

Full Length Research Paper

## Accelerated storage testing of freeze-dried *Pseudomonas fluorescens* BTP1, BB2 and PI9 strains

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Freeze-dried cultures of *Pseudomonas fluorescens* are used in agriculture and microbiological industry. However, *P. fluorescens* is very susceptible to damage during freeze-drying and subsequent storage and it would be useful to increase culture viability during storage. The viability of freeze-dried *P. fluorescens* strains (BTP1, PI9 and BB2) was evaluated by using the Arrhenius model. This model was described by measuring the reaction rate constants (D or k) and temperature sensitivity of rate constant (z or Ea). The freeze-dried *P. fluorescens* strains were stored in glass tubes at 60, 37 and 4°C for 8 h, 28 days and two months, respectively. D value decreased or k increased with an increase of the storage temperature. By comparing their decimal reduction time (D), we observed that BB2 strain was more resistant than BTP1 and PI9 at 37 and 60°C. The activation energy of all *P. fluorescens* strains were not significantly different and thermal inactivation may occur by the same mechanism. Thus, it was possible to compare rate constants of survival for the freeze-dried *P. fluorescens* strains. These results will be useful to the development of improved reference materials and samples held in culture collections.

**Key words:** Arrhenius model, accelerated storage testing (AST), freeze-drying, storage stability.

### INTRODUCTION

Freeze drying is a more effective and gentle method to dry and preserve organisms compared to spray drying or fluidizing. It involves freezing the sample and then removing the water under vacuum through sublimation (Portner et al., 2007). To develop this methodology further, it is therefore important to identify potential hazards and to perform accurate quantitative assessments of constraints, eventually by using probabilistic modelling approaches (Achour et al., 2001).

Accelerated storage testing (AST) is a widely used

method for the prediction of storage stability, storage quality and estimation of shelf-life of bacterial products (An-Erl King et al., 1998; Hernández et al., 2006; Hernandez et al., 2009). Such tests are performed by incubating the samples at temperatures higher than the usual storage temperature. Thus, data obtained under accelerated tests are used for performing extrapolation studies and prediction of the real-time behaviour. One disadvantage of this approach is that it considers only the assessment of viable cells, while the residual water content is omitted (Hernandez et al., 2009). The Arrhenius equation is the most common and generally valid assumption for the temperature-dependence of the deterioration rate. By means of the Arrhenius relationship, the stability of freeze-dried bacteria was successfully predicted using the accelerated storage test method (An-Erl King et al., 1998; Ziadi et al., 2005; Yao

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**Abbreviations:** AST, Accelerated storage testing; CWBI, Wallon Center of Industrial Biology; Ea, activation energy; D, decimal reduction time.

et al., 2008; Aguirre et al., 2009). AST can be also used to study the stability of freeze-dried bacteria. Supportive work was previously reported (An-Erl King et al., 1998; Achour et al., 2001; Ziadi et al., 2005; Hernández et al., 2006; Aguirre et al., 2009). The effect of different temperature on the survival of Gram positive bacteria was studied. The applicability of the Arrhenius method in studying and predicting the survival loss rate of the strains during storage may be applicable.

In this model, resistance to heat is described by two parameters: D and z. D is the time needed to reduce viable cell numbers by one log unit at a specified temperature, and z is the temperature change needed to bring about a tenfold change in D. Thermal inactivation of microorganisms may also be described by methods based on chemical reaction kinetics (Yao et al., 2008). The Arrhenius equation that is usually employed describes the temperature dependence of the rate constant of inactivation (k), in which the key parameter is the activation energy ( $E_a$ ). Using the Arrhenius model, it has shown that stability of freeze-dried lactic acid bacteria stored at low temperature may be quantitatively predicted on the results of short-term degradation studies at higher temperatures (Achour et al., 2001; Yao et al., 2008). Furthermore, the physicochemical state of freeze-dried bacteria was well correlated to temperature (Hutchinson, 2004). However, z-value and Arrhenius models are useful aids in the evaluation of freeze-dried bacteria stability during storage (Achour et al., 2001; Tsen et al., 2007).

The aim of the present work was to study how temperature influences survival of *P. fluorescens* strains freeze-dried during storage by using the Arrhenius model. To do this, we subjected three freeze-dried *P. fluorescens* strains to mortality accelerated test to determine their decimal reduction time (D) and activation energy ( $E_a$ ).

## MATERIALS AND METHODS

### Strains and pre-culture conditions

The strains used in our study were *P. fluorescens* BTP1 of Wallon Center of Industrial Biology laboratory (CWBI) (Ongena, 1996; Mputu Kanyinda et al., 2012) and *P. fluorescens* (PI9 and BB2) from Algeria to Saad Dahlab of Blida University. The King B agar (20 mg of casein peptone, 1.5 g of anhydrous  $K_2HPO_4$ , 1.5 g of  $MgSO_4 \cdot H_2O$ , 15 g of glycerol and 12 g of agar agar) was prepared for the maintenance of *P. fluorescens* strains. A colony from King B agar was inoculated into a pre-culture medium (King B liquid medium) in 250 ml Erlenmeyer flask. This pre-culture was incubated at 30°C for 24 h and inoculated into a 5000 ml Erlenmeyer flask containing 3000 ml King B medium.

### Production and freeze-drying

The strain was grown in 20 L bioreactor (Biolafite) containing 15 L of King B medium, for 24 h and then concentrated 20 times by centrifugation at 4000 rpm. The drying operation lasted approximately 46 to 48 h including two main periods: freezing and

sublimation. During freezing, the sample temperature decreased to a value of -25°C and then progressively increased back to 25°C at 0.9 mbar pressures. Cells were freeze-dried in a Low freeze-drier (LOUW KOELTECHNIEK BVBA) and stored in glass tube (Yao et al., 2008).

### Packaging and storage

Samples of the freeze-dried *P. fluorescens* were stored in glass tube at 60, 37 and 4°C. For each temperature, the dried samples (1 g) were rehydrated in 9 ml of peptone water solution, and sequential dilutions were prepared (Palmfeldt et al., 2003; Santivarangkna et al., 2007). The resulting colonies from samples taken during storage were counted on solid King B medium, and the number of viable cells was measured (Tsen et al., 2007).

### Thermal treatment

Freeze-dried pellet was stored in glass tube for accelerated storage test at 60 and 37°C. At 60°C, sample was removed at 2 h intervals for 10 h of exposure; at 37°C, sample was removed at seven days intervals for 28 days of exposure. Freeze-dried sample was also stored at 4°C to determine the number of viable cells each 15 days during two months. Kinetics parameters (z-value, activation energy, decimal reduction time and the rate constant of temperature) were determined by the method as described by (Mottar, 1984; Yao et al., 2008; Hernandez et al., 2009).

### Kinetics parameters

For an irreversible first-order reaction at constant temperature, the time dependent kinetic parameter can be expressed as the D-value as followed:

$$\text{Log } N = \text{Log } N_0 - \frac{t}{D} \quad (1)$$

The z-value represents a temperature range between which D value changes 10-folds and can be calculated from the D values as shown (1):

$$\text{Log } D = \text{Log } D^* - \frac{T - T^*}{z} \quad (2)$$

The time dependent kinetic parameter can also be expressed as k, which is inversely related to the decimal reduction time (D) as followed:

$$k = 2.303 \frac{1}{D} \quad (3)$$

The rate constant of temperature can be expressed by the Arrhenius model:

$$k = k^* e^{-\frac{E_a}{RT}} \quad (4)$$

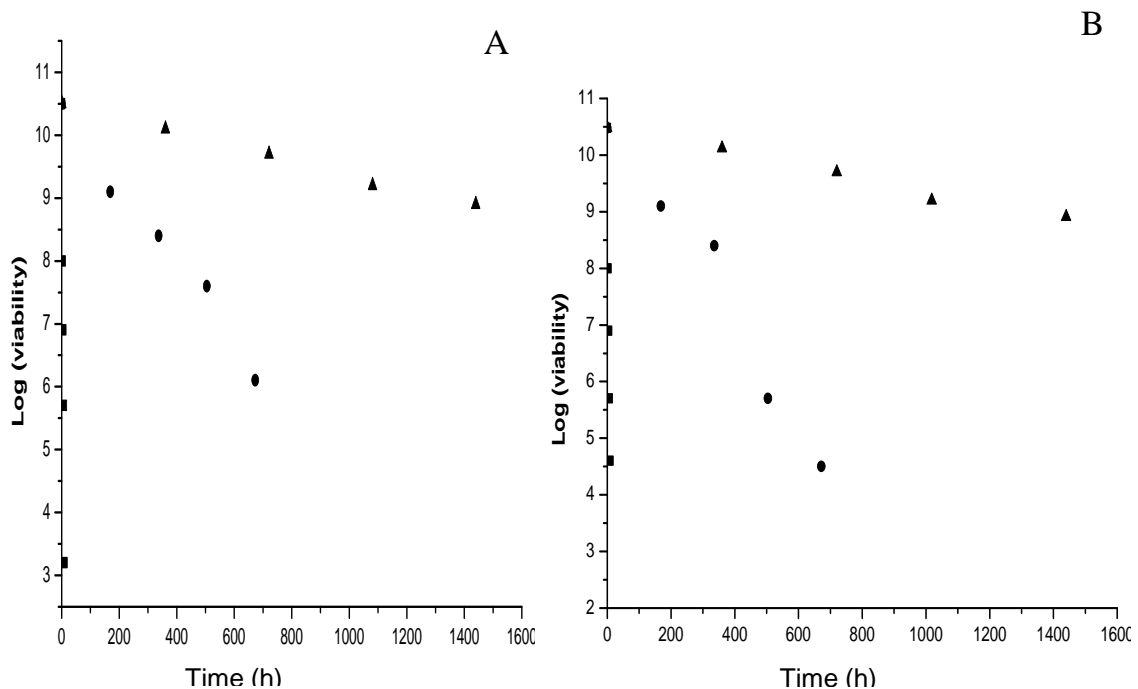
Where, k is the rate constant at the T temperature,  $k^*$  is the pre-exponential term,  $E_a$  is the activation energy of the degradation reaction and R is the ideal gas constant.

### Statistical analysis

Data from three replications were analysed by using analysis of

**Table 1.** Decimal reduction time (D) or rate constant (k) for freeze-dried *P. fluorescens* strains.

<i>P. fluorescens</i> strain	Storage (°C)	D (h)	k (S <sup>-1</sup> )	E <sub>a</sub> (kJmol <sup>-1</sup> )	z (°C)
BTP1	4	1200	0.0019	6.1	32.0±1.1
	37	56	0.021		
	60	1.4	1.67		
PI9	4	1000	0.0023	7.0	49.3±3.5
	37	64	0.036		
	60	1.4	1.69		
BB2	4	3000	0.0008	8.4	27.1±3.5
	37	109.3	0.021		
	60	1.3	1.3		

**Figure 1.** Thermal mortality curves of freeze-dried *P. fluorescens* strains (A) BTP1, (B) PI9, (C) BB2 at 4°C (▲), 37°C (●) and 60°C (■). Each point represents the mean of three counts.

variance to determine if significant differences ( $P \leq 0.05$ ) existed between mean values.

## RESULTS AND DISCUSSION

The stability of freeze-dried bacteria largely depends on the storage temperature. In order to identify with time some parameters related to conservation of freeze-drying *P. fluorescens* strains, we used the Arrhenius theory and the value of  $z$  at 60, 37 and 4°C (Yao et al., 2008). Viable cell counts in glass tubes stored at 60°C for 8 h declined too rapidly to be monitored, and demonstrated that *P. fluorescens* strains BTP1, BB2 and

PI9 are very sensitive to elevated temperature. At 4 and 37°C, *P. fluorescens* (BTP1, BB2, PI9) survived; with far the best survival at 4°C. Figure 1 shows the thermal mortality curves of freeze-dried *P. fluorescens* BTP1 at 60, 37 and 4°C. At each storage temperature, linear regressions were conducted for  $\text{Log}_{10}$  (viability) in function of storage time according to Equation (1). For example, *P. fluorescens* BTP1 decreased from 10.30  $\text{log}_{10}$  to 4.5  $\text{log}_{10}$  at 60°C after 8 h storage, to 5.7  $\text{log}_{10}$  after 28 days storage at 37°C and to 9.2  $\text{log}_{10}$  after 60 days storage at 4°C.

The present results basically confirm literature data (An-Erl King et al., 1998; Achour et al., 2001; Picot and

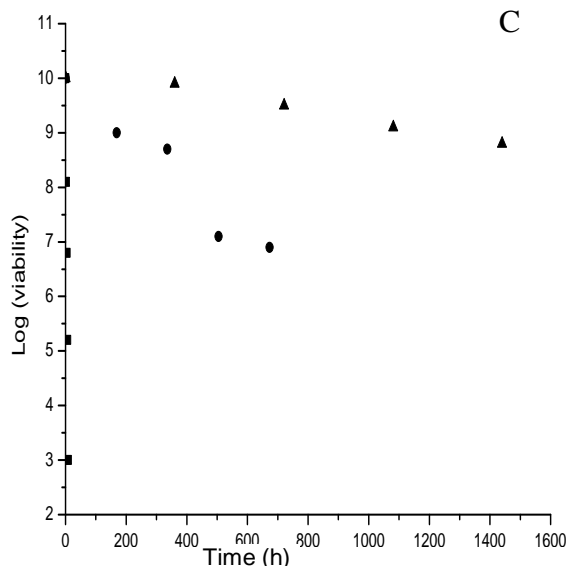


Figure 1. Continued.

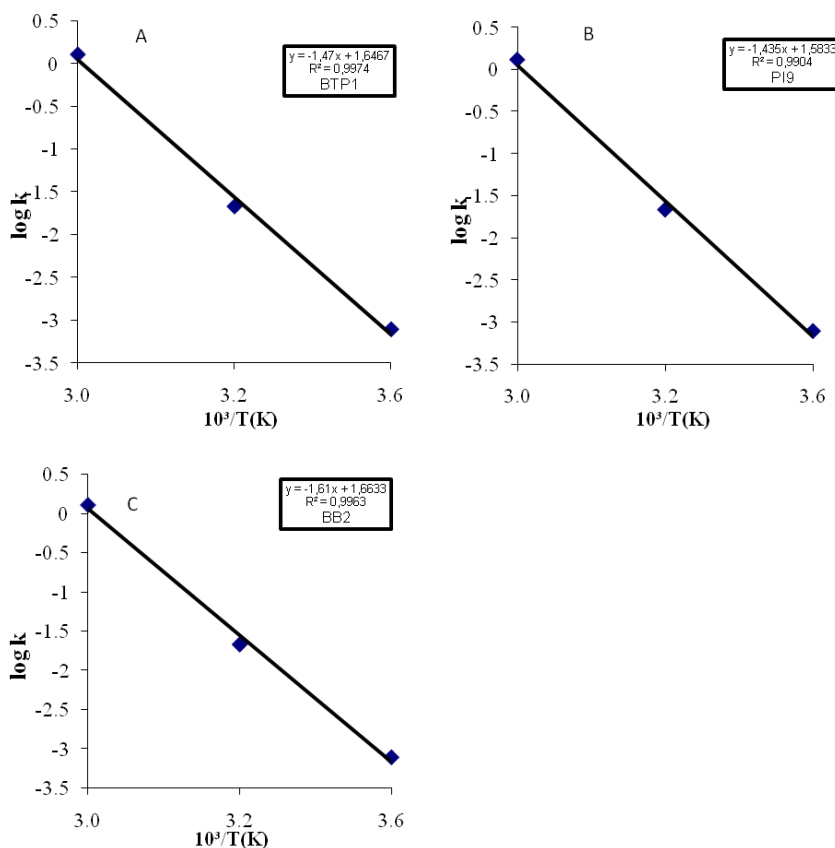


Figure 2. Determination of activation energy ( $E_a$ ) of freeze-drier *P. fluorescens* strains. ♦, BTP1; ■, BB2; ▲, PI9.

Lacroix, 2003; Ziadi et al., 2005). Strain BB2 performs better than BTP1 and PI9 at 37 and 60°C. Some work demonstrated, that experimental determination of bacterial survival at higher temperatures can be

successfully used to predict bacterial survival for lower temperature (Achour et al., 2001; Hernández et al., 2006; Ziadi et al., 2005; Yao et al., 2008). Figure 2 shows the survival rate curves of *P. fluorescens* strains.

The data obtained were used to calculate decimal reduction times (D), inactivation constant (k), activation energy and value of thermal resistance (z) according to equations 2 to 4. z is the change in temperature required to obtain a tenfold increase or decrease to a tenth of the value D, for our strains (BTP1, BB2, PI9), z value obtained were  $32.04 \pm 1$ ,  $1^\circ\text{C}$ ;  $27.10 \pm 1$ ;  $3^\circ\text{C}$  and  $49.3 \pm 3$ ,  $5^\circ\text{C}$ , respectively. The activation energy values (Kj/mol) for the degradation process of the powders stored at glass tube were 6.1 for BTP1, 8.4 for BB2 and 7.0 for PI9. The kinetic parameters k and D calculated from equations (1) and (4), respectively show that the decrease in decimal reduction time (D) or the increase of k temperature dependent (Yao et al., 2008).

The  $E_a$  and z value are inter-related. D and z values were experimentally obtained as given in Table 1 and used to design the heat treatments in order to obtain several inactivation intensities. Increasing the storage temperature causes an increase in cell death, and this shows that the freeze-dried powders of *P. fluorescens* strains does not keep viability for very long time during storage at temperatures above  $30^\circ\text{C}$ .

## Conclusion

Freeze-drying is a useful technology for drying *P. fluorescens* strains. Our results demonstrate that the z-value or Arrhenius models could be used to predict storage stability of freeze-dried *P. fluorescens* at various temperatures. The  $R^2$  value (Figure 2) close to unity is an indication of the applicability of the Arrhenius law, indicating an exponential effect of the storage temperature on the survival rate loss of the studying and predicting the survival rate loss of the strains during storage. Storage temperature at  $60^\circ\text{C}$  had a destructive effect on the viability of the freeze-dried bacteria of all three tested strains.

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