



Isolation and characterization of osmophilic fermentative yeasts from Bangladeshi honeys

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ABSTRACT: Despite the medicinal values and economic importance, honey is also a source of osmophilic fermentative yeasts. So, it can be utilized for the isolation of local fermentative yeast. Here, 1ml honey was inoculated in yeast extract peptone dextrose (YPD) broth containing 4% dextrose. After overnight incubation, the broth was streaked on an YPD agar plate to purify yeast colonies. In this study, total eight strains were isolated from seven physio-chemically defined florally diversified honey samples of Bangladesh. Microscopic morphology, plasmid profile, growth pattern and fermentative capacity of these isolates were determined. Under light microscope, these yeasts had one of three distinct shapes: ovoid, spherical, or cylindrical. The cytoplasm in young, actively reproducing cells occupied most of the interior and looked homogeneous. Two plasmids of around 3kb and 2.1 kb were common in these strains except for one. While compared with Baker's yeast, these strains showed faster growth. Five of them were attributed to high fermentative potency. Yeast 2 showed the highest fermentative potency yielding 33.48% (v/v) ethanol. We suggest that these strains have potentialities for efficient bioethanol production to meet the increasing demand of biofuel.

KEYWORDS: Honey, growth curve, osmophilic fermentative yeast, plasmid profile.

INTRODUCTION

World production of ethanol, often referred to as biofuel, has increased dramatically in recent years since it offers a valuable energy alternative to fossil fuels which are nonrenewable and significantly contribute to atmospheric pollution. Owing to the depleting reserves and competing industrial needs of petrochemical feed stocks, there is global emphasis in bio-ethanol production by fermentation process [1, 2]. Though *Saccharomyces cerevisiae* is regarded as an industrial working horse for ethanol production, still its ethanol productivity and fermentation rate are not satisfactory to meet up the increasing demand of bioethanol production worldwide [3, 4]. Therefore, it has become essential to find out potential alternative strains of yeast which will provide a valuable means of increasing the efficiency of ethanol production. Besides, a serious economic concern is that most industrial microorganisms are patented and

may not be available for use outside their country of origin which does not allow for rapid expansion of fermentation industries [5]. Hence, the need to source for indigenous and suitable yeast strains from local substrates for sustainable ethanol production has become a serious issue.

History of isolating various strains of indigenous yeasts capable of producing ethanol from different local sources started back to more than 3500 years [6]. Yeasts have been isolated from soil and food samples [7, 8], cereal based foods [9-11], various milk [12-16], or cheese [17] and ripe banana peels [18] in various regions of the world.

Honey contains yeasts naturally and primary sources of honey yeasts are likely to include pollen, the digestive tracts of honey bees, dust, air, earth and nectar [19]. Honey yeasts are osmophilic and protect themselves

against the high osmotic pressure by the synthesis of osmoprotectants such as alcohols and amino acids [20]. Various yeast strains have been isolated from honey of different regions in the world. A total of 30 osmophilic yeast colonies from 45 honey samples were recovered in a study in Saudi Arabia [21]. Yeasts were detected in three honeys in Serbia [22]. Yeasts were found in four out of 30 samples from the honey of Argentina in a recent study [23].

Since honeys of various floral sources are readily available in Bangladesh, it can be utilized as a potential source for the isolation of local fermentative yeast strains for efficient bioethanol production. But so far, it has been underutilized as potential growth medium for isolating local yeast strains. Therefore, this study was carried out to isolate and characterize osmophilic fermentative yeasts from different honey samples of Bangladesh.

MATERIALS AND METHODS

Sample collection and preparation

Seven different amber honey samples were collected from different regions of Bangladesh. Natural honeys were collected from the comb and commercially available honeys were bought from the market (Table 1). All samples were stored at room temperature (20-25°C) before analysis and were treated similarly. Individual samples were properly mixed with cold milli-Q water as 1:1 (v/v), and then were filtered through 0.45µm syringe filter to remove particles.

Isolation and maintenance of honey yeasts

Isolation and maintenance of yeasts from the honey samples were done according to methods of Nasir *et al.*, 2017 [8] and Qvirist *et al.*, 2016 [16] respectively with slight modifications. Each honey sample was inoculated in both of YPD (Yeast Peptone Dextrose) broths (Sigma, USA) and 4% dextrose (Oxoid, UK) solution from which one set was incubated aerobically for seven days, and other set was incubated anaerobically for fifteen days at 37°C. After that, the cultures were streaked on YPD agar plates and then overnight incubation at 37°C was done both aerobically and anaerobically. Individual colonies were observed under microscope to identify the yeast colonies. Yeast single colonies were picked and transferred to fresh YPD plates and YPD broths. Stock cultures of the isolates were prepared in 80% glycerol (Scharlau, Spain) solution and preserved at -20°C and at -80°C in cryotubes. Isolates were also maintained in YPD slants at 4°C.

Observation of morphology and growth of yeast isolates

The morphology of the yeasts was examined according to the modified method of Nofemele *et al.* 2012 [24]. Single colony was picked and a light suspension of cells from very young cultures was prepared in normal saline 0.9% (w/v) NaCl (normal saline) and then examined under a 40X objective of the phase contrast microscope (Nikon, UK). Photomicrograph was done using Nikon E995 digital camera.

To observe the growth characteristics of the isolates, after overnight growth, each of the yeast cultures was diluted in fresh sterile YPD broths to match 0.5 McFarland turbidity standard and the optical density (OD) was adjusted to 0.1 at 600 nm, which corresponds to 10⁸cfu/ml. Then it was kept incubated at 37°C with agitation at 120 rpm and sampling was done time to time at every 30 minutes intervals under aseptic condition to measure the absorbance at 600 nm using spectrophotometer until it reached to the stationary phase. The experiment was repeated three times and a mean of the three readings was taken where baker's yeast (*S. cerevisiae*) was used as control. Then the growth curve was drawn by plotting time along the X-axis and OD value along Y-axis using Microsoft Excel 2007.

Fermentation capacity determination of yeast isolates

Fermentation was carried out following the method of modified Periyasamy *et al.*, 2009 [25]. 1ml of yeast culture (10⁸cfu/ml) was transferred in an autoclaved 250ml conical flask containing 240ml fresh sterile fermentation broth containing Sucrose 10% (w/v), NaCl 0.05% (w/v) and Lemon Extract 4% (v/v) of pH 6.5±0.2 under aseptic condition and kept incubated at 37°C anaerobically with agitation (120 rpm) inside the fermenter (Fermentec, Korea) for 21 days for fermentation. Then the ethanol produced by the fermentation was extracted using IKA® RV 10 rotary evaporator (200mBar pressure, 70°C temperature and 100 rpm) and the volume of ethanol was measured. We used baker's yeast (*S. cerevisiae*) as our control. All the tests were conducted in triplicates.

Plasmid profile of yeast isolates

1.5 ml of yeast culture, after overnight growth, was centrifuged at 10,000 rpm for 10 minutes using a bench top centrifuge machine (Hettich, UK) and the supernatant was removed. The pellet was washed with normal saline that is NaCl 0.85% (w/v). Then 200µl lysis buffer comprising 100mM NaCl, 10mM Tris-HCl

of pH 8.0, 1mM EDTA of pH 8.0, and SDS 0.1% (w/v) (Sigma, USA) was added and mixed properly. An equal amount of acid washed glass beads of 0.45mm diameter (Ace Glass Inc., USA) was added just before the solution surface and vortexed at top speed for 2 minutes. 200µl PCI; Phenol:Chloroform:Isoamylalcohol (25:24:1) (Merck, India) was added and mixed well. After centrifugation at 10,000 rpm for 10 minutes, the aqueous phase was collected. Plasmid DNA was extracted with equal amount of isopropanol (Merck, India) and washed with 70% ethanol (v/v) (Scharlau, Spain). The DNA pellet was allowed for air dry at room temperature for 20 minutes and then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Finally, it was stored at -20°C. Plasmid was isolated for three times for each yeast isolate.

Isolated plasmid was analyzed on a 0.8% agarose gel containing ethidium bromide (Amresco, USA) added to a final concentration of 0.5, followed by electrophoresis using 0.5X TAE buffer system (20 mM Tris, 20 mM acetic acid, 1 mM EDTA) (Amresco, USA). 5µl plasmid was added to 1µl 6X loading dye (TOYOBO, Japan, containing 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl, pH 7.5 and 50 mM EDTA) before loading and 1Kb plus DNA was used as ladder. Visualization was performed under UV transilluminator (Hettich, UK) after electrophoresis where Photomicrograph was done. Electrophoresis was done for three times with each of three individual plasmid samples.

Table 1. Details of seven different honey samples (BDH=Bangladeshi Honey)

Sample	Source	Type
BDH 1	The Sundarbans	Multifloral (Natural)
BDH 2	University of Dhaka	Multifloral (Natural)
BDH 3	Jessore	Multifloral (Natural)
BDH 4	Savar	<i>Nigella sativa</i> (Natural)
BDH 5	Gazipur	<i>Brassica campestris</i> (Natural)
BDH 6	Local Market	Multifloral (Commercial)
BDH 7	Local Market	Multifloral (Commercial)

Statistical analysis

The assays were carried out in triplicate, and the results were expressed as mean values and the standard deviation (SD). The data were analyzed using Microsoft Excel spread sheet 2007. All the statistical analysis like average, standard deviation, standard error of mean was performed using Microsoft Excel 2007. All the graphs

and bar diagram were drawn using Microsoft Excel 2007.

RESULTS

Morphology and growth characteristics of yeast isolates

Total eight yeasts were isolated from the five raw honey samples and no yeast was found in the two commercial honey samples in this study. Colonies of the yeast isolates 1 to 7 were off white colored while isolate 8 colony was a bit pinkish. Also, the colony size was larger for the yeast isolates of 1, 2, 6 and 8 whereas isolates 3, 4, 5 and 7 had comparatively smaller colonies. Moreover, seven isolates, from 1 to 7, grew well at a temperature between 30°C to 37°C and the remaining isolate 8 grew better at a temperature between 4°C to 20°C. Furthermore, cells of the isolates were of 4 to 8 µ in diameter where the yeast isolates 1, 2 and 6 were ovoid shaped; isolates 3, 4 and 5 were spherical shaped; and yeast isolates 7 and 8 had cylindrical shaped cells (Figure 1). The three main parts of the cell were the cell wall, the cytoplasm, and the nucleus, bedded into the cytoplasm. The cytoplasm in young, actively reproducing cells occupied most of the interior and looked homogeneous for yeast isolates 1, 2 and 6 or somewhat granular for yeast 3, 4, 5, 7 and 8. Yeast isolate 8 was longer and contained two large vacuoles of equal size symmetrically located toward the two ends. The vacuoles were spherical in shape filled with transparent homogeneous liquid containing bodies with various sizes from cell to cell, small and hardly visible in young cells and large and quite conspicuous in older or resting cells. Additionally, budding cells showed an outgrowth from the parent cell pinches off, producing a daughter cell (Figure 2A). In old cultures, yeasts also formed filament-like, elongated sausage shaped cells to split evenly into two daughter cells by fission (Figure 2B).

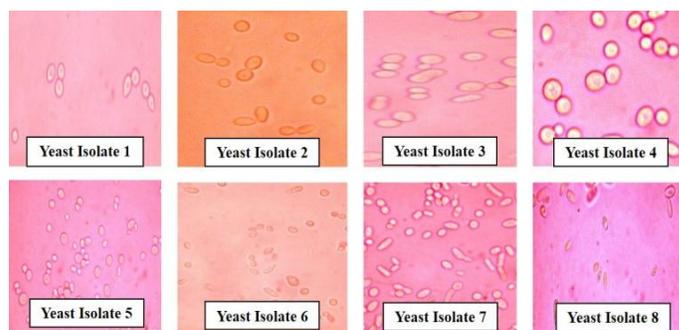


Figure 1. Microscopic images of the yeast isolates.

In addition, the yeast isolates were observed, in this study, to enter the stationary phase earlier than Baker's yeast (control) which indicated that these isolates could start fermentation earlier and thus could help to increase the yield of ethanol production as well as lessen the time and cost required for fermentation. Results showed that yeast 1, yeast 2, yeast 3, yeast 4, yeast 5, yeast 6, yeast 7 and yeast 8 entered the stationary phase within 6.92 ± 0.02 hours, 6.12 ± 0.01 hours, 9.5 ± 0.04 hours, 10.42 ± 0.01 hours, 9 ± 0.04 hours, 9 ± 0.03 hours, 9.42 ± 0.02 hours and 14 ± 0.03 hours respectively whereas Baker's yeast (*S. cerevisiae*) enters into the stationary phase after 14 ± 0.02 hours (Figure 3). As Yeast 2 entered the stationary phase within the shortest time, it could be assumed that this yeast could have the best fermentation capacity. Yeast 1, Yeast 3, Yeast 5 and Yeast 6 could also attribute better fermentation potency as their growth rates were also higher.

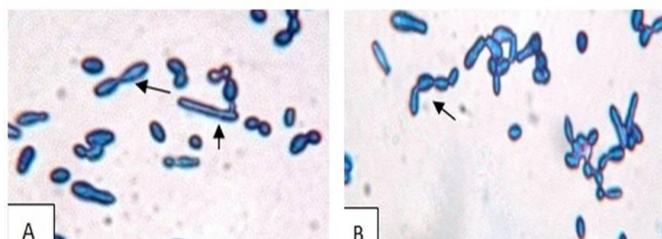


Figure 2. Yeast cells become elongated and cylindrical with elliptical edges to split evenly into two daughter cells by fission in old cultures (A). Chain of yeast cells (pseudomycelium) produced by budding in young cultures (B).

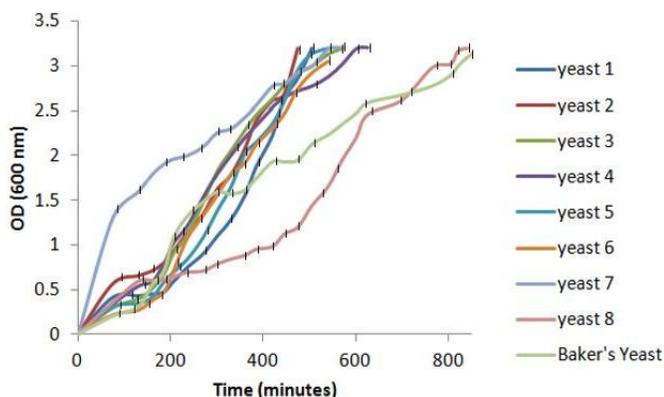


Figure 3. Comparison of the growth curves of the yeast isolates.

Fermentation capacity of yeast isolates

Five of the eight yeast isolates attributed very high fermentation capacity (Figure 4). Yeast 2 attributed the highest fermentative potency yielding $33.48\pm 1.35\%$ (v/v) ethanol. Besides, Yeast 1, Yeast 3, Yeast 5 and Yeast 6 were found to have high fermentative potency,

yielding $23.91\pm 1.64\%$ (v/v), $31.31\pm 1.68\%$ (v/v), $24.35\pm 1.65\%$ (v/v) and $20.44\pm 1.85\%$ (v/v) ethanol respectively, while baker's yeast was found to yield only $4.35\pm 1.31\%$ (v/v) ethanol. However, the yeast isolates 4, 7 and yeast 8 were found to be non-fermentative with a poor yield of ethanol $0.98\pm 0.36\%$ (v/v), $2.18\pm 0.83\%$ (v/v) and $0.34\pm 0.29\%$ (v/v) respectively.

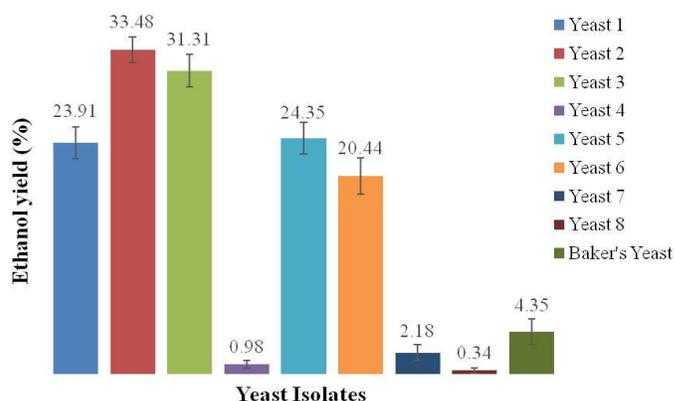


Figure 4. Fermentation capacity of the yeast isolates expressed as percent (v/v) of produced ethanol

Plasmid profile of yeast isolates

Eight yeast isolates derived from honey samples were screened for the presence of plasmid DNA. Yeast 8 was plasmid free and seven yeasts (yeast 1, yeast 2, yeast 3, yeast 4, yeast 5, yeast 6 and yeast 7) possessed plasmids with molecular weights ranging from around 1 kb to 12 kb as extrapolated from 1kb+ DNA ladder for electrophoresis (Figure 5). A total of three different molecular weights of plasmids (around 1kb, around 1.65 kb and around 12 kb) were obtained for the five isolates (yeast 1, yeast 2, yeast 3, yeast 4 and yeast 5). Yeast 6 contained plasmids of around 12 kb molecular weights. Yeast 7 contained plasmids of two different molecular weights (around 1kb and around 1.65kb). The molecular weights of the plasmids were ranging from 2.1 kb to 30 kb using previous data obtained from *E. coli* V517 molecular weight marker.

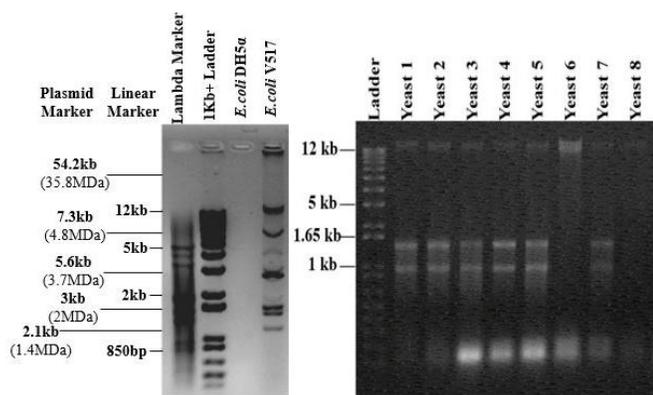


Figure 5. Agarose gel electrophoresis of the isolated plasmids from the yeast isolates.

DISCUSSION

Specific growth rate is one of the important process parameters which represent the dynamic behavior of yeasts in a fermenter. It is important to find out which yeast strain reaches to the stationary phase earlier to assume whether the yeast has good fermentative potency. If the yeast requires a low period of time to reach the stationary phase, it attributes better fermentation potency. Therefore, the growth characteristics of the yeast isolates were observed in this study. From the results, yeast 2 was found to have the fastest growth rate following the yeast isolates 1, 5, 6 and 3 (Figure 3). Surprisingly, the data from fermentation capacity measurement showed yeast 2 to possessing the best fermentative potency following the yeasts 1, 5, 6 and 3 among the isolates (Figure 4). So, it was found in this study that there is a significant relation between the required time by yeasts to reach stationary phase of growth and the fermentative capacity. Yeast 2 reached stationary phase most rapidly and it attributed the highest fermentation potency, while Yeast 8 entered the stationary phase most slowly and possessed the lowest fermentation potency. The arrangement for the time required to reach stationary phase by the isolates was: yeast 2 < yeast 1 < yeast 5 < yeast 6 < yeast 3 < yeast 7 < yeast 4 < yeast 8; while the arrangement for the yield of ethanol fermentation by the isolates was: yeast 2 > yeast 1 > yeast 5 > yeast 6 > yeast 3 > yeast 7 > yeast 4 > yeast 8. However, as yeast 8 grew well at low temperature (4°C to 20°C), its growth rate became slow with increasing temperature and thus it entered the stationary phase after a long period. So, this yeast isolate cannot be used as a good fermentative agent for industrial ethanol production because an ideal microorganism used for ethanol production must have good thermo-tolerance for rapid fermentative potential.

In a previous study, the ethanol production capacity of yeast strains *Saccharomyces unisporous* (P), *Saccharomyces cerevisiae* (C) and (T), isolated from

agro-industrial waste of Bangladesh, were found 15.00% for P, 12.50% for C and 10.15% for T [26]. In another study in Bangladesh, the ethanol production rate of the yeasts isolated from sugarcane, date juice and vegetable peels, ranged from 1.71% to 6.23% [27]. However, in this present study, the ethanol production capacity of five yeast isolates exhibiting high fermentative potency ranged from 23.91% to 33.48% (Figure 4). Therefore, the ethanol production potency of the yeast isolated from honey is much better than that of yeasts isolated from agro-industrial or kitchen wastes. Our findings also showed that the fermentation capacity of the yeast isolates was much higher than that of yeast strains from Nigeria and Greece where *S. cerevisiae* R-2, *S. cerevisiae* R-8, *S. cerevisiae* T-7, *S. kluyveri* K-6 and *D. hansenii* B-2 strains were isolated from banana peel and their ethanol fermentation yield was 4.6%, 7.2%, 4.3%, 4.8% and 3.6% [18]. Other study reported 7.8% of (m/v) ethanol production using *Saccharomyces cerevisiae* strains from sugarcane molasses [24].

Many metabolic engineering and genetic engineering applications in yeast rely on the use of its plasmids [28]. Since five of the yeast isolates in this study attributed high fermentative potency, information regarding their plasmid profile could be helpful to determine the reason for their efficient ethanol fermentation and the molecular relatedness of the yeast isolates. Therefore, plasmid profiling of the isolated honey yeasts was performed in this study. Seven of the eight isolates possessed plasmids with molecular weights ranging from around 1 kb to 12 kb (Figure 5).

CONCLUSION

This study indicated that Bangladeshi honeys could be utilized as potential sources for local fermentative yeast strains. Since the ethanol production capacity of the yeast isolates was higher than baker's yeast, they could be used for increased biofuel production to meet up the increasing demand of biofuel due to the scarcity of fossil fuels. The osmotolerant character makes honey yeasts a good fermentative agent for industrial ethanol production because during industrial fermentation yeasts have to tolerate a high sugar substrate concentration. The study also showed that some yeast isolates from honey possessed plasmids indicating that further studies on plasmid and molecular identification of yeast isolates from honey could be carried out. However, the study is ongoing for the molecular characterization of the yeast isolates. 16S rDNA sequencing and phylogenetic analysis is under consideration for the strain identification of the isolates to develop novel fermentative strains with efficient ethanol production capability.

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AUTHOR CONTRIBUTIONS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. MMKC and RFM were involved in conception and design of the experiments. RFM and MMKC contributed to perform the experiments. RFM and MMKC analyzed data. RFM contributed to drafting the article. MMKC and MAU contributed to revising it critically for important intellectual content. All authors reviewed and approved the manuscript for publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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