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THERMAL DEATH EVALUATION OF MULTI-STRAINS PROBIOTIC INOCULANT FOR SHELF-LIFE PREDICTION

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ABSTRACT

This study aimed to determine the thermal death kinetic of *Lactobacillus plantarum* 7-40, *Bacillus subtilis* E20, and *Saccharomyces cerevisiae* P13 contained in probiotic product and to study the relation of specific death rate (k_d) and temperature following the Arrhenius equation for shelf-life prediction. The result showed that the viability of probiotic strains was differently affected by storage temperature. The *B. subtilis* E20 could survival more than *L. plantarum* 7-40 and *S. cerevisiae* P13 under high storage temperature. It was also found that the lowest k_d was provided by *B. subtilis*. This result demonstrated that the *B. subtilis* was more stable than *L. plantarum* and *S. cerevisiae* during keeping under high-temperature warehouse. Thereby, the storage temperature of the multi-strains probiotics inoculant should be considered carefully. Also, the natural logarithm of k_d was well related to the storage temperature as the Arrhenius equation. The Arrhenius model could be applied for predicting the shelf-life of probiotic inoculant as well. This study could be used for managing the stock of a multi-strains probiotic product in the high-temperature warehouse.

Key words: Arrhenius equation, probiotic, shelf-life prediction, thermal death

INTRODUCTION

Probiotic is a Latin and Greek-derived word, meaning "for life", a definition of probiotics as substances secreted by one microorganism that stimulate the growth of another (Musikasang *et al*., 2009; Yusuf, 2012). In 2002, an FAO/WHO joint panel defined probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Hossain *et al*., 2017). Most probiotics are bacteria Gram-positive, among which lactic acid bacteria (LAB) but a few molds and yeasts can also be used as probiotics to increase food safety and consumer health by preventing and reducing pathogenic bacteria (Oyetayo & Oyetayo, 2005). Currently, probiotics are used in feeds animal to promoting good digestion, boosting immune function, including inhibiting the growth of pathogenic. Popularity use probiotic powder because ease of use, stability, and flexibility to use (Huang *et al*., 2017). For a good performance of probiotic product is necessary to have high initial probiotics in the digestive tract, probiotic may be reduced, resulting in inadequate animal health promotion (Wirunpan *et al*., 2016). Moreover, storage time, temperature including various factors in the production process (Huang *et al*., 2017). The aims of this study were focused to study the influence of temperature on the survival rate of microorganism contained in probiotic inoculant and to establish the simple equation for predicting the shelf-life of probiotic product.

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MATERIALS AND METHODS

Microorganisms and starter preparation

The *Lactobacillus plantarum* 7-40 was cultured in de Man, Rogosa and Sharpe (MRS) broth and kept at -80°C as a master stock. One milliliter of *L. plantarum* 7-40 was reactivated in 20 mL of MRS broth and incubated at 37°C for 24 hr. Subsequently, the cultured 7-40 strain was scaled up by transferring to 500 mL of MRS broth and incubated at the previous condition for using as a starter for the next experiment.

The *Bacillus subtilis* E20 was re-cultured in 20 mL of medium contained with 20 g of molasses, 5 g of glucose, 0.2 g of KH_2PO_4 and 0.2 g $MgSO_4$ in 1 L of water. Then, the E20 strain was incubated at 37°C, 150 rpm of shaking rate for 24 hr. Next, incubated at 37°C shaking at 150 rpm for 24 hr.

The *Saccharomyces cerevisiae* P13 was the third of probiotic strain was used in this study. It was grown in 20 mL of Yeast Maintenance (YM) broth and cultured at 30°C, 150 rpm of shaking rate for 24 hr.

Solid state fermentation

The probiotic strains were grown separately in soybean meal (SBM) with solid state fermentation (SSF). The starter of strain 7-40 and E20 was separately inoculated to 5 kg of SBM which was mixed with 5 L of water and sterilized at 121°C for 15 min and incubated at 30°C and 35°C for 24 hr, respectively. The cultured yeast P13 was transferred to 5 kg of SBM which added with 5 L of 5% molasses solution and incubated at 30°C for 24 hr. All experiments used 500 mL of starter for SSF. At the end of SSF, the number of viable cell count (VCC) of 7-40, E20 and P13 were increased to 9.0, 8.0 and 7.0 log CFU g^{-1}, respectively.

Production of multi-strains probiotic inoculant

The fermented SBM (FSBM) was dried at 40°C until the moisture content was lower than 10% dry basis in hot air oven. The dried FSBM was then grounded by grinder with 80 mesh size. The powder of each strain was mixed together. The mixed power was added with premixed component (cassava starch and skim milk). The multi-strains probiotic product was further filled in aluminium zip bag for 500 g per bag and used for the next experiment.

Thermal death of multi-strains probiotic inoculant

The multi-strains probiotic inoculant contained in aluminium zip bag was stored at 4, 25, 35, 45 and 55°C. The sample was withdrawn during the time interval for measuring the VCC by pour plate technique with a specific medium. The experiments were done triplicates.

Thermal death kinetic and activation energy

The specific death rate (k_d, day^{-1}) of each strain in product was determined following equation (1).

$$\ln\left(\frac{N_t}{N_0}\right) = -k_d t \quad (1)$$

Where,
N_0 = viable cell number at initial time (CFU g^{-1}).
N_t = viable cell number at t (CFU g^{-1}).
k_d = specific death rate (day^{-1}).
t = storage time (day).

The minimum energy required to achieve the death of microorganisms and proposes the sensitivity of the microorganism response to temperature transpose was expressed as the thermal death activation energy (E_a, J $mole^{-1}$) (Yan *et al*., 2014). The E_a of probiotic strains was calculated through the slope of an Arrhenius plot of $\ln(k_d)$ versus the reciprocal of the absolute temperature ($1/T$) of storage as follows (2) (Hallman *et al*., 2005):

$$\ln(k_d) = \ln A - \left(\frac{E_a}{RT}\right) \quad (2)$$

Where,
A = Arrhenius constant.
E_a = activation energy (J $mole^{-1}$).
R = gas constant (8.314 J $K^{-1}mole^{-1}$).
T = absolute temperature (°K).

RESULTS AND DISCUSSION

Effect of storage temperature on survival of microorganisms contained in probiotic inoculant

The different storage temperatures were experimented for studying the survival of *L. plantarum* 7-40, *B. subtilis* E20, and *S. cerevisiae* P13 during the prolonged storage. Figure 1A-1C presented the loss of viability of probiotic strains during storage. The decline of viable cell number bacterial load was represented by the survival fractions (%) after different storage times. The survival of *L. plantarum* 7-40 (Figure 1A), *B. subtilis* E20 (Figure 1B), and *S. cerevisiae* P13 (Figure 1C) were slightly decreased when the storage temperature lower than 45°C for 55th day except for the P13 for 45th day. However, at 55°C, the survival of *L. plantarum* 7-40, *B. subtilis* E20 were reduced to approx. 60 and 80%, respectively at the end of experiment. Moreover, the survival of *S. cerevisiae* P13 was dramatically decreased to 60% approximately within 18 days and 3 days at 45°C and 55°C, respectively.

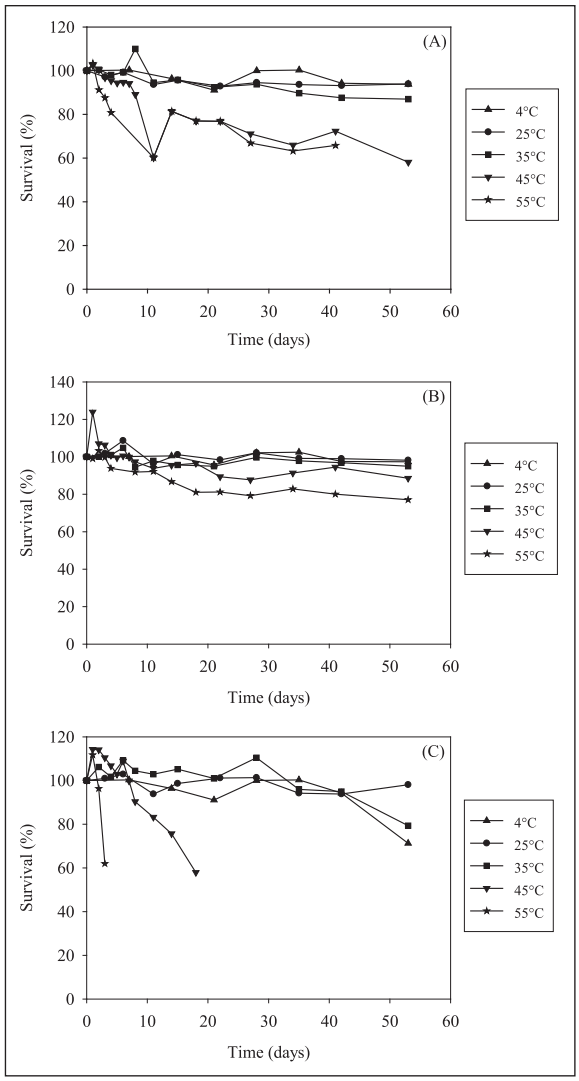

Fig. 1. Survival of probiotic strains 7–40 (A), E30 (B), and P13 (C) at various storage temperature.

The results denoted that the decreasing of viability was accelerated at higher storage temperature. The survival decreasing of *B. subtilis* E20 was the lowest compared with *L. plantarum* 7-40 and *S. cerevisiae* P13. While the survival of *S. cerevisiae* P13 was greatest decreased at higher storage temperature. The optimum temperature of *L. plantarum* 7-40 and *S. cerevisiae* P13 were reported for 37°C (Chiu *et al.*, 2007) and 30°C (Chiu *et al.*, 2010), respectively, while the optimum temperature of *B. subtilis* E20 was 40°C. The results demonstrated that *B. subtilis* could produce the endospore and multi-layered shell for protecting the bacterial genome in stress conditions (Liu *et al.*, 2009). Thereby, the *B. subtilis* would exhibit the thermotolerance characterization more than *L. plantarum* and *S. cerevisiae*.

Thermal death kinetic and activation energy

Equation (1) was used to describe the survival curves which are inherently based on the complex biochemical changes in the microorganisms when subjected to heat. The k_d was the kinetic value of (1) which presented the rate of microbial degeneration during storage at different temperature. The results in Table 1 showed that the k_d of all microorganism was increased with the increase in storage temperature. The highest k_d was observed in *S. cerevisiae* P13 for 0.0300 day^{-1} (Table 1) at 55°C of storage temperature. It was demonstrated that yeast P13 was rapidly destroyed with high temperature more than *L. plantarum* 7-40 and *B. subtilis* E20. This suggested that *S. cerevisiae* P13 was the mesophilic yeast which could not survive under high temperature.

The k_d value from Table 1 was calculated as ln(k_d) and presented with temperature (Kelvin, °K), in term of 1/T (Table 2). The thermal-death-time (TDT) curve for probiotic strains was shown in Figure 2. The curve for *L. plantarum*, *B. subtilis*, and *S. cerevisiae* was described by the linear regression equation in Table 3. The slope of the regression equation was converted to E_a by (2). The highest E_a was obtained from *S. cerevisiae* for 36.22 kJ/mole (Table 4). While the E_a of *L. plantarum* and *B. subtilis* were 19.21 kJ/mole and 8.05 kJ/mole, respectively (Table 4). This indicated that *S. cerevisiae* was more sensitive to temperature than *L. plantarum* and *B. subtilis*. Thereby, the survival rate of *S. cerevisiae* could be used as the indicator for monitoring the shelf-life of probiotic product.

Shelf-life prediction of probiotic strains by the Arrhenius equation

The k_d of probiotic strains at storage temperature was expressed by (2). The obtained k_d was used to construct the simple linear model for estimating the viable cell number during storage. Then, the shelf-life of probiotic strains was determined by (1). Table 5 presented the simple prediction model for estimating the shelf-life of probiotic inoculant based on the VCC. The N_t and N_0 were the VCC of microorganism at the storage time (t) and the initial time, respectively.

Table 1. The specific death rate at 4, 25, 35, 45 and 55°C

Strains	Temperature (°C)	k_d (day^{-1})
L. plantarum 7-40	4	0.0035
	25	0.0050
	35	0.0060
	45	0.0100
	55	0.0129
B. subtilis E20	4	0.0035
	25	0.0040
	35	0.0050
	45	0.0050
	55	0.0061
S. cerevisiae P13	4	0.0026
	25	0.0050
	35	0.0060
	45	0.0190
	55	0.0300

Table 2. Natural logarithm of death rate of micro-organisms at various storage temperature

Strains	Temp (°K)	(1/T)* 1000	ln(k_d)
L. plantarum 7-40	277.15	3.608	-5.6550
	298.15	3.354	-5.2983
	308.15	3.245	-5.2983
	318.15	3.143	-4.6052
	328.15	3.047	-4.3477
B. subtilis E20	277.15	3.608	-5.6550
	298.15	3.354	-5.5215
	308.15	3.245	-5.2983
	318.15	3.143	-5.2983
	328.15	3.047	-5.0936
S. cerevisiae P13	277.15	3.608	-5.9410
	298.15	3.354	-5.2980
	308.15	3.245	-5.1160
	318.15	3.143	-3.9630
	328.15	3.047	-3.5070

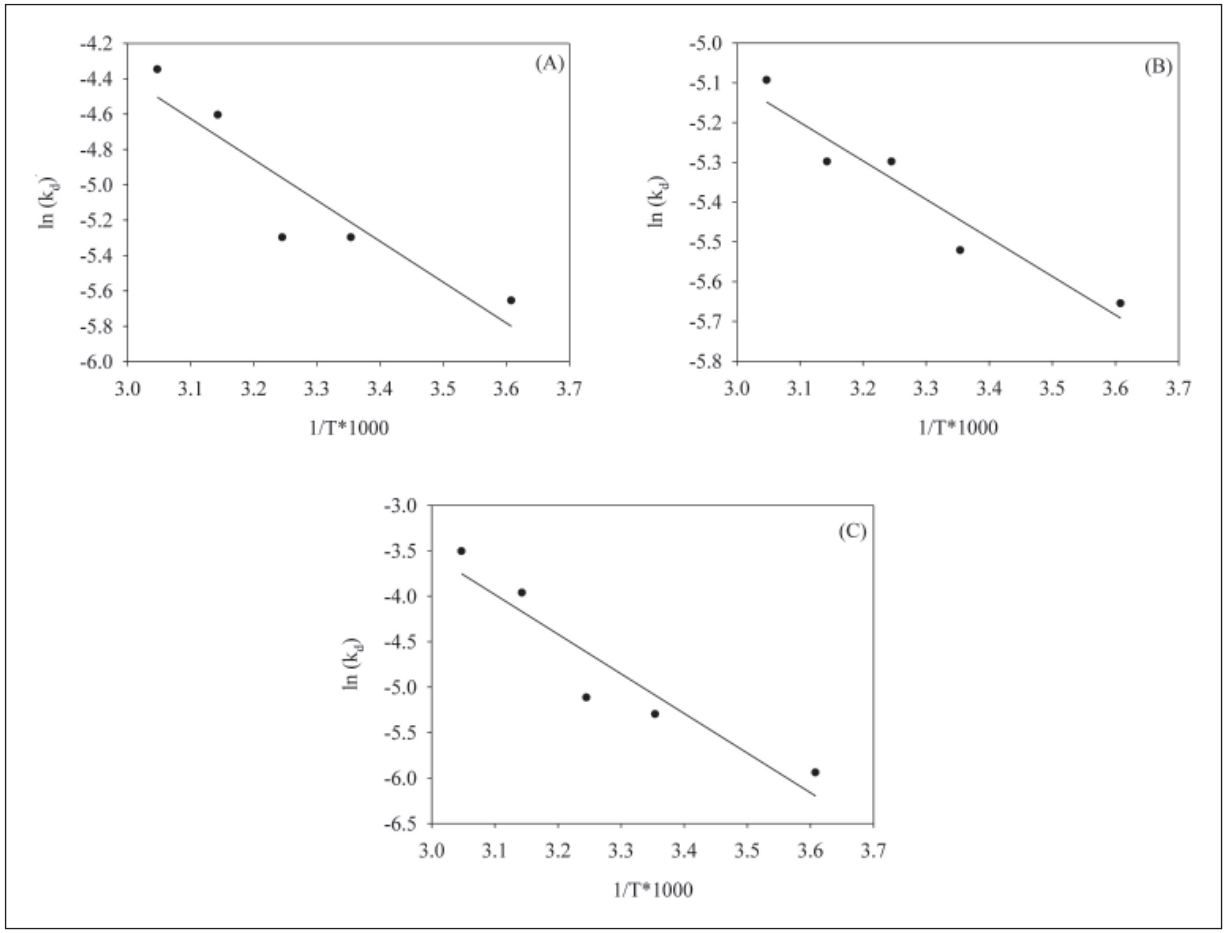

Fig. 2. Arrhenius plot for temperature effects on thermal death rates of probiotic strains of (A) *L. plantarum*, (B) *B. subtilis*, and (C) *S. cerevisiae.*

Table 3. Arrhenius equation of probiotic strains

Strains	Arrhenius equation	R^2
L. plantarum 7-40	$\ln k_d = -2.3101(1/T) + 2.5353$	0.8467
B. subtilis E20	$\ln k_d = -0.9686(1/T) - 0.9207$	0.9207
S. cerevisiae P13	$\ln k_d = -4.3571(1/T) + 9.5242$	0.8846

Table 4. Activation energy of microorganisms in probiotic inoculant

Strains	Activation energy, E_a (kJ mole^{-1})
L. plantarum 7-40	19.21
B. subtilis E20	8.05
S. cerevisiae P13	36.22

Table 5. Simple shelf-life prediction model of probiotic strains at 55°C of storage temperature

Strains	Simple shelf-life prediction model
L. plantarum 7-40	$\log N_t = \log N_0 - (0.0110)t$
B. subtilis E20	$\log N_t = \log N_0 - (0.0058)t$
S. cerevisiae P13	$\log N_t = \log N_0 - (0.0234)t$

CONCLUSIONS

This study provided a thermal death model which explained the response of probiotic strains to storage temperatures. The E_a of each strain was derived from the linear model and were useful to compare the relative heat. The storage temperature affected the survival of microorganisms in all kind of probiotic product. The increasing of temperature would increase the specific death rate of bacteria and yeast probiotic. Thereby the probiotic product needs to avoid high storage temperature for extending the shelf-life of the product.

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