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Fuel ethanol production using xylose assimilating and high ethanol producing thermosensitive *Saccharomyces cerevisiae* isolated from date palm juice in Bangladesh

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ABSTRACT

Four yeasts were isolated from Khejurer Rosh [an overnight natural fermented date palm (*Phoenix dactylifera*) juice/sap] at low temperature (~5–15 °C) to produce bioethanol. Cultural, morphological, physiological, biochemical and genetic analysis were carried out under various physiological conditions. All 4-strains (Dj-1, Dj-2, Dj-3, and Dj-4) could produce bioethanol and their production rates were further investigated under various carbon sources, growth temperatures, and pHs. Among them, the highest 10% (v/v) bioethanol was estimated from the thermosensitive yeast strain Dj-3, which was grown in the medium containing 18% of total sugars and 0.05% $(NH_4)_2SO_4$ at optimum temperature and pH of 25 °C and 6.0, respectively. Microscopic study and a partial 26S rDNA (D1/D2 region) sequencing identified Dj-1, and Dj-3 as *Saccharomyces cerevisiae*, whereas, Dj-2 and Dj-4 strains were, *Pichia kudriavzevii*, and *Debaryomyces hansenii*, respectively. The strains Dj-3 and Dj-4 could grow well in the medium containing xylose as the sole carbon source. Our results conclude that the strain Dj-3 is a natural mutant strain of *Saccharomyces cerevisiae*, which would be an industrially potential candidate for bioethanol production.

1. Introduction

The uncertainty of fossil fuel supply and efforts to diminish carbon dioxide emissions are playing the key role in the upsurge of renewable energy like biofuel in recent years (Balat and Balat, 2009). Bioethanol has become one of the most promising environment-friendly biofuels because of its less carbon dioxide emission (Li et al., 2015). Countries like United States, Brazil or Sweden are already using bioethanol and the global production of bioethanol is estimated to reach almost 134.5 billion liters (Bln L) by 2024 (Coyle, 2007; Martines-Filho et al., 2006).

Researchers are evaluating the biomass potential for lignocellulosic bioethanol production from various agricultural residues such as wheat straw, rye straw, oat straw, corn stover, pine wood chips, sugar bagasse etc. (Das and Singh, 2004; Saravanakumar et al., 2013). During the winter season of Bangladesh (December–January), where temperature goes down from summer ~35–40 °C to ~ 3–17 °C, many natural traditional fermented products are available including Khejurer Rosh (known as an overnight natural fermented date palm juice/sap). The date palm juice contains a huge amount of carbohydrate, reducing sugar, crude lipid, crude protein and other nutrition (Salah et al., 2011). So, this date palm juice can be a suitable source to isolate some thermosensitive microorganisms to produce fermented products like bioethanol (Salah et al., 2011). During fermentation, industries invest a lot of money for cooling and aeration (Curran et al., 1989). Use of thermosensitive strains for fermentation, can significantly, reduce this cost.

In this work, we tried to find some thermosensitive high ethanol producing strains from natural resources. Along with that, we also checked these strains capability to utilize diverse sugars. Lignocellulosic pentose sugar, Xylose, is a potential substrate for bioethanol production (Nogué and Karhumaa, 2015; Tantirungkij et al., 1993). Generally, *Candida shehatae*, and *Pachysolen tannophilus* found to utilized xylose (Van Zyl et al., 1989), but according to some reports, *Saccharomyces cerevisiae* cannot utilize xylose, but can increase their biomass on it (Saravanakumar et al., 2013). As the yeast *Saccharomyces cerevisiae* has

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high ethanol tolerance and growth rates, they are the better choice for industrial applications than the other microorganisms (Alfenore et al., 2002; Saravanakumar et al., 2013). Thus, engineered *Saccharomyces cerevisiae* are developed with the introduction of three enzymes of *Pichia stipites*, i.e. xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulokinase (XK) for commercial bioethanol production (Le Borgne, 2012; Peng et al., 2012; Tantirungkij et al., 1993). In some cases, xylose isomerase (XI) can replace both xylose reductase (XR) and Xylitol dehydrogenase (XDH) (Peng et al., 2012). During bioethanol production, some strains don't survive and inhibited by high ethanol (Uden, 1985). So, high ethanol-tolerant strains isolation is another challenge for efficient bioethanol production in the industry.

In this work, we have isolated four ethanol-producing strain from Khejurer Rosh. Interestingly, one isolate, Dj-3, was found to be high ethanol tolerant and as well producer, which may be a natural mutant of xylose utilizer.

2. Materials and Methods

2.1. Sample collection and inoculum preparation

Yeasts used in this study were isolated from Khejurer Rosh (an overnight natural fermented date palm juice/sap), which was collected from various locations of Sirajganj district, Bangladesh during January 2016. Generally, Khejurer Rosh contain: Glucose (~35%), fructose (~25%), sucrose (6–10%), fat, ash, mineral, vitamin etc. (Tang et al., 2013). YPD solid and liquid media (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) were used for inoculum preparation. Initially, Khejurer Rosh was inoculated in YPD liquid media and cultures were incubated at 25 °C for 48 h. The enriched cultures were then streaked on YPD solid agar plates (15 g/l agar) and kept in the same temperature for 24 h. The isolates were stored in glycerol broth solution (40%) at −80 °C in cryovial tubes.

2.2. Morphological characterization

Yeast cells were grown overnight at 25 °C in YPD liquid media, followed by 200-fold dilution in fresh YPD liquid media and then harvested by mild centrifugation (5000 rpm for 5 min) for microscopic study. In brief, cells were washed, fixed and stained with 5 µg/ml of DAPI (4′, 6-diamidino-2-phenylindole dihydrochloride) which is a DNA/Nucleoid specific dye that binds tightly to the nucleic acid. This dye enables the visualization of nucleoid/chromosome through a fluorescent microscope. The fixed cells (5 µl) were dropped into the well of a 10-well multi-test microscope slide (76 × 26 mm with 24 × 60 mm coverslip; Matsunami glass Ind., Ldt., Japan) and air dried at 27 °C. An immunofluorescent microscope was used (in Yamaguchi University, Japan) for characterization thermosensitive yeasts morphologically, especially shape, size and visualization of nucleoid area of the cell as described previously (Azam et al. 2000).

2.3. Genetical characterization by DNA sequencing

Yeast cells were cultured in YPD liquid media for DNA extraction. 1 ml of cell suspension was collected on to the 1.50 ml microcentrifuge tube. After centrifugation, the excess medium was removed from the microcentrifuge tube and the cells were stored in a freezer (−20 °C) until further use. Genomic DNA was separated and purified by using DNA extraction kits (Takara, Japan).

The yeast D1/D2 domain of the 26S rDNA was amplified by PCR with forward primer NL-1 and NL-4 (Forward Primer NL-1: 5′-GCATA TCAATAAGCGGAGGAAAAG-3'. Reverse Primer NL-4: 5′-GGTCCGTGT TTCAAGACGG-3' (Techaparin et al., 2017). The PCR product was collected, purified and checked by Agarose gel electrophoresis. The sequences were determined with PRISON BIO genetic Analyzer (Applied Biosystems) according to the instructions of the manufacturer. The sequence was compared pair-wise using the BLAST homology search. DNA sequencing experiments were done at Yamaguchi University, Japan.

2.4. Physiological characterization

Physiological characterizations of isolated yeasts were carried out in growth medium containing various carbon sources, temperatures and pHs. To characterize physiologically with three different carbon sources, YPD/X/A medium (10 g/l yeast extract and 20 g/l peptone) were prepared and supplemented with 20 g/l of Dextrose/Xylose/Arabinose, which were designated as YPD, YPX, and YPA, respectively. At first, one loop full yeast colony was inoculated from a fresh YPD plate into a test tube containing 3 ml of YPD broth and incubated at 30 °C for 24 h in a shaking water bath (incubator). Then yeast cells were streaked separately on the YPD, YPX and YPA agar plates and incubated at 25 °C for 48 h.

For screening the optimum pH of 4 yeast strains, yeasts were grown in YPD broth media at different pHs (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) for 0–72 h. YPD broth was prepared in seven different conical flasks and pH was adjusted to pH 4.0, pH 4.5, pH 5.0, pH 5.5, pH 6.0, pH 6.5 and pH 7.0 using concentrated sulfuric acid (H_2SO_4) and 2N Sodium hydroxide (NaOH). Three ml of YPD broth was distributed into 10 ml screw cap test tubes and were inoculated with each yeast strain from actively growing 24 h culture. All cultures in test tubes were incubated at 25 °C until 72hrs (Limtong et al., 2007). The initial optical density of each of the seven test tubes were read off on a spectrophotometer (Specord UV/Visible Spectrophotometer, Analytic Jena, Germany) at 600 nm against the YPD broth medium as a blank. The increase in optical density in a test tube was recorded as evidence of growth. At various time periods, the OD was recorded to observe the effects of acidic to basic pH change on the growth of the selected yeast strains.

For screening the optimum temperature of 4 yeast strains, yeast cells were cultured in YPD and YPX solid plate and broth, respectively. In brief, one loop full yeast colony was inoculated from a fresh YPD or YPX plate into test tubes containing 3 ml of YPD or YPX broth and incubated at 30 °C for 24 h in a shaking water bath (incubator). Cells about 50 µl (100-fold dilution) from a young actively growing culture were inoculated into test tubes containing 5 ml of YPD or YPX broth and then incubated at various temperatures (25–42 °C) for 0 h to at least 72 h. During incubation, cell suspension was collected in every 4 h interval, seized the cell growth by putting sample on ice box and the optical density of each test tubes containing sample was read off on spectrophotometer (Specord UV/Visible Spectrophotometer, Analytic Jena, Germany) at 600 nm against either the YPD or the YPX broth medium as blank.

2.5. Biochemical characterization

2.5.1. Bioethanol fermentation

Bioethanol fermentation of each strain was conducted independently under various medium temperatures and pHs. Various yeast inoculums were prepared by transferring one loopful of 24 h old culture grown on a plate of YPD agar medium to an Erlenmeyer flask containing 50 ml of a sugar cane juice medium (Basal medium) containing 18% of total sugar (Talukder et al., 2015). The inoculums were transferred at the rate of 0.5% to the screening medium, followed by incubation on a rotary shaker (160 rpm) at various temperatures ranging from 20 to 42 °C for 0 h to at least 72 h in 250 ml Erlenmeyer flasks containing 100 ml of a basal medium, supplemented with 18% glucose and 0.05% $(NH_4)_2SO_4$.

2.5.2. Bioethanol estimation

The total bioethanol content was measured using the method reported elsewhere (Miah et al., 2017). Briefly, the amount of ethanol in the fermentation medium was assessed by Potassium Dichromate

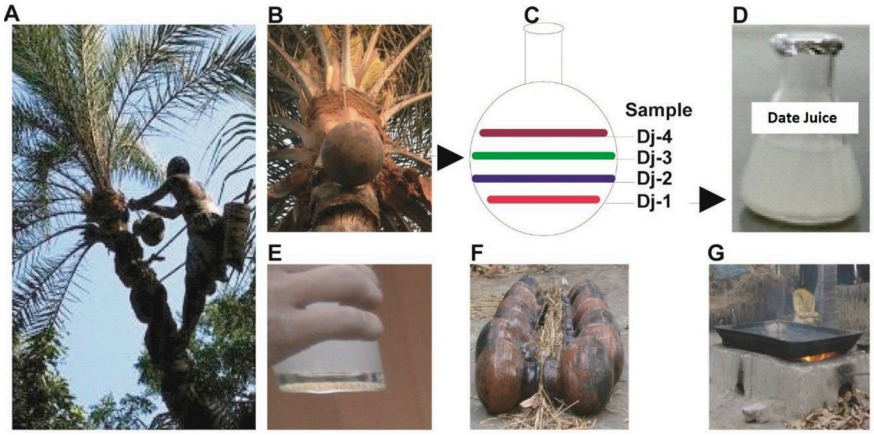

Fig. 1. Schematic procedure for collection of various yeasts from the Khejurer Rosh (Overnight natural fermented Date Palm juice). Here, **A** represents the date palm tree with Gachhis, a person who extracts date juice from trees. The gachhis extracts juice from date palm trees by first scraping the soft xylem tissue of the tree just below the cluster of leaves with a pillar and then inserting a short bamboo pipe into xylem tissue of the tree, which allows the flow of juice in drops into a vessel attached to the tree below the scrape as shown in **B**. **C** is a diagrammatic representation of sampling vessel and the locations of each of 4-different samples on the vessel designated as Dj-1, Dj-2, Dj-3 and Dj-4 (Date Palm juice 1–4). **D** represents a small amount of sample juice is transferred from sample jar to sterile conical flask. Date Palm juice can be drunk directly (**E**), or can be kept at the natural environment for traditional fermentation as shown in **F**. Fermented juice of the date palms is largely turned into molasses by being boiled in a vat as shown in **G**.

Oxidation method. For ethanol concentration measurement freshly prepared potassium dichromate (33.882 g/l), ferrous ammonium sulfate (135.5 g/l) and diphenylamine (0.5 g/100 ml concentrated H_2SO_4) solutions were used. The fermented sample was first diluted (ten-fold) with distilled water. Then, 10 mL of this diluted sample was distilled against $K_2Cr_2O_7$ (10 ml) containing concentrated H_2SO_4 (5–6 ml). Later, the distilled product was titrated against freshly prepared ferrous ammonium sulfate solution with diphenylamine as an indicator. The green colour appearance indicated the end-point of the titration. The amount of ferrous ammonium sulfate needed was recorded to calculate the amount (in percentage) of bioethanol present in the sample.

3. Results

3.1. Sample collection and isolation of thermosensitive yeasts from Khejurer Rosh

Detailed sample collection procedures, sources, site, time and temperatures are shown in Fig. 1 and Table 1. From each sampling tree, we have collected total 4-samples; first sample collection started from 7 p.m. (evening) and in every 4-h interval, remaining 3-samples were collected until 7 a.m. (next morning). Fig. 1A shows the date palm tree used for sampling. The sample number and the position of each 4 samples in a sample jar/pot are shown in Fig. 1C; typical sample number 1, which was collected at 7 p.m. at temperature 11 °C (Table 1). In each of 4-sampling time, sample jar was removed from the date palm tree and 5–10 ml sample was collected in a sterile conical flask with a sterilized pipette (Fig. 1D). The sample was kept at an ice bucket or 0 °C until further use. This sampling technique was followed for the collection of the rest of the samples from different date palm trees.

3.2. Morphological characterization

Fluorescence microscope was used for characterization yeasts morphologically (especially shape, size and visualization of nucleoid area of cell). With the aid of Fluorescent microscope in Yamaguchi University, Japan, Fluorescent microscopic images of various yeasts isolated from the Khejurer Rosh were clearly visualized (Fig. 2). We have identified three different types of yeast cells from Khejurer Rosh. The strains Dj-1 and Dj-3 are found to be morphologically similar in cell shape, size and nucleoid after comparing them with two remaining strains Dj-2 and Dj-4. Tari-6 was a thermotolerant yeast strain *Pichia galeiformis* identified recently from palm juice/sap (*Phonex Sylvestries*) used as an internal control in this study (Talukder et al., 2015). These results indicate that the various yeast samples isolated from date palm juice have different strain background, especially, in genus level and these yeast strains supposed to involve in the succession process during date juice preparation. Because the taste of date juice is changed gradually from sweeter to sour and these changes may be led by environmental factors like temperatures and pHs. Therefore, it is important to check the effect of medium temperatures and pHs, in addition to various carbon sources, on the growth of yeast strains as shown below.

3.3. Genetical characterization

The PCR product of yeast which was collected after DNA extraction, purified and checked by Agarose gel electrophoresis. Finally, we have sequenced 4-yeast strains (Dj-1, Dj-2, Dj-3, and Dj-4) isolated from the Khejurer Rosh, in order to identify their genus as well as taxonomic position. Partial nucleotide sequence information revealed that the yeast strains Dj-1, Dj-2, Dj-3 and Dj-4 isolated from overnight natural fermented date palm juice encoded *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Saccharomyces cerevisiae,* and *Debaryomyces hansenii*, respectively (Fig. 3). Our results are consistent with the report published by Chandrasekaran and Bahkali (2013) revealed that not only yeast but also other diverse groups of microorganisms present in the date palm juice, which are a potential source for bioethanol and other compound production.

3.4. Physiological characterization

Physiological characterizations of isolated yeasts were carried out in medium containing various carbon sources (YPD, YPX, and YPA);

Table 1
Various yeasts collected from the naturally fermented date palm juice.

Sample ID	Sample Source	Sample collection site/time/temperature	Colony Morphology	Comment
Dj-1	Date palm juice, bottom layer-1	Sirajganj, Bangladesh/7:00 p.m.,4th January 2016/11 °C	Glistering white, oval and smooth.	Yeast
Dj-2	Date palm juice, bottom layer-2	Sirajganj, Bangladesh/11:00 pm,4thJanuary 2016/9 °C	White, oval/big round, smooth.	Yeast
Dj-3	Date palm juice, middle layer	Sirajganj, Bangladesh/3:00 a.m., 4thJanuary 2016/5 °C	Glistering white, oval and smooth.	Yeast
Dj-4	Date palm juice, upper layer	Sirajganj, Bangladesh/7:00 a.m., 5th January 2016/8 °C.	Glistering white, round, small/smooth.	Yeast

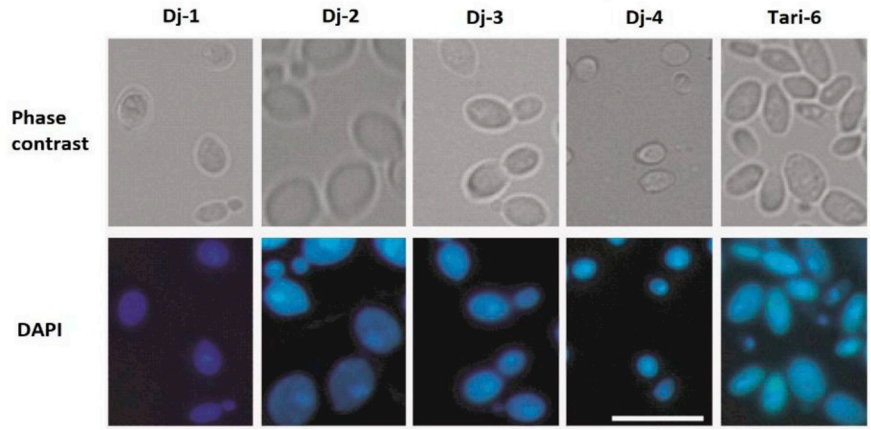

Fig. 2. Fluorescent microscopic images of various yeast cells isolated from Khejurer Rosh. Various yeast cells were grown overnight at 25 °C in YPD liquid medium, fixed and stained with DAPI. The strain Tari-6 (thermotolerant yeast) used as an internal control which could grow well even at 42 °C. Top and bottom panels of 5-photographs represent the images of phase-contrast (the first row) and DAPI-staining (the second row), respectively. Phase contrast image represents the shape and size of the yeast cells, whereas, DAPI represents the nucleoid shape, size and the position within the cell. The scale bar represents 7 μm.

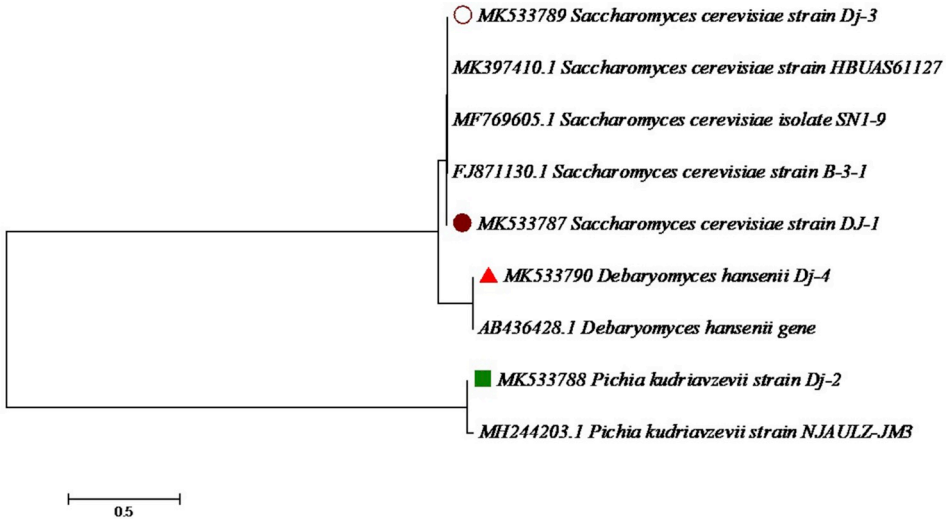

Fig. 3. Phylogenetic study of thermosensitive yeast isolated from Khejurer Rosh. The cladogram tree was constructed by using MEGA7. Neighbor- Joining method with 1000 bootstrap value was used to construct the tree. Isolates Dj-1 and Dj-3 were identified as *Saccharomyces cerevisiae*, whereas Dj-2 and Dj-4 as *Pichia kudriavzevii* and *Debaryomyces hansenii,* respectively.

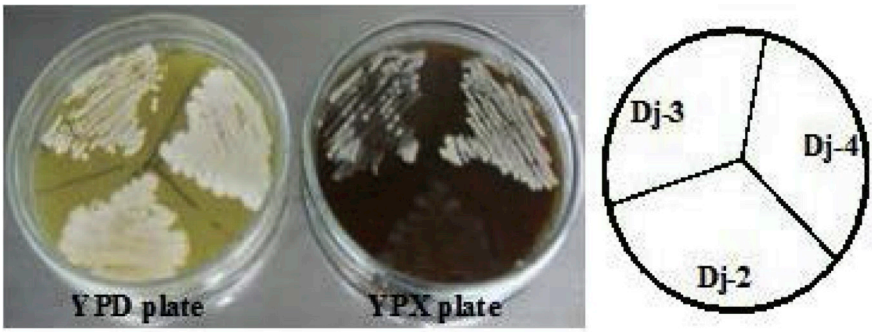

Fig. 4. Substrate specific growth of various yeasts isolated from Khejurer Rosh. Various yeast cells were grown either in YPD (left) or in YPX (right) Agar plate. Here, in left and right plates represent 2-days old YPD and YPX cultures, respectively, which were incubated at 25 °C. The strain Dj-3 (*Saccharomyces cerevisiae*) and the strain Dj-4 (*Debaryomyces hansenii*) isolated from the overnight natural fermented date palm juice could grow well in both YPD and YPX media. Strain Dj-2 (*Pichia kudriavzevii*) is a Xylose sensitive strain that is why no growth was observed in YPX Agar plate. Media preparation and yeast samples inoculation procedures were described in detail in the Materials and Methods.

temperatures (20, 25, 30, 37 and 42 °C) and pHs (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) are shown in Figs. 4, 5 and Table 2, respectively. It was found that all strains could grow well in Dextrose or Arabinose as carbon sources (data not shown). However, only two strains in Dj-3 and Dj-4 could grow in medium containing Xylose (Fig. 4). Various temperatures dependent cell growth was also recorded. The growth was gradually decreased with an increase in temperatures, except control thermotolerant strain, Tari-6 (Fig. 5). This observation indicates that the strains isolated from date palm juice are thermosensitive as we expected, because, our natural fermented samples were collected in winter (Table 1). The maximum and the minimum cell growth were estimated at 20 °C and 42 °C, respectively, in the case of Dj-3 strain (Fig. 5, left growth curve). All most reverse temperatures dependent cell growth was observed in the case of Tari-6 strain (Fig. 5, right growth curve), which bears thermotolerant yeast (Talukder et al., 2015). On the other hand, all strains could grow well in the medium pHs ranges from pH 5.5–6.5 (Table 2). Among the various strains analyzed here, in presence of various treatments and conditions, the optimum medium carbon source, temperature and pH were found to be Dextrose, 25 °C and 5.5/6.0, respectively, in Dj-3 strain (Figs. 4, 5, and Table 2).

3.5. Biochemical characterization

3.5.1. Ethanol tolerance

As in this work the strains were used for ethanol production, here we checked whether the strains could tolerate a high concentration of ethanol or not. To do so we cultured the strains in presence of 0%, 7%, 8% and 10% ethanol at 30 °C and 42 °C. As a control here we used

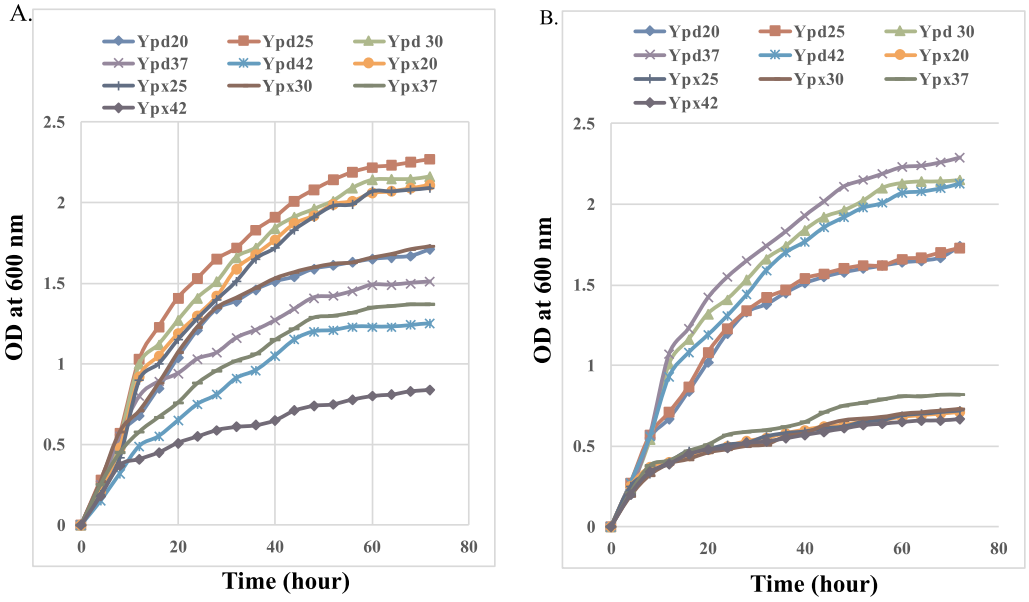

Fig. 5. Growth curve of two yeast samples collected from the naturally fermented product of Bangladesh. Sample collection, screening, growth conditions were described in detail in Materials and Methods. Yeast samples were grown to both the YPD and YPX broth at various temperatures from 0 h to 72 h. Left and right panel represents the growth curves of strain Dj-3 and Tari-6 (thermotolerant yeast), respectively. Samples were collected in various indicated time points and seized the growth and then their growth were measured by a spectrophotometer (Specord UV/Visible Spectrophotometer, Analytic Jena, Germany) at 600 nm against the YPD or YPX broth as blank.

Table 2
Effect of various medium pHs on growth of Yeast cells in YPD plate at 25 °C.

Strain No.	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0
Dj-1	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + +
Dj-2	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + +
Dj-3	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + +
Dj-4	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + +
Tari-6	+ +	+ + + +	+ + + +	+ + +	+ + +	+ +	+-

Here, YPD = Yeast-Peptone-Dextrose medium; YPX = Yeast-Peptone-Xylose medium; + + + + = EG (Excellent Growth); + + + = GG (Good Growth); + + = SG (Slow Growth); +- = VSG (Very Slow Growth) and – = NG (No Growth). Tari-6 (*Pichia galeiformis*) is a thermotolerant yeast was used as an internal control.

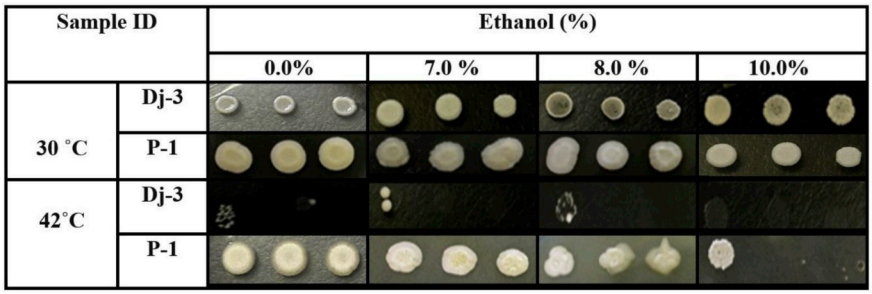

Fig. 6. Ethanol stress tolerance activity of bioethanol producing strain Dj-3. Here, top and bottom panel images represent ethanol tolerance activity of two strains Dj-3 and P-1 either at 30 °C or at 42 °C, respectively. Here, Dj-3 (*Saccharomyces cerevisiae*) is a high yield thermosensitive ethanol resistance strain and P-1 (*Pichia kudriavzevii*) is a thermotolerant strain which was used as an internal control. In each 3-spots from left to right represent sample spot applied for 5 µL of 10, 100 and 1000-folds diluted sample, respectively. Experiments were carried out at least 3-times independently and suitable ethanol tolerant spots are shown.

thermotolerant yeast P-1 (*Pichia kudriavzevii*). It was found that at 30 °C Dj-3 could tolerate even 10% ethanol in the medium same as the control, but as at 42 °C, the strain Dj-3 couldn't survive (Fig. 6). From here, it was confirmed that the isolate Dj-3 is thermosensitive and can tolerate high ethanol concentration, making it an ideal choice for ethanol production.

3.5.2. Bioethanol fermentation and estimation

We have checked the effect of temperatures (25, 30 and 37 °C) and pHs (4.5, 5.0, 5.5 and 6.0) on bioethanol fermentation as shown Fig. 7 below. Cells were grown in a basal medium. Bioethanol fermentation and estimation procedures were described in the Materials and Methods. The effect of various medium pHs for bioethanol fermentation was also investigated. All strains produced the highest concentration of bioethanol at pH 6.0 except in Dj-2 (optimum pH was 5.0 at 25 °C). Interestingly, it was found that at pH 6.0 Dj-3 produced about 10% ethanol, highest among all the conditioned checked. However, increasing the fermentation temperature of these four strains from 25 °C to 37 °C resulted in gradually decreased bioethanol concentrations, indicated that the yeast strains isolated from date palm juice are thermosensitive, those love to grow and produce bioethanol at relatively low temperature as we have confirmed in this study. On the other hand, bioethanol content was found to be lowest and highest at 25 °C and 37 °C, respectively, in the case of internal thermotolerant strain Tari-6 (Fig. 7).

4. Discussion

Like all microorganisms, yeasts exhibit diverse characteristics when growing on various ranges of temperatures, sometimes referred to as thermotolerant or thermosensitive yeasts (Pizarro et al., 2008; Talukder et al., 2016). Many groups around the world are trying to identify thermotolerant as well as thermophilic yeasts for industrial benefits (Arora et al., 2015). However, at present, a clear picture of how thermosensitive yeasts can play important roles in biotechnology industries and other human benefits are not understood properly, because the

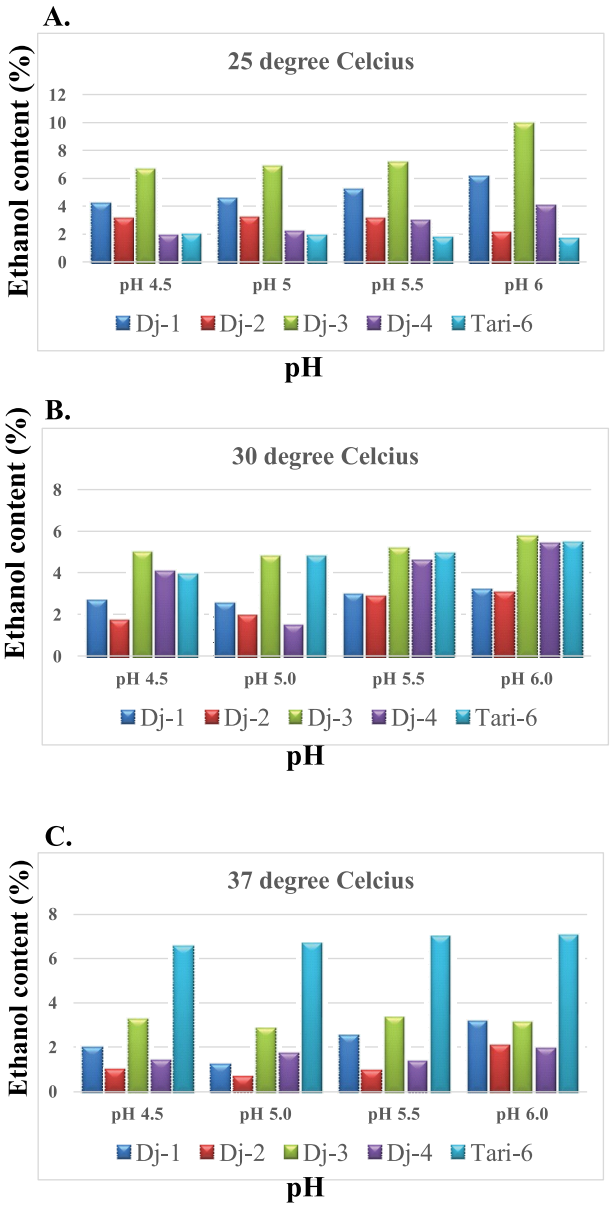

Fig. 7. Effect of medium temperatures and pHs on bioethanol production. Growth medium, inoculum preparation, and bioethanol fermentation procedures were described in Materials and Methods. Strains Dj1-4 were collected from Khejurer Rosh and the strain Tari-6 is a thermotolerant strain used as an internal control. Yeast strains were grown to 4-different medium pHs (4.5, 5.0, 5.5 and 6.0) in 3-different growth temperatures (25, 30 and 37 °C) as indicated. Yeast samples were collected after 72 h from the fermented medium and their bioethanol concentrations were measured.

identification, characterization, and application of their products were not well documented. Although, energy saving low-temperature bioethanol conversion can have a great impact (Shigechi et al., 2004). Moreover, xylose is a potential substrate for bioethanol production. Due to various industrial advantages of *Saccharomyces cerevisiae*, they are engineered to convert xylose into ethanol (Le Borgne, 2012; Peng et al., 2012 Tantirungkij et al., 1993). In doing so, enzyme systems of *Pichia stipitis* are introduced into *Saccharomyces cerevisiae* for better yield. As natural *Saccharomyces cerevisiae* do have the enzyme system, but due to poor yield they are subjected to genetic engineering. Interestingly, although our Dj-3 strain is a *Saccharomyces cerevisiae* but utilized xylose to a similar degree as dextrose. In our next work, further biochemical characterization will be done to find the xylose to ethanol conversion capability of Dj-3. Furthermore, genes responsible for xylose utilization will be explored.

Here, we have identified and characterized 4 yeast strains under various conditions. Among them, Dj-1 and Dj-3 both encoded *Saccharomyces cerevisiae* and the remaining two strains, Dj-2 and Dj-4 encoded well-known bioethanol producing yeast *Pichia kudriavzevii* and *Debaryomyces hansenii* (Chamnipa et al., 2018; Nobre et al., 1999; Techaparin et al., 2017). Interestingly, strain Dj-3 could grow well in presence of xylose. This result may indicate that Dj-3 could be a natural mutant of Saccharomyces *cerevisiae*. Moreover, Dj-3 was found to produce about 10% (v/v) ethanol and could tolerate this high concentration at low temperatures (Figs. 6 and 7). This strain could be an important agent for the brewing industry, because, the conditions and broad substrate spectrum, we have used here are suitable for bioethanol production. Further study might be required in the future to enhance the production of bioethanol in the laboratory as well as in industrial scale using this strain.

Conflicts of interest

The authors declare that they have no conflict of interest.

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