

## STRESS TOLERANCE YEAST STRAIN FROM PAPAYA WASTES FOR BIOETHANOL PRODUCTION

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**ABSTRACT:** Papaya wastes is naturally fermented and potentially overgrown by microorganisms such yeasts. The research aims to determine the presence of ethanol-fermenting yeasts on papaya wastes which has the ability to tolerate high glucose and ethanol contents. Yeasts isolated from papaya wastes with using Potato Dextrose Agar/PDA which modified with 3% yeast extract/YE and 10 ppm Amoxicillin, then incubated for 48h at room temperature. The isolates were identified macroscopic and microscopically then the yeast-like isolates cultured on Nutrient Broth/NB with the addition of 3% YE, 10 ppm Amoxicillin and 30% glucose or alcohol to be tested for the tolerance ability towards high glucose and alcohol. Yeasts presence on high glucose and alcohol media was determine by UV-Vis spectrophotometer by measuring optical density (OD) for UV absorbance at  $\lambda=600\text{nm}$ . The isolate with highest OD at glucose and alcohol media grown at papaya wastes for 72h and the ethanol contents measured by chromium dichromate oxidation methods every 24h. Species identification performed using sequence analysis of the rRNA gene internal transcribed spacer (ITS) region with using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), the sequences compared with the GenBank database using Basic Local Alignment Search Tools/BLAST algorithm. The results showed that three yeast-like isolates found from papaya wastes, with Y1 isolate identified as best isolate with the OD of 0.4699 at 30% glucose media and OD of 0.0960 at 30% alcohol media, with the highest ethanol fermented at 48h was 4.34%. The rate of isolate identification by sequence analysis resulted 96.20% (531/552) identical with *Pichia sp.* strain AQGWD 7.

**Keywords:** Ethanol, Stress tolerance, Papaya wastes, Yeasts

### 1. INTRODUCTION

Papaya (*Carica papaya*) waste is the second highest fruit wastes found in wet market particularly in West Java, Indonesia. As one of the featured product of West Java, papaya is constantly available in every season. However, the number of underutilized papaya wastes had demonstrated the potential usage or unsettling influence that can be come about.Ppa

The plenitude of papaya waste has been raising an approach to increase its economic value. Papaya waste commonly utilized for livestock feed or crude material of compost [1] [2]. Others started to create papaya as a biomass of crude materials for the making of sustainable power sources such biogas [3] [4]. Other sustainable power source that can be created from papaya biomass is ethanol [5] [6].

Papaya wastes contain high complex saccharide in a form of lignocelluloses which could be hydrolyzed into D-glucose and D-xylose then converted furthermore into ethanol by microorganism [7]. However, papaya wastes potential in ethanol fermentation is not just resulted from its complex-cellulose compound yet in addition from the biological aspect that represented by the existence of indigenous microorganisms

which naturally fermenting ethanol when the putrefaction occurs [8].

The potential of indigenous microorganisms isolated from papaya wastes is still rarely revealed especially for the ability in ethanol production. Kowser, et al. [8] has been isolated the potential acetic acid bacteria from rotten papaya that potential in utilizing ethanol for producing acetic acid. The latest research has been isolated yeast from various agriculture wastes including papaya that has xylose-utilizing ability in producing ethanol [9].

Yeasts has been known as the best microorganisms for ethanol producing activity, however yeasts with the ability in tolerating stress when the ethanol production occurs still hard to find [10] [11]. High sugar and ethanol stress was the common inhibitor when the ethanol fermentation occurs [12] [13] [14]. Both conditions could influence the osmotic pressure or membrane fluidity which disturbs yeasts growth and ethanol fermentation process [15]. Thusly, indigenous yeasts with the ability to tolerate high sugar and ethanol in ethanol fermentation still need to discover.

## 2. MATERIALS AND METHODS

### 2.1 Indigenous Yeasts Isolation from Papaya Waste

Papaya waste was randomly collected from the local market in Bandung City, Indonesia, blended aseptically then transferred into containers and stored in the refrigerator. Using Potato Dextrose Agar/PDA (Oxoid Ltd.) which modified with 3% yeast extract/YE (Kraft Inc.) and 10 ppm Amoxicillin, indigenous yeasts isolated from papaya wastes then incubated at room temperature for 48h. The morphological characteristics of indigenous yeasts was identified macroscopic and microscopically [15] [16].

### 2.2 Stress Tolerance Tests

To determine indigenous yeasts tolerance ability towards stress caused by high glucose and ethanol contents, 30% glucose or alcohol added into Nutrient Broth/NB (Oxoid Ltd.) modified with 3% yeast extract/YE (Kraft Inc.), 10 ppm Amoxicillin. Spectrophotometer UV-Vis used for measuring optical density (OD) at  $\lambda=600\text{nm}$  to determine yeasts presence on high glucose and alcohol media [15].

### 2.3 Ethanol Fermentation

Papaya waste weighed and blended aseptically with the dilution ratio of 1:1.5. Indigenous yeast with stress tolerance, cultured at papaya wastes in room temperature for 72h. The ethanol that resulted was measured by chromium dichromate oxidation methods every 24h [15] [16].

### 2.4 Indigenous Yeast Species Identification

rRNA gene internal transcribed spacer (ITS) region was used to identify indigenous yeast species, using sequence analysis of the with using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The gene sequencing has been done by Macrogen Inc., the sequence results then compared with the database of GenBank using BLAST algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) [17].

## 3. RESULTS AND DISCUSSIONS

### 3.1 Morphological Characteristic of Papaya Indigenous Yeasts

Indigenous yeasts isolation results showed that three yeast-like colonies have been identified from papaya wastes (Fig. 1). According to morphological characteristics, the three isolates could be categorized as yeast. The isolates has cell length of 1-5  $\mu\text{m}$  up to 20-50  $\mu\text{m}$ , width of 1-10  $\mu\text{m}$  also has

unicellular colonies, round, oval, long shaped with the establishment of pseudomycelium [18]. The results also in accordance with the characteristic of yeasts strain that isolated from papaya which has smooth surface, spherical or oval shape, creamy white color and presence of pseudomycelium [16].

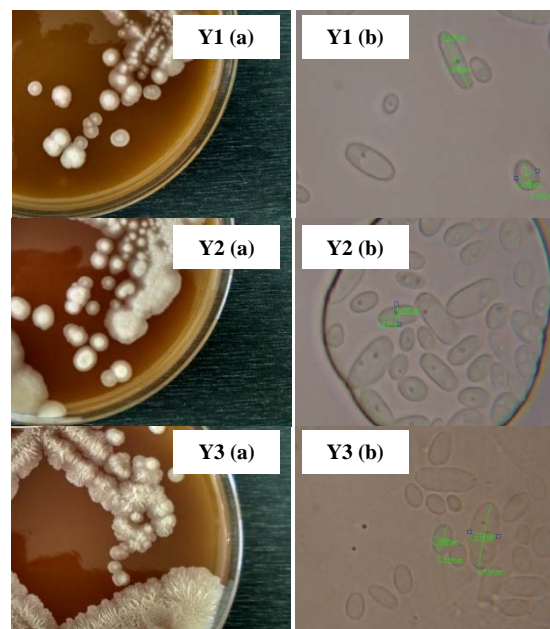


Fig. 1 (a) macro and (b) microscopic (1000x magnification) morphology of indigenous yeasts colony (Y1, Y2, Y3)

### 3.2 Yeasts Tolerance towards High Glucose

High glucose tolerance test results (Fig. 2) showed that all of the indigenous yeasts can be survived at 30% of glucose content media with Y1 and Y2 as the best isolates that shown OD of 0.4699 and 0.4656 respectively while Y3 reach OD of 0.4034.

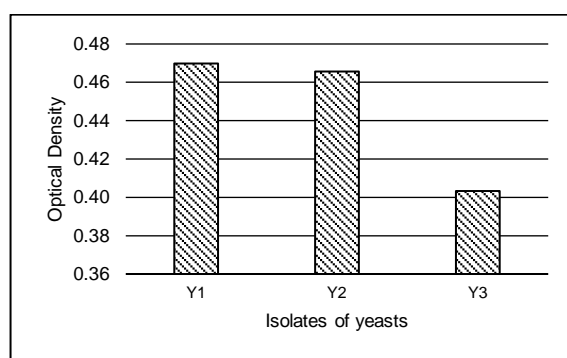


Fig. 2 Yeast optical density on high glucose media

Determination of glucose-tolerance was done to decide the capability of indigenous yeast isolated from papaya waste in generating ethanol on high osmotic pressure condition [19]. Mostly yeast

growth was inhibited by the osmotic pressure that resulted from high contents of sugar up to 30 % [20] [16].

However, higher ethanol also could be resulted from high sugar available because ethanol generated from sugar fermentation by microorganism [21]. The rate of ethanol generation was also influenced by sugar concentration [19]. Therefore, the ability in tolerating high glucose contents was important in resulting high ethanol.

At the beginning of fermentation, yeasts with the ability to tolerate osmotic pressure could consume glucose, doing glycerol synthesis and producing low acid [22]. High glucose osmotic stress can be combated by efficient glycerol transport into inside the yeasts cell [23]. Assimilation succinic and acetic acid by several *non-Saccharomyces* yeasts could help in surviving the osmotic stress condition [22] [24].

### 3.3 Yeasts Tolerance towards High Ethanol

Figure 3 has been shown all the indigenous yeast isolates had tolerance ability towards high ethanol up to 30% which determined by optical density of isolates Y1 (0.096), Y2 (0.075), and Y3 (0.070). The yeast presence on high ethanol environment due to normally papaya waste contains high sugar and the ethanol was accidentally generated when the putrefaction occurs.

High ethanol contents could delay the growth of yeasts [25]. Ethanol interrupt the permeability of yeast cell wall then the sorting and signaling function will be disturbed so that the growth, fermentation and viability of yeasts cell also decrease [26]. Therefore, ethanol tolerance test was performed to find out the indigenous yeast capability in tolerating stress resulting from high ethanol.

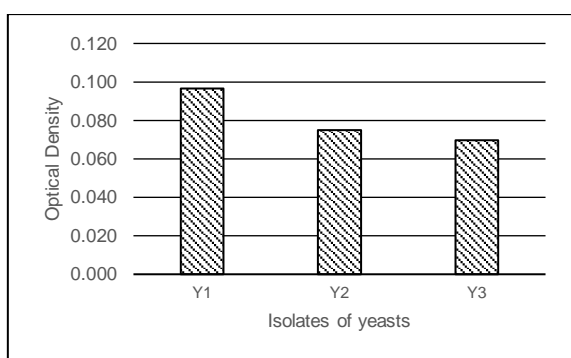


Fig. 3 Yeast optical density on high ethanol media

### 3.4 Ethanol fermentation

Indigenous yeast isolate of Y1 chosen as best isolate with the ability in tolerating glucose and ethanol up to 30% then used to ferment the papaya waste for resulting ethanol. The results (Fig. 4) showed that the ethanol contents tend to increase

until 48h with the contents of  $4.34 \pm 0.086$  %, then decrease at 72h ( $1.28 \pm 0.001$  %).

Natural ethanolic fermentation usually occurs and dominated by non-*Saccharomyces* yeasts at the early stages of fermentation [27]. The dominance will last for 2-3 days and affect the ethanol contents until 5% then decrease [28].

Early fermentation has shown the high rate of yeasts growth by utilizing amino acids and vitamins for continuing the ethanol fermentation [29]. Organic acids, esters, and ethanol dominated the early stage fermentation which resulted from  $\beta$ -glucosidase,  $\beta$ -xylosidase and some proteases enzyme [20].

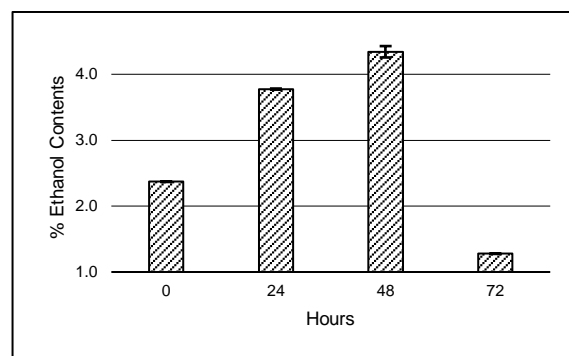


Fig. 4 Ethanol fermentation

After the growth end, non-*Saccharomyces* yeasts have respiration-fermentative regulatory mechanism that can decrease ethanol contents [31]. Carbon source available is consumed for respiration so that the ethanol decreases [32] [33].

### 3.5 Yeast Identification

Gene sequencing results (Table 1) showed that the ITS1 primer gained 725 of 756 (95.90%) sequences identical with *Pichia sp.* strains QAUPK01 (KT987926.1) and the nearest phylogenetic relatives which shown by the tree (Fig. 6). Meanwhile, the results of ITS 4 primer had shown higher identical sequences with 531 of 552 (96.20%) identical with *Pichia sp.* strains AQGWD7 (KP721590.1) with the nearest phylogenetic relatives shown by Fig. 7. The indigenous yeast isolate has close relatives with *Pichia kudriavzevii*, *Candida xylopsoci*, and *Issatchenkia sp.*

Table 1 Results of Yeast Identification

Primer	ITS1	ITS4
Identities	725/756	531/552
Percent Identities	95.90	96.20
Nearest phylogenetic relative	<i>Pichia sp.</i>	<i>Pichia sp.</i>
Strain	QAUPK01	AQGWD 7
Accession number	KT987926.1	KP721590.1

*Pichia kudriavzevii* previously known as *Issatchenkia orientalis* has been isolated from various natural sources and used for ethanol production. Some other strain of *Pichia kudriavzevii* also shown tolerance towards acid, ethanol, thermal and salt stress with higher thermotolerant activity than conventionally used ethanol-fermenting yeasts i.e. *S.cerevisiae* [34]. *Pichia kudriavzevii* is acidophilic yeasts which grow better under acidic conditions (pH of 4-6) that could lead the intracellular enzymes for optimal sugar conversion to ethanol and the other study

mentioned that the yeast presenting ethanol tolerance ability[35] [36].

The ability of indigenous non-*Saccharomyces* yeasts in naturally grown and producing ethanol at room temperature has been escalated since the ethanol-tolerance ability characterized [37]. Gidado, et al. [36] found that indigenous yeast species with identical sequence of 97-98% to *Pichia kudriavzevii* has the ability to grow and ferment ethanol up to 20% v/v. *Pichia kudriavzevii* strains GY1 was the most adaptive, efficient and effective in various sugars (fructose, galactose, glucose, lactose and sucrose) utilization for ethanol production [38].

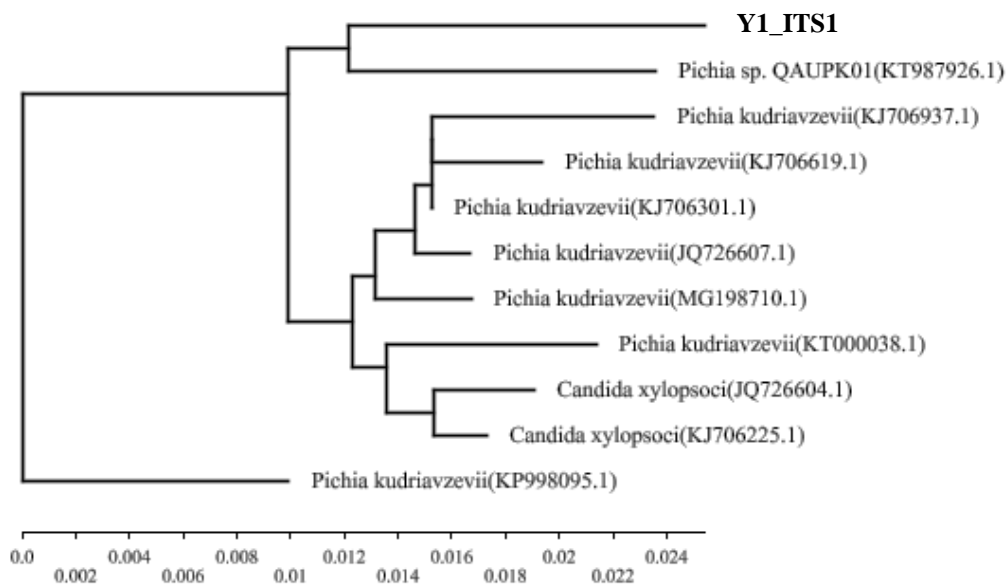


Fig. 6 ITS1 Phylogenetic Tree

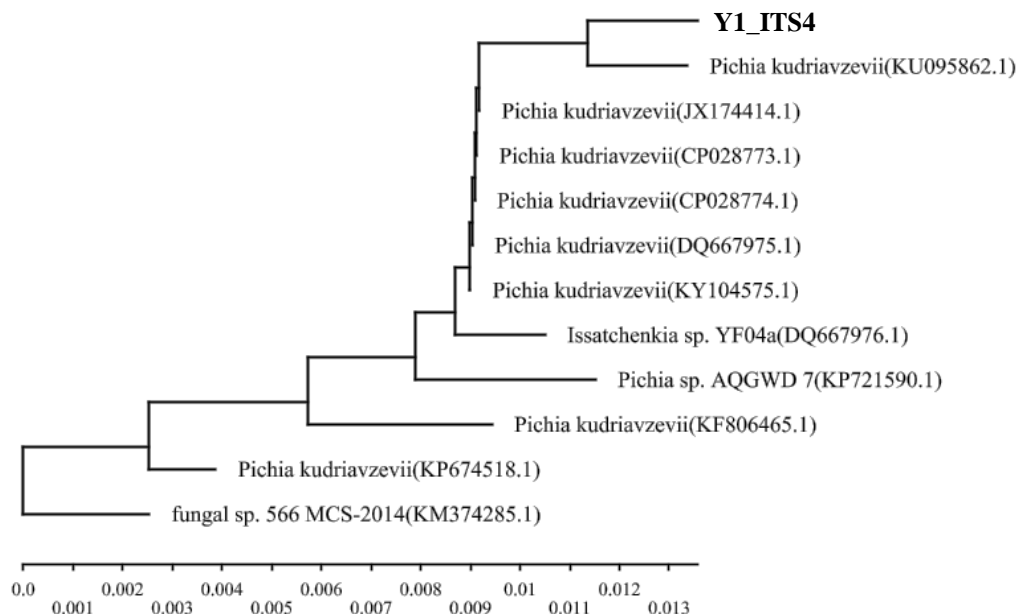


Fig. 7 ITS4 Phylogenetic Tree

#### 4. CONCLUSIONS

Three yeast-like colonies isolated from papaya wastes. Isolate Y1 chosen as best isolate in tolerating stress from glucose and ethanol contents up to 30% with the OD of 0.4699 and 0.0960 respectively. The chosen indigenous yeast isolates fermenting  $4.34 \pm 0.086\%$  v/v ethanol from papaya waste for 48h. Gene identification gained 96.20% (531/552) sequence identical with *Pichia sp.* strain AQGWD 7.

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