RESEARCH ARTICLE

Screening and characterization of bioethanol producing yeasts from various sources

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ABSTRACT

The use of bioethanol as an alternative, renewable and green source of fuel has been steadily increasing around the world. Ethanol can be made synthetically from petroleum or by microbial conversion of biomass through fermentation. In the present study, nine ethanol-producing yeasts were isolated from various sources like soil, sugarcane bagasse and rotten fleshy fruits. Morphological, microscopic and biochemical studies were carried out with these isolates. The yeasts were characterized for temperature tolerance, osmotolerance and growth with sugar sources at various pH. Ethanol production was estimated by Nitroblue tetrazolium assay. Different strains showed ethanol production with 2% of glucose, sucrose, galactose, lactose, maltose and fructose. One of the strains isolated showed high osmotolerance with growth up to 70% sugar. The strains grew at different pH ranging from 3.5 to 9 and temperature ranging from 20°C to 37°C. For one strain, maximum ethanol production was observed at 25°C, pH 5.5. This strain produced 43.9 mg/ml ethanol from 80 mg/ml of glucose after 72 hours of fermentation indicating that the strain has a potential for industrial application.

Keywords: Yeasts, Bioethanol, Fermentation, Nitroblue tetrazolium assay.

INTRODUCTION

Biofuels have great importance as it is directly related to the energy consumption in near future. Many research organizations and other energy related multi-national companies are now trying to focus their attention on production of biofuels with increase in its efficiency and reduction of harmful release products if any (Bai *et.al.*, 2008). Biofuel is mainly produced by sugar fermentation processes, which could be then used as a substitute for nonrenewable sources of fuels. Ethanol fuel is widely used in Brazil and United States, together producing almost 87.1% of world ethanol production in 2011. Recently, production of ethanol by microbial fermentation has received special attention and many strains of yeasts and bacteria are known to efficiently ferment

sugars from various sources to ethanol (Lin and Tanaka, 2006). However, there is constant quest for isolation of novel and robust strains of yeast that have potential for industrial application for efficient bioethanol production. With this in mind, the present study aims at isolation and screening of ethanol-producing yeasts from various sources that are robust and have high-yields.

MATERIAL AND METHODS

Screening of yeasts

The yeasts were isolated and screened from various sources like soil, sugarcane bagasse and flesh fruits like Kokum (*Gracinia indica*), Jamun *(Syzygium cumini)* and and toddy palm (*Borassus flabellifer).* Soil sample was collected from a vineyard from Nasik, sugarcane bagasse was collected from a local sugarcane juice vendor, and fleshy fruits were collected from different areas of Konkan, Maharahtra. The samples were collected in polypropylene bags, transported to the laboratory and kept refrigerated until further processing.

Isolation of yeasts

i. Soil samples: One gram of the soil sample was suspended in 9 ml sterile distilled water and mixed well. The suspension (0.1 ml) was plated on YPD agar media, pH 5.0 (1 g yeast extract, 2 g peptone, 2 g dextrose, 1.5 g agar in 100 mL of distilled water) and incubated for 48 hr at 25ºC.

ii. Sugarcane bagasse: A suspension was prepared by mixing 1 gram of bagasse with 5ml of autoclaved distilled water. The suspension (0.1 ml) was plated on YPD agar, pH 5.0 and incubated for 48 hr at 25°C.

iii. Fruit samples: A loop full of exudates form rotten fruits like Kokum (*Gracinia indica*), Jamun *(Syzygium cumini)* and toddy palm (*Borassus flabellifer)* were spread on YPD agar, pH 5.0 and incubated for 48 hr at 25°C.

The plates were checked for alcohol smell and the colonies suggestive of yeasts were picked from plates. These colonies were re-isolated on fresh YPD agar plates and preliminary identification was carried out by studying cell morphology and budding characteristics.

Assimilation of sugars

Fermentation was carried out in 5 ml BTB broth, pH 7.0 (0.45 g Yeast extract, 0.75 g peptone. 1.6 mg bromothymol blue in 100 ml distilled water) test tubes with Durhams tube inserted. In each test tube, 2% weight by volume of single sugar was taken. The sugar assimilation test was carried out using dextrose, galactose, sucrose, maltose, lactose, and fructose. The tubes were inoculated with 50 µl overnight grown culture of the various yeast isolates. The tubes were incubated at 25°C and checked every day until 98 hours for gas production, acid production and assimilation by turbidity only. For one yeast strain (AniX), other sugars that were also tested for assimilation were, D-xylose, L-arabinose, rhamnose, trehalose and mannitol (2% w/v). The tubes were inoculated with the yeasts isolates, incubated at 25°C and checked after 72 hours for sugar assimilation, growth and alcohol production.

Additionally, the following studies were carried out for only one (AniX) of the strains. These parameters will however have to be carried out for all the other strains.

Temperature and pH tolerance

The yeast strain was screened for growth at different temperatures and pH. The strain was streaked on YPD agar plates. The plates were incubated at different temperatures for 72 hours. The growth at different media pH between 3.5 and 9.5 was determined at 25°C. After 72 hours, the plates were observed for colony formation and the extent of growth was recorded.

Osmotolerance

The yeast strain was tested for osmotolerance by testing its growth in YPD broth containing different concentrations of glucose. Actively growing yeast culture (100 µl) was inoculated in 10 ml media and the tubes were incubated for 48 hours at 25°C. Samples were taken every 24 hours and optical density was recorded at 600 nm.

Bioethanol production

The amount of ethanol produced by AniX strain was determined in YPD broth containing different sugars, namely, dextrose, galactose, maltose and lactose (2% each). Ethanol was also quantitated at different concentrations of only dextrose (2%, 4%, 6%, 8% and 10%). The tubes were incubated at 25°C, and

bioethanol produced and residual sugars were estimated at 48 and 72 hours. Ethanol was estimated by Nitro-blue tetrazolium (NBT) assay (Fibla and Gonzalez-Duarte, 1993, Schhel, Lutke-Eversloh, 2013) and absorbance measured at 540 nm. Residual sugars were estimated by dinitrosalicylic acid (DNSA) method (Miller. 1959) and the absorbance was measured at 570 nm.

RESULTS AND DISCUSSION

Isolation and colony characteristics

From the various sources namely soil, sugarcane bagasse, and fermented fruits, nine different yeast strains were isolated that produced alcohols (Table 1).

Sugar assimilation

Six different hexose sugars (2%) in BTB media were used for the assimilation test. Ethanol production was confirmed by ethanol smell whereas the growth was seen by turbidity. The results are given in Table 2. It was observed that all the nine strains could assimilate glucose, fructose and sucrose, and showed acid and gas production after 48 and 72 hours. Four strains (J1, J2, J3 and AniX) could also assimilate maltose. Five of the strains (K1, K2, K3, J1, J2 and Ani X) could not assimilate galactose and lactose. Two strains, namely T1 and T2, could assimilate all the sugars but did not show gas production indicating that they could have higher efficiency of alcohol production and therefore could have potential application for industrial production of bioethanol. For the strain AniX, ability to utilize other sugars like xylose, arabinose, rhamnose, trehalose and mannitol was tested and not much of growth was observed in these sugars.

Temperature and pH tolerance

For the strain AniX, growth at different temperatures (20°C, 25°C, 37°C, 45°C and 55°C) was determined and the results are as shown in Table 3. The strain was able to grow at 20°C, 25°C, and 37°C whereas at 45°C and 55°C, the growth was totally inhibited. In prior studies (Patil and Patil, 2006; Ali and Khan, 2013), investigators have reported growth of yeasts up to 37°C and at higher temperature the growth was inhibited. Similar results were obtained in our study. The inability of yeasts to grow at 45°C is in agreement with the mesophilic character of yeasts.

Osmotolerance

The effect of sugar concentration on growth of the strain AniX was determined. The sugar concentration used was between 10% to 80% and growth was measured at 600nm. The results obtained are shown in Figure 1. The strain was able to grow in sugar concentration up to 80% but showed highest growth at 30%. A maximum of 20% sugar tolerance was reported in earlier studies (Osho, 2005; Ali and Khan, 2013). The tolerance of high sugar concentration by the strain indicates its robustness and potential for industrial production of bioethanol.

Table 1: Colony Characteristics of yeast isolates

Yeast isolate	Source	Colony colour	Colony nature	Elevation	Margin	Budding
S. cerevisiae	Lab strain	Whitish cream colour	Smooth and dry	Raised	Entire	Terminal
K1	Kokum	Off- white	Smooth and shiny	Convex	Entire	Terminal, bipolar
K2	Kokum	Off-white	Smooth and shiny	Convex	Entire	Terminal, bipolar
K3	Kokum	Off-white	Smooth and shiny	Convex	Entire	Terminal, bipolar
J1	Jamun	White	Smooth	Raised	Entire	Terminal
J2	Jamun	White	Smooth	Raised	Entire	ND
J3	Jamun	White	Smooth	Raised	Entire	Terminal
T1	Toddy palm	White	Dry	Flat	Wavy	ND
T2	Toddy palm	White	Dry	Flat	Wavy	ND
Anix	Sugarcane bagasse	White	Smooth	Raised	Entire	Terminal

Table 2: Assimilation and alcohol production of different sugars by the nine isolates

Sugar	Strain	*S. cerevisiae* (lab strain)			K1			K2			K3			J1		
	Time	AA	AP	GP	AA	AP	GP	AA	AP	GP	AA	AP	GP	AA	AP	GP
Glucose	24hr	+	+	+	+	+	+	+	+	++	+	+	++	+	+	-
	48hr	+++	**0**	+++	+++	**0**	+++	+	+	+++	+	+	+++	+	+	+++
	72hr	**0**	+	**0**	**0**	+	**0**	+	+	**0**	+	+	**0**	+	+	**0**
Galactose	24hr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	48hr	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72hr	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	24hr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	48hr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72hr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	24hr	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
	48hr	+++	+	+++	+	-	-	+	-	-	-	-	-	-	-	+
	72hr	**0**	+	**0**	+	-	-	+	-	-	+	-	-	-	-	+
Sucrose	24hr	+	+	+	_	-	-	+	+	-	+	+	-	+	+	+
	48hr	+++	+	+++	_	-	-	+	+	-	+	+	-	+	+	+
	72hr	**0**	+	**0**	+	**0**	-	+	+	-	+	+	-	+	**0**	+
Fructose	24hr	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48hr	+++	+	+++	+++	+	+++	+++	+	+++	+	+	+++	+	+	+++
	72hr	**0**	+	+++	**0**	+	+++	**0**	+	+++	+	+	+++	+	+	+++

Table 2: Continued…

Sugar	Strain	J2			J3			Anix			T1			T2		
	Time	AA	AP	GP	AA	AP	GP	AA	AP	GP	AA	AP	GP	AA	AP	GP
Glucose	24hr	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
	48hr	+	+	+++	+++	+	+++	+	+	+++	+	+++	-	+++	+	-
	72hr	+	+	**0**	**0**	+	**0**	+	+	**0**	+	**0**	-	**0**	+	-
Galactose	24hr	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
	48hr	-	-	-	+	-	-	-	-	-	+	+++	-	+++	+	-
	72hr	-	-	-	+	-	-	-	-	-	+	**0**	-	**0**	+	-
Lactose	24hr	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
	48hr	-	-	-	-	-	-	-	-	-	+	+++	-	+++	+	-
	72hr	-	-	-	-	-	-	-	-	-	+	**0**	-	**0**	+	-
Maltose	24hr	+	+	+	+	+	+	+	+		+	+	-	+	+	-
	48hr	+	+++	+	+	+++	+	+	+++		+	+++	-	+++	+	-
	72hr	+	**0**	+	+	**0**	+	+	**0**		+	**0**	-	**0**	+	-
Sucrose	24hr	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
	48hr	+	+++	+	+	+++	+	+++	+++	+++	+	+++	-	+++	+	-
	72hr	+	+++	+	+	+++	+	**0**	+++	**0**	+	**0**	-	**0**	+	-
Fructose	24hr	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
	48hr	+	+	+++	+++	+	+++	+++	+	+++	+	+++	-	+++	+	-
	72hr	+	+	+++	**0**	+	+++	**0**	+	+++	+	**0**	-	**0**	+	-

Key: '-' : nil; '+': mild; '++': moderate; '+++': strong

A=Assimilation (and acid production) | **AP=** Alcohol production | **GP**=Gas production

Table 3: Growth at different temperatures

Temperature	20°C	25°C	37°C	45°C	55°C
AniX	+++	+++	+++	-	-

Good growth: '+++', Moderate growth: '++', Weak growth: '+' and No growth: '-'

The strain AniX could grow between pH 3.5 and 7.5, but could not grow at pH 8.5 and 9.5. The results are as given in Table 4.

Table 4: Growth at different pH

pH	3.5	4.5	5.5	6.5	7.5	8.5	9.5
AniX	+++	+++	+++	+++	+++	-	-

Good growth: '+++', Moderate growth: '++', Weak growth: '+' and No growth: '-'

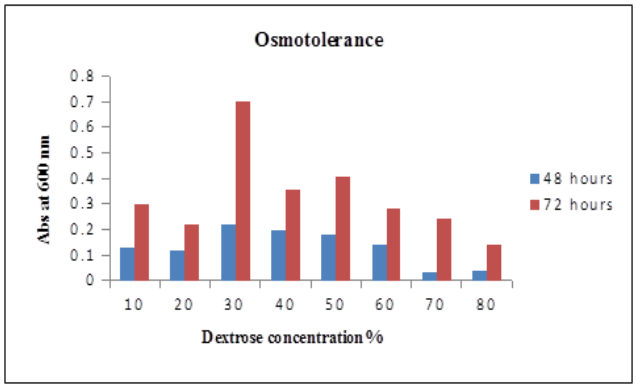

Figure 1: Determination of osmotolerance for AniX

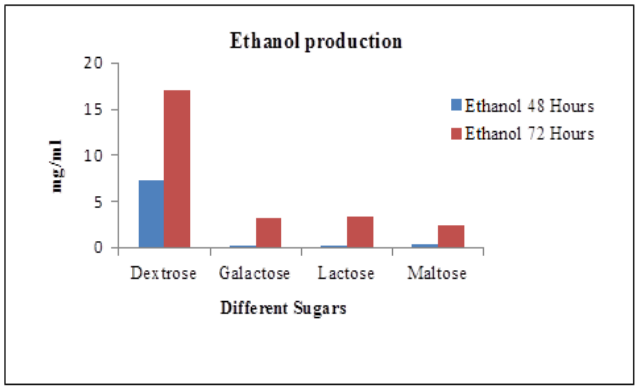

Figure 2A: Ethanol production from four sugars at 48 and 72 hours

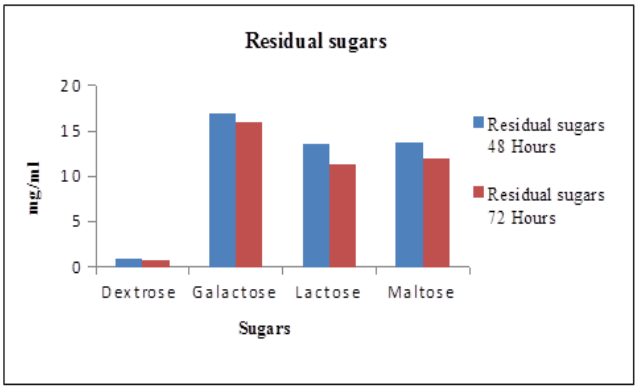

Figure 2B: Residual sugar concentration of four sugars at 48 and 72 hours.

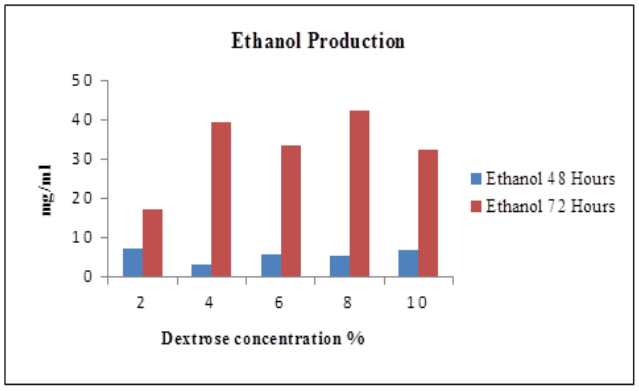

Figure 3A: Ethanol production with different dextrose concentrations at 48 and 72 hours

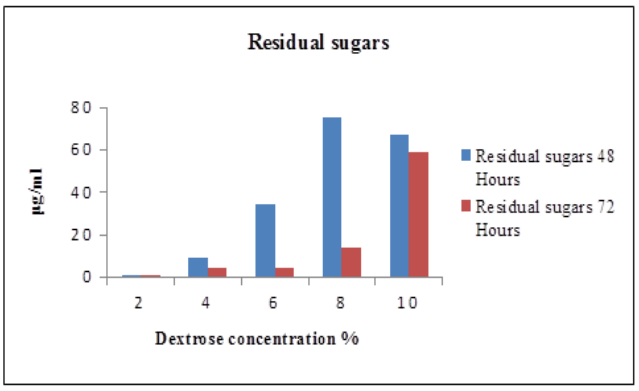

Figure 3B: Residual Dextrose concentration at 48 and 72 hours.

Bioethanol production

In order to understand the conversion efficiencies for different carbon sources, the amount of ethanol produced along with the amount of residual sugars was estimated. On the basis of sugar assimilation data, four reducing sugars namely 2% of Dextrose, Galactose, Lactose and Maltose were chosen for the ability of the stain AniX to produce ethanol. The quantity of ethanol produced and the amount of residual sugar were determined at 48 hours and 72 hours of fermentation. The results obtained are shown in Figure 2A and Figure 2B.

It was observed that with time there was an increase in ethanol production along with a decrease in residual sugar concentration. Among the four different sugars, it was found out that dextrose produced highest amount of ethanol of 17 mg/ml after 72 hours, followed by lactose, galactose and maltose. Maltose produced the least amount of ethanol of 2.5 mg/ml after 72 hours of fermentation. Since dextrose was found to be the preferred carbon source among the ones studied, further experiments to determine the ethanol production of the given isolate at different concentrations of dextrose was carried out (Figure 3A and 3B).

It was observed that as the concentration of dextrose increased there was an increase in the ethanol production. Maximum amount of ethanol produced was 42.5 mg/ml at 72 hours with 8% dextrose and 32.5 mg/ml at 10% dextrose concentration. The results indicate that, *Ani*X produces maximum alcohol at 8% and 10% concentrations of dextrose. It was also observed that, as the ethanol production increases with time there's a decrease in the residual sugar concentration indicating that sugars are being utilized by the organism for ethanol production. It was also observed that at each concentration, the amount of ethanol increased at a considerable rate from 48 hours to 72 hours. The results indicate that, the strain *Ani*X is efficient in production of ethanol than strains previously reported (Patil and Patil, 2006, Gupta *et.al*. 2009, Ali and Khan, 2013). Since it is also osmotolerant to high concentrations of dextrose, this strain has a potential to be used for industrial production of bioethanol.

CONCLUSION

In the present study, a total of nine strains of yeast were isolated from the various sources. Of these, two strains (T1 and T2) are capable of utilizing various hexose sugars without gas production. One other strain (AniX) was found to be highly osmotolerant and high ethanol producer. Further exploration and strain improvement could result in obtaining higher ethanol-producing strains. Thus, they could become promising candidates for efficient industrial production of bioethanol. Moreover, better culture methods, strain improvement techniques and cloning of cellulase genes in the isolates are options in the future, so that highly efficient production of bioethanol can be achieved using lignocellulosic wastes as substrates. To assist in this, sequencing the genome of the strains will also to be carried out.

Conflicts of interest: The authors stated that no conflicts of interest.

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