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Isolation, Identification, and Physiological Characterization of Indigenous Yeast Species Capable of Efficiently Utilizing Sugarcane Molasses as a Carbon Source

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ABSTRACT

Molasses, a byproduct of sugar production, contains sugars, ash, and inhibitors, limiting its microbial use. This study screened yeast species for efficient molasses utilization and inhibitor tolerance. Samples from four Khyber Pakhtunkhwa districts yielded 33 yeast strains after scrutiny. Following initial characterization, the strains were identified based on both morphological features and molecular methods involving the amplification of Internal Transcribed Spacer (ITS) regions. By the BLAST analysis, the ITS sequences for Candida tropicalis, Pichia kudriavzevii, Saccharomyces cerevisiae, Torulaspora delbrueckii, Trichosporon asahii, and Wickerhamomyces anomalus demonstrated 100% identity, whereas the sequence for Aspergillus fumigatus exhibited a maximum identity of 99.79% with the same species. In the phylogenetic analysis, these sequences were clustered with their respective corresponding species. Since molasses contain sucrose in major quantity, the physiological characterization of these isolated species in synthetic media containing sucrose as a sole carbon source reveals the higher growth efficiency of Torulaspora delbrueckii (OD600nm 5.24, µmax 0.0058 h-1) with second best performance of Trichosporon asahii (OD600nm 4.4, µmax 0.0049 h-1). The lowest grower was Saccharomyces cerevisiae (OD600nm 1.78 µmax 0.00016 h-1) while the remaining species i.e., Aspergillus fumigatus, Candida tropicalis, Pichia kudriavzevii, and Wickerhamomyces anomalus were of intermediate level (OD600nm 3.44, 3.89, 3.81, and 3.77, µmax was 0.0045 h-1, 0.0042 h-1, 0.0042 h-1, 0.0042 h-1 respectively). The isolated yeast species, known for utilizing non-molasses carbon sources, expand our understanding of substrate usage. Their potential as biofactories or genetic resources from natural evolution could aid in engineering industrial yeast strains for biofuel and biochemical production.

INTRODUCTION

With the rise of world's population, the economy needs to be shifted towards green and sustainable bioeconomy with reduced dependency on substrates derived from fossils. The choice of raw substrates for bioprocesses is crucial in terms of degrees of reduction, inhibiters, energy content, cost, and availability. The substrates can be individual compounds or complex in nature such as lignocellulosic biomass and waste from different industries (Kiselev *et al.,* 2022). The lignocellulosic biomass has high potential to provide carbon and energy to the microorganisms, however, their pretreatment is cumbersome process that also releases inhibitors like acetic acid and furan derivatives to the yeast-based industrial fermentation processes (Guaragnella and Bettiga 2021). On the other hand, the waste from agro-based industry such as molasses (byproduct from sugarcane and sugar beet refinery), is a rich source of carbon that can be valorized into a variety of useful single cell proteins and metabolites (Kumar, V. *et al.,* 2022). These wastes have the potential of polluting the environment, and their disposal practices results in elevated cost of consumable sugar as well as poses health hazards to human. Pakistan is the fourth largest producer of sugarcane (81.009 million tons annual production) after Brazil, China and India (https://www.fao.org/faostat/en/#data/QCL) and the production is projected to go beyond 100 million tons in next five years (Mehmood *et al.,* 2019). Typically, the composition of sugarcane molasses comprises of sucrose (30 –35%), fructose and glucose (10 –25%), free and

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bound acids as well as soluble gummy substances and non-sugar compounds (2–3%), mineral and moisture content (45–55%) (Solomon 2011). The sucrose, which is in the highest proportion in molasses, cannot be efficiently utilized by the model yeast - *Saccharomyces cerevisiae* (*S. cerevisiae*), since it is a 12-carbon containing compound and needed to be converted first to simpler sugars such glucose and fructose. Besides catabolite repression (due to the presence of low amount of glucose in molasses), the laboratory strains often find it difficult to consume sucrose as a carbon source, possibly, since sucrose must be hydrolyzed first into glucose and fructose by the invertase before its entry across the cell membrane (MWESIGYE and BARFORD 1996), in contrast to the reported wild- type yeast that has no such issue (Soares Rodrigues *et al.,* 2023). Besides the hindrance of sucrose utilization, the unwanted growth inhibitors present in this raw substrate may also halt the desired bioprocess by stopping the growth of yeast. Identification of robust yeast species that can efficiently utilize the sucrose and unwanted constituents in molasses is important so that it can be used directly in the bioprocess or indirectly by borrowing the genetic determinants and its translation into lab tested strains, in case the genetic-alteration tools are not developed. Many successful wildtype industrial strains have been reported to produce variety of compounds that has been adapted to specific environments due to the evolution by long time exposure to a specific environment (Le Borgne 2012). That suggest that the wild type mycobiota needed to be explored as it might be more resilient and robust in withstanding the challenges of sucrose utilization as well as the inhibitors that may be ultimately utilized in bioprocess. In this study, we screened wild type yeast for exploring their candidacy to be used in bioprocess, where molasses can be used as substrate. The present research work describes the isolation, identification, biochemical as well as physiological characterization of indigenous yeast that are efficiently capable of utilizing the sugar cane molasses as a carbon source. Different media composition containing molasses (obtained from different sugar refinery and jiggery plants) were designed to safely recover the yeast species capable of using molasses as an energy source. The identification was carried out based on nucleotides sequences of the conserved regions of ribosomal DNA (rDNA) complex, ITS1-5.8S –ITS4 rDNA spacers region (Hřibová *et al.,* 2011). Lastly, the identified yeast species were physiologically characterized and compared in synthetic defined medium containing sucrose as the sole carbon source.

MATERIAL AND METHODS

Sample Collection and Media Used

A total of 21 samples of molasses were collected from different locations (Figure 1) inside the molasses storage places of sugar refinery facilities in 4 districts of Khyber Pakhtunkhwa (KP), a province in northwest Pakistan, namely, Peshawar (34.028200°N, 71.569518°E), Mardan (34.179570°N, 72.058227°E), Charsadda (34.166131°N, 71.740271°E), and Bannu (32.987183°N, 70.688761°E). The sampling was based on different parameters such as pH, temperature, and stages of molasses production. The samples were collected in sterile 50mL falcon tubes and were stored at -4°C. The geographic coordinates for the collection sites were obtained using “Google Earth pro”, and the map was designed by ArcGIS V. 10.3.1 (ESRI, Redlands, CA, USA) (Figure 1).

Four serial dilutions (10^1,10^2,10^3, and 10^4) were prepared for each sample and 0.1mL of those dilutions were spread on plates and incubated at 30°C for 48 hours. The YPD (yeast extract 10g (Merck, Germany), peptone 15g (Merck, Germany), glucose 20g, and agar 15g (Biolife, USA) were used for routine propagation of yeast strains. However, for screening and isolation of yeast species based on their ability of molasses consumption as a carbon source, M1, M2, M3, and M4 were used with composition M1: contains 20 gL^{-1} molasses, 15 gL^{-1} agar at pH 5; M2: contains 20 gL^{-1} molasses, 5 gL^{-1} $(NH_4)_2SO_4$, 3 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $MgSO_4$, and 15 gL^{-1} agar at pH 5; M3: contains 10 gL^{-1} yeast extract, 20 gL^{-1} molasses, and 15 gL^{-1} agar at pH 5; M4: contains 10 gL^{-1} yeast extract, 10 gL^{-1} peptone, 20 gL^{-1} molasses, and 15 gL^{-1} agar at pH 5. To ensure the ability of the sole consumption of sucrose as carbon source by yeast, M-Synth media was formulated. M-Synth is a synthetic medium adopted from Verduyn (Verduyn 1992) that contain 5 gL^{-1} $(NH_4)_2SO_4$, 3 gL^{-1} KH_2PO_4, 4.5 mgL^{-1} $ZnSO_4 \cdot 7H_2O$, 0.84 mgL^{-1} $MnCl_2 \cdot 2H_2O$, 0.5 gL^{-1} $MgSO_4 \cdot 7H_2O$, 15 mgL^{-1} EDTA, 0.3 mgL^{-1} $CoCl_2 \cdot 6H_2O$, 0.4 mgL^{-1} $NaMoO_4 \cdot 2H_2O$, 4.5 mgL^{-1} $CaCl_2 \cdot 2H_2O$, 0.3 mgL^{-1} $CuSO_4 \cdot 5H_2O$, 3 mgL^{-1} $FeSO_4 \cdot 7H_2O$, 1 mgL^{-1} H_3BO_3, and 0.1 mgL^{-1} KI. After medium sterilization, filter-sterilized vitamins were added to a final concentration of 0.05 mgL^{-1} $D^{-(+)}$-biotin, 1 mgL^{-1} nicotinic acid, 25 mgL^{-1} myo-inositol, 1 mgL^{-1} D-pantothenic acid hemicalcium salt, 1 mgL^{-1} thiamine chloride hydrochloride, 0.2 mgL^{-1} 4-aminobenzoic acid, 1 mgL^{-1} pyridoxine hydro- chloride with 20 gL^{-1} of sucrose as a sole carbon source. For plate assay, 15 gL^{-1} of agar was added as a solidifying agent. The pH was adjusted to 6 with 2M KOH or with 2M H_3PO_4. To restrict the growth of bacteria and other undesired microorganisms, Chloramphenicol (Sigma Aldrich, Germany) at the final concentration of 100µg/mL was added to the plates when needed.

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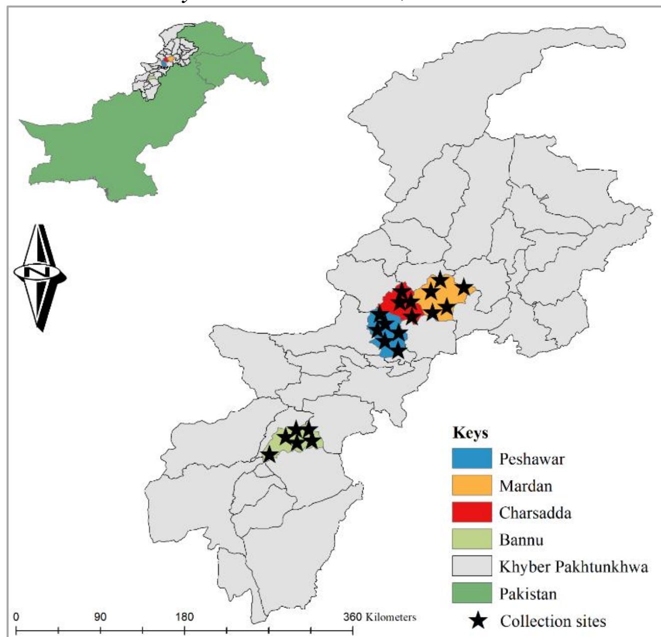
Figure 1
Map showing the sites where molasses samples were collected in Khyber Pakhtunkhwa, Pakistan.



Pre-treatment of Molasses

The molasses, before adding to the media, were pre-treated with modification as described by Raharja *et al.*, (Raharja *et al.,* 2019). Briefly, the molasses was diluted with ultra-distilled water in 1:1 ratio by adjusting pH to 5.2. H2SO4 solution (96.1 %) was added till the pH reached 3.9. The solution was then heated to 95°C for 10 minutes and kept overnight at room temperature. The precipitate was then removed by filtration. NaOH was added until the pH returned to initial value. Water dilution of the filtrate was carried out till 25 % brix sucrose concentration was achieved.

Morphology, Growth Curves and Maximum Growth Rates (μmax) Determination

The morphology of isolated yeasts was first investigated using alcian blue staining method (Lazcano *et al.,* 1993) and the stained cells were examined microscopically (BIOBASE, XS-208A, Jinan, China) by using an oil immersion lens at 100-400x magnification. Structure (mucoidal, watery or thick), height (flat and elevated), pigmentation (yellow, orange, and red), texture (sparkly or dark, even, coarse, and filamentous), and boundary (entire, undulating, lobed, and filaments) were noted down.

To determine the growth patterns of yeast species, an overnight culture of 5mL (from single cell colony) was prepared and was used to inoculate a second 75mL broth. The flasks were prepared in three biological replicates and were placed into an orbital shaker incubator with 300rpm on 30°C. Samples were taken each after 1 hour duration up to a span of 9 hours. A cuvette containing blank media served as a reference in the spectrophotometer (Genesys50 UV-VIS spectrophotometer). The optical density was recorded at a wavelength of 600nm. Different dilution of cell suspensions was used for precise measurement of OD_{600nm}. The maximum specific growth rate (μmax) was determined according to the formula described by Pylvänäinen (Pylvänäinen 2005).

Fungus Identification via ITS1-5.8S –ITS4 rDNA Spacers Sequencing

Genomic DNA extraction

Yeast cells biomass (0.1-1.0 mg) was scrapped using a sterile toothpick from a 2 day-old- plate grown on YPD and was transferred to 1.5 ml- Eppendorf tube. 0.3 g of glass beads having 212-300 µm diameter (Sigma Aldrich G-9143) were added along with 200 µL of lysis buffer (10Mm Tris at pH 8,1mM EDTA,100mM NaCl,1% SDS and 2%Triton X-100) and 200 µL of a 1:1 mix of phenol and chloroform. After vortexing at top speed (14,000 rpm) for 5 minutes, 200 µL of TE (10Mm Tris at pH 8, 1mM EDTA) was added and vortexed again for few second. The tube was then spined for 5 minutes (room temperature) at 14,000 rpm. Upper aqueous phase was transferred to a fresh Eppendorf tube while tube with glass beads was discarded. After that, 1mL of 100% ethanol was added at room temperature and was mixed thoroughly. Supernatant was discarded after spinning the tube for 5 minutes and 400 µL of TE, 3 µL of RNAase (10mg/mL) was added and incubated for 5 minutes at 37°C. In second last step, 10 µL of ammonium acetate (4M) and 1 mL of absolute ethanol was added followed by gentle mixing. Tube was again spined at top speed for 5 minutes and the supernatant was discarded, DNA was dried using a speed vac (Eppendorf) and was re-suspended in 40 µL of sterile distilled water

Amplification of ITS1-5.8S -ITS4 rDNA Spacers Regions through PCR

PCR amplification of ITS1-5.8S -ITS2 rDNA spacers regions were carried out with primers ITS1= 5′ TCCGTAGGTGAACCTGCGG 3′ and ITS4= 5′ TCCTCCGCTTATTGATATGC 3′. A total of 45 µl PCR mixture was prepared, out of which 30 µl was 10x PCR buffer (BU) (1x final concentration), 6 µl was dNTP mixture (10 mM) (dNTP) (final concentration will 200 µM of each dNTP), 3 µl each of 50 pmol/µl primer ITS1 and primer ITS4 (with final concentration of 0.5 µM), and Pfu DNA Polymerase 0.4 µl with end concentration of 0.05 U/µl and template DNA 10 ng. Thermocycler (Eppendorf GmbH, 22331, Hamburg, Germany) was programmed to the following sequence of events: Initial denaturation at 94°C for 2 min, and then for each cycle: denaturation at 94°C for 15 sec. Annealing was done at 55°C for 1 min, extension at 72°C for 60 sec, and terminal. extension at 72°C for 3 min with a total number of 32 cycles. PCR product was purified

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using Qia-QUICK (QIAGEN GmbH, 40724, Hilden) according to the manufacturer's instructions.

Electrophoresis was performed on 1% agarose gel which was prepared with 40 ml of 1% TAE buffer (10x TAE buffer contains (per liter): 48 g Tris-base,20 ml 0.5 M EDTA (pH 8.0), 11.4 ml Glacial Acetic acid, filled up to 1000 ml with distilled water and final pH was adjusted to 8.5 with NaOH). 10x TAE buffer was diluted to1% accordingly. 5 µl of each PCR product was mixed with 2 µl of loading dye (obtained from ready made available source having composition of sterile water, bromophenol blue, xylene cyanol, sucrose, SDS and EDTA at pH 8.0). 4µl of commercially available pre-stained DNA was used as a marker.100 V (7.5 V/cm) power supply was maintained for 50 minutes approx.

After electrophoresis, gel was stained with ethidium bromide (10 mg/ml solution in water) for 10- 15 minutes and visualized on a UV Transilluminator. The pictures were taken manually with camera device.

DNA Sequencing and Phylogenetic Analysis

A total of 33 amplified PCR products were submitted for DNA bidirectional sequencing. This process was carried out by a commercial company, Macrogen, Inc., based in Seoul, Korea, utilizing the Sanger sequencing method and the ABI 373XL system. The acquired sequences were cropped and assembled by using SeqMan v. 5, a software provided by DNASTAR, Inc. based in Madison, Wisconsin, USA. This process aimed to eliminate nucleotides with suboptimal readings. Multiple identical sequences for each species were treated as a consensus sequence. Subsequently, the trimmed sequences were subjected to the Basic Local Alignment Search Tool (BLASTn: https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.,* 1990) at National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/). The sequences were downloaded in FASTA format from NCBI, focusing on maximum identities. Subsequently, the acquired ITS partial sequences were aligned with downloaded sequences using ClustalW multiple alignments (Thompson *et al.,* 1994) in BioEdit Sequence Alignment Editor v. 7.0.5 (Hall *et al.,* 2011). The phylogenetic tree was constructed using the Maximum Likelihood (ML) method with the Kimura 2-parameter in Molecular Evolutionary Genetics Analysis (MEGA-X), employing a bootstrapping value of 1000 (Kumar, S. *et al.,* 2018).

RESULTS

Isolation of Yeast Species from Molasses Samples and Media Optimization

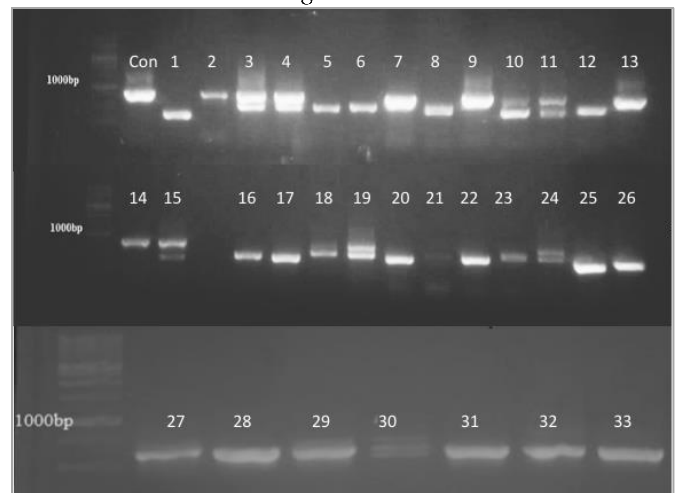
For initial screening, not to miss out any potential best yeast strain, four different media (M1-M4, described in materials and methods) were selected. The 21 samples (designated as 1-21) were grown on above 4 media. Besides visual observations to record the growth patterns on routine basis, the colonies obtained were re-streaked 3 times to obtain single and purified colonies. All the colonies at each step were subjected to microscopic examination to exclude the possibility of contamination and to identify them based on their morphology using alcian blue staining as described in materials and methods section. This helped us in removing the commonly occurring strains in all media. As a result, total of 33 colonies were purified based on their physical differences and hence subjected to subsequent investigation for their identification based on molecular markers i.e., ITS-1, the 5.8 S rDNA and ITS-4 region.

PCR Amplification of ITS1-5.8S -ITS4 rDNA Spacers Regions

To amplify the ITS1-5.8S -ITS4 rDNA, first the genomic DNA of all the 33 strains was extracted via the method described in material and methods section. PCR amplification of ITS1-5.8S -ITS4 rDNA spacers regions were carried out for all 33 strains. The PCR was performed according to the conditions and primers mentioned in material and methods section.

Figure 1

PCR amplification of rDNA gene cluster: Primers ITS-1 and ITS-4 were used for amplification of nearly 650 bp fragment of rDNA gene cluster, consisting of ITS-1, the 5.8 S rDNA and ITS-4 region.



The rDNA gene cluster, consisting of ITS-1, the 5.8 S rDNA and ITS-4 region is around 650 bp in length. Figure 2 shows the successful amplification of ~650 bp fragment.

Evolutionary Analysis

After removing the duplicates recurring sequences and the BLAST analysis of the obtained ITS partial sequences, *Candida tropicalis* exhibited a 100% identity match with the same species, followed by 98.24% identity with *Candida dubliniensis* and 97.74% with *Aureobasidium pullulans.* Phylogenetically, this sequence formed a cluster with corresponding species

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reported from India and China, while its sister clade was identified with *Candida dubliniensis* and *Aureobasidium pullulans*. For *Pichia kudriavzevii*, a 100% identity match was observed with corresponding species sequences, and it phylogenetically clustered with species reported from Netherlands, Japan, and Russia.

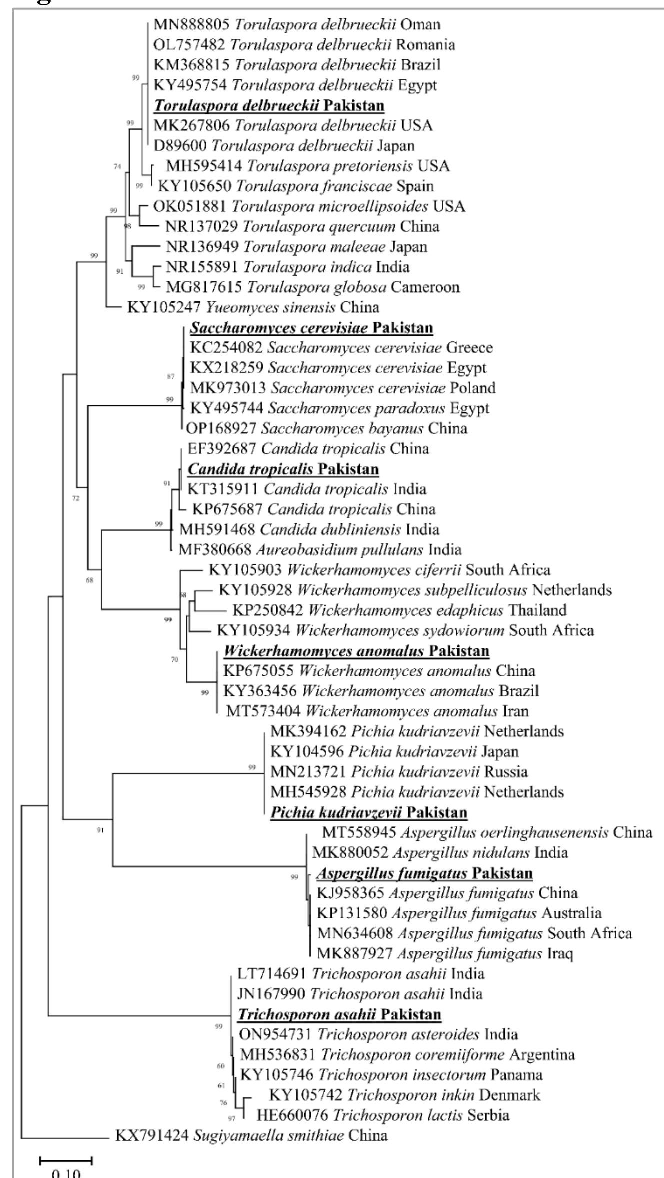
The *Saccharomyces cerevisiae* exhibited 100% identity with the same species, followed by 99.86% with *Saccharomyces bayanus* and 99.59% with *Saccharomyces paradoxus*. Phylogenetically, this sequence clustered with corresponding species reported from Greece, Egypt, and Poland, forming a sister clade with *Saccharomyces bayanus* and *Saccharomyces paradoxus*.

For *Torulaspora delbrueckii*, a 100% identity was observed with the corresponding species sequence, followed by 96.91% with *Torulaspora franciscae*, 96.62% with *Torulaspora pretoriensis*, 92.07% with *Torulaspora microellipsoides*, 91.48% with *Torulaspora quercuum*, 90.52% with *Torulaspora maleeae*, 89.65% with *Torulaspora globose*, and 89.27% with *Torulaspora indica*. Phylogenetically, it is clustered with corresponding sequences reported from Oman, Romania, Brazil, Egypt, the USA, and Japan, forming a sister clade with *Torulaspora franciscae, Torulaspora pretoriensis, Torulaspora microellipsoides, Torulaspora quercuum, Torulaspora maleeae, Torulaspora globose,* and *Torulaspora indica*.

Trichosporon asahii showed a 100% identity with the same species sequences, followed by 99.79% with *Trichosporon insectorum*, 99.59% with *Trichosporon coremiiforme*, 99.18% with *Trichosporon asteroids*, 98.33% with *Trichosporon lactis*, and 98.14% with *Trichosporon inkin*. Phylogenetically, it was clustered with corresponding sequences reported from India, forming a sister clade with *Trichosporon coremiiforme, Trichosporon insectorum, Trichosporon asteroids, Trichosporon lactis,* and *Trichosporon inkin*. The obtained *Wickerhamomyces anomalus* displayed 100% identity with corresponding species sequences, followed by 92.21% with *Wickerhamomyces sydowiorum*, 90.73% with *Wickerhamomyces ciferrii*, 90.54% with *Wickerhamomyces edaphicus*, and 89.10% with *Wickerhamomyces subpelliculosus*. Phylogenetically, it clustered with the same species sequences reported from Iran, Brazil, and China, forming a sister clade with *Wickerhamomyces sydowiorum, Wickerhamomyces ciferrii, Wickerhamomyces edaphicus,* and *Wickerhamomyces subpelliculosus*. The obtained ITS partial sequence for *Aspergillus fumigatus* displayed a maximum identity of 99.57-99.79% with the same species, followed by 99.36% with *Aspergillus oerlinghausenensis* and *Aspergillus nidulans*. Phylogenetically, it clustered with corresponding sequences reported from China, Australia, South Africa, and Iraq, forming a sister clade with *Aspergillus oerlinghausenensis* and *Aspergillus nidulans* (Figure 3).

All the acquired ITS partial sequences were deposited to the GenBank under accession numbers for *Aspergillus fumigatus* (PP062741), *Candida tropicalis* (PP062742), *Pichia kudriavzevii* (PP062745), *Saccharomyces cerevisiae* (PP062744), *Torulaspora delbrueckii* (PP062743), *Trichosporon asahii* (PP062746), and *Wickerhamomyces anomalus* (PP061541).

Figure 2



A Maximum Likelihood phylogenetic tree was constructed based on nucleotide sequences, specifically the ITS partial sequences for *Aspergillus fumigatus, Candida tropicalis, Pichia kudriavzevii, Saccharomyces cerevisiae, Torulaspora delbrueckii, Trichosporon asahii,* and *Wickerhamomyces anomalus.* The ITS sequence of *Suriyamaella smithiae* taken as an outgroup. Sequences are denoted by their GenBank accession number, followed by species names and countries (If

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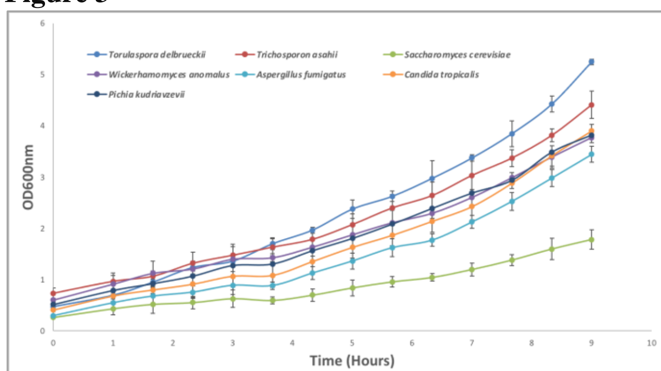
applicable). The obtained ITS partial sequences were highlighted in bold and underlined font.

Optical Density (OD_{600nm}) and Maximum Growth Rate (μ_{max}) of the Identified Species on M-Synth Media Containing Sucrose as a Sole Carbon Source

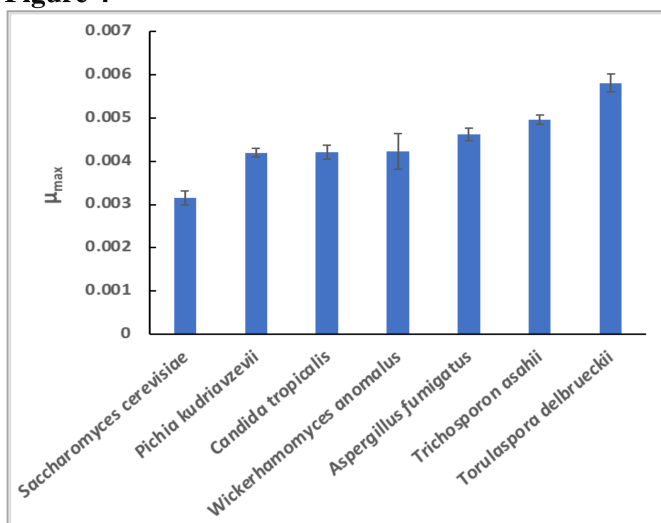
The 7 species identified were subjected to grow on M-Synth media (composition described in materials and methods) to check whether the strains growing on molasses can grow on sucrose as a sole carbon source. *Torulaspora delbrueckii* exhibits better performance in terms of final optical density (OD_{600nm}) and maximum growth rate (μ_{max}). The final OD_{600nm} attained was 5.24 with a μ_{max} 0.0058 h^{-1}. *Trichosporon asahii* being the second best with final OD_{600nm} of 4.4 h^{-1} and μ_{max} 0.0049 h^{-1}. *Aspergillus fumigatus, Candida tropicalis, Pichia kudriavzevii,* and *Wickerhamomyces anomalus* were having intermediate performance with OD_{600nm} 3.44, 3.89, 3.81, and 3.77 and μ_{max} of 0.0045 h^{-1}, 0.0042 h^{-1}, 0.0042 h^{-1}, 0.0042 h^{-1} respectively. The *Saccharomyces cerevisiae* having lowest OD_{600nm} of 1.78 with μ_{max} 0.00016 h^{-1}.

Figure 3



Growth curves of the *Aspergillus fumigatus, Candida tropicalis, Pichia kudriavzevii, Saccharomyces cerevisiae, Torulaspora delbrueckii, Trichosporon asahii* and *Wickerhamomyces anomalus* on sucrose as a sole carbon source.

Figure 4



Maximum growth rates (μ_{max}) of *Aspergillus fumigatus, Candida tropicalis*, *Pichia kudriavzevii*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Trichosporon asahii* and *Wickerhamomyces anomalus* on sucrose as a sole carbon source

DISCUSSION

Sugar cane molasses is the major byproduct of sugar manufacturing industry that comprises of sucrose (30 – 35%), fructose and glucose (10 –25%), free and bound acids, soluble gummy and non-sugar compounds (2–3%), as well as mineral and moisture content (45–55%) (Solomon 2011). The model *Saccharomyces cerevisiae*, being the principal cell factory, faces challenges in sucrose fermentation (Marques *et al.,* 2016) and its ability to properly ferment high-sucrose concentrations is not enough as compared to other fermentation processes (Jones *et al.,* 1994). For instance, half of the world's ethanol production needs effective fermentation of sucrose-containing raw materials like sugarcane molasses (Bušić *et al.,* 2018). This might be because of the poor sucrose fermentation pathway in *S. cerevisiae.* The present research work was carried out to isolate and identify the yeast strains that is naturally capable of efficiently using sugar cane molasses as energy source. In total, 21 samples of molasses were collected from inside the molasses storage places of sugar refinery facilities in 4 different districts of Khyber Pakhtunkhwa (KP Pakistan). The sampling criteria was based on different parameters such as pH, temperature, and stages of molasses production. After a detail culturing process on several media (M1-M4) parallelly and careful microscopic and biochemical characterization, 33 strains were isolated and purified by re-streaking. Each step was attentively accompanied by microscopic, visual, and biochemical (alcian blue staining) observations. To exclude the possibility of contaminations, the strains were re-streaked 3 or more times. Molecular techniques present superior advantages in identification, characterized by their swiftness, heightened sensitivity, and specificity. Notably, these methodologies rely on genetic traits, which exhibit greater endurance compared to phenotypic features. The Polymerase Chain Reaction (PCR) technique has ascended to become one of the preeminent tools in molecular biology due to its remarkable discriminatory prowess and reproducibility. This is attributable to its efficient nature, simplicity, and minimal specimen material requirements (Dos Santos *et al.,* 2004). The confirmation of thirty-three isolates through PCR for the identification of fungal species via the ITS region, employing ITS1 and ITS4 primers, unveiled anticipated outcomes. The fragment sizes of PCR products from diverse fungal species fell within the range of approximately 450-700 base pairs. These findings align with numerous studies advocating PCR as a dependable technique for species-specific

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identification. These studies emphasize the molecular methods utilizing the ITS regions of fungal ribosomal RNA, which harbor highly variable sequences. These sequences serve as precise markers, ensuring more accurate and specific discrimination of fungal species (Choudhry *et al.,* 1992); (Jahanbani *et al.,* 2009) (Martin and Rygiewicz 2005). Consequently, *Pichia kudriavzevii, Saccharomyces cerevisiae, Aspergillus fumigatus, Trichosporon asahii, Torulaspora delbrueckii, Wickerhamomyces anomalus*, and *Candida tropicalis* species sequences of the current study were subjected to molecular-based analysis involving ITS (ITS1 and ITS4) partial sequences. It is noteworthy that these primers were initially "intended to be specific to fungi," as underscored by Gardes and Bruns (Gardes and Bruns 1993). These fungal species' ITS partial sequences were analyzed via BLASTn, and revealed the respective species. The obtained sequences for *Pichia kudriavzevii, Saccharomyces cerevisiae, Trichosporon asahii, Torulaspora delbrueckii, Wickerhamomyces anomalus,* and *Candida tropicalis* signified the absence of genetic differentiation. The phylogenetic analysis of the acquired ITS partial sequences for fungal species substantiated a close phylogenetic resemblance to species documented in diverse zoogeographic regions in the world. Conversely, the *Aspergillus fumigatus* sequence displayed a 0.21% (468/469) genetic variation and was evaluated for its robust branch support linked to the respective species. The topologies of the phylogenetic trees for the obtained fungal species were then juxtaposed with those of the corresponding species and aligned with the conducted examination by Santiago *et al.* (2021) and Kurtzman *et al* (2008) The above identified strain are highly promising strains for biofuels production such as bioethanol, biodiesel, and other volatile compounds. For instance, *Pichia kudriavzevii* were reported by Chan *et al* (2012) Dhaliwal *et al* (2011) Sankh *et al* (2013) and Akita *et al* (2021) as highly potential strain for bioethanol and biodiesel production. On the hand *Wickerhamomyces anomalus* were reported by Oro *et al* (2018) Fan *et al* (2019) Ojha and Das (2018) and Czarnecka *et al* (2019) as potential strain to produce 42 organic volatile aromatic compounds, ethyl acetate, polyhydroxyalkanoates and used as biocontrol agent. *Candida tropicalis* were reported by Thangavelu *et al* (2021), Raj and Krishnan (2020), Shariq and Sohail (2019) to produce xylanase, bioethanol, biodiesel and as a bioconversion agent for wastewater treatment. *Torulaspora delbrueckii* were reported by Benito (2018), Pech-Canul *et al* (2019), Chua *et al* (2021) as a legendary strain for bioethanol, winemaking, and a competitor of *Saccharomyces cerevisiae* in fermentation industry.

Since the main purpose of this work was to use molasses as a carbon source, therefore, four versions of molasses-based media (M1-4) were used for isolation. The molasses was used because it can serve as a growth media since it can provide most of the essential nutrients needed for the growth of yeast. On the other hand, sucrose which is in highest proportion in molasses, cannot be efficiently utilized by the yeast since it is a12-carbon containing compound and needed to be converted to simpler sugars such glucose and fructose. The major question of efficient sucrose utilization was still not resolved. For that purpose, M-Synth synthetic media was adopted from (Verduyn 1992) that contain sucrose as a sole carbon source. M-Synth used as an anticipation to check whether the strains growing efficiently on molasses could also grow in the same manner as on sucrose as a sole carbon source. Since molasses contain other sugars and inhibitory materials, the M-Synth media would help in exploring the ability of identified species for sole utilization of sucrose as a carbon source. Among isolated and identified species, *Torulaspora delbrueckii* seems best of all in growth by reaching to an OD_{600nm} of 5.24 with a μ_{max} 0.0058 h^{-1}. Second best was *Trichosporon asahii* final OD_{600nm} of 4.4 h^{-1} and μ_{max} 0.0049 h^{-1}. *Aspergillus fumigatus, Candida tropicalis, Pichia kudriavzevii,* and *Wickerhamomyces anomalus* were having intermediate performance with OD_{600nm} 3.44, 3.89, 3.81, and 3.77 and μ_{max} of 0.0045 h^{-1}, 0.0042 h^{-1}, 0.0042 h^{-1}, 0.0042 h^{-1} respectively. Finally, the *Saccharomyces cerevisiae* isolate reached to an OD_{600nm} of 1.78 with μ_{max} 0.00016 h^{-1}. It is noteworthy that the isolated 7 yeast species almost exhibit resembling behaviour in other complex media i.e., M1, M2, M3, and M4 (data not given). That implies to the fact that since molasses contains highest proportion of sucrose, the isolated strains growth observed was mainly due to the efficient utilisation of sucrose in molasses the same way as replicated in M-Synth media.

CONCLUSION

21 samples of molasses collected from various localities in Khyber Pakhtunkhwa, Pakistan, from sugar processing plants resulted in the isolation of 33 yeast best strains in terms of growth and utilization of molasses. The strains were further identified based on their morphology and amplification of ITS (Internal transcribed spacer) regions. The identified isolates were mainly belonging to the genera *Pichia kudriavzevii, Saccharomyces cerevisiae, Aspergillus fumigatus, Trichosporon asahii, Torulaspora delbrueckii, Wickerhamomyces anomalus,* and *Candida tropicalis*. Physiological characterization of the species in defined media revealed their efficiency of sucrose utilization as a sole carbon source. Among the species, *Torulaspora delbrueckii* exhibited best performance in terms of growth with *Trichosporon asahii* as the second best. *Pichia kudriavzevii, Aspergillus fumigatus, Wickerhamomyces anomalus,* and *Candida tropicalis* were of moderate value and *Saccharomyces cerevisiae*

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being the least efficient in sucrose utilization for the growth. The isolated yeast species have been already reported for the production of important and industrially relevant compounds from carbon sources other than molasses and sucrose, and hence this work will serve an addition to the existing knowledge of substrates used for their growth. On the other hand, the current work also paves up the path towards sustainable bioprocess with a possibility to explore and translate the genetic determinants from these species into the laboratory or industrial yeast strains when sucrose or molasses are considered as energy source.

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