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Physicochemical & Microbial Characterization of Overripe Tempeh

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***Abstract*—** Recent research showed potencies of overripe tempeh for development as condiment, and therefore, requirement of guiding parameters related to sensory properties. In this study, four samples of overripe tempeh from single source of soybean with different processing method were observed. All samples were characterized with comparable brown color (L* 52.96, a* 6.84, and b* 18.52), and total number of lactic acid bacteria (8.30 – 8.46 log CFU/g). All samples also have similar trends of protein and soluble amino acid content (50.32 – 61.77 mg BSA eq/g dry base, 357.39 – 418.78 mg tyrosine eq/g dry base) compared to fresh tempeh (62.43 mg BSA eq/g dry base, 71.70 mg tyrosine eq/g dry base). The findings indicated suitability of above parameters as guidance in target overripe tempeh for condiment ingredients. Observation of total microbial and total yeast and mould showed that these parameters were affected with production method of overripe tempeh.

***Keywords*—** Overripe tempeh, guiding parameters, microbial characterization, physicochemical characterization

Nomenclature	
OT1	Overripe Tempeh (controlled production)
OT2,OT3,OT4	Overripe Tempeh (uncontrolled production)
TMC	Total Microbial Count
LAB	Lactic Acid Bacteria
YM	Yeast and Mould
SAA	Soluble Amino Acid

I. Introduction

Overripe tempeh is a term used to explain further fermented tempeh in which the solid state fermentation is taken over by bacterial fermentation. Overripe tempeh has pungent odor due to fat hydrolysis, brownish grey color, and unappetizing appearance compared to fresh tempeh. On the other hand, it is known to have the potencies to be main ingredient as an alternative flavor enhancer due to its abundant glutamic acid content (14.5%) [1] which is one of the taste-active components that is responsible for the umami taste. The previous research shows that the overripe tempeh which is further fermented for 72 h with temperature around 30˚C has shown the highest umami activity with dilution factor 256[2]. The production method of tempeh by each tempeh artisan might be uncontrolled and thus it is important to know the best characteristic of overripe tempeh based on its physicochemical and microbial characteristics.

II. Material and Method

A. Materials and Equipment

In this study, four fresh tempeh were used. The first fresh tempeh was made from yellow soybean which acquired from Pasar Modern, BSD. The soybean was washed and sorted, and then followed by overnight soaking (12 h) and de-hulling. The soybean was boiled in 100˚C for 30 min until cooked thoroughly. The boiled soybean was drained, dried, and cooled down. Afterwards, it was inoculated with tempeh starter Raprima obtained from Rumah Tempe Indonesia, Bogor. Inoculated beans were packed and incubated at 30˚C for 48 h to obtain fresh tempeh and furthered incubated for three days. The other three tempeh acquired from three different tempeh artisans which regarded as three different samples. These three tempeh were made from the soybean coming from the same source used in the first tempeh production mentioned above.

Chemical reagents used were Sodium chloride (Oxoid), Sodium carbonate (Analar, BDH, England), Potassium dihydrogen phosphate, Potassium phosphate dibasic anhydrous (Sinophan Chemical Reagent, Shanghai), Bovine Serum Albumin (Sigma-Aldrich, Germany), Standard Methods Agar (SMA) (Hardy Diagnostics, USA), Potato dextrose agar (PDA), deMan Rogosa Agar (MRSA), Trichloroacetic acid, Folin-Ciocalteu's phenol reagent, and Sodium hydroxide (Merck, Germany).

The equipments used were GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, USA), colorimeter PCE-SCM7 (England), autoclave (Hirayama HV-50, Japan), biosafety cabinet (ESCO Class II type A2), and incubator (Memmert, Germany).

B. Protein and Amino Acid Analysis

Frozen samples were crushed and grinded. The solvent used in the extraction was water with ratio 2 : 5 (40g samples in 100 ml distilled water). Extraction was done under room temperature with constant stirring for six hours and the mixture was filtered with cheese cloth. The solutions collected were centrifuged at 8000 rpm for 15 min and the supernatant were collected. The extract was stored under -20°C in the freezer.

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For total protein content analysis, extracted sample was diluted with distilled water until reached certain dilution level. Diluted sample was taken 0.3 ml and then mixed until homogenized with biuret reagent 1.5 ml. the mixtures incubated at room temperature for 10 minutes. Afterward 75 µl folin-cioclateu (1:2) reagent was added into it, homogenized and incubated for 30 minutes under room temperature. The samples were analyzed for the absorbance in 650 nm by using spectrophotometer. The absorbance were read and interpolated with the BSA standard curve.

For soluble amino acid analysis, extracted sample was added with 500 µl distilled water and added with phosphate buffer pH 8. The solutions were incubated for five min at 37ºC. 750 µl 10% of TCA was added and centrifuged at 10.000 rpm for 10 min. The supernatant was collected. In a test tube 300 µl supernatant was put, followed with the addition of 1000 µl 0.5M Na_2Co_3 and 200 µl Folin-Ciocalteu. The mixtures were transferred to one cm curette and with the UV Vis spectrophotometer at 578nm, the absorbance for each sample were read. To analyze the mixture, tyrosine was used to create a standard curve.

C. Microbial Growth Analysis

Sample was analyzed by using serial dilution and plate count method. Five grams sample was diluted in 45 ml sterilized distilled water and crushed by using hand blender and subsequently diluted in 0.85% sterilized saline solution. One ml sample was mix with the corresponding agar and incubated in incubator at 36°C for 48 h for TMC and LAB, while YM was incubated at 25°C for five days. Agar used for TMC, LAB, and YM are PCA, MRSA, and PDA respectively.

D. Color Analysis

Color analysis was applied to the sample by using colorimeter. The colorimeter was calibrated with white standard calibration kit and then measurement was taken for each sample. Data shown in the form of L*, a*, and b* in which L* shows brightness, a* shows color range from green to red, and b* shows color range from yellow to blue.

III. Result and Discussion

In order to create guiding parameters for an overripe tempeh, it is necessary to know the protein content and soluble amino acid content. It is chosen because content of protein and soluble amino acids directly related with the flavor or the taste of overripe tempeh.

Table 1 shows that different production method might cause variation in the protein content. Controlled overripe tempeh (OT1) was produced with single soaking for 12 hours and single boiling at 100ºC for 30 min and the uncontrolled overripe tempeh(OT2, OT3, OT4) was picked randomly from fresh tempeh sold at a local market and therefore the production method and treatment done until fresh tempeh obtained is unknown. Uncontrolled overripe tempeh might undergo longer soaking or boiling treatment which resulted in difference protein content [3].

The protein content was analysed by using Lowry method which detect color development by reaction of peptide bond of protein with alkaline cupric tartrate [4]. It means if the protein was denatured during boiling, the protein content found will be lower. [5] supported this phenomenon by pointing out the effect of boiling time was very real for protein content (two minute = 8.50% and six minute = 4.76%). Soaking and de-hulling might also caused the differences in protein content since it contributes loss of 48.2% nitrogen [6].

Table 1 Chemical Characteristics of Overripe Tempeh

Sample	**Protein Content (mg BSA eq/g dry base)**	**Soluble Amino Acid Content (mg Tyr eq/g drybase)**
OT1	56.26 ± 0.64^{a}	412.86 ± 26.53^{ac}
OT2	50.32 ± 0.29^{b}	357.39 ± 4.23^{b}
OT3	61.77 ± 0.67^{c}	418.78 ± 7.66^{a}
OT4	52.04 ± 0.95^{b}	376.54 ± 17.72^{bc}

*Different letters of within the column indicate significant differences at $p<0.05$ level.

SAA content was measured by using spectrophotometer method. In this method, folin-ciocalteau reagent was used and it makes blue complexes with amino acids such as tyrosine. Since the overripe tempeh was targeted to be a flavor enhancer, it is expected to have or contain high number of glutamic acid. Glutamic acid itself is one of the water soluble amino acid and it is the same for L-tyrosine[7]. When the proteins are broken down into amino acids, it can be release in a form of alanine, glutamic, serine, or the other amino acids that might exist in tempeh products. Thus the present considered tyrosine could also be the representative of the present of glutamic and aspartic which contributes to the umami flavor.

Table 1 also shows the content of soluble amino acid (SAA) content. The analysis was done using spectrophotometer method. SAA utilised the colour development of the reduction of phosphomolybdic-phosphotungstic components in Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan [4]. The presence of tyrosine and tryptophan assumed to be the representative of the presence of glutamic acid which contributes to the umami flavour. The controlled overripe tempeh shows significance different compare to uncontrolled overripe tempeh 2 and 4. Overripe tempeh 2 shows the lowest amino acid content which mean the effect from treatment before inoculation affect and at the least, it contains soluble amino acid 357.39 ± 4.23 mg Tyrosine eq/g dry base.

In summary, the lowest protein content (50.32 ± 0.29 mg BSA eq/g drybase) and soluble amino acid content (357.39 ± 4.23 mg Tyrosine eq/g drybase) were set as the bottom limit of protein and soluble amino acid content. Lower than these number, overripe tempeh is considered as not fully ripen.

1) Color Difference Test

The color property of overripe tempeh was also being determined. Determination of color creates easier and faster guide during the choosing of overripe tempeh ripeness because color intensity is related with the growth of microorganisms involved during solid state fermentation [3].

The result in Table 3 shows L* in between 48.96 – 56.97 for 72 hours overripe tempeh, compare to the result of [8] found L* 72.00 for 24 hours overripe tempeh, the result in this research is darker which is make sense since in this research the

Tempeh was fermented longer 72 hours compare to the journal reference (24 hours). Lower number of L* indicates more darkness which means prolonged fermentation lead to the darkening of overripe tempeh. This result is supported by the index value of fresh tempeh (L* 79.20) which is higher than the 24 hours overripe tempeh [8].

Maturation of mould, lower the enzymes production which causes the overripe tempeh to change color from white to brown. Immature mould was white in color and when it reached its maximum production at 24 hours fermentation it turns to darker color [8].

Since the color is related to the growth of microorganism, then the upper boundaries should be set to prevent over growth of microorganism. The color range set by taking the lowest and the highest color index number which are L* 48.96 - 56.97, a* 5.21- 8.27, and b* 16.14 - 21.54.

Table 2 Color Difference Test

Overripe Tempeh	Color Simulation	Index Value		
		L*	a*	b*
OT1		56.22	5.97	16.14
OT2		56.97	5.21	16.94
OT3		49.7	7.91	21.54
OT4		48.96	8.27	19.46

L* indicates lightness; a* indicates chromaticity coordinates of green (-) to red (+); b* indicates chromaticity coordinates of blue (-) to yellow (+).

2) Microbial Analysis in Overripe Tempeh

Table 2 shows total microbial count (TMC) of controlled overripe tempeh (OT1) is lower compared to the uncontrolled overripe tempeh (OT1, OT2, OT3). Lower number of TMC might be caused due to more hygienic conditions during beans preparation and also during incubation period. Uncontrolled overripe tempeh was made by tempeh artisan in a local market and there are some possibilities that the soybean was soaked by using unclean tap water or de-hulling by using foot. This unclean condition might lead to the high TMC result compare to the controlled overripe tempeh.

Cooking or boiling of soybean process reduces microbial populations in large numbers [9]. [10] also stated that the cooking process affect the microbial population in the next stage. At the same time, TMC is a method that measures all of the biological activity in a sample. It counts all including bacteria, fungi or mould, and yeast that are able to grow in aerobic or micro-aerophilic conditions. TPC used to estimate population levels in certain product. These mean, production of overripe tempeh with controlled process is able to suppress the number of microorganisms. By suppressing the number of microorganism, bitter taste caused by bacteria [11] is expected to be minimized.

Table 3 Microbial Characteristics of Overripe Tempeh

Sample	TMC (log CFU/g)	LAB (log CFU/g)	YM (log CFU/g)
OT1	8.96 ± 0.263^{a}	8.302 ± 0.08^{a}	7.86 ± 0.12^{a}
OT2	9.78 ± 0.18^{b}	8.370 ± 0.03^{a}	8.85 ± 0.10^{b}
OT3	9.89 ± 0.20^{b}	8.310 ± 0.07^{a}	9.03 ± 0.09^{c}
OT4	9.65 ± 0.34^{b}	8.462 ± 0.05^{a}	9.06 ± 0.08^{c}

Different letters within column indicate significant differences at $p<0.05$.
TMC: total microbial count; LAB: lactic acid bacteria; YM: yeast and mould

Total microbial count is highly related with hygiene. Higher numbers of microbial growth might be the result of less hygienic production site or different treatments to the soybean before incubation [12]. In the Table 2, it can be seen that the differences are not significant for LAB while for TMC and YM it is significant between several treatments.

During the production of tempeh TMC is increase significantly from fresh tempeh (8.91 CFU/g) to overripe tempeh (10.17 CFU/g)[13]. It indicates more micro-organisms involves in the fermentation.

The TMC and YM profile of OT1 is significantly lower compare to OT2, OT3, OT4. The growth of LAB is not affected by production method

IV. Conclusion

Protein content, soluble amino acid content, and the color index of overripe tempeh should be set by limit to obtain desired characters of taste and become more reproducible.

The bottom limit for protein content and soluble amino acid content is 50.32 – 61.77 mg BSA eq/g dry base and 357.39 – 418.78 mg tyrosine eq/g dry base respectively. Colour index range is L*: 52.96, a*: 6.84, and b*: 18.52. And the microbial growth for TMC, LAB, and YM is 8.96 – 9.89 log CFU/g, 8.3 – 8.46 log CFU/g, and 7.86 – 9.06 log CFU/g respectively.

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