G.J.L.S.B.R.,Vol.2(2):1-7 (April-June, 2016) ISSN: 2456-3102

GLOBAL JOURNAL OF LIFE SCIENCES AND BIOLOGICAL RESEARCH
(Published By: Global Institute for Research & Education)

www.gifre.org

Isolation and Screening of Ethanol Tolerant Yeast for Bio-ethanol Production in Ethiopia

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Abstract

Fifty seven yeasts isolates were isolated from different Honey samples selection for ethanol tolerance on yeast extract peptone dextrose agar medium. The aim of the study was to isolate and screening ethanol tolerant yeast to optimize maximal ethanol production from honey. All the yeast isolates were first tested for carbohydrate fermentation using Durham tube fermentation method in yeast extract peptone dextrose broth. Four isolates which were relatively high fermentative in Durham tube fermentation method were selected for testing of the isolates for ethanol tolerance. Ethanol tolerance was tested using different concentration made from 97% of absolute ethanol in yeast extract peptone dextrose broth and their growth was determined by measuring optical density of the cells in broth using spectrophotometer at 660nm. Two strains of isolates showed measurable growth in medium containing above 15% of ethanol were selected for further study. Strain HJ2 tolerates 16% ethanol and strain HG4 tolerates 16.5% of ethanol. According to the morphological and biochemical characterization, the selected yeast isolates were belonged to the genus saccharomyces. Under the optimum conditions the maximum yield of ethanol (8.32% and 9.13%) was obtained using HJ12 and HG33 yeast isolates respectively and 7.67 % ethanol by the standard baker's yeast.

Key words: *Durham tube, ethanol tolerant, fermentation parameters, optical density, S. cerevisiae and yeast isolates.*

Introduction

Nowadays, ethanol production from renewable resources has received great attention because of the increasing petroleum shortage (Nadir *et a*l, 2009). Biomass fuels such as ethanol are renewable and help reducing greenhouse gas emissions from fossil fuels (Ibeto *et al*, 2011).

Such renewable energy sources are indigenous and can therefore contribute to reducing dependency on oil imports and increasing security of supply (Jegannathan *et al*, 2011).

Bioethanol can contribute to a cleaner environment and with the implementation of environment protection laws in many countries; demand is increasing (Chaudhury and Qazi, 2006).

Saccharomyces cerevisiae is one of the oldest, most exploited and best studied microorganism in both old and new biotechnologies and is known to be the world's premier industrial microorganisms which readily convert sugar into alcohol and CO_2 in metabolic process called fermentation (Noor *et al.*, 2003).

Saccharomyces strains were used widely and traditionally for industrial ethanol production because of its ability to produce high concentrations of ethanol from hexoses and its high tolerance to ethanol and other inhibitory compounds (Somda *et al*, 2011a).

Bioethanol produced from renewable biomass, such as sugar, starch or lignocellulosic materials, is one of the alternative energy resources, which is both renewable and environmentally linked (Somda *et al*, 2011b). Tolerance to high temperatures and high ethanol concentrations are important properties of microorganisms of interest to industry (Somda *et al*, 2011c).

The ability of yeast to produce ethanol depends on many factors such as strains, growth factors and optimum environmental conditions (Khongsay *et al.*, 2010).

The aim of this study was to isolate and characterize ethanol tolerant yeast strains capable of producing high level of ethanol from Honey.

Materials and Methods

Sampling Site and Sample Collection

Fifty seven different sample of honey samples were collected from Southwestern Ethiopia, Jimma, Illuabba bora and West Wollega using sterile plastic bags and brought to the Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University and the samples were kept at 4 ℃ for further study.

Isolation and Screening of Ethanol Tolerant Yeasts

The yeasts were isolated from different honey samples after dilution followed by plating aliquots of appropriate dilution of samples on yeast extract peptone dextrose agar (YPDA). One ml of each of the sample was transferred to nine ml of sterile distilled water to be successively diluted to 10^{-1} up to 10^{-6}. Aliquots of 0.1 ml from final dilutions (10^{-3} and 10^{-4}) were spread onto YPDA (Pons *et al.*, 1986). The YPDA medium contains g/l of yeast extract 10, peptone 20, dextrose (glucose) 20, and agar 20. It supplemented with 0.1 mg/ml streptomycin sulphate antibiotics to inhibit bacterial growth (Osho, 2005). The plates were incubated at 37^0C for 24 and 48 hrs.

Morphologically distinguished colonies were then selected under a dissection microscope. Yeast isolates were purified by sub culturing on YPD medium by streaking. The Pure culture was kept on YPD slant agar and stored at 4℃ for further study.

Morphological Characterization of Ethanol Tolerant Yeast Isolates

The yeast isolate from honey of Jimma Zone were designated by HJ1-HJ23, the yeast isolate from honey of Illuabba bora were designated by HG24-46. The yeast isolates from honey of West Wollega were designated by HWW 47-57. The morphology of the vegetative cells determined by growing on solid culture media YPD (Kreger-van Rij, 1984; Kurtzman and Fell, 1997). Yeast cells were examined under microscope for size, shape and methods of vegetative reproduction.

Ascospore Formation

The selected yeast isolates were examined for ascospore formation using presporulation and sporulation according to Kurtzman and Fell (2005). The presporulation medium composed 20 g of glucose, 2 g of ammonium sulfate ($(NH_4)_2SO_4$), 2 g of potassium dihydrogen phosphate (KH_2PO_4) and 5 g of yeast extract in 1000 ml of distilled water and the medium was kept in sterile state for 7 days. The medium was inoculated with a loopful young culture of 48 hrs old and incubated at 25℃, on shaker for 3 days. The sporulation medium consisted of 1 g of glucose, 8.2 g of potassium acetate, 2.5 g of yeast extract and 1.86 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) in 1000 ml of distilled water. The medium was inoculated with a drop of yeasts from the presporulation medium and incubated at 25℃. The culture was examined microscopically for ascospores production at weakly intervals for 3 weeks. Ascospore formation was detected by staining the heat-fixed preparation with carbol-fuchsin (Kreger-van Rij, 1984). Slides were decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. They were slide was rinsed in water and counter stained with 1% methylene blue to observe the mature ascospores stain red and blue stains of the vegetative cells.

Testing of Yeast Isolates for Carbohydrate Fermentation

Durham tube was used for testing of yeasts for carbohydrate fermentation. Yeast fermentation YP broth was used characterization of the yeast isolates based on fermentation of specific carbohydrates. The carbohydrates used were; glucose, galactose, maltose, sucrose, lactose trehalose, fructose and xylose. Yeast fermentation broth was modification of media developed by Wickerham for the determination carbohydrate to detect the color of the medium and gas formation (Warren and Shadomy, 1991). Yeast fermentation broth with carbohydrate and Durham tube composed of 4.5 g of yeast extracts, 7.5 g of peptone, 80 g of lactose, 120 g of raffinose, 60 g other carbohydrates and 17 g of bromcresol blue per liter deionized filtered water and final pH 7.1 ± 2 at 25℃.

Screening of Ethanol Tolerance of the Yeast Isolates

The medium for the detection of ethanol tolerance of ethanol, sugar and thermo tolerant yeast was modified from Osho (2005). YPD liquid medium was used for the tests. The medium was sterilized at 121 ℃ for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol was taken from 5 to 25% (v/v), and transferred to different flasks (125ml). Forty ml of the medium was distributed into each flask and then inoculated with selected isolates. The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer.

The treatments were replicated three times and the blank was made of YPD medium without yeast inoculation. The OD is directly proportional to the cell mass or growth (one OD_{660} nm = $1.85\times10^{7cell/ml}$). All cultures were incubated at 30 ^{0}C for 3 days. The increase in optical density in a flask was recorded as evidence of growth.

Optimizations of Sugar Tolerance of the Yeast Isolates

Testing of glucose tolerance, YPD broth was prepared containing 10%, 15%, 30%, 45% and 50% of different sugar concentrations according to Fakruddin (2013). The initial optical density (OD) of each culture in flasks was read for UV absorbance at 660 nm using a Pye-Unicam SP6 spectrophotometer. The treatments were replicated three times and the blank was made of YPD medium without yeast inoculation. The OD is directly proportional to the cell mass or growth (one OD_{660} nm = $1.85\times10^{7cell/ml}$). All cultures were incubated at 30 ^{0}C for 48 hrs. The increase in optical density in a flask was recorded as evidence of growth.

Molasses Pretreatment

The molasses were collected from Wonji Shoa sugar factory in Ethiopia and used as nutrient source for the yeast isolates. Molasses were pretreated with sulfuric acid to remove particles, dirt and kill unwanted microbes and Urea was used as nitrogen source. 250 gm molasses is diluted with 1 L water and 0.10 gm urea and 30 ml concentrated Sulfuric acid were added. It was then heated to the boiling and kept standing for couple of hours before use (Fakruddin, 2012).

Fermentation Process

A 24 hrs cell suspension of loopful yeast isolates inoculated into 250ml of 250 ml of sterile pretreated fermentation media (in 1000ml Erlenmeyer flask) containing (g/l): glucose-20g; yeast extract-5g; peptone-2.5g. The pH of the fermentation medium was adjusted to pH-5. Then fermentation was carried out at 30°C for 72 hrs (Manikandan *et al*., 2010).

Ethanol Recovery and Distillation Method

Fermented solution 250 ml flask was heated at constant temperature (90℃) to force the lowest boiling material into the vapour phase.

The vapour was passed over fractional column and the bulb of a thermometer at which point vapour was determined (El-diwany *et al*., 1992). The vapour was condensed to a liquid in the horizontal condenser that was cooled with a flow of cold water. The distillate was collected in a receiver (Figure 1). The volume of the distillate was measured.

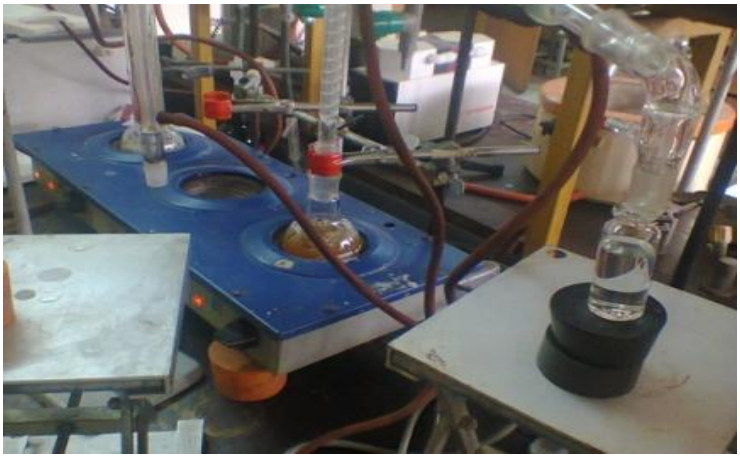

Figure 1. The distillated of the samples collected from distillation process

Determination of Bio-Ethanol Concentration

The Fourier Transform Infrared (FTIR) response was calibrated using different concentrations of 99.9% pure ethanol. The measurement was carried out in reflectance mode where ZnSe (Zinc selenide) window was used as a sample holder. After the absorbance the concentration of pure ethanol was recorded against the calibration curve and then converted to volume-to-volume concentration units.

Data Management and Statistical Analysis

All data were analyzed using one way analysis of variance (ANOVA) among treatment means at 5% level of significance were compared using the least significant difference (LCD) by SPSS software version 16 for windows.

Results and Discussions

Isolation and Screening of Ethanol Tolerant Yeasts

A total of 61 of yeast isolates were isolated from fifty seven samples of honey. It was possible to get pure colonies that to be used fermentation experiment most colonies were creamy (whitish) oval, convex and dome shaped (Figure 2).

Morphological and Physiological Characteristics of the Yeast Isolates

All of yeast isolates were observed under compound microscope and cell morphology was observed after 3 days of incubation, at 30℃, heavy, dry climbing pellicles were formed on the surface of YPD broth medium. The growth was butyrous with white cream color on YPD agar (Figure 2). The cell morphology of HJ12 and HG33 isolates was ovoidal to elongate and had single, pairs, or triple budding cells and Pseudo mycelia were also developed. Ascospore formed in ascospore forming media after incubating for 3 weeks at 25^0C (Figure 3). Similar result showed that filamentous pseudomycelium is characteristic of *Saccharomyces cerevisiae*, which is dimorphic, existing either in spherical, unicellular yeast like morphology or in a filamentous form (Gimeno *et al*., 2013).

Figure 2. Pure isolates from honey samples of yeast isolates

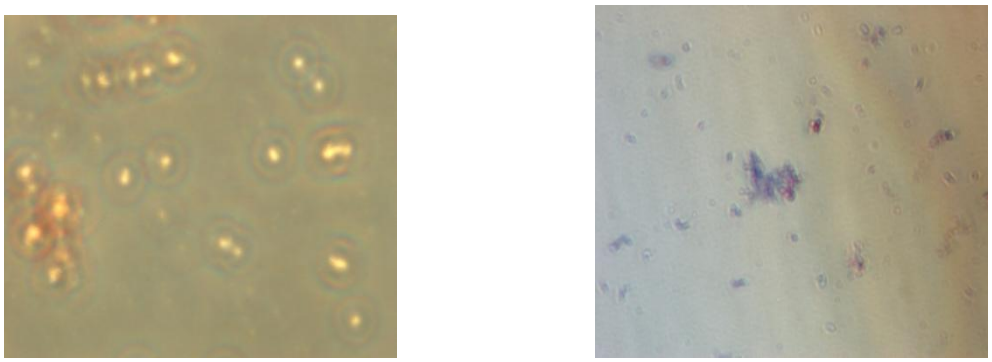

Figure 3. Ascospores of the ethanol tolerant yeast isolates

Yeast Isolates Test for Carbohydrate Fermentation

In this study, the yeast isolates showed variation in utilization of eight different sugars. Almost all isolates utilized glucose, galactose, sucrose, maltose, fructose and trehalose. All isolates failed to grow on xylose and lactose. The most vigorous isolates were HJ12 and HG33 growing on several of the test carbohydrates (Table 1). This was similar with results reported by Sathees Kumar *et al.* (2011).

Ethanol Tolerance of the Yeast Isolates

Yeast isolates (HJ12 and HG33) were able to grow in higher than 15 % (v/v) ethanol concentration (Figure 4). There were slight differences observed in growth rates while increasing the ethanol concentration from 5 to 10 % (v/v) and 15 to 25 % (v/v). The most ethanol tolerant yeast isolates (HJ33) that was ethanol tolerance to 16.5% (v/v) followed by the yeast isolate (HJ12) ethanol tolerant of 16% (v/v) ethanol in the broth medium (Figure 4). Accordingly, HJ12 and HG33 were relatively rapidly fermentative and the highest ethanol tolerant. Similarly, Fakruddin *et al*. (2013) showed that thermo tolerant yeast C, T and DB2 were grown at 0-20% (v/v) ethanol concentration. Certain strains P and C can grow well up to 18% (v/v) but T and DB2 were up to 15% (v/v). Also

Table 1. Sugar fermentation test for yeast isolates.

Type of sugar	Fermentation of sugar by yeast isolate
Glucose	+
Galactose	+
Sucrose	+
Sucrose	+
Maltose	+
Xylose	-
Lactose	-

(+) Positive, (-) Negative

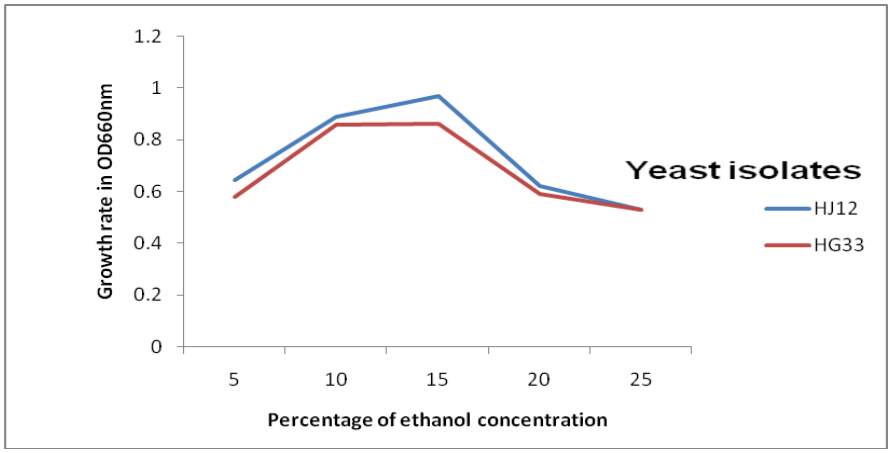

Figure 4. The growth of yeast isolates in relation to ethanol concentrations (dry weight in OD_{660} nm)

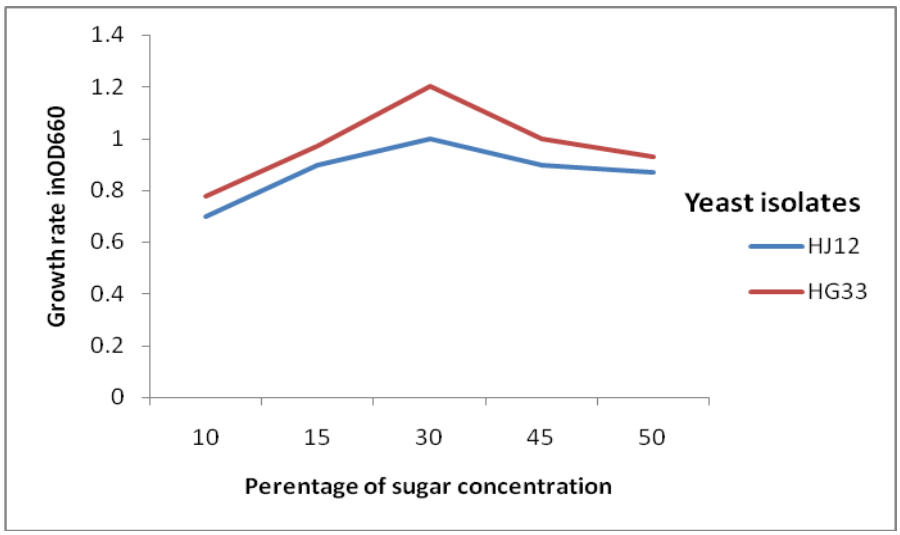

Figure 5. Yeast isolates growth rate response for different sugar concentrations (dry weight in OD_{660} nm).

Khaing *et al.* (2008) have reported that (KY1 and KY3) yeast isolates have tolerated up to 15% of ethanol in the medium and the yeast isolate (KY2) tolerated up to 20% of ethanol and maintained maximum ethanol production over a long incubation period.

Sugar Tolerance of the Yeast Isolates

Sugar tolerance the yeast isolates (HJ12 and HG33) increased in growth at an increasing concentration up to 30% but decreased slightly at concentration up to 45%

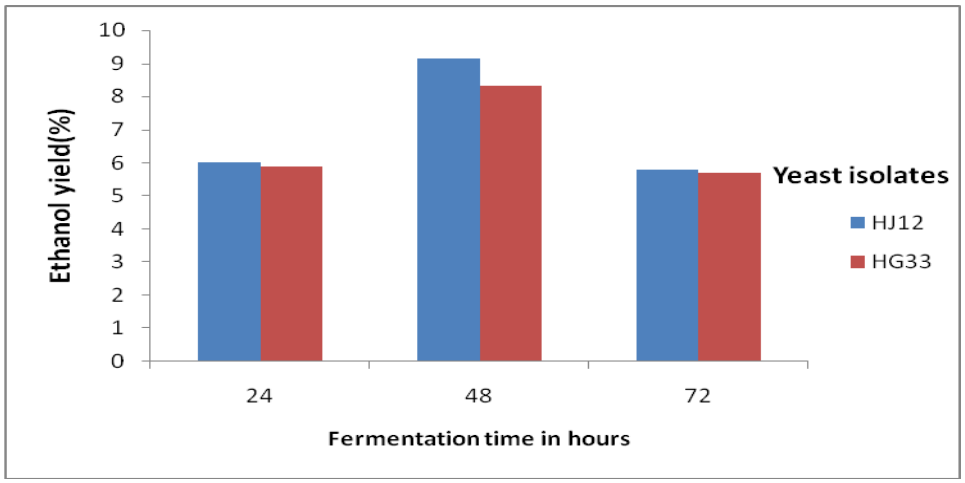

Figure 6. Ethanol yield formed after 24, 48 and 72 hrs fermentation time.

(Figure 5). The most sugar tolerant was HG33 with sugar tolerance capacity of up to 45% (v/v) followed by isolates HJ12 which was tolerant to 30% (v/v) sugar concentration in the broth media. In general HJ12 and HG33 were the highest sugar tolerant than other yeast isolates (Figure 5). Similarly, Osho (2005) showed that growth rate remain lowest in 25% (w/v) of sugar concentrations in all the yeast isolates. Dechasa Tolasa (2010) reported that the yeast isolates, TBY1, TGY2 and reference yeast were ability to grow best at the concentration from 24% to 32% of the medium containing sucrose. Their optical density were decreased as the sucrose concentration increased and their optical density at the concentration of 32% of sucrose deceased the growth of all yeast isolates in the first twelve hours and less than the initial optical density of the inoculums and increased gradually after twelve hours of incubation at 30^0C.

Ethanol yield

In present study, fermentation of the honey samples showed that the maximum ethanol yield (9.13% *v/v*) using yeast isolates HJ12 (Figure 6). The concentration of bio-ethanol increased with increasing fermentation time and decreased at the end of fermentation time. Maximum ethanol concentration, 9.13 % v/v was obtained at 48 hrs and the result started to decrease after 48 hrs of fermentation time (Figure 6). From the optimization experiment, the highest concentration of ethanol was achieved at 48 hrs of fermentation and started to level off (Figure 6). Similarly, Teramoto *et.al* (2005) showed that the analytical data for the resulting honey wine and the final concentrations of ethanol in honey wine made with ET99, W4, and K7 were 16.5, 17.5, and 17.5 % (v/v), respectively.

Conclusion and Recommendations

The highest ethanol tolerant yeasts were isolated from honey, HJ12and HG33 where able to tolerate ethanol concentration of 16.5 and 16 % (v/v) respectively. The isolates where able to tolerate 45% of sucrose as the substrate. The isolates were grouped under the genus *saccharomyces* depending up on their morphological and physiological characteristics and were closely related to *S. cerevisiae* .The optimum sugar concentration for ethanol production required for the isolates was 30 % at optimum pH 5. Generally both isolates were able to produce relatively high amount of ethanol from concentrated sugar solution. The result obtained from this study reveal a strong indication of yeast's great potential in the production of ethanol.

The potential of ethanol tolerant yeasts from different honey and sugar sources were not exploited. Therefore, efforts should have to be directed toward exploitation of ethanol tolerant yeasts especially from honey, honey wine and other sugar sources by collecting samples from different regions of the country for novel strain discovery and application of it for industrial ethanol production. Further studies should be conducted on characterization and utilization of the selected isolates.

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