Effect of NaCl Addition and The Incubation Time on Gallic Acid Concentration in Cabbage Fermentation using *Lactobacillus plantarum* and The Potential as Antioxidant

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Abstract. Cabbage is one type of vegetable that is often consumed. Cabbage contains gallic acid and sulforaphane compounds which potentially used as an antioxidant. Gallic acid and sulforaphane are produced from the hydrolysis reaction, which occurs by fermentation. Therefore, this research was focused on the effect of NaCl addition and the incubation time on the gallic acid concentration during cabbage fermentation using *L. plantarum*. The addition of NaCl used were 0 %, 0.5 %, 1.0 %, 1.5 %, and 2.0 % (w/v). While the incubation time used were 2, 3, 4, 5, and 6 days. The fermentation conditions used were 5 % (v/v) inoculum volume and pH 6. Gallic acid in fermented cabbage (biomass and filtrate) were determined using Folin-Denis method. The antioxidant analysis was determined using DPPH method. The optimum conditions were obtained at the addition of 1.0 % NaCl for 4 days fermentation. During these conditions, the gallic acid produced in biomass and filtrate were equal to 4.726 mg/100 g FW and 147.857 mg/100 g FW, respectively. Sulforaphane and gallic acid have potential as antioxidants with IC$_{50}$ values of 95.113 mg/L and 21.648 mg/L, respectively.

1. Introduction
Cabbage (*Brassica oleracea var. capitata*) contains tannin and glucoraphanin. Tannin could be degraded into glucose and gallic acid by tannase activity from bacteria, such as lactic acid bacteria [1], while glucoraphanin degraded into sulforaphane in the presence of myrosinase enzyme [2,3]. Gallic acid and sulforaphane reported as antioxidants [4,5].

![Structure of gallic acid (a) and sulforaphane (b)](image)

Figure 1. Structure of gallic acid (a) and sulforaphane (b)

Gallic acid can be an antioxidant compound because it has a hydroxyl group that can donate hydrogen atom to neutralize free radicals (see Figure 1 (a)) [6]. Sulforaphane can also be an antioxidant compound...
because oxygen atom in the methylene sulfoxide group can donate electrons to neutralize free radicals (see Figure 1 (b)) [7]. The myrosinase enzyme which functions to help hydrolysis glucosinolate can only be active due to cell wall damage. One way to prevent cell wall damage is the fermentation process [8]. Fermentation is a process of transformation, modification, or food processing, that cause changes in nutritional value, shelf life, and food safety [9]. Besides being able to hydrolysis glucoraphanin into sulforaphane, the fermentation process can also degrade antinutrients component in cabbage, namely tannin.

The fermentation of cabbage can be done using several types of lactic acid bacteria, such as: *Lactobacillus plantarum*, *Lactobacillus sakei*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Lactococcus lactis* N8, and *Lactobacillus lactis* LAC67. Based on these studies it was found that *Lactobacillus plantarum*, *Lactobacillus sakei*, and *Leuconostoc mesenteroides* have high effectiveness in the process of cabbage fermentation compared to other lactic acid bacteria [10]. Fermentation using *Lactobacillus plantarum* on media containing tannic acid 0.35 % (w/ v) at a temperature of 30 ºC, pH 6, and agitation speed of 125 rpm can induce the production of tanase enzymes at 9.29 U/mL enzyme activity [11]. The optimum conditions of fermentation using *Lactobacillus plantarum* bacteria were: 5% (v/v) inoculum volume, pH 6, at 25 ºC for 96 hours, can reduce tannin levels from 290.877 mg/g to 75.938 mg/g [12].

Besides several factors, such as inoculum volume, pH, and temperature, the fermented product also influenced by another factors, namely NaCl addition and incubation time [13,14]. Recent studies report the use of NaCl effect on microbial composition [15]. Excessive concentration of NaCl can damage the bacterial cell wall which then affect the ability of bacteria to secrete tannase enzymes which has the function to degrade tannins into reduced gallic acid [11,16]. Therefore, in this research, cabbage fermentation was carried out using *Lactobacillus plantarum* bacteria to hydrolysis one type of glucosinolate compound namely glucoraphanin to sulforaphane and degrade tannin to gallic acid which have potential as an antioxidant. The effect of NaCl addition and incubation time to fermentation product will also be studied.

2. Materials and Methods

2.1. Chemicals and Instrumentation
Ethanol 90 %, Na.CO._3_, dichloromethane, Folin-Denis reagent were obtained from Merck, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent was obtained from Sigma-Aldrich. The medium for *L. plantarum* cultivation was MRS media (de Mann Rogosa Sharpe) (OXOID). UV-Vis Spectrophotometry was used to determine gallic acid level and antioxidant potential.

2.2. Microorganism Preparation
*Lactobacillus plantarum* was supplied by Microbiology Laboratory, Brawijaya University. The bacteria were grown in MRSA and incubated for 48 h at 30 ºC. The bacteria from MRSA was transferred into MRS broth and incubated for 26 h at 30 ºC.

2.3. Fermentation
Fresh cabbage was prepared by slice off into strips and washing with water until clean. To study the effect of NaCl addition, each 100 g cabbage which had been prepared into five different fermentation vessels. Each vessel added with NaCl at various concentration of 0 %, 0.5 %, 1.0 %, 1.5 %, and 2.0 %. To study the effect of incubation time, five vessels of fermentation that have been filled with cabbage and NaCl in optimum condition were fermented with time variations from 2 until 6 days. Fermentation was carried out at room temperature, using an inoculum volume of 5 % (v/v), pH 6 in anaerobic conditions.

2.4. Gallic Acid Determination
2.5 g of biomass from cabbage fermentation was pureed and extracted using 20 mL of hot water. The extract was stirred and heated on a hotplate for 30 minutes. The extract was filtered and the filtrate was added with hot water until the volume reached 25 mL. After that, 0.2 mL of extract was added 0.6 mL of distilled water and 0.2 mL of Folin-Denis reagent. After 5 minutes, 1 mL of sodium carbonate solution (8%, w/v) and 1 mL of distilled water was added to the mixture. The solution was mixed and allowed to stand up to 30 minutes in a dark room. The solution was measured at a wavelength of 765 nm. Meanwhile, the filtrate from fermented cabbage was centrifuge for 10 minutes. The supernatant was used for gallic acid determination in the filtrate from fermentation using the same procedure with gallic acid determination in biomass. Gallic acid levels were determined by gallic acid standart curve (5 – 400 mg/L) [17].

2.5. Antioxidant Test
To test the antioxidant potential of gallic acid, the supernatant from the filtrate was diluted with various concentrations of 10 – 50 mg/ L. 6 mL of sample was added 4 mL of DPPH solution (20 µg/ mL). The solution was allowed to stand for 30 minutes in a dark room and measured at a wavelength of 515 nm. Meanwhile, to extract the sulforaphane, 2 g of the biomass was mashed using mortar and pestle. Then, the sample was extracted using dichloromethane (3 x 10 mL) solvent, and Na₂SO₄ solution was added. After that, the solution was evaporated using a rotary evaporator at 30 °C for 10 minutes. The extract was diluted with various concentrations of 20 – 100 mg/ L. The antioxidant potential determination in biomass was done using the same procedure as the antioxidant potential determination of gallic acid in filtrate.

Inhibition of DPPH was calculated using following formula [18]:

\[
\text{% inhibition} = \frac{(A_0 - A)}{A_0} \times 100
\]

notes: A = sample + DPPH absorbance; A₀ = sample absorbance

The results of calculations were plotted into a curve and used to determine the value of IC₅₀.

3. Result and Discussion

3.1. Optimization of NaCl Addition
The fermentation process of cabbage can cause cell wall damage. The addition of NaCl which used in this study using various concentration between 0 – 2.0 %.

![Figure 2](image-url)  The effect of various NaCl concentration on gallic acid level during cabbage fermentation
Based on Figure 2, fermentation process can increase the levels of gallic acid in the filtrate, whereas the levels of gallic acid in biomass was decreased (significant value: 0.000 < 0.05). This is because gallic acid is easily soluble in water [19], so there are more gallic acid levels in the filtrate than biomass. The amount of NaCl addition also affects the levels of gallic acid production during the fermentation process. The more amount of NaCl added until 1% (w/v), the more production of gallic acid, both in the filtrate and biomass. However, at the addition of NaCl concentration of 1.5%, gallic acid level was decreased, both in the filtrate and biomass. So, the optimum concentration of salt addition is 1.0%. At this condition, the total levels of gallic acid was increased from 4.983 mg/100 g FW into 97.236 mg/100 g FW.

3.2. Optimization of Incubation Time

Incubation time can affect fermented products. Therefore, various incubation time ranging from 2 – 6 days were studied for increase the resulting gallic acid.

![Graph showing the effect of various incubation time on gallic acid level during cabbage fermentation](image)

**Figure 3.** The effect of various incubation time on gallic acid level during cabbage fermentation

Figure 3 shows that the longer the incubation time, the levels of total gallic acid will also increase (significant value: 0.001 < 0.05). This is because the ability of *Lactobacillus plantarum* in producing tannase enzymes to degrade tannin will also increase. However, after 4 days, the resulting of total gallic acid was decreased. Based on the standard growth curve, *Lactobacillus plantarum* bacteria undergo a phase of death after 4 days, so that the activity of *Lactobacillus plantarum* in secreting the tannase enzyme and the concentration of gallic acid were decreased. From this result, it can be seen that the optimum incubation time to produce the highest levels of total gallic acid during the fermentation process is 4 days. For 4 days, total levels of gallic acid was increased from 6.462 mg/100 g FW into 152.583 mg/100 g FW.

3.3. The Antioxidant Potential

The main characteristic of antioxidants is their ability to capture the presence of free radicals and forming more stable free radicals [20]. To test the potential of compounds as antioxidants, it can be done using the DPPH method. Compounds that have potential as antioxidants can neutralize radicals from DPPH, where there is a change in the color of the solution from purple to colorless.
From Figure 4, it can be known that the IC₅₀ of filtrate and biomass values are 35.595 mg/L and 77.777 mg/L, respectively. The IC₅₀ values indicate how much concentration of compounds needed to inhibit 50% of free radicals. The higher the concentration of the sample, the higher the amount of DPPH reduction, which was marked by the decreasing of absorbance value.

Based on the results of determining gallic acid levels (figures 2 and 3), it can be seen that in the fermented filtrate at optimum conditions has high gallic acid levels, which is 147.857 mg/100 g FW. Thus, the possibility of active compounds in the filtrate that have the potential as antioxidants is gallic acid. In addition, there is also a study which states that gallic acid can act as an antioxidant [6].

![Gallic acid reaction as antioxidant](image)

**Figure 5.** Gallic acid reaction as antioxidant [6]
Gallic acid can neutralize free radicals by donating hydrogen atom from the OH group. Radicals formed in gallic acid can be stabilized by the presence of two OH groups around the radicals, so gallic acid radicals are formed which are more stable radicals (see figure 5) [6].

In figures 2 and 3 it can be seen that the levels of gallic acid in biomass at optimum conditions are less than in filtrate, which is 4.726 mg/100 g FW. Besides gallic acid in biomass which is an active compound as an antioxidant, sulforaphane has also been reported as an active compound that has the potential as an antioxidant [7]. The results showed that the highest sulforaphane levels were found in cabbage, which was 540 µg/g FW, whereas in broccoli and brussels sprout were 220 µg/g FW and 120 µg/g FW, respectively [21].

4. Conclusion
This study reveals that the NaCl addition and incubation time can affect gallic acid level in cabbage by simultaneous fermentation using Lactobacillus plantarum. The results showed that the most favourable conditions to increase total gallic acid level by L. plantarum fermentation were: NaCl 1 g and 4 d incubation time, as for total gallic acid increase from 6.462 mg/100 g FW into 152.583 mg/100 g FW. The antioxidant potential of filtrate and biomass were shown by their IC₅₀ values of 35.595 mg/L and 77.777 mg/L, respectively.

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