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The utilization of fruit and vegetable wastes for bioethanol production with the inoculation of indigenous yeasts consortium

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Abstract

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The aims of the study were to determine the utilization of fruit and vegetable wastes combination towards bioethanol production with the used of indigenous yeasts as starter. Banana, papaya and napa cabbage as dominates wastes were taken as materials from Central market around Bandung City. *Candida krusei* and *Hanseniaspora guilliermondii* were indigenous yeasts that isolated from wastes and determined as ethanol and glucose-tolerance isolates on preliminary research. The best ethanol fermenting activities of the isolates were tested into mixed wastes and resulted the consortium with ratio 1:1 of *C. krusei* and *H. guilliermondii* as the best starter with bioethanol production of 5.09% at 48 h fermentation. The isolates were grown in modified nutrient broth/NB (Oxoid Ltd.) with 3% yeast extract/YE (Kraft Foods) and 10 ppm amoxicillin for 48 h at room temperatures (23-28°C). Various fruit and vegetable wastes had been set into treatment combination of banana (W1), papaya (W2), napa cabbage (W3). Water was added for 1.5 times of wastes volumes and then mashed with blender until homogeny. Every treatment was inoculated with 3% of *C. krusei* and *H. guilliermondii* (1:1) then incubated for 72 h at room temperatures (23-28°C). The results shown the best bioethanol production gained by W1 at 24 h incubation with the bioethanol contents of 7.38% and the production of bioethanol tend to decrease after 24 h incubation.

Keywords: fruit wastes; vegetable wastes; bioethanol; *Candida krusei*; *Hanseniaspora guilliermondii*

Introduction

The demands in fruits and vegetables in many cities in Indonesia are also increase the volume of organic wastes. One of main distribution line of fruit and vegetables in big city is central market, such as Gedebage market in Bandung City. The accumulation of organic waste in the Gedebage market is dominated by vegetables and fruits which can reach 39.054 m^3/day with 86.86% wet weight and specific gravity of 0.202 kg/liter. Wastes from fruits and vegetables are the dominant type in traditional market especially in West Java.

It was found that banana, papaya and napa cabbage wastes were dominated the central market. The three commodities are some of the five leading commodities of West Java that are always available for the whole year which can be found in the small traditional markets and the biggest cen-

tral markets in the towns. The dominations of wastes affected the characteristics of organic wastes at traditional market with water content of 84.46%, dry matter 15.54%, and based on dry weight has a volatile content of 91.80%, ash content of 8.2%, C-organic content of 68,62%, total nitrogen level of 2,22% and C/N ratio 30,912 (Hidayat, 2004).

Organic wastes such as fruit and vegetables have biological and chemical potential in producing bioethanol (Utama, 2016; Utama et al., 2017). Some of the biological potentials are probability of indigenous microorganism such as *Candida spp.* (Cuc et al., 2010). Meanwhile, fruit and vegetable wastes also have chemical potentials due to high complex saccharide in a form of lignocellulose. High source of lignocellulose could be hydrolyzed into D-glucose and D-xylose which could further convert bioethanol by microorganism (Promon, 2015).

Lignocellulose consists of 30-50% of cellulose, 20-40% of hemicellulose and 10-15% lignin (Wilson and Lee, 2014). Cellulose is the main structure of lignocellulosic based biomass which is a glucose homologous polymer connected by b-1,4 glycosidic bond (Zhao et al. 2011; Promon, 2015). Cellulase enzymes such as exoglucanase, endoglucanase and b-glucosidase could be produced by microorganisms to hydrolyze cellulose into glucose (Sadhu and Maiti, 2011). After all the sugar sources are hydrolyzed into glucose and other simple sugars, the bioconversion continues until bioethanol is produced.

Indigenous yeast was isolated from Banana, Papaya and Napa Cabbage waste and the endurance was furthermore tested in extreme environment, such as high glucose and high alcohol environment. The yeast obtained were then used to ferment sugar in fruit and vegetables waste and produce bioethanol. The research is held to determine the effect of indigenous microorganism in vegetable and fruit waste in producing bioethanol.

Materials and Methods

Indigenous yeasts isolation. Fruit and vegetable wastes samples were cultured on modified PDA (Potato Dextrose Agar) containing 3% yeast extract (Kraft Inc.) and 100 µg per ml of amoxicillin to inhibit bacterial growth, and incubated for 48 h at room temperature. The colony identified as yeast was morphologically observed by microscope and subcultured on modified PDA and stored at 4°C (modification of Roostita et al., 2011).

Glucose and Alcohol-tolerance test. One loop of each isolate was inoculated into modified NB then was incubated for 48 h at room temperature (T = 23-27°C, RH = 77%). One ml of incubated isolate was mixed with 9 ml of modified NB with addition of 30% glucose or 30% of alcohol then was incubated for 48 h at room temperature (T = 23-27°C, RH = 77%). Qualitative analysis is done by looking at the sediment formed on the test tube. Quantitative analysis is done with optical density by using spectrophotometer at 600 nm wavelength (Fakruddin et al., 2013).

Yeast identification. The yeast identification was performed using RapID yeast plus system by thermo-scientific. The results analysed by Remel RapID Electronic Code Compendium (ERIC) Software (Pincus et al., 1999).

Bioethanol fermentation. The preliminary test for bioethanol fermentation was using modified NB with the addition of 15% glucose with the inoculation of the potential indigenous yeasts in glucose and alcohol tolerance and 1:1 consortium ratio of both. Fermentation is carried out for 72 h and the bioethanol production observed every 24 h. Bioethanol production continued with the variations of waste of banana waste (B), papaya waste (P), napa cabbage waste (N), with the third wastes combination in the ratio of 1:1:1. Fermentation is carried out with the addition of the best-treated isolates of the preliminary test and incubated for 72 h at room temperature then observed every 24 h. Bioethanol content analysed by dichromate oxidation method under acid conditions (Utama et al., 2016).

Results and Discussions

Glucose-tolerance test

Glucose-tolerance test was performed to determine the ability of indigenous yeast isolates isolated from fruit and vegetable waste to produced bioethanol in high osmotic environment (Thancharoen, 2015). The results (Fig. 1) showed that there are indigenous yeasts from banana, papaya, napa cabbage wastes grown in modified NB with 30% of glucose. Bioethanol were produced by fermentation activity of sugar using microorganism. High contents of sugar are usually still remaining in fruit and vegetable wastes. The higher the amount of sugar, the higher the amount of bioethanol that might be resulted. Therefore, it is very important that indigenous yeast used within this experiment show high tolerance in high sugar environment.

Each isolate was grown in modified NB medium with high concentration of sugar. The sediment shown in the bottom of test tubes is the indication that the isolates have tolerance towards high sugar contents. The highest bioethanol production from each isolate was chosen for further identification test. The isolates from banana, papaya, and napa cab-

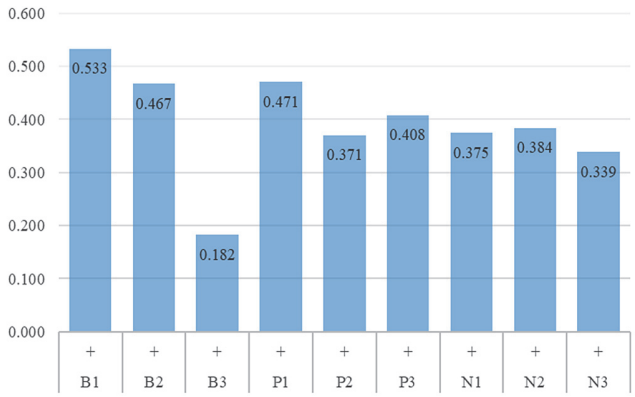

Fig. 1. Glucose-tolerance test towards fruit and vegetable wastes indigenous yeast isolates

Note: (+) = the presence of sediment with qualitative identification

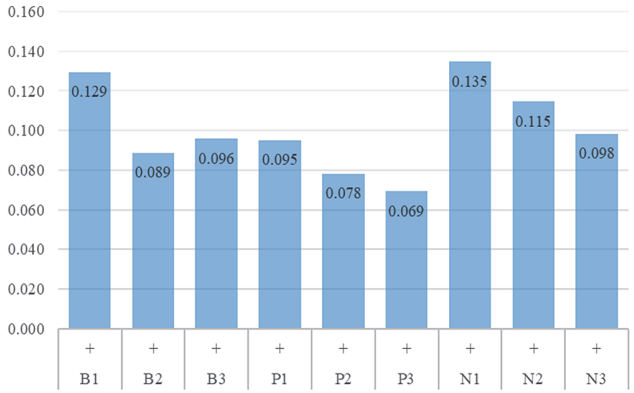

Fig. 2. Glucose-tolerance test towards fruit and vegetable wastes indigenous yeast isolates

Note: (+) = the presence of sediment with qualitative identification

bage wastes have high optical density which show tolerance in high glucose environment. Isolate B1 shows the highest optical density with the average of 0.533 that show the yeast ability to grew in 30% glucose medium.

High glucose refers to high osmotic pressure which can inhibit most yeast to grow (Utama, 2016). Sugar concentration is also critical factor in fermentation process and could also influence the rate of bioethanol production (Thancharoen, 2015). The high concentration of sugar leads to high osmotic pressure which causes the low growth rate of yeast (Attfield and Kletsas, 2000; Arroyo-López et al., 2009). Sugar concentrations from 20 to 30% could decrease the rate of yeast growth as shown in decreased sediment by all of the isolates (D'Amato et al., 2006). However, some yeast possess the ability to synthesize and utilize glycerol and may survive in substrates having high osmotic pressure due to high sugar concentrations (Myers et al., 1997).

Alcohol-tolerance test

Alcohol tolerance test was performed to determine the ability of indigenous yeast isolates isolated from fruit vegetable wastes to tolerate stress at high alcohol concentrations. High concentrations of alcohol can be role in delaying yeasts growth (Ali et al., 2014). The presence of alcohol can damage mitochondrial DNA in yeast cells and cause inactivation of hexokinase and dehydrogenase enzyme (Ibeas and Jimenez, 1997; Indah et al., 2015). Alcohol impaired cellular wall permeability disrupting sorting and signaling function so that decreases growth rates, fermentation rates and cell viability of yeasts (Navarro-Tapia et al., 2016).

Fig. 2 showed that all indigenous yeast isolates had alcohol tolerance which characterized by the occurrence of sediment after 48 h incubation. Quantitative analysis has shown that the optical density of isolates B1 from banana waste (0.129), P1 from papaya waste (0.095), and N1 from napa cabbage waste (0.135) were the highest of each sample that represent the tolerance towards alcohol concentrations up to 30%. The presence of yeast with high sugar and alcohol resistance in banana, papaya and napa cabagge wastes is due to naturally the three substrates already have high sugar content and in case of accidental fermentation will produce alcohol.

Some types of yeasts have the ability to survive in substrate with alcohol contents up to 12%-14% (Tikka et al., 2013). Meanwhile, it is found that some indigenous yeasts isolated from agro-industrial wastes can withstand up to 20% alcohol concentrations (Utama, 2016). The ability to defend against stress caused by high levels of alcohol is related to the fatty acid composition possessed by the yeast cell wall (Roostita and Fleet, 1999; You et al., 2003).

Potential indigenous yeast identification

Three isolates (B1, P1 and N1) were taken as best isolates for every wastes because their ability to tolerate high glucose and alcohol concentrations. Yeast identification was done using RapID Yeast Plus System and analyzed with ERIC.

Results (Table 1) identified that the three indigenous yeasts are *Cryptococcus albidus* (B1), *Candida krusei* (P1) and *Hanseniaspora guilliermondii* (N1). *Cryptococcus albidus*, *Candida spp.* and *Hanseniaspora sp*. were indigenous yeasts that commonly found on naturally or deliberately alcoholic fermentation and also had the ability to tolerate high concentrations of alcohol (Koulougliotis and Eriotou, 2016). The occurrence of *Candida* species is probably caused by hygiene deficiency or excessive moisture in the atmosphere

Table 1. RapID Yeast Plus System result with Electronic Code Compendium (ERIC) analysis

Wastes	Banana	Papaya	Napa cabbage
Isolate No.	B1	P1	N1
Glucose	+	+	+
Maltose	+	–	–
Sucrose	+	–	–
Trehalose	+	–	–
Raffinose	–	–	–
Lipid	–	–	–
NAGA	–	–	–
αGlucoside	+	–	–
βGlucoside	+	–	+
ONPG	–	–	–
αGalactoside	–	–	–
βFucoside	+	–	+
PHS	–	–	–
PCHO	–	–	–
Urea	+	–	–
Prolyne	+	–	–
Histidine	+	+	+
Leucyl-Gly	+	–	–
Yeast name	*Cr.albidus*	*C.krusei*	*H.guilliermondii*

(Rementeria et al., 2003; Balia et al., 2018). Actually the fruit and vegetable wastes were collected from the dump that was humid.

All of the isolates have the ability to hydrolyze glucose, only *Crypt. albidus* has the ability to hydrolyze other sugar such as maltose, sucrose, trehalose and raffinose. However, *Crypt. albidus* is grouped as weak ethanol producer because could ferment only < 25 g per g D-xylose which was lignocellulose derivatives (Rao et al., 2008). *Candida krusei* has the ability to produce bioethanol from glucose (Ando and Ikegami, 1998; Wang et al., 2002). *Candida krusei* also has succinic dehydrogenase activity to utilize succinic acid inside the Krebs cycle so that in semi-aerobic conditions it will be able to produce high bioethanol with low acid production (Kurtzman and Fell, 1998; Nakayama, et al., 2008). *Hanseniaspora guilliermondii* shows the activity of b-glucosidase and b-xylosidase enzymes which also show high levels of ethanol tolerance and glucose resistance that could be more effective in alcoholic fermentation (Lopez et al., 2015). Therefore, *C. krusei* and *H. guilliermondii* was chosen as potential indigenous yeast isolates for bioethanol fermentation from fruit and vegetable wastes.

Bioethanol production with the combination of indigenous yeasts

Preliminary tests of bioethanol production were conducted to determine the best isolates to be used in bioethanol

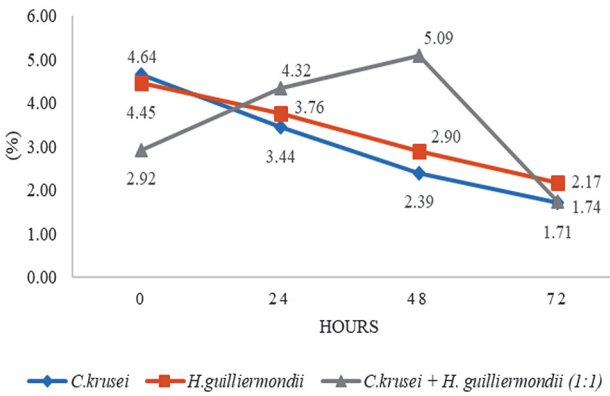

Fig. 3. Preliminary test for bioethanol fermentation with the combination of indigenous yeasts

fermentation from fruit and vegetable wastes. The results (Fig. 3) showed that consortium of *C. krusei* and *H. guilliermondii* (1:1) gave the best bioethanol contents of 5.09% at 48 h incubation. Meanwhile, single strains of *C. krusei* or *H. guilliermondii* have shown a decrease in the bioethanol content at 0-72 h incubation.

Candida spp. and *Hanseniaspora* (*Kloekcera*) is non-*Saccharomyces* yeasts that usually presence in of spontaneous alcoholic fermentation and dominated the early stages of fermentation (Romano et al., 2003; Wang et al., 2016). The yeasts have significant growth that influence the ethanol contents, however the growth limited until 2-3 days of fermentation or the ethanol contents reach 5% to 6% (v/v) then it will decrease (Romano et al., 2003).

The consortium of *C. krusei* and *H. guilliermondii* (1:1) has shown better bioethanol contents than single strains fermentation. Introducing different strain in fermentation can improve the rate of fermentation (Ciani and Comitini, 2015). The consortium of fermentation is likely modulate by nutrient availability and limitation, nutrient produces or utilize by one strain will relevant to another species or strain (Fleet, 2003). At the early stages, yeasts grow and utilize amino acids and vitamins, *Hanseniaspora spp.* has significant proteolytic activity that could generate amino acid, while the low tolerance to ethanol leads to early death and autolysis of yeasts cell wall which principally mannoprotein could be another nutrient source for another yeasts to grow (Hernawan and Fleet, 1995; Dizy and Bisson, 2000; Fleet, 2003; Ciani et al., 2016). Meanwhile, some strain of *Candida spp.* have high ethanol tolerance similar to *S. cerevisiae* so they can utilize the nutrients resulted before and continue the ethanol fermentation (Fleet, 2003; Utama et al., 2016; Utama et al., 2017).

Different with the consortium, single strain treatments shown decreased ethanol contents. In some occasion, non-*Saccharomyces* yeasts usually used in sequential separated fermentation in reducing alcohol contents in wine because of respiro-fermentative regulatory mechanism (Ciani et al., 2015). Available sugar is consumed via respiration rather than fermentation so that decrease the alcohol production (Quirós et al., 2014; Gobbi et al., 2014).

Bioethanol production from the combination of fruit and vegetable wastes with the inoculation of indigenous yeasts consortium

Continuing the bioethanol production, yeasts consortium is best treatment used in fermenting fruit and vegetable wastes. Fig. 4 showed that the best bioethanol contents resulted from banana wastes at 24 h fermentation with the bioethanol contents of 7.38% (v/v). The bioethanol contents increased at 24 h, then decreased until 72 h. Other wastes such napa cabbage wastes and mixed wastes showed the decrease of bioethanol contents from 24 h, while papaya wastes decreased after 48 h.

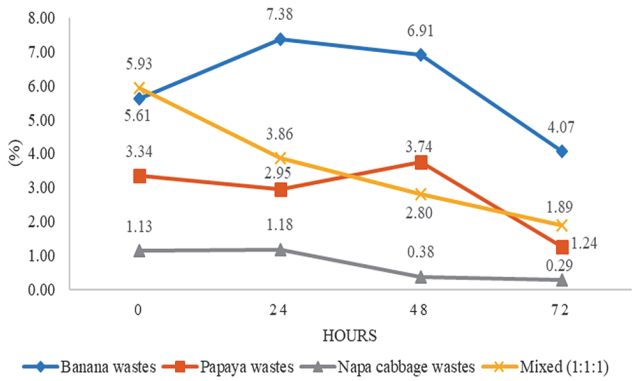

Fig. 4. Bioethanol production with the combination of fruit and vegetable wastes

All of the wastes fermented shown vary bioethanol contents. Banana wastes have the highest bioethanol content compared to other wastes. Bananas had high sucrose, glucose, and other simple sugar content. Bananas and papaya wastes had high source of energy and invert sugar suitable for bioethanol fermentation (Janani et al., 2013). Banana peels as one of banana wastes has 9% cellulose, 8% hemicellulose, 9% lignin, 3% starch, 2% glucose and 1% xylose as source for bioethanol fermentation (Gebregergs et al., 2016). Papaya contains simple sugar of 5.84% sucrose, 3.6% glucose, and 2.54% fructose with 0.38% crude fiber content (Villages, 1997; Fitriningrum et al., 2013). However, banana has higher total sugar contents than others so that the bioethanol resulted from papaya is lower than from banana-based fermentation (Janani et al., 2013; Vaitheki and Deepa, 2016).

The lowest production rate of bioethanol has occurred in the napa cabbage wastes. Napa cabbages contained 95% water, 0.25% fat, 1.3-1.7% protein and 0.7-1.3% fiber (Kallabis-Rippel, 2000; Rahmah et al., 2014). Low contents of carbohydrates tend to produce low bioethanol. In contrast to other substrates, napa cabbage has high amounts of antioxidants such as phenolic components, vitamins and chemopreventive components (Campas-Baypoli et al., 2009; Soengas et al., 2011). This could inhibit the yeast activity in producing bioethanol, so the substrate with the addition of napa cabbage produces lower bioethanol compared to other combinations of ingredients which also has shown a decrease of bioethanol contents at mixed substrates.

Conclusions

The results have shown that the best bioethanol production is gained from banana wastes at 24 h incubation and tend to decrease after 24 h. The highest bioethanol contents were 7.38% resulted from banana wastes with the inoculation of yeasts consortium of *C. krusei* and *H. guilliermondii*.

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