Half-life Estimation of Encapsulated *Enterococcus faecium* IS-27526 by Accelerated Shelf Life Testing (ASLT)

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Abstract. Probiotics are live microbes with beneficial effect to the host when administrated in adequate quantities. Ensuring the functionality of probiotics can be a challenge for manufacturers due to harsh environment conditions during processing, handling and storage. *Enterococcus faecium* IS-27526 is one of novel probiotic strain isolated from dadih, traditional fermented buffalo milk of West Sumatra. The purpose of this study was to determine the effect of microencapsulation, moisture absorber, and storage temperature on the half-life of probiotic powder estimated by Accelerated Shelf Life Testing (ASLT). There were two treatment groups, namely free cell (FC), and microencapsulated cell (EC), with additional treatment in each group, with addition of moisture absorber (MA) and without moisture absorber, stored at various temperatures (5°C, 27°C and 37°C) for 21 days and viable counts was assessed every 7 days. Storage temperature significantly affects the half-life of probiotic powder (p<0.05). At 5°C, EC significantly showed longer half-life than EC-MA, 140.14 ± 5.701 and 113.45 ± 3.242 days, respectively, at 27°C, EC significantly shorter half-life than EC-MA, 38.64 ± 6.831 and 42.35 ± 0.973 days, respectively. Microencapsulation and moisture absorber didn’t show significant effect on half-life (p>0.05) at various temperature storage.

Keywords: *Enterococcus faecium* IS-27526, half-life, microencapsulation, moisture absorber

1. Introduction

Probiotics are involved worldwide on production of functional food products [1][2]. Probiotics defined as microorganisms that influence the health of their hosts when administrated in adequate amounts [3]. Anti-carcinogens, anti-inflammatory, and enhancing immunity of the host are the benefits that probiotics can offer [4][5]. *Enterococcus faecium* IS-27526 is a novel probiotic strain isolated from “dadih”, a traditional fermented buffalo milk curd from West Sumatra, Indonesia [6]. Nowadays, increase demand on probiotic based functional foods is growth rapidly with increasing of awareness about the potential health benefits of probiotic. Viability of probiotics is considered as an important critical parameter during processing, handling and storage [7]. Hence, microencapsulation is needed to ensure the viability of probiotics during processing, handling and storage to protect microbes from environmental conditions ensuring stable viability [8], by incorporating sodium alginate and gelatin in lactic acid bacteria [9] and sodium alginate in lactic acid bacteria [10]. Estimated shelf life of probiotics is crucial for food manufacturers to ensure that probiotic viability at the end of shelf life still for its functionality and efficacy [11]. Several research on shelf life of probiotic based product has been done previously, such as probiotic in yoghurt [12]. Estimated shelf...
life with Accelerated Shelf Life Testing (ASLT) is a popular method in estimating half-life of probiotic products [13]. In the present studies, the viability loss of *Enterococcus faecium* IS-27526 were studied by applying ASLT method. The influence of microencapsulation on *Enterococcus faecium* IS-27526 half-life at different storage condition was also assessed.

2. Materials and Method

2.1. Materials
Free cells and microencapsulated *Enterococcus faecium* IS-27526 were obtained from PT. Ultrajaya Milk Industry (Indonesia). The viability of *Enterococcus faecium* IS-27526 were enumerated on De Man, Rogosa and Sharpe Agar (MRS agar) of Oxoid (Basingstoke, United Kingdom), and Bromocresol purple indicator of Merck (Germany). The moisture absorber used in this research was silica gel.

2.2. Sample Packaging and Storage
Prior to testing, the sample was stored in an aluminum standing pouch with airproof sealing. All samples originated from the same fluid bed-dried batch. The addition of silica gel as moisture absorber was done simultaneously with probiotic powder. Samples of probiotic powders were stored for 21 days at 5°C, 27°C, and 37°C. The experiments were done by two replications for each temperature and group of samples as shown at Table 1.

| Sample Name | Descriptions                          |
|-------------|---------------------------------------|
| FC          | Free-cell *Enterococcus faecium* IS-27526 |
| FC-MA       | Free-cell *Enterococcus faecium* IS-27526 + Silica Gel |
| EC          | Microencapsulated *Enterococcus faecium* IS-27526 |
| EC-MA       | Microencapsulated *Enterococcus faecium* IS-27526 + Silica Gel |

2.3. Viable counts
The viability of *Enterococcus faecium* IS-27526 were determined every 7 days for 21 days by culture dependent technique, a pour plate on MRS agar with addition of purple bromocresol as an indicator in CFU/g [14][15]. The samples for each temperature and group were rehydrated using ringer solution and sequential dilutions were prepared. The sample was incubated at 37°C for 48 hours before being enumerated by colony plate counter on the formation of yellow colonies of lactic acid bacteria [16].

2.4. Determination of Reaction Kinetic Model and Half-Life
Kinetic model of the survival rate during storage based on experimental data for the samples was determined following zero-order as in equation (1) and first-order kinetic models as in equation (2) [17]. The degradation reaction order was determined by the value of $R^2$ (coefficient determination) of the degradation rate at zero-order model (linear rate) and first-order kinetic models (exponential rate). The slope of the line is equal to the reaction rate constant of the degradation reaction.

$$C = C_0 - kt \quad (1)$$
$$C = C_0 \exp(-kt) \quad (2)$$

Where $C$ and $C_0$ are the viable counts of *Enterococcus faecium* IS-27526 at half-life period and at the initial storage time respectively; $t$ represents the half-life; $k$ represents the reaction rate constant. The half-life for each temperature and group of samples were calculated.
2.5. Estimation of Half-life at -20°C Storage Temperature

The estimation of half-life of probiotic powder at -20°C as a representative of frozen storage condition was done using extrapolation of reaction rate constant from 5°C as in equation (3)[18].

\[
\ln \frac{k_2}{k_1} = \frac{E_a}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)
\]  
(3)

Where \( k_2 \) and \( k_1 \) are the reaction rate constant at -20°C and 5°C respectively; \( T_2 \) and \( T_1 \) are the storage temperature at -20°C and 5°C respectively; \( \frac{E_a}{R} \) is a slope from the graph of reaction rate constant (Ln k) versus storage temperature (1000/K). The half-life for each group of samples at -20°C were calculated.

2.6. Statistical Analysis

The half-life data were evaluated by using analysis of variance (ANOVA) and followed by Duncan’s Multiple Range Test (DMRT) at a significance level of \( p<0.05 \) using SPSS (Statistical Product and Service Solutions) Version 21.0 (IBM, United States).

3. Results and Discussion

3.1. Determination of Kinetic Model Reaction on Loss of Viability

The parameters of degradation reaction were determined by assuming the order of the reaction then fitting the experimental data to the kinetic model. The viability of \textit{Enterococcus faecium} IS-27526 was used as critical parameter to determine the kinetic model reaction by following zero-order and first-order kinetics models as shown in Figure 1 with the reaction rate constant and \( R^2 \) (coefficient of determination) in Table 2.

| Sample | Storage Temperatures (°C) | Zero Order | First Order |
|--------|---------------------------|------------|-------------|
|        |                           | \( k \) (day\(^{-1}\)) | \( R^2 \) | \( k \) (day\(^{-1}\)) | \( R^2 \) |
| FC     | 5                         | 4.6908 x 10\(^7\) | 0.7928 | 7.3518 x 10\(^{-2}\) | 0.8400 |
|        | 27                        | 5.8486 x 10\(^7\) | 0.7906 | 1.7127 x 10\(^{-1}\) | 0.9311 |
|        | 37                        | 5.8968 x 10\(^7\) | 0.6480 | 3.4830 x 10\(^{-1}\) | 0.9843 |
| FC-MA  | 5                         | 4.9366 x 10\(^7\) | 0.8773 | 8.0274 x 10\(^{-2}\) | 0.7605 |
|        | 27                        | 5.8859 x 10\(^7\) | 0.8105 | 1.4861 x 10\(^{-1}\) | 0.9974 |
|        | 37                        | 5.8728 x 10\(^7\) | 0.6386 | 3.7201 x 10\(^{-1}\) | 0.9990 |
| EC     | 5                         | 4.9420 x 10\(^7\) | 0.7225 | 7.6044 x 10\(^{-2}\) | 0.7251 |
|        | 27                        | 7.0603 x 10\(^7\) | 0.8179 | 2.7641 x 10\(^{-1}\) | 0.9391 |
|        | 37                        | 6.205 x 10\(^7\) | 0.7564 | 3.7201 x 10\(^{-1}\) | 0.9734 |
| EC-MA  | 5                         | 5.9641 x 10\(^7\) | 0.8367 | 8.0274 x 10\(^{-2}\) | 0.7451 |
|        | 27                        | 6.8317 x 10\(^7\) | 0.8893 | 1.4861 x 10\(^{-1}\) | 0.9109 |
|        | 37                        | 7.4748 x 10\(^7\) | 0.8052 | 3.7201 x 10\(^{-1}\) | 0.9057 |
Figure 1. The fitted zero order (a, b, c, d) and first order (a', b', c', d') kinetic models of *Enterococcus faecium* IS-27526 viability loss during storage for 21 days at 5°C (●), 27°C (▲) and 37°C (■), for different treatment (a) FC, (b) FC-MA, (c) EC and (d) EC-MA.
The graphical representation of ln CFU/g versus storage time showed a straight line and coefficient of determination for a first order reaction close to unity indicating the kinetic model of degradation reaction was following first order kinetic model. The viability of all samples in general were decreased during storage at various rates depending on the storage temperature. The slope of the graph is equal to the reaction rate constant. Higher rate of viability loss is shown with steeper trendline. The viability loss reaction was characterized as first order kinetic model as shown by exponential decrease of viability during storage in accordance with previous research [19].

3.2. **Half-life of* Enterococcus faecium* IS-27526**
As the reaction rate constants were determined at three different temperatures, the principle of ASLT can be applied to predict the half-life of *Enterococcus faecium* IS-27526 at different storage conditions. The estimated time can alternatively be presented as the half-life period which corresponds to the time consumed when total population is equal to half of the initial population. The half-life at different storage temperatures and treatments were shown in Table 3.

**Table 3.** Effect of temperature and microencapsulation with addition of moisture absorber on the half-life values (in days) of *Enterococcus faecium* IS-27526.

| Storage Temperatures (°C) | Half-Life          |
|---------------------------|--------------------|
|                           | FC                 |
|                           | FC-MA              |
|                           | EC                 |
|                           | EC-MA              |
| 5                         | 143.21 ± 7.973a    |
|                           | 131.17± 6.822ab    |
|                           | 140.14 ± 5.701a    |
|                           | 113.45 ± 3.242b    |
| 27                        | 63.88 ± 7.881c     |
|                           | 70.74 ± 0.545c     |
|                           | 38.64 ± 6.831d     |
|                           | 42.35 ± 0.973d     |
| 37                        | 30.21 ± 1.211d     |
|                           | 28.25 ± 0.164d     |
|                           | 28.69 ± 0.092d     |
|                           | 27.71 ± 0.115d     |

Values followed with the different letters are significantly different (p<0.05).

EC has significant shorter half-life than the FC and FC-MA stored at 27°C (p<0.05) and at 5°C, EC-MA has significant shorter half-life than EC and FC (p<0.05). Meanwhile the probiotic powder stored at 37°C there was no significant difference on half-life in all treatment (p>0.05). The higher the storage temperature the lower the half-life period due to higher metabolic rate as shown by significant shorter half-life of probiotic powder at 37°C than other storage temperature (p<0.05). Higher storage temperature resulted in shorter lag phase and reached death phase faster. Hence, shorter half-life of probiotics powder [20]. Previous research discussed that application of microencapsulation could inhibit the oxygen exposure of the aerobic probiotic cells that could lower its viability during storage [21]. Meanwhile, moisture absorber didn’t show significant effect on half-life in all samples at each storage temperature. However, at 5°C the presence of moisture absorber significantly shortened the half-life of microencapsulated probiotic (p<0.05). Previous research about encapsulated *Escherichia coli* that stored with silica gel shown the decrease of viability [22]. Bacteria that stored with silica gel are exposed to osmotic pressure stress. Exposure to silica gel will promote the lysis of the cell membrane that will improve the diffusion rate through the membrane that will increase the reaction kinetic rate [23]. The result was on the contrary of general phenomena, addition of moisture absorber will be lowering water activity of product throughout half-life that will be slowing the metabolic rate of the probiotic bacteria which contribute to lengthen the half-life of probiotic powder by delaying the stationary and death phases [24].
The reaction rate constant and the estimated half-life at -20°C at several treatments is shown in Table 4. The estimation was based on the reaction rate constant at 5°C by Arrhenius equation. Microencapsulation and addition of moisture absorber did not significantly affect the estimated half-life at 20°C of each treatment (p>0.05), which were in a range of 143.41 ± 7.998, and 113.60 ± 3.249, for FC and EC-MA, respectively.

4. Conclusion
The viability loss of Enterococcus faecium IS-27526 at different treatments were determined under different storage condition. Total viable counts during storage were used to determine the apparent kinetics of the degradation rate. The reaction rate constant for entire group of samples were determined to find out the effect of storage temperature, application of microencapsulation and addition of moisture absorber on the stability of viable counts as estimated half-life. It was confirmed that storage temperature showed significant negative correlation with viability by accelerating the lag phase, moreover, microencapsulation did not show significant effect on the half-life period and addition of moisture absorber resulted in shorter half-life. ASLT is a valid method to estimate the half-life of Enterococcus faecium IS-27526 powder. The estimated half-life period of Enterococcus faecium IS-27526 stored at -20°C and 5°C were not significantly different, hence, optimal storage condition to maintain its viability is at 5°C.

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