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The Influenced of *Lactobacillus plantarum* Starter Addition and The Length Time of Fermentation Process on The Activity of Seaweed Antioxidant *Ulva lactuca* from Krakal Beach, Yogyakarta

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Abstract: Seaweed contains phenol compound functioning as antioxidant. *Lactobacillus plantarum* starter addition in a fermentation process was expected will increase the activity of antioxidant. The purpose of this research was to determine the influence of *L. plantarum* addition and the length of fermentation on the activity of antioxidant in *U. lactuca*. The experiment was conducted with factorial design. The first treatment consisted 2 different factors namely without *L. plantarum* addition and *L. plantarum* addition. While the second treatment were the different length fermentation time: 0, 12, 24, and 36 hours. Each treatment were done in triplicate. The data was analyzed using ANOVA and BNJ test was applied if there any differences between the treatments. The results showed that the fresh *U. lactuca* with *L. plantarum* addition for 36 hours fermentation had TPC BAL 9,83 CFU/ml, pH 4,26, phenol 231 ppm and antioxidant activity IC₅₀1375,12 ppm. Dried *U. lactuca* with *L. plantarum* addition that was fermented for 36 hours had TPC BAL 9,10 CFU/ml, pH 4,75, phenol 166,24 ppm and antioxidant activity IC₅₀4070,32 ppm. The fresh *U. lactuca* with *L. plantarum* addition for 36 hours fermentation was the best treatment since the antioxidant activity is IC₅₀1375,12 ppm. Although the antioxidant activity was categorized as weak but it was still showed an increase compared to the result of antioxidant activity with maceration method using n-hexana dissolver which was 11213,076 ppm, ethyl acetate 9770,285 ppm, and ethanol extract 4921,79 ppm.

Keywords: *Ulva lactuca*, Phenol, Antioxidant, *Lactobacillus plantarum*, Fermentation Length Time



1. Introduction

The usage of antioxidant compound recently is developing quickly, either for food or medical treatment. The usage as medicine and food are developing as equal as the knowledge development of free radical activity toward some degenerative diseases such as heart and cancer. The antioxidant sources can be classified into two, which are synthetic antioxidant (antioxidant which is obtained from chemical reaction synthesis result) and natural antioxidant (antioxidant which is the result of natural sources extraction). There are many synthetic commercial antioxidants such as butylated hydroxyl toluene (BHT), butylated hydroxy anisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) which are used to reduce the harmful effects of free radicals. However, these synthetic antioxidants may have other harmful effects [1].

Fermentation with lactat acid bacterium addition that was frequently used as a starter in probiotic drink production such as *Lactobacillus plantarum* which is able to increase the amount of antioxidant compound content, one of them is phenol compound. Phenol is the result of sugar destruction in seaweed *U. lactuca* becomes poliphenol. According to Primurdia and Kusnadi [2] the more fermentation time, the more lactat acid bacterium will reproduce, so that the bacterium ability to rend glucose becomes primary metabolit (lactat acid) and secondary metabolit (poliphenol) will be more.

Seaweed is one of the fishery results that contains phenol compound functioning as antioxidant, rich of Iodium fiber and other important minerals. Antioxidants are substances that prevent the process of oxidation by scavenging the free radicals in body cells, and may reduce potential mutations and thereby help prevent cancer and heart diseases [1]. *U. lactuca* is one of green algae that is mostly found in Waters Krakal Beach, Yogyakarta. The seaweed is named sea lettuce and it is categorized in father seaweed which is eatable seaweed, having antioxidant content, antibacterium, antifungal, and antitumor. Seaweed produces various types of antioxidant that counteract environmental stresses. Hence it is considered as a potential source of novel antioxidants [3].

The antioxidant activity increase in seaweed *U. lactuca* can be done with fermentation and starter bacterium addition such as *L. plantarum* that is able to increase functional value in seaweed *U. lactuca*. According to Aminin et al. [4] in his research about fermentation of seaweed *Euclidean cottoni* by adding bacterium starter *L. plantarum*, *Streptococcus thermophilus* can increase antioxidant content in seaweed. One of organic acids that is produced by BAL during fermentation process is lactat acid. Bisson [5] mentioned that lactat acid bacterium has the ability to produce phenol compound. One of organic acids that is produced by BAL during fermentation process is lactat acid. According to Kruszewska et al. [6] the antioxidant activity increase is also caused by the existence of secondary metabolit from bacterium metabolism.

2. Materials dan methods

2.1. Samples and Starter Preparation

The samples of sea lettuce *U. lactuca* was taken from waters Krakal Beach, Gunung Kidul, Yogyakarta. The samples were then washed by flowing water to remove the muck away. The samples was then placed in an open place and winded for more or less one week to get the dried samples. The samples were chopped in a small size more or less 1x1 centimeter. Starter bacterium used in this research was *L. plantarum* FNCC-0027 that is obtained from PAU UGM in the form of standing algae in a reaction tube. The recultured was done twice by taking one ose *L. plantarum* that was grew in 10-mL sterile MRS Broth media. It was then incubated in a temperature of 37°C for 24 hours inside the incubator.

2.2. The Calculation of Bacterium Cell Number

The calculation of bacterium cell number was done by using the method according to Gupta et al. [7]. Inoculum was prepared with 1 ml *L. plantarum* bacterium added 25 ml MRS Brorth and incubated for 24 hours. The media containing MRS, algae, CaCO₃, Na-azida that were incubated for 24 hours with a temperature of 37°C. Colony was counted by the amount of BAL total to know the number of bacterium colony *L. plantarum* in 1000 µl.

2.3. Fermentation Process *U. lactuca* with Bacterium *L. plantarum*

The fresh and dried seaweed *U. lactuca* are chopped in a small size at more or less 1 centimeter. Jar bottle filled with 24 ml aquades is heated by autoclave for 15 minutes with a temperature of 95°C. Five grams of fresh and dried seaweed *U. lactuca* are put into the different jar bottle. Then it was added 5% of starter *L. plantarum* that has been incubated for 24 hours in every glass jar containing aquades and seaweed *U. lactuca*. Fermentation process was done for 36 hours then it is filtered by filter paper and is done the test of the total amount of BAL, pH, total content of phenol, and antioxidant IC50 at 0, 12, 24, and 36 hours.

2.4. Data Analysis

The data of antioxidant IC50, the total amount of BAL, pH test, and total content of phenol test were statistically analyzed using one way ANOVA. Normality and homogeneity test are done first before ANOVA analysis to know the data characteristics [8]. If there any differences between treatments, the test was continued with BNJ in order to find out the variations.

2.5. Test Parameter

2.5.1. Total Amount of BAL Test

Total BAL content test was done by using the method according to Fardiaz [9]. The production of lactat acid by bacterium was done by using pour plate method using algae MRS media (MRS broth + agar 1,5% + CaCO₃ 0,80% + Na-azida 0,01%). 5 ml of samples was given the solution of 45 ml sterile NaCl 0,85% and homogenized in stomacher for one minute. 1 ml of samples that has been homogenized was put into the reaction tube containing 9 ml sterile solution (0,85%). The next step was, from each three series of the last thinning degree, we take 1 ml and put it into the sterile petry dish (every thinning uses twice replications). Then we pour 10-12 ml of algae MRS with a temperature of 45°C. After that, the homogenant and algae media are mixed by rubbed the dish on the table forming the number of eight (8). When media get solid, the dish was turn back and incubated it in a temperature of 37°C for 48-72 hours in a reverse position. The number of colony formed is calculated by colony counter.

2.5.2. pH Test

pH (acidity degree) test was using the pH meter that was based on pH measurement method of SNI 06-6989-11-2004.

2.5.3. Phenol Content Test

Phenol content was tested by using the method based on Orak [10]. Five grams of samples was put into the erlenmayer 100 ml. By using aquades, the samples was thinned in the measure jar until the volume reached 100 ml. The solution was filtered or centrifuged until get clear filtrate. 1 ml of solution or clear filtrate was put into reaction tube, then 0,5 ml of Follin Denis (1:1), poured and continued 1 ml of Na₂CO₃ solution, left it for 10 minutes. Another 10 ml aquades was poured, then the solution was vortexed until it becomes homogeneous. The samples absorbation was read by using spectrofotometer which the length of the wave 730 nm.

2.5.4. Antioxidant IC50 Test

a. Reagen Preparation

The solution of DPPH (2,2-difenil-1-pikrilhidrazil) was measured by Molyneux [11]. 1,97 mg of DPPH was dissolved by methanol in the measure jar until it becomes 100 ml so the solution which the number of concentration was 50 µm.

b. Samples Preparation

The samples was calculated in an amount of 5 grams and poured 24 ml of methanol that will be left for 3 days, that process was used as the stock solution.

c. The Antioxidant Activity Test

The antioxidant activity was analyzed by method based on Hanani [12]. Ten mg of extract was dissolved with 10 ml of methanol until the volume was reached 10 ml and the concentration number of 1 mg/ml. The methanol was added so samples concentration in a number of (50, 100, 150, dan 200 µg/ml). The antioxidant activity determination from each concentration was taken by using the drop pipette with a number 0,2 ml of samples solution with micro pipette and put it into the vial, then pour 3,8 ml of DPPH solution 50 µM. The solution was homogenized and left for 30 minutes in a dark place, the absorbtion was measured by using spektrofotometer UV – Vis with the wave length is 515 nm. As the comparison, ascorbic acid (concentrations of 2,3,4,5,6 µg/ml) was used with the same treatment for the curve standard samples. The determination of the DPPH maximum wave length [13]. The solution of DPPH 50 µM was taken by using a pipette in an amount of 3,8 ml and we pour 0,2 ml of methanol. After leaving it for 30 minutes in a dark place, the solution absorbtion was measured by spektrofotometer UV – Vis with the wave length was 400-800 nm.

3. Result and discussion

3.1. Total Amount of Lactat Acid Bacteria Test

The total amount of lactic acid bacteria was presented in Table 1.

Table 1. The Result of Total Amount Lactic Acid Bacteria in Fermented *U. lactuca* with *L. plantarum* (CFU/mL) Starter

Treatment	Length Time of Fermentation (hours)			
	0	12	24	36
A (fresh)	8,55±0,05 ^{ab}	8,71±0,07 ^b	9,50± 0,06 ^d	9,83± 0,10 ^e
B (dried)	8,31±0,05 ^a	8,54±0,11 ^{ab}	8,71± 0,13 ^b	9,10± 0,11 ^c

Note : The data was average from three time replications

The data followed by different lowercase letters indicates highly significant different (P<0.05)

ANOVA test showed an interaction between the addition of *L. plantarum* starter with the length time of fermentation had significantly effect the total amount of lactic acid bacteria (p<0,05)

The length time of fermentation significantly affected the growth of lactic acid bacteria. Table 1 showed that the amount of BAL value from fresh and dried seaweed *U. lactuca* seaweed samples at the beginning of fermentation has a value that was not significantly different but at the end of fermentation process has a significantly different value. When fermentation was started, the result of BAL amount on fresh samples is 8,55 CFU/mL and in the dried samples is 8,31 CFU/mL. At the end of fermentation (36 hours), the result of BAL amount on fresh samples was 9,85 CFU/mL and dried samples was 9,10 CFU/mL.

Table 1 showed that the fresh and dried samples in fermentation have a value that was not significantly different. This may happen because there is no activity that BAL does in the samples yet, so that when the test of BAL total was done, the ones counted is the amount of BAL that has just been put in the samples. In the end of fermentation, the fresh and dried samples have a value significant different. This may happen since BAL has done metabolism in the samples so that the amount of BAL increased. The difference of BAL amount in the end of fermentation in the fresh and dried samples may happen because the difference of growth media. The fresh samples has more BAL value compared

with the dried one so that BAL was easier to grow in the fresh samples. The fresh samples have more nutrition content for the growth media of BAL rather than in dried seaweed samples. According to Mallesha et al. [14], the growth of lactic acid bacteria increased by increasing the incubation time, the optimization of temperature, humidity, light, pH, and nutrition. This result was matched with the research of Luhur [15], about fermentation of seaweed *Glacilaria* sp. With starter *L. plantarum*, the amount of lactic acid bacteria in the end of fermentation increased because BAL has already activated metabolism and done the growth phase.

The samples of fresh seaweed *U. lactuca* have more amount of nutrition compared with the dried one. The nutrition was used for the growth media of lactic acid bacteria because in the fresh samples, of *U. lactuca*, there was no process yet that caused nutrition contained in *U. lactuca* becomes destroyed and decreased. In other hand different with dried samples of *U. lactuca* that has been done the heating process in *U. lactuca*, so that the approximation of nutrition contained in *U. lactuca* is decreased compared with the fresh *U. lactuca*. This is strengthened by the result of the research in table 1, that the more fermentation time, the more amount of lactic acid bacteria grow. In the dried samples, lactic acid bacteria is also increasing but not as much as in the fresh samples.

3.2. pH Test

The pH test was performed to determine the pH value of *U. lactuca* during fermentation seaweed process. The pH value was presented in Table 2.

Table 2. The Result of pH Test on Fresh *U. lactuca* and Dried *U. lactuca* Fermented with *L. plantarum* Starter

Treatment	Fermentation Length (hours)			
	0	12	24	36
A (fresh)	5,08 ±0,14 ^d	4,71 ± 0,15 ^b	4,49 ±0,07 ^a	4,26 ±0,05 ^a
B (dried)	5,24 ±0,05 ^e	5,04 ±0,10 ^d	4,87 ±0,08 ^c	4,75 ±0,05 ^c

Note : The data was average from three times replications

The data followed by different lowercase letters indicates highly significant different (P<0,05)

ANOVA test showed there was an interaction between samples (fresh and dried *U. lactuca*) on addition of *L. plantarum* with the length time of fermentation and it given significantly effect on the pH (p<0,05).

The result of pH value in the beginning time of fermentation, dried samples of *U. lactuca* fermented with the addition of *L. plantarum* bacteria has pH value that was significant different with dried samples of *U. lactuca* which is 5,08 and 5,24. This is because in the beginning of fermentation, the microorganisms inside either the seaweed or in starter haven't done the metabolism process yet to produce acid. In the end of fermentation process, the pH value in the fresh samples was significant different with the dried samples.

The data presented in Table 2 showed that the pH value at the beginning fermentation process in the fresh samples was lower than the dried one. This can be explained since in fresh form there was much nutrition that can be used by acid bacteria to proceed the metabolism process to produce acid compound, while in the dried samples, lactic acid bacteria have been decreased because of the nutrition decreased that can be used by the bacteria to grow because of the drying process. The pH both fresh and dried samples, were decreased during the fermentation process from the beginning until it was finished. The increased of fermentation length time in the fresh and dried samples with the addition of starter *L. plantarum* bacteria give significant different for the pH.

The result of ANOVA test showed there was an interaction between the samples type used and fermentation length time so that those are significant influenced toward the pH value decreased

($p < 0,05$). Kumalaningsih et al. [16] stated that pH decrease happens because of microorganism activity producing lactic acid from metabolism result. The accumulated lactic acid causes pH value becomes decreased during fermentation time. *L. plantarum* can decrease the pH of fermentation environment by changing the sugar becomes lactic acid (primary metabolite). This is strengthened by Nahariah et al. [17] that the pH decrease was caused by the fermentation activity that changes carbohydrate or sugar in food sources becomes acid and water and other final products. *L. plantarum* can digest complex compound becomes simple compound with the final result is lactic acid. This result just the same with Pratama et al. [18], on his research about casava fermentation with *L. plantarum* addition toward the total of lactic acid and pH. The longer fermentation time, the lesser pH value, because the bacteria metabolism so that they produce lactic acid.

3.3. The Amount of Phenol

The test of amount of phenol done to find out how much the amount of phenol from the fermentation of *U. lactuca* seaweed. The result of phenol content was presented in Table 3.

Table 3. The Results of Total Number of Phenols from *U. lactuca* Fermented with *L. plantarum* (ppm) Starter

Treatment	Fermentation length (hours)			
	0	12	24	36
A (fresh)	132,31±17,10 ^c	146,15±15,63 ^{cd}	170,8 ±7,15 ^e	231±14 ^f
B (dried)	52,9±5,06 ^a	97,59 ± 1,11 ^b	140,1±7,15 ^{cd}	166,24±7,35 ^{de}

Note : The data was average from three times replications.

The data followed by different lowercase letters indicated a highly significant different between samples ($P < 0,05$)

Length time of fermentation was really influenced toward the amount of phenol. Table 3 showed that the amount of phenol in the fresh and dried samples of seaweed *U. lactuca* in the beginning and the end of fermentation has the value that significantly different. This can be seen from phenol value in the fermentation result of fresh and dried *U. lactuca*, that there was a significant difference of phenol amount. The longer fermentation time, the more amount of *U. lactuca* phenol. This can be seen that in the hour of 0, the amount of phenol in fresh *U. lactuca* is 132,31 ppm, then it increased in the end of fermentation, which was 231 ppm. The dried *U. lactuca* that in the hour of 0, the amount of phenol is 52,9 ppm and it increased until the end time of fermentation. The increased of phenol amount was caused by the bacteria activity that digest the sugar contained in the samples that produces primary metabolit (lactic acid) and secondary metabolit (polyphenol), so that the content value is increasing. Fermentation length gives a significant influenced toward the increase of phenol value ($p < 0,05$). According to Primurdia and Kusnadi [2], the longer fermentation time, the more lactic acid bacteria that was reproduced, so that the bacteria ability to digest glucose becomes primary metabolit (lactic acid) and secondary metabolite (polyphenol) becomes higher. This result agreed with Hitayezu et al. [19], where polyphenolic and phenolic compounds are bioactive non-nutrient secondary metabolites present in fruits, vegetables, and cereals. These compounds protected the plant against pathogen attack or ultraviolet radiation. In food, these metabolites contributes not only to antioxidant activity but also to color, astringency, or bitterness.

3.4. Antioxidant IC50 Test

The IC50 antioxidant test was performed to find out how *U. lactuca* sea lettuce antioxidant activity to preventing free radicals at the beginning of fermentation and in the end of fermentation process. The results of IC50 antioxidant activity were presented in Table 4.

Table 4. The Results of Antioxidant Amounts of IC50 *U. lactuca* Sea Lettuce Fermented with *L. plantarum* (ppm) Starter

Treatment	Fermentation Length (hours)			
	0	12	24	36
A (fresh)	1917,18±1,23 ^d	1739,22±0,93 ^c	1532,33±0,97 ^b	1375,11±0,9 ^a
B (dried)	16512,12±0,83 ^h	15960,17±0,97 ^g	5571,21±0,83 ^f	4070,31± 0,59 ^e

Note : The data was average from three times replications.

The data followed by different lowercase letters indicated there was a highly significant different (P<0,05).

The length time of fermentation is significantly influenced toward the amount of antioxidant IC50. Table 4 showed that the amount of antioxidant in the fresh and dried seaweed *U. lactuca* in the beginning and the end of fermentation has the value that is significant different. This can be seen from the phenol value in fermentation result of the dried and wet *U. lactuca*, that there was a different amount of antioxidant IC50 that is significant. The longer fermentation time, the more antioxidant IC50 activity of *U. lactuca* that is increased. This can be seen that in the hour of 0, the wet *U. lactuca* has the value of 1917,18 ppm and it is increasing in the end of fermentation, which is 1375,11 ppm. It is same as the dried *U. lactuca*, that the amount of antioksidan IC50 in the hour of 0 is 16512,12 ppm and it is increasing until the end of fermentation time. The increase of antioksidan IC50 amount is caused by bacteria activity that digests the sugar contained in the samples that produces primary metabolit (lactat acid) and secondary metabolit (poliphenol). Phenol compound is the former compund of antioxidant. The more phenol amount, the more antioxidant IC50 activity. Although antioxidant IC50 activity increased during the fermentation process, but the antioxidant IC50 activity in seaweed *U. lactuca* was categorized very weak. This can be seen from the number of IC50, while the smaller number of IC50, the antioxidant activity is stronger. We can predict that the weak antioxidant content is caused by the fermentation time that is not enough, or the the concentration of starter bacteria has to be increased. According to Arditiana et al. [20] Inhibition Concentration (IC50) can be defined as samples compound concentration that will cause a reduction toward the DPPH activity 50%. The smaller number of IC50 means the antioxidant activity is higher. A compound can be considered as a very-strong antioxidant if the value of IC50 is less than 50 ppm. The antioxidant activity was strong if the IC50 value was between 50-100 ppm, it was average level if the IC50 value was between 100-150 ppm, and it was weak activity if the IC50 value was between 150-200 ppm.

Antioxidant activity of *U. lactuca* with *L. plantarum* addition, although it is still categorized weak, but it is increasing during the fermentation process and the activity was higher if it is compared with the result of antioxidant activity test with maceration method and using n-hexana solvent, which is 11213,076 ppm, ethyl acetat 9770,285 ppm and ethanol extract 4921,79 ppm [21]. It can be said that starter *L. plantarum* bacterium addition and the length time fermentation were significant influenced toward the actioxidant *U. lactuca* activity. This was agreed with Luhur [15] about fermentation of *Glacilaria* sp. with starter *L. plantarum* additon, he stated that the longer fermentation time, the bigger antioxidant (DPPH IC50) activity in defending the free radical.

4. Conclusion

Fermentation with starter *L. plantarum* increased the antioxidant amount of *U. lactuca*. The best value/number of fermentation was available in the hour of 36 of the fresh *U. lactuca* with the result of antioxidant IC50 test, which is 1375,117 ppm, and although the antioxidant activity is still categorized weak, but it was increased compared with the result of antioxiant activity test with maceration method using n-hexana solution, which was 11213,076 ppm, etil asetat 9770,285 ppm, and ethanol extract 4921,79 ppm.

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