Species Identification of Stress Resistance Yeasts Isolated from Banana Waste for Ethanol Production

G L Utama1,2,3, *, M O Kurniawan4, N Natiqoh4 and R L Balia5

1Graduate School on Sustainability Science, Universitas Padjadjaran  
2Centre for Environment and Sustainability Science – Universitas Padjadjaran  
3Centre for Environmental Innovation and Technology–Postgraduate School, Universitas Padjadjaran  
4Faculty of Agro-Industrial Technology, Universitas Padjadjaran  
5Faculty of Animal Husbandry, Universitas Padjadjaran  
*Corresponding author: g.l.utama@unpad.ac.id

Abstract. Banana waste putrefaction was naturally shown the potential activities of microorganisms in fermenting ethanol. Yeasts have been known as one of the potential microorganisms that wildly grown in banana waste that has the ability in producing ethanol. The objectives of the research were to isolate and identify yeasts with stress resistance ability towards high ethanol and glucose in the production of ethanol. Yeasts isolation has been done by using Potato Dextrose Agar/PDA (Oxoid Ltd.) that was modified with 3% yeast extract/YE (Kraft Inc.) and 10 ppm Amoxicillin, then incubated for 48h at room temperature. The yeast-like isolates were identified microscopic and macroscopically then cultured on Nutrient Broth/NB (Oxoid Ltd.) with the addition of 3% yeast extract/YE (Kraft Inc.), 10 ppm Amoxicillin and 30% glucose or ethanol for stress resistance ability test. The resistance ability of yeasts toward high glucose and ethanol media was determined by measuring optical density (OD) on UV-Vis spectrophotometer \( \lambda = 600\text{nm} \). The isolates species identified using sequence analysis of the rRNA gene internal transcribed spacer (ITS) region with the primers of ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’), the sequences compared with the GenBank database using the BLAST algorithm. The isolates were mixed and inoculated into banana wastes for 72h then the ethanol contents measured by chromium dichromate oxidation methods every 24h. The results showed that three wild yeasts (B1, B2, B3) were isolated from banana wastes, with the ability to produce ethanol with a concentration of 10.72±2.53% at 48h. Species identification showed that isolate B1, B2, and B3 were 98.99% (295/298) , 100% (330/330), 99.71% (695/597) identical with Pichia bruneiensis strain CLIB 1453, Kodamaea ohmeri F3 and Hanseniaspora sp. R2 respectively.

Keywords: banana, waste, yeasts, ethanol, species identification

1. Introduction
Banana (Musaceae) is one of the world's most developed fruit crops especially in tropical nations including Indonesia. This is why banana wastes were the highest fruit wastes found mainly in the Indonesian market. The tremendous wastes are still underutilized; on the other hand, banana waste is highly valuable for reuse material in different enterprises.

The utilization of banana waste has been done a lot such for the crude material of compost,
biofertilizer, cellulolytic enzyme generation and also renewable energy i.e. ethanol production [1] [2] [3]. All of the approaches have shown the vital role of microorganisms in utilizing the waste. Composting involves wild microorganisms or accidentally inoculated commercial mixed culture that decompose waste material into soil nutrient [4] [5]. Cellulolytic bacteria have a major role in generating a cellulolytic enzyme that yields potassic biofertilizer from banana waste [6]. Yeasts such S.cerevisiae (baker’s yeast) is commonly used in biofuels generation including from banana waste [3].

The increase of energy demand and high banana waste disposal has led the waste to energy utilization to become a major approach that expanded in several developing countries [7] [8]. However, the capabilities of microorganisms involved in ethanolic fermentation still need to increase. Yeasts have been shown as the best microorganisms for ethanolic fermentation, however still hard to find the best yeasts with stress resistance ability especially when ethanol fermentation occurs [9]. The osmotic stress resulting from high sugar and ethanol contents become the main inhibitor that disturbs the growth of yeasts and also the process of ethanol generation [10] [11]. Wild yeasts become the potential microorganisms that could lead to desired ethanolic fermentation, due to the capability in tolerating environmental stress including high sugar and ethanol concentration [12].

Isolation of wild yeasts from banana wastes are widely found. However, stress resistance to ethanol production is still low. Therefore, this research aimed to isolate and identify wild yeasts from banana wastes with high-stress resistance ability for ethanol production.

2. Materials and Methods
This research was done at the Laboratory of Food Microbiology, Faculty of Agro-Industrial Technology, Universitas Padjadjaran from March to August 2018. This was an experimental descriptive study. Banana waste collected from Bandung City local market, Indonesia, then aseptically blended and kept in the refrigerator. Potato Dextrose Agar/PDA (Oxoid Ltd.) added with 3% yeast extract/YE (Kraft Inc.) and 10 ppm Amoxicillin was used to isolate the wild yeasts, by incubating them for 48h at room temperature [13]. Morphological (macroscopic and microscopic) characteristics of the yeasts were identified. Then the isolates’ sequence analysis was done by sequencing rRNA gene internal transcribed spacer (ITS) region with a forward primer i.e. ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and a reverse primer i.e. ITS4 (5′-TCCTCCGCTTATTGATATG-3′). The results were analyzed using the BLAST algorithm. The nearest phylogenetic relatives tree was drawn using Treefinder [14].

2.1. Ethanol Fermentation
Banana waste was diluted by distilled water with the ratio of 1:1.5 then blended aseptically. One of the best isolates in tolerating high glucose and ethanol content was inoculated 3% v/v on banana wastes and incubated at room temperature for 72h. The oxidation of chromium dichromate was measured every 24h to determine the ethanol yield [11] [15].

3. Results and Discussions

3.1. Morphological Characteristic of Banana Waste Wild Yeasts
The isolation of wild yeasts from banana waste has been shown that there are three yeast-like colonies. Figure 1 showed that all of the isolates have unicellular colonies, round, oval, long formed with a cell length of 1-5 µm up to 20-50 µm and width of 1-10 µm [16]. In accordance with other research the characteristic of wild yeasts isolated from rotten banana has creamy white color; smooth or rough colony nature; opaque, transparent and translucent optical property; with raised a convex elevation; undulate and an entire margin; ellipsoidal, ogival, spherical or oval appearance [17]. Refers to the morphological characteristics, the three isolates could be sorted as yeast.
3.2. **Wild Yeasts Species Identification**

The amplification of three wild yeast isolates was giving 400–800 bp of PCR products (Fig. 2), in accordance with the local yeasts that were isolated from Malaysia environment [18].
Figure 3. Banana waste wild yeasts phylogenetic tree
The rRNA gene sequencing results showed that isolates B1 was 98.99% (295/298) identical with *Pichia bruneiensis* strain CLIB 1453 (LN909474.1), isolates B2 was 100% (330/330) identical with *Kodamaea ohmeri* F3 (MG827230.1), isolates B3 was 99.71% (695/597) identical with *Hanseniaspora sp.* R2 (JN084126.1) and the phylogenetic tree is shown below (Fig. 3).

*Pichia bruneiensis* is found in *Hibiscus rosa-sinensis* in Borneo, and forms colonies with budding yeast, rapidly grown, invasive and branching pseudohyphae, which has the closest relatives to *Pichia fermentans* [19] [20]. *Kodamaea* genus yeasts were previously located under *Pichia* genus and have been known to be able to metabolize pentose including xylose and arabinose [21]. Wild *Kodamaea ohmeri* has been reported to have good glucose fermentation ability and was isolated from *Theobroma cacao* beans fermentation and Australian *Hibiscus sp.* flower [22] [23]. *Hanseniaspora sp.* or formerly known as *Kloeckera sp.* is normally found in grapes with a prevalence of 70% in Germany, 70-90% in Greece, and 0-11.1% in Cyprus. *Hanseniaspora sp.* is also found in China on spontaneous ethanolic fermentation with the prevalence of 26.92-31.68% that depends on the climatic situation [24] [25] [26] [27].

3.3. *The Stress Resistance Ability of Yeasts*

Figure 4 showed that the three wild yeast isolates have resistance towards high glucose environment. Meanwhile, figure 5 showed the wild yeast resistance towards high ethanol media.

### Figure 4. Yeast optical density on 30% glucose media

![Yeast optical density on 30% glucose media](image)

### Figure 5. Yeast optical density on 30% ethanol media

![Yeast optical density on 30% ethanol media](image)
Glucose and ethanol resistance test were done to choose the ability of wild yeast isolated from the banana waste in fermenting ethanol on high osmotic condition with the presence of inhibitory compound (weak acid, furan aldehydes, a phenolic compound) [28]. The inhibition by osmotic pressure towards the yeast growth was caused by sugar and ethanol contents up to 30% [12] [29].

Non-Saccharomyces yeast has an ability in tolerating environmental stress. *Pichia sp.* can support high furan aldehyde fixations to tolerate multi-factor stress [30]. *Kodamaea ohmeri* has tolerant ability against several growth inhibitors [21]. *Hanseniaspora sp.* is multi-stress tolerant especially against ethanol [31].

The yeasts consume glucose, synthesizing glycerol and generating the low acid with efficient glycerol transport into the cell which effective in surviving the osmotic stress environment [28] [32]. Non-Saccharomyces yeasts could assimilate succinic and acetic acid in increasing the osmotolerant activities [33].

### 3.4. Ethanol Fermentation

Wild yeasts were mixed and cultured to ferment the banana waste into ethanol and the results showed that the highest ethanol contents gained at 48h incubation with 10.72±2.53% ethanol contents (Fig. 6).

![Figure 6. Ethanol fermentation from banana waste with wild yeasts consortium](image-url)

The yeasts have critical growth that affects the contents of ethanol, however, 2-3 days is the maximum limit of the growth of the yeast then it will decrease [34]. The mixed culture showed better ethanol fermentation because introducing different strain improves the fermentation rate [35]. The fermentation consortium is likely adjusted by nutrient accessibility and restriction; delivered nutrient by a certain yeast strain might be important for another species or strain [36].

*Hanseniaspora spp.* and *Kodamaea ohmeri* dominated early fermentation and generating amino acid, while the early death of yeasts occurs caused by low ethanol resistance then the release of mannoprotein occurs from the yeasts cell autolysis which can be utilized by another yeast to grow [21], [37], [38]. Meanwhile, *Pichia spp.* that has a high resistance against ethanol can utilize the nutrients resulted in the early fermentation to continue ethanol generation [36].

When the growth ends, the respiro-fermentative regulatory mechanism owned by non-Saccharomyces yeasts will decrease ethanol contents [39]. Every energy source including ethanol that is available will be used for respiration so that ethanol concentration decreases [40].
4. Conclusions
Three wild yeasts with high glucose and ethanol stress resistance ability were isolated from banana waste. The sequence identification showed the three isolates had similarity with *Pichia bruneiensis* CLIB 1453, *Kodamaea ohmeri* F3 and *Hanseniaspora sp.* R2. The wild yeasts consortium resulted in ethanol contents of 10.72±2.53% at 48h incubation at room temperature.

Acknowledgments
Authors would like to thank the Rector of Universitas Padjadjaran for Research Fundamental Unpad Grants and the Academic Leadership Grants. Also thanked the Student Research Group, Vivi Fadilla Sari, Isfari Dinika and Syarah Virgina who helped in the laboratory. This research/article’s publication is supported by the United States Agency for International Development (USAID) through the Sustainable Higher Education Research Alliance (SHERA) Program for Universitas Indonesia’s Scientific Modeling, Application, Research, and Training for City-centered Innovation and Technology (SMART CITY) Project, Grant #AID-497-A-1600004, Sub Grant #IIE-00000078-UI-1.

References
[1] Dabhi B K, Vyas R V and Shelat H N 2014 *Int. J. Curr. Microbiol. App. Sci.* 3 10
[2] Waghmare A G and Arya S S 2016 *Bioethanol* 2 146–156
[3] Gebregergs A, Gebresemati M and Sahu O 2016 *Pacific Science Review A: Natural Science and Engineering* 18 22–9
[4] Kadir A A, Rahman N A and Azhari N W 2016 *IOP Conf. Ser.: Mater. Sci. Eng.* 136 012055
[5] Juliastuti S R, Enhaperdhani D and Hasanah R U 2017 *KnE Life Sciences* 3 193–201
[6] Mahalakshmi R and Naveena M L 2016 *International Journal of Current Microbiology and Applied Sciences* 5 336–49
[7] Barve A and Tarfe K 2017 *Research & Reviews – A Journal of Life Science* 7 28–32
[8] Utama G L, Kurnani T B A, Sunardi S, Cahyandito F and Balia R L 2017 *Bulgarian Journal of Agricultural Science* 23 1016–20
[9] Utama G L, Kurnani T B A, Sunardi and Balia R L 2017 *International Journal of GEOMATE* 13 103–7
[10] Indah H, Putri F and Utama G L 2015 *International Journal on Advanced Science, Engineering and Information Technology* 5 107–109–109
[11] Utama G L, Kurnani T B A, Sunardi - and Balia R L 2016 *International Journal on Advanced Science, Engineering and Information Technology* 6 252-257–257
[12] De Matos M E, Bianchi Pedroni Medeiros A, De Melo Pereira G V, Thomaz Soccol V and Soccol C R 2017 *Fermentation* 3 62
[13] Balia R L, Kurnani T B A and Utama G L 2018 *International Journal on Advanced Science, Engineering and Information Technology* 8 1091–1097–1097
[14] Jobb G, von Haeseler A and Strimmer K 2004 *BMC Evol. Biol.* 4 18
[15] Parameswari K, Hemalatha M, Priyanka K and Kishori B 2015 *International Journal of Scientific and Engineering Research* 6 100–4
[16] Utba F, Balia R L and Utama G L 2018 *Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development* 18 8
[17] Thancharoen K 2015 *International Conference on Biological, Civil and Environmental Engineering* (Bali)
[18] Oslan S N, Salleh A B, Rahman R N Z R A, Basri M and Chor A L T 2012 *Acta Biochim. Pol.* 59 225–9
[19] Sipiczki M 2011 *FEMS Yeast Res.* 11 202–8
[20] Sipiczki M 2012 *Int. J. Syst. Evol. Microbiol.* 62 3099–104
[21] Sharma S, Arora A, Sharma P, Singh S, Nain L and Paul D 2018 *Chem Cent J* 12 8
[22] Lachance M A, Bowles J M, Starmer W T and Barker J S 1999 *Can. J. Microbiol.* 45 172–7
[23] Daniel H-M, Vrancken G, Takrama J F, Camu N, De Vos P and De Vuyst L 2009 *FEMS Yeast Res.* **9** 774–83
[24] Nisiotou A A and Nychas G-J E 2007 Y Greece *Appl. Environ. Microbiol.* **73** 2765–8
[25] Nisiotou A A, Spiropoulos A E and Nychas G-J E 2007 *Appl Environ Microbiol* **73** 6705–13
[26] Koulougliotis D and Eriotou E 2016 *Fermentation Technology* **05**
[27] Wang C, Mas A and Estève-Zarzoso B 2016 *Front Microbiol* **7**
[28] Mukherjee V, Radecka D, Aerts G, Verstrepen K J, Lievens B and Thevelein J M 2017 *Biotechnol Biofuels* **10** 216
[29] D’Amato D, Corbo M R, Nobile M A D and Sinigaglia M 2006 *International Journal of Food Science & Technology* **41** 1152–7
[30] Kwon Y-J, Ma A-Z, Li Q, Wang F, Zhuang G-Q and Liu C-Z 2011 *Bioresour. Technol.* **102** 8099–104
[31] López S, Mateo J J and Maicas S 2016 *Fermentation* **2** 1
[32] Rantsiou K, Dolci P, Giacosa S, Torchio F, Tofalo R, Torriani S, Suzzi G, Rolle L and Cocolin L 2012 *Appl. Environ. Microbiol.* **78** 1987–94
[33] Nakayama S, Morita T, Negishi H, Ikegami T, Sakaki K and Kitamoto D 2008 *FEMS Yeast Res.* **8** 706–14
[34] Romano P, Fiore C, Paraggio M, Caruso M and Capece A 2003 *International Journal of Food Microbiology* **86** 169–80
[35] Ciani M and Comitini F 2015 *Current Opinion in Food Science* **1** 1–6
[36] Fleet G H 2003 *Int. J. Food Microbiol.* **86** 11–22
[37] Hernawan T and Fleet G 1995 *Journal of Industrial Microbiology* **14** 440–50
[38] Dizy M and Bisson L F 2000 *Am J Enol Vitic.* **51** 155–67
[39] Ciani M, Capece A, Comitini F, Canonico L, Siesto G and Romano P 2016 *Front Microbiol* **7**
[40] Quirós M, Rojas V, Gonzalez R and Morales P 2014 *Int. J. Food Microbiol.* **181** 85–91