Influence of Mixed Cultures of *Saccharomyces cerevisiae* and *Acetobacter aceti* for Hydrolysis of Tannins in the Cabbage Fermentation (*Brassica oleracea L.var.capitata*)

Alfi Salamah¹, Arie Srihardyastutie¹*, Sasangka Prasetyawan¹, Anna Safitri¹²

¹Chemistry Department, Brawijaya University, Malang, 65151, Indonesia
²Research Centre for Smart Molecules of Natural Genetic Resources, Brawijaya

*Corresponding author email: arie_s@ub.ac.id

**Abstract.** This study aims to hydrolysis tannins contained in cabbage (*Brassica oleracea L.var.capitata*) using a mixture of *S.cerevisiae* and *A.aceti* cultures with submerged fermentation method. Determination of the best conditions for tannins hydrolysis carried out by stratified optimization with variation of the volume mixed inoculums (5%, 10%, 15% and 20% (v/v) in 100 mL distilled water) and pH values (4, 5, 6 and 7). Cabbage fermentation using a mixture of *S. cerevisiae* and *A. aceti* cultures with a ratio of 1:1 (v/v) and anaerobic incubation for 4 days at room temperature (25 ± 2 °C). The content of tannins before fermentation is 290.876 mg /100 g FW. After fermentation the tannins content decreased until the mixtures inoculum concentration was 15% and increased at 20%. The increase of tannins content due to the presence of high acetic acid on the fermentation medium so can inhibit tannase enzyme activity. The optimum volume of mixed inoculums at 15% produced tannins in the filtrate of 227.342 mg /100 mL and 193.619 mg /100 g FW biomass. Hydrolysis tannins can be carried out in acidic conditions. The optimum pH value at pH 4 produces tannins of 107.742 mg /100 g FW in biomass. Hydrolysis tannins can be catalyzed by tannase produced from *S.cerevisiae* and acetic acid by *A.aceti*.

**Keyword :** Cabbage (*Brassica oleracea L.var.capitata*), *Saccharomyces cerevisiae*, *Acetobacter aceti* and tannins.

1. **Introduction**

Cabbage (*Brassica oleracea L var.capitata*) contains variation components such as carbohydrates, fiber, lipids and proteins, as well as phyttonutrients such as glucosinolate, minerals and vitamins needed as a source of nutrition for the body. However, cabbage also has antinutrient substances such as phytic acid, oxalic acid, hydrogen cyanide and tannins. The content of tannins in cabbage is 2.84 mg/100 g FW [1,2].

Tannins are polyphenol which form complex compounds with polysaccharides and proteins via hydrogen bonds and covalent bonds. These compounds are more closely bound to proteins and produce tannins-protein complexes. Protein-tannins bond tends to settle and accumulate in kidney [2]. One of the method that capable to reduce the levels of tannins is fermentation. Tannins will be degraded through transesterification reactions by tannase to produce gallic acid and glucose [3]. Tannase can be produced from bacteria, fungi and yeast. In addition, tannins can be decomposed under acidic conditions and organic compounds such as methanol, acetone and ethyl acetate [4].
Saccharomyces cerevisiae can be produce tannase. Tannase produced by Saccharomyces cerevisiae has the ability to degrade hydrolyzed tannins and condensed tannins [5,6].

The adding of Saccharomyces cerevisiae in fermentation can be oxidize glucose and produce ethanol. The content of ethanol in food has negative impact on the body. Therefore, to reduce the ethanol content, ethanol will be oxidized to acetic acid by adding Acetobacter aceti. Chakraborty, et al. produced acetic acid of 60.08 g/L from bael (Aegle marmelos) using mixed cultures of Saccharomyces cerevisiae and Acetobacter aceti [7]. In this study, multilevel optimization is used the number of mixed inoculums and pH values with submerged fermentation using Saccharomyces cerevisiae and Acetobacter aceti to determine the best conditions for producing low tannins.

2. Materials and method

2.1. Chemicals and Reagents
All chemicals used in this study include glucose (Merck Pvt. Ltd, India), yeast extract (Merck Pvt. Ltd, India), peptone (Merck Pvt. Ltd, India), sodium carbonat (Merck Pvt. Ltd, India), etanol (Merck Pvt. Ltd, India), tannic acid and Folin-Denis reagent. All solutions, including freshly prepared doubled distilled water.

2.2 Plants collection and identification
Cabbage (Brassica oleracea L.var capitata) obtained from the village of Poncokusumo, Malang, East Java. The vegetable can be identified authenticated by Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University.

2.3 Starter culture preparation
The culture of Saccharomyces cerevisiae and Acetobacter aceti obtained from culture collection of Biotechnology Laboratory of Agricultural Technology Faculty, Brawijaya University. S. cerevisiae and A. aceti were inoculated in GYP medium. Medium for S. cerevisiae with a composition of 4 g glucose, 1 g yeast extract, 2 g peptone and 100 mL distilled water. Medium for A. aceti with a composition of 2 g glucose, 0.5 g yeast extract, 0.5 g peptone and 100 mL distilled water. The prepared media were sterilized at 121 °C for 15 min. S. cerevisiae incubated at 30 °C for 17 h while A. aceti shaking incubation at 25-30 °C, 100 rpm for 17 h. The inoculate are used for fermentation.

2.4 Fermentation process
Fermentation was carried out in submerged fermentation in 220 mL jar and incubated at room temperature (25 ± 2 °C) for 4 day. Fresh cabbage is cut into small pieces using a knife and weighed as much as 100 g. Then, washed, drained and put in a jar. Fermentation using a mixed of inoculum (S. cerevisiae and A. aceti) with a ratio of 1:1 (v/v) in 100 g of fresh cabbage. The volume of the different mixture inoculum is added (5%, 10%, 15% and 20% (v/v) in 100 mL distilled water) to the cabbage and mixed thoroughly. After 4 days of fermentation, samples were collected for analysis of tannins and sugar content. The samples were analyzed, include biomass and fermentation solution (filtrate).

Determine of the best conditions for tannins hydrolysis was performed by stratified optimization included the volume of mixed inoculum and the pH value. The variation used for volume of mixed inoculum were 5%, 10%, 15% and 20% (v/v) and the pH value were 4, 5, 6 and 7. The pH value was controlled by addition glacial acetic acid or NaOH. In the first step, determination of the best volume of mixed inoculum added to medium fermentation. Furthermore, the best pH value was determined using the volume mixed inoculum from the results of the first stage.

2.5 Determination of tannins contents
Determination of tannins content using the colorimetric method. Folin-Dennis reagent method by reading the absorbance at 760 nm with a UV–Vis spectrophotometer. Tannic acid is used as standard. Biomass (2.5 g) were grinded using mortal and extracted with 20 mL of distilled water. Then, heated
with stirring for 10 min, filtered using filter paper and washed with 2 mL of water. The filtrate is diluted in a 25 mL measuring flask. The filtrate was diluted with 25 mL flask with the addition of distilled water. Extract was piped 0.4 mL, put into a test tube, added 0.5 mL of Folin-Dennis reagent and 1 mL of saturated sodium bicarbonate solution. The mixture was vortexed, let stand for 30 min and measured the absorbance value at a wavelength of 760 nm using a UV-Vis spectrophotometer. The preparation for the fermentation solution was centrifuged at 8000 rpm for 10 min. After that, the filtrate was analyzed by the same procedure as the analysis on biomass.

2.6 Determination of sugar contents
Determination of sugar content using the UV-Vis spectrophotometry method with the addition of sulfate phenol at a wavelength of 485 nm. Biomass (0.1 g) were inserted into the tube, extracted with 5 mL ethanol 80% and closed by a tube. Furthermore, heated in a water bath at 80 °C for 1 hour and cooled. Extract was piped 1 mL and put in a test tube. After that, 1 mL phenol 15% was added, 5 mL of concentrated sulfuric acid and left for 10 minutes. Then, heat in a water bath with a temperature of 25-30 °C for 15 minutes and cool. The mixed solution measured the absorbance value at a wavelength of 485 nm using a UV-Vis spectrophotometer. The preparation for the fermentation solution was centrifuged at 8000 rpm for 10 min. After that, the filtrate was analyzed by the same procedure as the analysis on biomass.

3. Results and discussion
3.1 Influence of volume of mixed inoculum
This study using a mixture of inoculum ratios of *S. cerevisiae* and *A. aceti* with a ratio of 1:1 refers to Chakraborty, et.al. The high *A. aceti* ratio will reduce the production of acetic acid. This is due to the high *A. aceti* requiring more nutrients. Limited available nutrients will cause *A. aceti* cell growth and enzyme activity to be not optimal so that *A. aceti* metabolic process decreases and the resulting acetic acid level is low. The use of a high *S. cerevisiae* ratio causes ethanol levels to be higher than acetic acid under anaerobic conditions [7–9].

Variations in the volume of mixed inoculums include 5%, 10%, 15% and 20% (v/v) affecting the degradation of tannins in cabbage fermentation. The level of tannins in raw materials is 298.710 mg /100 g FW. Degradation or hydrolysis of tannins by tannase into gallic acid and glucose. Figure 1 show that the content of tannins and sugar from cabbage fermentation using mixed cultures of *S. cerevisiae* and *A. aceti* in hydrolysis of tannins. Based on Figure 1A shows that the higher the volume of mixed inoculum added to 15%, the fewer tannins present in biomass and filtrate. Trends of tannins content in biomass and filtrate are the same because this fermentation process produces ethanol. We assume that the presence of ethanol could dissolve tannins. The addition of a mixture of inoculums 20% showed an increase tannin content in biomass and filtrate. The presence of high acetic acid in the fermentation medium will inhibit the enzyme tannase activity in hydrolyzing the tannins-protein complex [4].

The addition of 15% mixed inoculums gave a lower tannins content in biomass compared to the addition of mixed inoculum 5%, 10% and 20%. This happened because the addition of a mixture of 5% and 10% inoculums could not produce sufficient tannase, so the rate tannins of hydrolysis became decreasing. However, the higher volume of mixed inoculums 20% showed an increase in tannins content of 456.177 mg/100 g FW (biomass) and 368.761 mg/100 mL, due to competition between microorganisms in utilizing nutrients contained in cabbage (sugar). This is also indicated that the sugar content in biomass with the addition 20% of mixed inoculums, smaller than the filtrate which is 208.228 mg/100 g FW.
Figure 1. Changes from hydrolysis of tannins in cabbage fermentation. (A) tannins and (b) sugar content by variations in the volume of mixed inoculums of *S. cerevisiae* and *A. aceti* (% v/v in 100 mL of distilled water).

Generally, sugar content in biomass decreasing with the addition of mixed inoculums. But, the addition 15% of mixed inoculums gave a contrast result, increasing total sugar content. It can be assumed that the addition 15% of mixed inoculums condition occurs with biosynthesis of tannins by *A. aceti* bacteria so that the sugar released smaller than in biomass. High glucose concentrations suppress tannase activity in tannins biosynthesis by bacteria and cause environmental conditions to be
anaerobic or microaerobic so there is a decrease in A. aceti activity in oxidation of ethanol to acetic acid while S. cerevisiae grows well to produce ethanol. However, A. aceti can be produced acetic acid under anaerobic conditions with little content. The content of acetic acid in the fermentation medium causes a decrease in tannase activity [4, 8, 9]. The biosynthesis of tannins by A. aceti is possible to begin to occur in the amount of mixed inoculum 15\% and 20\% due to the high sugar content in biomass. The biosynthetic process of tannins converts gallic acid to pyrogallol and ellagic acid [4]. In this study the fermentation of cabbage with mixed inoculums under anaerobic conditions can produce acetic acid even though A. aceti is an aerobic obligate bacterium. The best condition in tannin hydrolysis was the addition 15\% of mixed inoculums with tannins content in the filtrate at 227.342 mg /100 mL and biomass 193.619 mg /100 g FW.

3.2 Influence of pH
In this study pH variations were used to determine the activity of microorganisms (S. cerevisiae and A. aceti) in the hydrolysis of tannins. Variations in pH used include 4, 5, 6 and 7 under anaerobic fermentation conditions. The optimum pH range of S. cerevisiae growth was 4.5-6.5 while A. aceti pH 3.5-6. Both have a slightly similar range which causes competition in utilizing nutrients. The effect of pH in fermentation affects tannase activity. In addition, pH changes will affect the structure of the protein, catalytic side stability after the activity of the enzyme. Generally, optimum tannase activity at pH 4.5-7 [4, 8, 10]. Figure 2 shows that hydrolysis of tannins using a mixture of S. cerevisiae and A. aceti cultures can be carried out under acidic fermentation conditions. It is shown that the level of tannins in raw materials decreases with changes in pH from fermentation media. The level of tannins in cabbage raw materials is 290.876 mg /100 g FW. S. cerevisiae and A. aceti can grow at pH 7 but the cell population of both microorganisms decreases. Cell populations that are slightly unable to hydrolyze tannins so that the content of tannins contained in the filtrate is less. Hydrolysis of tannins at pH 7 produces tannins content which is 187.524 mg /100 g FW in biomass and filtrate at 211.471 mg /100 mL.

![Graph showing tannin content vs pH](A)
Figure 2. Changes from hydrolysis of tannins in (A) biomass and (B) filtrate by variations in pH.

Biomass at pH 5 and 7 produces the same tannins content of 188.524 and 187.902 mg/100 g FW. The difference between the two can be shown in the content of tannins in the filtrate, which are 211.471 mg/100 mL (pH 5) and 178.634 mg/100 mL (pH 7). The tannin content which is greater at pH 5 than pH 7 is influenced by the ethanol content. At pH 7 S. cerevisiae cannot grow optimally so that it will produce ethanol in low amounts. At pH 4 and 6 produce a lower tannins content compared to pH 5 and 7. This is because at pH 4 and 6, the growth activity of S. cerevisiae and A. aceti in mixed cultures is more stable, thus increasing the rate of hydrolysis of tannins. S. cerevisiae produces tannase in pH 6 [5,11]. The content of tannins hydrolyzed in biomass at pH 4 is less than pH 6 of 107.742 mg/100 g FW. In this study using the optimum conditions for anaerobic fermentation using mixed cultures of S. cerevisiae and A. aceti and can hydrolyze tannins in pH 4. At pH 4, tannins can be hydrolyzed due to the presence of acetic acid. Acetic acid is produced by S. cerevisiae and A. aceti.

4. Conclusion
The results of this study indicate that the volume of mixed inoculum and pH affects the hydrolysis of tannins in the fermentation of cabbage. The optimum conditions for hydrolysis of tannins in cabbage fermentation using mixed inoculums of S. cerevisiae and A. aceti are volume of mixed inoculum of 15% and pH 4. Hydrolysis of tannins using mixed cultures of S. cerevisiae and A. aceti requires acidic fermentation environmental conditions. Tannins can be hydrolyzed by tannase produced by S. cerevisiae and the presence of acetic acid in A. aceti fermentation acid.

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