

Effects of different *Pediococcus halophilus* level and fermentation time on chemical properties of fermented anchovy paste “*terasi ikan*”

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Abstract. The aim of this study was to investigate the *Pediococcus halophilus* addition on the chemical quality of *terasi ikan* (fermented anchovy paste) product. Two levels of bacterial concentration (10^6 CFU/mL and 10^9 CFU/ mL) were used as a single starter culture for the fermentation process. Changes in chemical characteristics were observed at day 7, 14 and 21. No differences ($p > 0.05$) in moisture and protein content were found in the analysis of variance within *terasi ikan* samples. The decrease in reducing sugar and L-lysine HCl during the fermentation was attributed to the formation of Maillard Reaction Products (MRPs) which was manifested in dark brown color of the end products. The interaction between *P. halophilus* and *terasi ikan* microbiota as well as their enzymatic activities were considered to affect vitamin B synthesis and degradation of protein into amino acids and amines. These findings facilitate further investigations on using *P. halophilus* as constituent of mixed culture, instead of as a single culture for *terasi* industry in order to produce products of well-controlled quality and safety.

1. Introduction

Terasi is a highly popular fermented seafood product among Indonesians. Its distinctive flavor and the presence of glutamic acid make it a common ingredient used in Southeast Asian dishes. *Terasi* is made of a half-dried small shrimp (*Acetes* sp.), termed as *terasi udang* or small fish /anchovies (*Stolephorus* sp.), termed as *terasi ikan*, mixed with 10-25% of solar salt, pounded, sun-dried and fermented for two days. After the first fermentation, the mixture is finely ground, sun-dried, shaped in cubes and wrapped in a banana leaves for the development of a special aroma. The second fermentation lasts about four weeks at ambient temperature (between 28°C and 30°C) leaving a soft-textured and dark-colored product. The fermentation takes place either spontaneously, or is orientated by adding a small percentage of fermented paste from the previous batch. The common practice in *terasi* making relies on spontaneous fermentation, although [1] observed the practice of using *terasi* starter which was composed of *Bacillus brevis*, *Bacillus pumilus*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus subtilis* and *Micrococcus kristinae* in the proportion of 39.1%, 26.1 %, 8.7%, 8.7%, 8.7%, and 8.7%, respectively. The halophilic lactic acid bacteria has also been isolated from autochthonous *terasi udang* paste and identified as *Tetragenococcus halophilus* and *T. muriaticus* [2]. The former species *Pediococcus halophilus* has been raised to genus level, forming the genus *Tetragenococcus* [3] which its species are important in lactic fermentation of high-salt-containing food. However, little is known regarding the use of this species as a part of mix starter cultures or single starter in *terasi ikan* production.



Lactic acid bacteria have been known for their ability to produce antibacterial substances such as bacteriocins which have a wide antibacterial spectrum with potential application as food preservatives. Study on the potential bacteriocin production by *Tetragenococcus halophilus* indicated that this type of lactic acid bacteria exhibited good antibacterial activity against *B. cereus*, *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, *V. parahaemolyticus* and *A. hydrophila* and had antibiotic resistance against streptomycin [4].

The addition of competitive starter cultures to begin the fermentation process is an effective method of controlling the growth of spoilage organisms, food-borne pathogens and preventing the formation of undesirable end-products. In traditional *terasi* production, the fermentation process is usually unstable. The proliferation of undesirable microorganisms also raises a quality control issue. The development of starters containing lactic acid bacteria species associated with *terasi* products seems promising since in general, lactic acid bacteria offer a potential alternative as natural food-grade biocontrol agents and have the ability to improve the safety, flavor, nutritional value, and structure of the products [5]. Therefore, the present study attempted to determine the effects of *P. halophilus* concentrations, as a single starter culture, and fermentation time on some quality characteristics of *terasi ikan* (fermented anchovy paste).

2. Experimental detail

2.1. Sample preparation

Fresh anchovies (*Stolephorus* sp.) were purchased from a local fisherman in Sungsang, South Sumatera. The fish were held in ice for at least 24h before arrival at the laboratory. They were then manually beheaded, gutted, washed and drained. After sun-dried, the half-dried anchovies was mixed with solar salt (20% w/w), ground and separated into three different batches. The first batch served as control, the second and the third batch was inoculated with 10^6 CFU/mL and 10^9 CFU/mL *Pediococcus halophilus* in saline water (0.85% NaCl) respectively. The pastes were fermented over an 18-h period in a closed jar, finely ground, formed into cubes shape and sun-dried for one day. Subsequently, the paste was wrapped tightly in banana leaves and left for fermentation process at 28-30°C and 95% humidity for 21 days. Samples were removed every week (day 7, 14 and 21) from the same processing batch for analysis.

2.2. Analytical methods

Amino acids analyses were performed by using Waters Acquity UPLC H Class Amino Acid Analysis System following the method outlined in [6]. Amino acids were identified by comparing retention times with a standard mixture of 15 authentic amino acids. The content of each amino acid was calculated on the basis of the standard curve of each authentic amino acid standard.

2.3 Statistical analyses

Main effects from a factorial model of *P. halophilus* culture concentrations, fermentation periods and their interactions were analyzed by analysis of variance (ANOVA). HSD was used to determine the differences of means. P values <0.05 were regarded as significant.

3. Results and discussion

Values of the moisture, protein content, reducing sugar, total volatile basic nitrogen (TVBN) and Vitamin B₁₂ (cyanocobalamin) in *terasi ikan* are presented in table 1. The levels of moisture and protein content in all samples ranged from 47.75% to 49.75%, and 12.72% to 15.48%, respectively. These results were in agreement with the previously reported data for fermented shrimp paste in Phillipines. The protein content derived from *terasi ikan* samples was similar to values reported for *bagoong alamang* which varied between 12.9–15.3 g/100g and dry matter content ranged from 38 to 53g/100g, equal to 47-62% moisture [7]. According to [8] as also stated in Indonesian National Standard (SNI-012716-1992), the typical *terasi udang* from Indonesia has 30-50% moisture.

No differences ($p > 0.05$) in moisture as well as protein content were found in the analysis of variance of main effects of *P. halophilus* culture combination or fermentation periods within *terasi ikan* samples. However, an increase in protein content was found between the 7th and the 14th day of fermentations on

control and samples inoculated with 10^6 CFU/mL *P. halophilus* which was attributed to the accumulation of bacterial cells and enzyme secretion due to rapid microbial growth. After that period, the protein content decreased which was possibly due to the proteolytic activity of the microorganisms and enzymes which degraded protein into amino acids, peptides and amines. Shift in moisture as fermentation prolonged, especially in inoculated samples was likely the result of biochemical process releasing water during the fermentation.

Table 1. Chemical characteristics of *terasi ikan*

Parameters	<i>P. halophilus</i> concentration	Fermentation time		
		7 d	14 d	21 d
Moisture (%)	Control	49.00±1.41 ^{Aa}	47.75 ±1.06 ^{Aa}	49 ^{Aa}
	10 ⁶ CFU/mL	48.50 ^{Aa}	49.00 ^{Aa}	49.75 ± 0.35 ^{Aa}
	10 ⁹ CFU/mL	49.75± 6.01 ^{Aa}	49.25±3.18 ^{Aa}	49.75±2.47 ^{Aa}
Protein (%)	Control	13.00±0.18 ^{Aa}	15.48±0.18 ^{Aa}	13.42±0.18 ^{Aa}
	10 ⁶ CFU/mL	13.38±0.14 ^{Aa}	15.58±1.40 ^{Aa}	13.94±1.41 ^{Aa}
	10 ⁹ CFU/mL	14.89±0.03 ^{Aa}	13.33±1.64 ^{Aa}	12.72±1.70 ^{Aa}
Reducing sugar (%)	Control	0.64±0.01 ^{Aa}	0.74±0.01 ^{Ab}	0.73±0.03 ^{Ab}
	10 ⁶ CFU/mL	0.24 ±0.06 ^{Aa}	0.23±0.05 ^{Aa}	0.25±0.06 ^{Aa}
	10 ⁹ CFU/mL	0.45±0.06 ^{Aa}	0.48±0.01 ^{Aa}	0.36±0.04 ^{Ab}
TVB (mg N/100g)	Control	102.60 ± 0.57 ^{Aa}	126.4±1.70 ^{Aa}	199.80±11.60 ^{Ab}
	10 ⁶ CFU/mL	107.80±3.11 ^{Aa}	82.10±0.42 ^{Ba}	198.40±42.25 ^{Ab}
	10 ⁹ CFU/mL	95.10±42.00 ^{Aa}	229.90±23.90 ^{Cb}	212.50±30.69 ^{Ab}
Vitamin B12 mcg/100g	Control	3.09	3.83	3.46
	10 ⁶ CFU/mL	3.11	3.74	3.98
	10 ⁹ CFU/mL	2.82	3.76	3.28

Results are expressed as mean ± standard deviation; n=2.

Values within a row with different superscripts (a-c) denotes significant differences from each other, as effected by different fermentation time, $p \leq 0.05$

Values within a column with different superscripts (A-C) denotes significant differences from each other as effected by different *P. halophilus* concentration, $p \leq 0.05$

During the *terasi ikan* fermentation, organic substances such as carbohydrates, fats and protein will be transformed into simpler compounds such as monosaccharides, disaccharides, fatty acids, peptides, amino acids, and other nitrogenous compounds either by the action of endogenous enzymes or microorganisms. The interaction between monosaccharides and disaccharides which are capable of acting as reducing agent, termed as reducing sugar and amino acids through Maillard reaction will decreased their availability. The formation of Maillard reaction products (MRPs) during fishery products fermentation and their correlation with antioxidant activity has been evaluated [9]. During the Maillard reaction, in which pH and temperature also play important roles, free amino groups react with carbonyl groups to form a reversible Schiff base continued by the formation of brown polymer melanoidins at the final stage [10]. The dark brown color formation in *terasi ikan* has been attributed to the formation of MRPs from reducing sugar and lysine, as this amino acid is very sensitive to Maillard reaction. The correlations between reducing sugar and L-Lysine HCl amino acid content in *terasi ikan* are depicted in figure 1 and figure 2 respectively.

The reducing sugar and L-lysine HCl content of *terasi ikan* fluctuated at different stages of the fermentation. A sharp decrease on reducing sugar was observed from day 7 to day 14 in both control and *P. halophilus*-inoculated *terasi ikan*. A similar pattern was observed in L-lysine HCl content which could be attributed to their interaction in Maillard reaction. As the sugar and protein decomposition continued, the amount of reducing sugar and amino acid increased. The formation of dark brown color on *terasi ikan* due to melanoidin production were similar to those reported in other studies on fermented

fish product, yu-lu [11] and Philippine salt-fermented shrimp paste [9]. The development of brown color formation on previous studies was assessed through absorbance measurement at 420 nm.

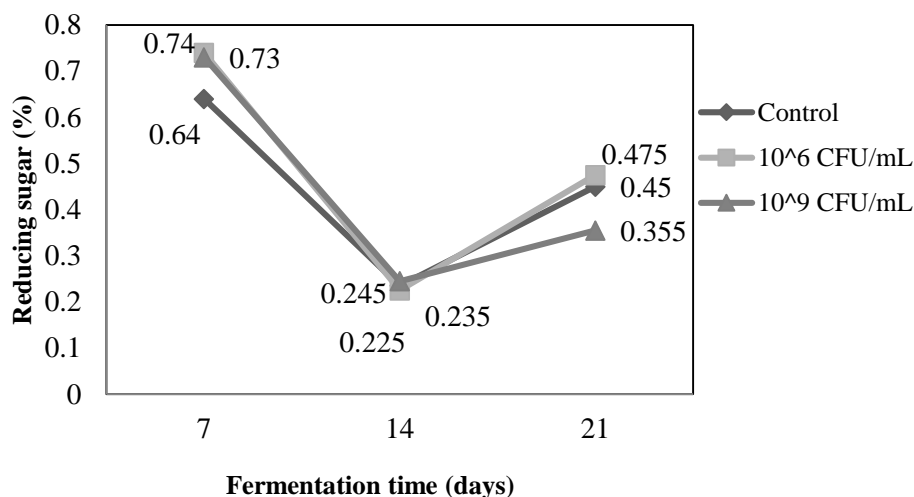


Figure 1. Changes in reducing sugar content of *terasi ikan* samples at 7, 14 and 21 days

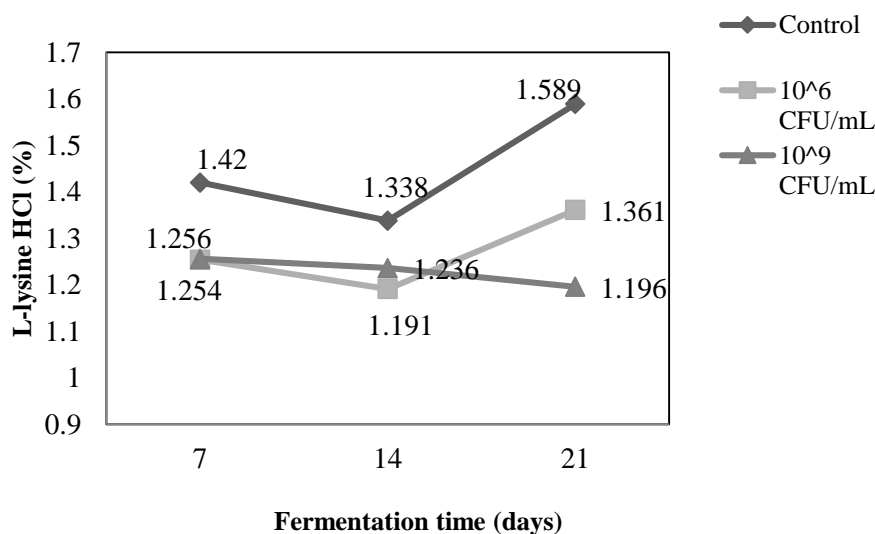


Figure 2. Changes in L-lysine HCl content of *terasi ikan* samples at 7, 14 and 21 days

During the microbial fermentation, formation of biogenic amines is considered as a result of metabolic activity in microorganisms. They are produced by the decarboxylation of free amino acids or by amination and transamination of aldehydes and ketones [12]. In case of *terasi ikan* fermentation, changes in l-lysine content might also be associated with its decarboxylation to produce cadaverine. Several groups of microorganisms has been known as biogenic amines producer, mainly Enterobacteriaceae, *Pseudomonas* spp., enterococci and some lactic acid bacteria [13]. Research conducted by [14] indicated the production of cadaverine by *Lactobacillus brevis* and *L. curvatus* but several strains of lactobacilli, *Leuconostoc* spp., *Weissella* spp., and pediococci showed no decarboxylase activity. The results suggested that the capability to produce amines was strain dependent. The use of *P. halophilus* as a starter and protective culture in this study and its further interaction with

indigenous bacteria in *terasi ikan* as a complex food system might have contributed to chemical changes including biogenic amines formation.

The results also indicated increase on TVB during the experiment. Both *P. halophilus* concentration and fermentation time significantly affected the TVB value ($P < 0.05$) which was expressed as mg N/100g dry matter. The TVB value of *terasi ikan* supplemented with 10^9 CFU/mL *P. halophilus* increased by 117.4 compared to 90.6 and 97.2 of the 10^6 CFU/mL-supplementation and the control group respectively. Total Volatile Bases Nitrogen (TVB-N) is one of the most widely used methods to estimate the degree of decomposition of fish. It includes the measurement of trimethylamine *dimethylamine*, *ammonia* and other volatile nitrogenous compounds associated with seafood spoilage [15]. The TVB-N concentration increased as the fermentation time prolonged ($p < 0.05$) despite the fluctuation over time of fermentation. When tested for *P. halophilus* culture concentration effect, there was no difference among culture concentration at day 7 and 28. The significant effect of *P. halophilus* concentration on TVB was only observed at day 14. The high TVB value on *terasi ikan* inoculated with 10^9 CFU/mL *P. halophilus* might be attributed to the rapid formation of trimethylamine which mainly originates from bacterial decomposition. Despite the high TVB values at day 21, they were still within the acceptable limits set, which are at 100-200 mg/100g for salted and dried fish products, according to FDA. A similar pattern has been reported for *terasi udang* which TVB value increased over fermentation time reaching 350 mg/100g after 28 days of fermentation [16].

The addition of microbes into the fermentation system in most cases significantly contributes to the nutritional value improvement of fermented product by taking part in the production of health-promoting compounds such as vitamins [17]. Certain LAB have capability to synthesize water-soluble vitamins specifically those included in the B-group including folic acid, riboflavin, cobalamin and biotin [18]. Vitamin B₁₂ (cobalamin) is one of the very important vitamins and is required for the metabolism of fatty acids, amino acids, nucleic acids and carbohydrates [19]. Cobalamin cannot be synthesized by animals, plants and fungi. It is the only vitamin that is exclusively produced by bacteria and archaea, particularly by anaerobes. *P. freudenreichii*, *Salmonella enterica* and *Bacillus megaterium* are examples of bacteria that could perform the anaerobic biosynthesis of cobalamin, while *Pseudomonas denitrificans* is the aerobic B₁₂-producing bacterium [20]. Lactobacilli have the ability to produce vitamin B₁₂, in particular a probiotic strains of *L. reuteri* which are shown to be capable of producing cobalamin [21]. The Vitamin B₁₂ values of *terasi ikan* supplemented with 10^6 CFU/mL *P. halophilus* had progressively increased over time of fermentation. It had an initial value of 3.11 and reached a level of 3.98 mcg/100g on the 21 days of fermentation. As for control and *terasi ikan* cultured with 10^9 CFU/mL *P. halophilus*, the values were fluctuated with a noticeable decrease at day 14. At the end of fermentation, the values of both samples increased but still lower than the 10^6 CFU/mL ones. The differences in cobalamine content was presumably due to the interaction between *P. halophilus* and several normal microbiota constituent of *terasi*, which can be originated from various species or strains including cobalamin producer such as *Pseudomonas* spp. and *Lactobacillus* spp..

table 2 presents the amino acid content of *terasi ikan* at various *P. halophilus* concentration and fermentation periods. The initial total of 15 amino acid content of raw anchovy sample was 14.68% with the major amino acids were L-glutamic acid, L-lysine HCl and L-aspartic acid. The research of [9] reported that the total content of free amino acid in shrimp paste changed during fermentation. It increased distinctly in the beginning, relatively stable in the mid fermentation period and then decreased as the fermentation prolonged. The same trend was observed in *terasi ikan* samples supplemented with 10^6 CFU/mL *P. halophilus*, while in control samples, the total count of amino acids still progressively increased after 21 days of fermentation. This result might indicate that the fermentation process can be terminated earlier with the use of lactic acid starter. The significant amount of *P. halophilus* may speed up the macromolecules conversion into their constituents such as amino acids, mono and disaccharides, and fatty acids. The high bacterial cell density in samples inoculated with 10^9 CFU/mL created competition between cells for oxygen and nutrients, leaving the survivors ruled the fermentation process. The decline in the amino acid content in both inoculates samples could be due to its degradation to

amines, volatile acids, and other nitrogenous substances as by-products of bacterial metabolism or enzymatic decomposition [9].

Table 2. Changes in amino acid content of *terasi ikan* as influenced by different *P. halophilus* concentration and fermentation time

<i>P. halophilus</i> concentration	Control			10 ⁶ CFU/mL			10 ⁹ CFU/mL			Fresh Anchovy
Fermentation time	7d	14d	21d	7d	14d	21d	7d	14d	21d	
Amino acids (in %)										
L-Aspartic acid	0.735	0.822	1.182	0.555	0.706	0.408	0.840	0.661	0.491	1.38
L-glutamic acid	0.832	0.956	2.076	0.546	0.504	0.468	0.865	0.699	0.552	2.38
L-Serine	0.050	0.054	0.471	0.025	0.037	0.022	0.038	0.037	0.034	0.61
Glycine	0.756	1.054	1.161	0.739	0.831	0.625	0.889	0.873	0.837	1.16
L-Alanine	0.570	0.610	0.531	0.591	0.568	0.563	0.570	0.579	0.589	0.98
L-Proline	0.531	0.665	0.665	0.518	0.553	0.498	0.577	0.597	0.594	0.65
L-Tyrosine	0.264	0.316	0.422	0.318	0.422	0.346	0.322	0.338	0.415	0.55
L-Histidine*	0.160	0.205	0.301	0.174	0.216	0.177	0.193	0.198	0.209	0.41
L-Arginine*	0.516	0.704	0.823	0.557	0.680	0.565	0.546	0.650	0.654	0.97
L-Isoleucine*	0.511	0.549	0.710	0.512	0.558	0.595	0.519	0.568	0.588	0.70
L-Leucine*	0.818	0.900	1.153	0.824	0.900	0.936	0.838	0.911	0.917	1.19
L-Lysine HCl*	1.420	1.338	1.589	1.254	1.191	1.361	1.256	1.236	1.196	1.51
L-Valine*	0.558	0.562	0.747	0.551	0.589	0.615	0.567	0.605	0.593	0.74
L-Phenylalanine*	0.315	0.399	0.547	0.369	0.478	0.392	0.465	0.411	0.489	0.77
L-Threonine*	0.244	0.240	0.659	0.198	0.234	0.202	0.223	0.232	0.223	0.69
Total	8.281	9.372	13.036	7.730	8.466	7.771	8.708	8.593	8.382	14.68

*essential amino acids

As also shown in table 2, glutamic acids in control samples was prominent, whereas lower content of the same amino acids were found in inoculated samples. Glutamate plays a central role in a wide range of metabolic processes in bacterial cells including protein synthesis, glycolysis, gluconeogenesis and the citric acid cycle [21]. Under the stress condition, such as acidic stress, certain microbes are able to metabolize glutamate through the action of glutamate dehydrogenase or glutamate decarboxylase (GAD). *Lactobacillus reuteri*, *Lactococcus lactis* and *Lactobacillus plantarum* are examples of lactic acid bacteria which possess enzyme that catalyzes the decarboxylation of glutamate to γ -aminobutyrate [22]. The variation in amino acids composition in each samples depended on microbial interaction within fermentation system. Some microbes secreted proteins decomposing-enzymes which would increase the amount of free amino acids while others would use the amino acids in their metabolism process.

4. Conclusions

The use of *P. halophilus* as a starter and protective culture and its further interaction with indigenous bacteria in *terasi ikan* as a complex food system might have contributed to chemical changes during the product's development. Some nutritional quality parameters including the content of reducing sugar, vitamin B and also the amino acid composition were changed over time and influenced by the concentration of *P. halophilus*. Roles of many other species of bacteria during the fermentation, however, can not be neglected. These findings facilitate further investigations on using *P. halophilus* as constituent of mixed culture, instead of as a single culture for *terasi* industry.

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