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Characterization of stress tolerant high potential ethanol producing yeast from agro-industrial waste

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Abstract: Bioethanol or biofuel as an alternative to fossil fuels has been expanded in the last few decades in the whole world. Use of bioethanol as a renewable transportation fuel will minimize the amounts of fossil-derived carbon dioxide (CO_2) to the Earth's atmosphere. Yeast is the most favorite organism for ethanol production because of its diverse substrate specificity and ease of production of ethanol under anaerobic condition. The main objective of this research work was to isolate & characterize stress tolerant, high potential ethanol producing yeast strains from agro industrial waste. In total 4 yeast isolates have been characterized on the basis of morphological and physico-chemical characters. Based on morphological appearance of vegetative cell under microscope, ascospore production, colony character and physico-chemical characters all the strains was identified to be Yeast. Phylogenetic identification by DNA sequencing confirmed that the strain P is *Saccharomyces Unisporus*, strain C is *Saccharomyces cerevisiae*, strain T is *Saccharomyces cerevisiae* & strain DB2 is *Candida piceae*. Most of the strains were thermotolerant, pH tolerant, ethanol tolerant as well as osmotolerant. They were resistant to cycloheximide at 0.0015g/100ml concentration, hydrogen peroxide (0.50%), Chloramphenicol (30µg/disc) but growth was inhibited in the presence of 1% acetic Acid. The strains P, C & T showed good Invertase activity & only the T strain was capable of producing killer toxin. They were capable of fermenting glucose, fructose, sucrose, amylose & trehalose. Ethanol producing capability of the strains was studied using sugarcane molasses as substrate. The bioethanol production capacity of the yeasts were found to be 15%, 14.5%, 12% & 8.15% for P, C, T & DB2 respectively at pH 6.0, 30^{o}C temperature in media with 5.5% initial reducing sugar concentration in shaking condition. Pilot scale ethanol production by P strain was 13.10%, C strain 11.15%, T strain 9.80% & DB2 strains 7.85% at 60 hours. These strains could be potential for ethanol production from cane molasses.

Keywords: Ethanol, Molasses, DNA Sequencing, Stress Tolerant

1. Introduction

Nowadays, ethanol production from renewable resources has received great attention because of the increasing petroleum shortage[(1)]. Biomass fuels such as ethanol are renewable and help reducing greenhouse gas emissions from fosssil fuels[(2)]. Such renewable energy sources are indigenous and can therefore contribute to reducing dependency on oil imports and increasing security of supply[(3)].

Bioethanol can contribute to a cleaner environment and with the implementation of environment protection laws in many countries; demand is increasing[(4)]. *Saccharomyces cerevisiae* is one of the oldest, most exploited and best studied microorganism in both old and new biotechnologies and is known to be the world's premier industrial microorganisms which readily convert sugar into alcohol and CO_2 in metabolic process called fermentation[(5)]. *Saccharomyces* strains were used widely and traditionally for industrial ethanol production because of its ability to produce high concentrations of ethanol from hexoses and its high tolerance to ethanol and other inhibitory compounds[(6)]. Bioethanol produced from renewable biomass, such as sugar, starch or lignocellulosic materials,

is one of the alternative energy resources, which is both renewable and environmentally linked [7]. Tolerance to high temperatures and high ethanol concentrations are important properties of microorganisms of interest to industry[8].The ability of yeast to produce ethanol depends on many factors such as strains, growth factors and optimum environmental conditions[9].

The aim of this study was to characterize stress tolerant yeast strains capable of producing high level of ethanol from molasses.

2. Material and Methods

2.1. Collection of Strain

The yeast strains were isolated from agro industrial wastes of Bangladesh. The strains were coded as P, C, T & DB2.

2.2. Identification of Yeast Isolates

The yeast strains were characterized based on their cultural characteristics (Colony shapes, pigment, elevation, edge and surface appearance). Morphological and biochemical characterization of the isolated yeasts was performed according to Boboye and Dayo-Owoyemi[10].

2.3. Ascospore Formation

Selected yeast strains were examined for ascospore formation according to Kurtzman *et al*. [11]

2.4. Pseudomycelium Formation

Following Kreger-van Rij[12] the formation of pseudomycelium was investigated by slide culture technique.

2.5. Sugar Fermentation

Yeast fermentation broth base with Durham tube was used for testing of yeasts for carbohydrate fermentation. The carbohydrates used were Glucose (dextrose), Fructose, Sucrose, Lactose, Galactose, Maltose, Trehalose, Raffinose, Ribose, Arabinose, Rhamnose and Xylose. The color of the medium changed from blue to yellow due to the formation of acids and gas produced[13].

2.6. Thermo tolerance

YPD liquid medium was used for determination of thermotolerance. The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 37°C, 40°C, 41°C, 42°C, 43°C, 44^0C, 46^0C and 48^0C for 48 hours for observing thermo tolerance of yeast strain. The increase in optical density in a tube was recorded as evidence of growth[14].

2.7. PH Tolerance

YEPD broth was prepared at different pH. Each McCartney contained 15 ml of YEPD media with different pH and blank media was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°c for 48 hrs. After 48 hrs cell density was further recorded at 600 nm for growth[15].

2.8. Ethanol Tolerance

YPD liquid medium was used for determination of ethanol tolerance. Concentrations of absolute ethanol was varied from 5 to 20% (v/v) and then added to different flask. The initial optical density of each flask was read off on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 30 °C for 48 hours. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts[16].

2.9. Osmo tolerance

YEPD broth was prepared containing 5%, 8%, 10%, 12%, 15%, 18% and 20% of NaCl. Each McCartney contained 15 ml of YEPD liquid media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°c for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

2.10. Sugar Tolerance

The procedure by Ekunsanmi and Odunfa[17] was employed for observation of sugar tolerance. YEPD broth was prepared containing 10%, 15%, 30%, 45% & 50% of different sugars. Each McCartney contained 15 ml of YEPD liquid media with appropriate concentration of salt and blank media was used as a control. Then each was inoculated by half loopful of Yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm. The increase in optical density in a flask was recorded as evidence of growth.

2.11. Acetic Acid Tolerance

YEPD broth was prepared containing 1% of Acetic Acid. Each McCartney contained 15 ml of YEPD liquid media with 1% concentration of Acetic Acid and blank media without Acetic Acid was used as a control. Then each was inoculated by half loopful of Yeast cell and the initial optical density were measured at 600 nm and incubated at 30°C for 48 hrs. After 48 hrs cell density was further recorded at 600 nm.

2.12. Cycloheximide Resistance

YEPD agar medium was used for determination of Cycloheximide resistance. Cycloheximide antibiotic (0.0015g) was added into 100 ml autoclaved YEPD agar media and inoculated by yeast cells and incubated for 48 hours.

2.13. Chloramphenicol Resistance

YPD agar medium was used for determination of Chloramphenicol resistance. Chloramphenicol antibiotic disc (30 µ/L) was placed into the center of the already inoculated petridish. Then the plate kept at 30°C for growing. The zone of inhibition by the disc was recorded as an evidence of Chloramphenicol sensitivity[18].

2.14. Hydrogen per Oxide Resistance

Petridish containing the solid YEPD agar media was inoculated by yeast cells. Then three discs containing 30 µl, 20 µl and 10 µl of Hydrogen peroxide were placed on difference places on the plate. Hydrogen peroxide containing plates were incubated at 30°c for 48 hours.

2.15. Invertase Activities

Yeast strains grown on the agar slants were harvested by pouring sterile distilled water into the slants and gently scraping with a wire loop. The cells were washed, centrifuged and 0.1 g wet weight of each was re-suspended in 10 ml of acetate buffer, pH 5.0[19], sucrose solution (4% w/v, 2 ml) in the same acetate buffer was inoculated with 1 ml of cell suspension for 5 min at 30^0 C. The amount of reducing sugar released was determined by dinitro-salicylic acid method[20]. The amount of enzyme which liberate 1 µmole reducing sugar per minute was defined as one unit of invertase activity.

2.16. Killer Toxin Production Capacity

Ribereau-Gayon et al.[21] described the action of a killer strain on a sensitive strain is easy to demonstrate in the laboratory on an agar culture medium at pH 4.2-4.7 at 20° C. The sensitive strain is inoculated into the mass of agar before it solidifies; then the strain to be tested is inoculated in streaks on the solidified medium. If it is a killer strain, a clear zone in which the sensitive strain cannot grow encircles the inoculums streaks.

2.17. DNA Extraction

DNA extraction was done according to the procedure described by Moslem et el.[22]. Yeast cell was harvested for 3 days on YPD broth. About 1.5-ml culture was centrifuged in micro-centrifuge tube and mixed well with 600 µl DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.6% SDS). 6 µl RNAse A (15 mg/ml final concentration) was added and vortexed and incubated at 65°C for 10 min. 140 µl Protein Precipitation solution (3 M sodium acetate, pH 5.3) was added and the contents were mixed thoroughly by inverting the tubes. Tubes were then incubated at -20°C for 5 min and after incubation centrifuged at 13,000 g for 5 min. 600 µl of the supernatant was carefully transferred to a new micro-centrifuge tube and 600 µl absolute isopropanol was added to it and mixed by inverting gently 30 times or by vortexing. The tube was stored at room temperature for 5 min and then Centrifuged at 12,000 g for 2 min at 4°C. DNA was visible as a pellet that ranges in color from off-white to light green. DNA precipitate was washed twice with 650 µl 75% ethanol. 90 µl DNA elution buffer (pre-warmed at 65°C) was added and DNA was stored at 4°C until usage[23].

2.18. DNA Quantification

DNA was quantified either by measuring the absorbance at 260 nm using the NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies).

2.19. Phylogenetic Identification

Primers used in this assay were nu-SSU-0817-5' (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1196-3' (TCTGGACCTGGTGAGTTTCC)[24]. PCR reaction mixer was composed of 1X PCR buffer, 25mM $MgCl_2$, 10 mM of each dNTP, 0.2 µl Taq polymerase (5U/ µl), 20 picomole of each of forward and reverse primer and 5 µl template DNA. Total volume of reaction mixer was 30 µl. PCR thermal cycler was set in the following program-94°C for 5 min, 35 cycles of 94°C for 30sec, 57°C for 1 min & 72°C for 1 min, final extension at 72°C for 10 min. After thermal cycling, 15 µl of the PCR product was loaded on the corresponding well of a 2% of agarose gel along with a 50-bp DNA ladder at 70 volts for about 1.5 hours and visualized by staining with 0.5% ethidium bromide and UV illumination at 302 nm to confirm PCR amplification.

Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABI-Prism 310 genetic analyzer. The chromatogram sequencing files were edited using Chromas 2.32. The homology of the 5.8S rDNA gene sequences was checked with the 5.8S rDNA gene sequences of other organisms that had already been submitted to GenBank database using the BLASTN (http:/ /www.ncbi.nih.gov/BLAST/) algorithm. Evolutionary analyses were conducted in MEGA5[25].

2.20. Molasses Pretreatment

Before the molasses is used for fermentation, it undergoes an initial treatment, the purpose of which is to remove the sludge (colloids, firm particles, sand and to kill unwanted organisms) etc. 1 kg molasses is diluted with 0.5 L water and 0.001% conc. Sulfuric acid were added. It was then heated to the boiling and kept standing for couple of hours before use[26].

2.21. Ethanol Production from Fermentation of Molasses

Composition of fermentation media was Molasses 250 gm/L, Urea 0.10 gm/L and Conc. (H_2SO_4) 0 .30 ml/L and pH 6.0 with initial reducing sugar conc. 5.50%. Fermentation was carried out in Erlenmeyer conical flasks. 250 ml fermentation media was taken into 500ml Erlenmeyer flasks and then added the homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was incubated at 30^0C in a shaking incubator at 115 rpm for 72 hours.

2.22. Reducing Sugar Estimation

The reducing substance (sugar) obtained due to the enzymatic reaction was determined by DNS method [27].

Fig 1. *Growth in YPD medium*

2.23. Alcohol Estimation

Alcohol percentage in the fermentation broth was measured by the method described by Conway[28].

3. Results

3.1. Identification of Yeast Isolates

Based on the colony characteristics (white and creamy texture) ovoid microscope shape, the presence of ascospore and budding pattern (multipolar) (fig-1 & fig-2), the selected isolate was found to belong S*acharomyces*type unicellular ascomycete according to Lodder [29] and Boekhout and Kurtzman[30].

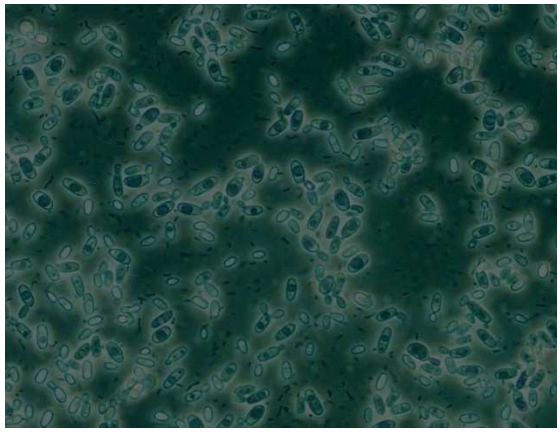

Fig 2. *Cell morphology under compound microscope*

3.2. Ascospore Formation

Following the method of Kreger-van Rij[12] and Kurtzman and Fell[31], ascospores formation by the yeast isolate P, C, T & DB_2 was detected for indication of the ascomycetous yeast. Ascospore formed in ascospore forming media after incubating for 3 weeks at 25 °C (fig-3).

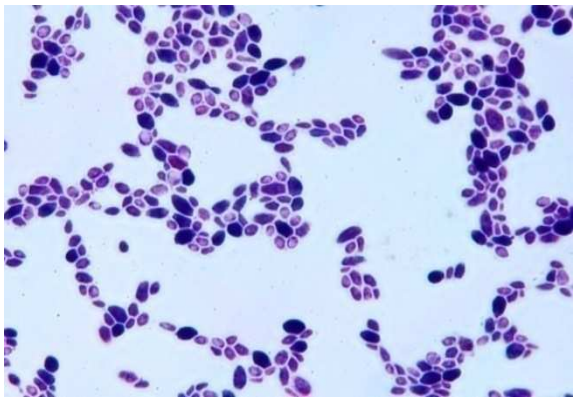

Fig 3. *Ascospore formation observation under compound microscope*

3.3. Pseudo mycelium Formation

The yeast isolate P, C, T & DB2 can produce pseudomycelium and showed in a filamentous form under microscope (Fig-5). Pseudomycelium formation is characteristic of *Saccharomyces cerevisiae,* which is dimorphic, existing either in a spherical, unicellular yeast-like morphology or in a filamentous form, termed pseudohyphae that results from elongated chains of cells that remain attached to one another[32] (fig-4).

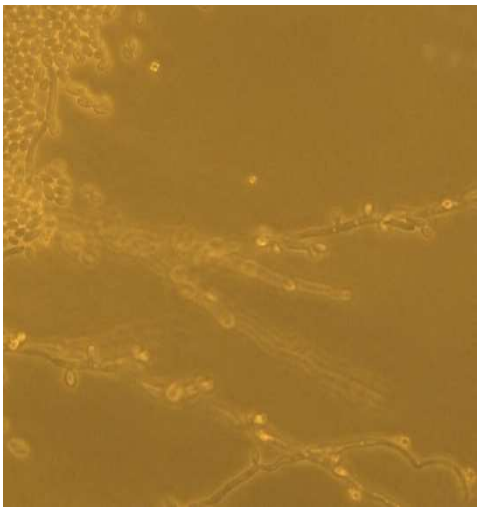

Fig 4. *Elongated chain of Yeast pseudo mycelium under microscope*

3.4. Sugar Fermentation

In this study, the yeast isolates P, C, T & DB2 showed variation of utilization of eight different sugars. They were utilized glucose, fructose, sucrose, maltose & trehalose but failed to grow on lactose and xylose, rhamnose, raffinose & arabinose[30].

3.5. Thermo tolerance

The thermotolerant yeast could promote high yield of ethanol at high temperature. Thermotolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries as well as under tropical climates [33]. The yeast isolates was found to be thermotolerant as they can grow up to 46 °c (fig-5).

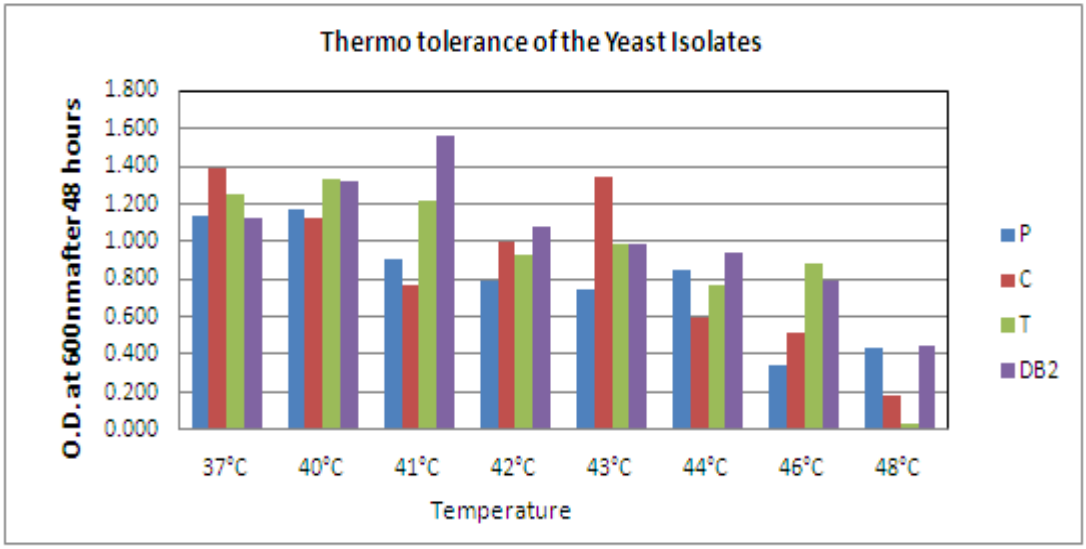

Fig 5. *Thermo tolerance of the yeast isolates*

3.6. PH Tolerance

The strains P, C, T & DB2 able to grow at wide range of pH (2 to 10). At pH 2, growth was decreased by highly acidic condition but can grow up to pH 10. Maximum growth was seen at pH 6 (fig-6).

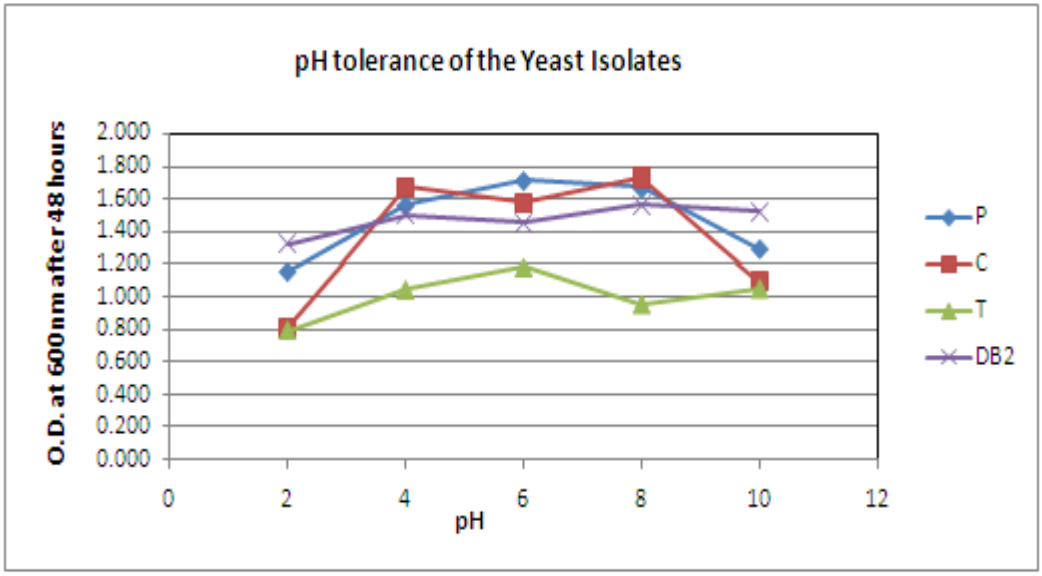

Fig 6. *PH tolerance of the yeast isolates*

3.7. Ethanol Tolerance

The thermotolerant yeast strains P, C, T & DB2 were grown at 0-20% (v/v) ethanol concentration. P & C strains can grow well upto 18% (v/v) and remained lowest in 20% (v/v) but T & DB2 were upto 15% (v/v). Only slight

differences were observed in the growth rates with increasing ethanol concentration from 15 to 20% (v/v) (fig-7).

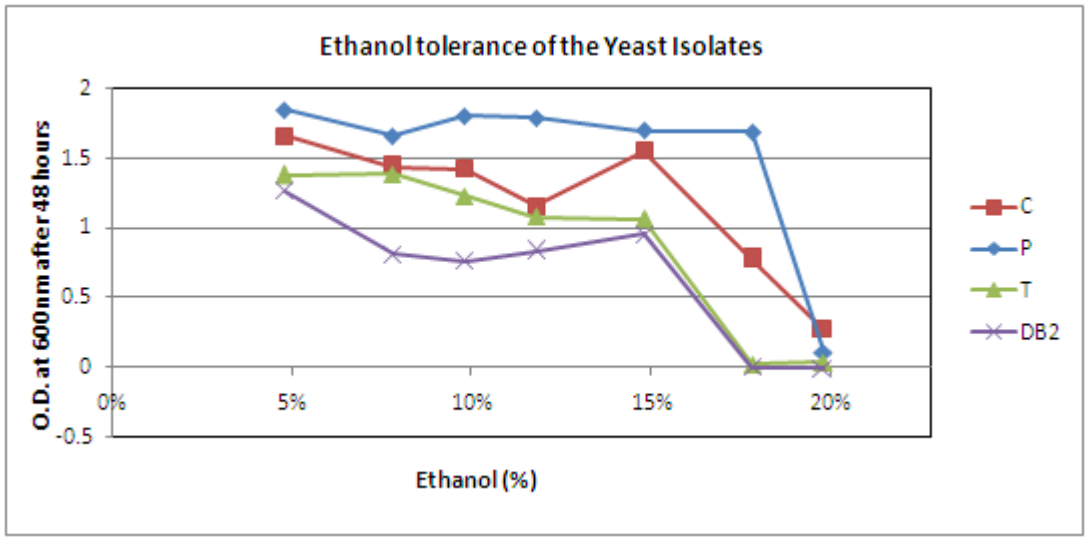

Fig 7. *Ethanol tolerance of the yeast isolates*

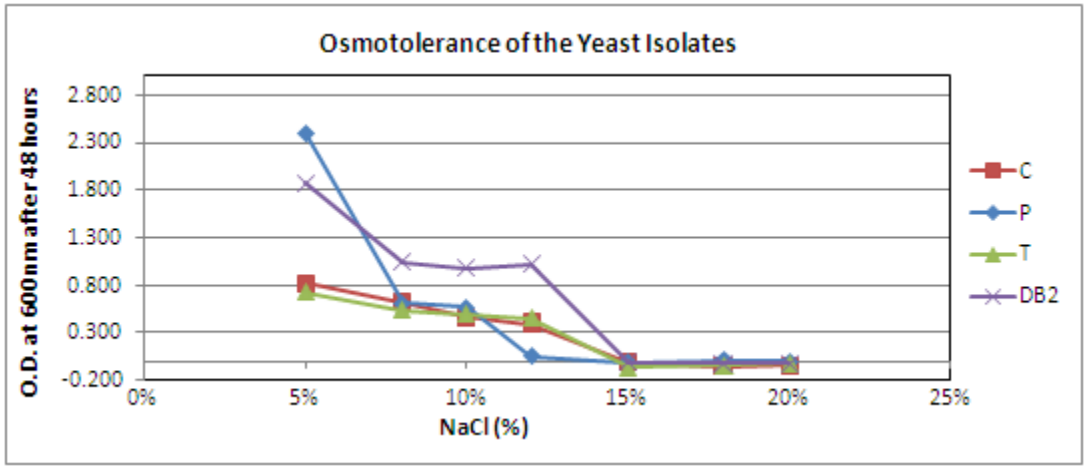

Fig 8. *Osmotolerance of the yeast isolates*

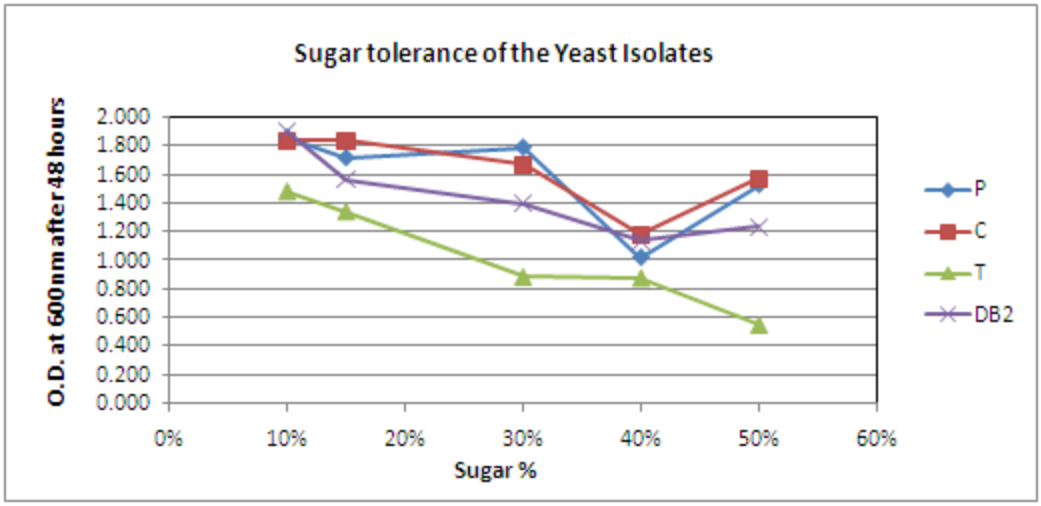

Fig 9. *Sugar tolerance of the yeast isolates*

3.8. Osmo Tolerance

The strains P, C, T & DB2 can tolerate up to 12% sodium chloride salt concentration but at higher concentration growth was reduced (fig-8).

3.9. Sugar Tolerance

The strains P, C, T & DB2 can toterate upto 50 % sugar & maximum growth was seen at 15 % (fig-9).

3.10. Acetic Acid Tolerance

1% Acetic acid usually inhibits the growth of yeasts. In this study, 1% Acetic acid inhibited the growth off all the 4 yeast strains in liquid YPED media.

3.11. Cycloheximide Resistance

The selected yeast strains were resistant to cycloheximide which is characteristic of *S. cerevisiae*[34]. It showed very good growth pattern on media with cycloheximide at 0.0015g/100ml concentration (Fig-10).

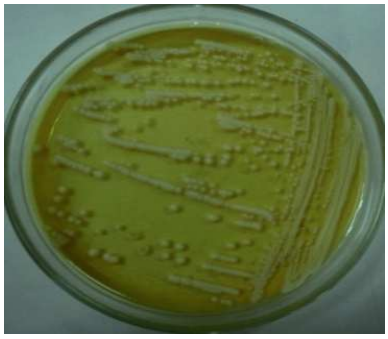

Fig 10. *Growth on Cycloheximide containing solid YEPD agar media at 30°C.*

3.12. Chloramphenicol Resistance

All the selected 4 yeast strains were resistant to Chloramphenicol at 30µg/ml concentration. They showed very good growth pattern against Chloramphenicol 30µg/ml disk in YPD plate (Fig-11).

Fig 11. *Growth on Chloramphenicol containing solid YEPD agar media at 30°C.*

3.13. Hydrogen per Oxide Resistance

Hydrogen peroxide usually inhibits the growth of *S. cerevisiae*[35]. All the yeast strains in this study were also resistance to hydrogen peroxide which is also a characteristic of *S. cerevisiae*[34].

3.14. Invertase Activity

All the isolates exhibited very good Invertase activity. They were capable of breakdown sucrose into Glucose & Fructose. Invertase activity of the isolates were presented in table 1.

Table 1. *Invertase activity of the selected Yeast Strains:*

Name of the Strains	Invertase activity (µmol/min)
DB2	7.79
P	17.86
C	17.14
T	15.50

3.15. Killer Toxin

Among all 4 strains only strain T was found to be capable of producing killer toxin & clear zone of inhibition was observed (fig 12).

Fig 12. *Observation of Zone of Inhibition due to Yeast killer toxin*

3.16. Phylogenetic Identification

Identification by DNA sequencing of the isolates was shown in table 2. Phylogenetic analysis by MEGA 5

showed that the isolates are phylogenetically distinct to other closely related species. Figure 13 shows the evolutionary positions of the isolates as revealed by 5.8s rDNA sequencing.

Table 2. *Phylogenetic Identification of the Yeast Strains:*

Strain Name	Identification	Accession number
P	*Saccharomyces unisporous*	AY046228.1
C	*Saccharomyces cerevisiae*	HM134859.1
T	*Saccharomyces cerevisiae*	DQ295800.1
DB2	*Candida piceae*	EF090821.1

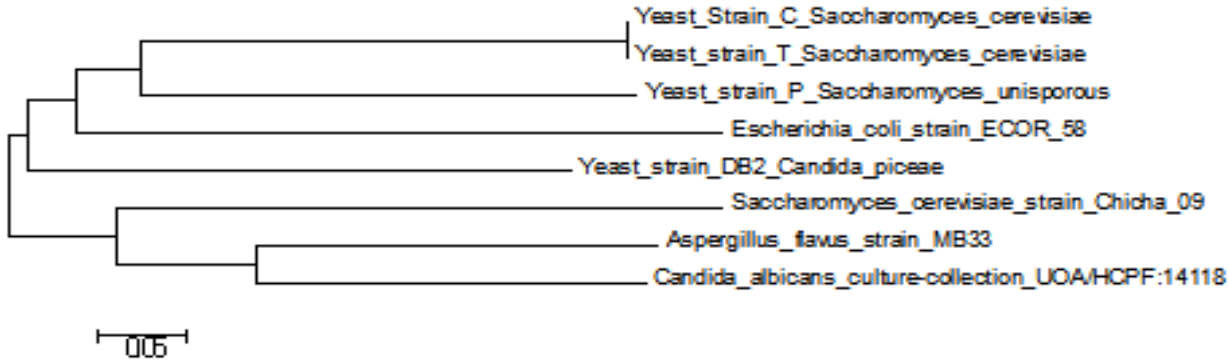

Figure 13. *Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method[36]. The optimal tree with the sum of branch length = 2.18969298 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method[37] and are in the units of the number of base differences per site. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 57 positions in the final dataset. Evolutionary analyses were conducted in MEGA5[25].*

3.17. Ethanol Production from Fermentation of Molasses

In Shaking condition (115 rpm), at 30°C using initial reducing sugar concentration of the fermentation media 5.50% and pH 6.0, maximum ethanol production was 15% by P strains, 14.50% by C strains, 12.00% by T & 8.15% by DB2 strains at 60 hours .

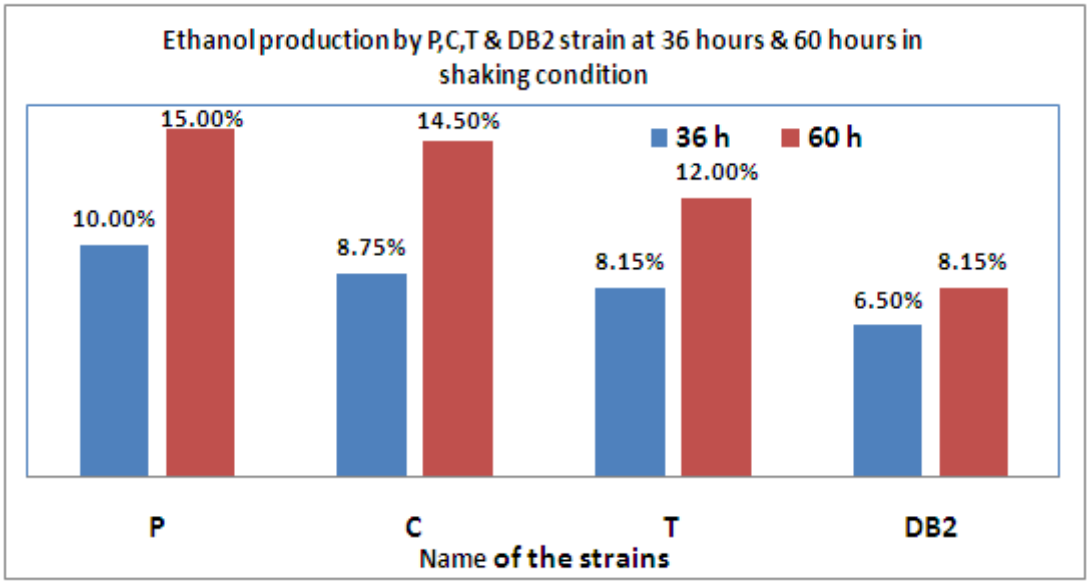

Fig 14. *Production of ethanol using initial reducing sugar concentration 5.50% at 30°C in shaking condition*

3.18. Pilot Scale Ethanol Production from Fermentation of Molasses

Pilot scale production by all the 4 strains was almost similar to that produced at small scale (Shake flask). Ethanol production by P strain was 13.10%, C strain

11.15%, T strain 9.80% & DB2 strains 7.85% at 60 hours.

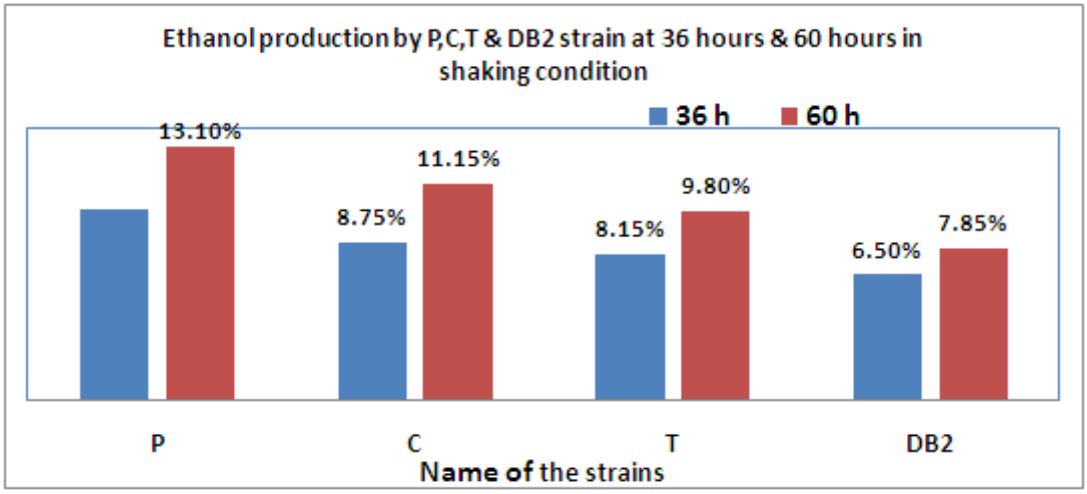

Fig 15. *Pilot scale ethanol Production of ethanol using initial reducing sugar concentration 5.50% at 30°C in shaking condition.*

4. Discussion

Samples were collected from different sources (Baggas, Silages) of agro industries. Based on some morphological & physiological characterization, presumptive yeast isolates has been selected. Based on the colony characteristics (white and creamy texture), ovoid microscope shape, the presence of ascospore and budding pattern (multipolar), the selected isolate was found to belong to *Saccharomyces* type unicellular ascomycete according to Lodder[29] and Boekhout and Kurtzman[30] (Fig-3). All the strains were able to produce pseudomycelium.

Molecular identification by DNA sequencing of 5.8s rDNA confirmed that strain P is *Saccharomyces Unisporus*, strain C& T are*Saccharomyces cerevisiae* & strain DB2 is *Candida piceae*. Phylogenetic analysis by MEGA 5 revealed that isolate P (identified to be *Saccharomyces unisporus*), isolate C & T (identified to be *Saccharomyces cerevisiae*) are evolutionarily distinct from other *Saccharomyces* (Fig 13).Isolate DB2 (identified to be *Candida piceae*) was also in evolutionary distinct position than *Candida albicans*.

All the strains were tested for fermentation of carbohydrates and they were capable of fermenting five sugars among 11 sugars used as substrates. Glucose, sucrose, maltose, dextrose and fructose were successfully fermented by all the strains but they couldn't ferment lactose, xylose, rhamnose, raffinose, ribose & arabinose.

There are several potential benefits of thermotolerant yeast to be used in the production of industrial alcohol[38]. Thermotolerant yeast exhibits rapid metabolic activity and a high fermentation rate with high product output & minimized contamination. All the selected strains in this study were thermotolerant & able to grow up to 46^0C temperature.

The rate of ethanol production by yeast cells is highly affected by the pH of the fermentation medium. Most of the yeasts generally showed maximum growth under acidic conditions. Both acidic and basic conditions retard the yeast metabolic pathways and hence the growth of cells[15]. All the selected strains were tolerant to wide range of pH. They were able to grow spontaneously from pH 2 to pH 10. Maximum growth was observed at pH 6.

A limitation of ethanol fermentation is the capacity of yeast to tolerate ethanol concentration, because ethanol inhibits alcoholic fermentation. Ekunsanmi and Odunfa[17] assert that the ethanol tolerance is an advantage when a yeast is being considered for industrial use especially where ethanol is being produced. Jimenez and Benitez[19] and Du Preez et al.[39] pointed out that ethanol tolerance is particularly important since ethanol tolerance can hardly be avoided during fermentation although substrate inhibition can be avoided through stepwise addition of substrate. The P, C, T & DB2 strain was screened for ethanol tolerance and P & C showed up to 18% ethanol tolerance in YPD liquid growth media but T & DB2 showed up to 15% . A slow growth rate was observed at 15-20% ethanol containing media.

All the strains were also osmotolerant as they could tolerate and grow up to 12% NaCl containing media. None of the 4 strains studied could tolerate 15 % salt concentration for growth.

The profitability of ethanol production is dependent on availability of sugar cane molasses, price and the quality of molasses (sugar %)[40]. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. However high substrate concentrations are inhibitory to fermentation due to osmotic stress[41]. Isolated yeast stains were able to grow up to 50% sugar (sucrose) containing liquid YEPD media. Maximum growth was seen in 15% sugar containing media for all the strains.

1% Acetic acid inhibited the growth of all the 4 yeast

strains. The selected strains were resistant to cycloheximide (0.0015g/100ml) & chloramphenicol (30µg/disc). The strains were also resistant to hydrogen peroxide (0.05%).

Invertase enzyme splits sucrose into glucose and fructose that are easily fermentable by yeast. A wide range of microorganisms produce invertase and can, thus, utilize sucrose as a nutrient. In this study good invertase activity was observed in most of the strains. Isolate P, C & T showed maximum invertase activity. So they were able to breakdown rapidly sucrose into glucose & fructose that is readily usable.

Yeast killer toxins are protein compounds, which are active against members of the same species or closely related species, and the activities of these toxins are analogous to the activities of bacteriocins in bacterial species[42]. The capability to produce killer toxin can confer an advantage over more sensitive competitive strains growing in a fermentative process[43]. In this study killer toxin producing capability of all the selected 4 strains were observed. Among all the strains only T strain produced killer toxin and clear zone of inhibition was observed.

In this study, isolate P produced 15.0%, C produced 14.50%, T produced 12.00% & DB2 produced 8.15% ethanol at pH 6.0, 30^0C temperature and 115 rpm agitation in molasses media with 5.50% initial reducing sugar concentration within 60 hours.

Semi-Pilot plant studies of ethanol production by these strains with optimized condition have conducted to assess their industrial suitability. Pilot scale production by all the 4 strains was almost similar to that produced at small scale (Shake flask). Isolate P produced 13.10%, C produced 11.15%, T produced 9.80% & DB2 produced 7.85% ethanol at 60 hours.

Productivity can also be improved by mutation through radiation or genetic manipulation. Metabolic pathway engineering to direct ethanol production may be a promising way to improve productivity.

5. Conclusion

Among all the 4 isolates the P, C and T would be useful to produce ethanol industrially in Bangladesh from molasses which is a very cheap and available raw material. Such industry will save foreign currency to import ethanol and will reduce dependency on fossil fuels. Productivity can also be improved by mutation through radiation or genetic manipulation. Metabolic pathway engineering to direct ethanol production may a promising way to improve productivity.

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