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Original Article

Effect of Various Parameters on the Growth and Ethanol Production by Yeasts Isolated from Natural Sources

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Two ethanol fermenting *Saccharomyces cerevisiae* were isolated from date juice and grapes and grown in YEPD medium. They were characterized for alcoholic fermentation using sugarcane molasses and their growth conditions were optimized with respect to pH and sugar concentration. Results revealed a temperature of 30ºC, pH 6.0 and 6.5% sugar concentration as optimum for fermentation. Stress tolerance tests showed that date juice isolate was highly tolerant to temperature, pH and high ethanol concentration in the medium. Under optimized conditions, *S. cerevisiae* isolated from date-juice produced 7.75% of ethanol in molasses as estimated by Conway method.

Key words: Ethanol, yeast, biofuel, *Saccharomyces cerevisiea*

Introduction

Over the last few decades, the negative impacts of fossil fuel on the environment and consequent global warming, progressive demand for energy, inevitable depletion of the world's energy supply, and the unstable oil market (such as the energy crisis of the 1970s) have renewed the interest in searching for alternative fuels[1]. The alternative fuels are expected to satisfy several requirements including substantial reduction of greenhouse gas emission, worldwide availability of raw materials, and capability of being produced from renewable feedstocks[2]. Production of bio-ethanol through fermentation is a potential alternative to traditional fossil fuel and can be utilized as a sole fuel in cars with dedicated engines or in fuel blends. Ethanol is currently produced from sugars, starches and cellulosic materials. The first two groups of raw materials are currently the main resources for ethanol production, but concomitant growth in demand for human feed similar to energy could make them potentially less competitive and perhaps expensive feedstocks in the near future, leaving the cellulosic materials as the only potential feedstock for production of ethanol[3].

In this study, yeast was isolated from date juice and grapes, fermentation was carried out in molasses and the characteristics of the yeast isolates in terms of ethanol production, temperature and pH tolerance were also analyzed.

Materials and methods

Isolation of yeast

The yeasts were isolated from rotten grapes and date-juice collected from markets around Dhaka. First the sample was grown in Yeast extract Peptone Dextrose (YPD) broth for 48 hour and then streaked on YPD agar medium. Suspensions from suspected colonies were observed under the light microscope to get desired yeasts. Yeast isolates which yielded more ethanol in preliminary screening were then identified by biochemical tests[4].

Identification of yeast isolates

The yeast isolates were characterized based on their cultural characteristics (colony morphology, pigment, elevation, edge and surface appearance). Morphological and biochemical characterization of the isolated yeasts was performed according to Boboye and Dayo-Owoyemi[5].

Maintenance of the Culture

The yeast isolates were cultured and maintained in Yeast Maintenance Medium (YMM, Difco, UK) and Yeast extract Peptone Dextrose medium (YPD, Difco, U K).

Inoculum Development

The yeast inoculum was prepared by transferring 2 to 3 yeast colonies in molasses broth and growing them in 250 ml conical flask containing 25 ml media at 30 °C at 130 rpm for 48 hours.

Cell count and Maintenance

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[6]. Mostly the cell count recorded was 10v cells/ml or a fraction higher.

Pretreatment of molasses

The molasses were collected from local market and used as nutrient source for the Yeast. Molasses were pretreated with

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sulfuric acid to remove particles, dirt and kill unwanted microbes and urea was used as nitrogen source. 250 g molasses was diluted with water to bring the final volume to 1 L and 0.10 g urea and 0.30 ml concentrated sulfuric acid was added and heated to boiling. This was kept standing for couple of hours before use.

Fermentation

Autoclaved molasses fermentation broth (10 ml) was inoculated with 48 hours grown slant culture of *S. cerevisiae* and the tube was placed in a rotary incubator at 30°C with vigorous shaking (180 rpm) to form a homogeneous suspension.

250 ml fermentation media was taken into 500 ml Erlenmeyer flasks and inoculated with homogenous suspension of yeast under aseptic condition. The flasks were incubated at different temperatures under both non-shaking and shaking condition.

Ethanol production procedure from fermentation of molasses

250 ml of sterile pretreated fermentation media was taken into 500 ml Erlenmeyer flasks and 1000 µl of 24 h old culture (10^8 CFU ml^{-1}) was added and incubated for 48 hours with different condition. The fermentation was carried out at varying temperature, pH, reducing sugar concentration and agitation.

Stress tolerances

Thermotolerance

Temperature is one of the most important factors that affect ethanol production by yeast using molasses as a carbon source[7]. The fermentation process is always accompanied with evolution of heat that raises the temperature of the fermenter. YPD liquid medium was used for detecting thermo-tolerance and growth in liquid media of selected yeast isolates. Ten ml portion of the medium was taken in McCartney tubes and inoculated with 48 hours old selected yeast isolates. The initial optical density of each tube was recorded using a 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 to observe thermo-tolerance of the yeast isolates. The increase in optical density in each tube was recorded as evidence of growth. Besides this, growth on YPD agar media at 25°C, 30°C, 37°C and 40°C was also observed to ensure thermo-tolerance.

Ethanol tolerance

One ml of various concentrations of absolute ethanol varying from 5 to 25% (v/v) were added to different flasks of Molasses fermentation medium to constitute varying percentages of ethanol: 5%, 10%, 12%, 15%, 18% 20% and 25% (v/v). Forty mililiter portion of the medium was taken in 125 ml flask and then inoculated with selected thermo-tolerant yeasts. The initial optical density of each flask was read in a 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.

pH tolerance

YPD liquid medium was used for detecting the ability of the yeast isolates to grow at different pH values. Sterilized YPD broth at different pH was prepared. Each test tube contained 13 ml of YPD media with different pH. Then each was inoculated with half loop-full of yeast colony. The initial optical density was measured at 600 nm and the tube was incubated at 30°C for 48 hrs. After 48 hrs cell density was again recorded at 600 nm for growth. Blank media was used as a negative control for growth.

Osmotolerance:

Growth impairment under conditions of high osmotic strength is often associated with defects in the cell wall or components of the cytoskeleton[8] . YPD broth was prepared containing 6%, 9%, 12%, 15%, 18% and 20% of NaCl. Each test-tube contained 13 ml of YPD medium with appropriate concentration of salt. Blank medium was used as negative control of growth. Each test tube was inoculated with half loop-full of yeast colony. Initial optical density was measured at 600 nm and the cells were incubated at 30°C for 48 hr. After 48 hr cell density was again recorded at 600 nm.

Estimation of reducing sugars

The concentration of reducing substances (sugar) of the fermentation medium was estimated by dinitrosalicylic acid (DNS) method[9]. A double beam UV/VIS –scanning spectrophotometer (Model-TGOU, Perkin Elmer,UK) was used for measuring absorbance at 540 nm.

Estimation of ethanol

The amount of ethanol was determined using the Conway method[10]. One ml of fermented solution was diluted 250, 500 and 1000 times with distilled water to estimate ethanol concentration and one ml of the diluted solution was taken as a sample. A Conway unit was used for ethanol detection by the following procedure.

One ml potassium dichromate was placed into the center of the Conway unit. The sample was placed around the center. The Conway unit was then covered by a glass plate for 24 hours for reaction. The water and ethanol slowly evaporated, came in contact with Potassium dichromate and then became oxidized. One Conway unit was used as a blank as a blank containing 1 ml distilled water. After allowing reaction for 24 hours the sample was titrated against 0.1 N Na_2SO_3.

Results and Discussion

Identification of yeast isolates

Based on the colony characteristics of (white and creamy texture) ovoid microscope shape, the presence of ascospore and budding pattern (multipolar), carbohydrate fermentation test, the selected isolates were found to belong to *Saccharomyces sp.*

Fermentation of carbohydrates

In this study, *Saccharomyces cerevisiae* showed variation in utilization of seven different sugars (Table 1). The date-juice isolate

utilized glucose, sucrose, fructose, lactose, maltose and trehalose but failed to grow on xylose. The isolate obtained from grapes utilized glucose, sucrose, fructose, lactose and trehalose but failed to grow on maltose and xylose.

Table 1. *Fermentation of different carbohydrates by selected date-juice isolate of S. cerevisiae.*

Carbohydrate	Before fermentation	After fermentation
Glucose/ Dextrose	Pink	+ (yellow), gas
Sucrose	Pink	+ (yellow), gas
Maltose	Pink	+ (yellow), gas
Lactose	Pink	+ (yellow), gas
Fructose	Pink	+ (yellow), gas
Xylose	Pink	- (no color change)
Trehalose	Pink	+(yellow), gas formed

Stress tolerance of isolated yeasts

Thermotolerance: Five YPD agar containing plates were streaked with Yeast colonies and incubated for 48 hours at 25°C, 30°C, 37°C, 40°C and 44°C. The date-juice isolate of yeast was able to grow at 25°-40°C but the grape isolate failed to grow at 44°C. To confirm the results obtained from solid media, thermotolerance study was repeated in liquid media. The results are shown in the given Table 3. It is evident that the Date-juice isolate was slightly thermo-tolerant as it had ability to grow up to 44°C and the most suitable condition for growth of the yeast was found to be 30°C.

Table 2. *Fermentation of different carbohydrates by selected grape isolate of S. cerevisiae.*

Carbohydrate	Before fermentation	After fermentation
Glucose/ dextrose	Pink	+ (yellow), gas
Sucrose	Pink	+ (yellow), gas
Maltose	Pink	- (no color change)
Lactose	Pink	+ (yellow),
Fructose	Pink	+ (yellow),
Xylose	Pink	- (no color change)
Trehalose	Pink	+ (yellow),

Ethanol tolerance: The two isolates DJ and G isolate were selected for screening of ethanol tolerance. It was found that the isolate could grow up to 20% ethanol containing liquid YEPD media. Maximum growth for the date-juice (DJ isolate) was observed at 5% ethanol containing media, but for the grape isolate growth occurred at 10% ethanol. Growth were recorded at 5%, 10%, 12%, 15%, 18%, 20%, and 25% of ethanol containing liquid media and O.D was recorded (Table 4).

Table 3. *Growth of date-juice and grap isolate at different temperatures in liquid media.*

Temperature	Strain	O.D.at Inoculation	O.D.after 24 hours	O.D.after 48 hours
25℃	Date-juice	0.559	1.501	2.290
	Grapes	0.519	1.424	2.063
30℃	Date-juice	0.515	1.848	2.311
	Grapes	0.441	1.790	2.162
37℃	Date-juice	0.671	1.744	2.058
	Grapes	0.523	1.025	1.901
40℃	Date-juice	0.465	1.456	1.918
	Grapes	0.350	0.301	1.261
44℃	Date-juice	0.511	0.693	0.802
	Grapes	0.687	0.530	0.471

Table 4. *Growth of date-juice and grape isolates in different concentrations of ethanol in the media.*

Ethanol %	Isolate	O.D.at Inoculation	O.D.after 24 hours	O.D.after 48 hours
5	Date-juice	0.364	0.654	1.920
	Grape	0.433	0.891	1.403
10	Date-juice	0.365	1.296	1.825
	Grape	0.445	0.638	1.452
12	Date-juice	0.247	0.588	1.206
	Grape	0.336	0.409	0.560
15	Date-juice	0.352	0.683	0.833
	Grape	0.246	0.288	0.305
18	Date-juice	0.269	0.670	1.293
	Grape	0.244	0.283	0.309
20	Date-juice	0.290	0.313	0.384
	Grape	0.192	0.277	0.503
25	Date-juice	0.220	0.201	0.166
	Grape	0.259	0.237	0.109

pH tolerance: The ethanol producing isolates were observed to have ability to grow at wide ranges of pH. At pH 2 the growth was not remarkable but the isolates survived the high acidic condition. They withstood the alkaline condition up to pH 10. Maximum growth was seen at pH 5. After 48 hours, cell density was recorded at 600 nm (Table 5). The optimal pH range for growth of the test yeast was found to vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen, and the strain of yeast. Similar observation was reported for pH tolerance of yeasts[11].This is possibly due to the optimum pH value for the activity of plasma membrane-bound proteins, including enzymes and transport proteins[11].

Osmotolerance: YEPD broth was prepared containing 6%, 9%, 12%, 15%, 18%, and 20% of NaCl. Growth was recorded at these salt concentrations by measurement of O.D at 600 nm (Table 6)

The results showed that both isolates were resistant to high concentration of NaCl indicating osmoltolerance. Both isolates showed highest growth at 6% NaCl containing media and tolerated up to 12% NaCl in the medium. Growth gradually declined at still higher concentration of salt (Table 6).

Kinetics of ethanol fermentation by the isolates under optimum growth condition

S. cerevisiae is capable of very rapid rates of glycolysis and ethanol production under optimal condition. The date juice isolated strain was grown in molasses media under optimum sugar concentration, temperature and two different pH and the results are shown in Figures 1 and 2. The production of alcohol was null at the initial stage of fermentation where the pH was fixed at 5 (Figure 1). After 24 hours, the level of glucose decreased and significant amount of ethanol was produced. Maximum alcohol was produced after 48 to72 hours of fermentation. Earlier experiments suggested more Ethanol was produced in shaking than non-shaking state[12]. After 96 hours of incubation under shaking condition fermentation yielded a maximum of 7.75% ethanol.

Table 5. *Growth of selected yeast isolates in liquid media at different pH*

pH	Isolate	O.D. at Inoculation	O.D. after 24 hours	O.D. after 48 hours
2	Date-juice	0.335	0.548	0.773
	Grapes	0.363	0.462	0.496
3	Date-juice	0.361	1.044	1.407
	Grapes	0.404	0.755	1.466
4	Date-juice	0.390	1.502	1.859
	Grapes	0.369	0.795	1.468
5	Date-juice	0.407	1.396	1.887
	Grapes	0.355	0.849	1.572
6	Date-juice	0.459	0.573	1.847
	Grapes	0.376	0.871	1.571
7	Date-juice	0.473	1.113	1.822
	Grapes	0.439	0.723	1.319
8	Date-juice	0.328	0.832	1.614
	Grapes	0.254	0.569	1.035
9	Date-juice	0.336	0.638	1.517
	Grapes	0.296	0.833	1.287
10	Date-juice	0.473	0.773	1.377
	Grapes	0.365	0.702	1.283

Table 6. *Growth in different concentrations of NaCl in the liquid media*

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

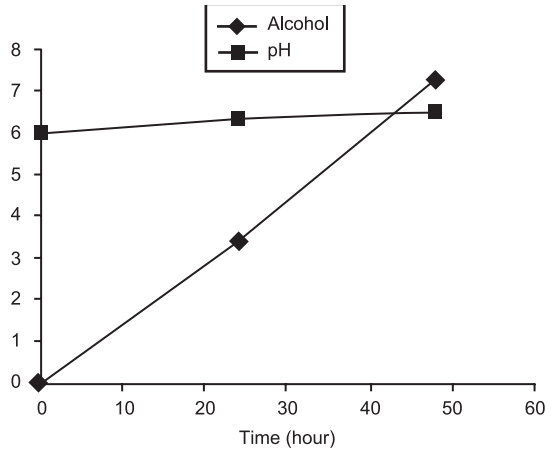

Figure 1. *Fermentation Kinetics of date-juice isolate in shake flasks at 6.5% reducing sugar and pH 6-.0 at 30° C temperature.*

At pH 5 glucose diminished rapidly and ethanol production rate was slow (Figure 2).

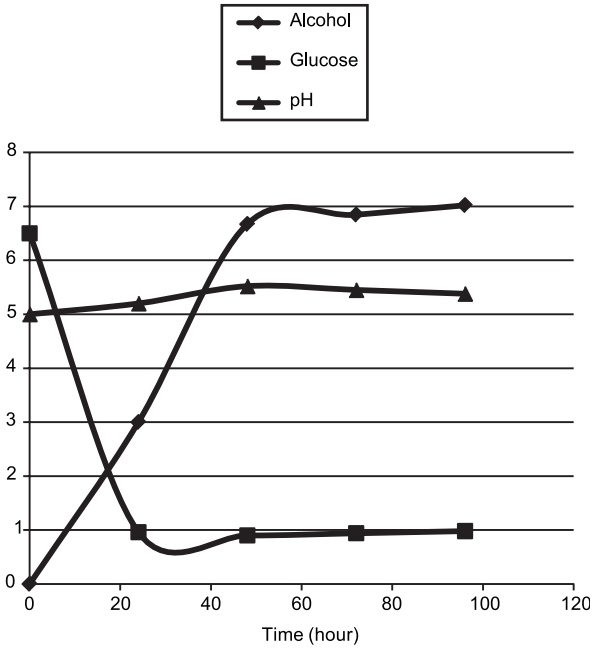

Figure 2. *Fermentation kinetics of date-juice isolate in shake flasks at 6.5% reducing sugar and pH 5.0 at 30° C temperature.*

Glucose concentration is pivotal in ethanol fermentation. Various sets of parameters showed that production of ethanol was favorable at 6.5% glucose (Table 7). In a similar study in Bangladesh five isolates were reported to produce alcohol by fermenting molasses at wide range of temperature (25-37°C). The production was maximal at 30°C after 48 hours of incubation. Using varying glucose concentrations (2.3 to 5.9%) as substrate in the fermented media alcohol production rate was reported to be maximal up to 36 hours[13].

Study showed the logarithmic relationship between time of fermentation and initial concentrations of sugar[14].

Table 7. *Fermentation by date-juice yeast isolate in shake flasks at different reducing sugar and pH levels at 30° C temperature.*

Glucose (%)	pH	Ethanol production (%)			
		24 hr	48 hr	72 hr	96 hr
6.5	5	3	6.67	6.845	7.02
6.5	6	3.73	7.75	7.205	6.66
7	5	2.27	6.15	5.19	5.19
7	6	2.07	6.83	6.66	6.66

In another experiment, ethanol production in the fermentation broth was increased with the addition of 5% (v/v) glucose but decreased beyond that. The final glucose utilization in the fermentation broth was found to be 5% (v/v), but above this concentration the final glucose utilization became quite appreciable[15]. The maximum specific growth rate and maximum ethanol concentration increased with an increase of glucose concentration from 5% (v/v). A reduction of ethanol production and growth of yeast were detected when glucose concentration was greater than 5% (v/v)[15]. Interestingly, similar findings were observed in the present study in which 6.5% and 7% glucose concentration proved to be the optimum sugar concentration for ethanol production. At 7.5% concentration of glucose production of ethanol plummeted. Date-juice isolates were found to be the most productive isolates than the grapes isolates.

Hydrogen ion concentration has a significant influence on industrial fermentation due as much to its importance in controlling bacterial contamination as its effect on yeast growth, fermentation rates and by-product formation[11].The best ethanol yields are generally obtained at pH 4.5-4.7. At higher pH, more glycerol and organic acids are formed at the expense of ethanol[16].pH 6.0 was found to be more suitable condition than pH 5.0 for the production of ethanol in this study.

Conclusion

Yeast isolates from date and grapes were tested for fermentation of carbohydrates. Date-juice isolate was capable of fermenting six out of the seven sugars tested. Glucose, Sucrose, Fructose, Lactose, Maltose and Trehalose were successfully fermented by this isolate but it failed to ferment Xylose. The Grapes isolate failed to ferment Maltose and Xylose, but utilized the other five carbohydrates, which proved the identity that both of the microorganisms are *Saccharomyces cerevisiae*.

Both isolates were screened for ethanol tolerance and showed up to 25% ethanol tolerance in YEPD liquid growth media. A slow growth rate was observed at 10-20% ethanol containing media.

The optimal pH range for growth of yeast can vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen, and the strain of yeast. In our study the date-juice isolate could grow in

a wide pH range between 2 and 10, but pH 5.0 was found to be the optimum pH for it. The grapes isolate showed growth at pH 3 to 10, but at pH 2, it grow at a slow rate. One of the most important findings of this experiment was the ability to grow at high acidic condition by the date-juice isolate.

Both isolates in this study had resistance against high osmotic pressure. Both isolates showed highest growth at 6% NaCl containing media. Growth gradually decreased at 15% and 20% NaCl containing media (Table 6).

Under shaking condition, the date-juice isolate showed highest ethanol production (5.93%) in the presence of 6.5% and 7% glucose. The grapes isolate produced 5.93% ethanol in 6% glucose containing medium. Under non-shaking condition, the date-juice isolate showed 5.53% ethanol production with 4% glucose and the grapes isolates produced maximum ethanol of 4.1% in the presence same glucose concentration (Table 7).

The 72 hours fermentation results showed the detailed characterization of the date-juice isolate, which proved to be the better isolate in every respect. A maximum of 7.75% alcohol production was recorded after forty-eight hours in the presence of 6.5% glucose and pH 6 (Table 7). This proved to be the highest production achieved in molasses media in this experiment.

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