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Optimization of Carbon Source and Concentration for *Lactobacillus acidophilus* Growth, Phenolic Production and Antioxidant Activity in Fermented Seaweed Extract

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ABSTRACT: The probiotic product available on the market is in the form of fermented milk or yogurt. Based on the diversity of biochemical compounds and functional bioactive that naturally exist in seaweed, a trend is now developing to make seaweed as functional food by fermentation process. The purpose of this study was to determine the effect of differences sugar types/carbon sources (sugar cane and palm sugar) and different concentrations (2%, 4% and 6%) sugar added in the fermentation of seaweed *Gelidium sp*. extract with *L. acidophilus* starter to the antioxidant activity. Sugar cane and palm sugar addition could increase the reducing sugar, total sugar, sucrose, total viable count, total phenol, antioxidant activity and decreased the pH and the lightness of the fermented seaweed extract. Both different sugar treatments gave the same result in the research. The highest total viable count was found in 4% sugar addition (1.54-2.47 × 10^8cfu ml^{-1}), but 6% gave the highest total phenol (214-259 ppm) and antioxidant activity (40.38-34.85 × 10^3 ppm). The challenge of this research was raising the starter viablitiy and the contribution that 4% sugar addition will result highest viable count however not the highest antioxidant activity.

Keywords: antioxidant activity, carbon source, fermentation, *Gelidiumsp*, *Lactobacillus acidophilus.*

Abbreviations: TPC, total plate count; DPPH, dyphenylpicrylhydrazil; L, for the lightness from black to white; a, from green to red; b, from blue to yellow

I. INTRODUCTION

It is reported that fermented seaweed higher antioxidant activity and anticoagulants compare to before fermentation [1]. Brown seaweed fermented with Monascus sp. showed increasing of reducing sugar, protein, essential fatty acids, lower IC50 values of antioxidant activities and lower IC50 values antidiabetic activities, increases phenolics and flavonoids content than those of unfermented extracts [2].

Gelidium sp. is red algae or rhodophyta that algae contains as R-phycoerythrin and R-phycocyanin both component is protein, and also contain polysaccharides [3]. *Gelidium sp*. contain monosaccaride mainly glucose, galactose, galacturonic acid and xylose [4-5]. *Gelidium sp*. is commonly found in Indonesian waters. This seaweed is a raw material in agar industry. *Gelidium sp*. produce secondary metabolite that have a function as antioxidant.

Fermentation seaweed has been carried out using lactic acid bacteria. *L. acidophilus* is one of starter from the lactic acid bacteria group. Some research showed that application starter with lactic acid bacteria could increase the secondary metabolic during the fermentation process. Lactobacillus acidophilus reported could produce moderate to high antioxidant activity and have higher phenolic content [6-8]. Higher number of lactic acid bacteria in the system fermentation will breakdown organic compound faster and increased secondary metabolite compound. Secondary metabolites produced by lactic acid bacteria, namely polyphenol are the result of the fermentation process [9].

In the previous study, some seaweed fermentation to make functional drink is using lactic acid bacteria namely *L. acidophilus*. The growth of starter lactic acid bacteria was not optimal. It is needed the suitable media to make a good growth of the starter. It need optimization the composition of media. Lactic acid bacteria need increase element like carbon source, N source, mineral dan vitamin for optimal growth [10-11]. Carbon source is very important to the growth of bacteria like lactic acid bacteria. Microorganism need energy from medium to grow and produce primary and secondary metabolite. Carbon is the main energy source for microorganism and play important role in the medium [11]. In the fermentation medium, generally carbon and nitrogen sources that very important. Cell division and metabolism directly effect by nutrient. The type and content of carbon sources can manage the secondary product as the catabolic mechanism. To increase the yield needed the optimization culture condition, good strain in yield production or using cheap medium [12].

Product formation is highly affected by the medium composition [13]. The forming of product in fermentation is related to the rate of glucose intake or growth rate of the microorganism. When the carbon source (glucose) concentration is in low level, the excretion of product will lower. The carbon source and concentration factors need to be optimized in fermentation [14]. Previous

research reported that sucrose is a promising source of carbon to produce bioactive compounds, especially antioxidants compared to other carbon sources [15]. Sugar cane and palm sugar have a high content of sucrose. The higher sugar is expected to be more carbon sources that can be used by starters and will produce more bioactive compounds especially antioxidants.

The purpose of this study was to determine the effect of differences sugar types/carbon sources and different concentration sugar added in the fermentation of seaweed *Gelidium sp*. extract with *L. acidophilus* starter to the antioxidant activity.

II. MATERIALS AND METHODS

Materials: Dried *Gelidium sp*. were obtained from GunungKidul Yogyakarta Indonesia. MRSA, NaCl, MRSB, Na-Azida, NaCl, $CaCO_3$, DPPH, Metanol, Alcohol, Follindenish, Na_2CO_3, Arsenomolibdat, Folin reagen, Nelson reagen (Merck, Germany).

Starter preparation: The bacterial starters used in this study were *L. acidophilus* FNCC-0051 obtain fromThe Centre Food and Nutrition Gajah Mada University Indonesia. Subculture was done 2 times before the starter used for fermentation. Starter was grown in sterile MRS broth media then incubated at 37ºC for 24 hours before the inoculation.

Seaweed extract fermentation: This research was conducted by fermentation treatment of *Gelidium sp*. with the addition of *L. acidophilus* concentration 5 %. Fermented seaweed extract made by soaked seaweed in water for 6 hours and cut into small pieces (1 cm). The sample was put into soymilk maker with a ratio of seaweed and water 1:12 to obtain seaweed extract. 200 ml seaweed extract put in sterile glass jar and added with 5% bacterial starter and incubated 24 hours in 37ºC

Methode of measurement: Measurement of sucrose, reducing sugar and total sugar 1 g sample of fermented seaweed homogenized with aquades until 100 ml. 1 ml filtrated adding with 1 ml Nelson C reagent. Heating that solution with 100ºC until 30 minutes in a water bath. Decrease the temperature and homogenized with 1 ml Arsenomolibdat. Adding aquades that increase the volume until 10 ml with vortex. That measurement can absorbance with 540 nm of spectrophotometer.

Measurement of pH: 5 ml from diluted sample testing by electrode pH meters. Result of fermented seaweed can be show on the screen of pH meters.

Measurement of TPC and viability of LAB: 10 g sample homogenized with 90 ml of physiological saline water for 60 s in stomacher. Aerobic, mesophilic counts were performed on Plate Count Agar, LAB on de Man, Rogosa and Sharpe (MRS) agar plates (Oxoid, CM361). Plates were incubated at 37ºC for 3 days.

Measurement of total phenolic: 5 g of sample diluted with 100 ml of aquades. Filtrated adding with solution of 0,5 ml Follindenish (1:1), 1 ml Na_2CO_3 and that all solution will centrifuge until 10 minutes. Adding aquadest that incrase the volume until 10 ml with vortex. That measurement can absorbance with 540 nm of spektofotometer.

Measurement of antioxidant activity: 5 g sample adding with 24 methanol and 3 days of maceration, this step repeated until 3 times, the end of day that dilution can be filtration so can use that filtrated. 10 mg filtrated diluted by 10 ml methanol so can take 1 mg/ml of concentration. Repeated that method obtained until 50, 100, 150, and 200 µg/ml of concentration. Dilution of sample was taken from 0,2 ml every concentration will be diluted by 3,8 ml DPPH 50 µM and homogenized until 30 minutes in the dark room. That measurement can absorb with 515 nm of spectrophotometer UV-Visual. The standard taking from comparison of ascorbate acid.

Measurement of colour: 5 ml of sample will be analyzed with Chroma Meter CR-200 that show that L, a, and b result.

Statistical analysis: A completely randomized design was used throughout this study and the experiments were done in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using honestly significance difference test. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

III. RESULTS AND DISCUSSION

A. Sugar content (reducing sugar, sucrose and total sugar) in fermented Gelidium sp. extract

Carbohydrates are structural compounds and play an important role in growth and also in the production of useful secondary metabolites [16]. Previous research reported that sucrose is a promising source of carbon to produce bioactive compounds, especially antioxidants compared to other carbon sources [15]. Sugar cane and palm sugar are rich on sucrose content. The data of reducing sugar, sucrose and total sugar in the fermented seaweed extract are showed on Table 1 for sugar cane addition and Table 2 for palm sugar addition.

Addition sugar will increase the sucrose content of the sample. The 6% sugar cane addition resulted 0.53% sucrose while in control without sugar was 0.13%. Whileaddition 6% palm sugar was showed 0.34% and 0.12% in control. Sucrose is non-reducing sugar because it does not have a reactive OH group, but during heating and under acidic conditions, sucrose will be hydrolyzed into inverted sugars (glucose and fructose) which are reducing sugars. The higher concentration sugar addition will give higher reducing sugar value [17-18].

The effect of different concentrations of sugar cane of fermented seaweed extracts showed that the increasing concentration of sugar added will make reducing sugar also rise (Table 1). The same phenomenon is also shown in the use of palm sugar (Table 2). Reducing sugar has a free aldehyde (aldose) or ketone (ketose) group. The reducing sugar in this experiment using sugar cane have value 0.27% to 1.32%. The reducing sugar in this experiment using palm sugar was 0.32% to 6.67%. Reducing sugar is used by microbes as a carbon source, glucose will be consumed first by lactic acid bacteria [19]. Lactic acid bacteria such as *L. acidophilus* and *L. bulgaricus* need energy sources and utilize reducing sugars such as glucose and fructose to produce lactic acid. The higher the reducing sugar is expected to be more carbon sources that can be used by starters and will produce more bioactive compounds especially antioxidants.

Total sugar is total all disaccharide and free monosaccharide [20]. Addition sugar cane and palm

sugar in fermented seaweed extract gave the similar phenomena. The higher sugar addition will make total sugar higher. The sugar from the palm sugar have higher value compare to sugar cane.

B. Viability of lactic acid bacteria in fermented Gelidium sp. extract

Sucrose contained in sugar cane and palm sugar can be hydrolyzed to produce glucose. *L. acidophilus* is a homofermentative that can break down glucose into lactic acid. Also, a type of homofermentative bacteria is a group of bacteria which ferment glucose into lactic acid as its main product in large quantities (85% or more) [21].

The addition of sugar will increase the number of lactic acid bacteria that grow (Table 1 and 2). This is consistent with the research of addition sugar can promote the growth of lactic acid bacteria [22]. Observe addition of sugar in dragon fruit juice to total lactic acid [23]. The increasing concentration of sucrose means the amount of nutrients that can be used for the metabolism of lactic acid bacteria will be even greater. The nutrient in the media will be used as an energy source for the growth of bacteria. Concentration 4% give the highest number bacteria in both types of sugars it is 1.54×10^8cfu ml^{-1} in sugar cane addition and 2.47×10^8 cfu ml^{-1} in palm sugar addition. 6% sugar addition give the lower number bacteria compare to 4% sugar addition. The results happen because the higher sugar concentration, the solution will become more concentrated.

Table 1: Reducing sugar, sucrose, total sugar, TPC LAB and pH value in fermented *Gelidium sp.* extract with sugar cane addition.

Sugar cane concentration	Reducing sugar (%)	Total sugar (%)	Sucrose (%)	TPC LAB (cfu/ml)	pH
0%	0.27 ± 0.03^{c}	0.40 ± 0.04^{c}	0.13 ± 0.05^{c}	$2.56 \times 10^{7\,d}$	$5{,}03 \pm 0.06^{a}$
2%	0.45 ± 0.08^{c}	0.47 ± 0.02^{c}	0.33 ± 0.48^{b}	$9.55 \times 10^{7\,b}$	$4{,}31 \pm 0.06^{c}$
4%	0.71 ± 0.08^{b}	0.96 ± 0.03^{b}	0.25 ± 0.04^{c}	$1.54 \times 10^{8\,a}$	$3{,}73 \pm 0.06^{d}$
6%	1.32 ± 0.13^{a}	1.85 ± 0.04^{a}	$0.53 \pm 0{,}13^{a}$	$5.80 \times 10^{7\,c}$	$4{,}53 \pm 0.06^{b}$

— Value represent mean ± SD from triplicate determination

Table 2: Reducing sugar, sucrose, total sugar, TPC LAB and pH value in fermented*Gelidium sp.* extract with palm sugar addition.

Palm sugar concentration	Reducing sugar (%)	Total sugar (%)	Sucrose (%)	TPC LAB (cfu/ml)	pH
0%	0.32 ± 0.03^{c}	0.39 ± 0.04^{d}	0.12 ± 0.05^{c}	$6.26 \times 10^{7\,d}$	5.10 ± 0.10^{a}
2%	0.41 ± 0.06^{c}	1.65 ± 0.08^{c}	1.27 ± 0.10^{b}	$1.12 \times 10^{8\,c}$	4.67 ± 0.11^{b}
4%	2.72 ± 0.41^{b}	5.29 ± 0.13^{b}	2.76 ± 0.13^{a}	$2.47 \times 10^{8\,a}$	3.93 ± 0.05^{d}
6%	6.57 ± 0.67^{a}	6.77 ± 1.87^{a}	0.34 ± 0.09^{c}	$1.78 \times 10^{8\,b}$	$4.30\ \ 0.10^{c}$

— Value represent mean ± SD from triplicate determination.

Table 3: Phenol, IC_{50} antioxidant activity and color value in fermented *Gelidium sp.* extract with sugar cane addition.

Sugar cane concentration	Phenol (ppm)	IC_{50} (10^3 ppm)	L*	a*	b*
0%	49.93 ± 1.92^{d}	50.97 ± 0.80^{a}	64.43 ± 0.51^{b}	15.63 ± 0.35^{a}	29.3 ± 0.89^{a}
2%	88.31 ± 2.56^{c}	47.93 ± 0.76^{b}	71.1 ± 0.85^{a}	15.4 ± 0.34^{a}	17.07 ± 0.92^{c}
4%	173.23 ± 1.64^{b}	42.48 ± 0.92^{c}	68.53 ± 1.36^{a}	15.87 ± 0.23^{b}	18.53 ± 0.50^{c}
6%	214.65 ± 2.89^{a}	40.38 ± 0.46^{d}	65.67 ± 1.15^{b}	14.3 ± 0.3^{b}	23.17 ± 1.25^{b}

— Value represent mean ± SD from triplicate determination.

Table 4: Phenol, IC_{50} antioxidant activity and color value in fermented *Gelidium sp.*Extract with palm sugar addition.

Palm sugar concentration	Phenol (ppm)	IC_{50} (10^3 ppm)	L*	a*	b*
0%	81.00 ± 2.65^{d}	49.06 ± 0.53^{a}	64.53 ± 0.40^{a}	15.76 ± 0.40^{a}	29.3 ± 0.89^{c}
2%	122.33 ± 2.51^{c}	46.37 ± 0.56^{b}	59.4 ± 0.34^{c}	13.73 ± 0.23^{d}	45.2 ± 1.36^{a}
4%	210.33 ± 1.53^{b}	39.18 ± 0.96^{c}	62.87 ± 0.23^{b}	14.87 ± 0.23^{c}	40.53 ± 0.50^{b}
6%	259.33 ± 2.52^{a}	34.85 ± 0.34^{d}	51.1 ± 0.17^{d}	15.5 ± 0.17^{b}	47.33 ± 1.53^{a}

Hypertonic solution condition can cause bacteria undergo plasmolysis, and making it difficult for bacteria to multiply. Also, too high of sucrose concentration can cause osmotic imbalance inside and outside bacteria cell. Hypertonic solution conditions can stimulate bacterial lysis and die [24].

C. pH value in fermented Gelidium sp. extract

The addition of sugar will reduce the pH at the end of the fermentation period (Table 1 and 2). The lowest pH value in each sugar types was in 4% sugar addition it is 3.73 in sugar cane addition and 3.93 in palm sugar addition. In this condition, sufficient carbon sources are available and environmental conditions also support the growth of the starter, *L. acidophilus*. The addition 4% sugar produces the highest number of bacteria due to the availability of sugar and the environment that supports starter growth. Addition of glucose will increase the cell density and reduce pH. A good growth of lactic acid bacteria starter will produce more acid and will reduce the pH value [25].

Glucose will be converted into lactic acid, while the addition of 6% sugar concentration will affect the solution to become hypertonic so the fluids in microorganism cells flow out which results in dehydration and shrinkage of microorganism (plasmolysis). Bacteria undergo plasmolysis cannot produce lactic acid, when the producing lactic acid is low, the pH value tends to be higher [26]. Bacterial cell membrane damage results in to the fail to absorb nutrient, so the metabolic process are inhibit [27].

D. Phenol content in fermented Gelidium sp. extract

Phenol content in the addition both sugar cane and palm sugar increased results with sugar concentration (Table 3 and 4). The highest value of total phenol is obtained in 6% both the samatypes of sugar addition. This phenomenon is because *L. acidophilus* use the compound in the media and produce primary metabolites (lactic acid) and secondary metabolites (polyphenols). The increase in phenol compounds during fermentation can be caused by microbial metabolism that produces secondary metabolic product, flavonoid compounds and also polyphenol [28]. The high content of sugar triggers the growth of lactic acid bacteria and the bacteria will convert sugar into primary metabolites product (lactic acid) and secondary metabolites (polyphenols). In addition conjugated phenolic compounds could be bio-tranformed to free form during fermentation [29].

Fermented *Arthrospira platensis* (Spirulina) with LAB showed higher in total phenol than unfermented Spirulina. The increasing phenol suggested because the algae were biodegrade by lactic acid bacteria [30]. Fermentation could produce gallic acid (poliphenols) or other metabolites that will increasing the antioxidant. In addition, the increasing phytochemical during fermentation due to the presence of hydrolytic enzymes produce by LAB and will hydrolyzed complex compound into simple compound [31-33].

Phenol content in 6% sugar addition give the highest value although the viability of the starter was not the highest. The highest viability of the starter was in the 4% sugar addition. This phenomenon might be cause by the bacteria generally excrete more secondary metabolites such as phenolic compound in environment disadvantages [34]. In sugar addition 6% the viability of sell is decrease. This probably because the solution become hypertonic and it means that the environmental is disadvantages and the cell produce more secondary metabolite like phenolic compound that observed in this experiment. The phenomena both the same in the addition of sugar cane and palm sugar

E. Antioxidant IC50 in fermented Gelidium sp. extract

Inhibition concentration (IC50) can be defined as the concentration of a sample solution that will cause a reduction in DPPH activity by 50%. Addition both sugar will decrease the IC50 value (Table 3 and 4). The lower antioxidant value of IC50 it means that the sample have higher antioxidant activity.

Fermentation proses will degrade macromolecule into smaller molecules that have higher antioxidant activities [35]. Antioxidant activity were enhanced in fermented apple juice with LAB although phenols and flavonoids were decreased. The enhanced antioxidant activities was suggested related to other compounds (phlorizin and caffeic acid).

The phenomena happen during fermentation both the same in sugar cane and palm sugar addition. The higher concentration sugar addition makes the low IC50 value. It is means that higher sugar addition will increase the activity of antioxidant in the product of fementation. The phenomena above is linier with the production of phenolic compound. The higher sugar addition will higher phenolic compound. Phenol are secondary metabolites and antioxidant [36]. Fermentation treatment with addition of *Lactobacillus* bacteria can increase the antioxidant activity [37]. The increase in antioxidant value is thought due to the degradation of compounds such as carbohydrates, proteins and fats to simple compound due to the metabolic activity of these bacteria. [38]reported that microorganism could increase antioxidant potential on Cystoseiratrinodis. Antioxidant properties could be enhanced during fermentation by lactic acid bacteria. Some species of lactic acid bacteria shown have antioxidative effects [39-42]. Antioxidant activity not only cause by phenol concentration but also the chemical structure [43].

Grouping of antioxidant activity consists of strong if the IC50 value is 50-100 ppm while average if the value is 101-150 ppm, and weak is 150-200 ppm [44]. The result of this experiment showed that the antioxidant activity was very weak. It might be cause by lack of fermentation time and need to be prolonged. In this study the fermentation time was 24 hours while in previous study showed that the antioxidant activity of fermented seaweed was highly increased after 36 hours fermentation time. The longer fermentation time it probably the starter adding the stationer phase or the death phase and the environment quality is decreased. The bacteria generally excrete more secondary metabolites such as phenolic compound in environment disadvantages.

F. Colour of the fermented Gelidium sp. extract

The L* value of fermented seaweed extract with addition with sugar cane indicate the lightness is not too dark and not too light. The ranges L* value is 64.43 to 71.1. Naturally the sugar cane colour was brownish yellow. The data shows that b* have positive value means the color of fermented seaweed extract with sugar cane

addition is yellow. Yogurt was made with seaweed extract showed higher yellowness *b* [45].
When the sugar addition is higher, the parameter L* is decreased means darker. This is in line with b* value the sample tent to red color although when the sugar is increased the red intensity become lower. Similar to the result that fermentation with LAB will result lower in L* and b* and higher in a* [42]. Palm sugar addition tends to be more brownish. Also, the value of L* and b* is inversely to the a*. The lower L* and b* will increase the a*.

IV. CONCLUSION

In conclusion, the addition sugar 6% in seaweed fermentation was able to increase the viability of starter, total phenolic and antioxidant activity. The study can be further extended for optimization addition nitrogen source for better viability of starter and longer fermentation period to increase the antioxidant activity.

V. FUTURE SCOPE

Future research is needed to determined the maximum level of sugar addition to increase the antioxidant activity and addition other media to increase the viability starter.

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