Isolation and characterization of thermo, osmotic and ethanol tolerant yeast for ethanol production from molasses-based media

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Abstract

Laboratory experiments were carried out to isolate and characterize the thermo tolerant yeast for ethanol production from molasses and to study some environmental condition and physiological parameters. Forty eight yeast stains were isolated from fruits (10 from banana (SB1-SB10), 7 from mango (SM1-SM7), 7 from orange (SO1-SO7), 8 from grape (SG1-SG8), 7 from apple (SA1-SA7) and 10 from date (SD1-SD10)), the fruits were collected from local market, Khartoum, Sudan. From the isolated isolate, two isolates (SD6 and SG3) were selected for further tests based on thermo tolerant, osmo tolerant and ethanol tolerant and identified by morphological and physiological characterization. Molasses was diluted by hot water (1:1). Fermentation was carried out in Erlenmeyer conical flasks. Fermentation media was taken into 500ml Erlenmeyer flasks and then the homogenous suspension of yeast inoculum was inoculated into the media in an aseptic condition. To increase ethanol yield, different amounts of nutrients were added to the molasses. The effectiveness of each supplement was evaluated at pH 6.0and temperature of 40^{o}C in incubation. The molasses contained 83.2% total soluble solids, 17.8% reducing sugars, 32.1% sucrose, 49.9% total sugars, 10.25% ash, 0.54% calcium, 0.28% sodium, 2.89% potassium and it had a pH value of 5.6. sugar concentrations on ethanol production efficiency by strain SD6 and SG3 showed the best result at 40% conc.

Keywords: Thermo tolerant yeast, fermentation, molasses, ethanol production.

Introduction

Yeasts are eukaryotic micro-organisms classified in the kingdom Fungi, with about 1,500 species [1]. They dominate fungal diversity in the oceans [2]. They are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body [3]. The yeast species are all characterized by a similar set of features, both morphological and physiological. This type of description, in which physiological characters are important, distinguishes yeast taxonomy from other fungal taxonomy [4]. *Saccharomyces uvarum* [5], *Schizosaccharomyces pombe* [6] and *Kluyueromyces* sp. [7], *Pachysolen tannophilus*, *Candida shehatae*, *Pichia stipitis*, and especially *Saccharomyces cerevisiae*, are the major yeast species currently used for industrial ethanol fermentation processes [8]. In general, industrial yeast isolates are able to grow and efficiently ferment ethanol at pH values of 3.5-6.0 and temperatures of 28-37^{o}C with

efficiency dropping off rapidly at higher temperature [9].

At the beginning of the 20th century, several kinds of raw materials were exploited for ethanol production, such as molasses or agricultural production. Carbohydrate-rich raw materials suitable for ethanol production can be classified into three groups of agricultural products: which all sugar, starch and lignocelluloses the first raw material group, sugar refers to sugar-beet as well as sugarcane and molasses. The second group, from such crops as cassava, cereals and potatoes. The last group, lignocelluloses, converts waste materials from the harvesting of agricultural crops such as rice straw, corn cob and sugarcane waste [10]. Molasses (alternatively termed final molasses or blackstrap molasses) can be defined as final effluent obtained in the preparation of sugar by repeated crystallization; it is residual syrup from which no crystalline sucrose can be obtained by smiles means [11]. World molasses production has increased markedly over the past 40 years from around 15 million Metric Tons (MT) in the early 1960s to around 45 million MT by 2000. The yield of molasses per ton of cane is approximately 2.7% but it is influenced by a number of factors and may within a wide range 2.2- 3.7% [12]. Sugar industry in Sudan offer large quantities of molasses as a by-product, which can be used as a raw material for the fermentation and ethanol industry with reasonable price [13]. Kenana Sugar Company (biggest Sugar Company in Sudan) utilizing 189.000 tons molaases per year 126.000 produced in Kenenasugar factory and the remains from Sudanese sugar company. Fermentation is a metabolic process characterized by: (a) incomplete oxidation, (b) the transformation of large amounts of substances by comparatively small amounts of organisms. In a plant the most common factors affecting the fermentations process including the yeasts isolates and species (thermoacido and osmo tolerant) yeast aeration during propagation, nutrients, pH, temperature alcohol concentration and contamination.

Ethanol is very important in the life the largest single use of ethanol is as a motor fuel and fuel additive, Alcoholic beverages vary considerably in their ethanol content and in the foodstuffs from which they are produced. Ethanol and 95% ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62%. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses, but is ineffective against bacterial spores.

The objectives of this study were to isolate selected thermo tolerant yeast for ethanol production, to study some environmental condition physiological parameters such as substrate concentration molasses medium and to study fermentation kinetics of ethanol production at laboratory level.

Materials and methods

Isolation and screening of different yeasts isolates

Forty eight yeast stains were isolated from fruits collected local market (ten isolates from banana (SB1-SB10), seven isolates from mango (SM1-SM7), seven isolates from orange (SO1-SO7), eight isolates from grape (SG1-SG8), seven stains from apple (SA1-SA7) and ten isolates from date (SD1-SD10). From each fruit sample, about 5.0 g were suspended in saline solution (0.9% w/v) in a test tube, shaked well and one ml of the suspension was streaked onto plates containing a yeast extract-malt extract (YM) agar, consisted of 3.0g yeast extract, 3g malt extract, 5g peptone, 10g glucose and 15gagar in 1 liter water, with an initial pH 5.5 [4]. The plates were incubated aerobically at 37°C for 3 days. Single colony formed was picked and streaked onto the same media and then observed under microscope. The cultures of yeast were maintained by sub-culturing on slants using YPD (Yeast Peptone Dextrose Media) media, incubating for 48 hrs at 37ºC and thereafter stored in a refrigerator at 4ºC for future use.

Selection of thermo tolerant, osmo tolerant and ethanol tolerant yeast

Growth ability of isolated yeast at high temperatures was analyzed using the modified method previously described [14]. The active cell of the isolated yeast was cultured in YPD agar at 40^{o}C until its growth reached. The ability of isolated yeast to withstand high ethanol concentration was also tested. Each of the isolated yeast was grown on YPD agar medium supplemented with different ethanol concentration 16 % (v/v) and incubated at 40^{O}C for 3 days. Growth of isolated yeast was recorded.

The ability to grow in the presence of high concentrations of sugar was tested by development on agar media distributed in Petri plates and on malt-yeast extract containing 50% D-glucose, respectively. The incubation was performed for 4 weeks at 25^{O}C.

From the isolated yeast, two isolates (SD6-SG3) were selected for further tests based on thermo tolerant, osmo tolerant and ethanol tolerant.

Detection of thermo-tolerance of selected yeast isolates (SD6 and SG3)

YEPD liquid medium was used for detecting thermo-tolerance and growth in liquid media of selected yeast isolates (SD6 and SG3). The medium was autoclaved at 121°C and 15 psi and cooled. 10 ml portion

of the medium was distributed into McCartney tubes, and then inoculated with 48 hours old selected yeast isolate.

The initial optical density of each tube was recorded on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 25°C, 30°C, 37°C, 40°C and 44°C for 2 days for observing thermo tolerance of yeast isolate. The increase in optical density in a tube was recorded as evidence of growth.

Detection of ethanol tolerance of selected yeast isolates (SD6 and SG3)

The medium for the detection of ethanol tolerance of thermo-tolerant yeast was modified and YEPD liquid medium was used for detecting yeasts for ethanol tolerance. The medium was sterilized at 121°C for 15 min in an autoclave and cooled. YEPD broth was prepared containing 5%, 10%, 12%, 15%, 18%, 20% and 25% of absolute ethanol. Forty milliliter portion of the medium was distributed into 125 ml flask, and then inoculated with selected thermo-tolerant yeasts. The initial optical density of each flask was read off on spectrophotometer at 600 nm against the medium as blank. All cultures were incubated at 40 °C for 5 days. 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.

The ability to grow in the presence of high concentrations of sugar

YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% of glucose. Each McCartney contained 15 ml of YEPD media with appropriate concentration of sugar and blank was used as a control. Then each was inoculated by half loop full of yeast cell and measured the initial optical density at 600 nm and incubated at 30°C for 48 h. After 48 h cell density was further recorded at 600 nm. Growth were recorded at 6%, 9%, 12%, 15%, 18% and 20% of salt containing media and O.D is given gradually.

Identification of the yeast

Morphological characterization

Yeast isolates were identified based on the morphological characters [4, .15].

Physiological characterization

Fermentation of carbohydrates

Yeast fermentation broth base with Durham tube was used for testing of yeasts for carbohydrate fermentation. Yeast fermentation broth media were used for identification yeasts based on fermentation of specific carbohydrates of fermentation pattern. The carbohydrate used were; glucose (dextrose), galactose, maltose, sucrose, lactose trehalose, fructose and xylose. Yeast fermentation broth was modification of media developed by Wicker ham for the determination of carbohydrate fermentation by yeasts. For fermented

carbohydrates by yeasts, the color of the medium changed from punk to yellow due to the formation of acids and gas produced [16].

Chemical Composition of Molasses

Clarification of molasses

Molasses samples were obtained from a local sugar factory (Kenana Sugar Company). Molasses was diluted by hot water (1: 1) and the pH value was adjusted to 4.5 – 5.0 by H_2SO_4 conc., and sterilize at 105ºc (0.5bar) for 45 minutes, then the molasses was transferred for another flask after 24 hours, the suspension was filtered and the residue was discard, pH, brix, sp.gr and sludge were checked, the molasses were transferred to the receiver flask.

The molasses samples were analyzed for pH using a pH-meter at ambient temperature according to [17] total soluble solids using a hand refract meter and reducing sugars content according to [18].

Inoculums development

From the selected yeast SD6 and SG3 inoculant were developed by serial dilution to give approximately, (10^6-10^7 cells/ml) were grown in a medium, containing (g/l): (0.3% yeast extract, 1% peptone, 2% glucose, 1.5% agar), adjusted to pH 5.0. The medium was autoclaved at 121°C and 15 psi and poured on Petri dish and cooled, then streaked by 48 hours old selected yeast isolate from slant.

After preparation of inoculums broth (g/l) (0.3% yeast extract, 1% peptone, 2% glucose) the medium was autoclaved at 121°C and 15 psi and poured on conical flask and cooled, then inoculated with 48 hours old selected yeast isolate from Petri dish and incubated at 40°C for 24 h. in vigorous shaking condition (180 rpm).

A hematocytometer was used to determine yeast cell counts in each conical flask. A 1 ml inoculum broth sample was serially diluted with a sterile saline solution (0.89% w/v NaCl) to a point where a reasonable number of cells could be counted [19]. Most of the time, the cell count recorded is 10^6 cells/ml or a fraction higher.

Fermentation of molasses

Fermentation was carried out in Erlenmeyer conical flasks, 250 ml fermentation media was taken into 500 ml Erlenmeyer flasks and then the homogenous suspension of yeast inoculums was inoculated into the media in an aseptic condition. The flask was cotton plugged and incubated at different temperature to an incubator in both non-shaking condition 250 rpm. To increase ethanol yield, different amounts of nutrients were added to the molasses. The effectiveness of each supplement was evaluated at pH 4.5 and temperature of 40C in terms biomass after 24, 48, 72 and 96h. incubation. Each

supplement was added according to [20]. The different treatments were:

Treatment1 (T1) -50- w/v % molasses + 0.5 % w/v $(NH_4)_2 SO_4$

Treatment2 (T2) -50--w/v% molasses + 0.5 % w/v $(NH_4)_2 SO_4$ + 0.3 % w/v $KH_2 PO_4$

Treatment3 (T3) -50--% w/v molasses +0.5% w/v $(NH_4)_2SO_4$ + 0.3% w/vKH_2PO_4 +2% w/v peptone.

Treatment4(T4) – 50- % w/v molasses + 0.5 % w/v $(NH_4)_2SO_4$ + 0.3% w/v KH_2PO_4 +1 % w/v yeast extract +0.05 % w/v Mg SO_4.7 H_2O+0.004% w/v $CaCl_2.2H_2O$.

Treatment5(T5) – 25-c % w/v molasses + 0.5 w/v $(NH_4)_2 SO_4$ + 0.3% w/v $KH_2 PO_4$ + 1% w/v yeast extract, 2%w/v peptone + 0.05% w/v $MgSO_4.7H_2O$ +0.004 % w/v $CaCl_2.2H_2O$ + 0.005. % w/v biotin + 0.0001% w/v calcium pantothenate (Vit-5_). *Biotin* is a water-soluble B-*vitamin*, also called *vitamin* B_7 and formerly known as *vitamin* H.

Effect of sugar concentration:

To study the effect of sugar concentration on ethanol production by SD6, the production media was prepared by diluting molasses to reduce sugar concentration 20, 30, 40 and 50% and fermentation was carried out in a volume of 250 ml media in a 500 ml conical flask. A twenty-four hour old inoculum of yeast was added at the medium. Samples were withdrawn at different time and estimated for residual sugars [21] as well as ethanol content in the media.

Results and discussion

Isolation of yeasts

Forty eight yeast stains were isolated from fruits collected from a local market in Khartoum, Sudan. (ten isolates from banana (SB1, SB2, SB3, SB4, SB5, SB6, SB7, SB8, SB9 and SB10), seven isolates from mango (SM1, SM2, SM3, SM4, SM5, SM6 and SM7), seven isolates from orange (SO1, SO2, SO3, SO4, SO5, SO6 and SO7), eight isolates from grape (SG1, SG2, SG3, SG4, SG5, SG6, SG7 and SG8), seven isolates from apple (SA1, SA2, SA3, SA4, SA5, SA6 and SA7) and ten isolates from date (SD1, SD2, SD3, SD4, SD5, SD6, SD7, SD8, SD9 and SD10).

Selection of thermo tolerant, ethanol tolerant and osmotic tolerant yeast

As shown in (Table 1) total of 48 yeast isolates were tested for thermo tolerant. The isolates vary in their tolerance to high temperature with 50% of the isolates grow at low temperature where as only 2% can grow at high temperature (40^{o}C). Likewise, the tested yeast isolates vary in their ethanol tolerance. Out of the 48 isolates only 2% were found to ethanol tolerant, 54% non ethanol tolerant and 44% showed low growth in ethanol concentration. The

isolates showed the same pattern for osmo tolerance as ethanol tolerance, 2% were osmo tolerant, 52% non osmo tolerant and 46% with low growth in high sugar concentration. In this study, two isolates thermo, osmo, ethanol tolerant ethanol fermentation were occurred at temperature higher than optimum temperature because yeast currently used for industrial fermentation are rapidly inactivated at 33-35^{o}C [22]. Temperature tolerance was also been found to depend upon sugar concentrations of the medium as Morimura [23] observed that fermentation of molasses at 35°C was possible when sugar concentration was 20% (w/v) with no fermentation when sugar concentration was 22% (w/v). Peres and Laluce [24] reported that final biomass formation declines for all concentrations of supplemented ethanol ranging from 0 to 9% (v/v) in the thermo tolerant yeasts they studied. From the isolates, isolates (SD6 and SG3) were found to be thermo tolerant, osmo tolerant and ethanol toleran and selected for further tests.

Thermo-toleranceof selected yeast isolates SD6 and SG3

After incubation in YPD media for 48 hours at 25°C, 30°C, 37°C, 40°C and 44°C, the two isolates grow in 25°C, 30°C and 37°C. Isolate SD6 of yeast was able to grow at up to 44°C, where as isolate SG3 failed to grow at 44°C. (Table 2). The growth of the two isolates showed significant differences with respect to temperatures with maximum growth at 30°C. No clear differences were observed between the two isolates. Fermentation in industries is usually carried out at ambient temperature of 25- 35°C but temperature exceeds 40°C during fermentation decreases the cell viability and productivity. Fermentation at 35-40°C or above has advantages such as ethanol recovery and significant savings on operational costs of refrigeration control in distilleries for alcohol production. Therefore many studies have been carried out for development of yeast to ferment at high temperature of up to 40-45°C. Laluce [25] studied the effects of temperature on fermentation capacity of three isolates; complete conversion of total sugar to ethanol was observed after 12 hrs of fermentation at 39-40°C. Above 40°C a strong inhibitory effect of temperature on ethanol production was observed. Du Preez [26] found that the ethanol production was reduced at 35°C for all isolates. As the evolution of heat at higher temperature during fermentation inhibited ethanol yield [23], selection of thermo- tolerant isolates should be adopted.

Table1. Selection of thermo, ethanol and osmo tolerant yeast from a total isolated (Growth at 40^{o}C temperature, 15% ethanol concentration and 15% NaCl in liquid media).

Source of isolate	Isolate	Thermo	Ethanol	Osmo
Date	SD1	++	+	-
	SD2	-	-	-
	SD3	+	+	-
	SD4	+	+	+
	SD5	-	-	-
	SD6	+++	++	++
	SD8	-	-	-
	SD9	++	+	-
	SD10	+	+	+
Apple	SA1	-	-	-
	SA2	+	+	-
	SA3	++	+	+
	SA4	-	-	-
	SA5	++	-	+
	SA6	+	-	-
	SA7	+	-	-
	SA8	++	+	+
Mango	SM1	+	+	+
	SM2	+	-	-
	SM3	+	-	-
	SM4	+	-	-
	SM5	++	+	+
	SM6	++	+	+
	SM7	-	-	-
Orange	SO1	+	-	-
	SO2	+	-	+
	SO3	+	-	-
	SO4	++	+	+
	SO5	-	-	-
	SO6	+	+	+
	SO7	+	-	-
Grape	SG1	++	+	+
	SG2	+	-	-
	SG3	+++	++	++
	SG4	+	-	+
	SG5	+	+	+
	SG6	-	-	-
	SG7	+	-	-
Banana	SB1	+	-	-
	SB2	+	+	+
	SB3	++	-	+
	SB4	+	+	+
	SB6	-	-	-
	SB7	-	-	-
	SB8	++	+	+
	SB9	+	-	+
	SB10	-	-	-

Table 2. Optical density at different temperature in liquid media.

Temperature °C	Isolate	O.D. at inoculation	O.D. after 24 hours	O.D. after 48 hours
25	SD6	0.559	1.501	2.290
	SG3	0.519	1.424	2.063
30	SD6	0.515	1.848	2.311
	SG3	0.441	1.790	2.162
37	SD6	0.671	1.744	2.058
	SG3	0.523	1.024	1.901
40	SD6	0.465	1.456	1.918
	SG3	0.350	0.301	1.261
44	SD6	0.511	0.693	0.802
	SG3	0.687	0.530	0.471

Ethanol-tolerance of selected yeast isolates SD6 and SG3

The isolate was selected for screening of ethanol tolerance yeast (Table 3). The isolate can grow up to 20% ethanol containing liquid YEPD media. Maximum growth for the date SD6 was seen in 5% ethanol containing media, but for the Grapes SG3 was 10%. Growth were recorded at 5%, 10%, 12%, 15%, 18%, 20%, and 25% of ethanol containing liquid media and O.D is given gradually. The maximum concentration of ethanol which can be produced by yeast varies with species up to 20% by volume. Ethanol, which is produced during fermentation, is rather inhibitory to cell growth than that from an exogenous source [27]. It was concluded that the effect of temperature on ethanol accumulation in *S. uvarum* growth was arrested when a critical intracellular ethanol concentration had been reached, and this intracellular accumulation was greater at higher temperatures [28]. The toxic effect of ethanol has also been attributed to damaging the cell membrane or changing its properties. The extent of ethanol tolerance of certain yeasts is highly isolate dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane [27].

Table 3. Optical density for different ethanol concentration containing media.

Ethanol %	Isolate	O.D. at inoculation	O.D. after 24 h.	O.D. after 48 h.
5	SD6	0.364	0.645	1.920
	SG3	0.433	0.891	1.403
10	SD6	0.365	1.296	1.825
	SG3	0.445	0.638	1.452
12	SD6	0.247	0.588	1.206
	SG3	0.336	0.409	0.560
15	SD6	0.352	0.638	0.833
	SG3	0.246	0.288	0.305
18	SD6	0.269	0.670	1.293
	SG3	0.244	0.283	0.309
20	SD6	0.290	0.313	0.384
	SG3	0.192	0.277	0.503
25	SD6	0.220	0.201	0.166
	SG3	0.259	0.150	0.109

Osmo-tolerance of selected yeast isolate (SD6 and SG3)

The two isolates vary significantly in their tolerance to sugar concentrations with isolate SD6 showing more tolerance after 24 or 48 hours of incubation (Table 4). Researchers studied fermentation with various initial concentrations of sugar and they demonstrated the logarithmic relationship between time of fermentation and initial concentrations of sugar [29]. Previously it was demonstrated that isolates were capable of fermenting up to 30% of sucrose efficiently [30].

Table 4. Optical density for different sugar concentration containing media.

Sugar %	Isolate	O.D. At Inoculation	O.D. after 24 hours	O.D. after 48 hours
6	SD6	0.206	0.569	1.325
	SG3	0.221	0.343	0.411
9	SD6	0.209	0.379	0.661
	SG3	0.226	0.267	0.315
12	SD6	0.218	0.258	0.329
	SG3	0.222	0.249	0.301
15	SD6	0.242	0.267	0.302
	SG3	0.260	0.254	0.294
18	SD6	0.252	0.281	0.314
	SG3	0.224	0.286	0.351
20	SG3	0.324	0.332	0.356
	SD6	0.227	0.222	0.341

Identification of the selected yeast

Fig (1) shows the features of the appearance of cultures when cells grown in YPD broth and on YPD agar. After 3days of incubation at 40^{o}C, heavy, dry climbing pellicles were formed on the surface of YPD medium.

The physiological characterization

Fermentation of carbohydrates

In this study, tow isolates showed variation of utilization of seven different sugars .For fermented carbohydrates by yeasts, the color of the medium changed from pink to yellow due to the formation of acids and gas The isolate obtained from date SD6 isolate utilized Glucose, Sucrose, Fructose, Lactose, Maltose and Trehalose but failed to grow on Xylose. The isolate obtained from Grapes GS3 isolate utilized Glucose, Sucrose, Fructose, Lactose and Trehalose but failed to grow on Maltose and Xylose. After 48 hours (Table 5).

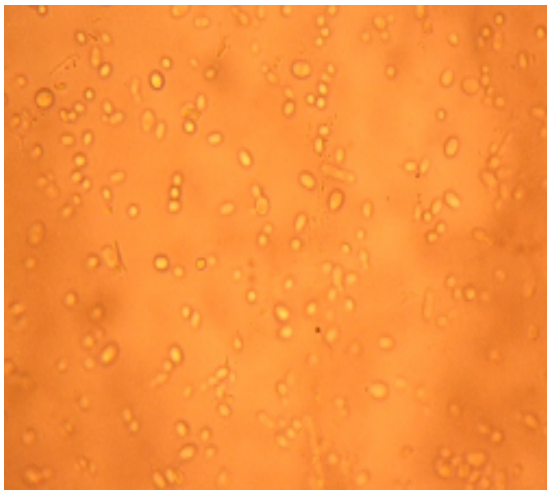

Figure 1. The cell morphology under compound microscope (**SG3 isolate**).

Table 5. Fermentation result of different carbohydrates for SD6 and SG3 isolate of yeast.

Carbohydrate	Isolate SD6	Isolate GS3
Glucose / Dextrose	+	+
Sucrose	+	+
Maltose	_	+
Lactose	+	+
Fructose	+	+
Xylose	_	_
Trehalose	+	+

The chemical composition of molasses is presented in Table (6). The molasses contained 83.2% total soluble solids, 17.6% reducing sugars, 37% sucrose, 54% total sugars, 13.25% ash, o.54% calcium, 0.28% sodium, 2.89% potassium, 36.31% pol, 43.58%purity and it had a pH value of 5.6. Most of the chemical parameters determined in this study were in close agreement with a previous report that molasses contained (45-55)% total sugars, (20-25)% reducing sugars, (25-35)% sucrose, (10-16)% ash, (0.4-0.8)% calcium, (0.1-0.4)% sodium, (1.5-5)% potassium and pH (5-5.5) [31]. On the other hand, it was found that molasses contained 52% total sugars, 16% reducing sugars, 34% sucrose, 12% ash and pH 5.0 [32].

Table 6. Chemical composition of molasses.

Parameter	Value %
Total soluble solids	83.20
Reducing sugars	17.60
Sucrose	37.00
Total sugars	54.00
Ash	13.25
pH	5.6.00
Purity	43.58
Calcium	0.54
Sodium	0.28
Potassium	2.89
Brix	82.60
Pol	36.31

Table (7) 5 treatment to the best tow isolate (SD6 and SG3) the efficiency best in treatment. The supplementation of additional nitrogen source (ammonium sulphate), phosphate source (KH_2PO_4) and peptone to the molasses medium alone (T3) was found to increase the ethanol production more than 4-folds. Table (8) showed the effect of sugar concentration on ethanol production by strain SD6 and SG3 showing the highest efficiency at 40% sugar concentration by both strains. The fermentation of molasses was optimized with respect to sugar concentration. Results revealed a temperature of 40^{o}C and 40% sugar concentration as optimum for

fermentation. Stress tolerance tests showed (DS6, SG3) isolates is highly thermo, smo and ethanol tolerant. Conway method for estimating percentage of ethanol was employed. Some researchers also studied viability of *Saccharomyces* sp. in 50% glucose and reported a viability of 10-98.8% in different isolates of yeast [33]. The detrimental effect of high sugar concentration on ethanol production was studied before in *Kluyveromycesmarxianus* and a sucrose concentration more than 23% in molasses was found to affect ethanol production [34]. Therefore, in the present study. growth and fermentation were carried out with sugar concentrations up to 20%.

Table 7. The efficiency of the five treatments to isolate (SD6 and SG3).

Treatment	Isolate code	Ethanol %	Efficiency %
T1	SD6	6.0	60
	SG3	5.7	57
T2	SD6	7.5	75
	SG3	7.0	70
T3	SD6	9.0	90
	SG3	8.2	82
T4	SD6	7.7	77
	SG3	7.9	79
T5	SD6	6.5	65
	SG3	7.0	70

Table 8. Effect of sugar concentration on ethanol production by (SD6, SG3) in 10^7 and T3.

Isolate code	Sugar conc. %	Ethanol conc.%	Efficiency %
SD6	20	5.7	57
SG3	20	5.3	53
SD6	30	7.5	75
SG3	30	6.9	69
SD6	40	9.0	90
SG3	40	8.2	82
SD6	50	7.9	79
SG3	50	7.7	77

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