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SCREENING AND CHARACTERIZATION OF POTENTIAL ETHANOL OXIDIZING YEAST ISOLATES FROM SUGAR-RICH ORGANIC WASTE RESOURCES USING SCHIFF'S-BASE REACTION

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ABSTRACT

Alcohol dehydrogenase is an oxidoreductase enzyme that carries both alcohol oxidation and aldehyde/ketone reduction activity. Alcohol dehydrogenase (ADH) activity of yeast cells were essential for industrial applications. Sugar-rich organic waste resources including spoiled fruits, fruit peel waste, spoiled vegetables, raw and composed sludge from the dairy industry, and cane molasses spill from the sugar industry, provide a favorable habitat for ethanol-oxidizing microorganisms with ADH activity. The total ethanol oxidizing microbial population of organic waste resources differs considerably from the overall microbial community. Yeast from the natural habitats has a great potential for developing novel enzymes for industrial uses. Under this backdrop, the present study was focused on screening and characterization of potential ethanol oxidizing yeast strains from sugar-rich organic waste resources. Totally seventeen ethanol oxidizing yeast strains belongs to *Pichia sp.* (9 strains), *Candida sp.* (2 strains) and *Debaryomyces sp.* (2 strains), *Hanseniaspora sp*, *Lachancea sp*, *Torulaspora sp*, and *Magnusiomyces sp.* have been identified. The ability of strains to oxidize ethanol into acetaldehyde was verified by the Schiff`s-base reaction through p-rosaniline assay. The selected five potential ethanol oxidizing strains were identified by 18S rRNA gene sequence as *Pichia kluyveri* FYES5, *Pichia kudriavzevii* FYES6, *Pichia kudriavzevii* VYES2, *Candida mengyuniae* CSYES1, and *Magnusiomyces capitatus* CSYES2 with the p-r index of 2.64±0.40, 3.79±0.54, 3.73±0.69, 2.28±0.24, and 2.87±0.58 respectively at 28°C. This is the first report to identify ethanol oxidizing yeast strains from sugar-rich organic waste resources and by this study found that the strain *Candida mengyuniae* has a better ability to oxidize ethanol.

Keywords: Organic waste; Yeast; Ethanol oxidation; Alcohol dehydrogenase; Schiff`s-base reaction

INTRODUCTION

Alcohol dehydrogenase (ADH) is a crucial enzyme in fermentable microorganisms that aid in converting sugar to ethanol **(Asokumar *et al*., 2018; Piriya *et al*., 2012)**. Alcohol dehydrogenase is an oxidoreductase enzyme present in all prokaryotes and eukaryotes that assists in ethanol fermentation and catabolism **(Karaoğlan *et al*., 2020; Simpson-Lavy and Kupiec, 2019)**. One of the most effective biotransformation processes utilized in the industry is bioreduction, mediated by alcohol dehydrogenase/reductase **(Hu *et al*., 2020)**. Ethanol is a kind of primary alcohol produced as a by-product of glycolysis under anaerobic conditions by the ADH enzyme, which also performs the reversible reaction of ethanol to acetaldehyde with a reduction of NAD^+ to NADH under aerobic conditions after glucose depletion **(De Smidt *et al*., 2008)**. However, ethanol to acetaldehyde oxidation occurs both with and without oxygen; they are most likely reactive in aerobic conditions **(Tagaino *et al*., 2019)**. Alcohol dehydrogenase is related to the oxidation of ethanol to acetaldehyde by a microbiological group that includes *Streptococci*, gram-positive aerobic bacteria, and yeast **(Sunanda *et al*., 2019)**. Yeast is a unicellular eukaryote that can tolerate high and low pH, temperature, ionic strength, heavy metals, and other external factors. Yeast cells have the potential to survive with or without nutrient supplement, however, it requires sugar-rich substrate for their growth. These special tolerance characteristics of yeast were due to its nature of sporulation. Thus the function of yeast cells persists for a long duration of time when used as a biocatalyst. *Candida albicans, Kluyveromyces lactis, K. marxianus, Saccharomyces cerevisiae, Pichia stipites, Lipomyces starkeyi, Hansenula polymorpha*, and *C. utilis* were the few yeast species containing ADH enzyme **(Karaoğlan *et al*., 2020; Suwannarangsee *et al*., 2010; Mazzoni *et al*., 1992; Verduyn *et al*., 1988; Heick and Barrette, 1970)**. Organic wastes are sugar-rich and low-cost substrates for ethanol production, as well as a desirable environment for ethanol-tolerant fermentable microorganisms **(Arshad *et al*., 2019; Tiwari *et al*., 2013)**. External factors and microbes' development usually damage fruits, and its low pH and high sugar content encourage yeast, molds, and acid-tolerant bacteria growth. Microorganisms affect the quality of fruits through the enzymatic reaction and production of lactic acid, acetic acid, ethanol, and acetaldehyde **(Erkmen and Bozoglu, 2016; Barth *et al*., 2009)**. Fresh vegetables that are less processed support the growth of yeast responsible for rotting. The spoilage due to contamination may be because of exposure to polluted environments during cultivation or poor hygienic conditions in processing **(Nguyen-the and Carlin, 2009)**. The dairy industry releases a huge volume of wastewater and active sludge. The treated sludge was used as a fertilizer, secondary feedstock for phosphate fertilizer granules, soil amendments and also possesses high respiratory, and dehydrogenases activity **(Shi *et al*., 2021; Ashekuzzaman *et al*., 2019; Frąc *et al*., 2017)**. Molasses rich in sugar spilled over soil decompose and release organic acids (acetic, propionic, butyric, and lactic) that neutralize the base and carbonates of the soil and stimulate microbial activity **(Escolar, 1967)**. Fermentable microorganisms from sugar industry's cane molasses, such as *Pichia veronae* and *Candida tropicalis* can ferment sugar into ethanol **(Hamouda *et al*., 2016)**.

The catalytic activity of ADH enzyme has been less important for the past few decades in the industry because of its poor availability, limited substrate scope, need for co-factors for activity, and low performance, researchers have overcome the limitation and now its use is currently increasing in industries **(de Miranda *et al*., 2022; Bartsch *et al*., 2020)**. Alcohol dehydrogenase was used as a biocatalyst in synthetic chemical production, for drug preparation in the pharma industry and ethanol-producing industry **(de Miranda *et al*., 2022; Bartsch *et al*., 2020; Asokumar *et al*., 2018; Shinde *et al*., 2018)**. Cofactor-dependent alcohol dehydrogenase is less stable outside the cell and more expensive for biocatalysis. Thus, whole cells are frequently used as the biocatalyst **(Hu *et al*., 2020)** in various analytical instruments like biosensors.

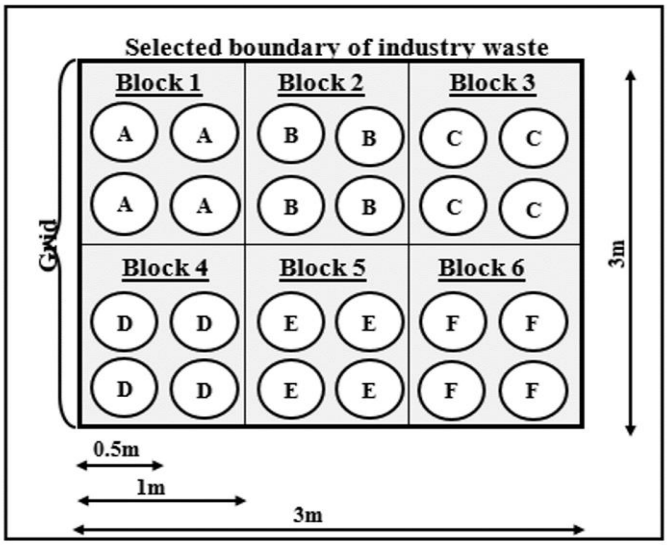
In this study, to isolate and identify the potential ethanol oxidizing strain with ADH activity from nature for industrial purposes, suitable sugar-rich cost-effective organic waste resources were chosen. The total microbial population habituated in organic waste and the specific microbial populations were able to oxidize ethanol were investigated. The overall microbial population and the total ethanol oxidizing microbial population found in the samples were hypothetically tested using Wilcoxon signed rank test.

MATERIALS AND METHODS

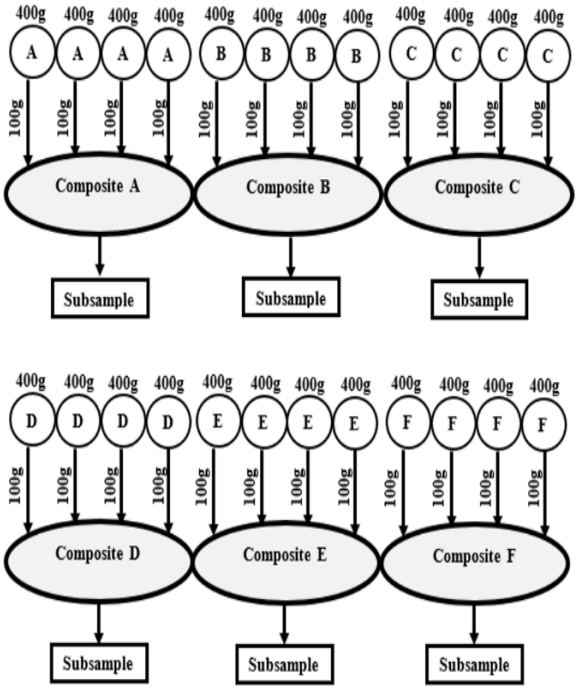
Collection of organic waste resources

Spoiled fruit waste: Spoiled fruit waste samples, including Mandarin (*Citrus reticulata*), Banana (*Musa acuminata*), Sapota (*Manilkara zapota*), Grapes (*Vitis vinifera*), Apple (*Malus domestica*), Cantaloupe (*Cucumis melo var. cantalupensis*), Pomegranate (*Punica granatum*) available in the fruit shop at

Chinnalapatti, Dindigul District, Tamil Nadu, India, were collected in an aseptic manner in a Plastic Ziplock pouch and brought to the laboratory for the further analysis.



(a)



(b)

Figure 1 (a) A systematic composite design within a grid block **(Adapted from USEPA, 2002)**. (b) Systematic grouping of each individual into composites and each composite into subsamples for analysis.

Fruit peel waste: Fruit peel waste of Pomegranate (*Punica granatum*), Cantaloupe (*Cucumis melo var. cantalupensis*), Mosambi *(Citrus limetta*) available in the fruit shop at Chinnalapatti, Dindigul District, Tamil Nadu, India, were collected in an aseptic manner in a Plastic Ziplock pouch and brought to the laboratory for further process. The obtained fruit peels were shattered into tiny shreds and weighed approximately 250g, then placed into each fermentation glass bottle in a triplet. Each of the bottles were sealed with a rubber cork hooked to a glass tube holding a rubber tube for CO_2 release, and the mouth end of the bottle was entirely sealed to sustain an anaerobic environment **(Vullo and Wachsman, 2005)**. To dissolve carbon dioxide, the bottle's rubber tube was placed in a tray of water. The bottles were entirely covered in aluminum foil to keep the environment dark, which promotes anaerobic ethanol production. The samples were fermented for approximately four months. The procedure was then carried out in aerobic conditions for a month to induce ethanol oxidizing microorganisms.

Spoiled vegetable waste: A collective of spoiled vegetable waste samples such as Peas (*Pisum sativum*), Bulb onion (*Allium cepa* L.), Small onion (*Allium parvum*), Tomato (*Solanum lycopersicum*), Carrot (*Daucus carota*), Potato (*Solanum tuberosum*), Beetroot (*Beta vulgaris*), Radish (*Raphanus sativus*) available in the vegetable market at Chinnalapatti, Dindigul District, Tamil Nadu, India, were collected in an aseptic manner in a Plastic Ziplock pouch and brought to the laboratory for the further analysis.

Raw sludge and composed sludge of dairy industry: Raw and composed sludge of dairy industry samples from SPS Dairy and Food Industry, Nagaiyargundampatti located at Dindigul District, Tamil Nadu, India were collected aseptically in an airtight Plastic Ziplock bag and brought to the laboratory for the further analysis.

Cane molasses spill of sugar industry: The boundaries of the soil amended with cane molasses spill of the sugar industry near Alanganallur, Madurai were initially identified and collected aseptically in an airtight Plastic Ziplock bag and transported to the laboratory for further examination. The soil sampling were conducted following the selected systematic composite design **(USEPA, 2002)** as shown in figure 1. A set of $n \times g$ (6×4) individual samples were collected, where n is the number of composites and g is the number of individual samples used to form each composite. The set of 6×4 individual samples was systematically grouped within grid blocks into 6 composite samples. Each composite sample was thoroughly mixed and homogenized. From each composite sample, one or more subsamples were collected and further processed for enumeration of microbial population.

Enumeration of the total microbial population of sugar-rich organic waste resources

The Total Plate Count (TPC) technique was used to count the total microbial diversity in spoiled fruits, fermented fruit peels, and spoiled vegetables. An extract was obtained by vortexing each sample individually in a pre-sterilized 0.85% saline **(Aneja, 2007)**, from which 10 mL of individual sample was aseptically suspended in 90 mL of saline (0.85%). Similarly, the Total Plate Count method was used to count the total microbial population in aseptically collected raw and composed sludge of the dairy industry as well as soil samples of cane molasses **(Carter and Gregorich, 2007; Foght and Aislabie, 2005)**. The sample should not dry out and should be analyzed within 24 h of collection. To prevent heterogeneity, 10 g of the sample was aseptically transferred to 90 mL of 0.85% saline with a sterile spatula **(Foght and Aislabie, 2005)**. All the suspensions were thoroughly mixed for 1 hour with the help of the orbital shaking incubator (REMI instrument Ltd.) before being cultured in three replicates after being serially diluted 10^{-1} to 10^{-7} on respective medium (Tab 1) using the spread plate method.

Table 1 Dilutions, incubation temperature, and time preferred for enumeration of the respective group of the microbial population by Total Plate Count method.

S. No.	Microorganism	Media for TMP	Media for TEMP[a]	Antibiotics	Dilutions	Incubation temperature	Incubation time
1.	Bacteria	TSA	Schiff`s-based TSA	-	10^{-6}	37◦C	1 day
2.	Yeast & molds	DRBC	Schiff`s-based PDA	Dichloran/ Chloramphenicol	10^{-3}	25◦C	3-5 days
3.	Yeast	YPD	Schiff`s-based YPD	Chloramphenicol	10^{-4}	25-30◦C	5 days
4.	Actinomycetes	SCA	Schiff`s-based SCA	Fluconazole/ Nalidixic acid	10^{-2}	28◦C	7 days

Legend: TMP- Total Microbial Population; TEMP- Total Ethanol oxidizing Microbial Population; Tryptic Soy Agar (TSA); Dichloran–Rose Bengal Chloramphenicol agar (DRBC); Potato Dextrose Agar (PDA); Yeast Extract-Peptone Dextrose Agar (YPD); Starch Casein Agar (SCA) **(Martin and JK, 1975; King Jr *et al*., 1979; Salfinger and Tortorello, 2015; Küster and Williams, 1964).**

[a]Respective media were supplemented with Schiff`s reagent by adapting **Conway *et al.* (1987)** method and 0.5% ethanol along with 0.1% carbon source.

Total ethanol oxidizing microbial population of sugar-rich organic waste resources

Total ethanol oxidizing microbial population viz., bacteria, yeast & molds, yeast, and actinomycetes of spoiled fruits, fermented fruit peels, spoiled vegetables, raw and composed sludge of dairy industry and soil amended with cane molasses were counted in triplicate on appropriate plates using the Total Plate Count technique **(Carter and Gregorich, 2007; Foght and Aislabie, 2005)** as described in table 1. The microbial colonies capable of oxidizing ethanol were counted using Schiff's reagent **(Conway *et al*., 1987)**. Schiff's reagent was a combination of p-rosaniline and sodium bisulphite. Conversion of ethanol into acetaldehyde in the presence of ADH enzyme was examined by preparing media with the addition of 2 mL of p-rosaniline reagent (0.0025 g mL^{-1} p-rosaniline in ethanol (95% (v/v)) and 0.025 g of sodium bisulphite to 100 mL suitable agar media under 45 ◦C that suspended with 0.5% (v v^{-1}) ethanol 0.1% (w v^{-1}) carbon source. p-rosaniline reacting with $NaHSO_3$ gets converted into the leuco form, making the media appear as a rose-colored medium. On agar plates, magenta-colored microbiological colonies indicate positive ethanol oxidation by ADH, whereas white-colored colonies suggest a negative outcome. If the organisms can oxidize ethanol to acetaldehyde, the leuco dye in the medium interacts with acetaldehyde to generate Schiff's base (red color). The reaction indicates that the equilibrium shifted ADH towards acetaldehyde synthesis that is dissolved and disseminated in an agar medium. The microbial count value of every sample measured thrice was reported as log cfu g^{-1}. Significant differences between total microbial counts and ethanol oxidizing microbial count in sugar-rich organic waste resources were determined using Wilcoxon signed rank test. This test is used to compare the two related measurements of the same sample obtained under different conditions **(Scheff, 2016)**.

Morphological, physiological, and biochemical characteristics of Yeast

For the morphologial identification the yeast colony color, texture, surface, elevation, and margin grown on Yeast-extract Peptone Dextrose (YPD) agar plate was noted. Under the microscope yeast cell shape, cell size (mean of 20 cells) measurement using ImageJ software, mode of reproduction, hyphae formation, and germ tube formation was observed and recorded according to **Kurtzman *et al*. (2011)**. Carbohydrate fermentation, growth assimilation on carbon and nitrogen compounds, and other growth tests includes growth in vitamin-free medium and vitamin requirements, growth at high osmotic pressure, growth at 37°C and 45°C, acid production from glucose, starch formation, urea hydrolysis, 1% acetic acid tolerance were the physiological and biochemical characteristics examined for the isolated yeast cells following **Kurtzman *et al*. (2011)** methods.

Qualitative screening for ADH activity by Schiff`s based basal medium for yeast under aerobic condition

The conversion of ethanol to acetaldehyde on oxidation by ADH under aerobic conditions was examined by Schiff`s based basal medium for yeast **(Conway *et al*., 1987; Wickerham, 1951)**. A loop of YPD slant culture of 24-48 hours was suspended in 2 mL of sterile distilled water in a sterile Eppendorf and evenly dispersed by the cyclic mixer (REMI equipments pvt Ltd.). The suspension was aseptically transferred into a basal medium **(Wickerham, 1951)** supplemented with 2% absolute ethanol to induce ADH expression in a serum bottle and incubated at 28°C in a shaking incubator (REMI Elektrotechnik Ltd.) with agitation 100 rpm under dark conditions for 3 days. Samples were prepared in triplicate and analyzed for ADH activity. Yeast cells grown in the basal medium were harvested by centrifugation process using a centrifuge (REMI Elektrotechnik Ltd.). The medium was taken for centrifugation process for 20 min at 3000 rpm (4°C). The collected cell debris was washed thrice and resuspended in sterile saline (0.85%). Further, the cell debris was dropped onto the aldehyde indicator plate containing Schiff`s based basal agar medium supplemented with 2% absolute ethanol and incubated at room temperature under dark condition. Development of magenta color in and around the colony indicates the conversion of ethanol to acetaldehyde which was examined at 20 minutes' interval from the time of inoculation till 12 hours and for 2 to 3 days.

Qualitative screening for ALDH activity by purple broth

The conversion of ethanol to acetic acid in presence of ALDH under aerobic conditions was examined by use of purple broth **(Pavlova *et al*., 2013; Wickerham, 1951)**. To confirm whether the yeast isolates produce ADH along with ALDH, the cell suspension of about 100 µL was added to the sterile 10 mL purple broth supplemented with 2% absolute ethanol and placed under dark condition for 2-3 days at 28°C. The color change from purple to yellow indicates the conversion of ethanol to acetic acid.

Quantitative screening of ADH activity by p-rosaniline assay

Yeast cells grown for 2 to 3 days in a basal medium supplemented with 2% ethanol were harvested using a centrifuge (REMI Elektrotechnik Ltd.) at 3000 rpm at 4°C for 20 minutes. From the collected cell suspension, a loop of culture was smeared at the center of the aldehyde indicator plate and incubated at 28°C under dark conditions for 3 days. The colony spots of each strain grown on the aldehyde indicator plate containing Schiff`s based basal agar medium supplemented with 2% absolute ethanol were recovered and transferred to 5 mL 0.85% saline solution. The suspended cells were homogenized vigorously by use of a cyclic mixer (REMI equipments pvt Ltd.) for 10 minutes. One aliquot was diluted into 1:100 dilutions in saline solution represented as 10^{-2} dilution and used to determine the colony forming unit (cfu mL^{-1}) by spread plate technique in basal agar medium supplemented with 2% absolute ethanol. Each homogenized cell suspension was made up to10 mL using absolute ethanol (99.9%) where p-rosaniline is miscible. The tubes were left over for 90 minutes at 28°C under dark conditions. After incubation, each cell suspension have been subjected to centrifugation for 5 min at 13, 000 rpm. The optical spectra were predicted by utilizing obtained supernatant. The optical spectra of leuco-rosaniline and shift base forms were determined at 420 nm and 548 nm. The appearance of high and less peak disappearance at the respective wavelength (λ) in magenta p-rosaniline form was observed using a spectrophotometer. A peaks at 420 nm and 548 nm were observed and the p-r index was calculated by the results of OD 548 nm/420 nm/ cfu mL^{-1}multiplied by the constant 10^5.

Molecular sequencing of potential ethanol-oxidizing yeast isolates

The DNA of potential ethanol oxidizing strains were extracted and the purity was checked by running gel electrophoresis (1% agarose). The 18S rRNA genome was then taken from the samples and amplified by PCR using forward (NS1) and reverse primers (NS4). The amplified genome band comprised 1050 bp validated by gel electrophoresis. To eliminate impurities, the amplified sequence was filtered. On the ABI 3730xl Genetic Analyzer, forward and reverse DNA sequencing reactions of PCR amplicons were performed with NS1 and NS4 primers using the BDT v3.1 Cycle sequencing kit (Symbiont Life Sciences). The strains' forward and reverse sequences were combined via embossing merger. The resulting sequence (18S rRNA) was BLASTed against the NCBI nucleotide database. The sequences with a high percentage of similarity were categorized using the Maximum Likelihood technique and the Kimura 2-parameter model in MEGA X **(Kumar *et al*., 2018, Kimura, 1980)**.

SEM analysis of potential ethanol oxidizing yeast isolates

The potential ethanol oxidizing yeast culture grown on YPD medium at 28°C for 2 to 3 days was centrifuged at 3000rpm for 20 minutes. The supernatant was discarded and added 4% of paraformaldehyde in 0.1M phosphate buffer (pH 7.4) to the cell debris and centrifuged again for 20 minutes at 3000rpm. Upon discarding the supernatant, the cell pellet was left without disturbing for 10 minutes after adding a small amount of 0.1M phosphate buffer (pH 7.4) **(Al Shehadat *et al*., 2018)**. The step was repeated twice, and the suspension was re-centrifuged. The collected cell biomass was dehydrated with 30% alcohol, 50% alcohol, 70% alcohol, 80% alcohol, 90% alcohol, and 100% alcohol (twice). Time taken for each dehydration process was about 10 minutes. After dehydration, cell biomass was carried out for SEM (TESCAN) analysis.

Statistical analysis

The mean and standard error of the measured value was calculated by Microsoft Excel 2016. The normality test (Kolmogorov-Smirnov and Shapiro-Wilk test) and Wilcoxon signed rank test for the measured values were analyzed using the statistics program IBM SPSS statistics 23 (SPSS Inc., Chicago, Ill., U.S.A.).

RESULTS AND DISCUSSION

Total microbial population in sugar-rich organic waste resources

Fruits are the most favorable environment for microbial proliferation. The present study examined total actinomycetes, yeast & molds, yeast and bacteria population in spoiled fruits (Mandarin, Banana, Sapota, Grapes, Apple, Cantaloupe, Pomegranate) to be 5.08±0.01, 5.76±0.03, 6.20±0.10, and 8.00±0.15 log cfu g^{-1} respectively (Tab 2). The total microbial population counted in this study is roughly in line with earlier findings **(Tango *et al*., 2018; Shokr *et al*., 2016; Akter *et al*., 2013)**. **Tango *et al*. (2018)** reported aerobic mesophilic bacteria (1.7 to 10.6 log cfu g^{-1}) and yeast & molds (5.5 to 7.9 log cfu g^{-1}) count in apple, tomato, and mandarin. In canned orange, grapes, peach, pineapple, cocktail, and cherry yeast & mold population vary from 1.0 to 1.85 log cfu g^{-1} **(Shokr *et al*., 2016)**. In this study it was reported that the bacterial and fungal populations in citrus fruits range from about 10^2 to 10^7 cfu g^{-1} and 1.2×10^3 to 3.6×10^3 cfu g^{-1} respectively **(Akter *et al*., 2013)**.

Table 2 Total microbial population in sugar-rich organic waste resources

Sugar-rich organic wastes		Microbial load (log cfu g^{-1})			
		Actinomycetes	**Yeast and molds**	**Yeast**	**Bacteria**
Fruits	Spoiled fruits	5.08±0.01	5.76±0.03	6.20±0.10	8.00±0.15
	Fermented fruit peels				
	FB1	NG	4.59±0.11	5.20±0.17	ND
	FB2	NG	5.36±0.05	6.78±0.02	7.30±0.30
	FB3	NG	4.46±0.15	6.01±0.06	7.69±0.09
Vegetables	Spoiled vegetables	4.30±0.02	6.24±0.00	6.92±0.01	8.75±0.01
Dairy Industry	Raw Sludge	4.95±0.01	ND	ND	7.63±0.06
	Composed sludge	5.07±0.01	5.14±0.02	5.80±0.04	8.00±0.01
Sugar industry	Cane molasses spill				
	A	4.70±0.02	ND	ND	7.67±0.06
	B	4.08±0.01	ND	5.26±0.24	ND
	C	4.44±0.02	4.10±0.17	ND	ND
	D	5.00±0.02	ND	ND	8.00±0.02
	E	4.68±0.05	ND	ND	ND
	F	4.14±0.06	ND	ND	ND

Legend: NG – Not Detected; The values in the tables were the mean of three replicates with ± Standard Deviation (SD)

In fruit peels, the bacterial count range from 2.96×10^5 to 3.2×10^9 and mold & yeast count range from 8.03×10^2 to 1.32×10^3 **(Gultie and Sahile, 2013)**. The fruit peels that were processed under required fermentation conditions possessing a population of yeast & molds, yeast and bacteria in the range of 4.46±0.15 to 5.36±0.05, 5.20±0.17 to 6.78±0.02, and 7.30±0.30 to 7.69±0.09 log cfu g^{-1} respectively (Tab 2) Generally, the bacterial load of fruits and vegetables was about 1.3×10^4 to 1.8×10^6 cfu g^{-1}, and fungal load ranges from 3.0×10^4 to 5.2×10^4 cfu g^{-1} **(Nwachukwu and Chukwu, 2013)**. **Chaturvedi *et al*. (2013)** examined the overall microbial population in onion (46.6±0.47 cfu g^{-1}), carrot (23.3±0.47 cfu g^{-1}), and radish, cauliflower, and peas (20.0±0.00 cfu g^{-1} each), potato (04.6±0.47 cfu g^{-1}). A group of actinomycetes, yeast & molds, yeast and bacteria populations present in a collective spoiled vegetable were 4.30±0.02, 6.24±0.00, 6.92±0.01, and 8.75±0.01 log cfu g^{-1} respectively (Tab 2) which were higher than the general count of the fresh vegetables. The dairy industry releases a huge amount of processed sludge rich in nutrients, organic compounds, minerals, metals, and microbes **(Shi *et al*., 2021; Frąc *et al*., 2017)**. **Mumtha *et al*. (2022)** evaluated the total bacterial load of dairy sludge from the dairy treatment plant to be about $3.47\pm0.12\times10^6$ cfu mL^{-1}. Actinomycetes and bacterial load of raw dairy sludge (4.95±0.01, 7.63±0.06 log cfu g^{-1} respectively) were lower than the sludge amended in soil i.e., composed sludge (5.07±0.01, 8.00±0.01 log cfu g^{-1} respectively) because the soil amended with dairy sludge enrich microbes with essential nutrients. Yeast & molds population of composed sludge ranges from 5.14±0.02 to 5.80±0.04 log cfu g^{-1} but these populations were absent in raw sludge of the dairy industry since the pH of the sludge differs according to the treatment and the environment. The total microbial groups of actinomycetes, yeast, and bacteria habited in the soil amended with cane molasses range from 4.08±0.01 to 5.00±0.02, 4.10±0.17, 5.26±0.24, and 7.67±0.06 to 8.00±0.02 log cfu g^{-1} respectively (Tab 2). **Abubaker *et al*. (2012)** evaluated that molasses samples diluted 10^{-1} and 10^{-2} have bacterial counts of 7×10^1 and 3×10^2 (cfu mL^{-1}) and yeast & mold counts of 8×10^1 and 2×10^2 (cfu mL^{-1}) respectively. The absence of microorganisms in molasses samples at greater dilutions and less microbial diversity was related to the high sugar concentration of molasses, which decreases water activity and osmotic pressure, therefore reducing the microbial population. However, in the current investigation, modest microbial development was detected.

Total ethanol oxidizing microbial population in sugar-rich organic waste resources

The investigation on ethanol oxidizing microorganisms was carried out under the idea of **Okuma *et al*. (1986)** and using the approach of **Conway *et al*. (1987)**. **Okuma *et al*. (1986)** obtained ethanol oxidizing yeast strain from a natural resource of soil or feces with both acid and ethanol tolerance. Various microbial groups capable of using ethanol and oxidizing it into acetaldehyde via ADH activity were investigated in this work. According to our knowledge, this was the first study to look at the ethanol-oxidizing microbial community in spoiled fruits, fermented fruit peels, spoiled vegetables, raw and composed sludge of the dairy industry, and cane molasses spills of the sugar industry on appropriate media using Schiff's base reaction. The toxicity of ethanol varies per microbial group, ranging from 2.5 to 70 % (v v^{-1}). Most microbes can grow at low ethanol concentrations of 0.1 to 3 % (v v^{-1}), and ethanol serves as both a carbon source and a signal that activates one or more pathways **(Dyrda *et al*., 2019; Chatterjee *et al*., 2006; Smith *et al*., 2004; Okuma *et al*., 1986)**. Distinct groups of microorganisms were studied in 0.5 % ethanol (v v^{-1}), an appropriate concentration for a range of microbial groups, to observe microorganism growth and capacity to oxidize ethanol.

Table 3 Total ethanol oxidizing microbial population in sugar-rich organic wastes

Sugar-rich organic wastes		Microbial load (log cfu g^{-1})			
		Actinomycetes	**Yeast and molds**	**Yeast**	**Bacteria**
Fruits	Spoiled fruits	4.84±0.02	5.37±0.08	5.56±0.07	7.36±0.10
	Fermented fruit peels				
	FB1	ND	4.50±0.17	5.26±0.24	ND
	FB2	ND	5.31±0.03	6.34±0.02	ND
	FB3	ND	4.26±0.24	5.20±0.17	ND
Vegetables	Spoiled vegetables	3.91±0.12	6.21±0.01	6.88±0.01	7.46±0.15
Dairy Industry	Raw Sludge	4.61±0.02	ND	ND	7.10±0.17
	Composed sludge	4.95±0.03	4.16±0.28	ND	7.28±0.06
Sugar industry	Cane molasses spill				
	A	3.42±0.06	ND	ND	ND
	B	4.62±0.03	ND	ND	ND
	C	ND	ND	ND	ND
	D	4.66±0.02	ND	ND	ND
	E	4.65±0.03	ND	ND	ND
	F	ND	ND	ND	ND

Legend: ND – Not Detected; The values in the tables were the mean of three replicates with ± Standard Deviation (SD)

Acetaldehyde is a transparent liquid with a pronounced fruity fragrance that occurs naturally in the human body and plants. Acetaldehyde is also present in products like fruits and juice, vegetables, dairy products, bread, and beverages like tea and soft drinks, beer, wine, and spirits. Microorganisms such as yeast, and bacteria that consume these products have the potential to produce acetaldehyde, which acts as a direct precursor to ethanol, and alcohol dehydrogenase accumulates during respiration **(Kalnenieks *et al*., 2019)**. Oxidation of ethanol to acetaldehyde by alcohol dehydrogenase is the main pathway of ethanol metabolism **(Edenberg, 2007)**. The ethanol oxidizing actinomycetes, yeast & molds, yeast, and bacteria populated in spoiled fruit samples were found to be 4.84 ± 0.01, 5.37 ± 0.05, 5.56 ± 0.04, 7.36 ± 0.06 log cfu/g respectively by the Total Plate Count method (Tab 3). Fermented fruit peels pose ethanol oxidizing yeast & molds population lies around 4.26±0.24 to 5.31±0.03 log cfu g^{-1} and yeast about 5.20±0.17 to 6.34±0.02 log cfu g^{-1}, there was no trace of growth of actinomycetes and bacteria on respective Schiff's based agar plates. Vegetables in a spoiled state constitute ethanol oxidizing actinomycetes, yeast & molds, yeast, and bacteria population of 3.91±0.12, 6.21±0.01, 6.88±0.01 and 7.46±0.15 log cfu g^{-1} respectively (Tab 3). Reduction, oxidation and various enzyme activities were higher in dairy sludge

(Frąc *et al*., 2017). Accordingly, in the present report, raw sludge from the dairy industry constitutes ethanol oxidizing actinomycetes and bacteria population of 4.61±0.02 and 7.10±0.17 log cfu g^{-1} whereas composed sludge comprising population load of actinomycetes, yeast & molds and bacteria of about 4.95±0.03, 4.16±0.28 and 7.28±0.06 log cfu g^{-1} respectively (Tab 3). The total ethanol oxidizing actinomycetes population habited in soil amended with cane molasses lies around 3.42 ± 0.06 to 4.66 ± 0.02 log cfu/g (Tab 3). In the case of total yeast and molds, yeast and bacteria, the population from soil amended with cane molasses was not detected. The lack of microorganisms on Schiff's-based medium showed that those microorganisms habited were unable to survive in the presence of 0.5% ethanol and lack ethanol oxidizing capacity. Ethanol was non-toxic at low doses, but only a few microbial species survived at 0.1% ethanol concentration **(Dyrda *et al*., 2019; Chatterjee *et al*., 2006)**.

Statistical Comparison between total microbial and total ethanol oxidizing microbial population in sugar-rich organic waste resources

Initially, the sample characteristics were checked for normal distribution before performing a parametric or non-parametric test. The Kolmogorov-Smirnov and Shapiro-Wilk test reported that the significant values of both total microbial population (TMP) and ethanol oxidizing microbial population (EOM`s) were less than 0.05 ($p<0.05$), indicating that the measures were not normally distributed. The differences between TMP and EOM`s of spoiled fruits, fruit peel waste, spoiled vegetables, raw and composed sludge of the dairy industry and cane molasses spill were tested hypothetically by Wilcoxon signed rank test (Tab 4).

Table 4 Relation between total microbial population and ethanol oxidizing microbial population of sugar-rich organic waste resources hypothetically tested by Wilcoxon signed rank test

Difference between total microbial population and ethanol oxidizing microbial population of sugar-rich organic wastes		Wilcoxon signed rank test statistics			
		Z	Sig. (2- tailed)	r	Effect
Fruits	Spoiled fruits	-3.059	.002	0.62	Large difference
	Fermented fruit peels				
	FB1	.000	1.000	0.00	No difference
	FB2	-2.490	.013	0.51	Large difference
	FB3	-2.666	.008	0.54	Large difference
Vegetables	Spoiled vegetables	-3.059	.002	0.62	Large difference
Dairy Industry	Raw Sludge	-2.201	.028	0.45	Medium difference
	Composed sludge	-3.062	.002	0.63	Large difference
Sugar industry	Cane molasses spill				
	A	-2.670	.008	0.55	Large difference
	B	-.943	.345	0.19	Less difference
	C	-2.214[b]	.027	0.45	Medium difference
	D	-2.201	.028	0.45	Medium difference
	E	-1.069	.285	0.22	Less difference
	F	-1.604	.109	0.33	Medium difference

Legend: Confidence level: $P<0.05$; r – Effect size

The difference between the total microbial population and ethanol oxidizing population of similar groups statistically states that all those microbes that habited naturally on the chosen sugar-rich organic waste were not able to oxidize ethanol. The microbial population of spoiled fruits, fruit peel wastes, spoiled vegetables, and composed sludge shows a large difference in their ability to oxidize ethanol whereas raw sludge and cane molasses spill shows a medium difference in their activity.

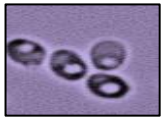
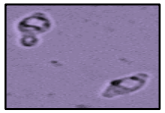
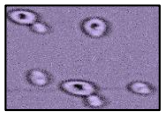
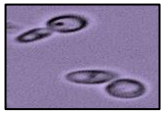
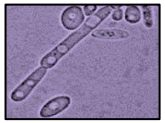
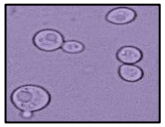
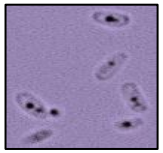
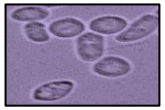
Identification of ethanol oxidizing yeast from sugar-rich organic waste resources

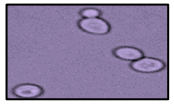
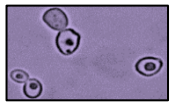
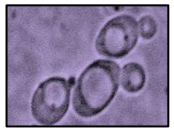
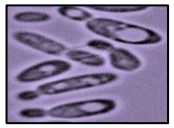
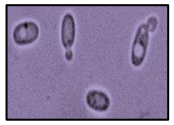
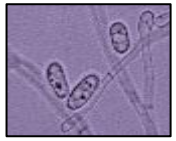
Fruits naturally contain yeasts which perform a variety of functions depending on their environment and can also cause rotting **(Gbeminiyi, 2022)**. Yeast species were diversified in different fruit samples. *Metschnikowia pulcherrima*, *Aureobasidium pullulans*, and *Hanseniaspora uvarum* were the three most prevalent yeast species in apple **(Wei *et al*., 2017; Mokhtari *et al*., 2012)**. *H. opuntiae* was found in sweet lime and fresh fruits of pineapple, watermelon, mango, banana, and orange were found to contain *Candida albicans*, *Saccharomyces cerevisiae*, *S. ellipsoideus*, and *Kloeckera apiculata* **(Gbeminiyi, 2022; Mokhtari *et al*., 2012)**. *Issatchenkia orientalis*, *C. tropicalis*, *C. viswanathii*, and *Pichia guilliermondii* were identified in Sapota, and *C. parapsilosis*, *I. orientalis* were found in wine grapes **(Rao *et al*., 2008)**.

In this study, ethanol oxidizing yeast isolated from sugar-rich organic waste materials on a YPD agar plate were identified based on colony color, texture, surface, elevation, and margin. Yeast cell shape, cell size, mode of reproduction, hyphae formation, and germ tube formation were all examined and evaluated under a microscope and recorded in table 5. In citrus fruits, *Cryptococcus spp.*, *Sporidiobolus pararoseus*, *Rhodotorula spp*., *C. adeliensis*, *Pichia guilliermondii*, *Candida membrafaciens* were the most common yeast isolates. A mixture of fruit salad frequently encountered yeast *Pichia sp*., *Candida sp.*, *Rhodotorula sp*, and *Debaryomyces sp*. **(Tournas *et al*., 2006)**. Citrus fruits (oranges, limes, mandarins) rotting was caused by *Kloeckera apiculata, Candida guilliermondii, C. stellata, Pichia kluyveri, P. fermentans*, and *Geotrichum candidum* **(Mokhtari *et al*., 2012; Spencer *et al*., 1992)**. The current study spotted that the five species of *Pichia*, as well as the *Candida sp*. *Hanseniaspora sp.* and *Lachancea sp*. of spoiled fruits such as mandarin, banana, sapota, grapes, apple, cantaloupe, and pomegranate, grow in presence of ethanol and functionally reduce ethanol to acetaldehyde (Tab 5). *Hanseniaspora uvarum* and *Pichia kudriavzevii* were isolated from pomegranate peels by **Utama *et al.* (2022)**. One of the possible causes of Pomegranate spots and spoiled symptoms was *Candida freyschussii* **(Patel *et al*., 2021)**. **Peng *et al.* (2018)** recovered *Hanseniaspora opuntiae* and *Meyerozyma guilliermondii* from the peel and pulp of mandarin fruit using an enrichment process. The fruit peels of pomegranate, cantaloupe, and sweet lime (mosambi) fermented under favorable fermentation conditions yielded two species of *Pichia* and genera *Torulaspora sp.* and *Debaryomyces sp*, able to oxidize ethanol.

Microorganisms naturally found in vegetables and develop rot under ambient conditions such as aeration, pH, temperature, and moisture. The yeast strains obtained from spoiled fermented vegetables were *Saccharomyces bayanus* and *S. unisporus* ***(Savard et al., 2002)***. Microorganisms, particularly yeast (*Saccharomyces cerevisiae*) and molds (*Rhizopus stolonifer*, *Alternaria alternata*, *Aspergillus niger*), cause onion bulb rot. Mold, *Aspergillus niger*, had the greatest percentage distribution in decaying onion bulbs, whereas yeast, *Saccharomyces cerevisiae*, had the lowest **(Samuel and Ifeanyi, 2015)**. The deterioration of grated raw carrots was caused by *Candida parapsilosis*, *C. tropicalis*, and *C. sake* **(Babic *et al*., 1992)**. *Geotrichum candidum* has been discovered as the cause of watery rot and soft rot on a carrot, cucumbers, tomatoes, and pumpkins **(Kim *et al*., 2011)**. Tomato spoilage was caused by the life processes of bacteria, yeast, and molds. Microorganisms consume the sugars in vegetables quickly and convert them to acetic acid, lactic acid, alcohol, and carbon dioxide **(Ogunniyi and Oladeojo, 2011)**. The isolates from freshly grated beetroots were dominated by Gram-negative bacteria and yeasts, with no signs of molds **(Lopez Osornio and Chaves, 1997)**. The current investigation established that two species of *Pichia* and the genus *Debaryomyces sp*. retrieved from spoiled vegetable waste including peas, bulb onion, small onion, tomato, carrot, potato, beetroot, and radish had the potential to oxidize ethanol. *Candida albicans*, *C. krusei*, *Lactobacillus plantarum,* and *L. casei* were identified from the dairy plant's activated sludge pond **(Keffala *et al*., 2017)**. *Trichosporon loubieri*, *Geotrichum* sp., and *T. montevideense* were obtained from a dairy industry's biological wastewater treatment facility **(Monteiro *et al*., 2010)**. After washing and fogging disinfection with alkyl amine/peracetic acid, the yeast *Rhodotorula mucilaginosa* was isolated from dairy production units **(Bore and Langsrud, 2005)**. The composed sludge of the dairy industry possesses ethanol-oxidizing yeast *Candida sp.* and *Magnusiomyces sp*. Dairy sludge, since rich in organic matter on applying to the soil enhances the nitrogen and phosphorous content as well as increases the microbial catabolic and respiratory activity **(Oszust *et al*., 2015)**. Hence, the present work obtained effective ethanol oxidizing yeast from composed sludge rather than raw sludge of dairy industry.

Table 5 Morphological Characterization of Yeasts

Strain no.	Source	Microscopic view of yeast isolates	Colony morphology					Microscopic observation				Genus identification
			Texture	Color	Surface	Elevation	Margin	Cell Shape	Cell size (µm)	Mode of reproduction	Hyphae formation	Germ tube formation	
FYES1	Spoiled fruits		Mucoid	White to cream	Smooth	Flat	Entire	Elongated ovoid	6.00 ± 0.38	Multilateral budding	Pseudohyphae	NF	*Pichia sp.*
FYES2			Butyrous	White	Smooth	Elevated	Entire	Ovoid	3.53 ± 0.23	Budding	True hyphae	NF	*Candida sp.*
FYES3			Mucoid	White	Smooth	Elevated	Entire	Ovoid	4.68 ± 0.39	Bipolar budding on a wide base	Absence of true hyphae	NF	*Hanseniaspora sp.*
FYES4			Butyrous	White	Smooth	Flat	Entire	Ovoid	5.68 ± 0.38	Budding	Absence of true hyphae	NF	*Pichia sp.*
FYES5			Butyrous	Tannish-white	Dull	Flat	Entire	Ovoid	5.43 ± 0.47	Budding on a narrow base	Absence of true hyphae	NF	*Pichia sp.*
FYES6			Butyrous	White to cream	Smooth	Elevated	Fringed	Elongated ovoid	7.55 ± 0.86	Sexual reproduction – by fusion	Pseudohyphae	NF	*Pichia sp.*
FYES7			Mucoid	White	Rough	Dom-like	Undulate	Sphere	6.10 ± 0.52	Budding	Absence of true hyphae	NF	*Lachancea sp.*
FYES8			Viscous	White	Smooth	Raised	Entire	Elongated ovoid	3.67 ± 0.28	Budding	Absence of true hyphae	NF	*Pichia sp.*
FPYES1	Fermented fruit peels		Mucoid	White	Rough	Flat	Entire	Ovoid	5.85 ± 0.67	Budding by fission	Absence of true hyphae	NF	*Pichia sp.*
FPYES2			Butyrous	White to cream	Glistening	Raised	Entire	Sphere	5.41 ± 0.60	Budding by binary fission	Absence of true hyphae	NF	*Torulaspora sp.*

FPYES3	Spoiled vegetables		Mucoid	White White to cream	Smooth	Raised	Entire	Sphere	5.49 ± 0.75	Budding	Absence of true hyphae	NF	*Pichia sp.*
FPYES4			Butyrous	White	Smooth	Flat	Entire	Ovoid	4.86 ± 0.53	Multilateral budding	Absence of true hyphae	NF	*Debaryomyces sp.*
VYES1			Viscous	White	Glistening	Raised	Entire	Ovoid	7.23 ± 0.79	Budding	Absence of true hyphae	NF	*Debaryomyces sp*
VYES2			Mucoid	White	Smooth	Raised	Ridged	Elongated ovoid	6.03 ± 0.63	Budding on a narrow base	Absence of true hyphae	NF	*Pichia sp.*
VYES3			Mucoid	White	Smooth	Depressed in the center	Entire	Elongated ovoid	5.70 ± 0.66	Sexual reproduction – by fusion	Pseudohyphae	NF	*Pichia sp.*
CSYES1	Composed sludge of dairy industry		Butyrous	White to dark yellow	Glistening	Raised	Entire	Ovoid	5.60 ± 0.57	Budding	Absence of true hyphae	NF	*Candida sp.*
CSYES2			Friable	White	Folded	Conical	Fringed	Elongated ovoid	10.12 ± 1.81	Budding	True hyphae	NF	*Magnusiomyces sp.*

Legend: NF – No formation; ±SD; FYES1-8 represents yeast strain from spoiled fruits; FPYES1-4 represents yeast strain from fruit peel waste; VYES1-3 represents yeast strain from spoiled vegetables; CSYES1&2 represents yeast strain from composed sludge

Physiological and biochemical characteristics of ethanol oxidizing yeast from sugar-rich organic waste resources

A Carbohydrate fermentation test was used to study the ability of the yeast strain to ferment sugars in the medium (Tab 6). *Pichia* species ferment all the tested sugars, glucose, sucrose, maltose, lactose, fructose, arabinose, and xylose and few of the species do not ferment all sugars. *Candida* species ferment only glucose, sucrose, and fructose among tested sugars. *Debaryomyces* species ferment glucose, sucrose, and fructose. *Hanseniaspora sp.* ferments only glucose and fructose. *Lachancea sp.* ferment glucose, sucrose and fructose. *Torulaspora sp*. ferment glucose, sucrose and fructose. *Magnusiomyces sp.* ferments all tested sugars except lactose. Since there is no reversible reaction during testing, the absorption of carbon and nitrogen sources is the primary test for yeast characterization rather than carbohydrate fermentation. Carbon, nitrogen, and vitamin ingestion as the sole source for the metabolic process were examined in an assimilation test. If the test was positive, the analyte remained positive, making the assimilation test more useful in both fermentative and non-fermentative yeast **(Devadas *et al*., 2017; Pincus *et al*., 2007; Wickerham and Burton, 1948)**. *Pichia sp*. can consume carbon from compounds such as β – fructosides, β – galactosides, α- glucosides, pentoses, simple alcohols, and polyols. *Candida sp*. consumes carbon from β – fructosides, α- glucosides, simple alcohols, and polyols. β – fructosides, α- glucosides, simple alcohols, polyols, and acids are among the carbon sources that *Debaryomyces* species ingest. All that *Hanseniaspora sp.* can digest are the carbon α- glucosides (maltose). Carbon β – fructosides and α- glucosides are assimilated by *Lachancea* species. β – fructosides, β – galactosides, α- glucosides, pentoses, and simple alcohols are among the carbon sources that *Magnusiomyces sp.* assimilates (Tab 6). *Hanseniaspora sp.* assimilates exclusively nitrogen aspargine while *Pichia sp*., *Candida sp*., *Debaryomyces sp.*, and *Magnusiomyces sp.* assimilate nitrogen ammonium sulphate and aspargine. In the presence of nitrogen *Lachancea sp.*, and *Torulaspora sp*. were exhibit modest growth. Additionally, the yeast *Pichia sp*., *Candida sp*., *Debaryomyces sp.*, *Magnusiomyces sp.* assimilate vitamins. In the presence of vitamins, *Hanseniaspora sp.*, *Lachancea sp.*, *Torulaspora sp*. were display modest growth.

Acetic acid is a microbial metabolic product that inhibits microbial growth. Stress resistance to acetic acid in yeast is crucial in commercial bioprocesses. Acetic acid is either a physiological substrate or a stressor in budding yeast **(Guaragnella and Bettiga, 2021, Palma *et al*., 2018)**. Osmotic pressure kills cells, thus isolated yeast strains that could resist osmotic pressure were examined using an osmotic pressure tolerance test under high concentrations of glucose and sodium chloride. Sugar and NaCl had osmotic responses that were comparable to water activity, but NaCl is harmful because K^+ may replace Na^+ in biomolecules, which causes the cells into detoxification mode **(Varelas *et al*., 2017; Hohmann, 2002)**. *Pichia sp*., *Candida sp*., and *Magnusiomyces sp.* tolerate acetic acid as well as osmotic pressure, but *Debaryomyces sp.*, *Hanseniaspora sp*., *Lachancea sp*., and *Torulaspora sp*. can tolerate only osmotic pressure. Acid is produced from glucose by the yeast *Pichia sp*., *Debaryomyces sp*., *Lachancea sp.*, and *Torulaspora sp*. *Candida sp*., and *Magnusiomyces sp.* both had positive urease test results. *Lachancea sp.* and *Magnusiomyces sp* provide positive results for the starch formation test. Few yeast strains most likely thrived well at 35, 40, and 45°C. The species of *Meyerozyma*, *Saccharomyces*, *Candida*, *Torulaspora*, and *Pichia* most probably resist high temperatures **(Phong *et al*., 2019)**. *P. kudriavzevii*, a thermotolerant yeast, can grow well at high temperatures. The strains of *Pichia sp*., *Candida sp*., *Debaryomyces sp.*, and *Magnusiomyces sp.* described in this study can thrive at high temperatures between 37 and 45°C (Tab 6).

Table 6 Physiological and biochemical characteristics of Yeast

Physiological and biochemical characteristics		***Pichia sp.***	***Candida sp***	***Hanseniaspora sp.***	***Pichia sp***	***Pichia sp***	***Pichia sp***	***Lachancea sp.***	***Pichia sp***	***Pichia sp***	***Torulaspora sp.***	***Pichia sp***	***Debaryomyces sp.***	***Debaryomyces sp.***	***Pichia sp***	***Pichia sp***	***Candida sp***	***Magnusiomyces sp.***
Carbohydrate fermentation																		
Glucose		+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*
Sucrose		+*	+*	-*	+*	+*	+*	+*	+*	-*	+*	+*	+*	-*	+*	+*	+*	+*
Maltose		+*	-*	-*	-*	+*	+*	-*	-*	-*	-*	-*	-*	+*	+*	-*	-*	+*
Lactose		+*	-*	-*	-*	+*	+*	-*	-*	-*	-*	-*	-*	-*	+*	+*	-*	-*
Fructose		+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*	+*	+*	+*	+*
Arabinose		+*	-*	-*	-*	+*	+*	-*	-*	-*	-*	-*	-*	-*	+*	+*	-*	+*
Xylose		+*	-*	-*	-*	+*	-*	-*	-*	-*	-*	-*	-*	+*	-*	+*	-*	+*
Carbon assimilation test																		
Hexose-based carbon sources																		
β - fructosides	Sucrose	+++	++	NA	+	+++	+++	+	+	NA	+++	+	+	+++	+++	+++	++	+++
β - galactosides	Lactose	+	NA	NA	NA	+++	+++	NA	NA	NA	NA	NA	NA	+++	+++	+++	NA	+++
α- glucosides	Maltose	+	NA	++	+	+++	+++	+	NA	NA	NA	NA	NA	+++	+++	+++	NA	+++
	Soluble starch	+++	+++	NA	+++	NA	+++	NA	NA	NA	NA	NA	+++	+++	+++	+++	+++	+++
Pentoses	D-Xylose	++	NA	NA	NA	+++	NA	NA	++	NA	NA	NA	NA	+++	NA	+++	NA	+++
	L-Arabinose	+	NA	NA	NA	+++	+++	NA	NA	NA	NA	NA	NA	+++	+++	+++	NA	+++
Alcohols																		
Simple alcohols	Methanol	+++	+++	+	+++	+	+	+	+	+	+	+	+++	+++	+++	+	+++	+++
	Ethanol	+++	+++	+	+++	++	+++	+	+	+	+	+	+++	+++	+++	+++	+++	+++
Polyols	Glycerol	+++	+++	+	+++	+	+++	+	+	+	+	+	+++	++	++	++	++	++
	D-Mannitol	++	+++	+	+++	+	+++	+	+	+	+	+	+++	+++	+	+	+	+
Acids	DL-Lactate	+++	++	+	+++	+	+++	+	+	+	+	+	+++	++	++	++	++	++
	Citrate	++	++	+	++	++	+++	+	+	+	+	+	++	++	++	+	+	++
Carbon-free medium		++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Nitrogen assimilation test																		
Potassium nitrate		++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	++
Ammonium sulfate		+++	+++	++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
Aspargine		+++	+++	+++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
Nitrogen free medium		++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Vitamins																		
Biotin		+++	++	++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
Calcium panthothenate		+++	++	++	+++	++	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++
Inositol		+++	++	++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++

Riboflavin	+++	+++	++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
Thiamine hydrochloride	+++	++	++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
Vitamin free medium	+++	+++	++	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
Other tests																	
Urease test	+*	-*	-*	-*	-*	+*	-*	-*	-*	-*	-*	-*	+*	+*	+*	-*	+*
Starch formation	+*	-*	-*	-*	-*	-*	+*	-*	-*	-*	-*	-*	-*	-*	-*	-*	+*
Acid production from glucose	+*	-*	-*	+*	-*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	+*	-*
Growth at 37°C	+++	ND	ND	+++	ND	+++	ND	ND	ND	ND	ND	ND	+++	+++	+++	+++	+++
Growth at 45°C	++	ND	ND	++	ND	++	ND	ND	ND	ND	ND	ND	++	++	++	++	++
Acetic acid tolerance (1%)	ND	ND	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	+++	+++	+++	+++
Osmotic pressure tolerance																	
Growth at 50% glucose	+++	+++	+++	+++	+++	+++	++	++	ND	ND	++	+++	+++	+++	+++	+++	+++
Growth at 60% glucose	+++	+++	+++	+++	+++	+++	++	++	ND	ND	+	+++	+++	+++	+++	+++	+++
Growth at 10% sodium chloride and 5% glucose	+++	+++	+++	+++	+	+++	+	+	ND	ND	++	+++	+++	+++	+++	+++	+++

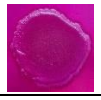
Legend: (+*) – Positive; (-*) – Negative; NA- No Assimilation (+++) – Excellent growth; (++) – Moderate growth; (+) – Poor growth; (ND) – Not Detected

Alcohol dehydrogenase and acetaldehyde dehydrogenase activity of ethanol oxidizing yeast strains

Acetaldehyde is a microbial bio-product, which is a metabolic catalyst of ethanol in ethanologenic microbes and it is very significant in the chemical industry **(Kalnenieks *et al.*, 2019)**. Microbial cells cultured in ethanol includes ADH enzyme, which may oxidize ethanol to acetaldehyde, as confirmed qualitatively by Schiff reagent (red color formation). Schiff reagent is a combination of p-rosaniline and sodium bisulphite ($NaHSO_3$) that is commonly used to detect aldehydes, sugars on glycoproteins following periodic acid oxidation, and microbes that produce aldehydes in a broth test. The isolated ethanol oxidizing yeast strains were tested for ADH activity using Schiff's based basal agar medium in this investigation. ADH activity was regarded as positive in magenta-colored colonies with dye diffusion and negative in pink-to-white colonies with halo zones (Tab 7).

Table 7 Qualitative analysis of ADH and ALDH activity of yeast strains

Strain no.	Strain name.	Magenta-colored yeast colonies	ADH activity	ADH reaction time	ALDH activity	ALDH reaction time
FYES1	*Pichia sp.*		+	20 minutes	+	24 hours
FYES2	*Candida sp.*		+	30 hours	+	72 hours
FYES3	*Hanseniaspora sp.*		+	24 hours	+	48 hours
FYES4	*Pichia sp.*		+	31 hours	+	72 hours
FYES5	*Pichia sp.*		+	20 minutes	+	72 hours
FYES6	*Pichia sp.*		+	20 minutes	+	72 hours
FYES7	*Lachancea sp.*		+	1hour 20 minutes	-	ND
FYES8	*Pichia sp..*		-	ND	-	ND
FPYES1	*Pichia sp.*		+	1 hour	-	ND
FPYES2	*Torulaspora sp.*		+	3 hours 40 minutes	-	ND
FPYES3	*Pichia sp.*		+	24 hours	-	ND
FPYES4	*Debaryomyces sp.*		-	ND	-	ND
CSYES1	*Candida sp.*		+	20 minutes	-	ND
CSYES2	*Magnusiomyces sp.*		+	24 hours	-	ND
VYES1	*Debaryomyces sp*		+	20 minutes	-	ND

VYES2	*Pichia sp.*		+	20 minutes	+	72 hours
VYES3	*Pichia sp.*		+	20 minutes	+	24 hours

Legend: (+) – Positive; (-) – Negative; ND – Not detected

Conway *et al.* (1987) were the first to develop an aldehyde indicator plate by incorporating Schiff reagent into a nontoxic solid medium. Para-rosaniline of Schiff reagent on reacting with $NaHSO_3$ attains leuco form giving the medium a rose color. The medium's leuco form acts as a sink, forming Schiff's base and a strongly magenta color when it reacts with acetaldehyde, suggesting that the ADH enzyme changed the balance toward acetaldehyde generation **(Tribelli *et al*. 2015)**. The aldehyde dehydrogenase activity's consumption of aldehydes and low levels of native alcohol dehydrogenase may contribute to the moderate color development in cell colonies on an aldehyde indicator plate **(Conway *et al*., 1987)**. The *adhB* gene encodes the ADH II enzyme, **Conway *et al*., (1987)** cloned the *adhB* gene in *Escherichia coli* from *Zymomonas mobilis*. The *adhB* expression activity was detected directly by using an aldehyde indicator plate (Schiff's agar plate). **Pavlova *et al*. (2013)** tested acetaldehyde formation by *Streptococcus gordonii* adh mutants from ethanol directly on the PBB Schiff's agar plate. **Tribelli *et al*. (2015)** examined ethanol dehydrogenase activity in *Pseudomonas extremaustralis* on LB-Schiff`s based agar medium supplemented with ethanol and sodium octanoate. **Rosca *et al*. (2016)** screened yeast strains for acetaldehyde production by Schiff's reagent, which was validated by the creation of violet coloration. The present work screened selected ethanol oxidizing strains on Schiff's based basal agar medium supplemented with 2% ethanol. *Pichia sp., Candida sp., Hanseniaspora sp., Lachancea sp., Torulaspora sp.*, and *Magnusiomyces sp.* can grow well in presence of 2% ethanol as well as oxidize ethanol into acetaldehyde effectively. The reaction time for ADH ranges from 20 min to 2 h at 37°C **(Conway *et al*., 1987)**. However, the present testing reveals that three species of *Pichia* and one species of *Candida* shows ADH reaction within 20 min under the dark condition at 28°C whereas other yeast strain shows delay in their reaction time.

Alcohol dehydrogenase (ADH) reduces ethanol to acetaldehyde and further to acetic acid ALDH. The full metabolization of ethanol into acetic acid occurs in microorganisms that have both active ADH and ALDH enzymes **(Tribelli *et al*., 2015)**. The purple broth changes into yellow color as ethanol is converted into acetic acid in the medium, which lowers the pH. If microbes only contain the ADH enzyme and not the ALDH enzyme, they only create aldehyde without further conversion to acid from ethanol. **Alam and Talukder (2015)** used 5% ethanol to screen acetic acid-generating bacteria on yeast-extract peptone glycerol (YPG) culture supplemented with bromocresol purple as a pH indicator. Bacteria that can make acetic acid will have halos or a yellow tint around the colony. To assess the synthesis of ALDH and ADH enzymes, isolated ethanol oxidizing yeast were tested for acetic acid production in purple broth with 2% ethanol. Six species of *Pichia*, one species of *Candida*, and *Hanseniaspora sp.* contain ALDH activity, and the color changes from purple to yellow, but other yeast strains with ADH activity show a negative result and remain purple. *Pichia sp.* and *Candida sp.* produce acetic acid between 24 to 72 hours but *Hanseniaspora sp* produces it within 48 hours (Tab 7).

Quantitative measurement of ADH activity of ethanol oxidizing yeast

The direct evaluation of ADH activity on an acetaldehyde indicator plate is a qualitative report. To pick out the effective ethanol oxidizing yeast strain among the isolated strains, adapted **Tribelli *et al*. (2015)** p-rosaniline assay method. To investigate any probability of oxidation reaction in LB infused with sodium octanoate extensively, **Tribelli *et al*. (2015)** performed a p-rosaniline experiment that revealed ADH activity. Magenta bacterial spot of *Pseudomonas extremaustralis* grown at 8 and 30°C in agar media plates treated with sodium octanoate showed a p-r of 5.97 ± 1.46 and 2.82 ± 1.33. In the present study, the magenta bacterial spot of FYES5 (*Pichia* sp.), and FYES6 (*Pichia* sp.) strains from spoiled fruits shows a p-r of 2.64±0.40 and 3.79±0.54, VYES2 (*Pichia* sp.) strain of spoiled vegetables and CSYES1 (*Candida sp.*), CSYES2 (*Magnusiomyces sp.*) from dairy industry grown on the aldehyde indicator plate, Schiff's based basal agar medium supplemented with 2% absolute ethanol shows a p-rosaniline index (p-r) of 3.73±0.69, 2.28±0.24, and 2.87±0.58 respectively at 28°C (Fig 2).

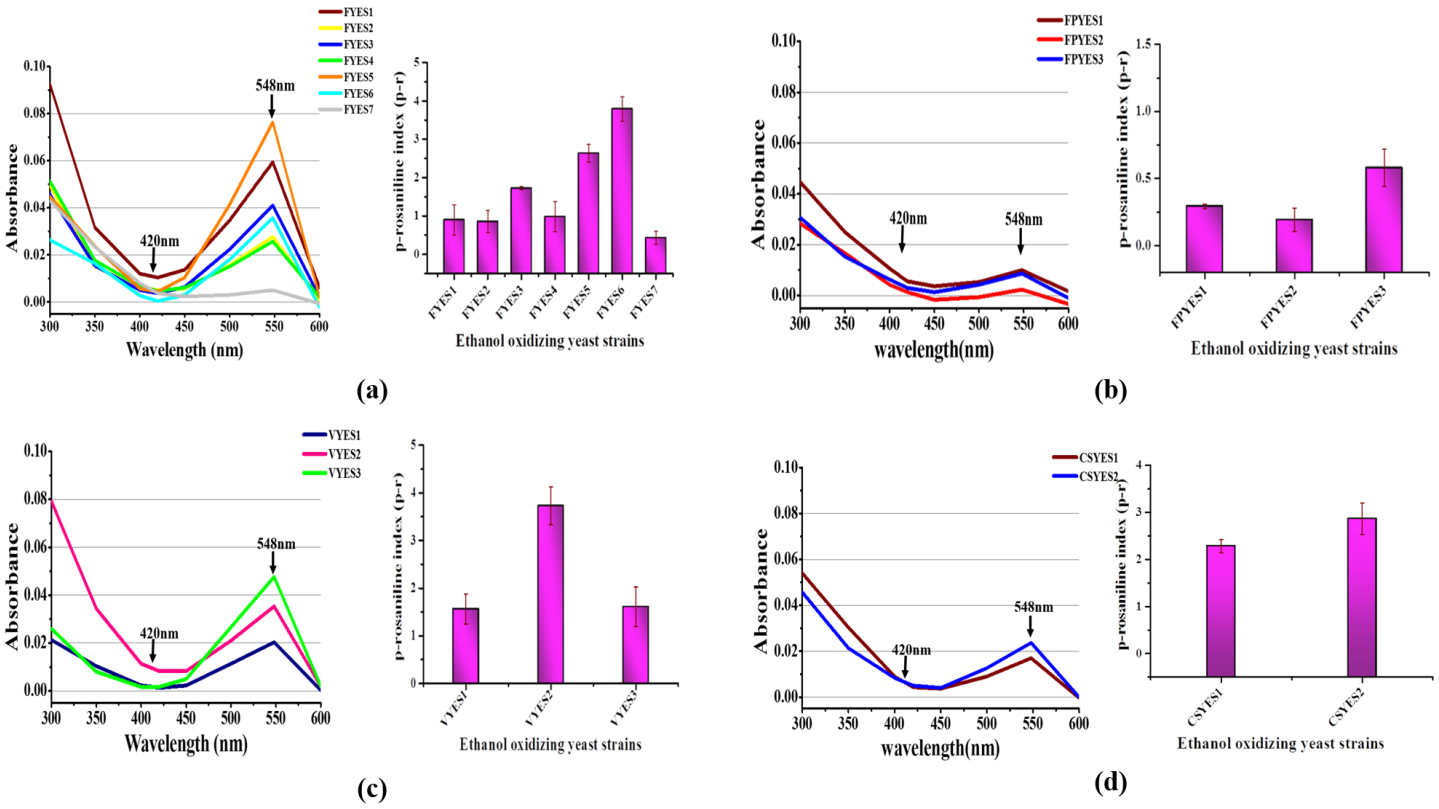


Figure 2 Absorption spectra of ethanol oxidizing yeast stains showing a minimal leco-rosaniline at 420nm and maximal shift base-rosaniline at 540nm in magenta rosaniline form and quantitative measurement of ADH activity by p-rosaniline assay: (a) Absorption spectrum and determination of p-rosaniline index of ethanol oxidizing yeast strains of spoiled fruits sample (b) Absorption spectrum and determination of the p-rosaniline index of ethanol oxidizing yeast strains of fermented fruit peel (c) Absorption spectrum and determination of the p-rosaniline index of ethanol oxidizing yeast stains of spoiled vegetable sample (d) Absorption spectrum and determination of the p-rosaniline index of ethanol oxidizing yeast strains of composed sludge sample from the dairy industry.

Molecular identification of potential ethanol oxidizing yeast strains

The potential ethanol-oxidizing yeast strain depending on morphological, physical, and biochemical characteristics were identified as *Pichia sp.*, *Candida sp.*, and *Magnusiomyces sp.* according to **Kurtzman *et al.* (2011)**. These isolated strains were identified as *Pichia Kluyveri* FYES5 (GenBank Accession no: OM731927), *Pichia Kudriavzevii* FYES6 (GenBank Accession no: OM736143), *Candida mengyuniae* CSYES1 (GenBank Accession no: OM736145), *Magnusiomyces capitatus* CSYES2 (GenBank Accession no: ON076010), and *Pichia Kudriavzevii* VYES2(GenBank Accession no: ON076032) based on phylogenetic analysis by *18S* rRNA gene sequencing. The Maximum Likelihood technique and the Kimura 2-parameter design were used to infer the phylogenetic origins **(Kimura, 1980)**. Figure 3 depicts the branch with the maximum log probability (-2380.52). The subset of branches with similar taxa grouped collectively can be seen adjacent to the branches. The first heuristic search nodes were constructed dynamically by executing the Neighbor-Join and BioNJ algorithms on a matrix of pairwise distance assessed by using the Maximum Composite Likelihood (MCL) approach and selecting the configuration with the greatest log-likelihood level. This study included 25 different genetic variants. The final dataset had 843 locations in total. The phylogenetic analysis was conducted through using MEGA X **(Kumar *et al.*, 2018)**.

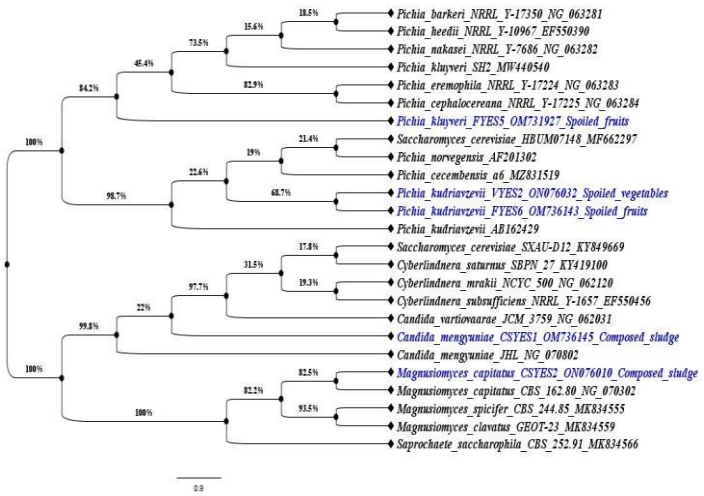


Figure 3 Phylogenic tree showing evolutionary relationship between the ethanol oxidizing yeast strains *Pichia Kluyveri* FYES5, *Pichia Kudriavzevii* FYES6, *Pichia Kudriavzevii* VYES2, *Candida mengyuniae* CSYES1, and *Magnusiomyces capitatus* CSYES2 based on 18S rRNA gene sequence. The species showing a high similarity percentage to the identified strains were retrieved from the BLASTn database and grouped by the Maximum Likelihood method and Kimura 2-parameter model.

Pichia kluyveri is a non-fermentable yeast that generates a lot of volatile chemicals. In wine production, P. kluyveri is one of the reasons for fragrance, glycerol, and ethanol synthesis, as well as inhibitory activity. Aromatic compound production by P. kluyveri is heavily regulated by culture medium, agitation, and temperature **(Ciani et al., 2022; Yıldırım, 2021; Vicente et al., 2021; Méndez-Zamora *et al.*, 2021)**. *P. kudriavzevii is a yeast with teleomorphic characteristics. P. kudriavzevii, on the other hand, is a non-pathogenic and industrially significant yeast that is identical to Candida krusei, with infections varying depending on the environment from which the strain was isolated* **(Douglass *et al.*, 2018; Cooper, 2011)**. *P. kudriavzevii* also acts as a plant growth promotor in the soil and forms biofilm **(Ramya *et al.*, 2021)**. **Elahi and Rehman (2018)** stated that *P. kudriavzevii* has both Pyruvate decarboxylase activity and alcohol dehydrogenase activity and can utilize hemicellulose and produces ethanol effectively. **Chen *et al.* (2009)** isolated *Candida mengyuniae* as a metsulfuron-methyl-resistant yeast strain from metsulfuron-methyl-contaminated soil. **Alcoba-Florez *et al.* (2011)** reported that *C. mengyuniae causes* intravenous catheter-associated fungemia. *Magnusiomyces capitatus* (*Geotrichum capitatum*) is a teleomorph yeast mainly isolated from woody areas, human & animal feces, and hot regions **(Cooper, 2011)**. *M. capitatus* produces lipase and grow well in Olive mill wastewaters under controlled conditions **(Salgado *et al.*, 2020)**. *M. capitatus* derived from the trash of the palm oil industry produces extracellular and cell-bound lipase and is used as a biocatalyst in biodiesel production **(Baloch *et al.*, 2021)**. Microorganisms *Geotrichum candidum, G. capitatum, G. eriense, G. fermentans, G. fragrans, G. klebahnii*, and *G. rectangulatum* were able to produce ADH II with a high range of substrate specificity. This kind of enzyme can produce and reduce ketones and alcohol effectively. **Fraser (1997)** extracted (S)-1,3-butanediol dehydrogenase enzyme from *G. candidum* which has high stereoselectivity, oxidizing 1,3-butanediol, phenylethanol, 3-hydroxybutyric acid ester and 2- octanol. **Yamada-Onodera *et al.* (2007)** purified ADH from *G. capitatum* which reduces *N*-benzyl-3-pyrrolidinone in presence of cofactor NAD^+. Effectively oxidize primary and secondary alcohol rather (S)-N-benzyl-3-pyrrolidinol. According to the literature, this is the first investigation to be published that the strain *P. kluyveri, P. kudriavzevii, C. mengyuniae, and M. capitatus* has specific ethanol oxidizing activity, i.e, conversion of ethanol into acetaldehyde by enzyme alcohol dehydrogenase.

Morphological confirmation of potential ethanol oxidizing yeast strains by SEM analysis

The SEM image as shown in figure 4, clearly reveals the specific shape of five selected ethanol oxidizing isolates. *Pichia kluyveri* was oval, both *Pichia kudriavzevii* FYES6 and VYES2 were elongated ellipsoidal, *Candida mengyuniae* cells were ellipsoidal, and *Magnusiomyces capitatus* are cylindrical conidiogenous long cells with expanded hyphae (Fig 4). The size of these isolated cells were 5.43 ± 0.47, 7.55 ± 0.86, 6.03 ± 0.63, 5.60 ± 0.57 and 10.12 ± 1.81 µm respectively (Tab 5).

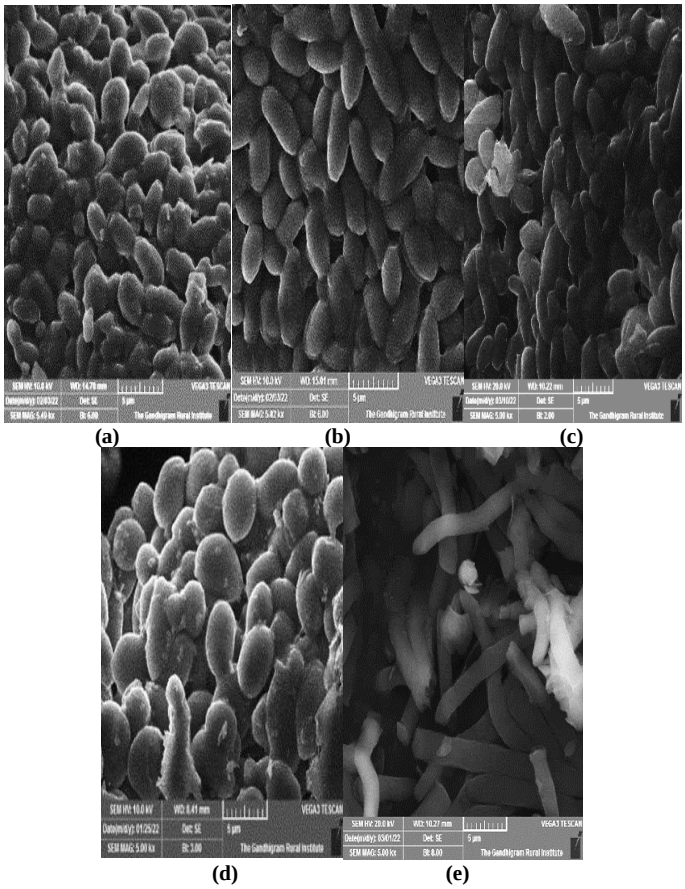


Figure 4 SEM image of potential ethanol oxidizing yeast strain: (a)*Pichia Kluyveri*_FYES5; (b) *Pichia Kudriavzevii* _FYES6; (c) *Pichia Kudriavzevii* _VYES2; (d) *Candida mengyuniae*_CSYES1; (e) *Magnusiomyces capitatus*_CSYES2

The shape of the reported yeast isolates of the current study was relevant to the previous report, *Pichia kluyveri* was slightly ovoid and 2–10 µm in size, and were hard to differentiate apart from the shape of *S. cerevisiae* or *S. ellipsoideus* cells. *P. kluyveri* form pseudohyphae in agar medium and broth medium and also produce hat-shaped ascospores **(Vicente *et al.*, 2021)**. *Pichia kudriavzevii* was an oval or elongated ellipsoidal shape, formed pseudohyphae, and possesses the multilateral type of budding **(Bshabshe *et al.*, 2019)**. *Candida mengyuniae* cells were ellipsoidal to elongate and 2.5–5.0 × 3.0–7.2 µm in size, appearing singly, in pairs, or groups. Budding was multilateral **(Chen *et al.*, 2009)**. *Magnusiomyces capitatus* were cylindrical conidiogenous long cells with expanded hyphae branched at acute angles, main branches are 7 µm wide, and lateral branches 2.5–3.5 µm wide. Blastoconidia was clavate with a truncate base, 2.5–3.5×7–10 µm **(De Hoog & Smith, 2011)**.

CONCLUSION

The current study shows that microbes adapted to sugar-rich organic waste resources can oxidize ethanol. The study statistically assured that not all the population of microbes habited in sugar-rich organic waste resources oxidize ethanol, only few of the isolates able to oxidize. Based on Schiff`s base reaction qualitatively the study explored that those species belonging to *Pichia, Candida, Hanseniaspora, Lachancea, Torulaspora,* and *Magnusiomyces* grow well in 2%

ethanol as well as oxidize ethanol into acetaldehyde. The existence of ADH enzyme was confirmed in ethanol oxidizing yeast isolates by p-r index and potential strains were identified as *Pichia kluyveri*, *Pichia kudriavzevii*, *Candida mengyuniae*, and *Magnusiomyces capitatus* using 18S rRNA sequencing. These strains can be researched further for ADH-related applications such as pharmaceutical production, ethanol synthesis, aldehyde or ketone reduction, alcohol (primary and secondary) oxidation, and bio-sensing. In this study, it was demonstrated for the first time that *Candida mengyuniae*, a metsulfuron-methyl-resistant yeast strain, has unsuspected alcohol dehydrogenase activity that needs to be clarified more to allow subsequent research.

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