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Candida tropicalis Isolated from *Tuak*, a North Sumatera-Indonesian Traditional Beverage, for Bioethanol Production

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Tuak is a traditional alcoholic beverage, one of the most widely known in the North Sumateran region of Indonesia. It is produced by a spontaneous fermentation process through the application of one or more several kinds of wood bark or root, called *raru* (*Xylocorpus* wood bark or a variety of forest mangosteen), into the sap water of sugar palm (*Arenga pinnata*) for 2–3 days. In this research, yeast that are potentially useful for ethanol production was isolated from *Tuak* and identified. Based on analysis of D1/D2 domain sequence of LSU (large subunit) rRNA genes, those isolated yeast strains, HT4, HT5, and HT10 were identified as *Candida tropicalis*. Fermentation test of these *C. tropicalis* isolates displayed an ability to produce 6.55% (v/v) and 4.58% ethanol at 30°C and 42°C, respectively. These results indicated *C. tropicalis* isolates more rapidly utilize glucose and obtain higher levels of the production of ethanol at the higher temperature of 42°C than *S. cerevisiae,* a common yeast used for bioethanol fermentation.

Keywords: Yeast, *Candida tropicalis*, *tuak*, bioethanol

Introduction

Ethanol has acquired much attention as bio-based alternative energy because of its friendly environment. Increasing of ethanol yield is required to meet a global demand, therefore development of cost-effective technology for bioethanol production is priority for many researchers and industries. Cost-effective technology depends on following factors; rapid and high yielding conversion of carbohydrate to ethanol [18]. Production process of bioethanol depends on raw materials. Various types of lignocellulosic materials such as empty palm bunch, wheat straw, sugarcane baggase, corncobs is treated in a more complex way than starch or grain raw materials. To produce ethanol from lignocellulosic material it is necessary to breakdown the chains of these carbohydrates (amylum, cellulose, hemicellulose, etc) for obtaining glucose, which can be converted into ethanol by yeast or other microorganism [4]. Usage of yeast *Saccharomyces cerevisiae* strain in the fermentation process to convert glucose into ethanol has been well established method. *S. cerevisiae* is a facutative anaerobe microorganism and it can ferment glucose into ethanol very high efficiency under anaerobic condition and thus is the most commonly used microorganism in industrial ethanol production [2, 15]. But, improvement of ethanol yield by using either genetically engineered *S. cerevisiae* strain or other yeast with some desirable traits may be required. Some desirable traits of yeast called superior strain such as tolerance to high temperature, high ethanol production (Htg), acidity tolerance (Acd), and high ethanol production (Hep) are indispensable for economic feasibility in ethanol production [1, 16]. Furthermore, when we apply lignocellulosic biomass as raw material, for cost-effective and eficient ethanol production, yeast strain should grow on not only glucose, but also xylose and arabinose, and it can be better if yeast strain can ferment those monosaccharides [20].

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Ethanol-producing yeast strains have been isolated from *Tuak,* North Sumatera Indonesia traditional beverage. *Tuak* is traditional alcoholic beverage of North Sumatera - Indonesia which is always used in social activities and several ceremonies. *Tuak* is produced by spontaneous fermentation of the sap water sugar palm (*Arenga pinnata*) in the presence of *raru* wood or several kinds of wood skin or roots (like nirih – *Xylocarpus* wood skin or a kind of forest mangosteen) for over night incubation (Toba pulp lestari website, 2013).

Three strains from *tuak* designed as HT4, HT5, and HT10 were isolated since they showed desirable trait for fermentation and showed interesting phenotypes. Based on analysis of D1/D2 domain sequence of Large Subunit (LSU) rRNA using two pairs of universal primer (ITS1-IT4 and NL1-NL4 pairs), these strains were identified as *Candida tropicalis*. Free and calcium alginate-immobilized *C. tropicalis* was used in production of bioethanol without the saccharification step [8]. Although *C. tropicalis* is a potentially useful agent for the commercial production of bioethanol, usage of *C. tropicalis* as ethanol-producing strains are very view reports.

Materials and Methods

Strains and media

Yeast strains (HT4, HT5, and HT5) were isolated from *Tuak* obtained from North Sumatera-Indonesia and these strains have been identified as *C. tropicalis.* Following procedure was used to obtain *C. tropicalis* isolates HT4, HT5, HT10 : 100 μl filtered sampel of a fresh *tuak* was spreaded on YPD agar media. Cells were incubated at 30°C for 2–3 days. If cell numbers were too much, filtrate should be diluted for 10×, 100×, or 1000× to obtain single colonies.

S. cerevisiae laboratory strain BY4741 with genopype *MAT*a *met15Δ0 his3Δ1 leu2Δ0 ura3Δ0* (Htg-). For routine subculturing and maintenance, the strains were grown on YPDA broth medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, 400 mg/l adenine) at 30°C, or on YPDA agar medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, 400 mg/l adenine, and 20 g/l bacto agar). YPXA agar consisted of 10 g/l yeast extract, 20 g/l peptone, 20 g/l xylose, 20 g/l bacto agar. YPAA agar consisted of 10 g/l yeast extract, 20 g/l peptone, 20 g/l arabinose, 20 g/l bacto agar. YPD10A broth medium consisted of 10 g/l yeast extract, 20 g/l peptone, 100 g/l glucose, 400 mg/l adenine.

Primers

Two universal primers pairs were used in this study: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) ; NL-1 (5′-GCATATCAATAAGCGGAGGA AAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) [11].

Phenotypic characteristics

Cells were grown on YPDA, YPXA and YPAA agar media, incubated at 30°C for 1 day to see cell growth in the presence of xylose and arabinose, respectively. Temperature sensitive phenotype was observed when cells grown on YPDA were incubated at 41°C for 2 days.

Genomic DNA extraction

Total genomic DNA was isolated from stationary-phase cultures according to previously reported procedure [14]. Cells were cultured in 50 ml centrifuge tubes containing 10 ml YPDA broth at 30°C with 200 rpm shaking overnight. Cells were harvested by centrifugation at 5000 × g at 4°C for 5 minutes, washed with 20 ml sterile water three times, resuspended in 200 μl lysis buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA and 0.5% SDS), and lysed by vortexing on a high speed vortex mixer with 0.2 g of glass bead (0.5 mm in size). After disruption of the cell, the lysed cells were centrifuged at 5000 × g and 4°C, for 5 minute. The supernatants were collected into a microcentrifuge, and 500 μl mixture of phenol:chloroform: isoamylalcohol in the ratio 25:24:1 (v/v/v) was added. Moreover, the mixture was briefly mixed by the vortex, and centrifuged at 12000 × g and 4°C for 10 minutes. The upper layer was withdrawn carefully and transferred to a new 1.5 ml microcentrifuge. One ml ice cold 95% (v/v) ethanol was added to the supernatant and briefly mixed by inversion. It was then stored at -20°C for 2 hours to precipitate the genomic DNA, and the sample was centrifuged at 12000 × g and 4°C for 10 minutes. The supernatant was carefully discarded to retain the genome DNA pellet. Afterwards, 1 ml 75% (v/v) ice cold ethanol was used to wash the genomic DNA pellet three times, and the DNA pellets were then dried by incubation at 37°C for 1 hour. The genomic DNA was resuspended in 200 μl of sterile water and stored at -20°C until use.

Gene sequencing and analysis

Two universal primer pairs consisted of ITS1 and ITS4

primer pair and NL-1 and NL-4 primer pair were used to amplify the DNA region containing the internal transcribed spacers (ITS). ITS1-ITS4 primer pair amplified the internal transcribed spacer region (ITS1-5.8S rDNA-ITS2), while NL1-NL4 primer pair amplified D1/D2 domain of 26S rRNA gene. PCR products were purified with the QIA quick purification kit (Qiagen). Moreover, primers ITS1 and NL1 were used for sequencing analysis. The nucleotide sequences of DNA fragments were determined using Autosequencers ABI Prism 310 or 3100 (Applied Biosystems). DNA sequencing data was analyzed through BLAST (Basic Local Alignment Search Tool) National Center for Biotechnology Information homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Fermentation test

The method used to determine fermentation ability was as described by previous report [1]. *C. tropicalis* isolates or *S.cerevisiae* BY4741 cells were cultured in YPDA medium at 30°C with shaking for overnight, and these cells were transferred to fermentation medium YPD10A, where the initial cell density was adjusted to OD_{600} = 0.1. Fermentation was performed using culture tubes (18 × 175 mm) with working volumes of 15 ml YPD10A medium, at two different temperatures 30°C and 42°C with shaking 150 rpm. The bioethanol concentration from fermentations were determined by using an Alcohol mechine AL-3 (Riken Keiki, Japan) every 10 hours. Glucose concentrations were measured using the Glucose C-II-test (Wako, Japan).

Results and Discussion

Nucletiodes sequencing analysis

In this study, three yeast isolates (HT4, HT5, and HT10) from *tuak* were further identified using molecular method. By PCR method using two universal primer pairs, ITS1-ITS4 and NL1-NL4 have succesfully amplified two parts of ribosomal regions. Ribosomal regions, such as the internal transcribed spacer (ITS) region and 28S rRNA, exhibit a low intraspecific polymorphism and a high interspecific variability, therefore these region is an ideal targets for species identification purpose [7]. Nucleotides sequencing analysis of the PCR products were carried out using each ITS1 and NL1 oligonucleotides separately. By using ITS1 oligonucleotide primer, nucleotide sequence of entire ITS1-5.8S rDNA-ITS2 of HT4, HT5, and HT10 isolates showed identical sequences with *C. tropicalis* as many as 95–99%, 94%, and 95–99%, respectively. While using NL1 oligonucleotide primer, nucleotide sequence analysis of LSU rRNA D1/D2 domain gene respectively showed similarity 95–99%, 99–100%, and 99%, with *C. tropicalis* (Fig. 1 and Fig. 2). All analysis of these nucleotides sequences were less than 100% similarity with *C. tropicalis*, it assumed that these differences reflected intraspecific diversity [19]. Therefore, based on these sequences producing significant alignments data indicated that all HT4, HT5, and HT10 isolates were identified as *C. tropicalis*. *C. tropicalis* was isolated from rotten fruits of sapota (*Achras sapota L.*) and strawberry (*Fragaria sp. L.*) which are capable to convert pentose sugar xylose into ethanol [17].

Phenotypic characteristic

Phenotypic tests of *C. tropicalis* isolates (HT4, HT5, and HT10) revealed that these isolates can grow in the presence of arabinose and xylose as well as glucose as sole carbon source on YPAD, YPAX, YPAD agar media, respectively (Fig. 3). This indicated that *C. tropicalis* isolates was able to utilize and consume both pentose sugars as well as glucose for growth. This trait is indispensable since both arabinose and xylose are present in high amounts in lignocellulosic biomass. Several genetic engineering strategis have been used in attempt to enable arabinose and xylose utilization by *S. cerevisiae* in which contributed to improved ethanol production [10]. In the presence of mixture of glucose, L-arabinose, and D-xylose as a carbon source in batch culture, the recombinant *S. cerevisiae* utilizes glucose first and completely consumed, L-arabinose and D-xylose consumption rates were higher after glucose depletion [3]. The arabinose- and xylose-growing strains is crucial to utilize lignocellulosic biomass for bioethanol production. The co-utilization of arabinose and xylose significantly reduced formation of the by-product xylitol, can improve bioethanol production, and make the fermentation process more cost-effective [10, 13]. Fig. 3 also showed that the growth of these *C. tropicalis* isolates at 30°C for 24 h were faster than *S.cerevesiae* strain as a common microbe for ethanol fermentation in glucose medium. Further observation, these *C. tropicalis* isolates showed tolerance to high temperature 41°C on YPD agar medium, while wild type *S. cerevisiae* strain showed sensitive phenotypic at this temperature. Tolerance to high temperature

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-TTANTAGCGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGCTCTTTCAGAGTC 59
--TAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGCTCTTTCAGAGTC 58
CTTAGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGCTCTTTCAGAGTC 60
  *  ************************************************************

CGAG-TTGTAATTTGAAGAAGGTATCTTTGGGTCTGGCTCTTGTCTATGTTTCTTGGAAC 118
CGAG-TTGTAATTTGAAGAAGGTATCTTTGGGTCTGGCTCTTGTCTATGTTTCTTGGAAC 117
CGANGTTGTAATTTGAAGAAGGTATCTTTGGGTCTGGCTCTTGTCTATGTTTCTTGGAAC 120
***  *** ************************************************************

AGAACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGATCCAGGCCTATGTAAAGTTC 178
AGAACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGATCCAGGCCTATGTAAAGTTC 177
AGAACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGATCCAGGCCTATGTAAAGTTC 180
************************************************************

CTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGGGGTAAATTCCATCTAAA 238
CTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAA 237
CTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAA 240
****************************************** ****************

GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC 298
GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC 297
GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC 300
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TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAAAC 358
TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGAC 357
TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGAC 360
************************************************************ **

TTGGTATTTTGTATGTTACTTCTTCGGGGGTGGCCTCTACAGTTTATCGGGCCAGCATCA 418
TTGGTATTTTGTATGTTACTTCTTCGGGGGTGGCCTCTACAGTTTATCGGGCCAGCATCA 417
TTGGTATTTTGTATGTTACTTCTTCGGGGGTGGCCTCTACAGTTTATCGGGCCAGCATCA 420
************************************************************

GTTTGGGCGGTAGGAGAATTGCGTTGGAATGTGGCACGGCTTCGGTTGTGTGTTATAGCC 478
GTTTGGGCGGTAGGAGAATTGCGTTGGAATGTGGCACGGCTTCGGTTGTGTGTTATAGCC 477
GTTTGGGCGGTAGGAGAATTGCGTTGGAATGTGGCACGGCTTCGGTTGTGTGTTATAGCC 480
************************************************************

TTCGTCGATACTGCCAGCCTAGACTGAGGACTGCGGTTTATACCTAGGATGTTGGCATAA 538
TTCGTCGATACTGCCAGCCTAGACTGAGGACTGCGGTTTATACCTAGGATGTTGGCATAA 537
TTCGTCGATACTGCCAGCCTAGACTGAGGACTGCGGTTTATACCTAGGATGTTGGCATAA 540
************************************************************

TGATCTTAAGTCGCCCGTCTTGAAACCACGGACCA 573
TGATCTTAAGTCGCCCGTCTTGAA---------- 561
TGATCTTAAGTCGCCCGTCTTG------------ 562
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Fig. 1. Alignment of nucleotides sequences of Internal transcribed spacer (ITS1-5.8S rDNA-ITS2). The deduced nucleotides sequences of Internal transcribed spacer (ITS1-5.8S rDNA-ITS2) of HT4, HT5, and HT10 were aligned using Clustal X. Asterisks and grey colour indicate identical nucleotide.

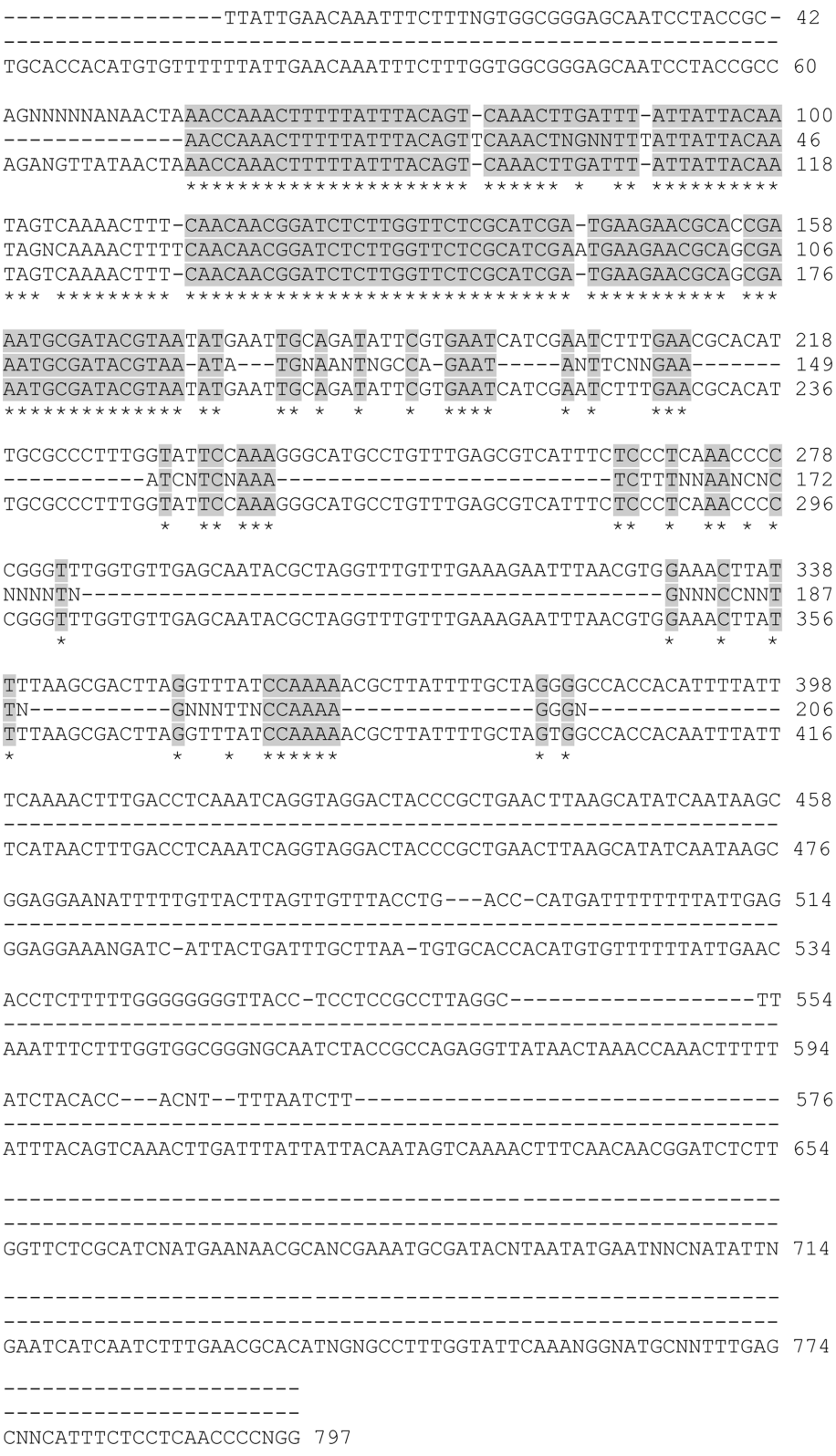

Fig. 2. Alignment of nucleotides sequences of 26S rDNA D1/D2 domain. The deduced nucleotides sequences of 26S rDNA D1/D2 domain of HT4, HT5, and HT10 were aligned using Clustal X. Asterisks and grey colour indicate identical nucleotide.

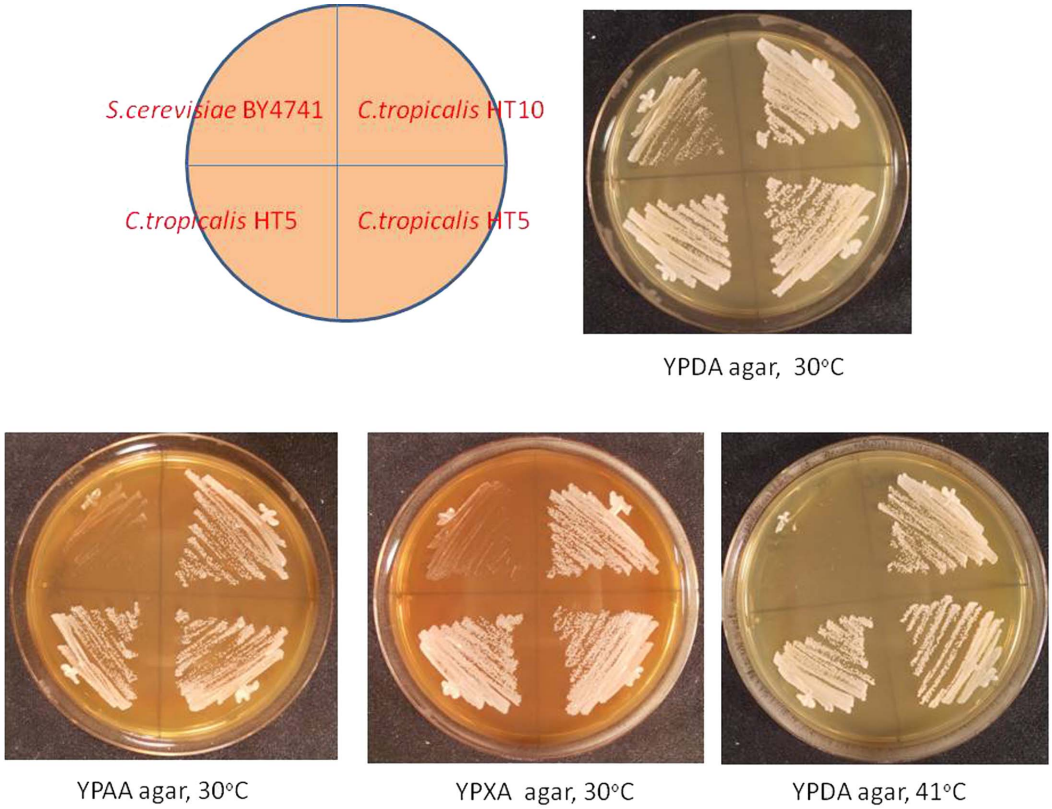

Fig. 3. Phenotypic test of *C. tropicalis* isolates. *C. tropicalis* isolates (HT4, HT5, and HT10) and wild type strain *S. cerevisiae*, BY4741 were streaked on YPDA (glucose), YPAA (arabinose), and YPXA (xylose) media incubated at 30°C and 41°C for 1–2 days.

(Htg$^+$) is required for bioethanol production, and another desirable traits are tolerance to acidity (Acd$^+$) and high ethanol productivity (Hep$^+$). Advantages using high-temperatures resistant yeast for fermentation can reduce the risk of contamination, cooling cost, and enabling easy recovery of ethanol [6].

Fermentation tests

To see whether *C. tropicalis* is potentially useful yeast for bioethanol production, fermentation tests for their capable fermenting arabinose, xyolse, and glucose incubated at 30°C and 42°C were performed. Although *C. tropicalis* isolates were able to grow on various carbon sources, not all carbon sources could be fermented and converted producing ethanol under anaerobic condition. They may produce different products of fermentation. As presented in Fig. 4A, at common working temperature, 30°C for 20 h capability of *C. tropicalis* isolates showed ethanol production comparable to that *S. cerevisiae* wild type BY4741 in fermenting glucose which is 6.5% (v/v). At this conditions, 100 g/l of glucoses were completely consumed by all *C. tropicalis* isolates while for *S. cerevisiae* strain BY4741 2.87 g/l of unutilized glucose remained as presented in Fig. 4B. In the presence of arabinose or glucose as a sole carbon source, similar to *S. cerevisiae* wild type BY4741, fermentation by *C. tropicalis* isolates did produce an ethanol (data not shown). This result supports published report that *C. tropicalis* converts xylose to ethanol under aerobic, but not anaerobic conditions [9]. *C. tropicalis* isolated from buffalo feces utilized xylose as a sole carbon source and preferred to produce xylitol more than ethanol [12]. Fermentation of starch with free and immobilized *C. tropicalis* cell in fed-batch mode by stirring and oxygen maintained at 450 rpm and 0.6 L/min respectively yielded ethanol concentration of 5.6% (w/v) [8]. Most of yeast species are able to convert L-arabinose to arabitol, while the L-arabinose and D-xylose pathways share the common intermediate xylitol, but whereas L-arabinose is converted to xylitol according to the following sequence : L-arabinose to arabitol to L-xylose to xylitol [5]. At higher temperature 42°C, fermentation of glu-

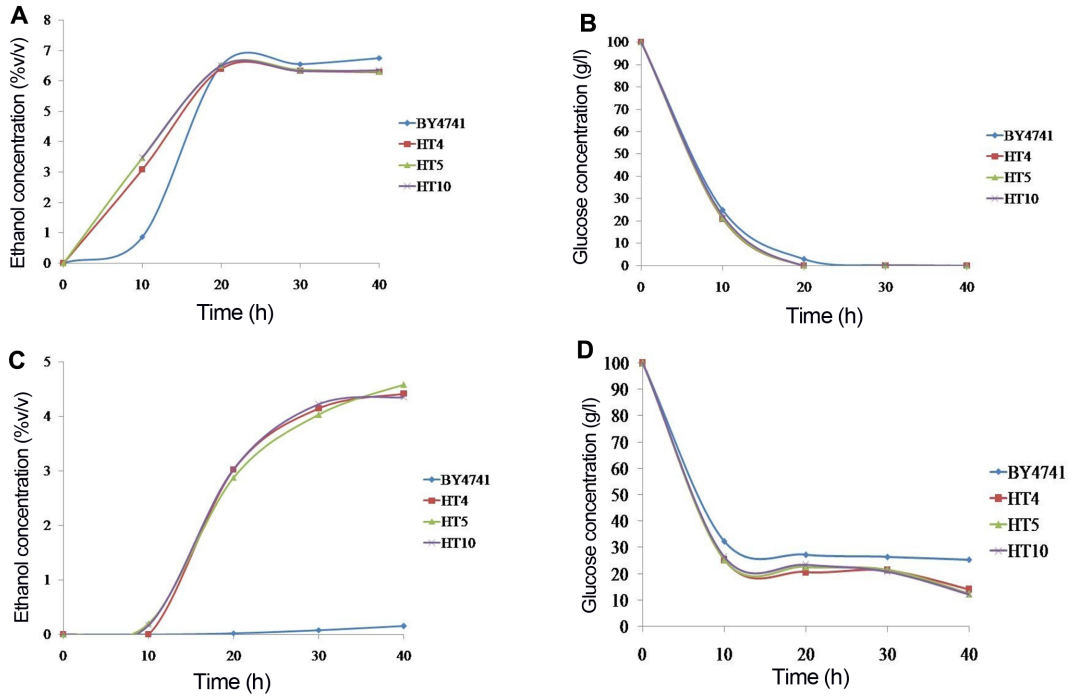

Fig. 4. Fermentation test. Fermentation performance of *C. tropicalis* isolates (HT4, HT5, and HT10) and *S. cerevisiae* strain BY4741. Inoculums of yeast culture grown in YPDA medium for overnight at 30°C was transferred to YPD10A containing 100 g/l glucose at initial OD_{660} = 0.1, and fermented at two different temperatures, 30°C and 42°C. Time course profiles of ethanol production and glucose concentration during fermentation were performed at 30°C (A and B) and 42°C (C and D), respectively. At indicated points, ethanol concentrations were determined using an Alcohol mechine AL-3 (Riken Keiki, Japan) and Glucose concentrations were measured using the Glucose C-II-test (Wako, Japan).

cose by *C. tropicalis* can result higher ethanol than by *S. cerevisiae* as presented in Fig. 4C, and ethanol production under high temperature at 42°C was lower than under common fermentation condition at 30°C.

As shown in Fig. 4C and Fig. 4D, by using *C. tropicalis* isolates HT4, HT5, HT10, after 20 hour of fermentation at 42°C from 100 g/l glucose fermentation, 20.72, 22.67, 23.42 g/l glucose remained unutilized although 3.02, 2.88, and 3.03% (v/v) of ethanol were respectively produced, while *S. cerevisiae* BY4741 remained 27.39 g/l unutilized glucose and produced 0.02% (v/v) ethanol. After 30 h of fermentation, 21.36, 21.63, and 20.91 g/l glucose remained unutilized; 4.15, 4.03, and 4.22% (v/v) ethanol were produced by *C. tropicalis* isolates HT4, HT5, and HT10 respectively. After 40 h of fermentation, ethanol production did not increase significantly, *C. tropicalis* isolates HT4, HT5, and HT10 produced 4.42, 4.58, and 4.35% (v/v) ethanol, with 14.16, 12.37, and 12.27 g/l glucose remained unutilized respectively. Ethanol production by *S. cerevisiae* after 30 and 40 h of fermentation were 0.08 and 0.15% (v/v), although glucose remained unutilized were 26.55 and 25.42 g/l, respectively. These results indicated that the ability of *C. tropicalis* isolates is more rapid in utilizing glucose and higher production of ethanol at high temperature 42°C as compared with *S. cerevisiae* a common yeast for bioethanol fermentation. Ethanol content produced by these *C. tropicalis* isolates were almost similar compared with previous report stating that *S. cerevisiae* (hybrid TJ14 strain) displayed ability to produce 4.65% (w/v) ethanol from 100 g/l glucose fermentation at 41°C [1].

In summary, this is the first report describing utilization of *Tuak*, a North Sumatera-Indonesia traditional beverage, focused on isolation and identification of yeasts for bioethanol production. Phenotypic observations showed that HT4, HT5, HT10 isolates grew well on glucose, xylose, and arabinose as sole carbon source, and these isolates have molecularly identified as *C. tropicalis*. Fermentation test of these *C. tropicalis* isolates displayed ability to produce 6.55% (v/v) and 4.58% ethanol from 100 g/l glucose fermentation at 30°C and 42°C, respectively.

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