Journal of Young Pharmacists 5 (2013) 191–194

Contents lists available at ScienceDirect

Journal of Young Pharmacists

journal homepage: www.elsevier.com/locate/jyp

Short communication

Isolation, biochemical and genetic characterizations of alcohol-producing yeasts from the flowers of *Woodfordia fruticosa*

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ARTICLE INFO

Article history:
Received 27 September 2013
Accepted 22 November 2013
Available online 16 December 2013

Keywords:
Woodfordia fruticosa
Saccharomyces cerevisiae Jm.20
Yeast
Alcohol

ABSTRACT

Objective: To isolate and characterize the alcohol-producing yeasts from *Woodfordia fruticosa* flowers, which are used for the induction and maintenance of fermentation in the making of *Ayurvedic* formulations.
Materials and methods: Initially twenty four yeasts strains were isolated on MGYP agar plate. Among them, four strains were selected for further studies on the basis of their alcohol generation capacity using jaggery media (50% w/v). Physiological, biochemical and genetic characterization (18S rRNA sequencing) of selected strains were carried out.
Results: Physiological, biochemical and genetic characterization (18S rRNA sequencing) confirmed the strains as *Saccharomycopsis fibuligera* Jm.8, *S. fibuligera* Jm.10, *S. fibuligera* Jm.16 and *Saccharomyces cerevisiae* Jm.20. Under the controlled conditions, *S. cerevisiae* Jm.20 produced 69.57 g/l of alcohol, whereas remaining strains produced the alcohol in the range of 6.04–7.32 g/l.
Conclusion: Among selected strains, strains *S. fibuligera* are a newer in the flowers. Kinetic study of alcohol generation revealed the strain *S. cerevisiae* Jm.20 can be efficiently used in making of fermented *Ayurvedic* formulations instead of use *W. fruticosa* flowers.

1. Introduction

Ayurveda is oldest system of medicine known to human which is based upon the use of plants (herbs) for the preparation of medicaments.[1,2] *Ayurvedic* formulations belonging to the category of asavas and arishthas are prepared by traditional fermentation processes using the flowers of *Woodfordia fruticosa*. Alcohol is the main product generated during this fermentation process.[3] This alcohol causes the extraction of water insoluble active ingredients from crude drugs. Traditionally, the flowers of *W. fruticosa* (Family-Lythraceae) are used as inoculums for the induction and maintenance of fermentation process.[1] This process uses the yeast *Saccharomyces cerevisiae* présent on the flowers.[4] For the complete extraction of phytoconstituents, about 40–80 g/l (i.e. 5–10% v/v) of alcohol is required.[5] The plant *W. fruticosa* is a large shrub widely distributed in tropical and subtropical region of South.[6] The yeasts on the flowers may vary depending on the source location. Literature survey revealed the presence of yeast species *S. cerevisiae* and *Pichia anamola* on the same flowers, but no one has evaluated their alcohol-producing ability in quantitative way.[4,7] Also, there may be some other species of yeasts that are yet to reveal but are responsible for the alcohol generation. Therefore, attempts were made to isolate and characterize such types of yeasts from the flowers.

2. Materials and methods

2.1. Collection of flowers

Five samples of dried flowers of *W. fruticosa* were collected from different parts of Solapur district of Maharashtra. The flowers were authenticated from Agharkar Research Institute, Pune.

2.2. Isolation of yeasts

Yeasts associated with the flowers of *W. fruticosa* were isolated by serial dilution method.[8] About 1 g of flowers was transferred to

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0975-1483/$ – see front matter
http://dx.doi.org/10.1016/j.jyp.2013.11.007

500 ml sterile Erlenmeyer flask containing 200 ml of sterile MGYP broth (3 g malt extract, 10 g glucose, 3 g yeast extract, 5 g peptone per liter of water, pH 4.5) and incubated at 32.5 °C for 24 h on a rotary shaker at 180 rpm. About 0.5 ml of above suspension was spread on MGYP agar plate and incubated for 24 h at 32.5 °C. After incubation, isolated colonies were directly examined and their purity was verified by visualizing yeast cells under microscope.

Colonies with distinct morphological differences such as color, shape and size were picked up and purified by streaking at least three times on MGYP agar (3 g malt extract, 10 g glucose, 3 g yeast extract, 5 g peptone and 20 g agar per liter of water, pH 4.5) plates. Subsequent streaking of each isolate was done on MGYP agar plate containing 100 ppm of streptomycin. Total 24 yeasts were obtained and were named as Jm.1–Jm.24. These yeasts were maintained on MGYP agar slants and preserved for routine use at 4 °C.

2.3. Screening for alcohol generation capacity

Screenings of yeasts for alcohol production were carried out using preanalyzed 50% w/v jaggery media. The inoculums for each strain was prepared by transferring one loopful of yeast into 100 ml of sterile 12°brix jaggery medium containing 0.01% of diaminophosphate and 0.01% of urea as a nitrogen source. The inoculums were incubated for 48 h at 32.5 °C in shaker incubator at 180 rpm. After incubation, about 10 ml of inoculum was added to 250 ml Erlenmeyer flasks containing 90 ml of the sterile 50% w/v jaggery medium and flasks were incubated at 32.5 °C for 14 days.

2.4. Characterization of yeasts

2.4.1. Morphological, physiological/biochemical characterization

Based on alcohol generation capacity, four strains i.e. Jm.8, Jm.10, Jm.16 and Jm.20 were selected for further studies. Identification of these yeasts up to the strain level was carried out based on standard morphological and physiological/biochemical tests.[9,10] Urea's test was carried out according to the reported method.[11] Identification of the selected strains was based on the comparative analysis of distinguishing morphological, physiological and biochemical characteristics. The results were compared with the information of reference yeast strains given in the literature.

2.4.2. Genetic characterization

Yeasts 18S rRNA sequences are attractive targets for developing identification methods because they represent conserved regions in all yeasts. Therefore, confirmation of strains was done by determining the 18S rRNA sequences of them. The strains were submitted to National Centre for Cell Sciences, Pune (India), for identification by PCR using ABI 9800 (Applied Biosystems, Foster City, USA) and primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The gene was extracted and purified according to procedure described in literature.[12]

The sequencing of gene 18S rRNA was carried out using same primers which were used for PCR and ABI BigDye Terminator Cycle Sequencing Reaction Kit in Automated ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, USA). The ITS1 and ITS4 sequence data of 18S rRNA obtained were blast at NCBI (http://www.ncbi.nlm.nih.gov) producing significant alignments. Multiple sequence alignments of these sequences were made by using ClustalW. The phylogenetic analysis was done using neighbor-joining method with the MEGA-3.1 program.[13]

2.5. Alcohol generation kinetics

The inoculums of selected strains were prepared by transferring one loopful of culture from agar slant into 100 ml of sterile 12°brix jaggery medium containing 0.01% diaminophosphate and 0.01% urea. The inoculums were incubated for 48 h at 32.5 °C in shaker incubator at 180 rpm. About 50 ml of inoculums of isolated yeast was added to 1000 ml Erlenmeyer flask containing 450 ml of sterile 50% w/v jaggery medium.

Another 1000 ml Erlenmeyer flask containing 500 ml of sterile 50% w/v jaggery medium was inoculated with 16 g of *W. fruticosa* flowers. All the flasks were incubated at 32.5 °C till the stopping of alcohol generation in any one of the fermentation flasks. At regular intervals, the fermentation broth was centrifuged for 20 min at 6000 rpm, and the supernatant liquid was used for analysis of alcohol and residual sugar.

2.6. Renaming of strains

After the confirmation of strains, strain Jm.8, Jm.10, Jm.16 and Jm.20 were named as *Saccharomycopsis fibuligera* Jm.8, *S. fibuligera* Jm.10, *S. fibuligera* Jm.16 and *S. cerevisiae* Jm.20, respectively.

2.7. Methods of analysis

The jaggery media was diluted appropriately and its sugar contents were determined by RP-HPLC (Waters, USA) using ultra amino column (150 × 4.6 mm, Restek) maintained at 35 °C. The mobile phase used was acetonitrile (75%) and deionised-water (25%) with a flow rate of 0.8 ml/min. Sample volume injected was 20 μl and detector used was refractive index detector (Waters 410, USA). For alcohol determination, fermentation broths were centrifuged at 6000 rpm for 20 min and supernatant liquids were used for alcohol determination by dichromate oxidation method.[14]

3. Results

3.1. Isolation and screening of yeast

Twenty four yeast strains were isolated from *W. fruticosa* flowers on MGYP agar at 32.5 °C and were named as Jm.1–Jm.24. All the strains were screened for their alcohol generation capacity using 100 ml fermentation media. Jaggery (50% w/v) was used as fermentation media and alcohol produced was determined after 14 days. The jaggery media (50% w/v) was analyzed by HPLC and found to contained fructose 3.17% w/v, glucose 3.22% w/v and sucrose 23.31% w/v. Based on the alcohol production ability, four strains i.e. Jm.8, Jm.10, Jm.16, and Jm.20 were selected for identification and characterization studies. The strains Jm.8, Jm.10 and Jm.16 have produced 11.01 g/l, 10.77 g/l, and 10.53 g/l of alcohol, respectively.

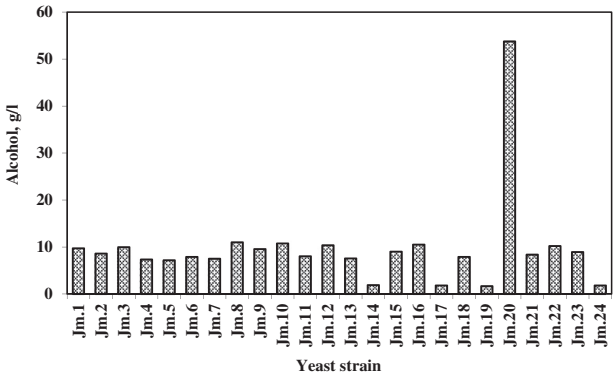

Fig. 1. Graphical representation of alcohol generation by isolated yeasts.

The strain Jm.20 has produced alcohol in highest amount i.e. 53.79 g/l (Fig. 1).

3.2. Characterization of yeasts

After 24 h on MGYP agar plate, the isolated colonies of yeasts strain Jm.8, Jm.10 and Jm.16 were sticky with cream color surface, while Jm.20 was smooth with cream color. The shape of cell was observed under phase contrast light microscope (Fig. 2). Morphological characteristics of strains were done by slide culture technique using MY agar and Fowell's acetate agar. The study results have shown that strain Jm.8, Jm.10 and Jm.16 produced pseudohyphae abundantly and consisted of filamentous cells with few blastospores on both the medium, whereas strain Jm.20 did not produce it.

Physiological and biochemical testing of selected strains were also carried out. All the strains fermented glucose and sucrose. In addition, strain Jm.20 fermented galactose, maltose, and raffinose. Testing of assimilation of carbon compounds and nitrogen compounds is essential criterion required for taxonomic study and identification of yeasts. All the strains assimilated glucose, sucrose, trehalose and ethanol. Strains Jm.8, Jm.10 and Jm.16 assimilate glycerol and D-Ribose, while strain Jm.20 assimilates maltose and raffinose. The strains Jm.8, Jm.10 and Jm.16 were also able to assimilate various nitrogen compounds like nitrate, L-lysine and ethylamine HCl. The strain Jm.20 did not assimilate any of nitrogen compound tested.

Also, some additional biochemical tests were done for the identification of yeasts. The tests included starch hydrolysis, cycloheximide resistance (0.1%), gelatin liquefaction etc. These results suggested that the strains Jm.8, Jm.10 and Jm.16 may belong to species *S. fibuligera*, whereas the strain Jm.20 may belong to *S. cerevisiae* (Tables 1 and 2).

3.3. Genetic characterization

18S RNA sequencing has confirmed the strains Jm.8, Jm.10 and Jm.16 belonging to the species *S. fibuligera* and the strain Jm.20 belonging to the species *S. cerevisiae*. Phylogenetic study was done by MEGA-3.1 Software program. The phylogenetic tree of each strain was made using sequences of ITS1 regions.

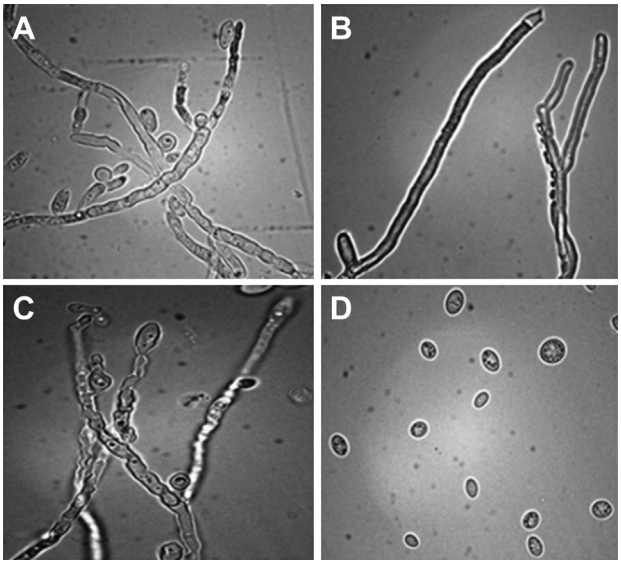

Fig. 2. Microscopical images of yeasts (A) Jm.8, (B) Jm.10, (C) Jm.16, and (D) Jm.20.

Table 1
Results of physiological and biochemical tests.

Yeast strain	*S. fibuligera*			*S. cerevisiae*
Test	Jm.8	Jm.10	Jm.16	Jm.20
Fermentation of sugars				
D-Glucose	+	+	+	+
D-Galactose	−	−	−	+
Sucrose	w/−	w/−	w/−	+
Raffinose	−	−	−	+
Lactose	−	−	−	−
Maltose	−	−	−	+
Trehalose	−	−	−	−
D-Xylose	−	−	−	−
Cellobiose	−	−	−	−
Melibiose	−	−	−	−
Assimilation of carbon compounds				
D-Glucose	+	+	+	+
D-Galactose	−	−	−	w/−
D-Sucrose	w/−	w/−	w/−	+
Maltose	−	−	−	+
Cellobiose	−	−	−	−
Trehalose	+	+	+	+
Lactose	−	−	−	−
Melibiose	−	−	−	w/−
Raffinose	−	−	−	+
Soluble starch	−	−	−	−
Inulin	−	−	−	−
D-Xylose	−	−	−	−
D-Arabinose	−	−	−	−
L-Arabinose	−	−	−	−
D-Ribose	+	+	+	−
Ethanol	+	+	+	+
Glycerol	+	+	+	−
D-Sorbitol	−	−	−	−
Assimilation of nitrogen compounds				
Potassium nitrate	+	+	+	−
Sodium nitrite	−	−	−	−
Ethylamine HCl	+	+	+	−
L-Lysine	+	+	+	−

Responses: + = positive; − = negative; w/− = weak or negative.

3.4. Alcohol generation kinetics

Batch type of fermentation process was employed because in continuous type, there is higher possibility of mutation and

Table 2
Results of additional tests.

Yeast strain	*S. fibuligera*			*S. cerevisiae*
Test	Jm.8	Jm.10	Jm.16	Jm.20
Production of ammonia from urea	−	−	−	−
Starch hydrolysis	+	+	+	−
Nitrate reduction	−	−	−	−
Cycloheximide resistance (0.1%)	+	+	+	−
Growth on 50% glucose-yeast extract agar	w/−	w/−	w/−	w/−
Growth in 10% NaCl plus 5% glucose solution	+	+	+	w/−
Tolerance of 1% acetic acid	−	−	−	−
Acid production from glucose	+	+	+	+
Citrate utilization	−	−	−	−
Gelatin liquefaction	+	+	+	−
Growth at				
37 °C	w/−	w/−	w/−	w/−
20 °C	+	+	+	+
25 °C	+	+	+	+
40 °C	−	−	−	−

Responses: + = positive; − = negative; w/− = weak or negative.

Table 3
Kinetic study of fermentation using isolated strains and *W. fruticosa* flowers.

Sr. No.	Inoculums	Initial total reducing sugar (g/l)	Rate of alcohol generation (g/l/h)	Alcohol generated after 168 h (g/l)
1	*S. fibuligera* (Jm.8)	444.20	0.036	6.050
2	*S. fibuligera* (Jm.10)	314.90	0.044	7.3232
3	*S. fibuligera* (Jm.16)	306.60	0.036	6.0496
4	*S. cerevisiae* (Jm.20)	283.70	0.414	69.57
5	*W. fruticosa* flowers[a]	345.00	0.166	27.93

[a] Alcohol generation was not stopped.

contamination. Results of flask level fermentation have shown that the strain *S. cerevisiae* (Jm.20) generates alcohol faster than the fermentation with the use of flowers. It utilizes nearly 60% of initial sugar within 168 h (7-days) for generation of alcohol (69.57 g/l), while flowers utilized only 24% of initial sugar and produce lesser amount of alcohol (27.93 g/l) within same period. Also, the rate of alcohol generation by isolated strain *S. cerevisiae* was about 2.5 times more than that of with flowers. Remaining 3 strains have produced very low alcohol from the range of 6.04 to 7.32 g/l (Table 3).

3.5. Analysis of jaggery

The jaggery (50%w/v) medium was found to contain three reducing sugars i.e. fructose (6.35%w/v), glucose (6.45%w/v) and sucrose (46.62%w/v).

4. Discussion

Total twenty four yeast strains were isolated from the flowers of *W. fruticosa*. These strains were tested for alcohol production capacity using jaggery as a source of sugar which is used in the preparation of *Ayurvedic* formulations. Out of them, four strains were selected on the basis of alcohol production and were used for identification study. Using biochemical test Jm.8, Jm.10 and Jm.16 were found to be from genus *S. fibuligera* while Jm.20 from genus *S. cerevisiae*. From the 18S rRNA sequencing, identification of the yeast strains was confirmed to be belonging to *S. fibuligera* and *S. cerevisiae* species. The species of *S. cerevisiae* are well known for the production of ethanol at industrial scale. Species *S. cerevisiae* was found before on the same flowers but the species *S. fibuligera* is newer on the flowers. The isolated yeast species were used to compare alcohol production with the traditional method of using flower of *W. fruticosa* as inoculums. Yeast strain *S. cerevisiae* (Jm.20) produced recommended amount of alcohol i.e. 40–80 g/l within a shorter period relative to traditional method of using flower. In using Jm.20, rate of alcohol generation and rate of sugar utilization were faster than other inoculums. In this case, fermentation process was stopped after 168 h. Yeast strain *S. fibuligera* was found to produce least amount of alcohol and rate of alcohol generation and sugar utilization was much less than others.

5. Conclusion

Thus, from the results it is clear that species *S. fibuligera* is a newer in the flowers, whereas the species *S. cerevisiae* has been found before. Kinetic study of alcohol generation demonstrated that the strain *S. cerevisiae* Jm.20 can be efficiently used as inoculums in making of fermented *Ayurvedic* formulations instead of using *W. fruticosa* flowers.

Conflicts of interest

All authors have none to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jyp.2013.11.007

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