Chemical properties of solo black garlic fermented by Saccharomyces cerevisiae

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Abstract. Several research report shown a fermentation could increase or produce a new compound in a material. Research of solo black garlic involved fermentation process of fresh garlic prior to aging process was limited. The aim of this research was to examine chemical properties of solo black garlic fermented in medium containing S. cerevisiae before its aged. The variance result shown that there was an interaction between fermentation and aging time on its antioxidant capacity, total flavonoids and total polyphenol significantly. The treatment of fermentation of fresh solo garlic in medium containing S. cerevisiae for 4 days and continued by aging for 21 days gave the best chemical properties on solo black garlic, with 95.88% of antioxidant capacity, 89.74 mg GAE/g of total flavonoid and 108.92 mg QE/g of total polyphenol. Comparing with control - without fermented by S. cerevisiae, those treatment gave a better chemical properties. Furthermore, profiling by LC-MS-QTOF revealed that several alkaloids, polyphenol and flavonoids compound was founded in those sample. That findings indicate that fermented by S. cerevisiae prior to aging process could be considered for increasing of solo black garlic’s functional properties.

Keywords: black garlic, fermented, S.cerevisiae.

1. Introduction
Utilization of nature-origin materials and herbs has been a back-to-nature major issue and been accepted widely almost around the globe. In conjunction with the aforementioned trend, Indonesia is well-known for its diverse and abundance of plant-originated natural medicine. Yet, this potential is still in waiting for a optimal development. One reason which hinders the development of these natural materials is that scientists have little information of bioactive element circulating in the plants. World Health Organization (WHO) stands in front as a supporter of initiatives strengthening natural medicine’s securities and efficacies. This organization suggest that traditional medicine poses less negative effect than the modern medicine does [1]. One herb, the male garlic, has been used as degenerative treatments [2].

Solo black garlic is a garlic known to be growing in extreme environment which make it unable to grow properly and concomitantly only has one [3]. Solo garlic keeps active high active compounds and extreme smell and taste compared to general garlic [2, 4]. In contrast to its well-known benefits, it is not a popular as a consumable herbs due to its spicy taste and strong odor, not to mention gastro-intestinal inconvenience. The latest method of garlic production is far from lacking but space for improvement is easily found e.g. steady tart taste and extreme smell that might harm human physiology. Hence, this study introduces a current star of processed-garlic products that is black garlic.
Black garlic is fresh garlics which have been stored and heated under controlled humidity and certain level of temperature for few days that is be called aging process [5]. Aging process allows black garlic having higher bioactivity relative to normal fresh garlic [6]. Based on the previous studies, it has been reported that the mentioned increase in bioactivity was between 4 – 10 times higher than that of fresh garlic is [7,8]. Several studies discovered that black garlic’s bioactivity functioned as antioxidant [9] and immunomodulator [10].

Jung et al. [11] stated that black garlic’s antioxidant is playing important role in decreasing bad cholesterol, significantly leveling down LDL in human’s body as well as boosting insulin production to optimize blood-sugar absorption before circulates it to the needed cells. Fermentation process in black garlic production is proven to level up its biological activities [11,12]. In a study conducted by [11], black garlic fermentation using Saccharomyces cerevisiae is performed after the aging process. However, another study by Kimura et al. [6] where they take usual garlic (multi clove) as the main material and Saccharomyces cerevisiae as the fermentation-agent microbe. In this research, we use immersion (fermentation) technique to undergo the fermentation process and solo garlic as an object.

2. Materials and Methods

2.1 Preparation of fermentation medium
The medium used in this fermentation process is composed of bean sprout extract, sugar and yeast extract. Bean sprout extract is made by boiling the bean in 1:1 composition. After being filtered, it then sterilized in 121 C temperature altogether with 40 of sugar and 10 g yeast. Solo garlic fermentation in a medium contained with S.cereviseae S. cereviseae culture is inoculated using the above solution and incubated in a room temperature for 48 hours. This solution is then used as fermentation medium of fresh solo garlic. Next, this fresh garlic was immersed/soaking in the fermentation medium with 0 hour, 2 days, 4 days and 6 days treatment.

2.2 Aging of solo garlic
After being fermented, it then wrapped using 3 pieces of aluminium foil hence it is protected by 3 layers of materials. The next stage is storing the garlic under a 70 – 72 C temperature and 80 – 85% humidity. Changes in chemical features was observed in day 0, 7, 14 and 21.

2.3 Determining antioxidant capacity [13]
As much as 200 microliter of sample and 800 DPPH 0,2 mM are stored in a 1,5 mL tube which then stirred slowly. Next, it is incubated for 30 minutes under room temperature and without light. After that, the sample is being centrifuged for 10 minutes at 9000 rpm. Sample’s absorbance is measured at wave-length of 517 nm. Blank form is made by replacing sample with methanol. Ascorbate acid is used as the standard solution. Antioxidant capacity is report through Ascorbic acid Equivalent Antioxidant Capacity (AEAC).

2.4 Determining total polyphenol content [14]
The 50 µl of sample added by 800 µl sterilized aquadest (distilled water) is filled in into a 1,5 mL microtube that has been layered by aluminum foil. As much as 50 µl of Folin Ciocalteu 10% reactants and 100 µl solution of Na2CO3 7% are put together in a ependorf tube which then stirred slowly. Next, it is incubated for 30 minutes under room temperature and without light. Sample’s absorbance is measured at wave-length 750 nm. Galactic acid is used as the standard solution as concentration of 3,125 ppm, 6,25 ppm, 12,5 ppm, 25 ppm, 50 ppm, and 100 ppm. The procedure to measure total phenolic in the standard solution is made by replacing sample with galactic acid solution. Total phenolic compound is stated using Gallic Acid Equivalent (GAE).

2.5 Determining total flavonoid content [15]
To obtain a correct measurement of flavonoid rate, we mix 50 µl of garlic extract, 300 µl absolute ethanol, 50 µl AICI3%, 50 µl potassium acetate 1 M and 600 µl sterilized distilled-water. This solution
is incubated under room temperature for 30 minutes. Then, it’s absorbance is measured at 415 nm wave-length.

2.6 Profiling bioactive compound using LC-MS
A sample is measured to obtain 0.1 g inside the centrifuge tube. Then add a 3-5 ml of hexane. After that, the sample is stirred for 1 minute. Further step is extraction. This step is done by adding another hexane as much as 3 ml. move methanol phase into a 25 ml evaporation tube. Then this extraction is repeated by adding 3 ml of methanol twice. Combination of solution derived from extraction is then undergoes ultrasonic method for 15 minutes. Before being rinsed using 1 ml of methanol, the result of the ultrasonic method is filtrated using GHP membrane. What derived from the rinse process is mixed with what derived from extraction process. The sample which has gone through several processes then being evaporated using turbovap that assisted by nitrogen. After it dried, add 1 ml of methanol and perform divortex method. The sample then moved to 2 ml vial and injected by LC-MS-QTOF.

3. Results and Discussion
3.1 Antioxidant capacity
The antioxidant capacity of solo black garlic increased with aging time, and the highest was with a 21-day aging treatment. From Table 1, it can be seen that the highest antioxidant capacity was shown by solo garlic fermented in S. cerevisiae medium for 4 days and followed by aging treatment for 21 days, with a value of 95.88%. The result showed about 1.1 times greater than solo garlic fermented for 2 days and followed by aging treatment for 21 days.

Based on the results of analysis of variance, it is known that there was an interaction between fermentation time and aging time on the resulted solo black garlic. Further statistical analysis showed that the fermentation time significantly affected the antioxidant capacity (p<0.05). From Table 1, it can be seen that the antioxidant activity value of solo black garlic was different between each time of fermentation, and the fermentation treatment for 4 days was the most optimal one. Furthermore, the aging time also significantly affected the antioxidant activity. It can be seen from the antioxidant activity which increased with aging time. Kimura et al. [6] stated that the aging process that involved a thermal process in the processing of black garlic could increase its antioxidant compared to fresh garlic. This was also confirmed by Zhang et al. [16]. Thermal processes could cause changes or the new formation of compounds through non-enzymatic reactions that produced hydroxymethylfurfural (HMF) and melanoidins. These two compounds contributed to antioxidant activity [17]. In addition, the aging process changed color and reduced off-flavor compounds in fresh garlic, and changed unstable components such as alliin, into stable and water-soluble components such as S-Allylcysteine (SAC) [18]. SAC is one of the main components thought to be responsible as antioxidants.

Compared to the treatment of solo black garlic which was not fermented in S. cerevisiae medium, it was seen that the overall antioxidant capacity increased through the fermentation process by S. cerevisiae. This was clearly seen at aging points of 14 and 21, where the antioxidant capacity value was 2 times greater than the unfermented solo black garlic (Table 1). The results of this study were in line with the research conducted by [19] which fermented solo black in a medium containing S. kluyveri before the aging process.

The presence of organic acids produced by S. cerevisiae was thought to be the cause of the increasing antioxidant activity in solo black garlic. The research conducted by Jung et al. [11] reported that yeast metabolites could change components in food and converted sugar into alcohol and lactic acid which could increase the bioactivity of onion components. Lu et al. [20] also stated that the presence of lactic acid was a strong antioxidant, so it could increase the antioxidant activity in garlic.
Table 1. Antioxidant capacity of unfermented and fermented solo black garlic.

| Soaking period (days) | Aging period (days) | Antioxidant capacity (% inhibition of DPPH 0.2mM) |
|-----------------------|---------------------|-----------------------------------------------|
| 0                     | 0                   | 3.66 ±1.14                                    |
| 0                     | 7                   | 8.24 ± 0.66                                  |
| 0                     | 14                  | 42.92 ± 1.44                                 |
| 0                     | 21                  | 49.53 ± 0.13                                 |
| 2                     | 0                   | 4.08 ± 0.02                                  |
| 2                     | 7                   | 20.78 ± 0.04                                 |
| 2                     | 14                  | 74.14 ± 0.04                                 |
| 2                     | 21                  | 86.61 ± 0.32                                 |
| 4                     | 0                   | 9.44 ± 0.28                                  |
| 4                     | 7                   | 21.47 ± 0.20                                 |
| 4                     | 14                  | 88.37 ± 1.30                                 |
| 4                     | 21                  | 95.88 ± 0.06                                 |
| 6                     | 0                   | 14.47 ± 0.22                                 |
| 6                     | 7                   | 15.19 ± 0.24                                 |
| 6                     | 14                  | 84.99 ± 0.11                                 |
| 6                     | 21                  | 94.69 ± 0.13                                 |

3.2 Total polyphenol content
The high antioxidant capacity was affected by the presence of total polyphenols and flavonoids, therefore, an analysis on total polyphenol content was carried out to clarify the results of the antioxidant compounds obtained. The analysis on total polyphenol content was carried out using the modified Folin-Ciocalteu method [14]. From Table 2, it can be seen that the total polyphenols increased with the aging time. Slightly different from the antioxidant capacity, solo black garlic fermented for 2 days and then aged for 21 days showed the highest total polyphenol content compared to all treatments, which was 115.64 mg GAE/g.

Based on the results of analysis of variance, it was found that there was an interaction between fermentation time and aging time on the total polyphenols content of solo black garlic significantly (p<0.05). From further statistical analysis, it was known that the fermentation time did not have significant effect on total polyphenols content. This can be seen from the total polyphenols content that was similar between fermentation times at the same each aging point (Table 2). Furthermore, the aging time had a significant effect on the total polyphenols content (p<0.05). This can be seen from the increase in total polyphenols content along with the length of aging. Compared with the solo garlic which had not been aged yet, the total polyphenols content increase in the aged solo black garlic reached was about 4 times higher.

In Table 2, there was a total polyphenols content of solo black garlic that was not fermented with S. cerevisiae. Compared with solo black garlic fermented with S. cerevisiae, there was an increase in total polyphenols content of 1.2 – 1.3 times higher. This shows that the application of fermentation with S. cerevisiae was able to increase the total polyphenols content of solo black garlic. This increase was thought to be due to the metabolism of microorganisms such as organic acids which then remodeled compounds into sources of polyphenols [21]. The same mechanism was also revealed by Hur et al. [22] who stated that phytochemical structural changes could occur due to the influence of microbial secondary metabolites.
Table 2. Total polyphenol content of unfermented and fermented-solo black garlic.

| Soaking period (days) | aging period (days) | Total phenolic content (mg GAE/g) |
|-----------------------|---------------------|----------------------------------|
| 0                     | 0                   | 21.14±1.06                       |
| 0                     | 7                   | 75.65±3.89                       |
| 0                     | 14                  | 90.16±0.24                       |
| 0                     | 21                  | 92.78±0.59                       |
| 2                     | 0                   | 30.84±0.06                       |
| 2                     | 7                   | 86.34±3.24                       |
| 2                     | 14                  | 108.47±2.9                       |
| 2                     | 21                  | 115.64±6.42                      |
| 4                     | 0                   | 31.72±5.07                       |
| 4                     | 7                   | 94.18±2.71                       |
| 4                     | 14                  | 106.59±1.59                      |
| 4                     | 21                  | 108.93±6.42                      |
| 6                     | 0                   | 36.68±4.42                       |
| 6                     | 7                   | 92.55±2.89                       |
| 6                     | 14                  | 101.05±2.83                      |
| 6                     | 21                  | 113.26±1.00                      |

Compared to the fresh form, the aging process increased in the total polyphenols content (Table 2). The increase in the total polyphenols content in the aging process occurred due to: (1) the process of breaking the glycoside and ester bonds into freer forms, (2) the formation of complex polyphenols during the Maillard reaction and (3) the decrease or inhibition of enzymatic oxidation involving antioxidant compounds in the raw material [23]. Some of the polyphenolic compounds in solo black garlic are p-hydroxybenzoic acid, gallic acid, vanillic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, m-coumaric acid, o-coumaric acid [24].

3.3 Total flavonoid content
The antioxidant capacity could also be affected by the flavonoid content in a material. According to research by Kim et al. [24], flavonoids in black garlic are catechin, epicatechin, epigallocatechin gallate, flavones, quercitin, apigenin, flavonols, myricetin, resveratrol, morin, quercetin, kaempferol, flavanones, naringenin. From Table 3, it can be seen that the total flavonoids content in solo black garlic increased along with aging time. This was in line with the statistical analysis which stated that aging had a significant effect on total flavonoids content (p<0.05). The average increase in total flavonoids content compared to before the aging process was 20 times at the aging of 21 days, with values ranging from 87 to 89 mQE/g. This increased total flavonoids during the aging process due to the release of flavonoid components bound in the matrix [25]. Research by Ioannou et al. [26] also stated that thermal processes, time, temperature, psychochemical materials had great effect on the value of flavonoids.

Based on the statistical analysis, it was also known that the fermentation time had significant effect on total flavonoids content (p<0.05). From the three fermentation times, the solo black garlic produced from a 4-day fermentation was solo black garlic with the highest total flavonoids content after getting aged for 21 days, which was 89.74% mg QE/g. The results of analysis of variance also showed that there was a significant interaction between fermentation time and aging time. This shows that both treatments had significant effect on total flavonoids content of solo black garlic.

Compared with solo black garlic produced without fermentation, it was clear that the fermentation treatment increased the total flavonoids content in solo black garlic (Table 3). The fermentation treatment increased the total flavonoids about 3 times after 21 days of aging. The increase in total
flavonoids was thought to be related to the metabolites produced by yeast that broke down components in onions. Furthermore, Prabhu et al. [27] reported that the increase in flavonoids value was thought to be due to an increase in acid during fermentation which freed the bound flavonoid component in the sample.

3.4 Profiling Bioactive Compound using LC-MS-QTOF
From the above treatment, solo black garlic was selected which was produced from a 4-day fermentation process followed by 21 days of aging as a treatment that provided optimal functional value. From the results of the LC-MS-QTOF analysis, several alkaloids, phenolic and flavonoid compounds were identified, as shown in Table 4. Identification by LC-MS-QTOF did not show S-allyl cysteine. It could be caused by the of injection or extraction methods which was used.

Table 3. Total flavonoid content of unfermented and fermented-solo black garlic.

| Soaking period (days) | Aging period (days) | Total phenolic content (mg QE/g) |
|-----------------------|---------------------|---------------------------------|
| 0                     | 0                   | 0.63±0.52                       |
| 0                     | 7                   | 8.97±1.42                       |
| 0                     | 14                  | 23.67±1.34                      |
| 0                     | 21                  | 30.17±1.24                      |
| 2                     | 0                   | 4.54±0.02                       |
| 2                     | 7                   | 12.77±0.28                      |
| 2                     | 14                  | 37.62±0.37                      |
| 2                     | 21                  | 87.53±0.06                      |
| 4                     | 0                   | 9.51±0.11                       |
| 4                     | 7                   | 17.71±0.15                      |
| 4                     | 14                  | 47.57±0.17                      |
| 4                     | 21                  | 89.74±0.22                      |
| 6                     | 0                   | 17.28±0.06                      |
| 6                     | 7                   | 17.36±0.39                      |
| 6                     | 14                  | 45.84±0.22                      |
| 6                     | 21                  | 89.14±0.02                      |

Table 4. The major compound detected in the optimum treatment of solo black garlic.

| Retention time | Compound          | Remarks     |
|----------------|-------------------|-------------|
| 0.45           | Trigoneline       | Polyphenol  |
| 3.12           | Tran-Feruldehyde  | Polyphenol  |
| 3.24           | Maltol            | Flavonoid   |
| 6.68           | Isosalosine       | Alkaloid    |
| 8.76           | Strictosamido     | Alkaloid    |
| 11.88          | 2-Minaline        | Alkaloid    |

4. Conclusion
Fermentation by soaking garlic in S. cerevisae media can increase antioxidant capacity, total polyphenols, and total flavonoids. Fermentation time significantly increased antioxidant capacity but had no effect on flavonoid and polyphenols. During aging proses, there was an increase in antioxidant capacity, total polyphenols, and total flavonoids. Four days-fermentation and 21 days-aging gave the optimal functional value of black garlic. The functional properties of black garlic are due to the presence of polyphenolic, alkaloids, and flavonoids compounds.
References

[1] World Health Organization 2018 Traditional medicine http://www.who.int/mediacentre/factsheets/fs134/en/

[2] Utami P and Mardiana L 2013 Umbi ajaib tumpas penyakit (Jakarta): Penebar Swadaya

[3] Untari I 2010 J Gaster. 71

[4] Kalikulla PDK 2016 Uji aktivitas antibakteri dari ekstrak bawang lanang (Allium sativum L.) terhadap pertumbuhan bakteri Straphylococcus aureus dan Escherichia coli Skripsi (Yogyakarta: Universits Sanata Dharma)

[5] Angeles TM, Jesus PA, Rafael MR, Tania MA 2016 Food Chemistry 199 135-9

[6] Kimura S, Tung YC, Pan MH, Su NW, Lai YJ, Cheng KC 2016 J Food Drug Anal. 25(1) 62-70

[7] Liang T, Wei F, Lu Y, Kodani Y, Nakada M, Takuya M and Mazsaru T 2015 J Agri Food Chem. 63(2) 683-91

[8] Mun Su K, Min Ju K, Woo Suk B, Keun Sung K, and Sung Soo P 2012 J Korean Soc Food Sci Nutrition 41(5) 661-65

[9] Queiroz YS, Ishimoto EY, Bastos DHM, Sampiao GR, and Torres EAF S 2009. Food Chem 115(1) 371-74

[10] Wang D, Feng Y, Liu J, Yan J, Wang M Sasaki J, and Lu C 2010 Medic Aroma Plant Sci Biotech. 4(1) 37

[11] Jung YM, Lee SH, Lee DS, You MJ, Chung IK, Cheon WH, Kwon YS, Lee YJ, and Ku SK 2011 Nutr Res. 31 387-96

[12] Kim S, Park SL, Lee S, Lee SY, Ko S, and Yoo M 2016 Food Chem. 211 555-59

[13] Muanda F, Kone D, Dicko A, Souliman R, and Younous C Evid Based Compl Altern Med 2011 674320, p 8 DOI 10.1093/ecam/nep109

[14] Choi S, Cha HS, and Lee YS 2014 Molecules 19 16811-16823

[15] Chang CC, Yang MH, Wen HM, and Chern JC 2002 J Food Drug Anal. 10 178-182

[16] Zhang Z, Lei M, Liu R, Gao Y, Xu M, and Zhang M 2014 J Food Biochem. 39 39-47

[17] Sun Yue-E and Wang W 2017 J Food Sci Technol. 55(2) 479-88

[18] Bae SE, Cho SY, Won YD, Lee SH, and Park HJ 2014 LWT Food Sci Technol. 55 (1) 397-402

[19] Setiyoningrum F, Priadi G, Afiafi A, Herlina N, Solikhin A, and Lisani N 2018 Asian J. Agric. 248–51

[20] Lu X, Li N, Qiao X, Qiu Z, and Liu P 2017 J Food Drug Anal. 25 340-49

[21] Tai HN, John VC, Smagghe G, and Raes K 2014 Int J Mol Sci. 15(11) 19369-19388

[22] Hur SJ, Lee SY, Kim YC, Choi I, and Kim GB 2013 Food Chem. 160 346–56

[23] Dewanto V, Wu X, Adam KK, and Liu RH 2002 J Agric Food Chem. 50 3010-3014

[24] Kim JS, Kang OJ, and Gweon OC 2013 steps J Uncti Foods. 5 80-86

[25] Choi Y, Lee SM, Chun J, Lee HB, and Lee J 2006 Food Chem. 99(2) 381–87

[26] Ioannou I, Hafsa I, Hamdi S, Charbonnel C, and Ghou M 2012 J Food Engin. 111(2) 208–17

[27] Prabhu A, Mrudula CM, and Rajesh J 2014 Int J Agri Food Sci. 4(1) 59-65