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

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 *B. coagulans* and *B. 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 effort in

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

2-MATERIAL AND METHODS

2-1-Strain and culture media:

Bacillus cereus ATC11966 and *Bacillus subtilis* DMS210 were used in this work. Nutrient Broth (Oxoid, Basingstoke, UK) was used for the propagation of the species. Regarding sporulation media, 1L of sporulating agar medium was prepared by dissolving, 5 g of peptone, 5 g of sodium chloride, 1 g of meat extract, 2 g of yeast extract, 15 g of agar, 0.5 g of dissodic phosphate, 0,1g of calcium chloride, 0,04g of manganese sulphate and the pH adjusted to 7±0.2 before sterilization at 121°C for 15 min [20]. The medium was poured into sterile Petri dishes. Nutrient agar (oxoid, Basingstoke, UK) was used for spore germination.

2-2-Spore production:

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

2-3-Spore activation:

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

Table1: real values of variables tested of CCD design for thermal and alcohol activation of 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

2-4-Spore Germination and Outgrowth Assessment:

2-4-1-Theoretical background

According to Baranyi et al. 1995 the difference in the time taken by two bacteria with the same growth rate and initial concentration to reach a given load is proportional to the difference between their latent phase times. This reflects in the case of cells from the same strain a difference in their history or the previous environment in which they originate. We hence verified in a preliminary experiment, that vegetative cells from the same bacterial strain have the same growth rate as their respective spores during their outgrowth (Figure 1). This permitted us to deduce that the difference in time taken by two spore cells to form a visible colony (about 10^6 cells) is proportional to their germination time (Figure 1). During spore counting, the time of appearance of visible colonies on the agar surface is hence proportional to their germination time. Thus the kinetics of single spore appearance within a population can be obtained by the cumulative form of the time to colonies appearance distribution. Thereby the activated spores were counted after their outgrowth and cumulatively expressed in terms of colonies appearing in function of time as earlier demonstrated in our previous work [13]. In this regards the time to first colony appearance is used, in place of the single spore germination time due to the protocol used.

2-4-2-Protocol

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

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

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

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

the following equation:

$$\text{Outgrowth ratio} = Nt/N0 \quad (2)$$

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

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

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

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

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

2-5-Statistical Analysis:

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

3-Results:

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

Table 2: Spores population outgrowth parameters (rate, P.lag) of Bacillus cereus and *Bacillus 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

*B.s=*B.subtilis* and B.c=*B.cereus*); **Fitting Baranyi and Roberts (1994) model to the data gave and R^2 >0.99 in general and permitted the estimation of Rate and Plag .

In order to best assess spore population behavior during germination and outgrowth, the distribution of the time to single spore colony appearance where obtained through the Weibull PDF function. The detailed data are presented in Table3 and table 4. Globally, the mode and mean time to colony appearance of ethanol activated spores of *Bacillus subtilis* were higher than those of *Bacillus cereus* (Table3 and Figure 3a), indicating that *Bacillus subtilis* spores germinated quickly than *Bacillus cereus* spores. Outgrowth distribution mode after ethanol activation was only influenced by strain (*P*=0.03) while pH (*P*=0.04) affected the mean colony appearance time. Regarding the spores time to colony appearance distribution mode and mean after thermal activation (table4), in absolute terms we can observe a higher value of mode and mean for *Bacillus subtilis* in comparison to *Bacillus cereus* (Figure 3b). The strain was the main factor which affected the mode (*P=0.02*) and mean (*P*=0.01) after thermal activation. It was generally observed that the mode *Bacillus subtilis* after ethanol activation were lower than those obtained after thermal activation (Figure 3c). In the case of *Bacillus cereus* spores it was at the contrary thermal activation that induced lower values of the time distribution mode (Figure 3d). The analysis of independent factors (pH) and other factor like strain and activations methods (data not shown) shows that the mode is significantly affected by strain (*P*=0,01) and the activation pH (*P*=0,03). On other hand the distribution mean is only influenced by strain (*P*=0.01).

Table 3: Distribution parameters (mode, mean) for *Bacillus cereus* and *Bacillus subtilis* after 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
5	40	70	**S-5-40-70**	6.8	7.1	C-5-40-70	8.4	12.3

***sample code**: strain (S=*B.subtilis*; C=*B.cereus*)-pH-time-alcohol percentage; **Fitting of $S(t) = \exp(-bt^n)$ to the data gave and R^2 >0.98

Table4: Distribution parameters (mode, mean) of *Bacillus cereus* and *Bacillus subtilis* after 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

***sample code**: strain (S=*B.subtilis*; C=*B.cereus*)-pH-time-alcohol percentage; ****Fitting of $S(t) = \exp(-bt^n)$ to the data gave and $R^2 > 0.98$

The germination yields of *Bacillus cereus* and *Bacillus subtilis* spores obtained after thermal and ethanol activation are presented in table 5. In the case of ethanol activation, we generally observed for the two strain that the germination yield was increased when the percentage of ethanol exposure decreased. For *Bacillus cereus* the lowest yield (16.3%) is obtained at 70% after 60min of activation at pH5, while for *Bacillus subtilis* the lower yield (21.9%) is obtained at 70% after 80min of exposure at pH9. On the other hand the best germination yield for the two strains is also obtained at the same percentage of ethanol (30%) after 60min of exposure at pH7. Regarding thermal activation, we can say that germination yield of *Bacillus subtilis* are higher than those of *Bacillus cereus* (Table 5). We can also observe that the increase of exposure time generally reduce de germination yields. The best germination yield (80.0%) for *Bacillus cereus* and (87.7%) for *Bacillus subtilis* are obtained when spores are exposed at 80°C for 10 min at pH7. The lower value of yield (8.2%) for *Bacillus cereus* is obtained at temperature of 90°C after 18 min of activation at pH9. For *Bacillus subtilis* (16.1%) is obtained after activation at 80°C, during 18min at pH7.

Table 5: Experimental conditions for assessing outgrowth colony appearance kinetics and germination yield of *Bacillus cereus* and *Bacillus subtilis* spores after ethanol and thermal 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

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

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

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

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

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

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

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 latence 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 in 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.

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

permit the viscosity of the bacterial spores to change. 60 min activation appears to be the ideal time to get the best germination yields. [8] and [12] obtained similar results in the activation of spores.

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

5-Conclusion

In terms of findings, spore germination yields can be said to be heavily influenced by the activation conditions. Exposure of spores to 30% ethanol, pH 7 for 60 min is the best condition spore activation for the two strain studied. Therefore ethanol activation offers new opportunities in research and during spores counting in foods. The rate of colony appearance after ethanol activation is higher than that observed after heat activation, leading to higher germination yields. All this indicate that activation condition should be taken in account in the choice of methods for the enumeration of bacillus spores, particularly the pH of activation media that should be adjusted to neutrality.

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

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Figure legend

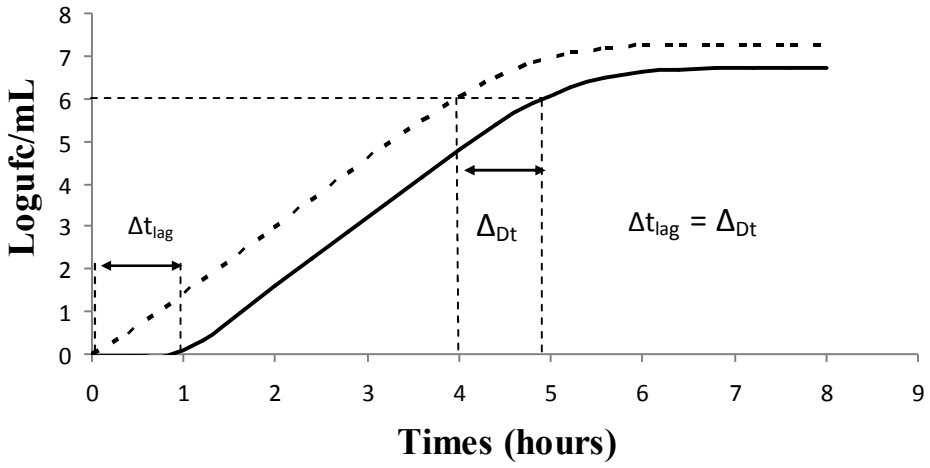
Figure1: Germination and outgrowth kinetic of vegetative cell (interrupted line) and spore (thick line) of the same bacteria with the same initial load. Δt_{lag} is the difference between the spore germination +lag time and the vegetative cell lag time; Δ_{Dt} is the difference in detection time at 6Log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

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

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

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

Figure 1:



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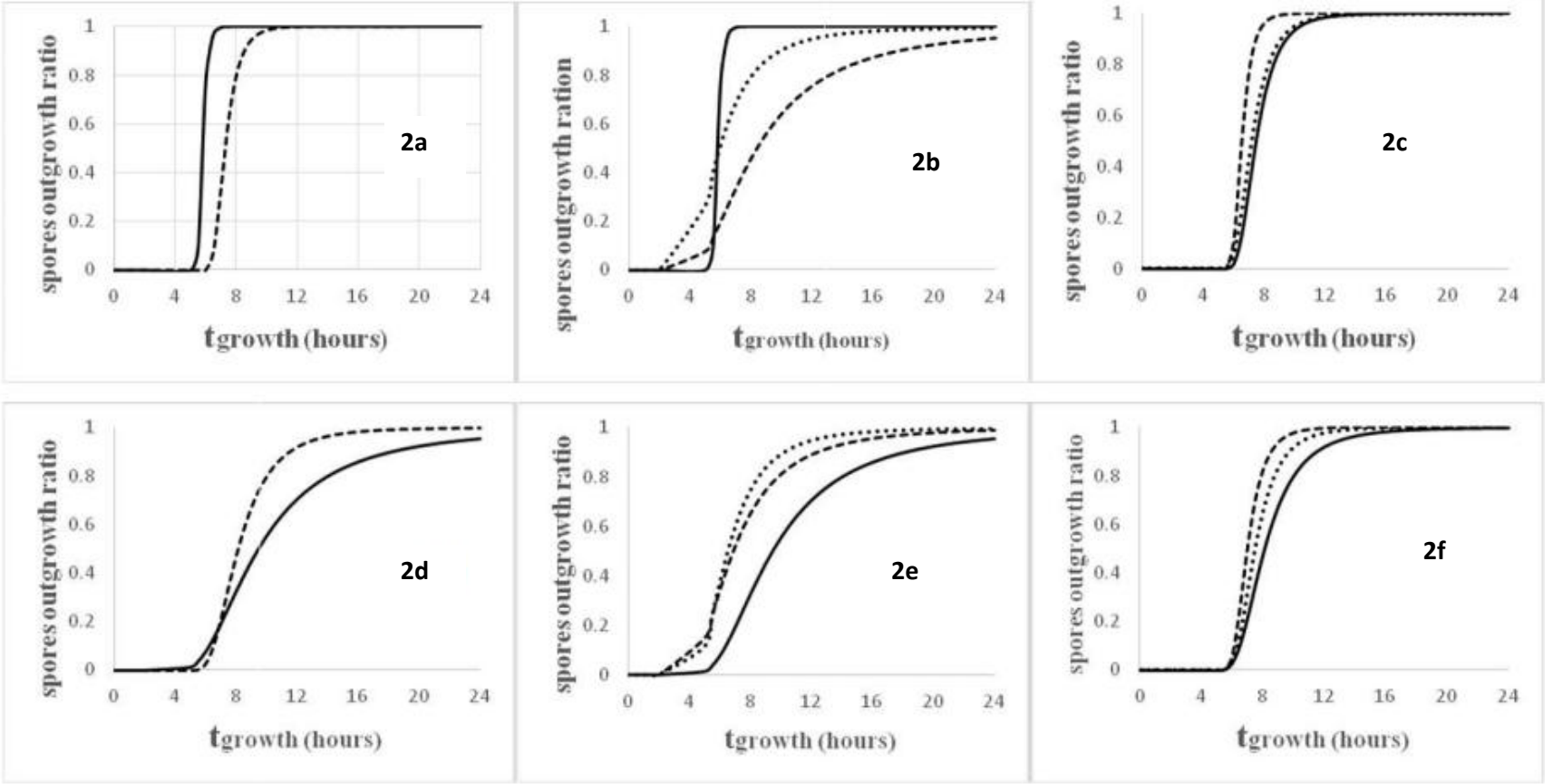

499 Figure 3

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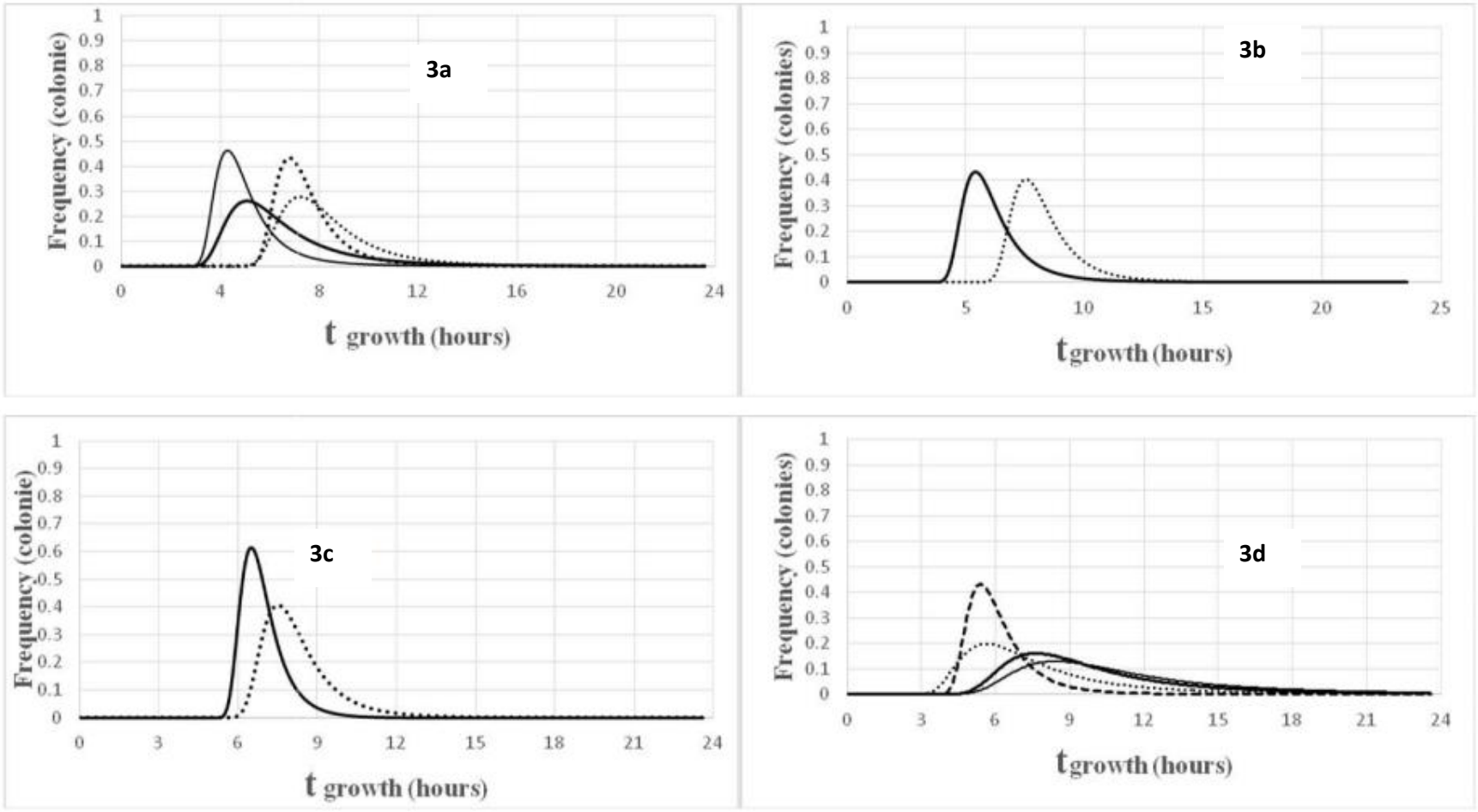

504 Figure 4

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