Characterization of ethanol producing yeasts isolated from *Garcinia indica* and *Syzygium cumini*

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Abstract
Biofuels are commonly recognized as the prospective rivals fighting against our age-old reliance on fossil fuels. Rising demand for fuel economy, environmental sustainability and availability of energy has drawn global attention to liquid biofuels. In India, sugarcane molasses is the main resource for bioethanol development and raw material supply inconsistency holds the major responsibility for sluggish response to blending targets. As the biofuel mass production is under developmental stage, the need for alternative ways to produce the sustainable energy is encouraged all across the globe. Therefore, the main objective of this study is to report the bioethanol producing ability of the yeasts isolated from natural sources.

This study was conducted to characterize the three isolates obtained each from Garcinia indica (kokum) and Syzygium cumini (jamun) extracts. The isolated yeasts (here referred to as K1, K2 and K3 from kokum; J1, J2 and J3 from jamun) were subjected to various assays to determine their qualities for industrial applications. The microscopic, morphological and biochemical characteristics of all the strains were studied. All the yeast strains were found tolerant against pH range from 3 to 10 and were able to grow at temperatures like 25ºC and 37ºC suggesting them as being mesophilic. All the strains could also utilize the provided eight carbon sources (sugars) after a varied interval of incubation. The most favored sources of carbon were fructose and dextrose. Among kokum-isolated yeasts, K2 yielded the highest alcohol yield (51.34 mg / ml) while J3 produced maximum alcohol (54.20 mg / ml) among the jamun strains.

Keywords: Yeasts, Biofuels, Bioethanol, Fermentation, Characterization.

Introduction

Yeasts are commonly found in aquatic, terrestrial and aerial habitats[1]. Chemoorganotrophs acquire carbon mainly from hexose sugars such as glucose and fructose or from disaccharides such as sucrose and maltose. Pentose sugars such as xylose, alcohol and amino acids may also be metabolized by certain species. Yeast species either require oxygen for aerobic cellular respiration (obligate aerobes) or they are anaerobic but also have aerobic methods of energy production (facultative anaerobes)[4,8]. Most species of *S. cerevisiae* and a few bacterial species like that of *Zymomonas mobilis*, are found to be producing ethanol by fermentation[9,10]. Fermentation of carbohydrates in fruits, grains and other biomass to ethanol by *S. cerevisiae* is the critical process for a wide range of products from fine wines to gasoline additives[1,14].

As per the International Energy Agency (IEA), Paris, a biofuel is fuel originating from living matter upon carbon fixation. There exist a few sorts of biofuels-bioethanol, biodiesel and biomethane, produced by enzymatic change, esterification of vegetable oils and animal fats and getting biogas from anaerobic digesters and landfills. Biofuels are a result of carbon fixation that is, a process where inorganic carbon is converted into organic forms. When this process occurs inside a live cell, it is called 'biological carbon fixation'. Combustion of fossil fuels generates CO_2, carbon monoxide and methane which not only cause harm to pollution but also generate waste whereas biofuels made of oxygen molecules tend to reduce the generation of toxic by-products.

In the carbon cycle, the natural recovery of fossil resources is significantly slower than their present exploitation rate. The vast reserves of fossil fuels are held by a limited number of countries, further increasing the unsustainability of their production. In addition, increased greenhouse gas emissions arise from the burning of fossil fuels and land use shifts as a result of human activity, resulting in an escalation of the global warming crisis. By replacing fossil with renewable energy, which are more evenly spread and cause less environmental and social problems, current global warming situation and other fossil-based issues could be successfully altered[6]. There is already a consensus that it is necessary to shift the global energy scenario to maximize the share of renewable sources and that ethanol will inevitably be a significant biofuel.

The need to create cheap green fuels to replace fossil fuels is identified in several countries' political agendas[13]. Among all the three biofuels, bioethanol has around 90% usage worldwide. The major consumers of bioethanol are the USA, Brazil and China[6]. Conventional crops like corn, sugarcane, potatoes along with fermentation of waste are used to produce biofuels. This helps in diminishing the generation cost. In order to promote biofuels in India, a National Policy on Biofuels was made by Ministry of New and Renewable Energy during the year 2009[12]. The goal of the National

Biofuels Policy (2018) is to enable availability of biofuels in the market thereby increasing its blending percentage. Currently the ethanol blending percentage in petrol is around 2.0% and biodiesel blending percentage in diesel is less than 0.1%. An indicative target of 20% blending of ethanol in petrol and 5% blending of biodiesel in diesel is proposed by 2030[12].

This study focuses on the ability of yeasts to ferment sugars and produce ethanol by fermentation. The strains used in this study are isolated from kokum (*Garcinia indica*) (K1, K2 and K3) and jamun (*Syzygium cumini)* (J1, J2 and J3)[16]. In the current study, the organisms will be tested for sugar utilization, alcohol production, alcohol tolerance and for also other parameters that will determine their industrial application potential.

Material and Methods

Isolation of yeast: All the six strains were isolated from natural fruit extracts. The three strains from *Garcinia indica* and three strains from *Syzygium cumini* were isolated on yeast extract peptone dextrose (YPD) agar plates. These strains are expected to be alcohol producing, according to the results found in the study[16]. The isolated strains were used for further studies.

Temperature and pH tolerance: The yeast strains were streaked on YPD agar plates, pH 5.0 and incubated at 4ºC, 25ºC, 37ºC, 40ºC and 45ºC respectively to examine temperature tolerance. Their growth was recorded for 24 hours, 48 hours and 72 hours.

For pH tolerance, the isolates were plated on YPD agar plates having different pH values, ranging from pH 3.0 to pH 10.0. Their growth was observed for 24 hours, 48 hours and 72 hours. The isolates were also inoculated in YPD broth tubes, having the same pH range and their culture densities (OD) were measured at 600 nm after 24 and 48 hours.

Ethanol production: The residual sugars and bioethanol production assays were carried out at 48 hours and 72 hours. Four sugars were chosen for both the assays, based on the sugar assimilation profile. These four sugars were dextrose, fructose, maltose and lactose. A final concentration of 2% sugar was incorporated in the YP broth. The supernatant from the growing isolates was aliquoted and centrifuged at 14000rpm for 5 minutes at 4ºC. This supernatant was then used for the ethanol assays.

Bioethanol estimation was done by NBT assay which was made using 1mg/ml stocks of each ingredient made in 100 mM Tris-HCl, pH 8.6, except NBT stock solution which was made using water. A 1:1 diluted supernatant of the cultures was made to react with 1.7 ml of the assay solution and then incubated at 37ºC for 10 minutes, after which the reaction was stopped using 360 µl of 1M KH_2PO_4[15,16]. The absorbance was measured at 570nm using a UV Spectrophotometer (Shimadzu Corp.).

Osmotolerance: The yeast strains were tested for osmo tolerance by testing their growth in YPD broth with ranging concentrations of dextrose (2%, 5%, 10% and 20%). Actively growing yeast culture (50 µl) was inoculated in 5 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.

Results and Discussion

The strains of yeast isolated from Garcinia indica (K1, K2 and K3) and *Syzygium cumini* (J1, J2 and J3) were tested for pH tolerance, temperature tolerance, sugar assimilation, alcohol tolerance and their morphology was analysed by staining techniques. All the strains were discovered to produce alcohol in high quantity when compared to reports found in the literature.

Colony Characteristics and Morphology: All the strains were isolated on sterile YPD agar plates and incubated for 48 hours at 25ºC. The colony characteristics and morphology (Table 1) were then determined which were found to match with the data provided in earlier studies[16].

Sugar Utilization: The isolated yeast strains were allowed to grow in the sterile YP broth, each containing different carbon sources at 25ºC. It was observed that all the six strains could utilize all the sources of carbon after 48 hrs of incubation (Table 2, Table 3).

Table 1
Colony characteristics after incubation at 25ºC for 48 hours.

Strain	K1	K2	K3	J1	J2	J3
Color	Pale white	Pale white	Pale white	White	White	White
Size	2 mm	2-3 mm	2 mm	2 mm	2-3 mm	2-3 mm
Surface and Consistency	Smooth	Smooth	Smooth	Smooth	Mucoid	Smooth
Margin	Entire	Entire	Entire	Entire	Entire	Wavy
Elevation	Slightly raised	Slightly raised	Raised	Raised	Flat with raised centre	Raised
Morphology	Spherical/ Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
Budding	Terminal, bipolar	Terminal, bipolar	Terminal, bipolar	Terminal	Terminal	Terminal

Table 2
Sugar assimilation profile of K isolates

Strain	K1			K2			K3		
Sugar	**24 Hr**	**48 Hr**	**72 Hr**	**24 Hr**	**48 Hr**	**72 Hr**	**24 Hr**	**48 Hr**	**72 Hr**
Dextrose	+	++	+++	+	+++	+++	+	++	+++
Fructose	+	+++	+++	+	+++	+++	-	++	+++
Maltose	-	+	++	+	++	++	-	+	++
Lactose	-	+	++	-	+	++	-	+	++
Galactose	+	+	++	+	+	++	+	+	++
Sucrose	+	+	++	+	+	++	+	+	+
Mannitol	+	+	++	+	+	++	+	++	++
Cellobiose	+	++	+++	+	++	+++	+	++	++

Table 3
Sugar assimilation profile of the J isolates

Sugars	**J1**			**J2**			**J3**		
	24hrs	**48hrs**	**72hrs**	**24hrs**	**48hrs**	**72hrs**	**24hrs**	**48hrs**	72hrs
Fructose	+++	+++	+++	+++	+++	+++	+++	+++	+++
Maltose	+	++	+++	+	++	+++	+	++	+++
Lactose	+	++	+++	+	++	+++	+	++	+++
Galactose	++	+++	+++	++	+++	+++	++	+++	+++
Sucrose	++	+++	+++	++	+++	+++	++	+++	+++
Cellobiose	++	+++	+++	++	+++	+++	++	+++	+++
Mannitol	++	+++	+++	++	+++	+++	+++	+++	+++
Dextrose	+++	+++	+++	+++	+++	+++	+++	+++	+++

KEY: - No growth + Slight growth
++ Heavy growth +++ Growth with thick precipitate of cells

Table 4
pH tolerance as seen after 24 hours of incubation at 25ºC.

Strain	**pH**							
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**
K1	+	+	++	+	+	+	+	+
K2	+	+	++	+	+	+	+	+
K3	+	+	++	+	+	+	+	+
J1	+	+	++	+	+	+	+	+
J2	+	+	++	+	+	+	+	+
J3	+	+	++	+	+	+	+	+

Key: (+) Growth (++) Heavy growth (*) Variable

Table 5
Results obtained after incubation for 72 hours at different temperatures.

Temperatures	**4ºC**	**25ºC**	**37ºC**	**40ºC**	**45ºC**
J1	-	+++	+++	-	-
J2	-	+++	+++	-	-
J3	-	+++	+++	-	-
K1	-	+++	+++	-	-
K2	-	+++	+++	-	-
K3	-	+++	+++	-	-

KEY: + Growth- Pinpoint colonies
++ Growth- 2-3mm diameter colonies
+++ Overgrowth
- No growth

pH Tolerance: In sterile YPD broth and YPD agar plates, which were adjusted to different pH, the organisms were inoculated. The broth aliquot was collected and growth was detected at 600 nm by taking OD. At pH 3, 4, 5, 6, 7 and 8, all six strains showed growth. It was also seen that at pH 9 and 10, the organisms did not demonstrate conclusive results (Table 4). In the industrial application of these yeasts, this data can be very advantageous as they do not show inhibitory activity at any given pH.

Temperature Tolerance: The temperature tolerance of the strains was tested by streaking the culture on sterile YPD agar plates and then incubating at different temperatures. It was found that all the strains were able to grow at 25ºC and 37ºC after 48 hours of incubation. It was also noted that no organism shows growth above 40ºC, suggesting the mesophilic nature of the yeast strains (Table 5).

Ethanol Production: Ethanol production was the mainstay of the study, which was measured using the NBT assay method. The standard plot of ethanol estimated by NBT assay method is given in figure 1 based on which the determination of ethanol produced by the various samples was carried out. The highest ethanol producing sugar was found to be dextrose followed by fructose, maltose and lactose, for all the strains, except the K3 strain which showed maximum ethanol production using fructose.

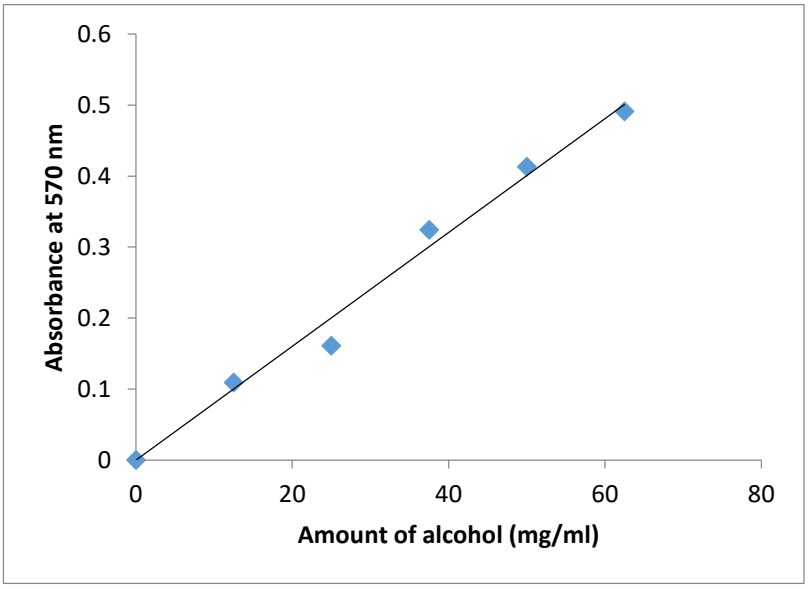
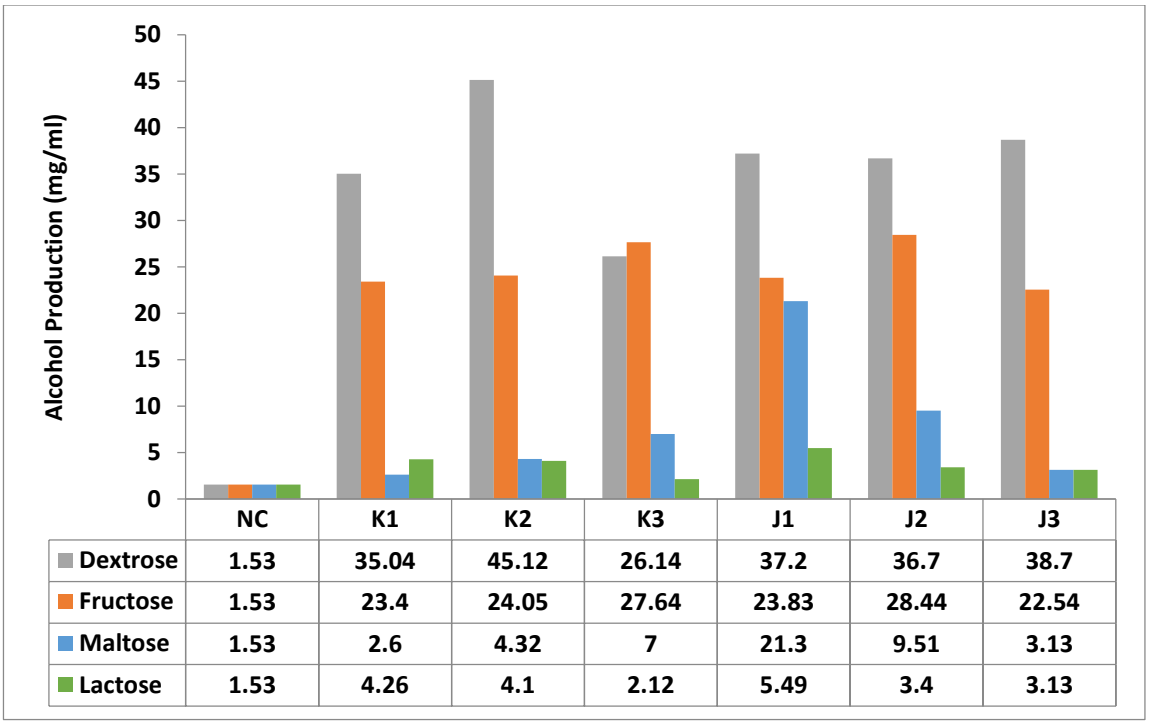
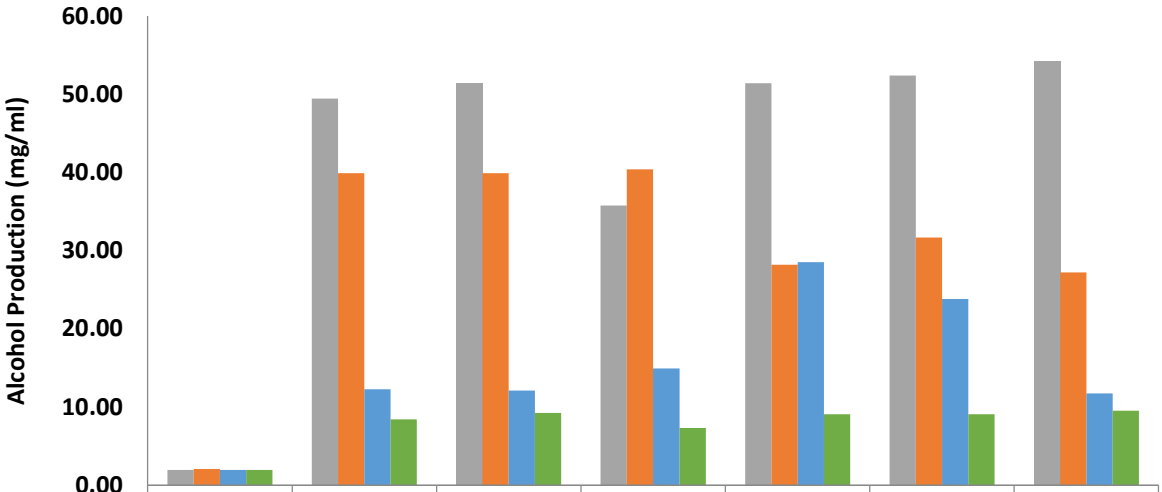


Figure 1: Standard plot of alcohol (NBT Assay)



	NC	K1	K2	K3	J1	J2	J3
Dextrose	1.53	35.04	45.12	26.14	37.2	36.7	38.7
Fructose	1.53	23.4	24.05	27.64	23.83	28.44	22.54
Maltose	1.53	2.6	4.32	7	21.3	9.51	3.13
Lactose	1.53	4.26	4.1	2.12	5.49	3.4	3.13

Figure 2: Ethanol production after 48 hours of incubation



	NC	K1	K2	K3	J1	J2	J3
Dextrose	1.94	49.45	51.34	35.75	51.40	52.40	54.20
Fructose	2.07	39.91	39.91	40.40	28.17	31.65	27.19
Maltose	1.94	12.28	12.09	14.91	28.50	23.83	11.72
Lactose	1.94	8.42	9.22	7.32	9.09	9.09	9.52

Figure 3: Ethanol production after 72 hours of incubation

The highest ethanol producers among all the three J and K strains were J3 and K2 respectively, using dextrose. Ethanol production after 72 hours (as seen in figure 3) was found to be slightly higher compared to the 48-hour value for dextrose (as seen in figure 2), while ethanol production using lactose was lower for all of the isolates.

Conclusion

In the present study, the three isolates obtained from *Garcinia indica*, (Kokum fruit) extract and three isolates from *Syzygium cumini* (Jamun fruit) extract, were characterized. In order to determine their characteristics for industrial applications such as bioethanol production, the six isolates were subjected to different tests and assays. The yeasts were found to grow well over a wide range of pH but, better in acidic than in alkaline[5] conditions. Studies have demonstrated changes in the expression of hundred genes in *S. cerevisiae* following alterations in pH[7]. The responses of *S. cerevisiae* to alkaline pH have been reviewed by Ariño[3] involving various signaling pathways.

All strains were found to be resistant to the pH range of 3 to 10, indicating possible industrial applications. The optimal fermentation temperature of traditional brewing yeast is 28–33°C, generally no more than 36°C, which restricts the industrial production of ethanol due to dramatical raise of the cost for cooling[11]. Likewise, the strains under study were able to grow only at temperatures 25ºC and 37ºC suggesting them as being mesophilic in nature. The osmo-tolerance results of all the isolates were also suggestive of their robustness. For characterization, assimilation of carbon is one of the main factors and the isolates under this study were subjected to eight different sources of carbon for growth. It was observed that all the strains could assimilate the sources with varied incubation period. Among all the different sugars tested for ethanol production, the monosaccharides fructose and dextrose showed the highest conversion efficiency of fermenting sugars into ethanol. The isolates K2 and J2 showed the highest ethanol production amongst their respective groups. Further investigations for assessing their toxic ethanol concentration should also be tested because it has previously been reported that after a certain concentration of ethanol, the yeast cells undergo oxidative stress and impact their ethanol production[2].

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