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# Honey Mead Fermentation from Thai Stingless Bee (*Tetragonula leaviceps*) Honey using Ethanol Tolerant Yeast

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## Abstract

Honey mead is a well-known conventional alcoholic beverage made by microbial fermentation of diluted honey. The selection of prospective yeasts for inoculation of honey-must with regard to honey mead quality determines the quality of mead production. The yeast consortium tolerant to ethanol stress was selected for this study using an enrichment technique. The activity of the invertase enzyme and the level of ethanol tolerance have been investigated. Thai stingless bee honey was used as a substrate, and the selected ethanol tolerant yeast consortium was used for mead fermentation. The results revealed that the PP03 had the highest invertase activity of 75.13±9.16 U/mL and the highest ethanol tolerance level of 12%. This is the first study using an ethanol tolerant yeast consortium to ferment honey mead from Thai stingless bee honey.

**Keywords:** stingless bee, honey wine, mead, ethanol tolerant yeasts, fermented food, beverage

## 1 Introduction

Beverages have played an important role in human history in terms of improving food nutrition and food preservation (Shiby and Misha, 2013). Alcoholic beverages and their non-alcoholic equivalents include beers, wine, spirits, cider, mead, sake, and others (Makwana and Hati, 2019; Hornsey, 2007). Alcoholic beverages are mostly made from saccharide ingredients, such as mead from honey, beer from grain, wine from fruit, and sake from rice (Hornsey, 2007). Equation (1) depicts the conversion of sugar (glucose) to alcohol (ethanol), which typically takes 2–4 weeks at 20–30 °C (Walker and Stewart, 2016).

$$C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2 \quad (1)$$

Honey mead, also known as honey wine, is a favourite alcoholic drink made from bee honey, water, herbs (clove, cinnamon, nutmeg, oregano, chamomile, and lavender), and some fruit (blackberry, strawberry, and raspberry) (Gupta and Sharma, 2009). Honey mead fermentation consists of two major stages: (1) fermentation at 15–25 °C for 3–6 weeks and (2) aging in oak barrels at 10–15 °C for up to 10 years (de Simon et al., 2014). This beverage has received positive feedback in terms of human health, such as a lower risk of heart disease, diabetes, and stroke (Chang et al., 2016).

The stingless bee (*Tetragonula leaviceps*) is a highly domesticated bee found in tropical and subtropical territories such as Thailand (Suntiparapop et al., 2011; Jame, 2004). The stingless bee collects the plant sugar solution and converts it into bee honey through bee hypopharyngeal gland enzymes such as diastase, amylase (EC 1.2.1.1), glucosidase (EC 3.2.1.20) and glucose oxidase (EC 1.1.3.). Diastase and amylase catalyses starch breakdown into shorter carbohydrate chains or maltose while glucosidase transform sucrose into glucose and fructose. Glucose oxidase is responsible for antimicrobial activity due to the

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oxidation of glucose to hydrogen peroxide or D-gluconolactones (Edelhauser and Bergner, 1987). Stingless bee honey is composed of 80–85% of carbohydrates (46–72% of glucose, 7–61% of fructose, and 1–11% of sucrose), 15–17% of water, 0.3% of proteins (arginine, histidine, isoleucine, lysine, methionine, threonine, tryptophan, valine and other amines), and 0.2% of ash. Furthermore, Thai stingless bee honey also contains vitamins B1, B6, and niacin. It has been consumed in the form of raw honey (Lim et al., 2018; Sawatthum et al., 2009; De Groot, 1953).

During ethanol fermentation, a great increase in the ethanol concentration ranging between 23.7 and 94.7 g/L limits yeast growth rate and rate of ethanol production by 5.2 times. However, ethanol tolerant yeasts can improve the efficiency of fermentation under ethanol stress (Nguyen et al., 2015). The ethanol tolerant yeast has been found in a variety of sugar-rich materials, including fruit, distillery effluent, and molasses (Tikka et al., 2013). It has been reported to be used in the fermentation of rice wine (Flor and Hayashida, 1983), grape wine (Sumby et al., 2019), and pineapple wine (Tyokusa and Owuama, 2018). The goal of this research is to find an ethanol tolerant yeast consortium for use in the production of honey mead from stingless bee honey. This is the first research on using Thai stingless bee honey as a raw material for honey mead production.

## 2 Material and Methods

### 2.1 Culture source

Traditional culture starter balls composed of spontaneous mixed culture (Look-Pang) and fresh pineapple fruits were gathered from Thailand's various provinces. Fresh pineapples were carefully washed in sterile tap water to remove impurities. The peels of pineapple were separated and used as a culture source. Table 1 shows the specifics of the material type and location.

10% (w/v) of material used as the potential source of required yeast consortium (Table 1) was added into 200 mL of sterile liquid media, pH 7.0 containing 10% (w/v) sucrose, and 0.1% (w/v) Brewer's yeast extract (Cooray et al., 2017). The 10 samples containing materials stated in Table 1 were incubated at 30 °C for 3 days without shaking in triplicate.

### 2.2 Selection of Ethanol Tolerant Consortium

Ethanol tolerant yeast was selected by inoculating 1 mL of previously obtained culture ($1.0 \times 10^8$ cell/mL) into 9 mL of sterile liquid media composed of 10% (w/v) sucrose, 0.1% (w/v) Brewer's yeast extract, and 5% (v/v) food-grade ethanol and with pH 7, then incubating it at 30 °C for 3 days. The original spontaneous culture was re-inoculated seven times to ensure that only an ethanol tolerant culture could be obtained. A suitable consortium of ethanol-producing yeasts was chosen.

### 2.3 Determination of Invertase Activity

The selected yeast consortia were grown in sterile liquid media containing 10% (w/v) sucrose, 0.1% (w/v) Brewer's yeast extract, and 5% (v/v) food-grade ethanol with pH 7 for three days at 30 °C. The invertase activity was determined using modified method according to Jimenez and Benitez (1986). In brief, 1 mL of cell solution was mixed with 2 mL of 4% (w/v) sucrose in acetate buffer, pH 5. All samples were incubated for 5 minutes at 30 °C. A commercial wine yeasts *Saccharomyces cerevisiae* Davis904 was used as a control. The dinitro-salicylic acid method was used to calculate the amount of reducing sugar released. Invertase activity was expressed in international enzyme unit defined as the amount of enzyme that catalyses a conversion of 1 mole of reducing sugar per minute. Invertase activity and ethanol content were measured every 12 hours for three days. The consortium with the highest invertase activity and ethanol yield was chosen.

**Table 1** *Details of material collection used as a source of ethanol tolerant yeast consortium*

| Sample Type | Location | Collection Date | Code |
|---|---|---|---|
| Sriracha pineapple | Chon Buri, Thailand | April, 2021 | PP01 |
| Srithong pineapple | Trat, Thailand | April, 2021 | PP02 |
| Wild pineapple | Phang Nga, Thailand | April, 2021 | PP03 |
| Wild pineapple | Trang, Thailand | April, 2021 | PP04 |
| Wild pineapple | Chumphon, Thailand | April, 2021 | PP05 |
| Culture starter balls | Surat Thani, Thailand | April, 2021 | LP01 |
| Culture starter balls | Nakhonsi Thammarat, Thailand | April, 2021 | LP02 |
| Culture starter balls | Phatthalung, Thailand | April, 2021 | LP03 |
| Culture starter balls | Phang Nga, Thailand | April, 2021 | LP04 |
| Culture starter balls | Narathiwat, Thailand | April, 2021 | LP05 |

### 2.4 Determination of Ethanol Tolerance

Ethanol tolerance of a selected consortium was investigated by allowing the consortium to grow in sterile liquid media containing 10% (w/v) sucrose and 0.1% (w/v) Brewer's yeast extract with varying concentrations of food-grade ethanol such as 0%, 3%, 6%, 9%, 12%, and 15% (v/v) took over from Tikka et al. (2013) and modified. The 10% (v/v) culture ($1.0 \times 10^8$ cell/mL) was mixed with 90% (v/v) liquid medium and incubated at 30 °C for 3 days. At 24 hrs of growth, the optical density at 600 nm (OD600) was measured.

### 2.5 Honey Mead Fermentation

The stingless bee honey used in this experiment (Table 2) was obtained from a local beekeeper in Thailand's southern region. Pereira et al. (2013) and Mendes-Ferreira et al. (2013) described a method of honey-must preparation for fermentation with a selected yeast consortium. The honey was diluted in commercial natural spring water (Table 3) to 37% (w/v) solution and autoclaved to remove contaminating microbes to produce an alcoholic beverage with approximately 11% (v/v) ethanol. The fermentation took place in a 250 mL glass-bottom filled to 70% of its total volume with no additions and shaken at 30 °C. Determination of carbon dioxide ($CO_2$) production was used to monitor the fermentation processes.

### 2.6 Specific Gravity

A sample was taken from the honey mead at the end of the ethanol fermentation. A densitometer was used to determine the specific gravity (SG). The wine's percentage alcohol content (%), calories (calories/oz), residue sugar (%), apparent fermentation degree (%), and fermentative capacity (g/L) were calculated as follows (Balogu and Towobola, 2017):

$$\text{Percent alcohol by volume (\% ABV)} = [(\text{initial SG} - \text{final SG}) / 7.36] \times 1000 \quad (2)$$

$$\text{Residual sugar (\% RS)} = 231.3\,[1 - (1 / \text{final SG})] \quad (3)$$

$$\text{Apparent fermentation degree (\% AFD)} = [(\text{initial SG} - \text{final SG}) / \text{initial SG}] \times 100 \quad (4)$$

$$\text{Fermentative capacity (g/L)} = \text{initial RS} - \text{final RS} \quad (5)$$

### 2.7 Proximate Compositional and Physiochemical Analysis

The Association of Official Chemists (AOAC) methods were used to establish the proximate nutritional compositions such as moisture content (%), crude protein content (%), crude fibre (%), crude fat (%), and total carbohydrate (%). The physicochemical parameters such as titratable acidity, volatile acidity, and pH were determined using the method described by Balogu et al. (2017). Briefly, the total titratable acidity (TTA) was measured by titrating 10 mL of a sample with 0.1 M NaOH (4 g of NaOH in 1,000 mL of distilled water) until a neutral pH was reached. The TTA and volatile acidity (VA) were calculated as follows:

**Table 2** *Characteristics of raw stingless bee honey used in this experiment.*

| Characteristics | Value in raw honey | Unit |
|---|---|---|
| Moisture content | 17.10±0.03 | % |
| Ash | 0.33±0.10 | % |
| Crude carbohydrate content | 83.00±0.02 | % |
| Sugar content | 82.73±0.01 | % |
| Crude protein content | ND | % |
| Crude fibre content | ND | % |
| Crude fat content | ND | % |
| pH | 4.10±0.02 | – |

*ND = not determined*

**Table 3** *Characteristics of commercial mineral water used in this experiment.*

| Characteristics | Value in mineral water | Unit |
|---|---|---|
| Silica | 45.50 | mg/L |
| Calcium | 57.00 | mg/L |
| Potassium | 1.50 | mg/L |
| Magnesium | 6.50 | mg/L |
| Bi-carbonate | 220.00 | mg/L |
| Chloride | 1.00 | mg/L |
| Fluoride | 0.39 | mg/L |
| Sulphate | 4.00 | mg/L |
| Sodium | 4.00 | mg/L |
| pH | | – |

$$\text{TTA (g/L)} = 7.5 \times 0.1 \times \text{volume of NaOH} \quad (6)$$

$$\text{VA (g/L)} = 6 \times 0.1 \times \text{volume of NaOH} \quad (7)$$

The acetic acid content (g/L) was determined using the AOAC method, which involved mixing 2 mL of a sample with 25 mL of distilled water and titrating with 0.1 M NaOH. As an indicator, a 1% (w/v) phenolphthalein solution (0.01 g of phenolphthalein in 10 g of absolute ethanol) was used. The acetic acid concentration (%) was calculated as follows:

$$\text{Acetic acid concentration (\%)} = (\text{volume of NaOH} \times 0.1 \times 6{,}000) / 2{,}000 \quad (8)$$

**Table 4** *An overview of sources of ethanol tolerant yeasts*

| Source | Yeast | Reference |
|---|---|---|
| Orchard soil | *Saccharomyces* sp. Orc6 | Moneke et al., 2008 |
| Molasses and Cashew apple | *S. cerevisiae* | Priya et al., 2011 |
| Winery soil | *S. cerevisiae* ATKU132 | Thammasittirong et al., 2012 |
| Wine | *S. cerevisiae* | Anderson et al., 2012 |
| Soil | *S. cerevisiae* UVNR56 | Thammasittirong et al., 2013 |
| Pineapple peel | Yeast consortium PP02 and PP03 | This study |

## 3 Results and Discussion

### 3.1 Selection of Ethanol Tolerant Consortium

Four ethanol-tolerant yeast strains were isolated from the drainage area of a winery in Nakhon Pathom, Thailand, using an enrichment technique in a liquid medium containing 10% (v/v) ethanol (Thammasittirong et al., 2012). Two ethanol tolerant yeast consortia, designated as PP02 and PP03, were achieved from pineapple peels, while no ethanol tolerant yeast was gained from traditional culture starter balls when they were enriched in a liquid medium containing 5% (v/v) ethanol. Table 4 shows the source of ethanol tolerant yeast.

### 3.2 Determination of Invertase Activity

The glycoprotein invertase, also known as β-fructofuranosidase (E.C. 3.2.1.26), cleaves the terminal non-reducing β-fructofurannoside residues. It is found in a wide range of organisms, including plants and microorganisms like *S. cerevisiae*, *Candida utilis*, and *Aspergillus niger* (Kulshrestha et al., 2013). Figure 1 depicts the invertase activity of PP02, PP03, and the control (*S. cerevisiae* Davis904) during cultivation in liquid medium containing sucrose as a sole carbon source at 30 °C, pH 7. The results show that the maximal invertase activity of PP03 is 75.13±9.16 U/mL, which is 20.05% and 24.57% more than the control commercial strain (60.07±3.23 U/mL) and the PP02 (56.67±4.06 U/mL), respectively. Furthermore, the time course of invertase activity of the PP02 consortium is very similar to the course of the control strain, while the maximum determined value of PP02 is even slightly higher than at the control strain. The invertase activity highly depends on cultivation conditions such as sucrose concentration, N-source, pH, aeration, type of cultivation (submerged × surface; batch × fed-batch × continuous) etc. (Vitolo et al., 1995). That is why it is difficult to compare the obtained invertase activity with literature data. The reported data of several papers dealing with invertase activity of *S. cerevisiae* under similar conditions as in this study indicate that there is a number of other (genetic) aspects influencing invertase activity. The studies were conducted at the media composed of sucrose 1–4%, yeast extract 0.2–0.3% and peptone 0.25–0.5%, pH 5–7, temperature 22–36 °C and the incubation time 48–72 hrs. Haq et al. (2005) informed how cultivation conditions can influence the invertase activity of five wild strains of *S. cerevisiae* isolated from date palm fruit (*Phoenix dactylifera*) and found invertase activity ranging in 42.02–59.61 U/mL depending on the set cultivation parameters. Ali et al. (2016) detected that the wild-type of *S. cerevisiae* isolated from soil reached only 17 U/mL but its chemically induced mutant reached between 23–74 U/mL of invertase activity again depending on cultivation parameters. On the other hand, Shatif et al. (2002) studied the effect of mineral nutrients on the invertase activity of the strain *S. cerevisiae* GCB-K but the maximal value was only 12.68 U/mL.

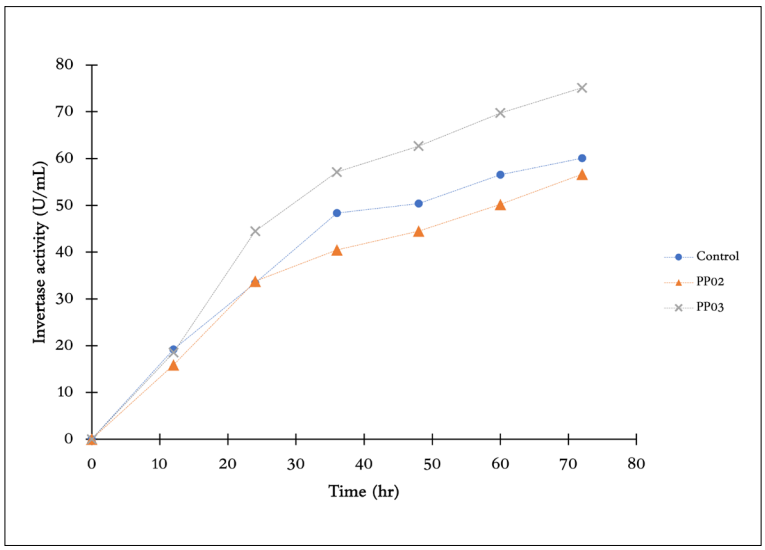


**Figure 1** *Invertase activity of selected consortium and control (*S. cerevisiae *Davis904) when they were incubated at 30 °C for 3 days in a liquid medium.*

Kumar and Kesavapillai (2012) demonstrated that *S. cerevisiae* isolated from ethanol fermentation slurry can produce even 107.5 U/mL extracellular invertase activity in 72 hrs via solid substrate fermentation at 40 °C and pH 5. This extreme value exceeds the invertase activity of our consortium PP03 by 30.11%. However, that experiment was conducted under completely different culture conditions which in our case have not been tested.

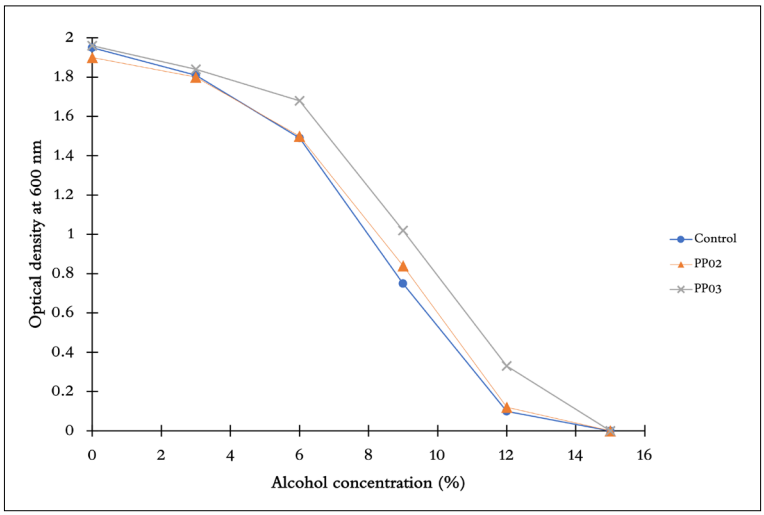


**Figure 2** *Ethanol tolerance level of selected consortium and control (S. cerevisiae Davis904).*

### *3.3 Determination of Ethanol Tolerance*

The ethanol tolerant consortia PP02 and PP03, as well as the control strain, were inoculated into the media containing different ethanol concentration ranging between 0–15%. After 3 days of incubation without shaking, the tolerance levels of PP02 and the control of up to 6% of ethanol were determined by tracking the growth potential at $OD_{600}$. The strain PP03 showed substantially better tolerance to ethanol up to the level of 12% (Figure 2). It is well known that many representatives of *S. cerevisiae* are highly tolerant to ethanol accumulated in their environment (Chi and Arneborg, 2000). However, the level of ethanol tolerance greatly varies among individual strains as shown in Table 5 depending on genetics and the ability to adapt to increasing ethanol concentration in their environment. Other factors such as temperature and osmotic pressure also interfere with the yeast resistance to ethanol stress (da Silva et al., 2013). That is why winemakers and researcher can describe low, high and very high ethanol tolerant yeasts. In addition to the biologically distinct abilities to tolerate certain levels of ethanol, it is necessary to point out the issue of the lack of precise ethanol tolerance definition. Usually, viability or surviving ability of the yeast cells, that are exposed to different concentrations of ethanol, is used for comparative studies. The most similar case to our study is the research on nine yeast isolates from sugarcane juice from a Brazilian distillery. The authors found that some yeasts showed fermentative activity between 6 and 8% of ethanol at 30 °C and that at 10% of ethanol no fermentation was detected at all (da Silva et al., 2013). These findings closely correspond to our results for PP02 and the control strain. Moreover, the same authors detected one more resistant strain among the wild strains, which was able to ferment at the ethanol contents of 10 and 12% at 30 °C similarly to our PP03 strain. Also Tikka et al. (2013) reported that seven strains of *S. cerevisiae* obtained from different fruit displayed the ethanol tolerance levels between 7–12 %.

However, some *Saccharomyces* strains are able to produce extremely high concentrations of ethanol and thus they are commonly considered to be extensively ethanol tolerant. In traditional practice of fermentation beverages,

**Table 5** *Comparison of ethanol tolerance level of yeasts in this study with others.*

| Yeast strain / Consortium | Tolerance level (%) | Reference |
|---|---|---|
| *H. uvarum* | 25 | Pina et al., 2004 |
| *Saccharomyces* sp. | 9 | Osho, 2005 |
| *S. cerevisiae* YEPD | 7 | Stanley et al., 2010 |
| *S. cerevisiae* SH631 | 8 | Park et al., 2012 |
| mixed culture of *S. cerevisiae* and *K. lactis* | 20 | Yamaoka et al., 2014 |
| *S. cerevisiae* | 7 | Ganucci et al., 2018 |
| *Lachancea cidri* | 8 | Villarreal et al., 2021 |
| *S. cerevisiae* Davis 904 (Control) | 6 | This study |
| PP02 | 6 | This study |
| PP03 | 12 | This study |

the following order relating to ethanol production/tolerance is accepted: sake yeasts > wine yeasts > distillers' yeasts > brewers' yeasts (Casey and Ingledew, 1986). The examples of extreme tolerance/resistance to ethanol levels can be the study of Pina et al. (2004). The research team showed that *Hanseniaspora uvarum* isolated from grape must used for Port wine production can survive even at 25% ethanol concentration. Moreover, the work of Yamaoka et al. (2014) indicated that the mixed culture of *S. cerevisiae* and *Kluyveromyces lactis* can grow at 20% ethanol concentration when they were incubated for 8–15 days.

### 3.4 Honey Mead Fermentation

The PP03 yeast consortium, which displayed a better tolerance to ethanol, was used as a starter culture for honey mead fermentation ($1.0 \times 10^8$ cell/mL). The fermentation process of stingless bee honey diluted with mineral water was carried out at 30 °C for 21 days without shaking. Microbial activity was detected by monitoring $CO_2$ production. After 21 days of incubation, the enological properties of honey mead were determined. The results showed the TTA of 12.85 g/L, VA of 10.28 g/L, acetic acid concentration of 5.14%, ABV of 9.90%, RS of 34.41%, AFD of 6.71%, and fermentative capacity of 190.59 g/L were achieved. This study yielded a slightly higher TTA than the previous study by Pereira et al. (2019), which yielded 5.87 g/L when the mead was fermented by a single strain of *S. cerevisiae*. However, our work still provides the same level of TTA as the study, which produced mead by *S. cerevisiae* and honey must was prepared from honey and coconut water (Balogu and Towobola, 2017).

Table 6 presents basic nutritional and physiochemical parameters.

Pereira et al. (2013) previously used *S. cerevisiae* ($1.0 \times 10^8$ cell/mL) for mead fermentation. The ABV was 10.03±0.38% when fermented at 22 °C with mild shaking (120 rpm/min) supplemented with 5 g/L potassium tartrate, and pH adjusted with malic acid. On the other hand, the low-density yeast cell with $1.0 \times 10^6$ cell/mL has been used for mead fermentation. The results show that the optimal range of microbial growth was 30 days at 25 °C with the addition of 5mL of 0.02% (w/v) citric acid (Balogu and Towobola, 2017).

**Table 6** *Nutritional and physiochemical properties of honey mead from Thai stingless bee honey using ethanol tolerant yeast consortium PP03*

| Parameters | Honey mead | Unit |
|---|---|---|
| Total carbohydrate | 6.85 | % |
| Moisture content | 93.12 | % |
| Ash | 0.03 | % |
| Crude fat content | ND | % |
| Crude fibre content | ND | % |
| Total protein content | ND | % |
| pH | 4.10 | – |

## 4 Conclusion

The PP03 yeast consortium was isolated from wild pineapple peel collected from the Phang Nga province, Thailand using an enrichment technique. This consortium had a higher invertase activity of 75.13±9.16 U/mL and ethanol tolerance of up to 12% v/v than the other isolate of PP02 and commercial control strain of *S. cerevisiae*. We demonstrated a promising ability of the PP03 consortium to grow under ethanol stress conditions. The isolate was also used to produce the first experimental honey mead from stingless bee honey. However, this is a preliminary research that needs to be complemented by further microbiological and technological data.

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