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Original Article

Fuel Ethanol Production from Molasses by Some Indigenous Yeast Isolates

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In view of the anticipated shortage of the traditional supplies of fossil fuels there is a great deal of interest in production of ethanol as an alternative biofuel in recent years. The present report describes the search for potential yeast isolates from various ferments capable of producing ethanol. Twenty-one indigenous yeast isolates were recovered from various sources. Thirteen of them were found to produce ethanol belonging to the genera *Saccharomyces, Zygosaccharomyces* and *Kluyveromyces.* Comparative studies of the desirable properties of yeast showed that five isolates were promising from the industrial viewpoint, of them *S. cerevisiae* EP-17 was the most potent isolate for ethanol production. The isolates showed good tolerance of ethanol. Ethanol production by the selected yeasts was highest at pH 4.5 and 30°C in molasses medium with initial sugar concentration of 15%. *S. cerevisiae* EP-17 produced 7.89% ethanol using an inoculum size of 10^8 cells/ml. In larger fermentation tank, the yeast isolate produced 6.65% ethanol. After one-step distillation, 210 ml ethanol was obtained from 3 l molasses ferments and the concentration of ethanol was 65.56%. This study further revealed that indigenous yeast isolates could be used to benefit the fuel ethanol, spirit and industrial alcohol industries.

Keywords: Biofuel, Ethanol, Indigenous yeasts, *Saccharomyces cerevisiae*

Introduction

Because of upward spiral in the price of fossil fuel, coupled with impending shortages of the classical energy sources and environmental problems, there has been much interest in investigations of non-conventional energy sources during the last few dacades[1]. The production of ethanol or ethyl alcohol from starch or sugar-based feedstock is among man's earliest ventures into value-added processing. There are three main uses for ethanol (industrial, beverage and fuel), while the basic steps remain the same, the process has been considerably refined in recent years, leading to a very efficient process. Historically, ethanol has been used as a fuel in times of crisis in a number of countries, and this role is now being re-examined worldwide[2]. Ethanol fuel is the same type of alcohol found in alcoholic beverages. It can be used as a fuel, mainly as a biofuel alternative to gasoline, and is widely used in cars in Brazil[3]. Because it is easy to manufacture and process, and can be made from very common materials, such as sugar cane, it is steadily becoming a promising alternative to gasoline throughout much of the world[4].

In order to fulfil the increasing demand for ethanol increased production of ethanol is necessayl[5]. To achieve this target through fermentation it is essential to evolve good or potential yeasts isolates. It is also essential to study the production capacity of selected isolates for ethanol production fermentation from cheap and readily available raw materials like molasses in comparison with other substrates[6-7]. Two-third of the total molasses (about 50 thousand tonnes) obtained as a by-product of our sugar mills, remains unutilized every year due to lack of modern technology for fermentation process for ethanol production in Bangladesh[8]. In order to develop a practical process based on molasses, clarification or pre-treatment of molasses is required to reduce the inhibitory components that interfere with ethanol production[9-10]. The culture conditions like pH, temperature, sugar concentration, salt concentration, etc. together with effect of additives, if any, which have profound effect on ethanol production[11]. With a view to increasing productivity of ethanol by indigenous yeast isolates utilizing abundantly available cheap raw materials like cane molasses, process parameters like media composition, environmental factors etc. had been studied in this proposed project.

The specific objectives of this work were to isolate ethanol-producing indigenous yeasts, to evaluate the efficiency of yeast isolates for ethanol production in cane molasses under various conditions, to determine the effects of different substrates and their concentration on ethanol production, and to optimize the conditions for maximal ethanol production.

Material and Methods

Growth of microorganisms

Growth of yeast in liquid media was carried out in shake-flasks using an orbital shaking incubator. Inoculated solid and liquid media were incubated at different temperatures using an incubator.

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Yeast cells were separated from culture supernatant by centrifugation at 2,000 rpm for 10 min. A phase-contrast microscope was used for the determination of cell number and microscopic characteristics of the yeas isolates.

Maintenance of cultures

The pure yeast cultures were routinely maintained on malt extract-yeast extract-glucose-peptone (MYGP) agar medium in screw cap vials. The cultures were stored at 4°C until required. Subcultures were prepared monthly on fresh agar. For long term preservation the stock vials were filled up with paraffin oil, capped tightly and kept at 4°C.

Substrate preparation

The viscous molasses was diluted prior to use. The molasses was then acidified with hydrochloric acid to pH 4.5 and heated to100°C for 1 min in order to precipitate some organic materials. It was then clarified by heating the solution for half an hour at 100°C. The clarified molasses was kept at room temperature for overnight for sedimentation of suspended particles. In order to remove the coarse particles in the solution, it was filtered though absorbance cotton and the sediment were discarded. Sugar concentration in the treated molasses was then determined by refractometer and finally the clarified molasses was diluted with distilled water to adjust the desirable concentration of sugar for fermentation.

Fermentation medium

Raw molasses of sugar mill industries was used as the main substrate for ethanol fermentation. Commercially obtained glucose was also used as substrate to determine the comparative fermentation ability of the yeast isolates.

Isolation of ethanol-producing yeasts

Twenty samples from five different sources such as fermented sugarcane juice, mango juice, apple juice, decomposed bullocks heart and pineapple were selected for isolation of yeast isolates. Two millilitre of each sample was taken into McCartney bottle containing 15 ml extract-yeast extract-molasses-peptone (MYMP) medium with pH 5.0 and the medium plus the sample were mixed by vortexing and allowed to incubate at 30°C for 24 h on a rotary shaker. After sufficient growth was achieved, 1 ml of culture suspension from each bottle was re-inoculated into a 250-ml conical flask containing 50 ml of MYMP broth and incubated again under same condition. After three times of repeated inoculation and incubation, the active and enriched yeast isolates were isolated. After incubation, morphologically dissimilar discrete colonies were picked up from the culture plates by sterile needle and sub-cultured repeatedly following streak plate technique to get the pure culture.

Morphological characterizations

The yeast colonies grown on MYGP agar for 24 h at 30°C were characterized morphologically in terms of size, shape, colour, opacity, consistency, margin, elevation, texture and pigment formation. MYGP broth was used for examining the other characteristics like pellicle formation, turbidity, flocculation, gas-formation, alcohol-production and reproductive pattern (bud-formation).

Physiological and biochemical characterizations

For identification of the yeast isolates, various physiological and biochemical test such as carbohydrate fermentation and nitrate reductase activity test were performed.

Growth measurement

Yeast count was performed using Neubaur counting chamber. Viability was determined by staining of the cells with methylene blue reagent. The viable yeast cells contained some active enzymes. Yeast that decolorized methylene blue dyes when it prepared the cells. Dead yeast cells, which contained inactive enzymes, stain blue with the dye. The percentage of unstained cells is, therefore, a measure of viability. The methylene blue dye solution was mixed with an equal volume of cell suspension on a cover slip examine under a light microscope.

Estimation of ethanol

Ethanol produced in the fermentation medium was estimated by potassium dichomate oxidation method[12]. Potassium dichomate (33.882 g/l), ferrous ammonium sulphate (135.5 g/l) and diphenylamine (0.5 g/100 ml concentrated H_2SO_4) solutions were used as reagent for estimation of ethanol concentration. The fermented sample was diluted ten times with distilled water. Ten millilitre of the diluted sample was distilled against $K_2Cr_2O_7$ (10 ml) containing concentrated H_2SO_4 (5-6 ml). Then distilled was titrated against freshly prepared ferrous ammonium sulphate solution with diphenylamine as an indicator. Appearance of green colour indicated the end point of the titration. Burette reading (amount ferrous ammonium sulphate) was recorded to calculate the amount (in percentage) of ethanol present in the sample.

Estimation of reducing sugar

Reducing sugar in the wart and ferments was determined by dinitrosalicylic acid (DNS) method[13] using glucose as standard.

Ethanol fermentation

One loop-full of yeast colony taken from 24-h-old fresh culture into sterile 10 ml distilled water in test tube was shaken well by vortex mixer to dispense the cells into a homogenous suspension. One millilitre of suspension (about 10^{-8} viable cells) was inoculated to 100 ml MYGP broth in 250-ml conical flask and was incubated at 30°C on a rotary shaker for 24-72 h. The yeast count was determined by using Neubauer counting chamber. The standard concentration of the inoculum (10^8 cells/ml) was prepared by the appropriate dilution with sterile distilled water.

For scale up trial, fermentation medium was prepared in a 10-l aspirator bottle containing 3 l. Medium and inoculum was prepared as stated. The fermentation was carried out at 30°C for

72 h. The fermented medium was collected for distillation of ethanol. Only one yeast isolate designated as *Saccharomyces cerevisae* EP-17 was employed for this trial since it performed the best alcohol fermentation among the isolates studied.

Distillation process

Distillation was carried out using a distillation apparatus. Heating of fermented materials was carried out at 78°C. The condensation product was collected in a flask and used for estimation of ethanol concentration[12].

Results and Discussion

It is believed that ethanol has several advantages in biotechnological applications synthesized by microbial fermentation. A screening program was undertaken to obtain potential yeast isolates capable of producing ethanol from agro-industrial residues like molasses. Potential isolates were further studied to find out their optimum fermentation conditions for ethanol production.

Most of the yeast isolates capable of producing ethanol are usually found in habitats where sugar is decomposed under natural conditions. Samples of 5 different natural ferments were incubated with MYGP broth at 30°C under static condition for 24 h for the fermentation and enrichment of the ethanol-producing yeast isolates. After two successive transfers of the ferments to the fresh medium and incubation under same conditions, the enriched and activated yeast were isolated on MYGP agar plate. A total of 21 different yeast isolates were isolated on the basis of their good growth and ethanol production. Among the yeast isolates, 13 were flocculent yeasts, but 5 isolates formed turbid and 3 formed surface growth. The flocculent yeasts isolates were purified and preserved for further study. Though all isolates produced ethanol and gas, only 13 isolates produced a considerable amount of ethanol (2.5-7.23% v/v) under the given fermentation conditions. The isolates were obtained from fermented sugarcane juice, mango juice, apple juice, or pineapple juice samples.

Thirteen potential isolates were selected for characterization and systematic study to comprehend their relationship to other isolates published in literature. Each of these isolates were grown in different media for identification, based mainly on their cultural characteristics, morphological studies and physiological and biochemical analyses. All the isolates grew well in MYGP medium with distinct colonial characteristics. From the cultural characteristics including colonial morphology on MYGP agar medium, the microscopic characteristics including shape, reproduction system and ascospores and physiological and biochemical analyses such as fermentation of glucose, galactose, maltose, sucrose, lactose and raffinose, utilization of nitrate, the isolates were characterized for identification up to the genus level. The isolates were members of the family Saccharomycetaceae including *Zygosaccharomyces* (EP-01, EP-02, EP-05 and EP-08) *Saccharomyces* (EP-03, EP-04, EP-06, EP-11, EP-12 and EP-17) and *Kluyveromyces* (EP-10, EP-13 and EP-15).

The isolates were used for ethanol production in MYMP broth medium at pH 5.0 and 30°C for 72 h. The initial inoculum concentration was same for all the isolates. Six isolates of *Saccharomyces*, produced 3.47-6.42% of ethanol. The isolate *Saccharomyces* EP--17 produced the highest amount (6.42% v/v) of ethanol. *Zygosaccharomyces* isolates, produced 3.67-5.62% ethanol. Among them *Zygosaccharomyces* EP-02 produced considerable amount (5.62%) of ethanol. The isolates of *Kluyveromyces* were slow fermenters and produced 2.45-3.04% ethanol. Indigenous non-*Saccharomyces* yeast species grow during the early stages of many wine fermentations[14-15]. As the fermentation progresses, these non-*Saccharomyces* species die off leaving the more ethanol tolerant *S. cerevisiae* to redominate and complete the fermentation[16]. The extent to which indigenous yeasts contribute to the overall fermentation will be determined by their growth rates and maximum cell biomass relative to those of *S. cerevisiae*, as well as their ethanol tolerance[16].

The effect of sugar concentrations (5-25% w/v) on the production of ethanol by ten selected indigenous yeast isolates, capable of producing considerable amount of ethanol rapidly, was carried out for 72 h using MYMP broth as basal medium. Ethanol production increased with the increase of sugar concentration up to 15%, and thereafter, the production decreased (Figure 1). All isolates produced maximum amount of ethanol at 15% sugar concentration. High initial sugar concentrations can affect yeast growth by increasing the lag phase, decreasing the growth rate, decreasing the maximum cell population and decreasing the ethanol tolerance in later stages of fermentation[17-19]. The amount of ethanol in the fermentation broths ranged from 4.46-7.58%. Five isolates, *viz*., *Zygosaccharomyces* EP-02, *Zygosaccharomyces* EP-03, *Saccharomyces* EP-04, *Saccharomyces* EP-11 and *Saccharomyces* EP-17) produced high amount (>6%) of ethanol and were selected for further study.

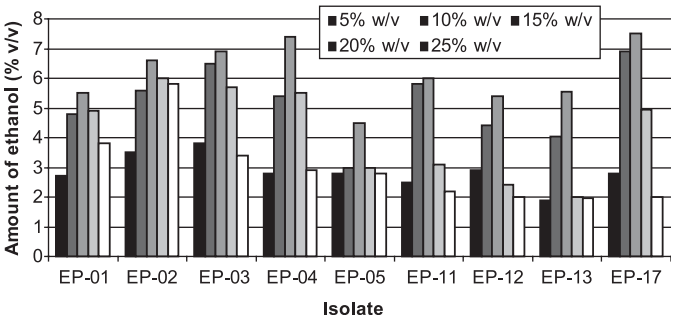

Figure 1: *Effect of sugar concentration on production of ethanol on selected indigenous yeast isolates*

Economic feasible of ethanol production largely depends on cost of fermentable substrate[20]. Molasses, a cheap raw material, was used for the production of ethanol in this study. For comparison, the fermentation was carried out using glucose-containing medium (MYGP) and molasses-containing (MYMP) medium, with sugar

concentration of 15%. The selected five isolates produced appreciable amounts of ethanol in both media. Ethanol production in both media was comparable, although slightly better production was obtained in MYGP medium (6.96-8.12% in MYGR vs. 6.54-7.58% in MYMP (Figure 2) and this result is comparable to that reported by Mendoza *et a1*.[21]. Among the yeast isolates, *Saccharomyces* EP-17 was found to be the best organism for the production of ethanol that produced 8.12% ethanol in MYGP and 7.58% in MYMP broth.

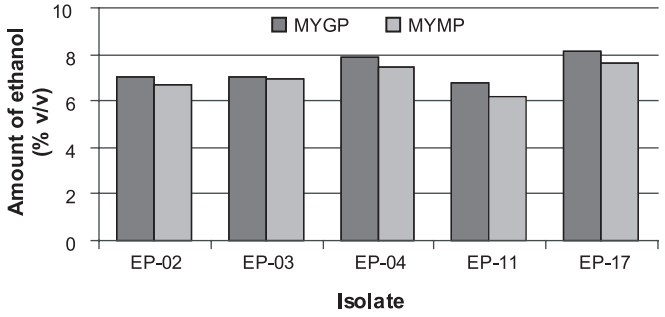

Figure 2: *Comparative ethanol production by five selected yeast isolates in malt extract-yeast extract-glucose-peptone (MYGP) malt extract-yeast extract-molasses-peptone (MYMP) broth media*

Several factors, such as ethanol tolerance, culture pH, substrate concentration, temperature and inoculum size affect the microbial growth and ethanol production[16,22]. For determination of ethanol tolerance by selected yeast isolates, the organisms were incubated in MYMP medium containing different concentrations of ethanol (0-20% v/v) at 30°C for 24 h. The initial yeast cell count used was fixed (10^5 cell/ml) for all the flasks. The flasks were then placed in a rotary shaker for incubation at 140 rpm under aerobic condition. It was found that the isolates were tolerant up to 10% ethanol concentration, and above this limit the number of viable yeast counts decreased remarkably (Table 1).

Table 1: *Effect of ethanol concentration on the viability of selected yeast isolates*

Yeast isolate	Viable yeast count (Cells/ml) at various concentration of ethanol (% v/v)				
	0	5	10	15	20
Zygosaccharomyces EP-02	3.6 x 10^8	3.1 x 10^7	4.9 x 10^4	1.6 x 10^3	Nil
Zygosaccharomyces EP-03	1.9 x 10^8	2.6 x 10^7	1.7 x 10^5	2.8 x 10^3	2
Saccharomyces EP-04	2.3 x 10^8	3.3 x 10^7	1.3 x 10^5	3.3 x 10^2	2
Saccharomyces EP-11	2.2 x 10^8	1.7 x 10^7	2.5 x 10^5	5.5 x 10^2	Nil
Saccharomyces EP-17	4.7 x 10^8	3.0 x 10^7	2.6 x 10^6	3.7 x 10^3	3

To investigate the effect of initial pH and temperature, ethanol fermentation was carried out at various pH values (4.0-5.5) and temperatures (20-40°C) for 24 h at in MYMP broth medium using the selected yeast isolates. All of the isolates produced maximum amount of ethanol (2.56-4.70%) at pH 4.5 and 30°C. There are some evidences that the rates of yeast growth and fermentation are decreased as the initial pH is decreased towards 3.0[23-25], however the yeast strains examined in another study[16] exhibited similar growth behaviour at either pH 3.0, 3.5 or 4.0, and there was no firm evidence that initial pH over the range 3.0 to 4.0, would provide a selective growth advantage to any one particular species.

The initial sugar concentration of the fermentation medium is an important factor for maximum ethanol production[5]. The effect of substrate concentration on the production of ethanol was studied using *Saccharomyces* EP-17 isolate. Fermentation was carried out in 250-ml conical flasks containing MYMP broth plus molasses with various sugar concentrations (11-15%) at 30ºC for 72 h. Ethanol production increased with the increase of sugar concentration and the highest amount of ethanol (7.58%) was obtained in the fermentation broth containing the highest amount (15%) of sugar (Figure 3). Usually isolates of yeast need 11-15% sugar in the molasses medium[5]. Too much sugar reacts adversely, creating undesirable osmotic effect on yeast cells, which results in lower ethanol production[11,26].

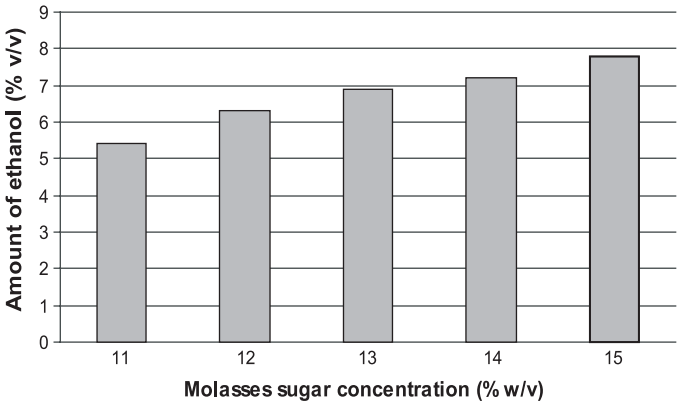

Figure 3: *Effect of sugar concentration on the production of ethanol by Saccharomyces EP17 isolate*

In all the previous experiments, an inoculum size of 10^8 cells/ml was used. D'Amore and colleagues[27] reported that the rate and extent of ethanol production increased with increasing inoculum size. It is commonly known that increasing size of inoculum curtails the period for maximal level of ethanol production[28], but use of excessive inoculum in the culture medium has been reported to interfere with production and recovery of ethanol. It was therefore, required to find out a suitable inoculum size for efficient production of ethanol. Fermentations were carried out using *Saccharomyces* EP-17 isolate with different inoculum size (10^5-10^9 cells/ml) in MYMP broth medium containing 15% sugar with an initial pH 4.5 under static condition at 30ºC for 72 h. The production of ethanol increased with increase of inoculum size up to 10^8 cells/ml, however further increase of inoculum size caused decreased production of ethanol (Figure 4). The highest amount of ethanol estimated was 7.89% using inoculum concentration of 10^8 cells/ml.

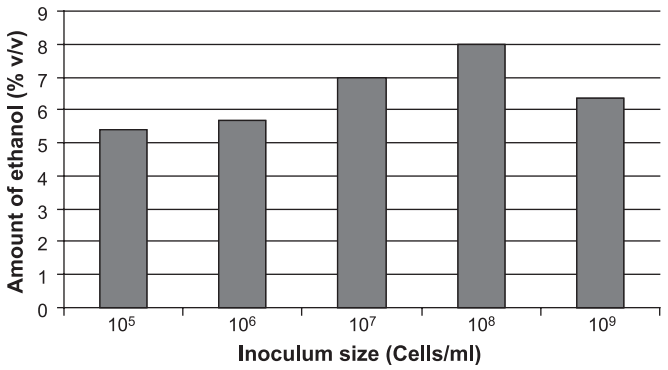

Figure 4: *Effect of inoculum size on production of ethanol by Saccharomyces EP-17 isolate in MYMP broth medium*

An attempt was undertaken to scale up ethanol fermentation under laboratory conditions using *Saccharomyces* EP-17 isolate. Fermentations were carried out in 10-l aspirator flask with a working volume of 3 l using MYMP broth containing 15% sugar. After 72 h fermentation at 30°C, the yeast isolate produced 6.65% ethanol, which was only 12% lower than that achieved with small laboratory scale (100 ml) experiments. The lower production might be due to the high volumetric pressure on the yeast cell as they existed as flocculent and without any shaking. After a single distillation, 210 ml ethanol was obtained from 3 l molasses ferments and the concentration of ethanol achieved was about 65.56%.

Present study showed that some indigenous yeast isolates can be used for ethanol production. Among the yeast isolates tested *Saccharomyces* EP-17 was the most potent isolate for production of ethanol from molasses. Ethanol production from molasses by *Saccharomyces* EP-17 is greatly influenced by the fermentation conditions like temperature, pH, inoculum size and concentration of substrate.

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