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ISOLATION OF *SACCHAROMYCES CEREVISIAE* FROM PINEAPPLE AND ORANGE AND STUDY OF METAL'S EFFECTIVENESS ON ETHANOL PRODUCTION

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In view of the anticipated shortage of the traditional supplies of fossil fuels, there is a great deal of interest in the production of ethanol as an alternative biofuel in recent years. The main objective of this research work was to isolate and characterize stress tolerant, high potential ethanol producing yeast strains from various fruit peel. Two yeast isolates from pineapple (Pa) and orange (Or) have been isolated, characterized on the basis of morphological and physic-chemical characters and optimized on ethanol producing capability using sugarcane molasses as substrate. Ethanol production percentage was estimated by Conway method. Isolates were thermotolerant, pH tolerant, ethanol tolerant as well as osmotolerant. They were resistant to Chloramphenicol (30 µg/disc) and Nalidixic acid (30 µg/disc). The isolates showed no killer toxin activity against *E. coli*. The highest production capacity of the yeasts was found to be 7.39% and 5.02% for Pa and Or, respectively, at pH 5.0, 30 °C temperature in media with an initial reducing sugar concentration of 6.5% for Pa and 5.5% for Or (shaking). Addition of metal ions increased the rate of ethanol production highest to 10.61% by KH_2PO_4. This study revealed that indigenous yeast isolates could be used to benefit the fuel demand and industrial alcohol industries.

Keywords: yeast, *Saccharomyces cerevisiae,* fermentation, metal's effectiveness

Abbreviations: Pa = yeast isolates from pineapple; Or = yeast isolates from orange; pH = negative logarithm of hydrogen ion concentration; DNS = 3,5-dinitro salicylic acid; OD = optical density; YPD = yeast extract peptone dextrose; YMM = yeast maintenance media; $CuSO_4$ = copper sulfate; $K_2Cr_2O_7$ = potassium dichromate; $MgCl_2$ = magnesium chloride; $CaCl_2$ = calcium chloride

Introduction

The energy crisis necessitates studying and discovering new processes involved in the production of renewable compounds as alternative energy sources among which fermentation of ethanol using renewable resources represents a significant alternative. In fact, ethanol is being widely investigated as a renewable fuel source because, in many respects, it is comparable to gasoline fuel [1]. This situation has led many countries including Brazil to use ethanol as a fuel especially from food crop. In Bangladesh, sugarcane resource can be used to produce a variety of commercial products that can be marketed domestically, regionally, and internationally. In economic and environmental terms, the three products that have special significance are sugar, ethanol, and electricity. Bangladesh, through its potential in developing large sugarcane production, can develop a proper strategy of using ethanol as a fuel especially from cane sources. Yeast alcohol is the most valuable product for the biotechnology industry with respect to both value and revenue. Approximately 80% of ethanol is produced by anaerobic fermentation of various sugar sources by *Saccharomyces cerevisiae*, and the technology has undergone significant improvements during the last decade although

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profit margins are not attractive. Contamination, limited availability of raw materials, and proper design of fermentation process are the major limitations causing reduced alcohol yields and quality. In view of the importance of alcohol as an alternative for liquid fuel, several investigations in ethanol fermentations are currently reported. The price of the sugar source is an important parameter when considering the overall economy of production, and it is of great interest to optimize alcohol yields to ensure an efficient utilization of carbon sources [2, 3]. Combustion of ethanol results in a relatively low emission of volatile organic compounds, carbon monoxide, and nitrogen oxides. The emission and toxicity of ethanol are lower than those of fossil fuels such as petroleum, diesel etc. [4, 5]. Molasses, a cheap byproduct of sugarcane or sugar beet processing industries, is widely used as a raw material for the production of ethanol for economic reasons, and different strains of yeast have been selected for efficient ethanol production [6–8]. Obtained after sugar beet processing, molasses contains about 60% sucrose and 40% other components. The non-sucrose substances include inorganic salts, raffinose, ketose, organic acids, and nitrogen-containing compounds. Molasses is used in the production of baker's yeast, ethanol, citric, lactic, and gluconic acids, as well as glycerol, butanol, and acetone production, as an ingredient of mixed feeds and in the production of amino acids [9, 10]. The fermentative yeast *S. cerevisiae* is largely used in ethanol production using such renewable biomass as sugar cane or sugar beet molasses as the main carbon source [11, 12]. In the present study, these types of *S. cerevisiae* were selected as production microorganisms on account of their commercial availability and an extensive application in food industry. After detailed characterization and optimization of physicochemical parameters for ethanol production of the selected yeast isolates, these can be used as a potential strain for ethanol production industrially as expected results.

Materials and methods

In this study, two yeasts were isolated from pineapple and orange peel. Based on their colony characteristics (white and creamy texture), ovoid microscopic shape, and the presence of budding pattern (multipolar), all isolates that were found belongs to *saccharomyces* type unicellular ascomycete according to standard methods [13, 14]. The isolates were tested for fermentation of carbohydrates; isolate Pa was able to utilize 6 sugars out of the seven tested sugars, and Or isolate was able to utilize 5 sugars, indicating that they were diverse in sugar utilization.

Isolation and screening of stress tolerance

Fruit samples (pineapple and orange) were collected from local market, and their peels were extracted. One gram of sample was soaked in 250 ml yeast maintenance media (YMM) broth at 30 °C for 3 days. After 3-day incubation, each 100 µl of suspension was spread on a plate containing YMM, which consisted of 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, and 15 g agar, in 1 l water, initial pH 5.5 [15], and was incubated aerobically at 30 °C for 3 days. Single colony formed was picked, and the cells were observed under a microscope.

Maintenance of culture

The culture of yeast was maintained by subculturing on slants using YMM, incubating for 48 h at 30 °C, and, thereafter, storing in a refrigerator at −4 °C for future use [16].

Growth of isolates

In the present study, the morphology of cells of the selected isolates and their appearance on *yeast extract peptone dextrose* (YPD) agar media was examined [15–19]. The medium was autoclaved at 121 °C and 15 psi and poured on a petri dish and cooled. After cooling, the plates were inoculated with 48-hour-old yeast strain and incubated at 30 °C for 48 h. The following features of the appearance of cultures were recorded: texture, color, and surface of colonies.

Selected isolates Pa and Or were cultured in YPD liquid medium. The medium was autoclaved at 121 °C and 15 psi and cooled. Fifteen milliliters of the medium was distributed into McCartney tubes, then inoculated with a half loop-full of 48-hour-old selected yeast strain, and incubated at 30 °C for 3 days. The culture was examined for the growth visually on the surface of YPD liquid medium, and the shape of cells, by compound microscope (Olympus, Japan) [20].

Fermentation of carbohydrates

The ability to ferment seven different carbohydrates (glucose [dextrose], maltose, sucrose, lactose, trehalose, fructose, and xylose) of selected isolates was examined anaerobically and was assessed by looking for the formation of gas (CO_2) in Durham tube and color change of the fermentation media. Yeast fermentation broth was modification of media developed by Wickerham; the color of the medium changed from red to yellow due to the formation of acids and gas produced [21]. In addition, the medium was prepared from 10 g peptone, 5 g NaCl, phenol red (Indicator/Chromogen), and 5 g carbohydrate and by making the volume up to 1000 ml with distilled water. A volume of 15 ml aliquots dispensed in different McCartney tubes were autoclaved 121 °C and 15 psi, after cooling the media, and then inoculated with 48-hour-old selected yeast strain and fermented for 72 h.

Detection of thermotolerance

YPD liquid medium was used for detecting thermotolerance and growth in liquid media of selected yeast isolates [22]. The medium was autoclaved and cooled. Ten milliliters of the medium was distributed into McCartney tubes and then inoculated with a half loop-full of 48-hour-old selected yeast isolates. The initial optical density of each tube was recorded on a spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25 °C, 30 °C, 32 °C, 37 °C, 40 °C, and 44 °C for 3 days for observing thermotolerance of the yeast strain. The increase in optical density in a tube was recorded as evidence of growth. Moreover, growth on YPD agar media at 25 °C, 30 °C, 32 °C, 37 °C, 40 °C, and 44 °C was also observed to ensure thermotolerance of the strain.

Detection of ethanol tolerance

The medium for the detection of ethanol tolerance of thermotolerant yeast was modified [23]. YPD liquid medium was used for detecting yeasts for ethanol tolerance. The medium was autoclaved and cooled. One milliliter of various concentrations of absolute ethanol was varied from 5% to 20% (5%, 6%, 9%, 10%, 12%, 15%, 18%, and 20%) (*v*/*v*) and added to different flask of the same medium to constitute varying percentages of ethanol differing by 1–3% (*v*/*v*) from one flask to the others. Fifteen milliliters of the medium was distributed into the 125-ml flask and then inoculated with a half loop-full of selected yeasts. The initial optical density of each flask was read off on a spectrophotometer at 600 nm against the medium as the blank. All cultures were incubated at 30 °C for 48 h. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts.

Growth at different pH in liquid media

YPD liquid medium was used for detecting the ability to grow at different pH [22]. The medium was autoclaved and cooled. YPD broth was prepared at pH 2–10. Each McCartney contained 15 ml of YPD media with different pH, and blank media were used as a control. Then, each was inoculated with a half loop-full of the yeast cell (the initial optical density at 600 nm was measured) and then incubated at 30 °C for 48 h. After 48 h, cell density was further recorded at 600 nm for growth.

Osmotolerance observation

YPD broth was prepared containing 6%, 9%, 12%, 15%, 18%, and 20% of NaCl. Each McCartney contained 15 ml of YPD liquid media with appropriate concentration of salt, and blank media were used as a control. Then, each was inoculated with a half loop-full of the yeast cell (the initial optical density at 600 nm was measured) and then incubated at 30 °C for 48 h. After 48 h, cell density was further recorded at 600 nm [22].

Chloramphenicol and nalidixic acid resistance test

Sensitivity to chloramphenicol and nalidixic acid was evaluated by growing isolates in malt extract agar (MEA) in the presence of 30 µg/ml discs. Sample was collected using the method of Kirby et al. [24]. In this study, YPD agar medium was used for detecting yeasts for chloramphenicol and nalidixic acid resistance. Chloramphenicol and nalidixic acid disc (30 µ/l) was placed into the center of the already inoculated petri dish. Then, the plate was kept at 30 °C for growing. The zone of inhibition authenticates the resistance.

Determination of killer toxin production capacity of yeasts

First, the target bacterium was inoculated in nutrient broth for 24 h, and 10 ml molten agar (3%) was added to the broth. Then, it was poured on a plate and allowed to solidify. Yeast was streaked on a plate in 2–3 rows and incubated at 25 °C for 24 h. Clear zone of inhibition was observed to recognize the yeast strain as "killer strain" [25, 26].

Preparation of yeast cell suspension

A 48-hour-old slant culture of yeast cell was added aseptically to autoclaved molasses fermentation media (10 ml), and the tube was shaken gently to form a homogeneous suspension.

Fermentation of molasses

Fermentation was carried out in Erlenmeyer conical flasks. A total of 250 ml fermentation media were taken into 500-ml Erlenmeyer flasks and added the homogenous suspension of yeast was inoculated into the media in an aseptic condition. The flask was cotton plugged and incubated at different temperatures in an incubator under nonshaking and shaking condition [27].

Estimation of reducing sugars

The reducing substance (sugar) obtained due to the enzymatic reaction was determined by dinitrosalycylic acid (DNS) method [28]. A double beam UV scanning spectrophotometer was used for measuring absorbance. Reducing sugar contents before fermentation and after fermentation

were determined by taking 1.0 ml diluted solution (1 ml sample in 100 ml distilled water) with 3.0 ml of DNS reagent in a test tube. Blank containing 1.0 ml of distilled water and 3.0 ml of DNS was run parallel. The tubes were heated in a boiling water bath for 15 min. After cooling the tubes at room temperature, 8 ml of distilled water was added to each and absorbance was noted at 540 nm using the spectrophotometer. Reducing sugar concentration was estimated from the standard curve *(Fig. 1)*.

Estimation of ethanol: Conway method

Ethanol was determined by redox titration. In this method [29], ethanol is oxidized to ethanoic acid when ethanol reacted with excess of potassium dichromate solution (0.05 N) and unreacted dichromate is then determined by adding potassium iodide (50% KI) solution which is oxidized by the potassium dichromate. Potassium iodide reacts with potassium dichromate and creates iodine. Then, the iodine is titrated with a standard solution of sodium thiosulfate (0.1 N). The titration reading is used to calculate the ethanol content after fermentation. One milliliter fermented solution was diluted 250, 500, and 1000 times with distilled water, and each 1 ml diluted solution was taken as a sample. A Conway unit was used for ethanol detection by this procedure. One milliliter potassium dichromate was placed into the Conway unit center, and sample was placed around the center. The Conway unit was covered by a glass plate for 24 h for reaction. The water and ethanol slowly evaporate, come in contact with potassium dichromate, and then oxidized. More ethanol evaporates until, eventually, all the ethanol from the fermented dilute solution has left the sample and reacted with the dichromate. One Conway unit was used as a blank, and in that unit, 1 ml distilled was used as a sample.

Effect of sugar concentration

To study the effect of sugar concentration on ethanol production by *S. cerevisiae*, the production media was prepared by diluting molasses to reducing sugar concentration 4.5%, 5.50%, 6.0%, 6.50%, 7%, and 7.5%, and fermentation was carried out in a volume of 250 ml media in a 500-ml conical flask. A 48-hour-old inoculum of yeast was added to the medium. Samples were withdrawn at different times and estimated for residual sugars [28] as well as ethanol content in the media.

Effect of pH

To study the effect of pH on ethanol production by *S. cerevisiae*, fermentation media with different reducing sugar concentration were used for the production of ethanol. Fermentation was carried out at pH 5.0 in a volume of 250 ml media in a 500-ml conical flask. A 48-hour-old inoculum of yeast was added to the medium. Samples were withdrawn at different times and estimated for residual sugars [28] as well as ethanol content in the media.

Effect of metals

To determine the effect of metals on ethanol production, $MnCl_2$, $CuSO_4$, KH_2PO_4, and $ZnSO_4$ were added at different concentrations in 250 ml fermentation media and the fermentation was carried out in different 500-ml conical flasks in the presence of different metals. In every conical flask (0.10 g/250 ml), metals were added into the fermentation media. Ethanol production was observed at 30 °C, pH 5.0, and using the initial reducing sugar concentration of 7.5% in shaking condition [30].

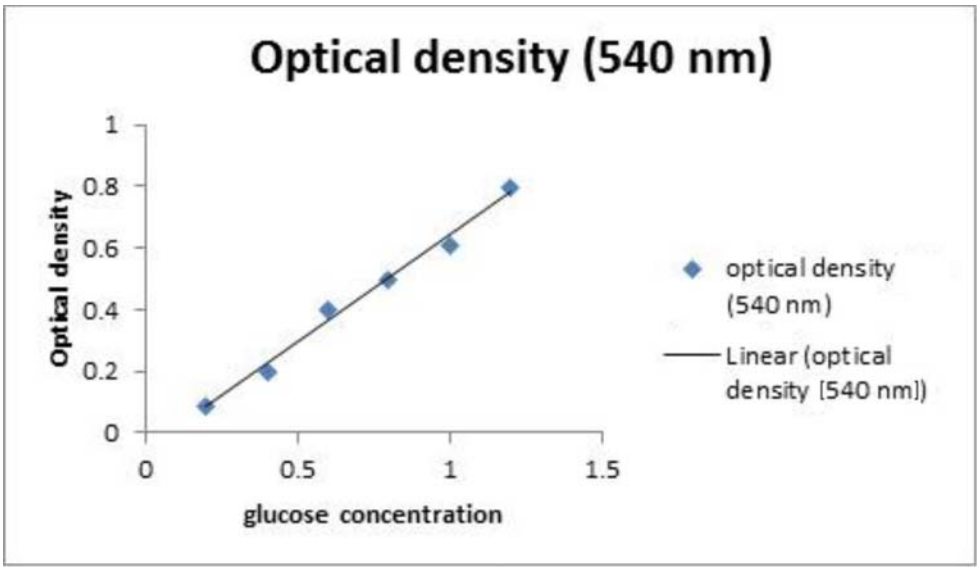

Fig. 1. Reducing sugar concentration was determined from the standard curve of glucose and multiplied by dilution factor. A standard curve of the glucose prepared was shown

Effect of agitation

To study the effect of agitation on ethanol production by *S. cerevisiae*, fermentation media with different reducing sugar concentration were used. The molasses was diluted, and fermentation was carried out at pH 5.0 and temperatures 25 °C, 30 °C, and 35 °C in a volume of 250 ml media in a 500-ml conical flask. A 48-hour-old inoculum of yeast was added to the medium, and then, flasks containing the same sugar concentration were kept in both shaking (120 rpm) and nonshaking condition, and thereby, ethanol content was measured after 48 h. To determine the optimum pH for fermentation, the solutions were kept consecutively at pH 5 and 6.

Results

Growth of isolates

The morphology of the vegetative cells of yeast was observed grown in liquid and on solid media according to the standard method [21, 31] *(Fig. 2a)*. After 3 days of incubation at 30 °C, heavy, dry climbing pellicles were formed on the surface of YPD liquid broth medium.

Microscopic observation

The cell morphology of the ethanol-tolerant Pa and Or isolates observed under the compound microscope showed an ovoidal to elongate having single, pairs, or triple budding cells. The budding stage of the isolates was observed after incubation for 48 h at 30 °C and confirmed to be yeast *(Fig. 2b)*.

Fermentation of carbohydrates

In this study, *S. cerevisiae* showed variation in terms of utilization of seven different sugars (*Tables 1* and *2*). The strain utilized glucose (dextrose), sucrose, maltose, fructose, xylose, and trehalose but failed to grow on lactose. Change in media color confirmed the fermentation. The results were recorded after 48-hour inoculation.

Tolerance of selected yeast strains to different environmental conditions

Effect of temperature on growth

Six YPD agar plates containing yeast cell were incubated for 48 h at 25 °C, 30 °C, 35 °C, 37 °C, 40 °C, and

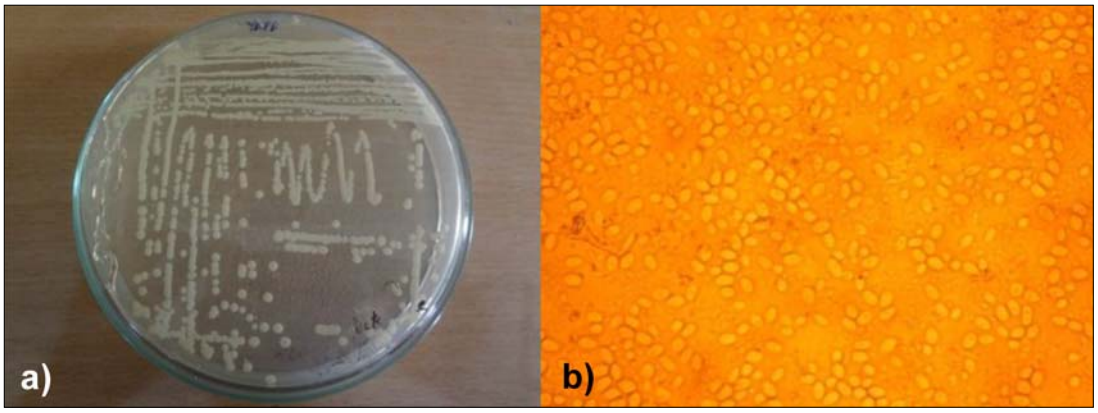

Fig. 2. a) Yeast isolates formed butyrous and smooth white raised colonies on YPD agar medium. Six petri dishes were used to confirm the identification. b) The cell morphology of Pa isolate under compound microscope (40×). The strain reproduces vegetatively by budding

Table 1. Carbohydrates fermentation by Pa isolate

Carbohydrate	Before fermentation	After fermentation	
	Color of the medium	Color of the medium	Gas production
Glucose	Pink	Yellow	Yes
Sucrose	Pink	Yellow	Yes
Maltose	Pink	Yellow	Yes
Lactose	Pink	No change	No
Fructose	Pink	Yellow	Yes
Xylose	Pink	Yellow	Yes
Trehalose	Pink	Yellow	Yes

Table 2. Carbohydrates fermentation by Or isolate

Carbohydrate	Before fermentation	After fermentation	
	Color of the medium	Color of the medium	Gas production
Glucose	Pink	Yellow	Yes
Sucrose	Pink	Yellow	Yes
Maltose	Pink	Yellow	No
Lactose	Pink	No change	No
Fructose	Pink	Yellow	No
Xylose	Pink	No change	No
Trehalose	Pink	Yellow	Yes

45 °C. Both isolates were able to grow at 25–44 °C. To confirm the results obtained from solid media, thermotolerance were repeated in liquid media (*Tables 3* and *4*).

From the tables, it is evident that both the yeast isolates were highly thermotolerant as Or showed ability to grow up to 40 °C and Pa showed ability to grow up to 44 °C.

Effect of ethanol concentration in the media on growth

The isolate was selected for the screening of yeasts tolerant to ethanol. It was observed that the Or isolate can grow in up to 12% and Pa isolate can grow in up to 15% ethanol containing liquid YPD media (*Tables 5* and *6*). Maximum growth for both isolates was seen in 5% ethanol containing media. Growth was reported by change in optical density

Table 3. Growth of Pa isolate in liquid media at different temperatures

Temperature (°C)	Initial (OD)	After 48 h of growth (OD)
25	0.269	1.435
30	0.346	1.537
32	0.322	1.730
37	0.441	2.236
40	0.461	2.079
44	0.482	0.848

Table 4. Growth of Or isolate in liquid media with different temperatures

Temperature (°C)	Initial (OD)	After 48 h of growth (OD)
25	0.356	1.112
30	0.211	0.717
32	0.255	1.488
37	0.559	2.420
40	0.519	2.171
44	0.515	0.501

Table 5. Growth of Pa isolate in liquid media with different ethanol conc.

Ethanol percentage	Initial (OD)	After 48 h of growth (OD)
5	0.328	1.851
6	0.187	1.542
9	0.242	1.406
10	0.336	1.314
12	0.184	0.736
15	0.317	0.339
18	0.287	0.282
20	0.220	0.210

Table 6. Growth of Or isolate in liquid media with different ethanol concentration

Ethanol percentage	Initial (OD)	After 48 h of growth (OD)
5	0.254	0.687
6	0.236	0.557
9	0.298	0.530
10	0.250	0.456
12	0.229	0.236
15	0.223	0.163
18	0.174	0.151
20	0.259	0.153

Table 7. Growth of Pa isolate in liquid media with different pH

pH	Initial (OD)	After 48 h of growth (OD)
2	0.349	0.868
3	0.430	1.813
4	0.390	1.893
6	0.405	1.789
7	0.199	1.442
8	0.200	1.460
9	0.279	1.210
10	0.418	1.425

Table 8. Growth of Or isolate in liquid media with different pH

pH	Initial (OD)	After 48 h of growth (OD)
2	0.359	0.863
3	0.382	1.487
4	0.274	1.370
6	0.304	1.489
7	0.197	1.320
8	0.229	1.265
9	0.217	1.215
10	0.425	1.207

(OD) after 48 h at 5%, 8%, 10%, 12%, 15%, 18%, and 20% of ethanol containing liquid media.

Effect of pH on growth

In the study, both Pa and Or isolates showed ability to grow in a wide range of pH, from 2 to 10. Both isolates withstood pH 10, but pH 4.0 for Pa isolate and pH 6 for Or isolate showed optimum result. After 48 h, cell density was recorded at 600 nm and given gradually for evidence of growth (*Tables 7* and *8*). The data were in agreement with the results from Silva et al. [32]**.**

Effect of salt concentration in the media on growth

The Pa isolate successfully tolerated up to 9% sodium chloride salt in the media, and this was an index of osmotolerance. However, at higher concentration, the growth reduced. The Or isolate showed optimum at up to 12% sodium chloride salt concentration (*Tables 9* and *10*).

Killer toxin test

Killer toxin test was carried out for both the Pa and Or strains against *Escherichia coli*. Presence of clear inhibition zone in a petri dish was considered to be the key evidence of this property, which was not observed. Investigations revealed that the occurrence of the killer phenotype in yeast is widespread in alcohol fermentations for beverage production such as in breweries, wine, plants, and, more recently, in sugar-producing plants [26]. The concentration of sensitive cells influenced the sensitivity of the bioassay. In addition, the composition of the medium and buffer solution may have contributed to the sensitivity of the assay [33].

Table 9. Growth of Pa isolate in liquid media with different salt concentration

NaCl percentage	Initial (OD)	After 48 h of growth (OD)
6	0.232	1.372
9	0.219	0.648
12	0.267	0.443
15	0.202	0.229
18	0.269	0.263
20	0.254	0.237

Table 10. Growth of Or isolate in liquid media with different salt concentration

NaCl percentage	Initial (OD)	After 48 h of growth (OD)
6	0.204	0.877
9	0.237	0.246
12	0.242	0.248
15	0.197	0.154
18	0.290	0.184
20	0.257	0.197

Antibiotic resistance test by chloramphenicol and nalidixic acid

Antibiotic resistance test showed that both the strains were resistant to chloramphenicol and nalidixic acid. The effects of nalidixic acid appeared to be principally, if not exclusively, on mitochondrial processes: the drug affects the biosynthesis of respiratory enzymes, especially of cytochrome oxidase, and, depending on the concentration used, it has been reported either to bring about or interfere with the (ethidium bromide-mediated) induction of the mitochondrial *petite* mutation [34].

Ethanol production under shaking and nonshaking condition

Based on the results of tolerance and ethanol production at different sugar concentrations at 30 °C temperature and pH 5 in shaking and nonshaking condition, Pa isolate showed better results than Or. Thus, a series of experiments had been conducted at different physico-chemical conditions to optimize ethanol production by the Pa isolate. To know the optimum shaking, condition experiments were carried out at different rpm (120, 130, and 140) and it was showed that the optimum result was 7.02% ethanol production at 130 rpm (*Figs. 3* and *4;*

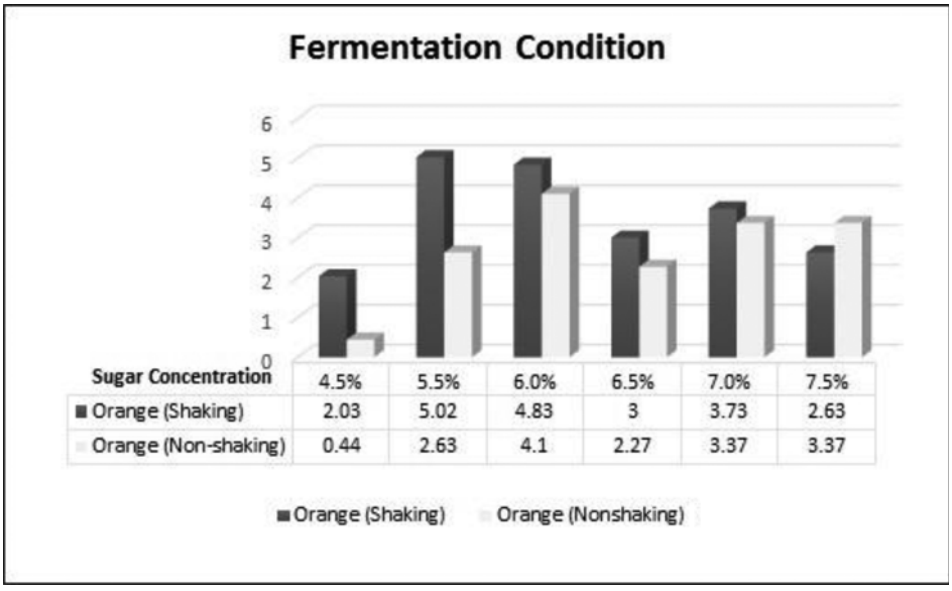

Fig. 3. Ethanol production under shaking and nonshaking condition by Or isolate at initial pH 5 and with different sugar concentration. The highest production recorded (5.02%) at sugar conc. of 5.5%. The data shown are the average of three individual experiments under given condition

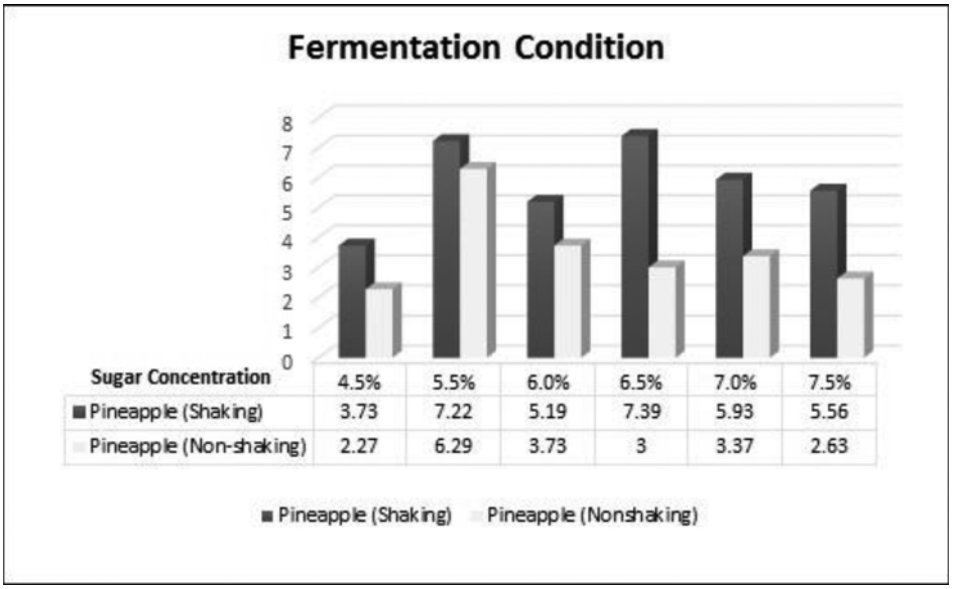

Fig. 4. Ethanol production under shaking and nonshaking condition by Pa isolate at initial pH 5 and with various sugar conc. The highest production recorded (7.39%) at sugar conc. of 6.5%. These data are the average of three individual experiments, where the condition kept unchanged

Tables 11, 12, and *13*). The production rate is in agreement with the previous studies [35, 36]. In a recent study, Thammasittirong et al. reported 8.6% (*v*/*v*) production for wild-type strain [37].

Pa isolate at 96-hour fermentation with 6.5% glucose and pH 6

At 30 °C temperature, using initial reducing sugar concentration of 6.5% of the fermentation media, and pH 5.0, maximum ethanol production was 4.83% at 48 h in shaking condition (120 rpm) *(Fig. 5)*.

Pa isolate at 96-hour fermentation with 7.5% glucose and pH 6

Under shaking condition (130 rpm), at 30 °C temperature, using initial reducing sugar concentration of 7.5% of the fermentation media, and pH 6.0, maximum ethanol production was 7.02% at 96 h *(Fig. 6)*. The condition stands

Table 11. Alcohol production comparison at different parameters

Glucose concentration (%)	Orange (shaking)	Orange (nonshaking)	Pineapple (shaking)	Pineapple (nonshaking)
4.5	2.03	0.44	3.73	2.27
5.5	5.02	2.63	7.22	6.29
6	4.83	4.1	5.19	3.73
6.5	3	2.27	7.39	3
7	3.73	3.37	5.93	3.37
7.5	2.63	3.37	5.56	2.63

Table 12. Glucose depletion comparison at different parameters

Glucose concentration (%)	Orange (shaking)	Orange (nonshaking)	Pineapple (shaking)	Pineapple (nonshaking)
4.5	1.56 → 0.56	1.73 → 1.37	0.33 → 0.25	1.47 → 1.01
5.5	3.19 → 1.73	4.05 → 3.99	0.63 → 0.62	3.60 → 1.35
6	0.82 → 0.72	4.89 → 3.27	0.85 → 0.75	3.84 → 1.72
6.5	4.28 → 1.80	5.55 → 5.41	0.81 → 0.60	4.77 → 3.42
7	4.29 → 3.44	6.35 → 5.85	1.09 → 0.92	5.21 → 3.97
7.5	6.32 → 4.48	6.68 → 4.82	2.66 → 1.02	5.54 → 3.84

Table 13. pH fall comparison at different parameters

Glucose concentration (%)	Orange (shaking)	Orange (nonshaking)	Pineapple (shaking)	Pineapple (nonshaking)
4.5	4.61 → 4.73	4.69 → 4.65	4.80 → 4.83	4.72 → 4.73
5.5	4.56 → 4.50	4.73 → 4.77	5.18 → 5.27	4.84 → 4.98
6	4.86 → 5.07	4.87 → 4.85	5.24 → 5.18	4.90 → 4.95
6.5	4.75 → 4.57	4.88 → 4.85	5.20 → 5.24	4.93 → 4.97
7	4.87 → 4.61	4.93 → 4.89	5.09 → 5.37	5.00 → 5.05
7.5	4.64 → 4.62	4.75 → 4.76	4.87 → 5.15	4.81 → 4.91

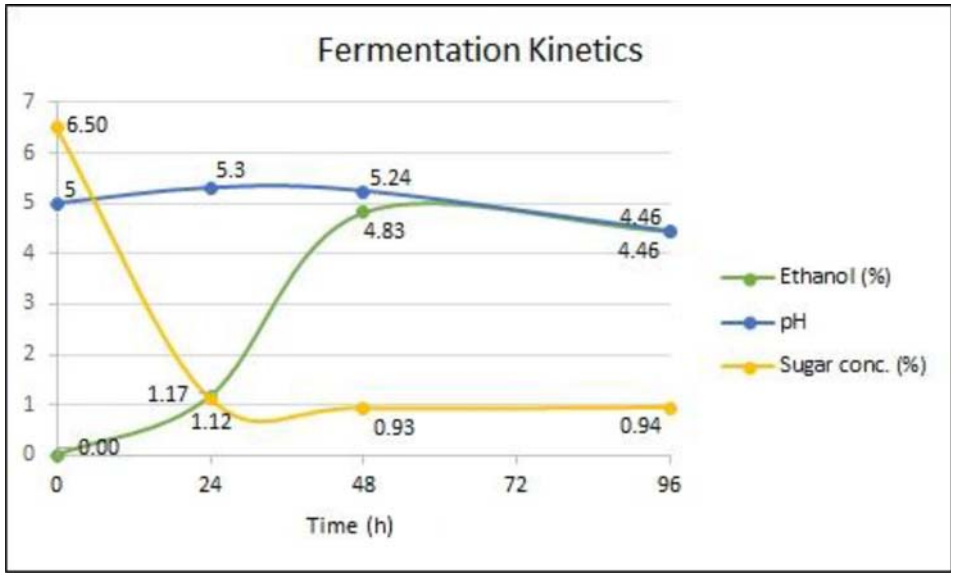

Fig. 5. Fermentation kinetics is showing ethanol production, pH vacillation, and sugar depletion by Pa isolate at initial sugar conc. of 6.5%. The highest ethanol production recorded was 4.83%. Two 500-ml Erlenmeyer flasks were used in parallel for fermentation with three consecutive experiments. The representative data are the average of all results

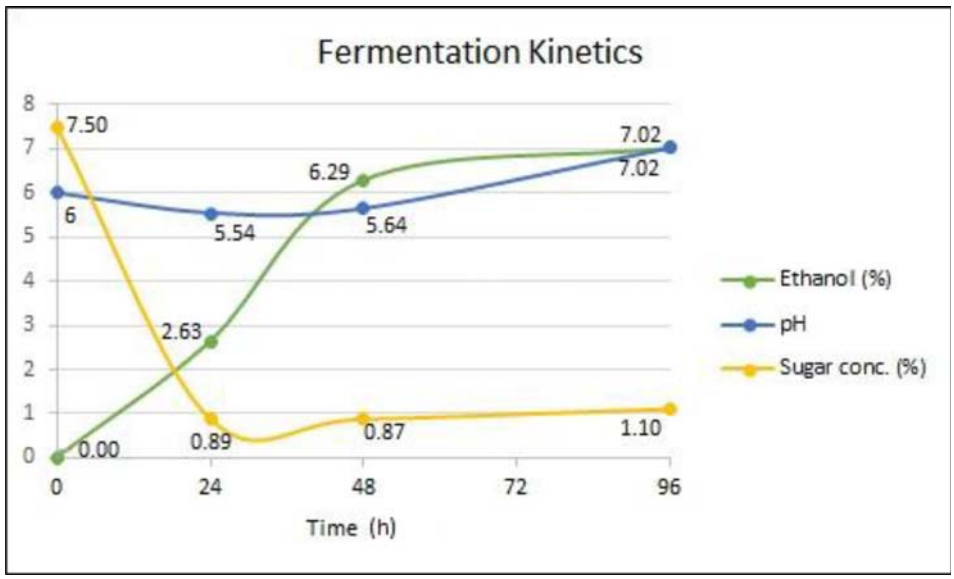

Fig. 6. Fermentation kinetics is showing ethanol production, pH vacillation, and sugar depletion by Pa isolate at initial sugar conc. of 7.5%. The highest ethanol production recorded was 7.02%. Two 500-ml Erlenmeyer flasks were used in parallel for fermentation with three consecutive experiments. The data represent the average of all results

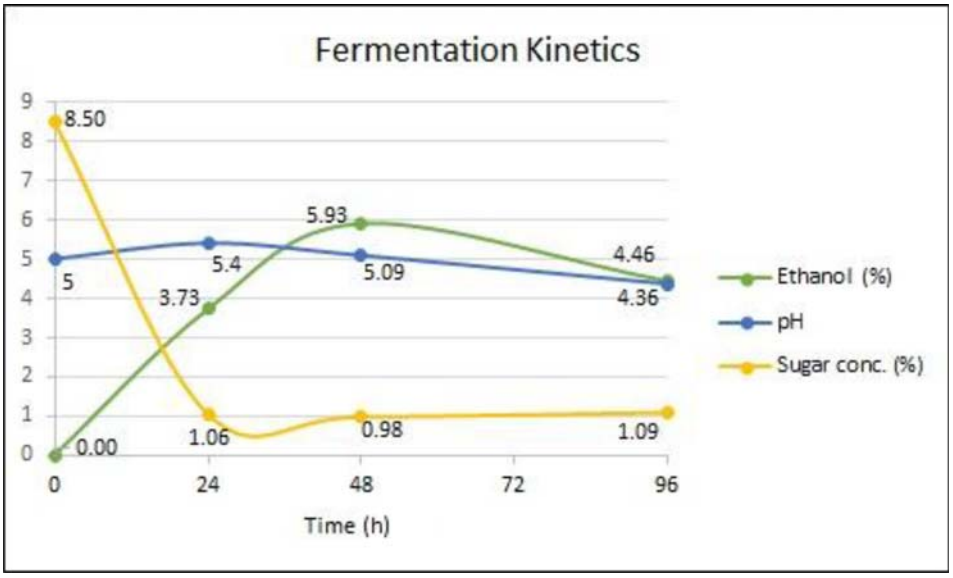

Fig. 7. Fermentation kinetics is showing ethanol production, pH vacillation, and sugar depletion by Pa isolate at initial sugar conc. of 8.5%. The highest ethanol production recorded was 5.93%. Two 500-ml Erlenmeyer flasks were used in parallel for fermentation with three consecutive experiments. The data are the average of all results

as optimum for the strain and in close agreement with others [38].

Pa isolate at 96-hour fermentation with 8.5% glucose and pH 5

After 48 h, under shaking condition (140 rpm), at 30 °C temperature, using initial reducing sugar concentration of 8.5%, and pH 5.0, maximum ethanol production was 5.93% *(Fig. 7)*.

Pa isolate at 96-hour fermentation with 9.5% glucose and pH 5

Under shaking condition (130 rpm), at 30 °C temperature, using initial reducing sugar concentration of 8.5% of the fermentation media, and pH 6.0, maximum ethanol productivity was 6.66% at 48 h *(Fig. 8)*.

Effect of metals on ethanol production

The optimization of ethanol production by additional nutrient supplements as effect of metals ($MnCl_2$, $CuSO_4$,

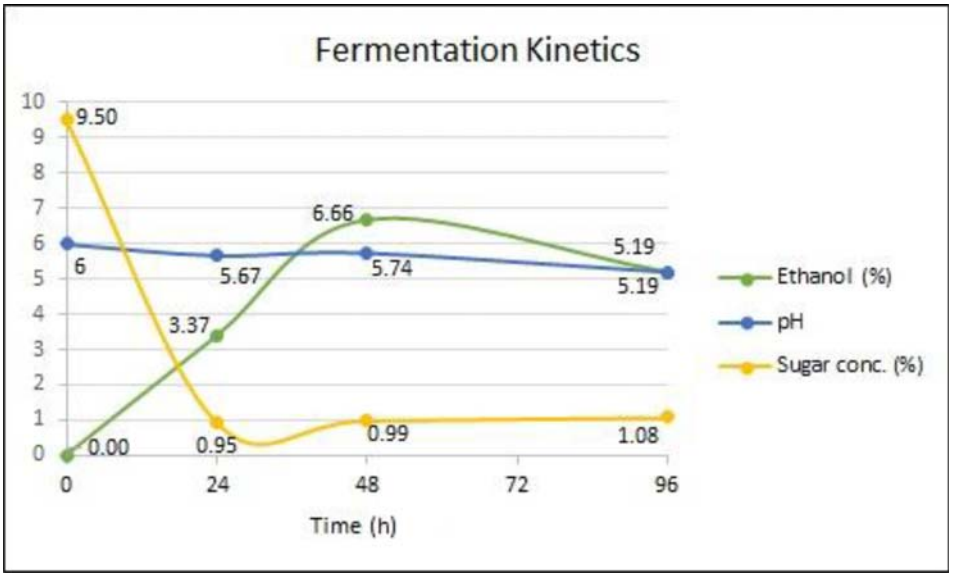

Fig. 8. Fermentation kinetics is showing ethanol production, pH vacillation, and sugar depletion by Pa isolate at initial sugar conc. of 9.5%. The highest ethanol production recorded was 6.66%. Two 500-ml Erlenmeyer flasks were used in parallel for fermentation with three repeated experiments. The data are the average of all results

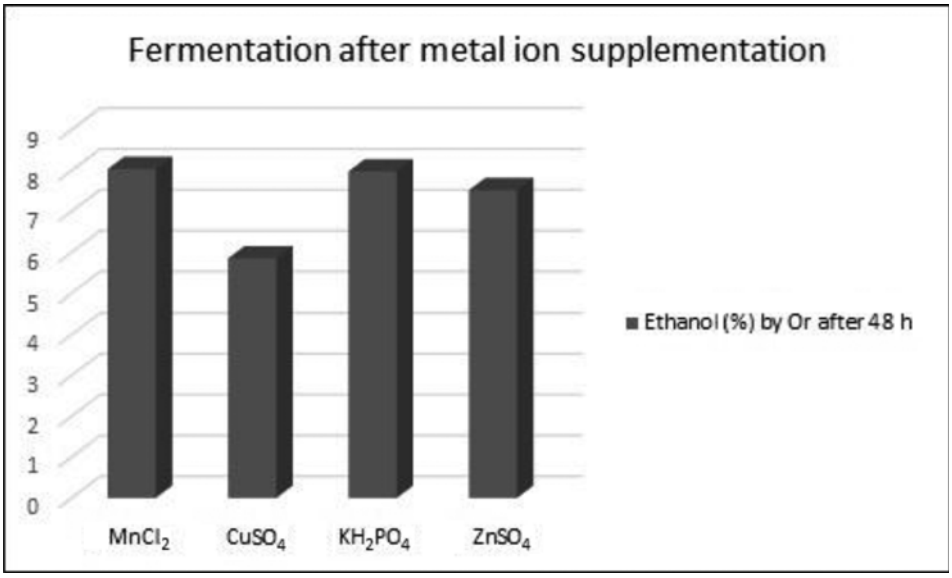

Fig. 9. Ethanol production of Or isolate in presence of metal ions at 30 °C temperature (shaking condition 120 rpm) after 48-hour fermentation. Three consecutive experiments carried out in similar condition. Two 500-ml Erlenmeyer flasks were used, and the average of all results is represented

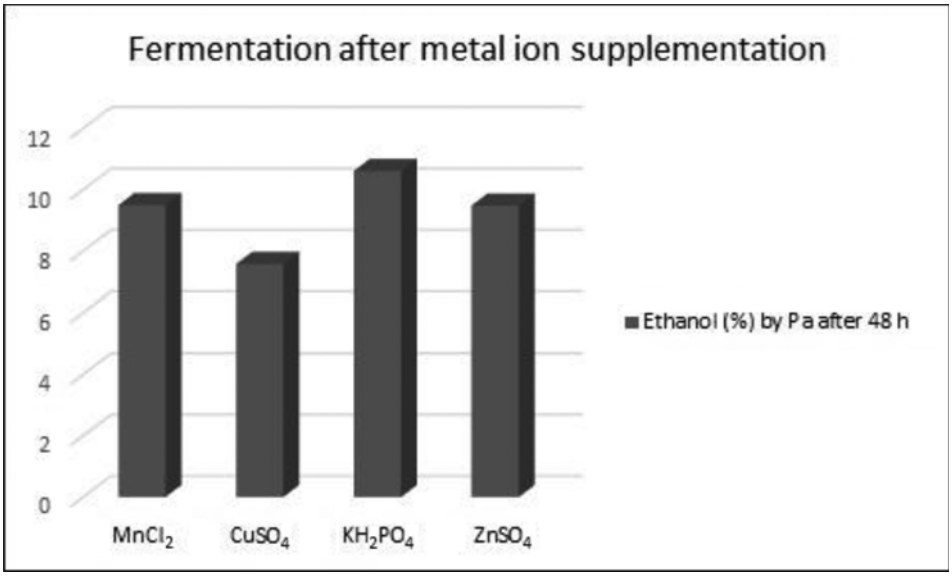

Fig. 10. Ethanol production of Pa isolate in presence of metal ions at 30 °C temperature (shaking condition 120 rpm) after 48-hour fermentation. Three consecutive experiments conducted in similar condition. Two 500-ml Erlenmeyer flasks were used, and the average of all results is shown

KH_2PO_4, and $ZnSO_4$) was analyzed. Both isolates showed high productivity after supplementation, as anticipated [39]. The highest ethanol production observed (10.61%) was by Pa with KH_2PO_4 supplements in shaking condition. Or isolate produced 8.04% ethanol after $MnCl_2$ supplementation in shaking condition (*Figs. 9* and *10; Table 14*).

Table 14. Production of ethanol by Pa and Or at 30 °C temperature in presence of metals (shaking condition 120 rpm)

Metals	Ethanol (%) by Pa (48 h)	Ethanol (%) by Or (48 h)
$MnCl_2$	9.50	8.04
$CuSO_4$	7.58	5.85
KH_2PO_4	10.61	7.98
$ZnSO_4$	9.48	7.52

Discussion

In this study, two yeasts were isolated from pineapple and orange peel. Based on their colony characteristics (white and creamy texture), ovoid microscopic shape, and the presence of budding pattern (multipolar), both isolates that were found belong to *saccharomyces* type unicellular ascomycete [13, 14] *(Fig. 2b)*.

The isolates were tested for fermentation of carbohydrates; isolate Pa was able to utilize 6 sugars out of the seven tested sugars, and Or isolate was able to utilize 5 sugars, indicating that they were diverse in sugar utilization (*Tables 1* and *2*).

In general, yeasts are mesophilic with upper limit growth temperature between 28 °C and 38 °C. This is the reason why the operating temperature must be maintained between 30 and 35 °C in typical yeast fermentation reactors [40]. Previous investigation strongly indicated that high temperature had a negative effect on yeast cell viability [39]. However, there are only a limited number of reports on the successful selection and isolation of yeasts capable of growth or fermentation at or above 40 °C [41–44]. As the temperature increases, productivity decreases sharply because of greater ethanol inhibition [45]. Moreover, previous studies reported that thermotolerant yeast can produce >6% ethanol within 24 h at 40 °C. From the result of the present study (*Tables 3* and *4*), the isolates can be regarded as mild thermotolerant [46, 48].

The ethanol concentrations are the major influencing factors during the fermentation process. Extremely high ethanol concentrations in the fermentation cultures have been shown to inhibit or depress the fermentation process. Study revealed particular genes expression, such as *Erg2, Prs3, Rpb4, Vma8,* and *Erg24,* may be responsible for the recalcitrance [49]. A growth inhibition at ethanol levels of less than 3% *v*/*v* [50] has been reported for *Clostridium thermocellum.* Like the palm wine yeast, TBY1 and TGY2 isolates exhibited remarkably high ethanol tolerance comparable to industrial yeasts such as sake and distiller's yeasts associated with high level of ethanol tolerance [51, 52]. The level of ethanol tolerance of 15% (*v*/*v*) by the yeast strain UVNR56 [37] is in agreement with previous studies [53, 54]. In our present study, we have seen the similar result for Pa and Or isolates that tolerated up to 12% ethanol, which is much more consistent with the other results (*Tables 5* and *6*).

Roukas [55] found that the optimum pH range for ethanol production from carob pod extract by Ca alginate-immobilized *S. cerevisiae* was 3.5–5.5 and reported that this was due to the good yeast growth over the pH range of 3.5–6.5. Pa and Or isolate grew in a wide pH range, from 2 to 10 in the present study, but pH 4.0 for Pa isolate and pH 6 for Or isolate demonstrated optimum result (*Tables 7* and *8*), and this range is due to the optimum pH value for the activity of plasma membrane-bound proteins, including enzymes and transport proteins [56].

The Pa yeast isolate showed better tolerance than the Or yeast isolate at up to 15% sodium chloride equivalent of osmotic pressure (*Tables 9* and *10*). Some studies [57, 58] have reported changes in the growth dynamics of *Saccharomyces* genus upon exposure to various osmotic stress conditions. The decrease in logarithmic growth rate constants of the test yeasts in relation to increasing osmotic pressure is therefore consistent with the views expressed by these workers [23]. Increase in the medium sugar level is believed to affect the relative proportion of total medium sugar converted to alcohol [57, 59, 60]. The decline in yeast ethanol productivity at high medium glucose levels as observed in this study is in close agreement with the finding of several other researchers of the *Saccharomyces* genus in medium of high osmotic pressures [57, 58].

Yeast killer toxins are protein compound; the property of which was not detected in the experiments. The capability to produce killer toxin can confer an advantage over more sensitive competitive strains growing in a fermentative process [26].

Based on the results of tolerance and ethanol production at different sugar concentration at 30 °C temperature and pH 5 in shaking and nonshaking condition, Pa isolate showed a better result than Or isolate. Thus, a series of experiments had been conducted at different physicochemical conditions to optimize ethanol production by the Pa isolate. To know the optimum pH for ethanol fermentation, the solutions were kept at pH 5 and 6 with different initial sugar concentration. Previous studies acknowledged that fermentation glucose tolerance can be increased to (20% *w*/*v*) [25], and the higher the ability of a strain to consume a certain type of sugar, the lower the concentration of residual sugar left in the broth after fermentation [61]. The molasses was diluted, and fermentation was carried out in 500-ml flasks. In shaking condition (130 rpm), at 30 °C temperature, using initial reducing sugar concentration of the fermentation media at 7.5%, and pH 6.0, maximum ethanol productions were 7.02% at 96 h *(Fig. 6)*. In shaking condition, a low ethanol yield of 4.83% was observed at ambient temperature in 48 h and maximum ethanol produced was 7.02% at 96 h at ambient temperature using initial reducing sugar concentration of 6.5% and 7.5% (shown in *Figs. 5* and *6*).

Shaking is a vital factor that influences ethanol fermentation [62]. To determine the optimum shaking rate for ethanol production through incubation, the fermentation media at different shaking rates (0, 120, 130, and 140 rpm) were investigated. The optimum shaking rate for ethanol production was at 130 rpm, which showed increasing the shaking rate is favorable for yeast growth [63].

After the optimization of substrate concentration, pH, and temperature, additional nutrient supplements were also tested further to increase the ethanol yield. Data from other studies also supported this additional investigation [30, 39]. Consequently, effect of metals ($MnCl_2$, $CuSO_4$, KH_2PO_4, and $ZnSO_4$) on ethanol production was analyzed. All the isolates produced the high ethanol after supplementation with the metals. Highest ethanol production of 10.61% observed was by Pa yeast isolate with KH_2PO_4 supplementation in shaking condition. Or isolate showed

highest ethanol production of 8.04% by $MnCl_2$ supplementation in shaking condition (*Figs. 9* and *10; Table 14*).

The experimental results reported in the present study revealed that both the Pa and Or isolates are highly ethanol tolerant and thermotolerant as well as moderate osmotolerant and can survive at various pH ranges. The ability to produce ethanol from sugarcane molasses was tested for both isolates. These findings stated that the isolates could be used at industrial level for fermentation of various raw materials in order to obtain an increased production of bioethanol.

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Authors' contribution

A.N. carried out the collection and all experimental studies, analyzed and interpreted data, and drafted the thesis. S.S.R. carried out the collection and all experimental studies; analyzed and interpreted data; drafted, edited, finalized, and revised the article; and helped to coordinate the article to the publisher. M.M.H. and N.C. participated in designing, supervising, and coordinating the study and helped to revise the draft. All authors read and approved the final article.

Competing interests

The authors declare that they have no competing interests.

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References

1. Jones AM, Ingledew WM: Fuel Alcohol production: appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. Appl Environ Microbiol 60(3), 1048–1051 (1994)
2. Bai ZG, Dent DL, Olsson L, Schaepman ME: Proxy global assessment of land degradation. Soil Use Manage 24, 223–234 (2008)
3. Rolz C, de Leon R: Ethanol fermentation from sugarcane at different maturities. Ind Crops Prod 33(2), 333–337 (2011)
4. Hansen AC, Zhang Q, Lyne PWL: Ethanol diesel fuel blends – a review. Bioresour Technol 96, 277–285 (2005)
5. Wyman CE, Hinman ND: Ethanol, Fundamental of production from renewable feedstocks and use as transportation fuel. Appl Biochem Biotechnol 24–25, 735–750 (1990)
6. Takeshige K, Ouchi K: Factors affecting the ethanol productivity of yeast in molasses. J Ferment Bioeng 79, 449–452 (1995)
7. Beuchat LR: Influence of water activity on growth metabolic activities and survival of yeasts and molds. J Food Prot 46, 135–141 (1983)
8. Hahn-Haegerdal B, Larrson M, Mattiasson B: Shift in metabolism towards ethanol production in *Saccharomyces cerevisiae* using alterations of the physical-chemical microenvironment. Biotechnol Bioeng Symp 12, 199–202 (1982)
9. Belitz HD, Grosch W, Schieberle P (2009): Food Chemistry, 4th edn., Springer
10. Satyanarayana T, Kunze G (2009): Yeast Biotechnology: Diversity and Applications, Springer
11. Echegaray O, Carvalho J, Fernandes A, Sato S, Aquarone E, Vitolo M: Fed batch culture of *Saccharomyces cerevisiae* in sugarcane blackstrap molasses: invertase activity of intact cells in ethanol fermentation. Biomass Bioenergy 19, 39–50 (2000)
12. Sanchez OJ, Cardona CA: Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99, 5270–5295 (2008)
13. Lodder J (1970): General classification of the yeasts. In: The Yeasts, A Taxonomic Study, 2nd edn., ed. Lodder J, North Holland, Amsterdam, pp. 1–33
14. Boekhout T, Kurtzman CP (1996): Principles and methods used in yeast classification, and overview of currently accepted genera. In: Non-conventional Yeasts in Biotechnology: A Handbook, ed. Wolf K, Springer Verlag, Berlin, Heidelberg, pp. 1–8
15. Kreger-Van Rij NJW (1984): The Yeasts: A Taxonomic Study. Elsevier Science Publishing Company, New York, p. 1082
16. Nofemele Z, Shukla P, Trussler A, Permaul K, Singh S: Improvement of ethanol production from sugarcane molasses through enhanced nutrient supplementation using *Saccharomyces cerevisiae*. J Brew Distill 3(2), 29–35 (2012). DOI: 10.5897/JBD12.003
17. Kurtzman CP, Fell JW (1997): The Yeasts: A Taxonomic Study, 4th edn., Elsevier Science Publishing Company, Amsterdam, p. 1055
18. Mesa JJ, Infante JJ, Rebordinos L, Cantoral JM: Characterization of yeasts involved in the biological ageing of sherry wines. Lebensm–Wiss Technol 32, 114–120 (1999)
19. Ortiz-Muñiz B, Carvajal-Zarrabal O, Torrestiana-Sanchez B, Aguilar-Uscanga MG: Kinetic study on ethanol production using *Saccharomyces cerevisiae* ITV-01 yeast isolated from sugar cane molasses. J Chem Technol Biotechnol 85(10), 1361–1367 (2010)
20. Lorenz MC, Cutler NS, Heitman J: Characterization of alcohol-induced filamentous growth in *Saccharomyces cerevisiae*. Mol Biol Cell 11(1), 183–199 (2000)
21. Warren P, Shadomy L (1991): Yeast Fermentation Broth Base with Carbohydrate and Durham Tube. In: Manual of Clinical Microbiology, 5th edn., Washington D.C.
22. Fakruddin M, Islam MA, Quayum MA, Ahmed MM, Chowdhury N: Characterization of Stress tolerant high po-

tential ethanol producing yeast from agro-industrial waste. Am J BioSci 1(2), 24–34 (2013). DOI: 10.11648/j.ajbio.20130102.11
23. Osho A: Ethanol and sugar tolerance of wine yeasts isolated from fermenting cashew apple juice. Afr J Biotechnol 4(7), 660–662 (2005)
24. Bauer AW, Kirby WMM, Sheris JC, Truck M: Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol 145, 225–230 (1966)
25. Ortiz-Zamora O, Cortès-García R, Ramírez-Lepe M, Gómez-Rodríguez J, Aguilar-Uscanga MG: Isolation and selection of ethanol-resistant and osmotolerant yeasts from regional agricultural sources in Mexico. J Food Process Eng 32, 775–786 (2009)
26. Soares GAM, Sato HH: Characterization of the *Saccharomyces cerevisiae* Y500-4L killer toxin. Braz J Microbiol 31, 291–297 (2000)
27. Periyasamy S, Venkatachalam S, Ramasamy S, Srinivasan V: Production of bio-ethanol from sugar molasses using *Saccharomyces Cerevisiae*. Mod Appl Sci 3(8), 32–37 (2009)
28. Miller GL: Use of dinitrosalycylic acid reagent for determination of reducing sugar. Anal Chem 31, 426–428 (1959)
29. Sharif K: Conway Microdiffusion Analysis. Available at https://www.physicsforums.com/threads/conway-microdiffusion-analysis.255771/
30. Palukurty MA, Telgana NK, Bora HSR, Mulampaka SN: Screening and optimization of metal ions to enhance ethanol production using statistical experimental designs. Afr J Microbiol Res 2, 87–94 (2008)
31. Kurtzman CP, Fell JW (1998): Definition, classification and nomenclature of the yeasts. In: The Yeasts, A Taxonomic Study, 4th edn., eds. Kurtzman CP, Fell JW, Elsevier Science BV, Amsterdam, The Netherlands, pp. 3–5
32. Silva JB, Sauvageau D: Bacteriophages as antimicrobial agents against bacterial contaminants in yeast fermentation processes. Biotechnol Biofuels 7(123), (2014)
33. Panchal CJ, Meacher C, Van Oostrom J, Stewart GG: Phenotypic expression of *Kluyveromyces lactis* killer toxin against *Saccharomyces* spp. Appl Environ Microbiol 50(2), 257–260 (1985)
34. Mahler HR, Johnson J: Specific effects of nalidixic acid on mitochondrial gene expression in *Saccharomyces cerevisiae*. Mol Gen Genet 176, 25–31 (1979)
35. Rahman SS, Sarkar MKI, Islam MR, Hossain K, Nahar K, Roy CK, Uddin ME, Choudhury N: Isolation of yeasts from raisins and palm-juice and ethanol production in molasses medium. Indian J Sci Technol 9(12), (2016). DOI: 10.17485/ijst/2016/v9i12/85509
36. Khan AR, Malek MA, Choudhury N, Khan SI: Alcohol production from molasses and liquid sugar using some indigenous yeast isolates. Bangladesh J Microbiol 6(1), 37–42 (1989)
37. Thammasittirong SN, Thirasaktana T, Thammasittirong A, Srisodsuk M: Improvement of ethanol production by ethanol-tolerant *Saccharomyces cerevisiae* UVNR56. SpringerPlus 2(583), (2013)
38. Zohri AA, Ragab WSM, Ali MMA: Ethanol production from Egyptian sugar cane molasses by six yeast strains using batch fermentation. J Basic Appl Mycol 5, 43–49 (2014)
39. Deesuth O, Laopaiboon P, Jaisil P, Laopaiboon L: Optimization of nitrogen and metal ions supplementation for very high gravity bioethanol fermentation from sweet sorghum juice using an orthogonal array design. Energies 5, 3178–3197 (2012)
40. Ueno R, Urano N, Kimura S: Effect of temperature and cell density on ethanol fermentation by a thermotolerant aquatic yeast strain isolated from hot spring environment. Fish Sci 68, 571–578 (2002)
41. Pellegrini L, Cardinali G, Martini A: Selection of *Saccharomyces cerevisiae* strains able to ferment at supraoptimal temperatures. Appl Microbiol Enzymol 49, 55–65 (1999)
42. Sree NK, Sridhar M, Suresh K, Bharat IM, Rao LV: High alcohol production by repeated batch fermentation using immobilized osmotolerant *S. cerevisiae*. J Indust Microbiol Biotechnol 24, 222–226 (2000)
43. Rahman SS, Hossain MM, Choudhury N: Effect of Various Parameters on the Growth and Ethanol Production by Yeasts Isolated from Natural Sources. Bangladesh Jo Microbiol 30(1–2), 49–54 (2013). DOI: 10.3329/bjm.v30i1-2.28453
44. Choudhary J, Singh S, Nain L: Thermotolerant fermenting yeasts for simultaneous saccharification fermentation of lignocellulosic biomass. Electron J Biotechnol 21, 82–92 (2016)
45. Sree NK, Sridhar M, Suresh K, Banat IM, Rao LV: Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. Bioresour Technol 72, 43–46 (2000)
46. Anderson PJ, McNeil K, Watson K: High-efficiency carbohydrate fermentation to ethanol at the temperature above 40 °C by *Kluyveromyces marxianus* var. marxianus isolated from sugar mills. J Appl Environ Microbiol 51(6), 1314–1320 (1986)
47. Ueno R, Hamada-Sato N, Urano N: Fermentation of molasses by several yeasts from hot spring drain and phylogeny of the unique isolate producing ethanol at 55 °C. J Tokyo Univ Fish 90, 23–30 (2003)
48. Pereira FB, Guimarães PMR, Gomes DG, Mira NP, Teixeira MC, Sá-Correia I, Domingues L: Identification of candidate genes for yeast engineering to improve bioethanol production in very high gravity and lignocellulosic biomass industrial fermentations. Biotechnol Biofuels 4(57) (2011)
49. Canganella, F, Wiegel J (1993): The potential of thermophilic clostridia in biotechnology. In: The Clostridia and Biotechnology, ed. Woods DR, Butterworth-Heinemann, Boston, pp. 393–429
50. Casey GP, Ingledew WM: Ethanol tolerance in yeasts. CRC Crit Rev Microbiol 13, 219–280 (1986)
51. Hayashida S, Feng DD, Hungo M: Functions of high concentration of alcohol-producing factor. Agric Biol Chem 38, 2001–2020 (1974)
52. Nwachukwu IN, Ibekwe VI, Nwabueze RN, Anyanwu BN: Characterization of palm wine yeast isolates from industrial utilization. Afr J Biotechnol 5(19), 1725–1728 (2006)
53. Teramoto Y, Sato R, Ueda S: Characteristics of fermentation yeast isolated from traditional Ethiopian honey wine, ogol. Afr J Biotechnol 4(2), 160–163 (2005)
54. Roukas T: Continuous ethanol production from carob pod extract by immobilized *Saccharomyces cerevisiae* in a packed-bed bioreactor. J Chem Technol Biotechnol 59, 387–393 (1994)
55. Narendranath NV, Power R: Relationship between pH and medium dissolved solids in terms of growth and meta-

bolism of *Lactobacilli* and *Saccharomyces cerevisiae* during ethanol production. Appl Environ Microbiol 71(5), 2239–2243 (2005)
56. D'Amore T, Panchal CJ, Stewart GG: Intracellular ethanol accumulation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 54, 110–114 (1988)
57. Dombek KM, Ingram LO: Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation. Appl Environ Microbiol 52(5), 975–981 (1986)
58. Jimenez J, Benitez T: Characterization of wine yeasts for ethanol production. Appl Microbiol Biotechnol 25, 150–154 (1986)
59. Nagodawithana TW, Steinkraus K: Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in rapid fermentation. Appl Environ Microbiol 31, 158–162 (1976)
60. Jasman, Prijambada ID, Hidayat C, Widianto D: Selection of yeast strains for ethanol fermentation of glucose-fructose-sucrose mixture. Indones J Biotechnol 17(2), 114–120 (2012)
61. Jayus, Nurhayati, Mayzuhroh A, Arindhani S, Caroenchai C: Studies on bioethanol production of commercial baker's and alcohol yeast under aerated culture using sugarcane molasses as the media. Agric Agric Sci Procedia 9, 493–499 (2016)
62. Osman ME, Khattab OH, Hammad IA, EI-Hussieny NI: Optimization of bio-fuel production by *Saccharomyces cerevisiae* isolated from sugar cane bagasse. J Am Sci 7(5), (2011)