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BIOPROSPECTING OF THERMOTOLERANT AND ETHANOL TOLERANT FERMENTATIVE POTENTIAL YEASTS FROM INDIGENOUS FERMENTED FOODS.

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Author Contribution:

SJP, GP designed the study. GP carried out the laboratory work and wrote the manuscript. KBR and GGT analyzed the data. All authors read and approved the final version of the manuscript.

ABSTRACT

The yeast with stress tolerance (robust) traits plays a vital role in bioethanol production. It offers advantages such as lower cooling costs and a reduced risk of contamination. Using a yeast strain that can tolerate ethanol and high temperatures is crucial for the benefit of biofuel industries. Our study isolated yeast strains from locally available fermented foods. These strains were initially identified and characterized based on cell morphology and microscopic observations, including colony features, mode of cell division, and cell shape. Screening for ethanol and thermal tolerance was performed using YPD (yeast extract, peptone, dextrose) broth, with optical density measured at 600 nm. Durham's tube method was used to evaluate the fermentative ability of the isolates. Two strains, DBS-I (3) and DBS-II (2), were selected based on their maximum tolerance to ethanol at 30% (v/v) and temperatures up to 50°C, along with superior fermentation activity. Biochemical tests were carried out for these selected isolates, DBS-I (3) and DBS-II (2), which were identified as Pichia Kudriavzevii and Candida tropicalis, respectively, using molecular identification methods.

Key words: *Candida Tropicalis*, Ethanol-tolerant, Indigenous yeast, *Pichia kudriavzevii*, Thermotolerant.

INTRODUCTION

Yeasts are unicellular fungi that can ferment carbohydrates to produce carbon dioxide, ethanol or acids. Their fermentative properties make them useful in the food industry, bioethanol production, and brewing (Walker and Stewart, 2016). The global imbalance between the supply and demand for crude oil has grown significantly as a result of industrialization and population growth. Researchers must investigate indigenous, inexhaustible, and non-polluting renewable energy sources to address the world's energy needs because they can be derived from sustainable resources, whereas traditional fossil fuels are limited (Choudhary *et al*., 2017). Global warming and sharp increases in oil prices have escalated to dangerous levels. Thus, there has been a strong push to increase the usage of

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biofuels (Evcan & Tari, 2015).

The increasing demand for sustainable energy sources has intensified interest in bioethanol production, with yeasts playing a central role due to their fermentative abilities (Chansom *et al*., 2016). Wild yeasts that have evolved under stressful fermentation conditions are among the various microbial communities found in traditional fermented foods like Dosa, Idli, and Jalebi batter, etc. (Tamang, 2022).

Effective screening involves examining isolates for growth at high temperatures and ethanol concentrations. Yeast strains with greater stress tolerance may be found by screening yeasts from local fermented foods, offering sustainable alternatives for industrial biotechnology. Despite advances, few studies focus on isolating robust yeasts from regional fermented foods. The purpose of this study is to fill that gap by identifying and describing possible strains originating locally. The selected strains will be characterized based on fermentation performance and endurance to stress.

MATERIALS AND METHODS

Sample collection

Samples were collected in sterile screw cap tubes or bottles placed in ice boxes from random hotels and other places in Davangere City and brought to the laboratory, then kept in the refrigerator for further studies.

Isolation of yeast from samples

Homogenized the collected samples (one gram of each sample dissolved in 10 ml of sterile 0.85% physiological saline). Homogenized samples are then serially diluted up to 9-fold (10-9) with sterile 0.85percent saline. Then, inoculate the 100 µl serially diluted samples onto the YEPD (Yeast extract Peptone Dextrose) agar medium with 0.05 percent chloramphenicol as an antibacterial through the spread plate method (Kumari *et al.* 2019). Inoculated plates are incubated at 27°C - 30°C for 48 hrs. Following incubation, the colonies and their morphology are examined under a microscope. Color, texture, border, and elevation were all used to investigate morphological traits (Mamun-Or-Rashid *et al.,* 2022). Simple staining was used to observe the shape and organization of yeast cells during budding. Then, we obtained the pure cultures by subculturing the morphologically distinct yeast colonies and keeping the pure cultures in refrigerators for future work.

Morphological and Microscopic observation of isolated strains

The tested isolates were inoculated onto YEPD (Yeast extract Peptone Dextrose) Agar and Broth and incubated for 48 hrs at 30°C. After incubation, colony morphology was observed and noted on agar plates, then cell morphology was observed and recorded under the microscope with 100X magnification using a broth culture (Dalawai 2017).

Ethanol tolerance screening

The ethanol tolerance of isolates was tested by inoculating 1 ml of a 48-hour broth culture of each strain into tubes containing YEPD (Yeast Extract Peptone Dextrose) broth containing 1 ml of 10 percent to 30 percent ethanol stock solution (v/v) in triplicate. After inoculation, flasks were incubated at 30°C for 48 hours, and the optical density was recorded at 600 nm. Mean values were considered after each experiment was carried out in triplicate (Martins *et al*., 2015). Further spot tests

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will be performed to confirm ethanol tolerance (Nurcholis, Setiawan, *et al*., 2021).

Screening for thermo-tolerance

Thermotolerance was determined using the YEPD (Yeast extract Peptone Dextrose) liquid media. Each tube's initial optical density was measured at 600 nm using a spectrophotometer in comparison to the blank medium. To test the yeast strain's thermotolerance, all cultures were incubated at 40°C, 45°C, 50°C, and 55°C for 48 hours. The rise in optical density was noted as an indication of growth (Fakruddin, 2013).

Fermentation test

The ability of the isolates to ferment was examined. Using methyl red indicator and an inverted Durham tube, 100 µl of the isolates' 48-hour culture was inoculated into a test tube that contained yeast extract, peptone water, and 1percent or 2 percent sugar (glucose). The incubation period is 24 to 48 hours. The media's color changing from pink to yellow and the release of bubbles in Durham's tube are indications that yeast has the capacity to ferment. It is considered that the presence of gas or bubbles indicates a comparatively high rate of fermentative activity (Tesfaw *et al*., 2021).

Biochemical Assays

Ascospore formation

After streaking the test isolates on 5 percent Malt Extract Agar (MEA), they were incubated for two to five days at 25°C. Lactophenol cotton blue was used to stain, and the presence of ascospores was examined under a microscope (Srividya D. *et al*., 2023).

Urease test

Test cultures were inoculated into Stuart's Urea broth. To suspend the culture, gently shake the tube. The tubes' caps were loosened, and then they were incubated for 48 hours at 35°C. Check for a bright pink color throughout the broth after incubation, as this indicates the production of urease.
(Srividya D. *et al*., 2023).

Nitrogen assimilation test

Auxanography technique was used to test the nitrogen assimilation, base medium and agar were used and different nitrogen compounds such as Ammonium sulphate, potassium Nitrate, and Sodium Nitrate are placed as small discs on the agar plate then incubate plates for 24 to 48 hrs at 30°C, examined the plates for visible growth zones around the nitrogen source discs, indicating ability to utilize that specific nitrogen source (Wickerham,1946).

Carbohydrate assimilation test

Dhrum`s tube method was used to test the Carbohydrates assimilation, 5ml of yeast extract and peptone water containing 1 percent or 2 percent carbohydrates such as Glucose, Fructose, Sucrose, Galactose, Lactose and Maltose and 0.01 percent methyl red indicator in a 10 ml test tubes, placed the Dhrum`s tubes invertedly into test tubes then autoclaved the test tubes, after the autoclave inoculated the100µl of the 48 hrs fresh test cultures and incubated for 24-48-78 hrs at 30°C, observed for color change in medium from red or pink to yellow and air bubbles and gas trapped inside the Dhrum`s tube (Tesfaw *et al*., 2021).

Molecular characterization

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Isolated the genomic DNA through the standard protocol and quantified the DNA using the NanoDrop Spectrophotometer. The ITS gene with universal primers was employed for PCR, and then forward and reverse sequencing were conducted to obtain the ITS region sequence. Using the NCBI tools and MEGA software to construct a phylogenetic tree and identify the isolates based on their similarities (Tamura K. and Nei M., 1993; Tamura *et al*., 2021).

RESULTS

Sample collection

Dosa batter, Idli batter, and jalebi batter samples were collected from random places in Davangere city.

Isolation

14 yeast strains were isolated from the collected samples, 8 strains from Dosa batter, 2 strains from Idli batter, and 4 strains from Jalebi batter using YEPD (Yeast extract Peptone Dextrose) media. Then, pure cultures were obtained and maintained by the subculturing method. Isolated strains were named as DBS-I (1-3), DBS-II (1-3), DBS-III (1-2), IBS-I (1-2), and JBS-I (1-4).

Morphological and Microscopic observation

Colony morphology of the 14 isolated strains was observed on YEPD agar plates based on their color, texture, border, and elevation.

The cell morphology of the isolated strains was observed through a microscope using 40X and 100X magnification, and all the strains were identified as yeast based on their oval and round shapes, large size, and budding in cells.

Ethanol tolerance

The isolated 14 strains were screened for ethanol tolerance at different concentrations, such as 10, 15, 20, 25, and 30 percent. Only two strains, DBS-I (3) and DBS-II (2), exhibited a nearly 50 percent survival rate at a 30% concentration of ethanol, and a spot test was also conducted for confirmation, as shown in Figure 1.

Screening for thermo-tolerance

Thermo-tolerance screening test has been conducted for 14 isolated strains at different temperatures, such as 40°C, 45°C, 50°C, and 55°C. All the isolates showed good growth up to 40°C to 45°C, and a few strains showed considerable growth at 50°C, but no growth occurred at 55°C, as shown in Figure 2.

Fermentation test

The 8 strains were selected based on their Thermo and ethanol tolerance and were screened for their fermentative ability by using YEPD broth and Durham's tubes. All strains showed good fermentation ability, meaning they fermented the glucose within 48 hrs, but two strains, DBS-I (3) and DBS-II (2), showed prime fermentation ability, meaning they fermented the glucose within 24 hrs, as mentioned in Table 1.

After three screening tests, including ethanol tolerance, thermo tolerance, and fermentation test, two isolates, DBS-I (3) and DBS-II (2), were selected for further work.

Biochemical Assays

Ascospore formation

The two selected strains were tested for ascospore formation on 5 percent malt extract agar plates. After a 2 to 5-day incubation, both strains produced 2 to 4 ascospores in single cells were observed under the microscope. It was shown in Figure 3.

Urease test

Urease test was screened for two selected isolates named as DBS-I (3) and DBS-II (2), after 48 hrs of incubation both strains exhibited a negative result means there is no color change in media it remains yellowish, which indicates the isolates don't have urease, so this negative result is evident for the avirulence of the isolates, as shown in Figure 4.

Nitrogen assimilation test

Three nitrogen sources, including sodium nitrate, potassium nitrate, and ammonium sulfate, were used to screen for the two selected isolates in the nitrogen assimilation test. Following incubation, two strains had only a growth zone around the ammonium sulfate disc; no growth zone was noticed around the control or the other nitrogen discs. It means that the isolates that were examined can only use ammonium sulfate as a nitrogen source, not in the form of nitrate. It was mentioned in Table 2.

Carbohydrate assimilation test

Two isolates, DBS-I (3) and DBS-II (2), were screened for assimilation and fermentation of carbohydrates using 6 carbohydrates, such as Glucose, Fructose, Sucrose, Galactose, Lactose, and Maltose in YPED Broth media. Firstly, DBS-I (3) assimilated all carbohydrates within 48 hrs except lactose, and for fermentation, 4 carbohydrates, such as Glucose, Fructose, Sucrose, and Maltose, were fermented within 24 hrs of the incubation period, but galactose and lactose remain unfermented. DBS-II (2) assimilated all carbohydrates within 24 hours except lactose, and for fermentation, 4 carbohydrates, such as Glucose, Fructose, Sucrose, and Maltose, were fermented within 48 hrs, but galactose and lactose remain unfermented. It was in Table 3.

Molecular Identification

After genomic DNA was extracted, a NanoDrop Spectrophotometer was used to evaluate its quantity, and a 0.8 percent agarose gel was used to assess its quality. There was only one band of high-molecular-weight, undamaged, and transparent DNA visible. ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) universal primers were used for PCR of the ITS gene. When the PCR amplicon was resolved on an agarose gel, a single distinct band of about ~550 bp was observed, as shown in Figure 5 (a).

Following the elution and purification of the fungal ITS region PCR amplicon, forward and reverse primers were used to perform forward and reverse DNA sequencing on the PCR amplicon. The forward and reverse sequences were combined to create the ITS region's consensus sequence. After the procedure, based on the similarities and phylogenetic trees, the isolate DBS-I (3) was found to be *Pichia kudriavzevii* (GenBank accession: PV658848), and isolate DBS-II (2) was found to be *Candida tropicalis* (GenBank accession: PV621843), as shown in Figure 5 (b) and (c).

DISCUSSION

This study focused on local fermented foods like dosa, Idli, and jalebi as reservoirs of thermo and ethanol-tolerant yeast strains. Many previous studies have isolated the ethanol-tolerant and thermotolerant yeast strains from a variety of samples, such as soil, tree, etc., including fermented and non-fermented foods. In this study, 14 yeast strains were isolated from 3 different local fermented foods, such as dosa, Idli, and jalebi batters. In some previous studies Nurcholis, Setiawan, *et al*., (2021), isolated the 7 yeast strains from Indonesian local fermented foods like *Peuyeum*, Balinese *Brem*, Solid *Brem*, Cassava *Tape*, etc. Nurcholis, Kurniawan, *et al*., (2021) isolated the 11 yeast strains from local vegetables, like as eggplants, cabbage, potatoes, mustard greens, bitter melon, chayote, green beans, etc., The isolated 14 strains were screened for ethanol tolerance at different concentrations, such as 10% to 30%. At 30% of ethanol, two isolates, DBS-I (3) and DBS-II (2), showed a nearly 50% survival rate and it is the highest tolerance as compared to previous studies. Dalawai, (2017) achieved ethanol tolerance, up to 14 % to 15 % by the two yeast isolates, namely YGP and YSJ. A 20% ethanol tolerance was reported by Kechkar *et al*., (2019) from two isolates. For thermotolerance, all isolates were screened; all isolates exhibited good growth at 40°C to 45°C, but few isolates had growth up to 50°C, and no growth was observed at 55°C. This thermo tolerance was the highest compared to the other studies. Hawaz *et al*., (2022) conclude that thermotolerance was only up to 45°C. Nasir *et al*., (2017) reported that only 44°C is the maximum thermotolerance of their isolates. after these two screening tests, 8 isolates were selected for fermentation screening based on their maximum tolerance to both ethanol and temperature. Among the 8 isolates, only two isolates, DBS-I (3) and DBS-II (2), exhibited prime fermentation (fermentation occurred within 24 hours) activity. Two isolates, namely DBS-I (3) and DBS-II (2), were selected. The biochemical assays, such as the ascospore formation test, the urease test, the nitrogen assimilation test, and the carbohydrate assimilation test, were performed for those two isolates. Ascospores typically develop to survive severe heat, starvation, and other biological and physiological stress (Srividya D. *et al*., 2023). Both isolates proved a positive result by producing 2 to 4 ascospores in a single cell. Both isolates showed a positive result by producing 2 to 4 ascospores in a single cell. For the urease test, both isolates showed a negative result, and it was evident for the avirulence of the isolates. Both isolates can only utilize the ammonium sulphate as a nitrogen source, but not in the form of nitrates. The same result for the urease test and nitrogen assimilation test was reported by Srividya D. *et al*., (2023). Carbohydrate assimilation was tested for both isolates using six different carbohydrates, such as Glucose, Fructose, Sucrose, Galactose, Lactose, and Maltose. Both isolates assimilated all carbohydrates except lactose within 48 hrs, and both isolates fermented glucose, fructose, sucrose, and maltose within 24 hours, while galactose and lactose were not fermented. the molecular identification of both isolates, using the universal primers ITS1 and ITS4 and the Sanger sequencing method. The isolates DBS-I (3) and DBS-II (2) were identified as *Pichia Kudriavzevii* and *Candida tropicalis,* respectively. The findings of this study support further exploration of indigenous yeasts for industrial use.

CONCLUSION

Identified yeast isolates, such as *Pichia kudriavzevii* and *Candida tropicalis,* were found to be useful for various industrial fermentation processes. Exploring local microbial diversity opens doors to innovation and new solutions for industrial challenges. The outcomes could result in more reliable and efficient methods for bioethanol production, which would have a big impact on the fermentation sector. Beyond bioethanol, these yeasts hold promise for use in food, beverage, and pharmaceutical fermentations where stress tolerance is crucial for consistent yields. Industrial fermentations can operate in harsh conditions due to their physiological endurance, which subsequently increases both operational sustainability and economic efficiency. Additional investigation is required to maximize the utilization of these yeasts, including genetic analyses to understand their mechanisms better and improve their fermentation productivity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Fermentation test

Isolates	Sugar (Glucose)
DBS-I(1)	+++
DBS- I(2)	+++
DBS- I(3)	++++
DBS-II(1)	+++
DBS- II(2)	++++
DBS- II(3)	+++
DBS- III(1)	+++
DBS- III(2)	+++

Note:-
"-" No fermentation ,**"+"** Weak fermentation (after 72 hrs), **"++"** Moderate fermentation (after 48 hrs)
"+++" Good fermentation (after 24 hrs), **"++++"** Prime fermentation (within 24 hrs)

Table 2. Nitrogen assimilation test

Sl. No	Isolates	Nitrogen sources (Bases)			
		Control (DW)	Ammonium Sulphate	Potassium Nitrate	Sodium Nitrate
1	DBS-I (3)	Negative	Positive	Negative	Negative
2	DBS-II (2)	Negative	Positive	Negative	Negative

Table 3. carbohydrate assimilation test

Carbohydrates	Isolates			
	DBS-I (3)		DBS-II (2)	
	Assimilation	Fermentation	Assimilation	Fermentation
D-Glucose	++	++	++	++
Fructose	++	++	++	++
Sucrose	++	++	++	++
Galactose	+	-	++	-
Lactose	-	-	-	-

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Maltose	++	++	++	++

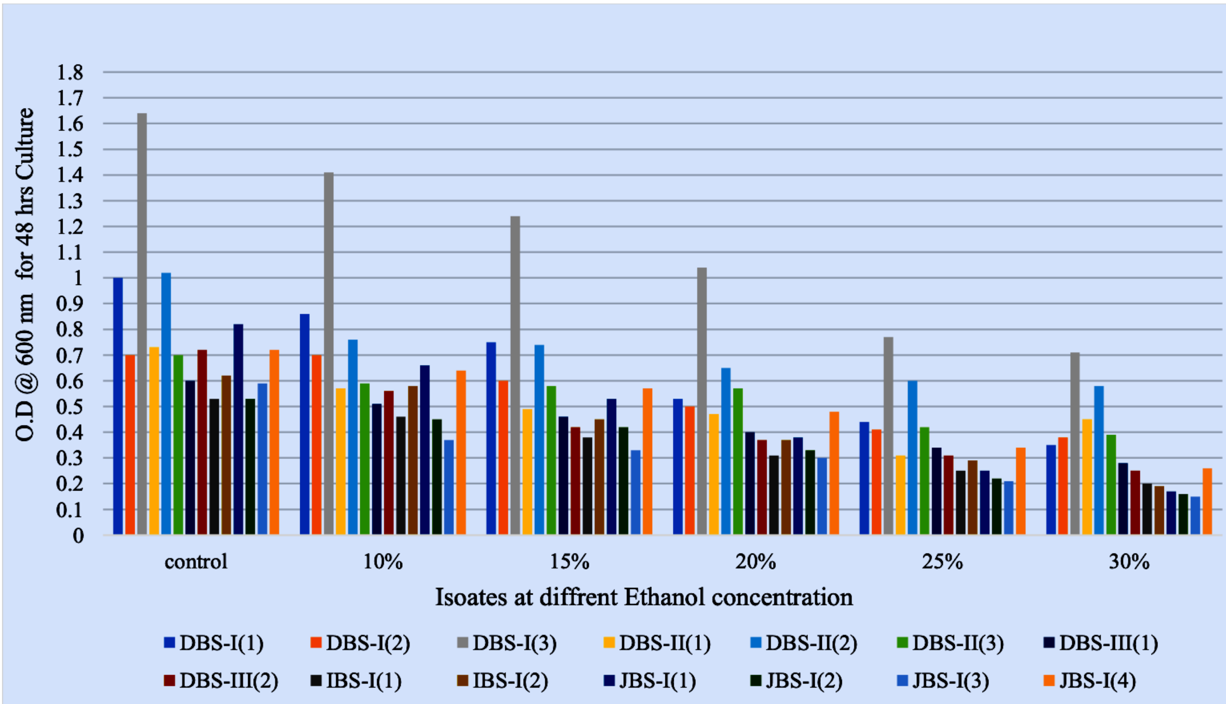

Figure 1: chart for ethanol tolerance

Note: - "-" negative result, **"+"** after 24 hrs within 48 hrs, **"++"** within 24hrs.

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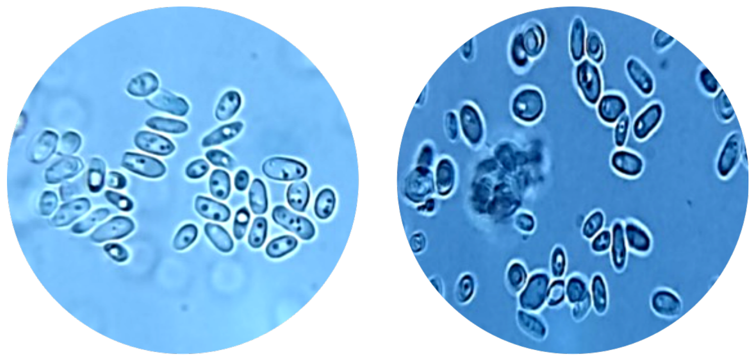

DBS-I (3) DBS-II (2)

Figure 3: Ascospore formation test

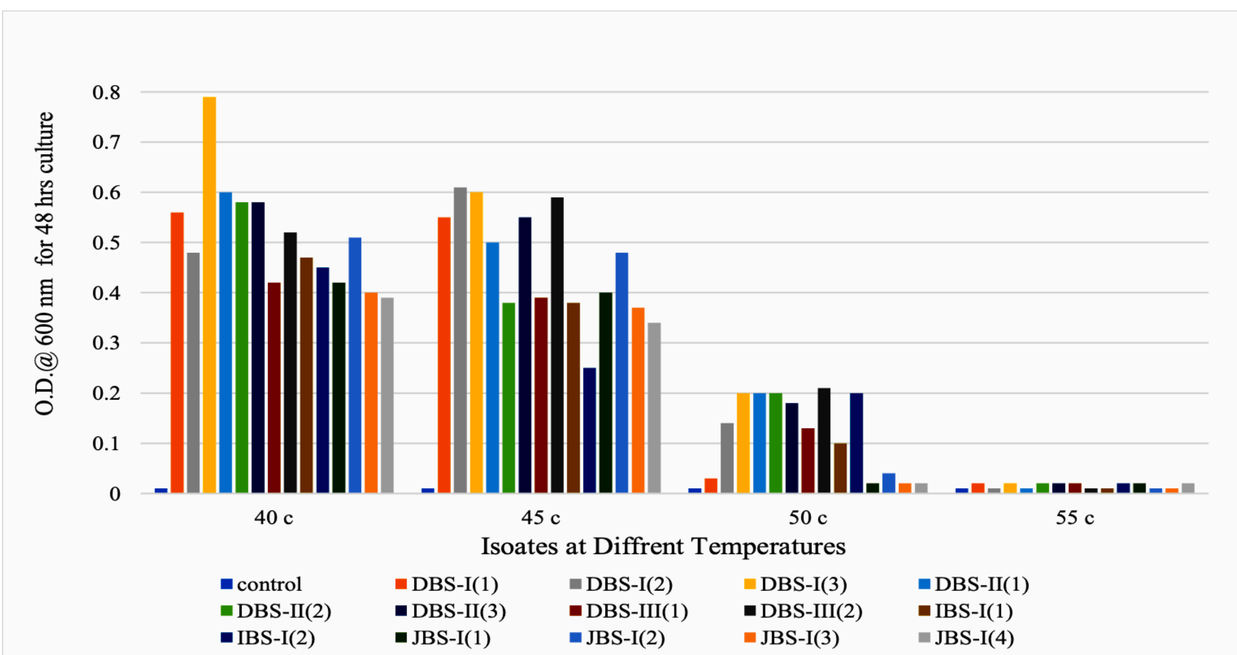

Figure 2: chart for thermo tolerance

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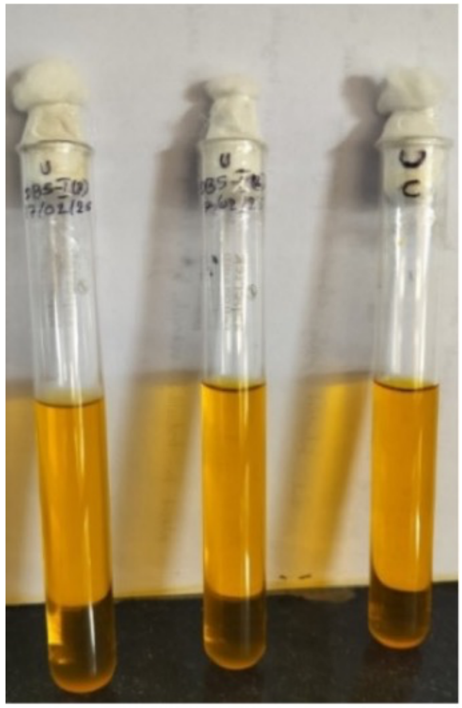
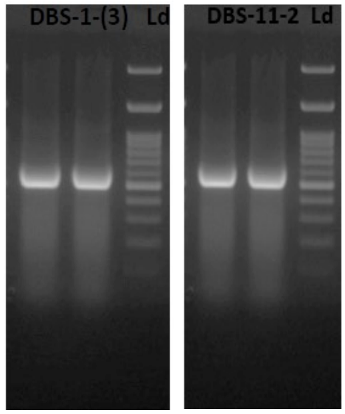


Figure 4: Urease test

(a)



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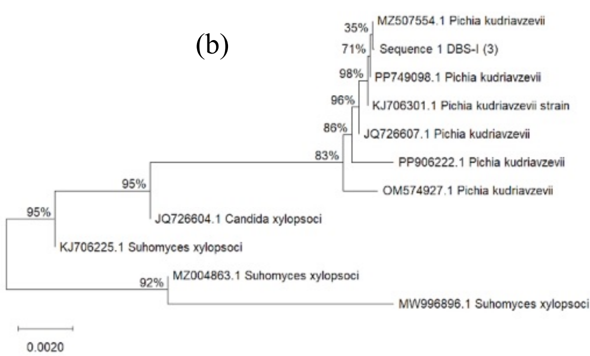

(c)

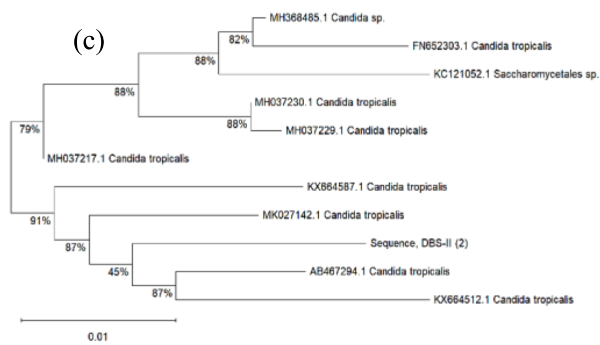

Figure 5: (a) Agarose (1.5%) gel electrophoresis of PCR using ITS1 and ITS4 isolate sample, (b) phylogenetic tree of DBS-I (3) and (c) phylogenetic tree of DBS-II (2).