Bioethanol-Producing Yeast Isolated from Fermented Cocoa

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**Abstract.** Corn and sugarcane-base bioethanol dominantly contributes to the 25 billion gallons of bioethanol worldwide. Recent researches focused on the potential microbes and biomasses for optimum production. This study is, therefore, aimed to screen the bioethanol generating yeast strains of Biotechnology Culture Collection (BTCC), isolated from chocolate fermentation in several medium containing various carbon sources. A total of 72 yeast strains were grown in the media containing sugarcane juice, sorghum juice, and molasses, which served as carbon sources. Based on 26S rDNA gene analysis, these species were included in 9 genera, encompassing Saccharomyces (63.9%), Hanseniaspora (9.7%), Candida (0.7%), Torulaspora (0.4%), Pichia (0.8%), Issatchenkia (0.1%), Wickerhamomyces (0.3%), Metschnikowia (0.1%), and Rhodotorula (0.1%). Therefore, spectrophotometer UV-Vis was used to analyze cell growth, while the fermentation products (sugars and ethanol) were evaluated using the HPLC, and about 70 strains produced bioethanol. The highest yields were obtained during fermentation, using sugarcane juice, molasses, molasses waste, and sorghum juice, at concentrations of 43, 50, and 7 g/L, respectively. Furthermore, the *Saccharomyces cerevisiae* strain were the most significant producers, as the genus was able to generate various concentrations from several carbon sources. However, the only genus without the ability to yield any related products during fermentation was Pichia (0.8%). Based on these results, it is necessary to further develop the yeast strains from chocolate fermentation, due to the potential for bioethanol production from biomasses.

**Key words:** bioethanol, yeast, fermentation, sugarcane molasses, sorghum juice

1. **Introduction**

   Feedstock-base Bioethanol is the most renewable biofuel globally, and is capable of reducing greenhouse gas by up to 80%, compared with conventional fossil-base fuels. In addition, the raw
materials for production are broadly classified as (i) sucrose-containing (sugarcane, sugar beet and sweet sorghum), (ii) starch-containing (wheat, corn and cassava) and (iii) cellulosic feedstock (straw, grasses, wood, stover, agricultural wastes, paper, etc.) [1] However, the main source is from traditional food crops, including corn (USA), sugar cane (Brazil), wheat (France, England, Germany, and Spain), cassava (Thailand, Nigeria) and sorghum (India), depending on the location and dominant agriculture output [2]. In addition, most current processes utilize more readily degradable biomass, including cereals (corn or grain) and sugar cane juice. The yield from sucrose-and starch-containing feedstock is classified as 1st generation bioethanol (ethanol from corn and sugarcane), while the utilization of edible agricultural crops conflicts with food and feed production [3].

The conversion of sugar to bioethanol is facilitated by yeast, especially the species *Saccharomyces cerevisiae* [4]. In addition, *Pachysolen tannophilus*, *P. stipitis*, *Candida tropicalis*, and *C. shehatae* are known to ferment one of the most abundant pentoses sugars (xylose) to ethanol, although the wild-type strains are unable to simultaneously convert both the hexoses and pentoses. This challenge was resolved through metabolic engineering, and then the co-culture of bacteria and yeast was also studied to prove the potential synergistic use of different microorganism metabolic pathway [5].

The yeast strains from Biotechnology Culture Collection (BTCC) were screened and evaluated in media containing Inhibitory Chemical Complex (ICC), and *S. cerevisiae* BTCC3 produced the highest bioethanol yield, compared to other strains [6]. However, no information concerning the potency of BTCC yeast in bioethanol fermentation was obtained for sugarcane juice, molasses, molasses waste, and sorghum juice. The purpose of this research, therefore, was to screen the yeast strains from BTCC for bioethanol fermentation tendency, using sugarcane juice, molasses, molasses waste, and sorghum juice as biomass. These potential yeasts are expected to be applied as 1st generation producers.

2. **Experimentals**

2.1. Yeast strains and media

The yeasts were obtained from the Biotechnology Culture Collection (BTCC), Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). These were further subjected to routine subculturing and maintenance, by growing on Yeast Peptone Glucose (YPD) agar plate containing 10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L glucose, and 20 g/L agar. Moreover, the pre-culture was prepared by inoculating a single colony of each strain from the YPD plate into a 12 mL YPD liquid medium in 100 mL erlenmeyer flask. The culture was subsequently incubated overnight at 30 °C and 150 rpm.

2.2. Screening yeast in various media

The yeasts were cultured in 1000 uL of YPD (yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L) media. The fermentation process was performed overnight under conditions of 1400 rpm at 30 °C
in the deep well plate, and incubated in a deep well maximizer, from Taitec Bioshaker M BR-0224UP (Japan). Furthermore, 100 uL of each culture were transferred into 900 uL of the main culture containing YM medium (yeast extract 1 g/L, Mg(NO₃)₂·6H₂O 0.15 g/L, KH₂PO₄ 5 g/L, CO(NH₂)₂ 3 g/L, and the variety of carbon sources, including sugarcane juice, molasses 15% (w/v), sugarcane molasses waste, sorghum juice, glucose, and xylose). All experiments were performed in triplicate.

2.3. Fermentation product analysis

The culture growth was then analyzed by measuring the dry cell weight after dehydrating with Freeze Dry Alpha 1-2 LD plus (Germany). Therefore, the fermentation parameters, including glucose consumption and ethanol production were evaluated using HPLC (High Performance Liquid Chromatography) (Shimadzu LC-20AB, Japan). The refractive index detector (RID) encompassing the Aminex HPX-87H column from Bio-Rad as a stationary phase, was used to detect compounds. In addition, the following conditions were maintained: flow rate of 0.6 mL/min, column oven temperature of 60ºC, injection volume of 20µL, 30 minutes elution time, and 5mM H₂SO₄ as eluent. The ethanol yield was calculated using the following equation [7]:

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\text{Ethanol yield} = \frac{\text{Measured ethanol in sample (g)}}{\text{Theoretical ethanol yield (g)}}
\]

Theoretical ethanol yield (g) = 0.5x amount of initial sugar (g) in fermentation solution

3. Results and Discussion

The BTCC yeast strains were screened in a minimum medium with added minerals, and two types of C-sources were applied in this step. These include synthetic (glucose and xylose) and derived sugars from feedstocks, encompassing sugarcane (juice, molasses and molasses waste), and sorghum juice.

Generally, a total of 72 yeast strains grew in the sugars. Based on ethanol yield, this occurred only in medium containing glucose, sugarcane juice, sugarcane molasses, and molasses waste, and most species were able to produce bioethanol. However, none of strains were able to generate the product from xylose (Table 1), and the BTCC strains are thus assumed to assimilate xylose, which does not produce ethanol during metabolism.

**Table 1.** The cell growth and ethanol production of BTCC yeast strains after incubation in medium YM containing different C-sources

| No. | Code    | Spesies             | Ethanol (g/L) | DCW (g/L) |
|-----|---------|---------------------|---------------|-----------|
| 1   | IDI-Y001| *Saccharomyces cerevisiae* L121 |               |           |
| 2   | IDI-Y002| *Saccharomyces cerevisiae* L122 |               |           |
| 3   | IDI-Y003| *Saccharomyces cerevisiae* L123 |               |           |
The Yeasts BTCC with potency to produce bioethanol in the medium fortified with sugarcane juice were BTCC 2, 27, 33, 40, and 50, estimated to generate about 34.4; 43.25; 21.69; 33.8; and 35.4 g/L, respectively, within 24 hours. The highest yield was identified in BTCC 27. However, only BTCC 3 was able to produce bioethanol with the yield of 7.3 g/L in the media consisting of sorghum juice.

Strain BTCC 2, 18, 21, and 29 generated 47; 28.65; 44.9; and 45.4 g/L of product in the media containing sugarcane molasses, with the highest yield in BTCC 2, among others. In addition, strain numbers BTCC 8, 13, and 27 generated the highest bioethanol compared to other strains with the similar amount, 19; 19.2; and 19.2 g/L, in sugarcane molasses waste, while BTCC 3, 13, 14, 29, 32, and 43 produced 17.1; 17.5; 12.9; 17.1; 17.9; and 17.3 g/L in the 50 g/L glucose medium.

The results showed BTCC 2, in the medium containing sugarcane molasses, as the highest producer of bioethanol yield, compared to other strains. This contains 62.3 g/L glucose content, from 10% sample (data not shown), responsible for the high amount of convertible sugar. In addition, molasses
are the non-crystallizable residue after sucrose purification, commonly used as a feedstock for bioethanol production. This is known to possess some advantages, including the relatively inexpensive raw material and ready availability. Also, direct fermentation is used as the resources do not require starch hydrolysis and pre-treatments. The results also showed the relatively greater resistance of BTCC 2 strain under osmotic stress, and this phenomenon was attributed to the high sugar content in the molasses culture. Meanwhile, *S. cerevisiae* experienced several forms of stress, including glucose depletion, severe heat shock as well as ethanol stress, and also induced formation of SGs with a pronounced repression of translation, resulting from the exposure to sodium azide (NaN₃) [8,9,10,11].

![Figure 1. Diversity and bioethanol production of BTCC yeast strain in several media](image)

The yeast strains with the capacity to produce bioethanol in 5 types of sugar were mostly *S. cerevisiae* species. Figure 1 showed the for 65, 50, 84, 69, and 65% conversion potential of the *Saccharomyces* genus on sugarcane juice, sorghum juice, sugarcane molasses, sugarcane molasses waste, and glucose (Figure 1). These species are well known as a bioethanol producing yeast, and *S. cerevisiae* is mainly applied in the fermentation of ethanol, using renewable biomass from sugarcane or sugar beet molasses as the main carbon source [12,13]. Therefore, the most effective
microorganisms observed in this experiment were *S. cerevisiae*, based on several advantages. These include high ethanol productivity and tolerance, as well as resistance towards inhibitory compounds [14].

4. Conclusion

Based on the results and discussion, the strain of *Saccharomyces cerevisiae* was identified as the most significant bioethanol producer among the BTCC yeast. This genus is capable to generating varying concentrations of yield with several carbon sources. However, *Pichia* (0.8%) was the only genus devoid of production capacity. The two potential yeast strains of *S. cerevisiae* BTCC2 and BTCC3 grew quickly and yielded relatively higher products than others, hence the potential for development as first generation bioethanol fermenters. This is attainable following future research on strain characterization and fermentation optimization with various sugar sources.

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