Homogeneity and stability of a secondary microbiological reference material candidate for *Salmonella* in fish matrix

E Kurniawati1,2*, B Ibrahim2 and Desniar2

1Ministry of Marine Affairs and Fisheries Republic of Indonesia, Indonesia
2Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University (IPB University), Bogor, Indonesia

*E-mail: ethie_tea@yahoo.com

Abstract. Reference material (RM) is defined as a material, sufficiently homogeneous and stable with respect to one or more specified properties which has been established to be fit for its intended use in a measurement process. RM is one of the tools used to assess laboratory performance on an ongoing basis, as quality control in conducting testing and can be used to calibrate an equipment and to validate or verify the testing method. Currently microbiological reference materials with fish matrices are not much available so that there is a need for RM with fisheries matrices specification. Comprehensive studies of homogeneity and stability are need to be able to develop of any reference material. Initially, a feasibility study revealed a suitable processing procedure for fish matrix, the fish protein hydrolysate (FPH) was prepared by enzymatic hydrolysis, freeze-drying, and milling. Microbiological reference material with fish matrix were shown to be sufficiently homogeneous, and storage at temperatures of -20°C for 4 weeks statistically showed the effect of storage time (P value that was significant at 5% significance level). These results provide the basis for the development of a RM for *Salmonella* in fish matrix.

Keywords: FPH, homogeneity, microbiological reference material, stability

1. Introduction

Testing laboratories that have been accredited with ISO / IEC 17025: 2017 are required to be able to provide reliable test results through a quality assurance of test results, one of which is the use of Certified Reference Material (CRM) and internal quality control using materials secondary reference (SNI 2008). Reference material is a material which has one or more homogeneous material properties and is stable enough to be used in equipment calibration, assessment of test methods or as a standard in confirmation or sample analysis (ISO 2015). The reference material used, in addition to being homogeneous and stable, must also be representative that is in accordance with or approaching the sample matrix of routine tests conducted. What often becomes an obstacle is the availability of reference materials in accordance with the expected testing, namely test parameters, test matrices, concentrations and prices. The use of reference materials in Indonesia is constrained due to the absence of a provider of CRM, so that testing laboratories rely on CRM from overseas providers. At present the microbiology secondary reference material with a fish matrix in Indonesia is not yet
available so there is a need from the fisheries laboratory for the microbiology reference material with a specific fisheries matrix.

The production of microbiological secondary reference material is basically an encapsulation process, which is a process of coating a core material, in this case a microbe, using a certain encapsulation material. Freeze drying is a method commonly used to preserve bacterial culture (Sumanti et al 2016). Pispan et al (2010) stated that protein especially milk protein is suitable to be used as an encapsulation material for lactic acid bacteria because protein has good binding properties to form cell wall material so that the damage to microbial cells due to the freezing process can be minimized. Rizqiati et al (2008) stated that the use of protein as a coating can maintain bacterial resistance. The role of proteins and sugars is believed to be important in cell stabilization because they are believed to interact with cell membranes. The development of making microbiological reference materials is often hampered by the instability of living organisms and the inherent statistical variability of microbiological procedures. The making of microbiological reference materials is still widely used in skim milk (Mooijman and Havelaar 1997, Jarvis 2014, In’t Veld et al 1998, Phillip et al 2007) as a bacterial coating material.

Fish protein hydrolysates (FPH) are products of hydrolysis reaction on peptide bonds in proteins and result in shorter peptides that contain 2–20 amino acids (Parvathy et al 2018b). Generally, protein can be hydrolyzed by chemical process or protease enzymes. Chalamaiah et al (2010) said that FPH have potential application as functional ingredients in different foods because they possess numerous important and unique properties such as water holding capacity, oil absorption capacity, protein solubility, gelling activity, foaming capacity and emulsification ability. FPH can be used as an excellent source of nitrogen for maintaining the growth of different microorganisms. Ghorbel et al (2005) used various FPH from sardinelle (Sardinella aurita) as nitrogen sources for the production of extracellular lipase by the filamentous fungus Rhizopus oryzae and reported that the best results were obtained with defatted fish protein hydrolysates. In another study by Safari et al (2012) showed that the hydrolysates generated from yellowfin tuna (Thunnus albacares) head waste have been shown to be effective in promoting the growth of lactic acid bacteria better than the commercial MRS media. Studies related to FPH as a matrix in the manufacture of secondary microbiological reference materials have not been done much. Tuna protein hydrolysate (TPH) is expected to be a matrix in making microbiological secondary reference materials. This study aims to determine the characteristics of TPH used as a matrix and determine the best TPH concentration in order to produce a candidate microbiological secondary reference material that is homogeneous and stable.

2. Materials and methods

This research was conducted in January to May 2019 at The National Center for Examination of Fisheries Product Implementation (BBP2HP), Jl. Raya Setu No.70 Cipayung East Jakarta and PT. Saraswanti Indo Genetech, Bogor.

2.1. Materials

Tuna (Euthynnus affinis) (weight range 1-1.4 kg) in this research were obtained from fish cold storage in Muara Baru, North Jakarta Indonesia and enzyme papain water soluble 30,000 USP-U/mg (EC 3.4.22.2) was purchased from Merckmillipore with specific activity 500 U/mg. For the development of a reference material, we use a strain Salmonella enteritica sv Enteritidis (ATCC 13076) from American Type Culture Collection.

2.2. Methods

2.2.1. Preparation of raw materials. The tuna fish samples were weighed, filled without skin, and separated by meat to be used as raw material for fish protein hydrolysis. The tuna fish meat was then
mashed using a food processor, then the proximate composition test (moisture content, ash content, protein content and fat content) was tested.

2.2.2. **Fish protein hydrolysate preparation.** In this study, fish protein hydrolysate preparation refers to research conducted by Nurhayati et al (2013) that has been modified using a sample of tuna fish with the enzyme concentration of papain 5% (w/w) and hydrolysis time of 5 hours. Fish meat that has been crushed, then homogenized with distilled water (ratio 1:2) using a shaker for 2 minutes. The pH value of the mixture was adjusted to reach pH 7.0 by adding 1 M NaOH solution or 1 M HCl solution. Furthermore, the fish meat mixture was added with papain enzyme with a concentration of 5% (w/w). The hydrolysis was carried out at 55°C using waterbath shaker for 5 hour. After the hydrolysis process is completed, the enzyme papain was inactivated at 80°C for 20 minutes to stop the hydrolysis process. Samples were centrifuged at a speed of 5,000 rpm for 20 minutes at 4°C in order to separate the supernatant and pellet which thereafter the supernatant was dried using freeze dryer for 96 hours at -40±2°C. The fish protein hydrolysate was tested for the proximate composition (moisture content, ash content, protein content and fat content) following method of AOAC (2005).

2.2.3. **Preparation of bacterial strains.** Preparation of bacterial culture was preceded by a preliminary test to determine the purity and population of *Salmonella* bacteria. The population of *Salmonella* bacteria was carried out by inoculation in a 10 mL BHI broth and incubated for 18-24 hours at 35±2°C, then TPC testing was carried out according to SNI (2015). The number of bacterial populations inoculated into the reference material was 10^2 CFU/mL.

2.2.4. **The production of microbiology secondary reference material.** The secondary microbiology reference material was processed refers to research conducted by the National Center for Examination of Fisheries Product Implementation (2017). After the bacterial culture was ready, the candidate of microbiological reference materials processed with a fish matrix with 5 different concentrations (10%, 11%, 12%, 13%, and 14%). The tuna fish hydrolysate was weighed according to each concentration and dissolved in distilled water. The solution was added with sodium glutamate, 40% glucose solution, gelatin and homogenized using a magnetic stirrer. The pH of homogenate solution reached up to pH 7.2 and it was sterilized using an autoclave at 110°C for 15 minutes. The sterile homogenate solution was inoculated with *Salmonella* bacterial culture (10^2 CFU/mL) and then piped and put into vials using a sterile pipette. The vials were frozen using dry ice and 95% absolute ethanol for ±1.5 hours then freeze dried using a freeze dryer at -40±2°C for 36-48 hours. The candidate of secondary microbiological reference material that has been dried is then tested for homogeneity and stability.

2.2.5. **Viability test.** Viability test was done by calculating the bacterial population before the freeze drying process and immediately after the freeze drying process is completed. Viability of bacterial cells was calculated using the TPC method with a series of dilutions in buffer phosphate buffered solution, then it was grown in PCA culture media. Bacterial survival was determined by calculating colony-forming units per ml (CFU/mL). The percentage of bacterial viability was calculated by using the following equation (Rizqiati et al 2008).

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\text{Viability} \% = \frac{\log_{10} \text{CFU/mL after freeze dry}}{\log_{10} \text{CFU/mL before freeze dry}} \times 100 \%
\] (1)

2.2.6. **Homogeneity test.** Homogeneity test was done by taking 10 vials randomly from the batch produced and tested by duplicate for each vial. The total plate count (TPC) method was carried out according to SNI 2332-3-2015 (SNI 2015). Homogeneity studies were carried out using statistical evaluations to compare the dispersion of observations on several units of reference material, with the accuracy of measurement procedures to determine the standard deviations between units. The
distribution of results was checked using normal probability plots and histograms. Finally, an analysis of variance (ANOVA) was performed to quantify the within-bottle standard deviation and the between-bottle standard deviation.

**Stability test.** There are two types of stability test for microbiological reference material candidate, a stability test at -20°C and at higher temperatures. The stability of the material stored at higher temperatures was determined at two different temperatures. The tested temperatures were -20°C, 30°C, and 37°C. Once a week, over a period of 4 weeks, three vials from each storage temperature were examined in duplicate on PCA medium.

3. Results and discussion

The chemical composition of tuna fish meat was determined from the proximate composition in the form of moisture content, ash content, protein content and fat content which can be seen in table 1.

| Proximate composition | % wet basis | Red meat of tuna fish (%)* | White meat of tuna fish (%)* |
|-----------------------|-------------|---------------------------|----------------------------|
| Moisture              | 73.18±0.03  | 72.94±0.23                | 74.54±0.46                |
| Ash                   | 1.60±0.02   | 1.34±0.10                 | 1.12±0.02                 |
| Protein               | 24.60±0.40  | 28.34±1.63                | 26.34±0.79                |
| Fat                   | 0.57±0.23   | 1.11±0.04                 | 1.10±0.07                 |

* Parvathy *et al* (2018b)

As shown in table 1, based on the raw material of tuna meat wet weight that used, it has a 73.18%, moisture content. The base on wet weight of tuna meat, it has 1.60%, ash content, 24.60%, protein content and 0.57% fat content. Tuna contain high amounts of protein, in this study, tuna meat which was used as the raw material for preparation of TPH contained 24.60% protein. Parvathy *et al* (2018a) said that the protein content of most tuna species ranged between 15-30%. In this study, tuna meat protein content is smaller than the results of research conducted by Parvathy *et al* (2018b), the protein content of white meat and red meat of tuna fish is shown to be 26.34% and 28.34%.

3.1. Proximate analysis of tuna protein hydrolysate

The hydrolysis of tuna fish meat was carried out using the commercial papain enzyme (Merck, Kenilworth, USA) with the used enzyme activity of papain at 500 U/mg, this means that 1 mg of the papain 5% (w/w) enzyme can catalyze the hydrolysis reaction to convert 500 µmol of protein substrate per minute into tuna fish protein hydrolysate products. The yield of tuna protein hydrolysate obtained was 12.09%. In the process of hydrolysis, not all substrate is destroyed and hydrolyzed but some substrates are precipitates. Wijayanti *et al* (2015) said that the enzyme concentration had a significant effect on the yield of hydrolyzed milkfish FPH using the enzyme papain. The results of hydrolysis in the form of supernatant were dried with freeze drying so that the protein hydrolysate powder of fish was obtained. Tuna protein hydrolysate that obtained from the hydrolysis process is the form of clean yellowish white color powder as seen in figure 1.

The proximate content of tuna protein hydrolysate can be seen in table 2. Several studies of fish protein hydrolysates were made from tuna fish raw materials have been carried out with different chemical compositions. This is influenced by the concentration of enzymes and the type of fish used.
Figure 1. Tuna protein hydrolysate.

Table 2. Proximate compositions of tuna protein hydrolysates (E. affinis).

| Parameters        | TPH   | TPH (E. affinis) * | TWPH (E. affinis) ** | TRPH (E. affinis) ** |
|-------------------|-------|--------------------|----------------------|----------------------|
| Moisture content (%) | 4.65±0.04 | 1.35±0.15          | 11.96±0.12           | 12.95±0.14           |
| Ash content (%)   | 7.41±0.03 | 4.03±0.02          | 6.31±0.80            | 7.32±0.61            |
| Protein content (%) | 87.50±0.14 | 89.90±0.60         | 78.01±1.09           | 75.17±1.55           |
| Fat content (%)   | 0.25±0.01 | 2.71±0.06          | 4.28±0.49            | 4.05±0.48            |

*Parvathy et al (2018a); ** Parvathy et al (2018b)

The tuna protein hydrolysate has 4.65% moisture content. The moisture level difference on tuna fish protein hydrolysates is very dependent on the drying method. The low moisture content of tuna fish protein hydrolysates was related to the freeze drying method that was used in this study, there are samples experience loss of moisture content during the drying process (Seniman et al 2014). The level of TPH ash was 7.41%, greater than the ash content of the research results by Parvathy et al (2018a) and Parvathy et al (2018b). Several studies have reported that FPH ash content varies depending on the type of fish and the process. Salamah et al (2012) said that high ash content in FPH is caused by the addition of alkali compounds such as NaOH and/or acid compounds for example HCl, in the process of protein hydrolysis that aimed at achieving the optimum pH value of the enzyme and keep the pH constant during the hydrolysis process, so that the enzyme peptide’s breaks can continue. The mixture of acid and alkali compounds in the protein hydrolysate solvent will cause the formation of salt compounds so that they can increase the ash content of the protein hydrolysate.

Parvathy et al (2018a) said that many researchers have reported that the protein content of fish protein hydrolysate range from 60% to 90% of total composition and that the high protein content was due to solubilization of protein during hydrolysis and removal of insoluble solid matter by centrifugation. The fat content of tuna fish protein hydrolysate shows lower value at 0.25%. Wijayanti et al (2016) said that low fat content was generally more stable and durable when compared to high fat protein hydrolysate products. Furthermore, Seniman et al (2014) said that the low fat content in tuna fish protein hydrolysates was caused by the removal of insoluble fat at centrifugation at 4°C.

3.2. Amino acid composition of TPH

The amino acid composition of fish protein hydrolysates is important because of the nutritional value and the influence on the functional properties. In addition, the composition of amino acids plays a role in the functional. Burges and Shaw (1986) in Sari (2008) reported that papain enzyme can break the peptide bond in the residue asparagine-glutamine, glutamate-alanine, valine and leucine-phenylalanine-tyrosine. Seniman et al (2014) said that the major amino acids of hydrolysates prepared
by Alcalase and Papain were Glu, His, Lys and Asp. The amino acid composition of tuna fish protein hydrolysate is presented in figure 2. The highest amino acid content is glutamic acid of 44.12 mg/g, histidine is 31.20 mg/g and aspartic acid is 27.41 mg/g.

![Amino acid composition of tuna protein hydrolysate](image)

**Figure 2.** Amino acid composition of tuna protein hydrolysate.

3.3. Viability test of Salmonella sp.

Viability test was carried out to determine the resistance of Salmonella bacteria in the process of making microbiological reference material that is after the freeze drying process. The viability of Salmonella bacteria after freeze drying at each concentration can be seen in figure 3.

![Viability of Salmonella sp. in candidate microbiological reference material](image)

**Figure 3.** Viability of Salmonella sp. in candidate microbiological reference material.

Microbiological secondary reference material with a fish matrix in this study was made by freeze drying technique. Dimitrellou et al (2016) states that among the drying methods, freeze drying is the most commonly used method to preserve microorganisms, because freeze drying provides protection from spoilage and contamination, ease of storage and longer survival. Martin et al (2015) stated that freeze drying (lyophilization) can be divided into 3 steps, namely freezing, primary drying and secondary drying. Ice crystals form during freezing so they can damage bacterial cells. The formation of ice crystals depends on the speed and freezing temperature. A quick freezing rate is preferred over a slow freezing rate, since it will lead to the formation of smaller ice crystals avoiding extensive cellular damage (Fowler and Toner 2005). In addition to the formation of ice crystals that can damage bacterial cells, damage to substances dissolved in the fraction that remains when water crystallizes can also harm bacteria. At the primary drying stage, frozen water is removed by sublimation under vacuum conditions, whereas at the secondary drying stage, non-frozen water is removed by desorption (Maltesen and van de Weert 2008). Removal of water from bacterial cells can cause extensive damage to surface proteins, cell walls and cell membranes, and reduce its viability after drying (Castro et al 1997, Teixeira et al 1994).
The results showed that the viability of Salmonella at 5 different FPH concentrations of tuna fish was above 50%. The highest viability of Salmonella was obtained at a concentration of 14% in the amount of (67.08±1.21)%. Salmonella viability tends to increase with the amount of TPH concentration of tuna fish used. Production of candidate microbiological secondary reference material is basically a process of encapsulation using TPH as a matrix and coating material for microorganisms. Chen et al (2006) said that proteins have the ability to interact, protect, and reverse bonds with various active compounds through their functional groups. Furthermore Martin et al (2015) said that various protectors (cryoprotectants) were added to the drying media before the freeze drying process to protect the survival of probiotics during dehydration. FPH has the ability as a cryoprotectant to maintain the quality of frozen fish mince as reported by Cheung et al (2009). The positive effect of the use of protein on dry microorganisms due to its capacity to protect cells by stabilizing cell membrane constituents and to make porous structures in freeze dried products that make rehydration easier (Selmer-Olsen et al 1999).

3.4. Homogeneity test of candidate microbiological reference material

Homogeneity is an important requirement for reference materials including homogeneity within and between units. Homogeneity between units and within units is important to ensure that each unit of reference material carries the same value so that if a subsample is taken by the user, the reference material has the same value. Maier et al (1993) stated that prepare material is homogeneous is the process that is difficult and expensive. Homogeneity test results of the reference material with a fish matrix (TPH) are presented in table 3.

| Table 3. Homogeneity test of candidate of microbiological reference materials with a fish matrix. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Concentration of TPH (%) | 10% | 11% | 12% | 13% | 14% |
| RSD a | 0.16 | 0.15 | 0.06 | 0.46 | 0.04 |
| $S_{bb}$ b | 0.17 | 0.21 | 0.08 | 0.42 | 0.03 |
| $S_w$ c | 0.12 | 0.09 | 0.06 | 0.13 | 0.05 |
| P-value (ANOVA)d | 0.0116 | 0.0003 | 0.0110 | 0.00003 | 0.1463 |

a Relatif standard deviation of all measurement  
b between-bottle standard deviation  
c within-bottle standard deviation  
d P-value

Homogeneity is an important requirement for all reference materials and includes both within- and between-unit homogeneity. Between-unit homogeneity is important to ensure that each RM unit carries the same value for each property; within-unit homogeneity is important where subsample can be taken for measurement by users of the material. ISO 17034 accordingly requires the assessment of the homogeneity of a reference material. Homogeneity can be determined one of them by confirmation using a one-way ANOVA design, that the between unit term is not statistically significant at the 95% confidence. The results showed that only at 14% concentrations of TPH showed homogeneous conditions (P-value > 0.05). This is possible because with higher concentrations of FPH of tuna fish, Salmonella bacteria can be encapsulated better than lower concentrations of TPH of tuna so that the distribution of bacteria in the reference material is better. Sugindro et al (2008) showed that the higher the concentration of the coating material, the encapsulation efficiency is increasing, the wall layer is getting better and stronger so that it can protect the core material well.

3.5. Stability study of microbiological reference material candidate

Reference materials should be sufficiently stable for their intended use, so that the end user can rely on the assigned value at any point within the period of validity of the certificate. The value of each property can change over time for variety of reason, to different degrees, and at different rates
depending on the conditions. Three sets of conditions are particularly important: conditions during long-term storage at the RM producer’s, conditions during transport to the user’s premises, and the specified conditions of storage and use at the user’s premises (ISO 2017). The results of the stability test of reference material candidates with the fish matrix can be seen in figure 4.

**Figure 4.** Result of stability tests of candidate microbiological reference material with fish matrix stored at various temperatures during 4 weeks: -20°C (♦), 30°C (■) and 37°C (▲).

An isochronous short-term stability study revealed no significant trends for storage temperature of -20°C (P-value > 0.05). The stability of the reference material stored at 30°C was reached only until the second week of storage, but could not be achieved at storage temperature 37°C.

The making of this reference material candidate was carried out by adding glucose, monosodium glutamate and gelatin as lyoprotectant to increase the stability of the candidate reference material. Addition of lyoprotectant from type of protein or carbohydrate does not always result in increased viability after freeze drying. Carvalho et al (2002) observed no significant differences in the survival of *L. plantarum* (LR-ESB) and *L. rhamnosus* (LR-ESB) after freeze drying with the addition of inositol, sorbitol, fructose, trehalose, monosodium glutamate and propyl gallate, but continued survival life is higher during storage at 20°C. Similar observations were made by Savini et al (2010), when freezing dried *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502, addition of inulin, glycerol, dextrin, mannitol, sorbitol or starch Crystalean 1 produced insignificant differences in survival rates after drying. Broeckx et al (2016) said that however, when the powder is kept at room temperature, viability is far better in the presence of glycerol and mannitol, compared to that which is only added to skim milk. Therefore, it is possible that after freeze drying, skim milk shows sufficient protective capacity to protect probiotics during freeze drying. When the powder is kept at room temperature, additional excipients need to be added to maintain viability and stability (Broeckx et al 2016).

The results showed a good stability performance in storage at -20°C. Mooijman and Havelaar (1997) reported a certification study of certified reference materials for *E coli*, showing good stability at -20°C and low stability with increasing temperatures. Research conducted by Jarvis (2014) states that the microbiological reference material for Salmonella is stable at a storage temperature of -20°C for several years but for storage the temperature of 5°C is stable for only about 6 months.

4. Conclusion

Studies indicated that tuna protein hydrolysate can be used as a matrix of microbiological secondary reference material. The concentration of 14% TPH is the best concentration in producing the highest viability of *Salmonella* bacteria. The TPH exhibited satisfactory homogeneity for their intended use.
At a storage temperature of -20ºC the stability of Salmonella has also been reached. The reference materials once available will be valuable quality assurance tools for fisheries laboratories in particular and will contribute to establish and maintain reliable measurement results for effective consumer protection.

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