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Effect of Mixed Inoculums Volume and pH on Anti Nutritional Level in Cabbage Fermentation using *Saccharomyces cerevisiae* and *Lactobacillus plantarum*

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Abstract. Cabbage (*Brassica oleracea* var. *capitata*) that is one of the widely consumed vegetables, contains significant amounts of anti-nutritional components, such as tannin. This component can be reduced by fermentation, where bacterial tannase activity is able to degrade tannin into glucose and gallic acid. A simultaneously mixed cultures fermentation using *Saccharomyces cerevisiae* and *Lactobacillus plantarum* has been developed because they possess some mutualism interaction that can be beneficial in the fermentation process. Therefore, this research was focused on the effect of mixed inoculums volume of *S. cerevisiae* and *L. plantarum* and initial culture pH on the tannin and glucose level during cabbage fermentation. The sliced cabbage was inoculated with simultaneously mixed cultures (volume ratio of yeast and bacteria used was 1:1) at a different volume range 5%-25% (v/v) and also various pH range from 4 to 7, and then incubated at room temperature in anaerobic condition for 4 days. Tannin and glucose level in fermented cabbage (both biomass and filtrate) were determined using Folin-Denis and phenol-sulphuric method, respectively. The using of 5% mixed inoculums volume and initial pH around 7 were found to be the optimum condition for tannin degradation in cabbage. The remained tannin level in these fermented cabbage decreased up to 18.58% (54.061 mg/100 g FW). This experiment reveals that the amounts of inoculums and initial culture pH affected tannin degradation during cabbage fermentation using mixed cultures of yeast and lactic acid bacteria.

Keywords: Cabbage, Tannin, Fermentation, *Lactobacillus plantarum*, *Saccharomyces cerevisiae*

1. Introduction

Cabbage (*Brassica oleracea* var. *capitata*) is one of the most widely consumed vegetables in the world. This vegetable is an excellent source of variety vitamin, mineral, protein, and dietary fiber [1,2]. Despite their abundant supply of health-promoting compounds, cabbage also contains anti-nutritional components, such as tannin. Mosha, et al. [3] reported that cabbage contains 1266 mg tannic acid (hydrolysable tannins) per 100 g FW. This compound decreases protein digestibility in humans,



probably by forming a non-absorbable complexes with protein and disrupts its availability or binding directly to digestive enzymes, such as lipase, amylase, trypsin, and chemotrypsin [4,5].

An extensively studies has been conducted to degrade tannin through fermentation using *Lactobacillus plantarum*. This process is facilitated by tannin-degrading enzyme produced by bacteria, commonly known as tannase. This enzyme hydrolyzes the galloyl ester bonds of tannin and produces gallic acid and glucose [6-8]. Natarajan, et al. [6] reported that *L. plantarum* was able to produce tannase under medium containing 3.5% tannic acid with the optimum temperature 30 °C and pH 6. Moreover, Matsuda, et al. [7] showed that tannase production from *L. plantarum* in 0.1 % tannic acid-containing medium had optimal condition as follows: temperature around 30 °C and pH was 8.

Nowadays, a mixed cultures system using lactic acid bacteria and yeast in fermentation process has been developed, because they possess some mutualism interaction that can be beneficial for their growth. Yeast secretes amino acids (glutamine, threonine, phenylalanine, tryptophan, and serine) for stimulate *Lactobacillus plantarum*'s growth [9], while *L. plantarum* provides a lactose-degrading enzyme to release glucose and galactose, and also lactic acid that act as energy source for *S. cerevisiae* [10]. However, the main difficulty in mixed cultures fermentation is the different condition for each microorganism, such as pH and the amounts of inoculums added. Since yeast and lactic bacteria may have different optimum condition for their growth, this fermentation was carried out at various inoculums volume and initial culture pH to evaluate its tannin degradation performance in cabbage.

2. Materials and Methods

2.1. Chemicals and Instrumentation

Glucose, H₂SO₄ 95-98%, phenol, ethanol 99.9%, Na₂CO₃, Folin-Denis reagent, were utilized. All chemicals and regents used in this study were of analytical grade and purchased from Merck. The medium for *Lactobacillus plantarum* cultivation was MRS media (de Mann Rogosa Sharpe) (OXOID) and supplemented agar for multiplication plate. PDA (Potatoes Dextrose Agar) (OXOID) and GYP (Glucose Yeast Pepton) were used to multiply and culture *Saccharomyces cerevisiae*. Determinations of glucose and tannin level were conducted using spectrophotometer UV-Vis.

2.2. Plant Material

Cabbage (*Brassica oleracea var. capitata*) samples were collected from Malang, East Java. The plant was identified and authenticated by plant taxonomist of the Microbiology Laboratory, Department of Biology, Brawijaya University.

2.3. Microorganism and Inoculums Preparation

Saccharomyces cerevisiae and *Lactobacillus plantarum* were supplied by Microbiology Laboratory, Department of Food Technology, Brawijaya University. The yeast and bacteria were multiplied in PDA and MRSA, respectively, and incubated for 48 h at 30 °C. The cultured yeast from PDA was transferred into YPD broth and incubated for 17 h at 30 °C, whereas the bacteria from MRSA was cultivated in MRS broth and incubated for 26 h at 30 °C.

2.4. Fermentation

Fresh cabbage was prepared by removing the core and outer layers. The cabbage leaves were then sliced into strips using knife and washed under running tap water. Subsequently, each 100 gram of the sliced cabbage leaves was transferred into fermentation container. To study the effect of inoculums volume, sliced cabbage was inoculated with simultaneously mixed cultures of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (volume ratio 1:1) at various volumes ranging from 5% to 25% (v/v). To determine the optimal pH, the inoculation amount of yeast and bacteria were set at 5% and the initial pH of both medium (YPD and MRS broth) were set as 4, 5, 6, 7. The pH was adjusted by adding 0.1 M NaOH or acetic acid glacial. Fermentation was carried out at room temperature for 4 days in anaerobic condition.

2.5. Tannin Determination

Tannin content in the biomass and filtrate of fermented cabbage was determined according to AOAC (1995). A 2.5 g of the fermented cabbage biomass was crushed using pestle and mortar and extracted with 20 mL distilled water in an 50 mL Erlenmeyer flask by stirring and heating for 10 min. Then, the mixture was filtered and rinsed with 2 mL additional distilled water and the volume was adjusted to 25 mL. After that, 0.4 mL of this extract was transferred into test tube, then added with 0.5 mL Folin-Denis reagent and 1 mL Na_2CO_3 solution. The mixture was made up to the 10 mL and the colour was measured after 30 min at 760 nm using a spectrophotometer, the reagent blank was prepared in similar manner without sample solution. Meanwhile, the filtrate from fermented cabbage was centrifuged for 10 min. The supernatant was used for tannin determination in filtrate using the same procedure as those used for biomass. Tannin level was determined by a tannic acid standard curve and expressed as milligrams per 100 g of fresh weight sample.

2.6. Glucose Determination

Glucose content in biomass and filtrate of fermented cabbage was extracted and measured according to Dubois, et al. (1965) [11]. In total, 0.1 g of fermented cabbage biomass was added with 5 mL of 80% ethanol in the test tube and heated in water bath for 1 h at 70-80 °C. Then, 1 mL of the sample extract was delivered into another test tube and added with 1 mL of 5% phenol. After that, 5 mL of H_2SO_4 95-98% was added rapidly directed against the liquid surface. The tube was allowed to stand 10 min, and then kept in water bath at 25-30 °C for 15-20 min. The absorbance of this solution was read at 485 nm using spectrophotometer. Blank was initially prepared with all reagents without sample solution. After that, the filtrate from fermented cabbage was centrifuged for 10 min. The supernatant obtained was diluted 10 times using distilled water. One millilitres of this solution sample was taken for glucose determination in filtrate using the same procedure as those used for biomass. Glucose level was measured using a glucose standard curve and expressed as milligrams per 100 g fresh weight.

3. Results and Discussion

3.1. Optimization of Mixed Inoculums Volume

Since the cabbage fermentation was generated using *Saccharomyces cerevisiae* and *Lactobacillus plantarum* simultaneously, inoculums volume of the mixed cultures may affect their growth and activity. Therefore, various inoculums amounts ranging from 5 to 25% (v/v) were studied for tannin degradation.

Figure 1 shows that the different inoculums volume affected tannin level, both in fermented cabbage biomass and filtrate (1a). Either in biomass or filtrate, tannin level was reduced at 5% inoculums and increased at 10% inoculums. However, the addition of 15 and 20% inoculums accidentally resulted in tannin reduction levels again, and increased gradually when 25% of mixed inoculums were used. Although the level of tannin in biomass was tend to fluctuate at the inoculums volume above 5%, it can be seen that tannin level decreased slightly compared to the tannin level in fresh cabbage (290.877 mg/100 g FW). This indicated that the more mixed inoculums added will cause a competition between bacteria and yeast to survive, which promoted a reduction in their growth or activities. Moreover, the elevated ethanol content produced by *S. cerevisiae* was known to have opposite effects on the growth of another microorganism in mixed cultures system [12,13]. Therefore, it can be concluded that the ethanol slowly decreased the activity of *L. plantarum*, and then resulted in tannase activity reduction.

Due to the trends of tannin level were seem to fluctuate, and tannin hydrolysis by bacterial tannase may release glucose [8], the glucose level was also examined in this experiments. The tannin level was inversely proportional to the glucose level in the fermented cabbage biomass (Figures 1a and 1b). The similar trends were also observed between tannin and glucose levels in filtrate. These finding

confirmed the presence of tannase in the fermentation system that promoted tannin hydrolysis into glucose, and thus produced opposite relationship between tannin and glucose from all inoculums range. It was found that the optimal volume of mixed cultures inoculums was 5%, where tannin level reduced up to 62.69% (182.362 mg from 290.877 mg tannic acid per 100 g fresh weight of cabbage).

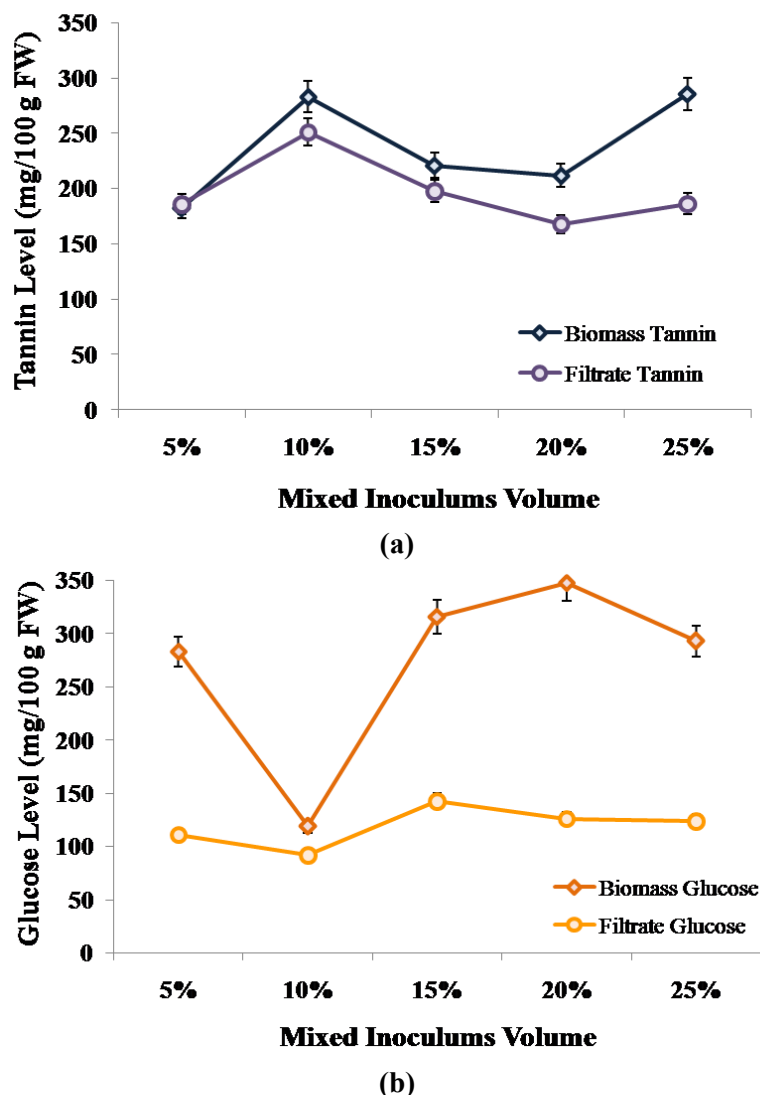


Figure 1. The effect of mixed inoculums volume of *S. cerevisiae* and *L. plantarum* on tannin level (a) and glucose level (b).

3.2. Optimization of pH Initial Culture

It was known that *S. cerevisiae* and *L. plantarum* have different optimum pH for their growth. In addition, some studies showed various optimum pH for tannase production by *L. plantarum*. For this reason, the effect of different pH of mixed cultures system on tannin degrading activity was examined using 5% of mixed culture inoculums.

Generally, the more acidic pH reduced tannin degradation (Figure 2). Despite the tannin level in filtrate was found to fluctuate along the pH value, the total tannin from both fermented cabbage biomass and filtrate was lower than that in raw cabbage, indicating that tannin was degraded into less-toxic compounds. The optimum pH for tannin degradation was found to be 7, where remained tannin

level in fermented cabbage biomass was 18.59% (54.061 mg remained tannin from 290.877 mg/100 g FW). This result is slightly different with earlier reports published by Natarajan, et al. [6] where *L. plantarum* showed optimum tannase activity in pH around 6 or those by Matsuda, et al. [7] that showed the optimum pH of tannase enzyme produced by *L. plantarum* was 8. However, according to Jimenez, et al. [8], *L. plantarum* had two types of tannase enzyme; called TanALp (extracellular tannase) with an optimum pH 6 and TanBLp (intracellular tannase) with optimum pH was 7-8. In this report, TanALp was able to degrade complex tannic acid outside the cell. The less complex tannin derived from TanALp would induce the expression of *tanB_{Lp}* gene, passed into the cell to be degraded by TanBLp, and thus released gallic acid and glucose. This report could at least explain why there was different optimum pH for tannase produced by *L. plantarum* in some researches. Which types of tannase enzyme activity that would dominate the others was not described in this experiment. However, since this experiments conducted using *S.cerevesiae*, the ethanol and organic acid produced by this yeast probably promoted of membrane integrity disruption of another microorganism [12,13], in this case was *L. plantarum*. The membrane disruption in *L.plantarum* thus will facilitate the transportation of the product originated from TanALp activity from outside into the cell cytoplasm to be more degraded.

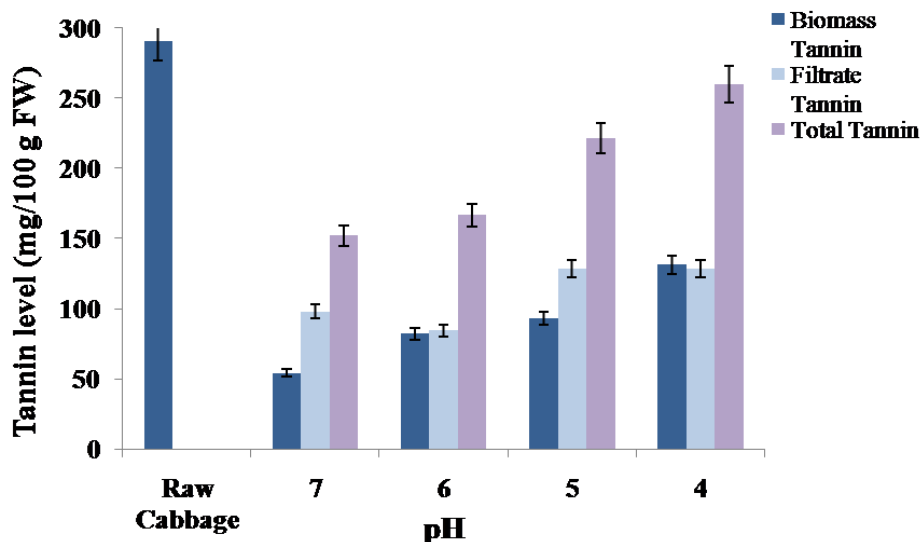


Figure 2. The effect of various pH on tannin level during cabbage fermentation. The dark blue bars represented tannin level in cabbage biomass, the lighter blue showed tannin level in filtrate, and the purple bars represented total tannin after fermentation.

As mentioned previously, tannin reduction gradually decreased below pH 7 (Figure 2). In the acidic environments, *S.cerevesiae* was tend to produce more ethanol [14]. In addition, *Lactobacilli* itself was also produce ethanol even in small quantities. Although this two microorganisms were known as ethanol and acid-tolerant, its growth, especially *L. plantarum* is still strongly inhibited by the elevated concentrations of ethanol or organic acid reached during fermentation [12]. Lee, et al. [15] reported that *L. plantarum* cell numbers were slightly decrease when it was grown in the 5% ($OD_{600}=1.6$) to 8% ($OD_{600}=1.2$) ethanol-containing medium for 60 h. Also, these bacteria did not grow at ethanol concentration around 10%. Furthermore, van de Veen, et al. [13] reported that *L. plantarum* had 5-fold-lower growth rate in 8% MRS containing ethanol compared with control medium without ethanol. Therefore, it was possible to suggest that the presence of ethanol and organic acid on fermentation medium could be negatively affected on the growth of *L. plantarum* and lead to the reduction of tannase production. Hence, the tannin degradation decreased proportionally with decreasing pH value.

4. Conclusion

In conclusion, this study reveals that the volume of mixed inoculums and initial culture pH affect tannin degradation in cabbage by simultaneously fermentation of *S.cerevisiae* and *L. plantarum*. Tannin level in fermented cabbage biomass or filtrate was inversely proportional glucose level, suggesting there was glucose release from tannin hydrolysis reaction by tannase enzyme produced by lactic acid bacteria. The results showed that the best favourable conditions for tannin degradation by mixed cultures fermentation were: inoculums volume 5% (yeast and bacteria volume ratio=1:1) and pH 7.

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