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Analysis of Total Acidity toward Bacterial and Endophytic Fungi Profile during Black Garlic Processing from Garlic (*Allium sativum* L.) and Shallot (*Allium ascalonicum* L.)

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Abstract

Black garlic or shallot are products of processed garlic and shallots obtained through a heating process conducted over a certain period. Black garlic/shallots have a mild aroma with a sweet and sour taste. The heating process causes chemical compound transition in the garlic, including acidity. In addition to the chemical process, the garlic's color and component changes are due to the role of microorganisms during black garlic processing. However, the presence and function of such microorganisms have not been identified. Therefore, this research explores the black garlic's microorganisms, their role in black garlic processing, and their relation to the total acidity changes. Total acidity test was completed using the potentiometric titration method, while the onion's microorganisms were explored through isolation and characterization. Data show that black garlic's total acidity of both garlic and shallot increases during the heating period day by day. Endophytic microbes that were successfully isolated during black garlic processing were observed on days 0 and 6. According to the rough data, the bacteria that emerged on day 0 are presumed to come from genus *Erwinia, Pseudomonas, Xanthomonas, Agrobacterium, Ralstonia, Xylophilus, Pantoea, Acidovorax, Burkholderia, Coryneform,* and *Streptomyces*, while the bacteria observed on day 6 are assumed to be generated from genus *Coryneform* and *Streptomyces*.

Keywords: black garlic, black shallot, total acidity, endophytic microbes, bacteria, fungi

Introduction

Garlic and shallot are tuber crops that are used as seasoning and medicinal ingredients because of their pharmacological properties, including antibacterial, anticancer, antioxidant, antitumor, and anti-asthma [1, 2]. Garlic and shallot have biological effects because they have flavonoid compounds. Similar to sulfur, these compounds act as an antioxidant that protects the cell from destruction. Aside from having flavonoids, garlic and shallot also have organosulfur compounds, such as *S*-alk(en)-il-l-cysteine sulfoxide (ACSOs), *S*-methyl-cysteine sulfoxide (methiin), *S*-(1-propenyl)-cysteine sulfoxide (isoalliin)), *S*-propyl-cysteine sulfoxide (propine), and (1S, 3R, 5S)-5-methyl-1,4-thianaze-3-carboxylic acid 1-oxide (cycloalliin) [3]. Some of those organosulfur compounds carry a robust smell and taste, which is why some people are reluctant to consume garlic and shallot directly.

Black garlic is made from garlic and shallots in a chamber at a temperature of 60 ℃ for 24 days. No extra compounds are added; thus, its water content [4] is reduced. The obtained black garlic has a greater antioxidant content than raw garlic and shallot, do, and it is reported to have anti-allergy, anti-diabetes, anti-inflammation, hypocholesterol, hypolipidemic, and anti-carcinogenic properties [5]. During the heating process, the garlic's components undergo transformation. For instance, its allicin compound transforms into an antioxidant compound, such as *S*-allyl cysteine, tetrahydro-β-carbolines, and alkaloids flavonoid, thus giving black garlic a sweeter taste and less strong aroma [6].

Ammelia *et al*. [7] analyzed the effect of the heating period on black garlic's organosulfur content. They found that a chemical compound has a different composition during the heating procedure in each procedure's stage. The chromatogram shows that volatile compounds evaporate, thus disappearing over the heating procedure, but new chemical compounds appear at a longer retention time. This finding indicates the presence of a recently relegated large molecule or a hardly evaporated component at the end of the process.

In addition to the heating effect, the garlic's color, taste, aroma, and component transformation are caused by the role of microorganisms during black garlic processing. Some studies discovered several endophytes within garlic, primarily identified as bacteria and fungi [8]. In recent years, many researchers have investigated the bioactive components within black and white garlic, such as phenol content [9], total water, pH, color intensity, and antioxidant activity [10]. However, information related to black garlic's bioactive component from shallot is rarely reported. Black garlic has relatively great nutrient content and similar compounds as garlic, as reported by [11]. At a temperature of 60 °C, garlic and shallots yield volatile organosulfur compounds and esters, as indicated by chromatograms. Aside from bioactive compound analysis, an investigation on black garlic's endophytic microbes generated from white garlic was also completed. Endophytic microbes that have been isolated and characterized are bacteria and fungi. Several dominant bacterial strains in black garlic include strains of *Bacillus subtilis*, *Bacillus methylotropphicus*, and *Bacillus amyloliquefaciens* [8]. [12] have successfully isolated one dominant fungal strain found in garlic, which was identified as *Trichoderma brevicompactum*. In another report, more than one fungal strain was isolated from garlic, including *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium sp 1*, and *Peniciullium sp 2* strains [13]. However, an analysis of endophytic microbes on shallot and garlic has never been performed, and the role of endophytic microbes during black garlic processing has not been identified. Therefore, further research that explores endophytic microbial strains on garlic and its role during the black garlic processing, as well as its relation to the total acidity changes of black garlic from shallots and white garlic, is required [8, 12, 13].

An enzyme is a compound with a high molecular weight, and it primarily consists of amino acid chains connected by peptide bonds. Enzymes facilitate the increase in the reaction rate without being involved in the reaction. Enzyme activity is affected by various aspects, such as temperature and pH. In the enzymatic reaction case, many enzymes are inactivated at a high temperature. At above 40 °C, mostenzymes encounter denaturation; as a result, the enzyme function does not work. Meanwhile, the optimum pH is the enzyme's ideal pH. An extraordinarily high or low pH causes the disappearance of most enzymes. pH also serves as the enzyme stability factor. Each enzyme has an area of stability optimum pH. The optimal pH value varies between enzymes [14, 15].

This topic is interesting to follow because of the interesting dynamics in the chemical content and because no one has reported the origin of the process. The Maillard reaction is a possibility, but fungi and bacteria were found in the system, which introduced new biological degradation routes of some components. New and fascinating research can always be conducted on the enzymes from the microbes that were indicated by some chemical changes during the process. Additional questions need to be answered with regard to shallot, which is a similar plant to garlic but with different chemical components.

Material and Methods

Preparation of shallot and garlic. The materials used in this study were 1 kg of shallot and garlic, which were obtained from one of the supermarkets in Malang, East Java, Indonesia, and were not peeled. Aquades was used as a solvent during the extraction process, while NaOH 0.05 M and formaldehyde were used in the titration process. Moreover, 75% alcohol, 0.3% NaOCl, potato dextrose agar (PDA) media, nutrient agar (NA) media, bacterial strains, isolated fungi, and distilled water were used in the isolation and characterization process of the bacteria and fungi.

The equipment used in this study was a Miyako MCM-606 rice cooker with temperature control Autonics TC4S-N4R and output solid-state relay with 15 A power connected using thermocouple cable FT-K-M6. The analysis instrument used was a Branson 2510 MTH ultrasonic cleaner and pH meter.

Black garlic processing. Each clove of 1 kg of shallot and garlic was wrapped with aluminum foil and then placed in the rice cooker at a temperature of 60 °C for 24 days. The sample was taken every 6 days for the total acidity test and endophytic microbe isolation. The total acidity test was performed with the potentiometric titration method, and its results were compared with the isolated and characterized endophytic microbe.

Acidity level analysis. The analysis of the acidity of black garlic was conducted by referring to the method applied by Zhang *et al*. (2016) [16]. A total of 5 g of black garlic sample was taken from shallot and garlic and mashed using a mortar and pestle. The sample was then moved to an Erlenmeyer flask, dissolved into 100 mL distilled water, and set aside for 24 hours. The solution was extracted using ultrasonication for 30 minutes at a temperature of 25 °C, set aside for a while, and then filtered. The 20 mL filtrate was dissolved into 60 mL distilled water in a 250 mL beaker glass, while the pH solution was measured. The solution was titrated with NaOH 0.05 M until its pH reached 8.2. A total of 10 mL of formaldehyde was added to the solution, and its pH was measured. Afterward, the solution was titrated using NaOH 0.05 M until 9.2 pH was attained.

Fungi and bacteria isolation. Endophytic bacteria were isolated during the black garlic processing from white garlic at a temperature of 60 °C. The black garlic was peeled and sterilized with 75% alcohol for 1 minute, then NaClO 5.3% solution for 3 minutes, and then placed in 75% alcohol for 30 seconds. The garlic was then dried using sterilized tissue. It was then inoculated by splitting it using a sterilized knife and placed on the surface of PDA and NA media. The incubation was performed at room temperature until the colony was formulated [17].

Isolate purification. After it was inoculated on PDA and NA, the fungi and bacterial colony grew around the sample cut. Afterward, the colony with a macroscopy difference was separated and cultured again on the new PDA and NA media until a macroscopically homogeneous fungus and bacterial culture was obtained. Endophytic fungi were purified by using an inoculating loop that was heated over a Bunsen burner and then placed again in new PDA and NA media and incubated at a temperature range of 27 °C – 29 °C for 3 to 5 days. The generated isolate was then placed in a slope [17].

Identification of endophytic microbe isolate. The morphological properties of the endophytic microbe that had been incubated for 3 to 5 days at 27 °C to 29 °C were observed both macroscopically and microscopically. Macroscopic observation was completed directly by identifying the colonies' surface form and color. Microscopic observation was performed by using a binocular microscope [17].

Results and Discussion

Fermentation of black garlic. Fermentation aims to generate black garlic from the shallot and white garlic at a high temperature with controlled moisture for a few days. The results of the fermentation procedure are presented in Figure 1 (a and b).

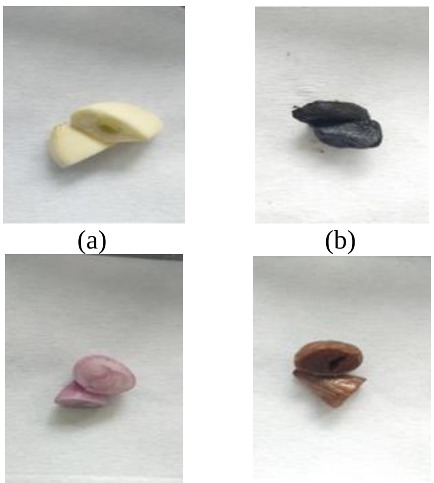


Figure 1. White Garlic before (a) and after Heating (b), Shallot before (c) and after Heating (d)

The color transformation on the onion is caused by the Maillard reaction and caramelization process. These two reactions happen hand in hand and are fixed by the heating process. The Maillard reaction occurs faster at a high temperature and slower at a low temperature. The higher temperature and longer heating process create a more concentrated blackish brown color due to the more significant number of melanoidin pigments. Therefore, the color intensity highly relies on the heating temperature and period [18]. Other than contributing to the color transformation, the Maillard reaction also advances the taste and aroma changes.

Analysis of total acidity. During the black garlic processing, the garlic's total acidity increases, as illustrated in Figure 2. Analysis of total acidity in black garlic is important for testing because it can be used as a parameter to produce black garlic products with good quality in terms of taste, shelf life, and bioactive content.

Figure 2 presents the black garlic's increase in total acidity compared with that of shallot during the heating process. This increase is affected by some factors. Reducing sugar in the garlic improves the total acidity because glucose, as a reducing sugar, experiences oxidation within the cell, thereby producing acids such as lactic acid, tartaric acid, succinic, malic acid, and acetic acid. Moreover, the produced ethanol from the fermentation is turned into acetaldehyde by the alcohol dehydrogenase enzyme. The generated acetaldehyde is oxidized into acetyl-coenzyme A (CoA) with the aldehyde dehydrogenase enzyme's help. The acetyl-coenzyme is changed into acetyl-phosphate by the phosphotransa-cetylase enzyme. After that, the acetyl-phosphate experiences dephosphorylation into acetic acid because of the enzyme acetate kinase [19].

Isolation and identification of endophytic microbes on black garlic. An isolation process was performed to reveal the existence and profile of endophytic microbes on the black garlic. The isolation results are shown in Table 1.

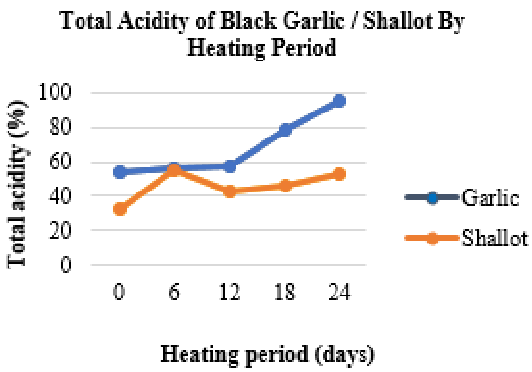


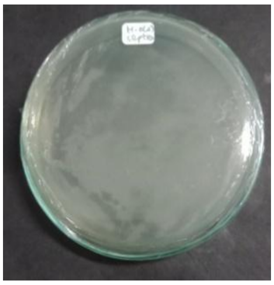
Figure 2. Black Garlic Total Acidity Transformation during the Heating Process

Table 1. Results of Endophytic Microbe Isolation on Black Garlic Sample

Type of Endophytic Microbes	Day	Isolate Number	Isolate Code
Endophytic Bacteria	0	2	H0.1
			H0.2
	6	1	H6

(Description: H0.1 = First day-0)

Table 2. Macroscopic Characterization of Endophytic Bacteria on Black Garlic

Isolate Code	H0.1	H0.2	H6
Purification Results			
Color	White	Transparent White	White
Shape	Irregular	Irregular	Irregular
Elevation	Flat	Flat	Flat
Edge	Irregular	Irregular	Irregular
Optic	Opaque	Transparent	Opaque
Surface	Smooth	Smooth	Smooth

According to Table 2, the three isolates have similar characteristics, such as their shape, color, elevation, edge, optic, and surface. However, the H0.2 isolate has color and optical differences from the H0.1 and H6 isolates.

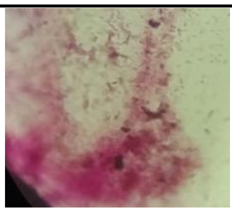
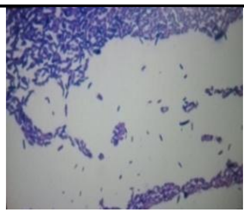
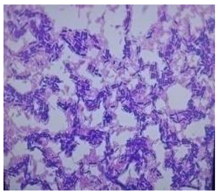
On the basis of the morphological features of each colony, the selected microscopic identification was performed through Gram staining to discover the Gram type of each bacterial isolate. The microscopic identification results are presented in Table 3.

According to Table 3, the H0.1 isolate has a different microscopic feature from the H0.2 and H6 isolates, which have the same microscopic features. Thus, the H0.2 and H6 isolates come from the same bacteria genus; meanwhile, H0.1 comes from different genus bacteria than the other two isolates.

The genus identification was completed on each isolate to prove its probability (endophytic bacteria) and the type of probability, such as *Bacillus subtilis*, *Bacillus methylotrophicus*, and *Bacillus amyloliquefaciens* [8]. The genus identification was carried out using the Schaad flowchart (Schaad, NW, Jones, JB, and Chun, W. 2001, Ed., *Laboratory Guide for Identification of Plantpathogenic Bacteria*. APS Press, Minnesota) [17]. Identification results indicate that isolate bacteria H0.1 is presumed to be included in the genus of *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Ralstonia*, *Xylophilus*, *Pantoea*, *Acidovorax*, and *Burkholderia*. The H0.2 and H6 isolates are assumed to be the group in genus *Coryneform* and *Streptomyces.*

Previous studies reported that several endophytic bacteria and fungi have been isolated and characterized from black garlic and garlic, such as *Bacillus subtilis*, *Bacillus methylotrophicus*, and *Bacillus amyloliquefaciens* [8]. The endophytic fungi include *Trichoderma brevicompactum* [12], *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium sp 1*, and *Penicillium sp 2* [13]. When compared with the results of previous studies, the results of the present study indicate significant differences in the types of bacteria and endophytic fungi produced. This finding may be influenced by several factors, such as the growing environment of garlic, the type of garlic used, and the processes that occur during the manufacture of black garlic.

Table 3. Microscopic Characterization of Endophytic Bacteria on Black Garlic

Isolate Code	H0.1	H0.2	BH H6
Picture			
Cell Shape	Bacilli	Bacilli	Bacilli
Size (µm)	1.4—3.7	1.2—3.4	2.1—3.6
Gram	Negative (-)	Positive (+)	Positive (+)
Endospores	Negative (−)	Negative (−)	Negative (−)
Confirmed KOH 10%	Negative (−); producing mucus after reacting with KOH	Positive (+); producing no mucus after reacting with KOH	Positive (+); producing no mucus after reacting with KOH

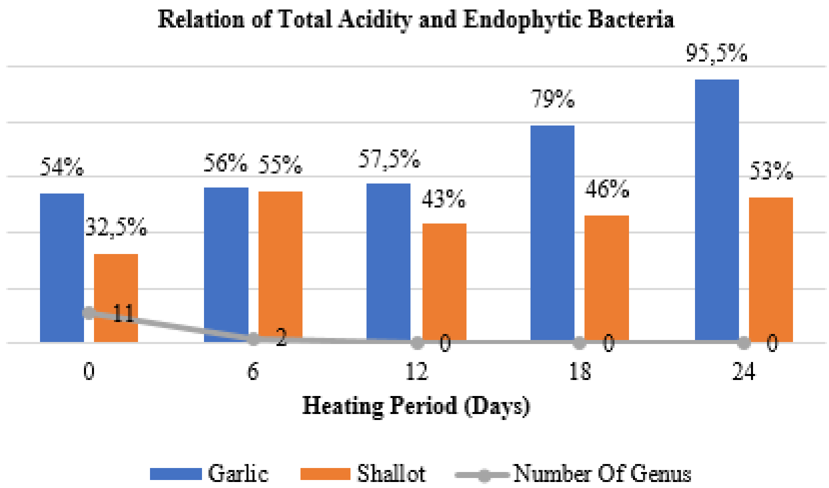


Figure 3. **Ratio of Total Acidity of Black Garlic from Shallot and White Garlic toward the Endophytic Bacteria Profile**

Relation of total acidity and endophytic bacteria on black garlic. Other than the sugar reducer and ethanol, the increase in the total acidity is caused by the endophytic microbe in the garlic. Figure 3 presents the relation among the successfully isolated endophytic bacteria toward the accelerated total acidity.

The expansion of total acidity of black garlic from the shallot and white garlic on days 0 and 6 is due to the role of endophytic bacteria. Each bacterium from that genus can produce organic acid compounds through the fermentation process [20]. The bacterium on black garlic creates various organic acid compounds through the carbohydrate fermentation process (polysaccharides, starch, cellulose, glycogen, sucrose, and maltose). The generated acid compounds are lactic acid, acetic acid, formic acid, propionic acid, glycolic acid, fumaric acid, and succinic acid.

From days 12 to 24, the colony of endophytic fungi and bacteria is not observed. However, the total acidity of the black garlic from the shallot and white garlic increases. The expanded total acidity is affected by the effect of glucose within the garlic. The glucose is obtained from the degradation of carbohydrates (polysaccharides, starch, cellulose, glycogen, and agarose) caused by some bacteria from the genus, which have been successfully isolated. Some genera that can degrade carbohydrates are the bacteria from genus *Xylophilus*, *Pseudomonas*, and *Xanthomonas*. The resultant glucose from the carbohydrate degradation then encounters the fermentation process by some bacteria such as *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Ralstonia*, *Xylophilus*, *Pantoea*, *Burkholderia*, *Coryneform*, and *Streptomyces* bacteria. It also undergoes a series of glycolysis procedures that generate acid compounds. The produced acid compounds include lactic acid. Therefore, the total acidity on days 12 to 24 increases due to the black garlic's glucose content.

In addition to the glucose, total acidity on days 12 to 24 is improved due to the consumption of a significant amount of the base group, such as the amino group in the amino acid during the Maillard reaction [21]. The high temperature also causes the hexose or pentose within the garlic to encounter hydrolysis and thus turn into organic acid by the division of α-dicarbonyl and β-dicarbonyl during the Maillard reaction [22].

Conclusion

The total acidity of the white garlic sample increases during the heating process, whereas that of shallot samples reduces on day 12 but escalates again the next day. The successfully isolated endophytic microbe during the black garlic processing is observed on days 0 and 6. On the basis of the raw data, the successfully isolated microbes on day 0 are assumed to come from genus *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Ralstonia*, *Xylophilus*, *Pantoea*, *Acidovorax*, *Burkholderia*, *Coryneform*, and *Streptomyces*. On day 6, the microbe is assumed to come from the genus *Coryneform* and *Streptomyces.* The accelerated total acidity of black garlic is affected by some factors, such as the endophytic microbe. This successfully isolated microbe then creates organic acid compounds (such as tartaric acid, succinic acid, malic acid, and acetic acid) through the glucose fermentation process. The microbes can degrade polysaccharides, which then become monosaccharides that produce organic acid compounds (such as lactic acid, acetic acid, formic acid, propionic acid, glycolic acid, fumaric acid and succinic acid).

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