed pH=7 presented in figure 4C and 4D, we can observe for *Bacillus cereus* that at constant value of pH (7) germination increases with time of exposure up to a maximum that depends on ethanol percentage. As ethanol percentage increases, time of exposure for maximum germination percentage decreases (Figure 4B). The yields of *Bacillus subtilis* germination increase with time exposure. This increase of yield is more important at low percentages of ethanol .The germination yield is higher around 60min of exposure to 30% of ethanol (Figure 4A). In the case of thermal activation, the yield of germination of *Bacillus cereus* spores increase with time up to 10min, the optimum yield is obtained at 80°C. Beyond this time germination decrease independently of the activation temperature (Figure 4D). Regarding *Bacillus subtilis* spores, increasing time of exposure over 12 min irrespective of the temperature reduce the germination yield. Decreasing temperature with low time of exposure decreases germination yield (Figure 4C).

4-Discussion:

Variability is known as a fundamental property of microbial population, a quantitative representation of this variability is a key aspect in explaining the outcome in many practical applications such as enumeration of bacterial spores in food. Heterogeneity of spore population have a great role on variability of spore germination. Some factor like activation method (heat or chemical), temperature, duration of activation are known to have an effect on the variability of spore germination lag time [24, 25, 26].We noted during this work, that the necessary time for the appearance of the first colony (P.lag) were based on the type of activation and the bacterial strain. Inherently *Bacillus cereus* has a generation time and a shorter lag time than that of *Bacillus subtilis* [27] this explains the significant effect of strain on the first colony appearance time. The difference in sensitivity of the two strains to treatment as observed by [28] explains the significant effect of the type of activation. In the case of colony appearance rates, it was generally observed that the speeds are higher after ethanol activation compared to thermal activation. It is important to observe that increase in rate of colony appearance from spore is an indication of a lower dispersion in single spore germination times within the population. In this regards, any factor reducing this rate, impacts the spore sensitivity by increasing individual spore dormancy. The heat acts on the DNA of the spores causing significant damage, therefore during germination damage repair times are very important [29, 30] compared to spores activated by ethanol which explains why the colony appearance speeds are higher after ethanol activation. A significant effect of pH and the bacterial strain was also observed on rate after ethanol activation. The difference in sensitivity and the intrinsic properties of the strains account for the significant effect of the bacterial strain on the rate of colony appearance. The denaturation of proteins is enhanced in acid and alkaline media [30, 31]. The pH values close to neutrality appear to be ideal for optimal activation of the spores.

The observation of spore germination dynamics in this work allowed us to estimate the outgrowth time with the highest frequency (mode) and the average time to appearance of outgrowth colonies within a spore population. In general the time to appearance distribution mode is about 5h in *Bacillus cereus* whereas in *Bacillus subtilis* it is 6h. This difference is due to intrinsic properties and strain sensitivity which varies according to the treatment [28] Regarding the mean of the distribution observed, there was no quite clear trend for the two bacterial strains. Sensitivity difference and heterogeneity of the spore population can be the main reason of the observed results [26]. The quality of the receiver may vary from one strain to another, and within the same population the amount of germination receptors varies from one individual to another[32] this contributes to the variation in the time of appearance of colonies. From a statistical point of view, the analysis of the effect of the tested factors and treatment mode reveals that only the strain has a significant effect on the mode and mean of the time to appearance distribution.

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303 Mathematical equations are increasingly being used in predicting the behavior of bacterial
304 spores [33,34,35]. In this study, multiple regression analysis allowed us to have spore germination
305 models based on factors (pH, times, ethanol proportion or activation temperature) tested. In the case
306 of the ethanol activation, it is observed that for *Bacillus cereus* and *subtilis* according to the obtained
307 models, germination efficiencies are inversely proportional to alcohol ratio used. High proportions of
308 alcohol cause a rupture of the bacterial spore's permeability barrier and a decrease in the viscosity of
309 the internal membranes of spores [31]. These cause a lot of damage in the spore cell and can lead to
310 death of the cell. [30].The best germination performance obtained with the lowest proportion of alcohol
311 could be because this proportion is less sporocidal. [31] demonstrated that 30% of alcohol does not
312 permit the viscosity of the bacterial spores to change. 60 min activation appears to be the ideal time to
313 get the best germination yields. [8] and [12] obtained similar results in the activation of spores.

314 In the case of thermal activation, in absolute terms, germination yields are higher in *Bacillus*
315 *cereus* compared to *Bacillus subtilis*. [28] attributed a major thermal sensitivity to *Bacillus cereus*
316 compared with *Bacillus subtilis*, this sensitivity facilitates the induction of spores of this germ under
317 thermal activation. Thermal activation at 80°C/10min seems to be the right balance for optimal
318 activation of spores of mesophilic bacteria which explains why it is recommended by the Food
319 Microbiology standards [36]. It was observed that germination yields are higher after alcoholic
320 activation than after thermal activation. The alcohol would facilitate the attachment of germination
321 effectors to germination receptors of spore membranes [12, 13]. In addition to this the effect of heat on
322 the DNA of spores explains the recovery of less spores compared to ethanol activation [37].

323 **5-Conclusion**

324 Spore germination yields can be said to be heavily influenced by the activation conditions.
325 Exposure of spores to 30% ethanol, pH 7 for 60 min is the best activation conditions for the two strain
326 studied. The rate of colony appearance after ethanol activation is higher than that observed after heat
327 activation, leading to higher germination yields. Therefore ethanol activation offers new opportunities
328 in research and during spores counting providing an alternative to heat activation.

329 .

330 **Conflict of Interest:** No conflict of interest declared by the authors

331 Ethical approval and consent are not applicable

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443 ## Figure legend

444 **Figure1:** Germination and outgrowth kinetic of vegetative cell (interrupted line) and spore (thick line) of the same
445 bacteria with the same initial load. Δt_{lag} is the difference between the spore germination +lag time and the
446 vegetative cell lag time; Δ_{Dt} is the difference in detection time at 6Log ufc/ml between the two kinetics. ($\Delta t_{lag} = \Delta_{Dt}$)

447 **Figure2**: Comparison of selected *Bacillus cereus* and *Bacillus subtilis* spores population outgrowth kinetics after
448 thermal and ethanol activation: **2a-** spores population outgrowth kinetics of *Bacillus cereus* (dotted line) and
449 *Bacillus sutilis* (tick line) after ethanol activation in conditions of (pH,time alcohol) , (7-60-30) for *B.cereus* and (9-
450 80-30) for *B.subtilis*; 2b –spores outgrowth kinetics of *B.cereus* after ethanol activation (30% for 60min) at different
451 pH7(dotted line), pH(5 tick line) and pH9(starry line); 2c-spores outgrowth kinetics of *B.subtilis* after ethanol
452 activation (30% for 60min) at different pH7(dotted line), pH(5 tick line) and pH9(starry line); **2d-** spores population
453 outgrowth kinetics of *Bacillus cereus* (dotted line) and *Bacillus subtilis* (tick line) after thermal activation and
454 previous activation condition of (pH,time temparature), (7-10-80) ; 2e –spores outgrowth kinetics of *B.cereus* after
455 thermal activation (80°C for 10min) at different pH7(dotted line), pH(5 tick line) and pH9(starry line); 2f-spores
456 outgrowth kinetics of *B.subtilis* after thermal activation (80°C for 10min) at different pH7(dotted line), pH(5 tick line)
457 and pH9(starry line)

458 **Figure 3:** Comparison of selected single spore outgrowth (tgrowth) distribution of *Bacillus cereus* and *Bacillus*
459 *subtilis* after thermal and ethanol activation.3a- single spores outgrowth distribution of *B.cereus* (dotted line) and
460 *B.subtilis* (tick line) after ethanol activation (fine line PH8-70min-40%; solid line pH9-60min-30%),3b- single spores
461 outgrowth distribution *of B.cereus* (dotted line) and *B.subtilis* (tick line) after thermal activation(pH7-10min -80°C);
462 3c-single spores outgrowth distribution of *B.subtilis* after thermal(tick line) and ethanol (pH7-60min-50%)
463 activation(dotted line); 3d single spores outgrowth distribution of *B.cereus* after thermal(solid tick line pH7-10min-
464 80°C; fine pH5-10min-80°C) tick line pH7-10min-80°C) and ethanol activation(solid dotted line pH5-60min-70%;
465 fine solid dotted line pH5-40min-30%)

466 **Figure4:** Germination yield of *Bacillus subtilis* (A) and *Bacillus cereus* (B) as function of ethanol proportion an
467 duration of activation at pH7, and germination yield as function of temperature an duration of activation at pH7 for
468 *Bacillus subtilis (C) and Bacillus cereus (D)*

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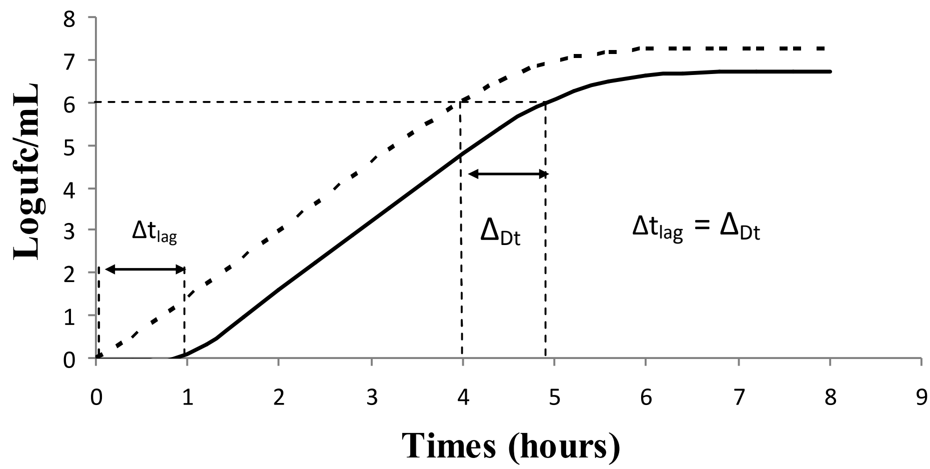

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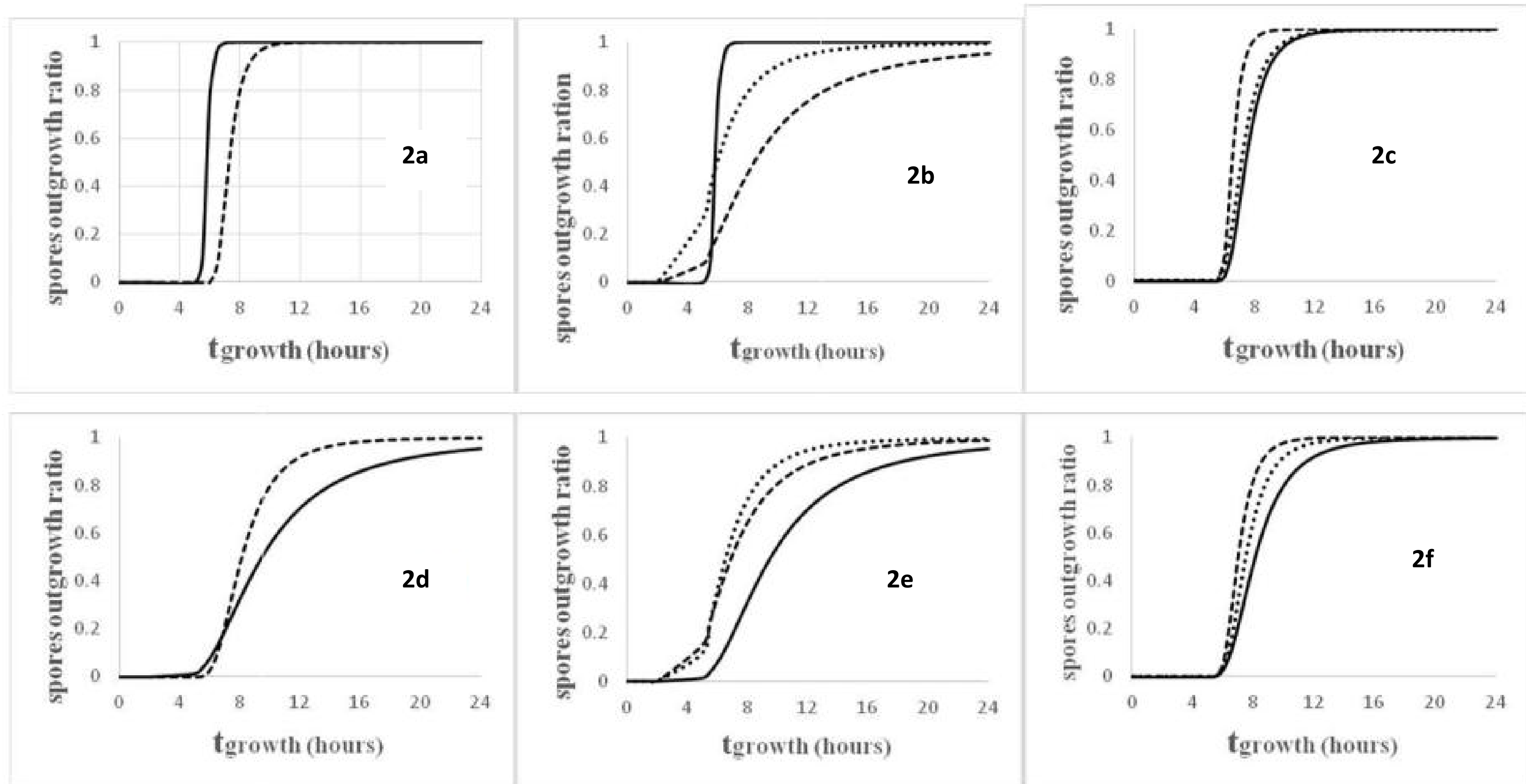

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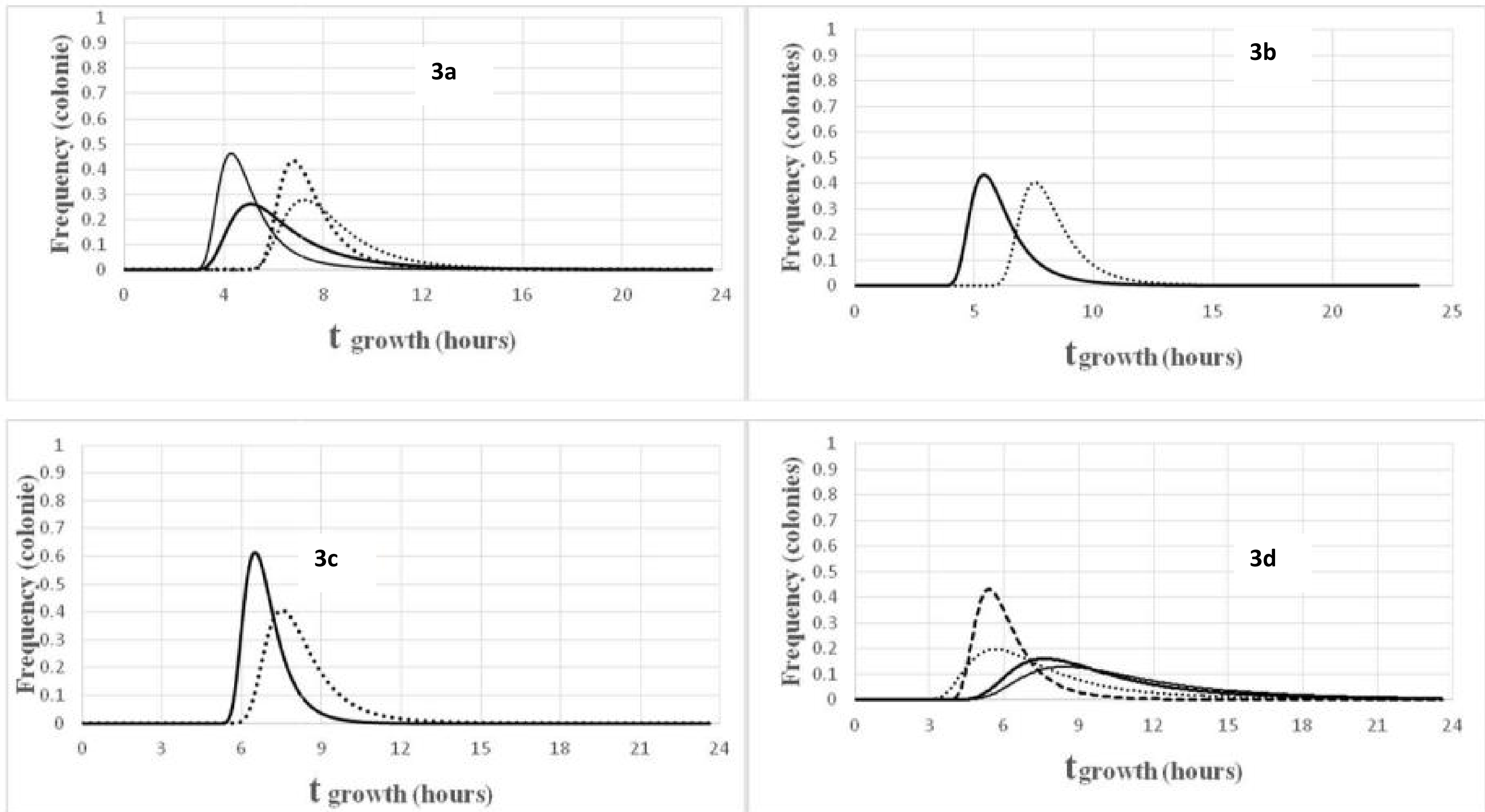

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