Ethanol Determination of Some Indonesian Medicines, Beverages and Various Tape Products by Enzymatic Assay

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ABSTRACT

Ethanol can be found in various medicines, beverages and also foods products. Enzymatic assay based on oxidation reaction of ethanol catalyzed by alcohol dehydrogenase is among the methods used for ethanol determination. The present study was intended to utilize this enzymatic assay to determine the ethanol content of some liquid medicines, beverages and also various tape products at different fermentation time points. The results of the present study indicate that ethanol content of the liquid medicines and beverages examined in this study were mainly in agreement with the information provided by manufacturer. Furthermore, in this study, the ethanol content of three *tape* types (black sticky rice, white sticky rice and cassava tuber) during seven days of fermentation were investigated. The ethanol content increased rapidly for the black and white sticky rice *tape* up to 5 days of fermentation, reached 9.5 and 8.1% (v/v), respectively. The ethanol content of black sticky rice *tape* remains constant up to the seventh day. However, the ethanol content of white sticky rice *tape* decreased at the sixth day of fermentation. While for cassava tuber *tape*, the ethanol content increased slowly during the first three days of fermentation and increased rapidly at the fourth day, followed by decrease of the ethanol content at the fifth day. The highest ethanol content of the cassava tuber *tape* was 4.5% (v/v) at the third day of fermentation.

Keywords: alcohol dehydrogenase, tape, alcoholic beverage, ethanol, liquid medicine

INTRODUCTION

Ethanol is one of important constituents in many everyday products including medicines, beverages and foods. Information of ethanol content provided in the label of particular products is sometimes not necessarily correct, and therefore need to be confirmed. Ethanol content of a product can be determined using volumetric titration (Friedmann and Klaas 1936), spectrophotometry (Magrí *et al.* 1997; Zanon *et al.* 2007), high performance liquid chromatography (HPLC) (Kudoh *et al.* 1984; Ishmayana 2011), gas chromatography (GC) (Penton 1985; Tangerman 1997) and Fourier transform infrared (FTIR) (Garrigues *et al.* 1997; Lachenmeier *et al.* 2010). Determination of ethanol content based on oxidation of ethanol by alcohol dehydrogenase was proposed by several authors (Bernt and Gutmann 1974; Ough and

Amerine 1988; Ishmayana *et al.* 2015). In this assay, alcohol dehydrogenase catalyzes the oxidation of ethanol accompanied by reduction of NAD^+ to NADH. The NADH formed, which is proportional to the amount of ethanol in a sample, can be detected by measuring absorbance at 340 nm. Semicarbazide is added to the assay buffer to force reaction to completion by binding acetaldehyde as the ethanol oxidation product, since the reaction is an equilibrium reaction (Ough and Amerine 1988). Zanon *et al*. (2007) also used alcohol dehydrogenase catalyzed assay with addition of phenazine methosulphate-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolimbromide (PMS-MTT) in the reaction buffer. In this assay, NADH formed from the enzymatic reaction oxidizes the PMS-MTT, forming a purple coloured MTT-formazan, which proportional to the amount of ethanol present in the sample (Zanon *et al.* 2007). Our group modify the proposed method by Brent and Gutmann (1974) and found that 4000 units/mL of alcohol dehydrogenase with incubation at 35°C for 40 minutes still gives accurate results of ethanol content of a sample (Ishmayana *et al.* 2015), and therefore this method will be used in the present study.

Tape is one of popular Indonesian foods. It is produced by fermentation of various substrate including steamed sticky rice and cassava tuber (Djien 1972). After several days (usually two to three days) of incubation at ambient temperature a soft, sweet, mildly alcoholic product is formed and ready for consumption (Gandjar 2003). Ethanol produced during tape fermentation can be varies depend on the starter culture used for fermentation, type of substrate and also environmental condition (Cronk *et al.* 1977; Gandjar 2003). The reported ethanol content of tape products are varies between one author to another. Cronk *et al*. (1977) reported that the highest ethanol content was 5.6% v/v at 4 days and 8.0% v/v at 6 days of fermentation when rice fermented using *Amylomyces rouxii* only and mixture with *Endomycospis burtonii*, respectively. Muchtaridi *et al*. (2012) followed the ethanol content after 3, 10, 17, 24 and 31 days of black sticky rice fermentation and found that the ethanol content never reach more than 5.3% v/v. In the present study we investigated the ethanol content of the tape product using enzymatic assay.

The objective of the present study was to determine the ethanol content of several medicines and alcoholic beverages using enzymatic assay. This study also intended to investigate the ethanol content of black sticky rice, white sticky rice and cassava tuber *tape* products within seven days of fermentation.

MATERIAL AND METHODS

Selection of medicines and beverages

Liquid medicines and beverages are selected based on their ethanol content representing non alcohol, low, mild and high ethanol content. All medicines were non prescribed liquid medicine and bought from local chemist store, while beverages were bought from local convenience store.

Preparation and fermentation of tape

Three type of substrate for tape fermentation were used in the present study, which are black sticky rice, white sticky rice and cassava tuber. Cassava tubers were peeled and washed followed by steaming until the tuber mildly soft. As for black and white sticky rice, after washing, the rice was steamed until mildly soft. The amount of substrate used in this experiment was about ~500 g. The steamed substrates were then

inoculated (0.1% w/w) with commercially available dry starter known as "*ragi*" obtained from local market. Each of the inoculated substrate was then divided into seven different containers, from which samples were taken from one container at everyday of experiment up until the seventh day of experiment.

Preparation of sample

Aqueous medicine and beverage that contain ethanol according the label were diluted to give ethanol concentration within acceptable range of 0.01 – 0.06% v/v as described elsewhere (Ishmayana *et al.* 2015). Samples from tape were collected by pressing the tape to give the liquid portion. The liquid portion was then centrifuged at 10,000 rpm and the clear supernatant was volumetrically diluted as required to give ethanol concentration at acceptable range. For diluted samples, the results of calculation from equation (1) were multiplied by the dilution factors.

Determination of ethanol content

To a tube containing 1.25 mL of semicarbazide buffer solution, 25 µL of the sample was added and mixed thoroughly. To the mixture 25 µL of 24 mM NAD^+ solution and 5 µL of 4000 units/mL alcohol dehydrogenase solution were added and mixed thoroughly. The mixture was then incubated at 35°C for 40 minutes. After incubation, the absorbance was read at 340 nm using Jenway 6305 UV-Vis Spectrophotometer after setting the spectrophotometer to zero with reagent blank. The concentration of ethanol in the sample was then calculated using equation (1) as previously described (Ishmayana *et al.* 2015).

$$\text{Ethanol concentration (\%v/v)} = \frac{A_{340nm}}{\varepsilon_{NADH}} \times 10^6 \times (1.305 \times 10^{-3}) \times \frac{1}{25} \times 46.08 \times 0.1 \times \frac{1}{0.789} \quad (1)$$

Where: A_{340nm} = Absorbance value at 340 nm
ε_{NADH} = Molar extinction coefficient of NADH (6,300 $M^{-1}cm^{-1}$)

RESULTS AND DISCUSSION

Ethanol content of liquid medicines and beverages

Liquid medicines used in the present study were cold medicine, and these types of medicine usually contain alcohol as described on their label. Three liquid medicines were chosen representing non alcohol (M1) and containing alcohol (M2 and M3) medicines. Four beverages were examined for their ethanol content, two of them were claimed non alcoholic beverages from known alcoholic beverages producer (B1 and B2), while two others were belong to class A (B3, ethanol content below 5% v/v) and class B (B4, ethanol content between 5 – 20% v/v) alcoholic beverages according to Indonesian government regulation.

The ethanol analysis results of the liquid medicines and beverages are presented on Table 1. The ethanol content of non alcoholic liquid medicine and beverage were in agreement with the information on their label, which are no ethanol in the products. If the decimal place of the calculation result is made to three decimal places, the value of ethanol concentration can be detected as 0.004, 0.002 and 0.002% v/v for M1, B1 and B2, respectively. However, according to previous results (Ishmayana *et al.*, 2015), ethanol concentration can be accurately measured in the range of 0.01 – 0.06% v/v

whereas outside of the proposed range high error can be found. The low ethanol concentration based on calculation may result from absorbance reading error, leading to absorbance reading even at blank. This can be predicted by low absorbance value (e.g. 0.084 AU for 0.004% v/v ethanol content). Therefore we report the ethanol content for M1, B1 and B2 as 0% ethanol content.

For the alcoholic liquid medicines and beverages, only one measurement was in agreement with the information on the label (M3). Measurement results of other samples (M2, B3 and B4) were found higher than that of the information provided by the manufacturer on the label. Further confirmation by other analysis methods is required to justify this result.

Table 1 Ethanol content of several liquid medicines and beverages determined by enzymatic assay using alcohol dehydrogenase. The values presented are means of two measurements followed by standard deviation.

Samples	Ethanol content on label (%v/v)	Measured ethanol content (%v/v)
Liquid medicine		
M1	0.0	0.00 ± 0.00
M2	4.8	5.50 ± 0.03
M3	2.0	2.05 ± 0.06
Beverage		
B1	0.0	0.00 ± 0.00
B2	0.0	0.00 ± 0.00
B3	4.7	5.81 ± 0. 31
B4	14.7	16.84 ± 0.63

Ethanol content of tape products

During tape fermentation, carbohydrate in the substrate are degraded into simple sugar and converted to ethanol by activity of microorganisms (Djien 1972; Cronk *et al.* 1977). Therefore, the ethanol content of the tape product will increase during fermentation as presented on Figure 1. The results of the present study indicate that there are differences of ethanol content during fermentation of the tape products.

The changes of ethanol content during seven days fermentation is presented on Figure 1. Black sticky rice tape has the highest ethanol content. Ethanol content at the fifth day of fermentation reached 9.14% v/v and relatively constant up to the seventh day. White sticky rice tape has lower ethanol content compared to black sticky rice tape but higher than cassava tuber tape. The highest ethanol content of white sticky rice tape (8.09% v/v) was reached at the fifth day of fermentation and on the sixth day of fermentation, it decreased to 6.26% v/v and remained constant until the seventh day. Ethanol content of cassava tuber tape slowly increased during the first three days of fermentation (~1% v/v) and reach the highest ethanol content at the fourth day (4.47% v/v) followed by decrease of the ethanol content at the fifth day back to ~1% v/v and remain constant until the end of fermentation process.

Different ethanol content of the tape product can be caused by different size of substrate. Black and white sticky rice has smaller size, and therefore more substrate exposed to the activity of microorganisms to convert carbohydrate to ethanol. Larger

size of cassava tuber causing less substrate exposed to the activity of microorganisms, and therefore lower ethanol produced during fermentation. Reduction of ethanol content after the highest ethanol content detected in white sticky rice and cassava tuber may be caused by diauxic shift as explained by Piskur *et al*. (2006). Microorganisms in *ragi* are generally mixture of yeast, bacteria and mold (Saono *et al.* 1974; Cronk *et al.* 1977; Suprianto *et al.* 1989) and depending on the property of the microorganisms, when it can not access sugar as their energy source, it may start using ethanol as their energy source known as diauxic shift (Piskur *et al.* 2006), and therefore the ethanol content can be reduced. It is interesting to follow up this finding by investigating more detail of the microorganism presence in the *ragi* starter and their activity toward different substrates.

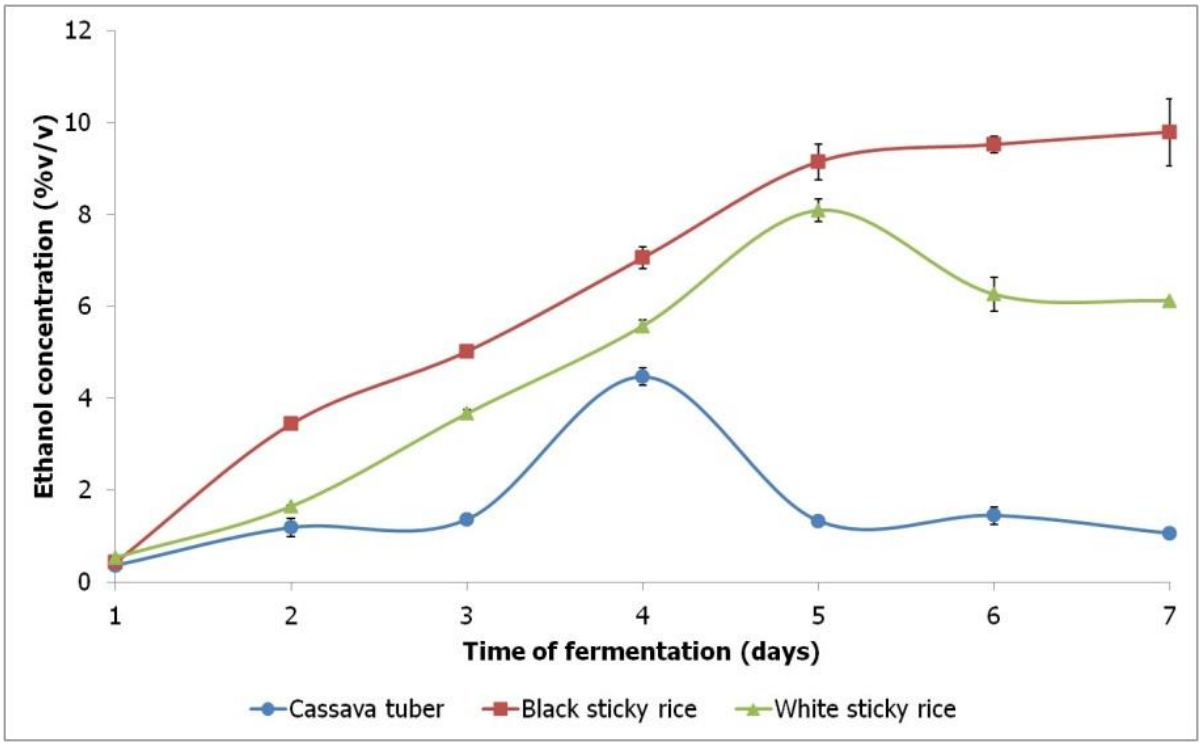

Figure 1 Ethanol content of various tape products (as indicated on the figure legends) during seven days of fermentation. Values presented are mean of two measurement and error bars indicate standard deviation.

CONCLUSIONS

Most of the ethanol concentration experiment results of the liquid medicines and beverages are in agreement with the information on label from the manufacturer. However, some of the measurement results indicate higher ethanol content. Therefore, measurement using different method is required to confirm the results.

Ethanol content of rice tape were higher compared to cassava tuber tape. This probably caused by different size of the substrate. The highest ethanol content was 9.78 (seventh day), 8.09 (fifth day), and 4.47% v/v (third day) for black sticky rice, white sticky rice and cassava tuber, respectively. Further investigation on the composition of

microorganism of the *ragi* and fermentation activity on different substrates are interesting aspects to be studied.

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