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The Effect of NaCl Concentration and Incubation Time on Oxalate and Total Acid in Fermented Cabbage using Various Microorganisms

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ABSTRACT

As the highest agricultural product, cabbage (*Brassica oleracea var. capitata*) remain to consider as a perishable vegetable and is also known to contain an antinutritional compound, called oxalate. The oxalate salts is a non-soluble compound in water, and thus settle on the human kidney. The preservation and improvement of the nutritional aspect of cabbage can be achieved by fermentation. Therefore, we study the effect of salt addition and incubation time, as the two important factors in commonly cabbage-based fermentation, combined with some oxalate-degrading-bacteria, which were *L. plantarum*, *L. plantarum* – *S. cerevisiae*, and *A. aceti* – *S. cerevisiae* on the oxalate and total acid level in cabbage fermentation. The fermentation was conducted in a submerged system at room temperature (25 – 27 °C) with the NaCl concentration addition ranging from 0 – 5% until 10 days. Those conditions were carried out for the three types of the tested cultures. The oxalate and total acid level were measured using the permanganometric and acid base titration, respectively. The results indicated that *L. plantarum*-induced fermentation combined with 3% NaCl during 10 days gave the lowest oxalate level in the fermented cabbage biomass, which was 0.005 mg/100 g FW. Moreover, this single-cultured fermentation was able to produce the highest total acid level in the brine solution, 1.270% at the 8 days of fermentation. This fermentation serves as an alternative method to improve cabbage consumption.

Keywords: cabbage, fermentation, oxalate, total acid

INTRODUCTION

Oxalate is a dicarboxylic acid mostly found in the consumed vegetables, fruits, nuts, and grains [1]. The oxalate salt inside the plant is produced by the formation of chelate compounds with metals, such as calcium and magnesium. This compound originally serves as a defense agent against fungi or pathogen microorganisms. In spite of that, the consumption of oxalate containing-vegetables can cause some health problems [1], [2].

Cabbage (*Brassica oleracea L. var capitata*) is one of the oxalate containing-vegetables and it has reported that the oxalate level in cabbage around 19.67 mg/100 g [3]. On the other hand, as the highest agricultural product in Indonesia, cabbage still consider as a perishable vegetable which decreases its consumption. One of the methods to preserve the shelf-life as well as degrade oxalate level in cabbage will be fermentation. The fermentation process is able to stimulate the bacteria to produce several oxalate-degrading enzymes [4] and facilitate the

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oxalate degradation into formic acid and CO_2 [5]. Those enzymes have been well-characterized in some microorganisms, such as *L. plantarum* [6], *S. cerevisiae* [7], and *A. aceti* [8].

Salt addition is an important step during common cabbage-based fermentation. It improves the taste quality of fermented product and inhibit the growth of pathogen microorganisms [9], [10]. Moreover, the salt addition also improves the organoleptic properties such as scent, taste, texture, water content, and organic acids composition [11], [12]. Apart from that, starter addition also improves the microbiological safety of fermented product. The organic acids resulted from bacteria metabolism are admitted to reduce the spoilage microorganism's growth [13], [14]. Taking together, the fermentation process in cabbage using some oxalate-degrading-bacteria combined with salt addition is potential to reduce the antinutritional compound as well as to highly preserve this vegetable.

Therefore, our research examined the effect of NaCl concentration along with the inoculation some microorganisms, which were *L. plantarum*, *L. plantarum – S. cerevisiae*, and *A. aceti – S. cerevisiae* on oxalate and total acids level in cabbage fermentation. Besides its antimicrobial effect, the latter compound was also measured as the benchmark of the presence of bacterial activity, since those three kinds of bacteria produce some organic acids as the major product during their metabolisms [15]–[17]. We also studied the effect of incubation time to optimize the bacterial activity in oxalate degradation and acid production.

EXPERIMENT

Sample and Chemicals

Cabbages (*Brassica oleracea var. capitata*) were obtained from Batu, East Java. The cultures *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, *Acetobacter aceti* used belongs to the culture collection of Biotechnology Laboratory, Department of Food Technology, University of Brawijaya.

The chemicals used for this research were D-glucose, yeast extract, peptone, sulfuric acid, pottasium permanganate, sodium oxalate, sodium hydroxide, and sodium chloride. All reagents were of analytical grade and purchased from Merck. In addition, we employed the medium for multiplication and cultivation of microorganisms from OXOID, such as de Mann Rogosa Agar/Broth (MRSA/MRSB), Nutrient Agar (NA), and Potatoes Dextrose Agar (PDA).

Procedure

The Preparation of Inoculums

The cultures *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, *Acetobacter aceti* were first subcultured in its respective solid medium: *L. plantarum* in MRSA, *S. cerevisiae* in PDA, and *A. aceti* in NA medium. Then, each microorganism was cultivated in broth medium as follows: *L. plantarum* in MRSB, *S. cerevisiae* in Yeast-Pepton-Dextrose (YPD) and *A. Aceti* in Glucose-Yeast-Peptone (GYP) medium. All medium were sterilized before at 121 °C for 15 min. The lactic acid bacteria and yeast were grown in anaerobic conditions at 30 °C for 17 h and 26 h, respectively. Meanwhile, *A.aceti* inoculum was prepared by maintained the bacteria at room temperature 25-27 °C for 17 h in shaker incubation.

The Fermentation Process

Cabbage was first prepared by trimming of the crop and outer layer. Then, the fresh cabbage was chopped into strips (around 2 mm) using a knife. About 100 g of chopped cabbage was washed in running tap water, dried for 10 minutes, and put into some fermentation jar. Some inoculum variations (v/v) [18]–[20]: *L. plantarum* 5%, *L. plantarum – S. cerevisiae* 5% and *A. aceti – S. cerevisiae* 15% were used separately in three different fermentation jar. After

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that, NaCl concentration ranging from (w/w): 0, 1, 2, 3, 4, 5% were added to each fermentation jar. The total acid and oxalate level were determined in the 10th day of fermentation. Meanwhile, to study the effect of incubation time, the total acid and oxalate level was daily measured until the 10th day of fermentation combined with the optimum NaCl concentration for each culture. The fermentation was conducted in anaerobic conditions at room temperature 25-27 °C using a submerged system. The cabbage fermentation without starter addition was also carried out as a comparison.

The Measurement of Total Acid Level

The total acid level in the brine solution from the fermented cabbage was determined using acid-base titration method. The brine solution was centrifuged first for 10 min. About 10 mL of the resulted supernatant was diluted in 100 mL volumetric flask using distilled water. After that, 10 mL of this solution was transferred into 250 mL Erlenmeyer flask and added with one drop of 1% phenolphtelein indicator solution. The mixture was titrated against 0,1 M sodium hydroxide until the pink-colored-solution formation. The total acid level was expressed as % v/v.

The Measurement of Oxalate Level

The measurement of oxalate level in the biomass of fermented cabage was done using the permanganometric titration method described by AOAC (1975). Briefly, 10 g of fermented cabbage biomass was mashed up with a blender and extracted using 50 mL of distilled water followed by stirring and heating for 15 min. Then, the extracted cabbage biomass was filtered and centrifuged for 10 min. About 5 mL of the resulted supernatant was put into a 250 mL Erlenmeyer flask and added with 5 mL of 2 N sulfuric acid. The mixture was stirred and titrated against 5 N potassium permanganate. The titration was allowed until the formed pink color lasted for 30 seconds. The remained oxalate level in the fermented cabbage biomass was expressed as mg/100 g FW.

Data Analysis

Data were showed as means ± standard deviations of four replicates. These data were analyzed using analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) at 5% level test to decided if there was a significant interaction between the treatments on observed parameters.

RESULT AND DISCUSSION

The Effect of NaCl Concentration on Total Acid Level

Figure 1 shows the effect of different cultures and NaCl concentration on total acid level in the brine solution. Overall, the highest total acid level in the brine solution, 1.040% was achieved by *L. plantarum – S. cerevisiae* fermentation mixed with 2% NaCl, followed by *L. plantarum* fermentation and 3% NaCl, which resulted about 1.010% total acid level. No significant differences ($P > 0.05$) between those two treatments. The NOB fermentation produced a stable total acid level in all NaCl ranges and it was significantly lower than the two former fermentations ($P < 0,05$). This fermentation generated the highest total acid level, only 0.536% combined with 1% NaCl. Similar to the NOB fermentation, the *A. aceti – S. cerevisiae*-induced fermentation tends to get the lowest total acid level, around 0.500% with 5% NaCl.

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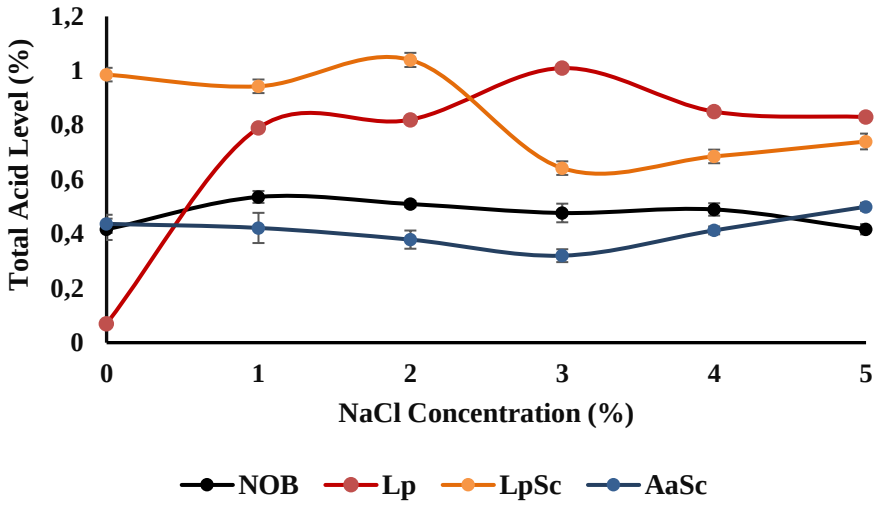

Figure 1. The effect of NaCl concentration addition on total acid level produced by various cultures in the brine of cabbage fermentation. The fermentation was conducted for 10 day. NOB: Naturally Occuring Bacteria; Lp: *L. plantarum*; LpSc: *L. plantarum – S. cerevisiae*; AaSc: *A. Aceti – S. cerevisiae*

The acidity in the brine originated from NOB fermentation was contributed by some spontaneous living bacteria. Wadamori, *et. al.* (2014) [21] and Suprihatin (2010) [22] reported that the spontaneous cabbage fermentation involves the growth of several lactic acid bacteria, such as *L. delbruckil*, *L. fermentum*, and *L. brevis*, *L. lactis*, *L. acidophilus*, *L. bulgaricus*, *L. mesenteroides*, and *L. dextranicum*. These species will stimulate the leaves decay by acid production. The stable acid produced by pure fermentation in all NaCl concentration, suggesting that the starter addition in another three fermentations had an effect in salt tolerant properties of the microbical population during cabbage fermentation.

The previous study also has reported that there was a different salt tolerance between Gram-positive and Gram-negative bacteria. The salt medium has a stronger growth inhibition properties toward Gram-negative bacteria compared to Gram-positive bacteria due to the hyper osmotic shock on the impermeable membrane of Gram-negative bacteria, which lead to the growth supppresion [23], [24]. Besides, it has been reported that *L. plantarum* was able to growth in up to 8% NaCl-enriched medium [25], whereas there are no growth of *A. aceti* inoculum in 2% NaCl-enriched medium [26]. On the other hand, the salt tolerance in yeast, *S. cerevisiae* has been studied by Park, *et.al* (2015) [27] and Subodinee, *et. al.*(2019) [28] which mentioned that this yeast still capable to grow in the presence of 10% NaCl. *S. cerevisiae* maintained Na^+ tolerant through ion homeostasis reached by ion transport and detoxification mechanisms and also osmotic adjustment by accumulating solutes inside the cell [29]. Taking together, those findings were in line with our research. The salt tolerance properties of *L. plantarum*, which was a Gram-positive bacteria and yeast in *L. plantarum*– and *L. plantarum – S. cerevisiae*-induced fermentation were contributed to its stable activity and metabolism. These stability would lead to the higher total acid produced. In contrast, due to the lower salt medium adaptation of acetic bacteria, *A. aceti* has a lower activity to generate some organic acid, and the limited total acid produced by *A. Aceti – S. cerevisiae*-induced fermentation was mostly generated only by *S. cerevisiae*.

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The Effect of Incubation Time on Total Acid Level

All cultures gave similar trend (**Figure 2**), where the increasing tendency of total acid level was directly proportional to the increasing of incubation time. There was a statistically significant interaction ($P < 0.05$) between culture types and incubation time on the total acid level. In combined with their respective optimum NaCl concentration addition (**Table 1**), the highest total acid level by NOB-, *L. plantarum*-, *L. plantarum* – *S. cerevisiae*-, and *A. Aceti* – *S. cerevisiae*-induced fermentation was achieved at 7, 8, 9, and 10 days, respectively. These data suggested that the starter addition also prolonged the optimum activity of microorganisms.

The NOB-induced fermentation gave the highest total acid around 0.755% at the 7th day. As mentioned above, pure culture in cabbage fermentation has contained some heterofermentative lactic acid bacteria. Wiander and Ryhanen (2004) [14] reported that those organic acids-producing-bacteria dominate the microflora at the beginning of fermentation and then it were replaced by more acid-tolerant homofermentative species, which accumulate more lactic acid at latter stages. Meanwhile, the induced *L. plantarum* in cabbage fermentation gave the highest total acid level among all culture used (**Table 1**), differs from our former finding, where *L. plantarum* – *S. cerevisiae* induction gave the highest total acid level (**Figure 1**). The acid production from *L. plantarum*, particularly as lactic acid, acetic acid, and formic acid came from the glucose metabolism in anaerobic condition [15]. On the other hand, the glucose metabolism of *S. cerevisiae* yield ethanol as the major product and only produce acid, mainly acetic acid as the minor product [16]. This ethanol evidently was known to have growth inhibiton and physiological damage effect to lactic acid bacteria cell. Lee *et. al.* (2012) [30] observed that ethanol destroyed the membrane function by changing the membrane structure, then induced the intracellular metabolic imbalance through hindering the carbon metabolism in the cell center or suppresing TCA cycle and glycolysis, which lead to the vitality reduction of bacteria. Hence, we can propose that the ethanol from yeast disturbed the cell activity in *L. plantarum* – *S. cerevisiae*-inoculated-cabbage fermentation, compared to the single cultured-cabbage fermentation and thus lead to the lower acid production. Lastly, instead of causing the growth inhibition effect toward *A. aceti*, the ethanol from *S. cerevisia*e actually can serve as the carbon source for oxidate fermentation of the acetic acid bacteria to release some organic acids, mainly acetic acid [17]. However, we have to consider that the NaCl addition affected its activity, and therefore this mixed culture still resulted in the lowest total acid level.

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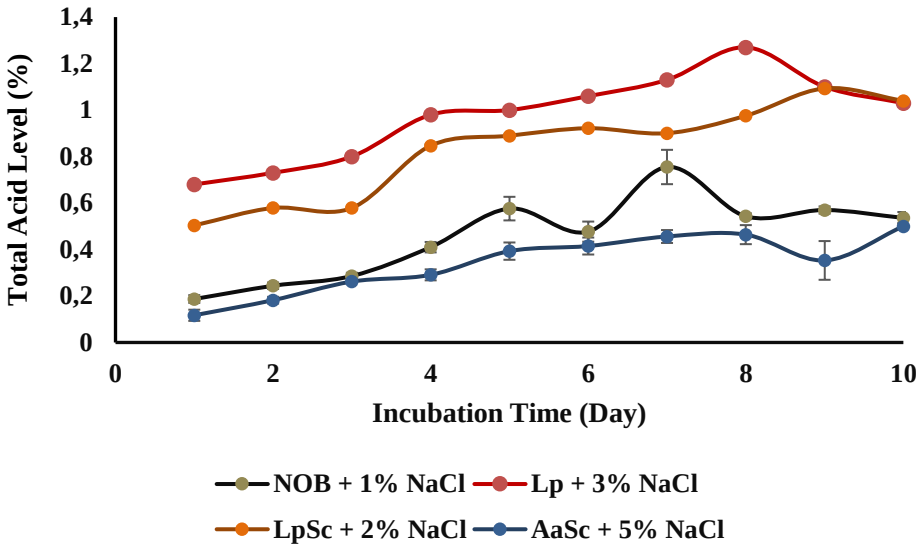

Figure 2. The effect of incubation time on total acid level produced by various cultures in the brine of cabbage fermentation in combination with its respective optimum NaCl addition. NOB: Naturally Occuring Bacteria; Lp: *L. plantarum*; LpSc: *L. plantarum – S. cerevisiae*; AaSc: *A. Aceti – S. cerevisiae*

Table 1. The optimum of each culture to produce the highest total acid level

Culture Type	NaCl Concentration	Incubation Time	Total Acid Level
Naturally Occuring Bacteria	1%	7	0.755 ± 0.073^{b} %
L. plantarum	3%	8	1.270 ± 0.001^{d} %
L. plantarum – S. cerevisiae	2%	9	1.093 ± 0.007^{c} %
A.aceti – S. cerevisiae	5%	10	0.500 ± 0.014^{a} %

*Data is expressed as means ± standard deviation. Different superscripts in the same column show a significant difference ($P < 0.05$)

The Effect of NaCl Concentration on Oxalate Level

Figure 3 illustrates the effect of culture types together with NaCl concentration addition ranging from 0 – 5% on the bacteria activity to degrade oxalate. Generally, the decrease of oxalate compound along with the increasing NaCl concentration addition in the fermented cabbage biomass was slightly constant for all inoculum types. However, *L. plantarum*-induced fermentation gave the lowest oxalate level compared to the all inoculum used ($P < 0.05$). The oxalate level originated from this single cultured-fermentation was 0.005 mg/100 g FW with 3% NaCl. The NOB- and *A. aceti – S. cerevisiae*-induced fermentation gave a similar results, where the lowest oxalate level achieved by using 0% NaCl (0.05 mg/100 g FW) and 1% NaCl (0.06 mg/100 g FW). No significant differences ($P > 0.05$) between those two kind of fermentations. Moreover, *L. plantarum – S. cerevisiae*-induced fermentation has a significant highest oxalate level among all inoculums ($P < 0.05$). The lowest oxalate level from this mixed culture fermentation, 0.139 mg/100 g FW was reached by using 3% NaCl.

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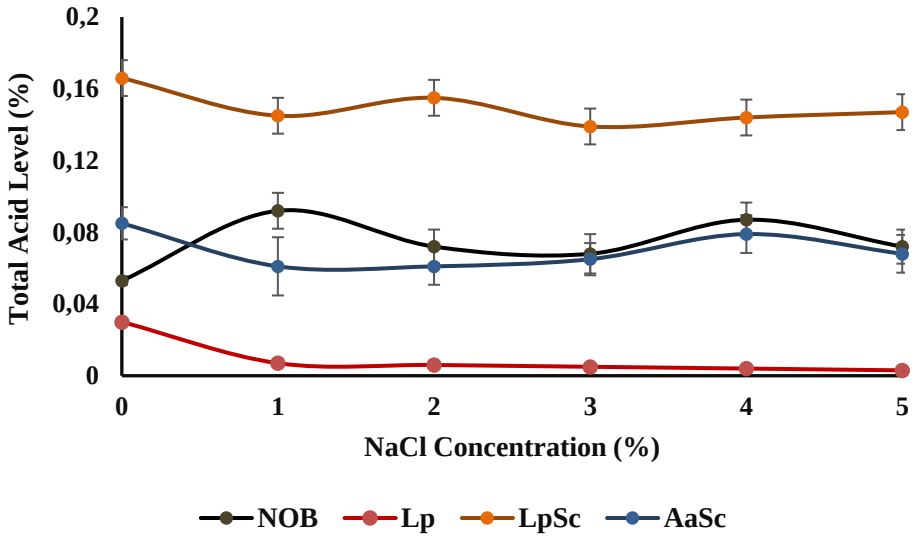

Figure 3. The effect of NaCl concentration on the oxalate level in fermented cabbage biomass. The fermentation was conducted for 10 day. NOB: Naturally Occuring Bacteria; Lp: *L. plantarum*; LpSc: *L. plantarum – S. cerevisiae*; AaSc: *A. Aceti – S. cerevisiae*

The three kind bacteria used for this cabbage fermentation: lactic acid bacteria, acetic acid bacteria, and yeast was known to have oxalate-degrading-enzyme. *L. plantarum* was able to produce oxalate decarboxylase enzymes [6], *S. cerevisiae* has a *Saccharomyces cerevisiae* acyl-scaae3 (ScAAE3) or called an oxalyl-coA-synthetase [7], whereas *A. aceti* has Formyl-coa:oxalate coA-transferase (FCOCT) and oxalyl-coA decarboxylase (OXC) [8]. On the other hand, NOB-induced fermentation developed the growth of some *Lactobacilli* and *Leuconostoc* species. However, Lee, *et. al.* (2018) [9] has reported that *Leuconostoc* species was immediately increase in salt-enriched-fermentation only after preparation step. The rapid growth of some bacteria cell in certain fermentation system with remained nutrition will undergo the reduction of bacterial population due to its carbon consumption and lack of nutrition [31]. Therefore, the increasing NaCl addition will lead to the rapid increase of bacterial population in NOB fermentation, but cause a reverse effect on its activity and mortality to release enzyme.

It was earlier highlighted that the inoculation of *L. plantarum* starter with 3-5% NaCl addition give the lowest oxalate level in fermented cabbage biomass, showing that this single-cultured-fermentation performed the best activity of oxalate-degrading enzymes compared to another bacteria used. According to Gomathi, *et. al.* (2014) [32], these enzyme can work optimally in acidic conditions. The acidic environment in the fermentation process stimulate the divalent oxalate ions ($C_2O_4^{2-}$) to deprotonate, and hence reduce its ability to bind with minerals to become insoluble oxalate salts [33]. These findings were correlated to our previous data that *L. plantarum* combined with 3% NaCl also produce the highest total acid level (**Figure 2**) that can stimulate the enzyme activity during fermentation. In spite of that, the addition of *L. plantarum – S. cerevisiae* exhibited the lowest enzyme activity based on the highest oxalate level. Taking to note, we could propose that the ethanol stress of *L. plantarum* due to the metabolism of *S. cerevisiae* as mentioned before also contributed to the lack of certain enzyme production, include oxalate-degrading enzyme. Furthermore, the optimum NaCl concentration to reduced oxalate level in *A. aceti – S. cerevisiae* slightly the same as observed in NOB

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fermentation, which is 1%. Again, the Gram-negative bacteria such as *A. aceti* was less resistent of NaCl exposure due to the thinner stucture of the cell wall that facilitated plasmolysis. Therefore, we assumed that the activity of oxalyl-coA decarboylase from the latter mixed culture was more dominant originated from *S. cerevisiae*.

The Effect of Incubation Time on Oxalate Level

Overall, all starter types give the similar results. The culture types had a significant effect on oxalate levels ($P < 0.05$), but no significant difference between incubation time on observed parameter ($P > 0.05$). The optimum incubation time for NOB, *L. plantarum*, *L. plantarum – S. cerevisiae*-, and *A. aceti – S. cerevisiae* to reduce oxalate level in fermented cabbage biomass were 8, 10, 10, 10 days, respectively (**Table 2**). Again, *L. plantarum* starter gave the lowest oxalate level, 0.005 mg/100 g FW, followed by non starter, which was 0.053 mg/100 g FW, and then mixed cultures *A. aceti – S. cerevisiae* (0.061 mg/100 g FW) and *L. plantarum – S . cerevisiae* (0.139 mg/100 g FW).

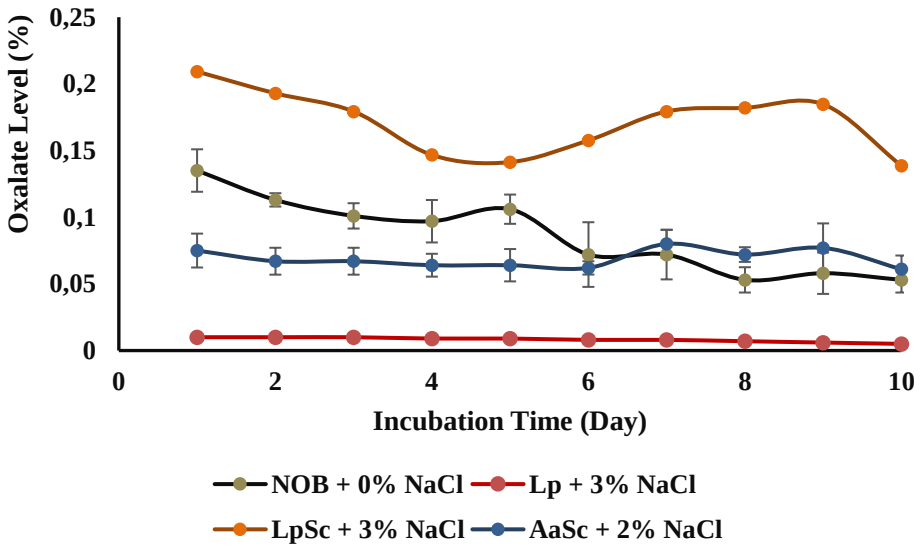

Figure 4. The effect of incubation time on the remained oxalate level in fermented cabbage biomass. NOB: Naturally Occuring Bacteria; Lp: *L. plantarum*; LpSc: *L. plantarum – S. cerevisiae*; AaSc: *A. Aceti – S. cerevisiae*

The trend observed between *L. plantarum*- and NOB-fermentation was quite the same, where the oxalate level decreased together with the increasing incubation time. It was indicated that the longer incubation time will allow the lactic acid bacteria in both fermentation to release more organic acids that stimulate the enzyme activity. These acid accumulations will lead to the increasing activity of both enzymes. In addition, the trend between the two mixed-cultures also had similarity. In the earlier stages, the oxalate level was reduced, and then there was a sligh elevation in oxalate level before it decreased again in the last day of fermentation. The slightly decrease of enzyme activity (based on the elevated oxalate level) in the middle stages of mixed culture fementation can be contributed by imbalance activity of the two microorganisms. The previous report has described that the worst problems in mixed culture fermentation is the control of the optimum balance among the microoganisms involved. Sometimes, it is difficult to define the dominant microoganism employed [34]. Therefore, the

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imbalance environment between two microorganisms contrastly affect their activity. Furthermore, we could suggest that the higher enzyme activity in the last stages was mostly contributed by the naturally ocurring lactic acid bacteria, which had been proven to have more salt and acid tolerance [23]–[25].

Table 2. The optimum condition to reduce oxalate in the fermented cabbage biomass for each culture

Culture Type	NaCl Concentration	Incubation Time	The Remained Oxalate Level
Naturally Occuring Bacteria	0%	8	0.053 ± 0.009^{c} mg/100 g FW
L. plantarum	3%	10	0.005 ± 0.001^{a} mg/100 g FW
L. plantarum – *S. cerevisiae*	3%	10	0.139 ± 0.000^{d} mg/100 g FW
A.aceti – *S. cerevisiae*	2%	10	0.061 ± 0.010^{b} mg/100 g FW

Data is expressed as means ± standard deviation. Different superscripts in the same column show a significant difference ($P < 0.05$)

CONCLUSION

The results of this study indicate that the various treatments of cabbage fermentation affect differently to the microorganism activity, either to produce total acid or reduce oxalate level. It was found that *L. plantarum*-induced fermentation combined with 3% NaCl during 10 days gave the lowest remained oxalate level, around 0.005 mg/100 g FW. Those single-cultured fermentations also produced the highest total acid level in the brine solution, 1.270% at the 8 days of fermentation. The variation of bacterial activity used in this experiment mostly could be contributed by the difference of the salt and acid-tolerant properties between *L. plantarum*, *S. cerevisiae*, and *A. aceti*.

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