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Brice Mvou Lekogo [1,2], Louis Coroller[1,2], Anne Gabrielle Mathot [1,2], Pierre Mafart[1,2] and Ivan Leguerinel[1,2*]

[1)] Université Européenne de Bretagne, France

[2)] Université de Brest, EA3882 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT 08.3 PHYSI'Opt, 6 rue de l'Université, 29000 Quimper, France.

[*]Corresponding author:

E-mail address: guerinel@univ-brest.fr

Abstract

Heat resistance of spores is affected by many factors such as temperature, pH, water activity (aw) and others. Previous studies have reported that free fatty acids can affect the germination and growth of bacterial spores. In this study, we investigated the influence of free fatty acids in heating medium or in recovery medium on the heat resistance of spores of *Bacillus cereus* NTCC 11145 and *Clostridium sporogenes* Pasteur 79.3. Four free fatty acids were studied: palmitic, palmitoleic, stearic and oleic acid. During thermal treatments, the impact of these FFA in heating media was generally low, but the presence of free fatty acids in the recovery medium highly decreases bacterial spore apparent heat resistance, particularly with unsaturated fatty acids. A mathematical model was developed to describe and quantify the influence of free fatty acids in recovery media on the D-values. The z'_{FFA} parameter values which quantify the impact of free fatty acids were determined. The variation of this parameter value according to the free fatty acid type was compared with MIC value variation given in the literature. The model enables the decrease in D-values in the presence of free fatty acids to be estimated. The high concentrations of free fatty acids in liver or canned duck may explain the microbial stability with low sterilization values applied.

Key words: free fatty acids, bacterial spores, heat resistance, recovery medium.

Introduction

Free fatty acids are a large group of non toxic compounds which present antimicrobial activity. It is well known that soaps composed of free fatty acid salt have a bactericidal effect. The bactericidal effect of free fatty acids has been extensively investigated and presented by Nieman (1954). More recently Kabara (1978) provided and analysed minimal inhibitory concentration values of saturated and unsaturated fatty acids for vegetative cells of pathogenic bacteria. For long chain fatty acids, the influence of free fatty acids varied according to acid type but did not correlate with carbon chain length. However, the carbon chain degree of unsaturation greatly influenced the MIC values.

The application of the bactericidal effect of fatty acids, given in the literature, mainly concerns soaps and the cosmetics industry. More recently, studies concerning the influence of free fatty acids on pathogens have examined food products. These studies focus on pathogens such as *Salmonella, Listeria* and *Staphylococcus*. However some spore-forming species have been studied such as *Bacillus cereus* (Ababouch et al., 1994; Lee et al., 2002), *Bacillus subtilis* (Tsuchido et al., 1992), *Clostridium botulinum* (Grecz et al., 1959) and *Clostridium perfringens* (Skřivanova et al., 2005).

In foods, free fatty acid concentrations are low, however in some foods containing fats and natural oils their concentrations are sufficient to affect bacterial growth. Free fatty acids in food can be considered as a hurdle usable for food preservation. In compliance with Leistner's concept (2000), this hurdle can be associated with other stresses such as temperature during heat treatment. The combination of heat treatment with pH, water activity or acids on bacterial heat resistance have been studied, modelled and presented in different publications (Blackburn et al., 1997; Coroller et al., 2001; Couvert et al., 1999; Davey, 1993; Juneja and Eblen, 1999; Leguérinel et al., 2005). These combinations affect surviving bacteria in two stages: during heat treatment and during recovery. In these publications, a Bigelow-

like model and z parameters were developed to quantify the impact of these environmental factors on the D-values.

Few studies quantify the influence of free fatty acids during heat treatment on bacterial spores. Tremoulet et al. (2002) observed for different free fatty acids (C16:0, C16:1, C18:1, C18:2 and C18:3) in heating media that their increasing concentration decreased the D-value for *Geobacillus stearothermophilus*. Concerning the presence of free fatty acids in recovery media following heat treatment Ababouch et al. (1994) have observed some influences.

The aim of this study was to quantify and model the influence of the concentration of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1), major free fatty acids in food, in heating and recovery media for *B. cereus* and *C. sporogenes* spores.

Material and methods

2.1 Microorganism and spore production

Spores of *B. cereus* NTCC11145 were obtained as follows: cells were precultivated at 37 °C for 24 h in Brain Heart Infusion broth (Difco). The preculture was used to inoculate nutrient agar plates (Biokar Diagnostics BK021) to which 40 mg/l of $MnSO_4$ and 100 mg/l of $CaCl_2$ were added to the surface. The plates were incubated at 37 °C for 5 days. Spores were then collected by scraping the surface of the agar. They were then suspended in sterile distilled water and washed three times by centrifugation (10,000 *g* for 15 min) (Bioblock Scientific, model Sigma 3K30). The final suspension (approximately 10^{10} spores/ml) was finally distributed into sterile Eppendorf microtubes and kept at 4 °C. For *C. sporogenes* Pasteur 79.3, spore production was obtained using the Third Method described by Goldoni et al. (1980).

2.2 Source and preparation of fatty acids

Palmitic and oleic acids were obtained from Alfa Aesar (Strasbourg, France) and palmitoleic and stearic acid from Fluka and reported as being more than 96%, 90%, 95% and 97% pure respectively. Stock emulsions with different concentrations were obtained by mixing (Polytron® PT-MR 2100, Kinematica AG, Switzerland) and microsonication (Branson Sonifier 250, Branson Ultrasonics, USA) in distilled water with 0.1% Tween™80 as a dispersant. These solutions were added to a nutrient broth (Biokar Diagnostic BK003HA) for heating media or a nutrient broth with Bacteriological Agar (15g) (Biokar Diagnostic A1010HA) for recovery media. For the different FFA, the concentrations studied were lower than their critical micelle concentration values (Freeman 1969, Mukerjee and Mysels, 1971). The concentrations of free fatty acids added to the heating media were 0.8mM or 2mM. In the recovery media, different concentrations were added from 0 to 2 mM according to their critical micelle concentration values and their influence on the D values determined. After sterilization by autoclaving at 110°C for 45 minutes as described by Marounek et al. (2003), the pH was adjusted to 7 and the aw value was also regulated. For better visibility of bacterial communities, 2,3,5-Triphenyl-2H-Tetrazolium Chloride (TTC) was added to the recovery medium containing free fatty acids. Tween™80 and TTC were obtained from Alfa Aesar (Strasbourg, France). The absence of influence of TTC and Tween ™80 on the bacterial heat resistance was checked (data not show).

2.3 Spore heat treatments

Firstly, 30 µl of spore suspension was diluted in 3 ml of adjusted heating medium. Capillary tubes of 200 µl (vitrex) were filled with 100 µl of sample, sealed, and subjected to a thermal treatment in a thermostated glycerol bath for different heating times. The heat treatment was stopped by cooling capillary tubes in a water/ice bath. Then they were broken at both ends and their contents poured into a tube containing 9 ml of sterile tryptone salt broth (Biokar

Diagnostics BK014HA) by rinsing with 0.9 ml tryptone salt broth. The viable spores were counted by duplicate plating in the recovery media and incubated 72h at 37°C for *B. cereus* and *C. sporogenes* strains.

2.4 Experimental design and data analysis

For each heating and recovery media condition, classical D-values and their associated standard deviation were estimated from survival kinetics. One concentration level was studied to assess the influence of free fatty acids in heating media. Concerning the influence of free fatty acids in recovery media, monofactorial design, without repeat, was carried out for each acid studied. The influence of free fatty acid concentrations in recovery media on the D-values was modelled by a simple Bigelow-like model as a secondary model (Eq1)

$$\log D = \log D^* - \frac{[acid]}{z'_{FFA}} \qquad \text{(Eq1)}$$

In this model z'_{FFA} values correspond to an increase in free fatty acid concentrations in the recovery media, leading to a 10-fold reduction in the D-value (D* corresponds to the D-values without free fatty acids in recovery media). This parameter quantifies the influence of fatty acid concentration in the recovery media on the heat resistance of bacterial spores.

The parameter values and their associated standard deviation were fitted using a non-linear module ("nlinfit" and "nlparci" Matlab 6.1, The Mathworks). The "nlparci" function used to assess standard deviation was based on the asymptotic normal distribution for the parameter estimates (Bates and Watts, 1988).

Results

For the two species studied, *B. cereus* and *C. sporogenes*, most of the surviving cell kinetics showed a linear relationship between heating time and the logarithm of survival cell numbers. Hence bacterial heat resistance values were quantified using the classical D-values.

Concerning heat treatment, D-values were determined in heating media in the presence of 4 free fatty acids (C16:0, C16:1, C18:0 and C18:1). Table 1 shows a low decrease in the D-values when free fatty acids were added whatever the carbon chain length or number of chain bonds for the two species studied.

The influence of free fatty acids in the recovery media on the apparent D-values was determined for different concentrations of each acid type and for each strain studied. The results are shown in Figures 1 & 2 and Tables 2 & 3. Concerning D values determined in media without fatty acid variability was observed. Bacterial spores coming from different productions explain this variability. The presence of free fatty acids in recovery media greatly reduced the apparent D-values for the two species studied. For the four free fatty acids studied, the evolutions in the D-values according to their concentrations are presented Figure 3. A linear relationship is observed between free fatty acid concentration and log of D-values for the 4 acids studied.

The z'_{FFA} values for the different acid types and for the two species studied are presented in Table 4. The results presented in this table show that the presence of unsaturated free fatty acids has a greater effect on D-values than saturated free fatty acids, with a greater sensitivity for *C. sporogenes* Pasteur 79.3 compared to *B. cereus* NTCC 11145. During food heat treatment, the environmental factors of heating and recovery media are identical. Table 5 presents the specific effects of palmitic and oleic acid in heating and recovery media on the D-value of *B. cereus* spores and their associated effects in heating and in recovery media. These results show the cumulative effect of free fatty acids during heat treatment and recovery.

Discussion

Free fatty acids in heating media enhance thermal inactivation of bacterial spores. The influence of the free fatty acids studied (palmitic, palmitoleic, stearic and oleic acid) reduced the D-value down to 30% for *B. cereus* and *C. sporogenes*. The D-values obtained appear lower than those observed by Tremoulet et al. (2002) for *G. stearothermophilus*. For 0.5mM of free fatty acid added to heating media, these authors observed D-value reduction ratios ranging from 50% to 75% according to the acid type used. As shown by the results presented, the chain length or unsaturated bond does not appear to influence this moderate effect. To the best of our knowledge, the literature gives no information to explain the mechanism of free fatty acid effects during heat treatment.

The benefit of the association of different stresses on bacterial inactivation has been described by Leistner (2000) through the hurdle concept. The combination of heat treatment with unfavourable environmental conditions of recovery media, low pH or aw, decreases the apparent heat resistance of bacterial spores. During heat treatment, the proportion of dead and injured spores increases with the heating time. In favourable and optimal environmental recovery conditions, most injured bacterial cells survive and grow, thus D-values appear higher. When recovery conditions are unfavourable, bacteria cells are stressed by the environmental conditions, low pH or aw, resulting in growth inhibition followed by death. Thus in unfavourable recovery conditions, D-values are lower than the D-values observed in optimal recovery conditions. The influence of low pH or aw values of recovery media on the apparent D-values has been quantified and modelled by Couvert et al. (1999) and Coroller et al. (2001). Like these two environmental factors, free fatty acids in growth media represent a stress for bacterial cell development and their presence in recovery media reduces the apparent D-values. It can be noted that for the different free fatty acids studied, their concentrations were below the CMI values and did not modify the initial population size observed without heat treatment. Thus for the concentrations studied, free fatty acids added

must be associated with a heat treatment or high thermal stress to show an inhibition activity. Free fatty acids present in recovery media affect thermally injured bacteria. This observation was made by Ababouch et al. (1994) on *B. cereus* heat stressed cells for additions of stearic and oleic acid.

This effect of fatty acid concentration is described and quantified by a Bigelow-like model (Eq1) where the z'_{FFA} parameter quantifies the impact of free fatty acid concentrations on the decrease in apparent heat resistance of bacterial spores. These effects of free fatty acids in recovery media have never been quantified in the literature. However z'_{FFA} values for different acids and for the two bacterial species studied can be compared to data concerning the impact of free fatty acid concentrations on bacteria spores or vegetative cells such as MIC values.

The z'_{FFA} values for the saturated free fatty acids palmitic and stearic acid show a limited effect. This observation for stearic acid corresponds to the observation of Ababouch et al. (1994) on *B. cereus* unheated and heat-shocked spores. Concerning the *Clostridium* species, the high MIC values for palmitic and stearic acid, greater than 5mM for *C. botulinum* (Skřivanova et al., 2005) and close to 10mM for *C. perfringens* (Kabara, 1978), concur with our observation of a moderate influence of saturated fatty acid in recovery media to reduce D-values. The impact of added unsaturated fatty acid on recovery media is higher than that of saturated fatty acid.

For the same carbon chain length, C18, the presence of unsaturated bonds in the carbon chain decreased the z'_{FFA} from 3.19mM (C18:0) to 0.89mM (C18:1) for *B. cereus* and from 2.36mM (C18:0) to 0.36mM (C18:1) for *C. sporogenes*. While no data is available in the literature concerning the influence of these unsaturated free fatty acids in recovery media on D-values, data exists for the MIC concentration concerning the impact of these acids on *Bacillus* or *Clostridium* growth. The presence of unsaturated bonds in the carbon chain

reduces the MIC values: 10mM (C18:0) to 0.75mM (C18:1) for *G. stearothermophilus* (Tremoulet et al., 2002), 0.4mM to 0.05mM for *B. megaterium* (Galbraith et al., 1971) 11.4mM (C18:0) to 1.1mM (C18:1) for *C. perfringens* (Kabara, 1978) or 20mM (C18:0) to 0.48-1.48mM (C18:1) for *C. perfringens* (Skrivanova et al., 2005). For C16 chain length, the influence of unsaturated bonds in the carbon chain on the decrease in z'_{FFA} values was also observed. This observation concurs with the difference in MIC values determined by Tremoulet et al. (2002): 10mM for palmitic acid, C16:0, and 1.25mM for palmitoleic acid, C16:1, for *G. stearothermophilus* strain. For these acids, similar observations were presented by Kabara et al. (1972) for different bacterial species such as *Streptococcus* or *Pneumococcus*. The presence of long chain free fatty acids, particularly unsaturated free fatty acids, in recovery media creates unfavourable conditions for the growth of injured heat-treated spores. Different mechanisms affect the physiology of bacterial spores or vegetative cells.

High adsorption of free fatty acids onto bacteria was observed by Maxcy and Dill (1967) and Galbraith and Miller (1973a). The accumulation of free fatty acids on the cell or spore surface is the first stage of the inhibition mechanism (Nieman 1954). Concerning bacterial spores, Foster and Wynne (1948) observed an inhibition of germination of *C. botulinum* spores in the presence of oleic acid. In 1982, Yasuda et al. assessed the inhibitory activity of short and long chain fatty acids on spore germination. The percentage inhibition of unsaturated oleic acid is higher than saturated fatty acids which present the same effect. These authors suggest that hydrophilic environments inhibit L-alanine receptors, keys to germination mechanisms described by Johnstone in 1994.

The difference in solubility of saturated and unsaturated fatty acids may explain the higher inhibitory effect of unsaturated fatty acids. In addition to germination inhibition, free fatty acids have long been recognized as growth inhibitors for Gram-positive bacteria (Kabara, 1978). Ababouch et al. (1994) studied the influence of stearic and oleic acids both on spore

and vegetative cells of *B. cereus* growth. For acid-treated spore or vegetative bacteria, morphological modifications appeared, giving long thin cells. Knapp and Melly (1986) made the same observations for *Staphylococcus aureus* in the presence of arachidonic acid. The influence of higher concentrations of free fatty acids in growth media causes membrane disruption and cell lysis (Tsuchido et al., 1992). Zheng et al. (2005) demonstrated specific action of palmitoleic and oleic acid on enol-acyl carrier protein reductase (Fab1) which inhibits fatty acid synthesis for *S. aureus* and *Escherichia coli*. Stearic acid does not present this effect.

Galbraith and Miller (1973b) showed that for *Bacillus megaterium* cells, low concentrations of fatty acids stimulate oxygen uptake, whereas high concentrations (0.2mM) of oleic acid inhibit oxygen intake. These authors observed that unsaturated fatty acids were more active than saturated acids. A similar decrease or inhibition of nutrient uptake was observed for amino acids glutamic acid and lysine for *B. megaterium* and *C. perfringens* vegetative cells (Galbraith and Miller, 1973b) and glucose and glycerol uptake for *Brochothrix thermosphacta* (Macaskie, 1982).

All these effects of free fatty acids on unheated bacteria were enhanced by the injury caused by heat treatment. Free fatty acids are present in natural oils such as olive oil or in fish and animal fats, for instance in canned fat duck liver. Thus the additive influences of free fatty acids in heating and particularly in recovery media can be taken into account to safely reduce heat treatment of bacterial spores. These major reductions in thermal resistance by fatty acids in recovery media may explain the sterility of canned fat duck liver which is heat treated with very low sterilization values, lower than 1. According to our results, the high concentrations of free fatty acids in fresh fat duck liver, 33.3mM of oleic acid and 2.4mM of palmitoleic acid (Tremoulet et al., 2002), are sufficient to greatly reduce the apparent heat resistance of bacterial spores. With these acid concentrations, the D-value calculated using equation 1 is

theoretically reduced by a factor of 10^{33} with oleic acid concentrations (33.3mM) and by a factor of $3*10^{10}$ with palmitoleic acid concentrations (2.4mM) for *B. cereus* NTCC11145 spores. The presence of free fatty acids in food associated with a heat treatment may be used to reduce heat treatment or increase food safety.

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Titles of figures and tables

Figure 1: Log N (colony-forming units, cfu) vs. time (min) for *Bacillus cereus* NTCC 11145 to 100°C (A & C) and *Clostridium sporogenes* Pasteur 79.3 to 90°C (B & D) with, in recovery media, different concentrations of palmitic acid (A & B) or stearic acid (C & D) at different concentrations: 0mM ●, 0.2mM □, 0.4mM ▲, (C18:1) 0.6mM ▽, 0.8mM ◆, 1mM ○, 1.5mM ■, 2mM △.

Figure 2: Log N (colony-forming units, cfu) vs. time (min) for *Bacillus cereus* NTCC 11145 heated to 100°C (A & C) and *Clostridium sporogenes* Pasteur 79.3 heated to 90°C (B & D) with, in recovery media, different concentrations of palmitoleic acid (A & B) or oleic acid (C & D) at different concentrations: 0mM ●, 0.05mM □, 0.1mM ▲, 0.2mM ◆, 0.4mM ○, 0.6mM ■, 0.8mM △.

Figure 3: Log D (minutes) vs. FFA concentration (µM) with respectively palmitic ●, palmitoleic ○, stearic ▲ and oleic △ acid in recovery medium for: spores *Bacillus cereus* NTCC 11145 heated to 100°C (A) and *Clostridium sporogenes* Pasteur 79.3 heated to 90°C (B).

Table 1: D-values (minutes) for *Bacillus cereus* NTCC 11145 heated to 100°C and *Clostridium sporogenes* Pasteur 79.3 heated to 90°C with palmitic, palmitoleic, stearic and oleic acids in heating medium.

Table 2: D-values (minutes) for *Bacillus cereus* NTCC 11145 heated to 100°C in the presence of different concentrations of palmitic, palmitoleic, stearic and oleic acid in recovery medium.

Table 3: D-values (minutes) for *Clostridium sporogenes* Pasteur 79.3 heated to 90°C in the presence of different concentrations of palmitic, palmitoleic, stearic and oleic acid in recovery medium.

Table 4: z-values for palmitic, palmitoleic, stearic and oleic acids in recovery medium for *Bacillus cereus* NTCC 11145 and *Clostridium sporogenes* Pasteur 79.3.

Table 5: Specific effect of the addition of 0.8mM of palmitic and oleic acid in heating and recovery media on the D-value of *Bacillus cereus* spores heated at 100°C and their associated effect in heating and recovery media.

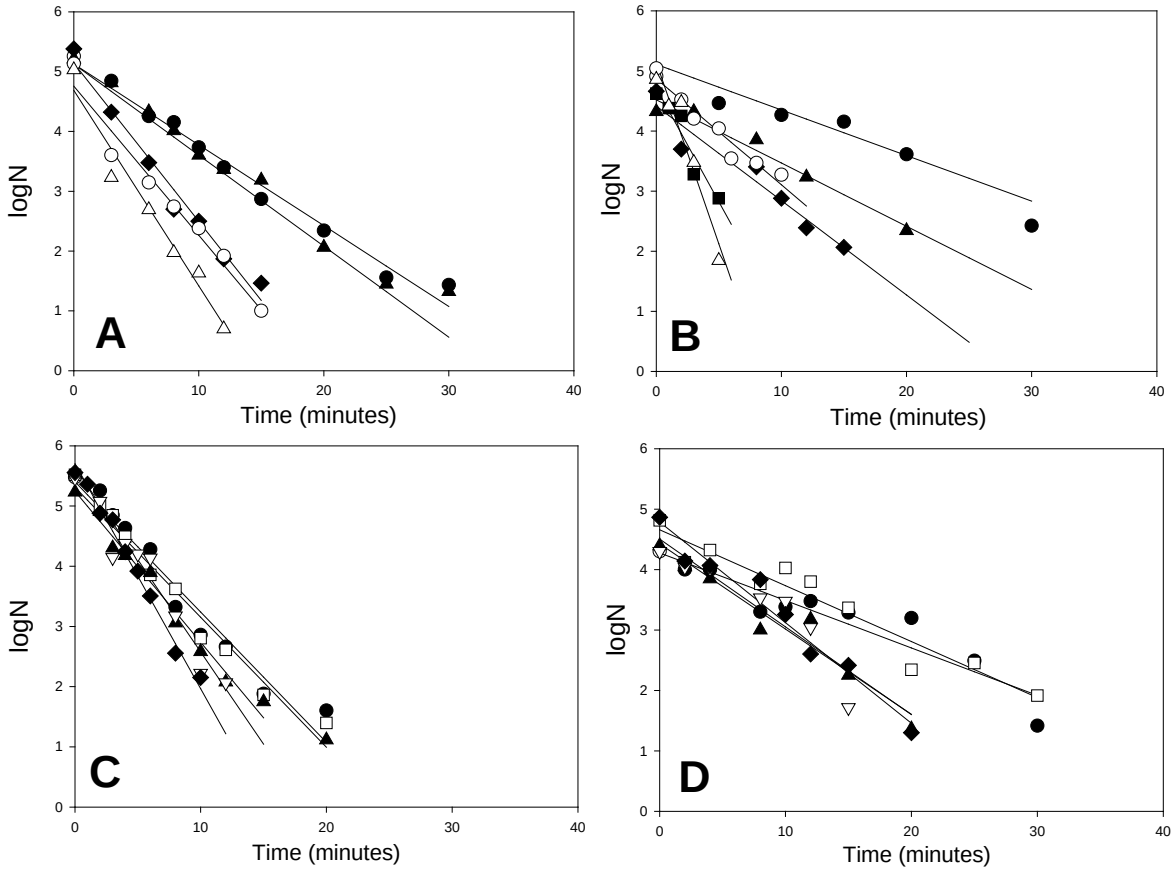

Figure 1

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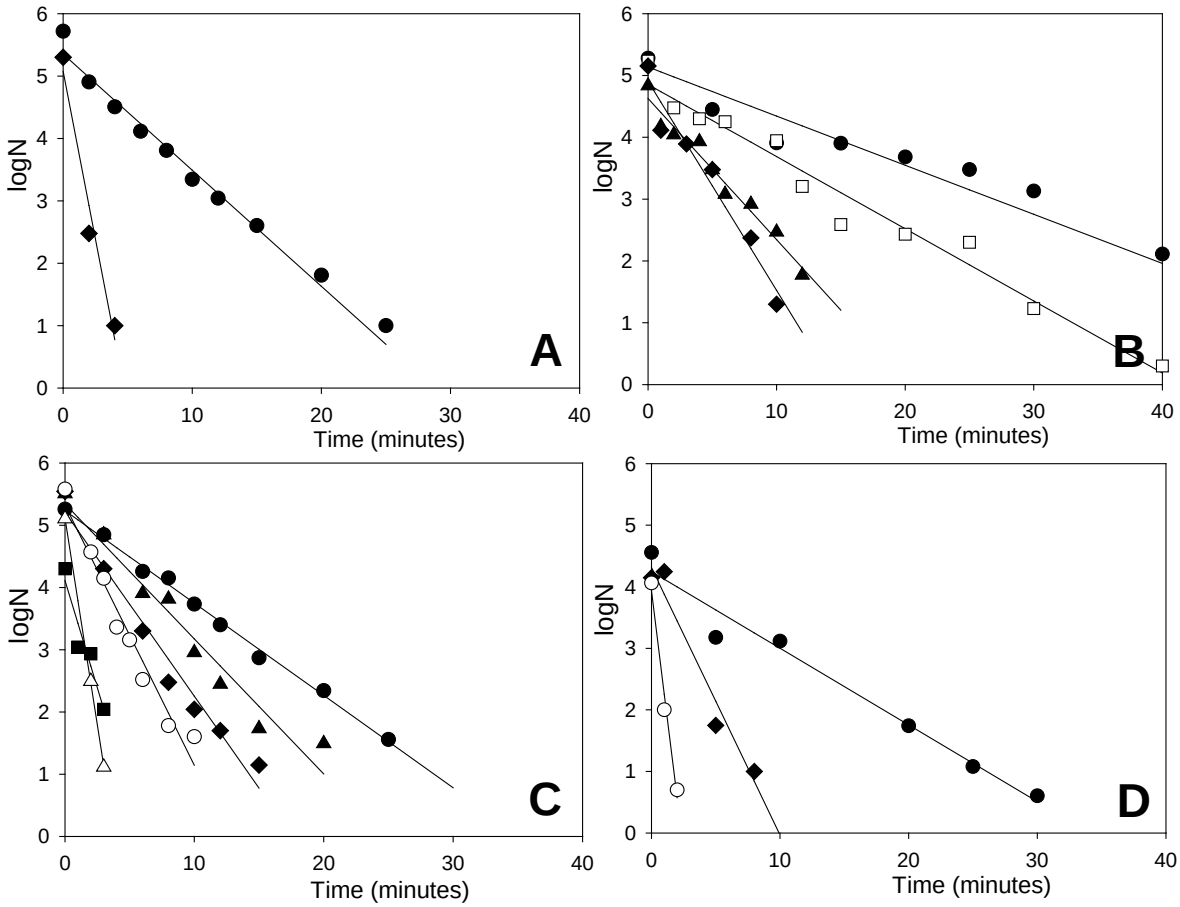

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397 Figure 2

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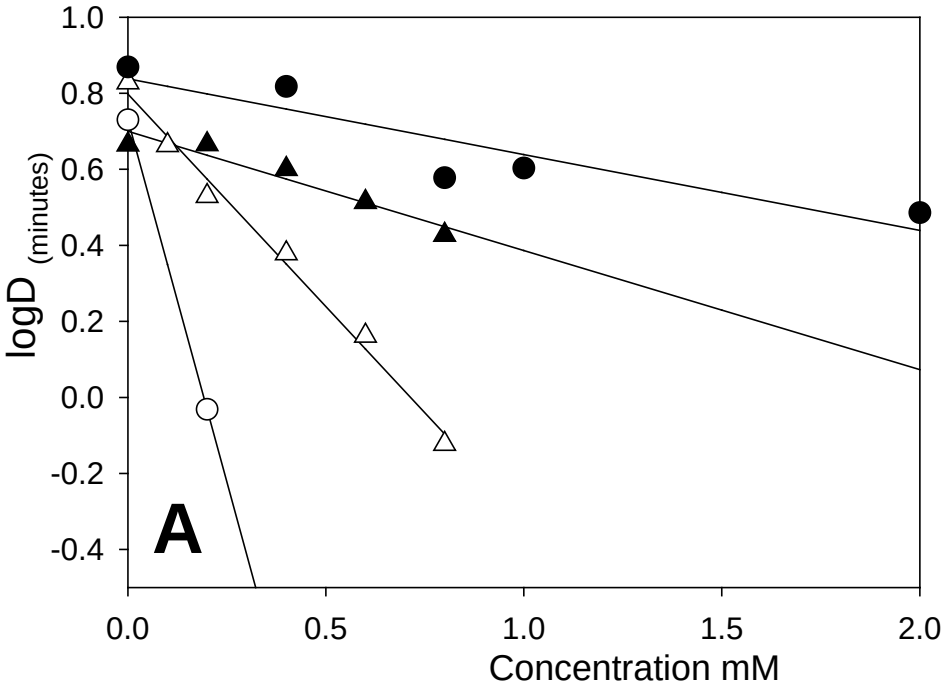

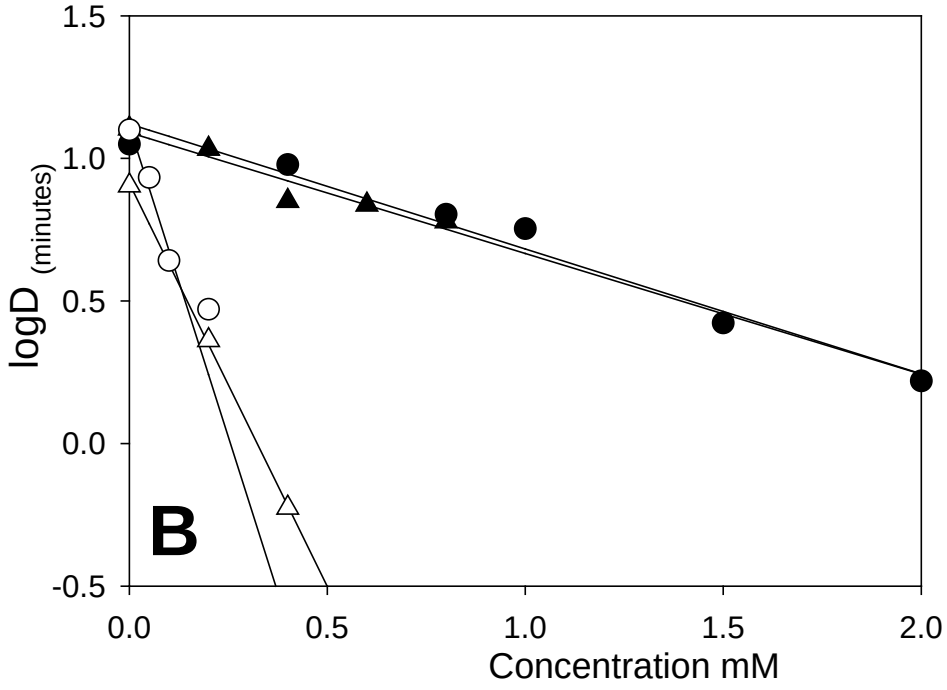

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401 Figure 3 A&B

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	concentration mM	Palmitic acid C16:0		Palmitoleic acid C16:1		Stearic acid C18:0		Oleic acid C18:1	
		D (min)	SD	D (min)	SD	D (min)	SD	D (min)	SD %
Bacillus cereus NTCC11150 Heating T°C: 100°C	0	6.74	0.21	6.74	0.21	6.99	0.21	6.74	0.21
	0.8	ND		5.21	0.32	ND		ND	
	2.0	3.55	0.27	ND		4.63	0.35	4.78	0.29
Clostridium sporogenes Pasteur 79.3 Heating T°C: 90°C	0	13.20	3.37	13.89	1.64	12.73	1.93	12.59	0.95
	0.8	ND		11.44	0.59	12.19	0.64	7.47	0.95
	2.0	4.78	0.29	ND		ND		ND	

405
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408 Table 1
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Concentration µM	Palmitic acid C16:0		Palmitoleic acid C16:1		Stearic acid C18:0		Oleic acid C18:1	
	$D_{100°C}$ (min)	SD	$D_{100°C}$ (min)	SD	$D_{100°C}$ (min)	SD	$D_{100°C}$ (min)	SD
0	7.40	0.42	5.37	0.35	4.62	0.47	6.74	0.72
0.1	ND		ND		ND		4.61	0.49
0.2	ND		0.93	1.09	4.63	0.35	3.38	0.35
0.4	6.57	0.26			3.99	0.28	2.39	0.24
0.6	ND				3.26	0.28	1.45	0.69
0.8	3.78	0.34			2.68	0.16	0.75	0.07
1.0	4.01	0.44						
1.5	ND							
2.0	3.06	0.70						

415

416 Table 2

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Concentration µM	Palmitic acid C16:0		Palmitoleic acid C16:1		Stearic acid C18:0		Oleic acid C18:1	
	$D_{90°C}$ (min)	SD	$D_{90°C}$ (min)	SD	$D_{90°C}$ (min)	SD	$D_{90°C}$ (min)	SD
0	11.22	2.27	12.59	0.95	12.73	1.93	8.03	0.98
0.05	ND		8.57	0.61	ND		ND	
0.1	ND		4.38	0.36	ND		ND	
0.2	ND		2.95	0.45	10.81	1.22	2.30	0.34
0.4	9.51	1.61			7.09	0.96	0.59	0.51
0.6	ND				6.88	1.78		
0.8	6.36	1.21			6.01	0.60		
1.0	5.67	0.86						
1.5	2.65	0.74						
2.0	1.66	0.46						

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421 Table 3

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423

	Palmitic acid C16:0		Palmitoleic acid C16:1		Stearic acid C18:0		Oleic acid C18:1	
	z' mM	SD	z' mM	SD	z' mM	SD	z' mM	SD
Bacillus cereus NTCC11145	5.01	2.10	0.26	-	3.19	0.85	0.89	0.7
Clostridium sporogenes Pasteur 79.3	2.28	0.27	0.23	0.04	2.36	0.68	0.36	0.5

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426 Table 4

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	Palmitic acid 2mM		Oleic acid 0.8mM	
	$D_{100°C}$ (min)	SD	$D_{100°C}$ (min)	SD
No fatty acid added to media	6.90 [(a)]	0.23	6.90 [(a)]	0.23
Fatty acid added to heating media	3.48 [(b)]	0.18	4.32 [(b)]	0.26
Fatty acid added to recovery media	2.04 [(c)]	0.15	0.98 [(c)]	0.14
Fatty acid added to both heating and recovery media	1.77	0.16	0.66	0.06
"Theoretical" or predicted value taking account D values (a), (b) and (c)	1.07		0.61	
"Theoretical" or predicted value taking account D values from Tables 1 and z'_{FFA} values from table 4	1.45		0.75	

430

431 Table 5

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