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Screening of yeast isolates from flowers for effective ethanol production

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Abstract: The use of alternative substrates to produce biofuel is a striking option nowadays. This study aimed to screen bioethanol-producing yeast strains. From different flowers, 65 yeasts were isolated. Twelve isolates assimilated glucose by liberation of CO_2 anaerobically. Out of these, only 5 yeast isolates fermented glucose in medium consisting of 0.8 g/L Mg^{2+} ions to produce 2.05 ± 0.03% ethanol. The selected five strains were identified as members of the genera *Metschnikowia* or *Meyerozyma* based on molecular characterization. Selected yeast strains were used for conversion of rice into bioethanol following dilute acid hydrolysis and fermentation. Consistent ethanol production was 1.80 ± 0.05% at days 2–4 by *Metschnikowia cibodasensis* Y34 and 2.20 ± 0.21% by *Meyerozyma caribica* Y42 at days 4–6 with a gradual decrease at the time of experiment termination (day 10). *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42 produced the highest ethanol at pH 3, i.e. 1.75 ± 0.14% at days 3 and 5 and 2.05 ± 0.14% at days 1 and 3, respectively, upon incubation with different pH levels and 1% NaCl. Growth and ethanol production at pH 4 and 5 was close to that at pH 3, with a slight increase in production by *Metschnikowia cibodasensis* Y34 at pH 4 up to day 3.

Key words: Ethanol-producing yeast, acid hydrolysis, *Oryza sativa*, bioethanol production, fermentation, biofuel

1. Introduction

The overconsumption of fuel and petroleum-derived products is a great threat to human society. Bioethanol production by fermentation has received widespread interest as a source of renewable energy. Ethanol appeared as an environmentally friendly alternative reducing the adverse effects and increasing costs of lead gasoline on human health (Sumari et al., 2010; Ghassem et al., 2012; Tikka et al., 2013).

Ethanol can be produced by exploiting several kinds of raw materials such as agricultural, industrial, and cellulosic wastes containing starch, sugars, and cellulose (Patle et al., 2008; Ibeto et al., 2011; Sirripattanakul-Ratpukdi, 2012). Sugar-rich materials such as molasses, sugar cane juice, or sugar beet and starchy substrates such as rice, wheat, potato, corn, cassava, millet, and sorghum have gained considerable attention (Roble et al., 2003; Bai et al., 2008; Gohel et al., 2012). Lignocellulosic materials such as rice straw, corn cob, and sugar cane waste were successfully applied in second-generation ethanol production (Tan et al., 2010). Rice (*Oryza sativa* L.) is considered one of the prominent food crops of the world and is native to Southeast Asia. Rice is second to wheat in terms of crops being used as food on the basis of annual production. Sixty percent of the world's population is dependent on the use of rice as food. Rice is primarily an Asian crop as about 95% is being cultivated and consumed in Southeast Asian countries ranging from India and Pakistan to Japan.

Ethanol producers have used broken rice, pearl millet, and sorghum for fuel purposes in India (Kleih et al., 2007; Gohel et al., 2012). Fermented rice noodle wastewater was also exploited for ethanol production in Thailand (Siripattanakul et al., 2010; Siripattanakul-Ratpukdi, 2012).

Starch is converted to glucose either by acid hydrolysis (Agu et al., 1997) or by enzymatic hydrolysis from bacterial amylolytic enzymes (Agrawal et al., 2005; Demirkan et al., 2005) and fungal enzymes (Omemu et al., 2005). Acid hydrolysis of starch had been widely used in the past. Recently, microbial fermentation has been exploited for ethanol production (Ward et al., 2002; Roble et al., 2003). For biosynthesis, in the fermentation process, yeast uses monosaccharide as a carbon source. *Saccharomyces cerevisiae* is now an important candidate for bioethanol production from agricultural resources because the yeast has a high fermentation rate and rapid metabolic activities (Verna et al., 2000; Pan and Lee, 2005; Limtong et al., 2007). Various yeast strains are found in varied

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environments such as plants, tree exudates, leaves, roots, necrotic tissues of plants, flowers, fruits, tanning liquors, mushrooms, animals (occasionally as pathogens), and soil and aquatic habitats (Lachance et al., 2001; Behera et al., 2010; Ghassem et al., 2012; Tikka et al., 2013). Bhadra et al. (2008) reported many yeasts from tree barks having the ability to assimilate xylose and arabinose, belonging to the genera *Metschnikowia*, *Pichia*, *Lodderomyces*, *Clavispora*, *Kluyveromyces*, *Debaryomyces*, *Sporidiobolus*, *Kodamaea*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, and *Guehomyces* and capable of growing on sugary media for alcoholic fermentation. This study focused on the screening and isolation of naturally occurring yeast strains from different flowers to evaluate their ability to ferment glucose in rice hydrolysate as the sole source of carbon and to produce ethanol. These yeast strains were members of the genera *Metschnikowia* and *Meyerozyma*.

2. Materials and methods

2.1. Isolation and identification of yeast strains

Different yeast strains were isolated from different flowers collected from different premises of Mie University, Tsu, Mie Prefecture, Japan. The flowers were sampled in the morning and were dispensed in 0.89% sterilized phosphate-buffered saline (pH 7.2–7.4), smashed by a glass rod to get the nectar. Yeast strains were isolated by spread-plating on MYG agar medium (g/L): yeast extract 3, malt extract 3, peptone 5, glucose 10, and agar 20, supplemented with chloramphenicol (50 µg/mL). All isolates were named temporarily and were subjected to preliminary microscopic investigations. From 30 flowers, 65 yeast strains were isolated.

2.2. Fermentation studies

MYG liquid medium with 5% glucose concentration was prepared and 12 mL was dispensed in glass test tubes, each with inverted Durham tubes. The medium was sterilized and was inoculated with different yeast cultures. The Durham tubes were observed daily for gas formation for 7 days. On the basis of fermentation, 12 strains were selected for further study. A brewing yeast strain, *Saccharomyces cerevisiae* K-7, was obtained from the Brewing Society of Japan in Tokyo, Japan.

2.3. Selection of yeast strains for bioethanol production from glucose

Nutrient supplementation and metal optimization play important roles in increasing the growth and fermentative activity of yeast. The Mg/Ca ratio, being antagonistic, has been implicated in improving the fermentation performance of yeast cells. Many industrial feed stocks such as cheese whey, molasses, and malt wort can be manipulated by Mg supplements to improve fermentation (Walker et al., 1996).

The synthetic mineral medium, with composition (g/L) of glucose 50, yeast extract 6.5, $(NH_4)_2SO_4$ 2.6, KH_2PO_4 2.72, $MgSO_4.7H_2O$ 0.8, $CaCl_2$ 0.3, $ZnCl_2$ 0.00042, citric acid 1.5, and sodium citrate 6, was prepared with minor modification (Bonciu et al., 2010). The medium was distributed in narrow-necked bottles and sterilized at 121 °C for 15 min. The medium was made with two ion compositions (g/L): a) $MgSO_4$ 0.8, $CaCl_2$ 0.3; b) $MgSO_4$ 0.5, $CaCl_2$ 0.5. Magnesium sulfate ($MgSO_4$) and $CaCl_2$ have antagonistic effects and can affect the yeast growth, too. Medium was inoculated with 5% yeast culture and incubated at 30 °C with mild shaking (125 rpm) for 7 days. The experiment was carried out in triplicates in narrow-necked glass bottles (capacity: 100 mL) containing 50 mL of medium and covered with aluminum foil. Samples were obtained after 24 h and evaluated for bioethanol production by an alcohol densitometer (Alcomate AL-2, Woodson Riken Keiki, Tokyo, Japan) (Isono et al., 2012). Cell density was measured at 600 nm. At the time of the experiment's start and termination, the pH of the fermented medium was observed.

2.4. Bioethanol production from xylose

For xylose, the abovementioned synthetic medium was used with some modifications (g/L): $MgSO_4.7H_2O$ 0.5, $CaCl_2$ 0.2, $ZnCl_2$ 0.05. Xylose (50 g/L) solution was autoclaved separately and inoculated in medium after sterilization. Yeast cultures (5%) were inoculated and incubated at 30 °C with mild shaking (125 rpm) for up to 10 days. The experiment proceeded in triplicates in narrow-necked glass bottles (capacity: 100 mL) containing 50 mL of medium and covered with aluminum foil. Samples were obtained at the time of inoculation and twice (days 5 and 10) during the experiment and evaluated for bioethanol production by an alcohol densitometer (Alcomate AL-2). Cell density of the sampled medium was measured at 600 nm.

2.5. Characterization of yeast strains

For the identification of yeast species, the study of physiological characteristics is as important as molecular characterization, but due to practical reasons we relied on cell characteristics and DNA sequences for identification (ITS5, ITS4) exclusively, which was accomplished by BLAST-querying the GenBank database at http://www.ncbi.nlm.nih.gov/blast. The BLAST searches generally achieved very high similarity scores (usually between 92% and 99%).

2.6. Acid hydrolysis of Japanese rice

The hydrolysate was prepared by dilute sulfuric acid hydrolysis of boiled Japanese rice (Arisa, 2005). The boiled Japanese rice (170 g wet weight containing 48% carbohydrates) was mashed with a glass rod. Mashed rice was mixed in 250 mL of sulfuric acid (0.4 M). The mixture was heated in an autoclave at 105 °C for 2 h. The sample

was then cooled after being removed from the autoclave. The extract was obtained by centrifugation at 3000 × *g* for 10 min. The volume was measured by collecting the extract. Charcoal (0.1%) was added in a measured volume and was incubated for 1 h. The mixture was then filtered by a low-pressure filtration technique and hydrolysate of rice was collected in a beaker and neutralized at 4 with 2 M NaOH. Glucose contents in hydrolysate were measured by the mutarotase GOD enzymatic method (Wako Autokit Glucose).

2.6.1. Yeast inoculum preparation and fermentation

The yeast was initiated in MYG medium at 30 °C. The 5 yeast colonies and one standard yeast (*Saccharomyces cerevisiae* K7) were cultured in synthetic mineral medium with 5% glucose concentration for 24 h at 150 rpm on a rotary shaker at 30 °C. The 5% inocula were used in the fermentation of rice lysate. Rice lysate contained 10% glucose content. Fermentation medium was prepared with 50% rice hydrolysate, 45% synthetic mineral medium, and 5% yeast inoculum in 250-mL Erlenmeyer flasks and flasks were covered with aluminum foil. The flasks were agitated at 150 rpm on a rotary shaker at 30 °C for 10 days. The experiment was performed in triplicates. Samples were collected daily in antiseptic conditions. Ethanol was evaluated by an alcohol densitometer (Alcomate AL-2) and absorbance was measured at 600 nm.

2.7. Evaluation of yeast in 1% NaCl concentration and low pH

To proceed with the fermentation of rice lysate in a bioreactor on a large scale in the future, the tendency of yeast cells was evaluated at 1% NaCl concentration and low pH because a bioreactor is an open system and fungi contamination is prohibited only by adjusting the medium to a low pH and 1% salt concentration. The synthetic mineral medium was supplemented with 1% NaCl and 5% glucose concentrations. The pH of the medium was adjusted to 3, 4, and 5 by 1 M H_2SO_4 and inoculated with 5% yeast inoculum. The medium was dispensed in 100-mL flasks and incubated for 9 days at 150 rpm on a rotary shaker at 30 °C. The experiment was carried out in triplicates. Samples were withdrawn after 48 h for ethanol estimation and absorbance was measured at 600 nm.

2.8. Statistical analysis

Experimental values were expressed as mean ± SEM for triplicates. All of the experimental data were analyzed using one-way analysis of variance followed by Duncan's multiple range test (SPSS 16.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Isolation of yeast strains based on fermentation

Sixty-five yeast strains were isolated from the nectar of 30 flowers on MYG medium. All yeast isolates were subjected to sugar assimilation and fermentation testing using 5% glucose in Durham tubes. With regard to fermentation of sugar, only 12 isolates were selected based on anaerobic liberation of CO_2 (Table 1) for the next step, i.e. ethanol production.

3.2. Evaluation of yeasts for ethanol production from glucose

The selected 12 yeast strains were further evaluated to test their alcohol-producing capabilities in synthetic mineral medium supplemented with 5% glucose under laboratory conditions. Yeast isolates exhibited growth and produced ethanol in both media containing $MgSO_4$:$CaCl_2$ (0.8:0.3 and 0.5:0.5 g/L) as recorded in Tables 2 and 3. All isolates utilized $MgSO_4$:$CaCl_2$ (0.8:0.3

Table 1. Gas production by fermentation of glucose by different yeast isolates.

Flower names	Selected isolates	Gas production (days)
Tagetes	Y25	+ (2)
	Y28	+ (3)
Abelia	Y30	+ (3)
	Y31	+ (3)
	Y32	+ (2)
	Y33	+ (2)
	Y34	+ (2)
Torenia	Y35	+ (2)
	Y37	+ (2)
	Y38	+ (2)
Zinnia	Y42	+ (5)
Melampodium paludosum	Y49	+ (5)

Table 2. Day-wise estimation of ethanol in fermentation medium containing 5% glucose with different ion concentrations of $MgSO_4$ and $CaCl_2$ ($MgSO_4$ 0.8 g/L, $CaCl_2$ 0.3 g/L) by different yeast isolates.

Yeast strains	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
K7	0.047 ± 0.01[a]	0.014 ± 0.02[a]	0.35 ± 0.03[a]	0.62 ± 0.05[a]	0.94 ± 0.06[a]	1.35 ± 0.03[a]	1.70 ± 0.06[a]
Y25	0.027 ± 0.02[a]	0.08 ± 0.01[ab]	0.24 ± 0.03[b]	0.48 ± 0.02[b]	0.70 ± 0.02[b]	1.02 ± 0.09[b]	1.20 ± 0.06[b]
Y28	0.00 ± 0.00[b]	0.01 ± 0.01[b]	0.06 ± 0.01[c]	0.14 ± 0.03[c]	0.32 ± 0.01[c]	0.86 ± 0.03[c]	0.85 ± 0.03[c]
Y30	0.00 ± 0.00[b]	0.00 ± 0.00[bc]	0.3 ± 0.04[ab]	0.35 ± 0.03[d]	0.61 ± 0.04[b]	0.72 ± 0.02[d]	1.05 ± 0.04[d]
Y31	0.11 ± 0.02[c]	0.19 ± 0.02[ac]	0.51 ± 0.02[d]	0.71 ± 0.04[a]	0.91 ± 0.05[a]	1.28 ± 0.04[a]	1.53 ± 0.03[e]
Y32	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.20 ± 0.02[be]	0.31 ± 0.01[cd]	0.59 ± 0.02[bd]	1.05 ± 0.03[b]	1.25 ± 0.09[b]
Y33	0.00 ± 0.00[b]	0.11 ± 0.01[a]	0.42 ± 0.04[ad]	0.51 ± 0.02[b]	0.70 ± 0.02[b]	0.71 ± 0.01[d]	0.75 ± 0.02[c]
Y34	0.22 ± 0.01[d]	0.67 ± 0.12[c]	1.00 ± 0.05[f]	1.43 ± 0.03[e]	1.71 ± 0.03[e]	1.81 ± 0.04[e]	2.00 ± 0.07[f]
Y35	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.20 ± 0.02[beg]	0.22 ± 0.03[c]	0.55 ± 0.04[bd]	0.64 ± 0.04[df]	0.90 ± 0.08[cd]
Y37	0.00 ± 0.00[b]	0.19 ± 0.01[ad]	0.25 ± 0.03[beg]	0.60 ± 0.03[ab]	0.70 ± 0.03[b]	0.83 ± 0.03[cd]	1.30 ± 0.06[b]
Y38	0.00 ± 0.00[b]	0.21 ± 0.01[ad]	0.25 ± 0.02[beg]	0.39 ± 0.03[bd]	0.65 ± 0.04[bd]	0.73 ± 0.02[d]	1.35 ± 0.03[b]
Y42	0.09 ± 0.01[e]	0.13 ± 0.02[ade]	0.48 ± 0.01[d]	0.76 ± 0.07[a]	1.05 ± 0.03[f]	1.78 ± 0.04[e]	2.05 ± 0.03[f]
Y49	0.00 ± 0.00[b]	0.07 ± 0.02[ab]	0.30 ± 0.02[a]	0.33 ± 0.03[d]	0.70 ± 0.05[b]	0.96 ± 0.03[bc]	1.25 ± 0.02[b]

All values represent means of three replicates ± SEM. Two values within a column not sharing a common letter differ significantly from standard and experimental strains. Values are significantly different at $P \leq 0.5$ in single-factor analysis of variance. K7: *Saccharomyces cerevisiae* K7(standard strain).

Table 3. Day-wise estimation of ethanol in fermentation medium containing 5% glucose with different ion concentrations of $MgSO_4$ and $CaCl_2$ ($MgSO_4$ 0.5 g/L, $CaCl_2$ 0.5 g/L) by different yeast isolates.

Yeast strains	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
K7	0.06 ± 0.01[a]	0.12 ± 0.02[a]	0.30 ± 0.02[a]	0.55 ± 0.02[a]	0.90 ± 0.06[af]	1.36 ± 0.03[a]	1.60 ± 0.02[a]
Y25	0.02 ± 0.01[b]	0.07 ± 0.02[b]	0.30 ± 0.02[a]	0.46 ± 0.03[b]	0.65 ± 0.03[b]	1.06 ± 0.07[b]	1.25 ± 0.03[b]
Y28	0.00 ± 0.00[b]	0.00 ± 0.00[c]	0.05 ± 0.01[b]	0.13 ± 0.02[c]	0.30 ± 0.01[c]	0.66 ± 0.03[c]	0.75 ± 0.02[c]
Y30	0.00 ± 0.00[b]	0.04 ± 0.01[b]	0.15 ± 0.01[c]	0.31 ± 0.01[df]	0.40 ± 0.03[d]	0.67 ± 0.01[c]	1.00 ± 0.03[d]
Y31	0.00 ± 0.00[b]	0.17 ± 0.02[d]	0.50 ± 0.02[d]	0.67 ± 0.02[e]	0.95 ± 0.03[a]	1.27 ± 0.02[a]	1.30 ± 0.03[b]
Y32	0.00 ± 0.00[b]	0.00 ± 0.00[c]	0.25 ± 0.01[a]	0.26 ± 0.01[df]	0.60 ± 0.01[b]	1.07 ± 0.05[b]	1.25 ± 0.03[b]
Y33	0.00 ± 0.00[b]	0.00 ± 0.00[c]	0.10 ± 0.03[bc]	0.13 ± 0.02[c]	0.40 ± 0.02[d]	0.58 ± 0.05[c]	0.75 ± 0.04[c]
Y34	0.10 ± 0.02[c]	0.17 ± 0.01[d]	0.40 ± 0.03[e]	0.72 ± 0.03[e]	1.10 ± 0.05[e]	1.28 ± 0.02[a]	1.50 ± 0.02[e]
Y35	0.00 ± 0.00[b]	0.00 ± 0.00[c]	0.20 ± 0.02[acf]	0.21 ± 0.01[f]	0.55 ± 0.03[b]	0.61 ± 0.03[cd]	0.80 ± 0.05[c]
Y37	0.00 ± 0.00[b]	0.17 ± 0.02[d]	0.25 ± 0.03[a]	0.57 ± 0.02[a]	0.65 ± 0.03[b]	0.73 ± 0.03[c]	1.15 ± 0.03[f]
Y38	0.00 ± 0.00[b]	0.15 ± 0.03[ad]	0.15 ± 0.02[c]	0.37 ± 0.03[d]	0.65 ± 0.02[b]	0.68 ± 0.01[c]	1.15 ± 0.03[f]
Y42	0.07 ± 0.01[a]	0.18 ± 0.01[de]	0.25 ± 0.03[a]	0.72 ± 0.04[e]	0.95 ± 0.08[a]	1.64 ± 0.02[e]	1.95 ± 0.04[g]
Y49	0.07 ± 0.02[a]	0.14 ± 0.02[ad]	0.45 ± 0.03[de]	0.66 ± 0.03[e]	0.80 ± 0.03[af]	0.93 ± 0.02[f]	1.15 ± 0.02[f]

All values represent means of three replicates ± SEM. Two values within a column not sharing a common letter differ significantly from standard and experimental strains. Values are significantly different at $P \leq 0.5$ in single-factor analysis of variance. K7: *Saccharomyces cerevisiae* K7(standard strain).

g/L) well as shown by good growth and good ethanol production; this was exhibited clearly by *Metschnikowia* sp. Y31, *Metschnikowia cibodasensis* Y34, *Metschnikowia* sp. Y37, *Metschnikowia* sp. Y38, and *Meyerozyma caribica* Y42, for which ethanol production was recorded as 1.53 ± 0.03%, 2.0 ± 0.07%, 1.30 ± 0.06%, 1.35 ± 0.03%, and 2.05 ± 0.03% on the 7th day, respectively. Significant ethanol was produced by *Metschnikowia cibodasensis* Y34 (days 1–7) and *Meyerozyma caribica* Y42 (days 3 and 5–7) (Table 2). The growth pattern is shown in Figure 1; the same growth pattern was observed in all isolates and a slight increase was observed in the medium containing $MgSO_4$:$CaCl_2$ (0.8:0.3 g/L). All yeast cells showed logarithmic growth up to day 5 and a somewhat stationary phase continued up to day 7 observing the same values. The pH of the fermentation broth dropped from 6 to 4.5–4.75 within 7 days.

3.3. Assimilation of xylose by yeasts

Twelve yeast strains capable of fermenting glucose were also used for fermentation of xylose to check the ability to degrade hemicelluloses. No strain was found to ferment xylose into bioethanol. All strains showed good growth by utilizing xylose with a prolonged stationary phase from day 5 to day 10, except Y28 and *Meyerozyma caribica* Y42. Y28 showed logarithmic growth (Figure 2).

3.4. Characterization of yeast isolates

On the basis of fermentation of glucose and ethanol production, 5 yeast strains were selected and characterized on a molecular basis. These strains belonged to the genera *Metschnikowia* and *Meyerozyma* (Table 4). The yeast strains exhibited high similarity scores in BLAST searches, such as 92% for *Metschnikowia cibodasensis* Y34; 94% for *Metschnikowia* sp. Y31, *Metschnikowia* sp. Y37, and *Metschnikowia* sp. Y38; and 99% for *Meyerozyma caribica* Y42.

3.5. Acid hydrolysis of rice

Acid hydrolysis was used to release reducing sugars in rice. By acid treatment, the polymers are converted into monomers and dimers of sugars. Table 5 shows the time course of ethanol production using the rice hydrolysate as a substrate. The ethanol production rate increased rapidly with all isolates after day 1 as was observed in the standard. A gradual decrease was then observed for all strains except *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42. In *Metschnikowia cibodasensis* Y34, consistent ethanol production was observed from day 2 to 4 (1.80 ± 0.05% to 1.75 ± 0.03%) and day 5 to 6 (1.65 ± 0.04%), leading to decrease afterwards. The highest percentage of ethanol, i.e. 2.2 ± 0.21%, was recorded for *Meyerozyma caribica* Y42 at day 4 but it remained consistent (2.05 ± 0.10%) up to day 6 and then a gradual decrease started. This indicated that *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42 were tolerant to the ethanol and the glucose concentration was consistent with the time period of ethanol production. The growth pattern of yeast isolates in rice hydrolysate is revealed in Figure 3. All yeasts and the standard strain showed rapid growth up to day 2, followed by a long stationary phase up to day 8, except *Metschnikowia* Y31, Y37, and Y38 (day 7), with a decline phase until the termination of the experiment on day 10.

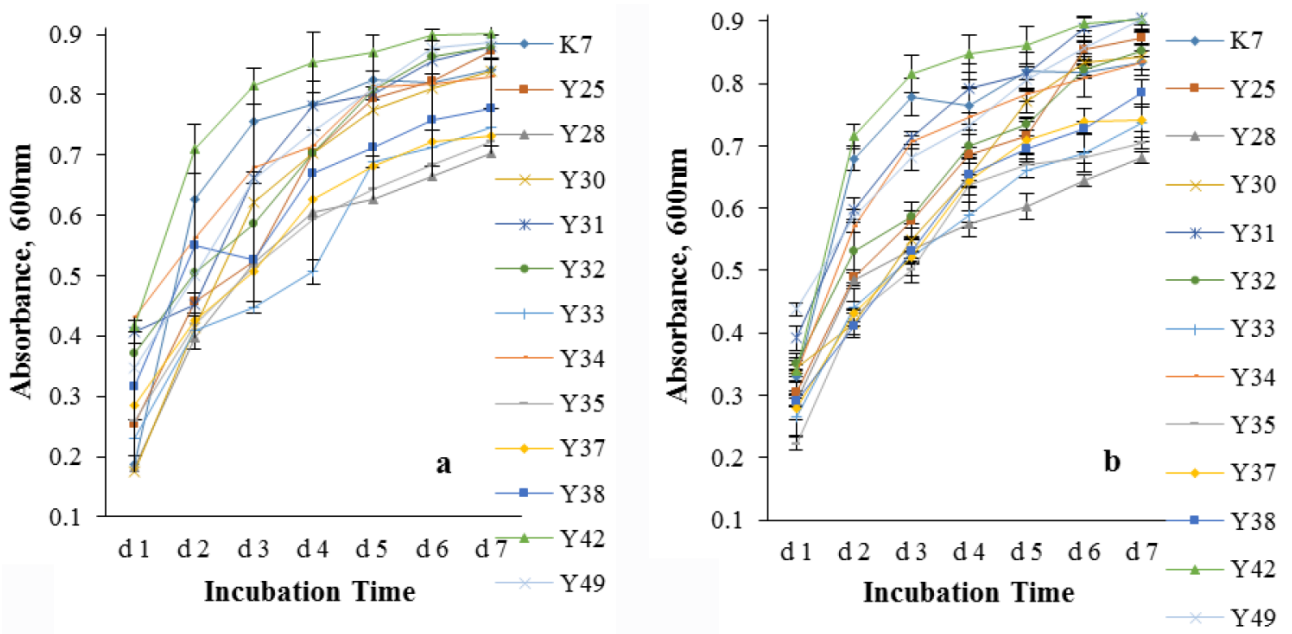

Figure 1. Growth pattern of different yeast isolates by fermenting glucose in two different media with different concentrations (g/L) of a) $MgSO_4$ (0.8), $CaCl_2$ (0.3); b) $MgSO_4$ (0.5), $CaCl_2$ (0.5). Bars represent SEM.

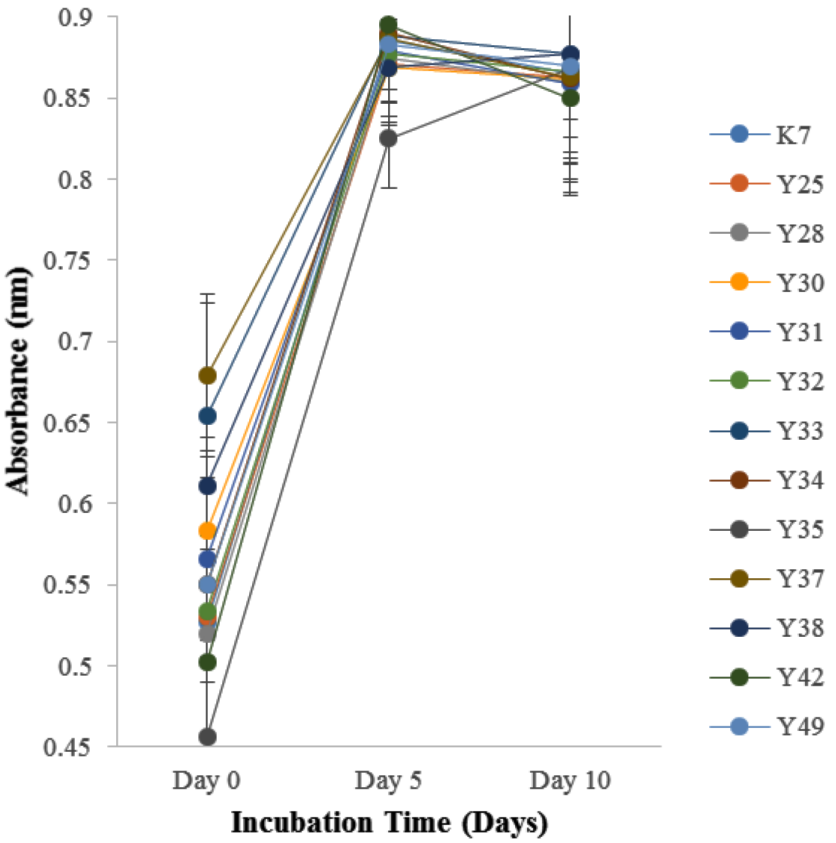

Figure 2. Growth pattern of different yeast isolates by fermenting xylose as a carbon source with synthetic mineral medium. Bars represent SEM.

Table 4. Identification of selected yeast isolates.

Flower names	Yeast strain code	Species	Accession numbers
Abelia	Y31	*Metschnikowia* sp.	AB 693153
	Y34	*Metschnikowia cibodasensis*	AB 693154
Torenia	Y37	*Metschnikowia* sp.	AB 693155
	Y38	*Metschnikowia* sp.	AB 693156
Zinnia	Y42	*Meyerozyma caribica*	AB 693157

3.6. Fermentation of rice hydrolysate in salt and a low pH

In *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42, good growth as well as the highest ethanol production was observed at pH 3, i.e. 1.75 ± 0.14 at days 3 and 5 and 2.05 ± 0.14 at days 1 and 3, respectively (Table 6; Figure 4). Growth and ethanol production at pH 4 and 5 was close to that at pH 3, while a slight increase was observed in *Metschnikowia cibodasensis* Y34 at pH 4 up to day 3. Zero percent ethanol was recorded at day 9 in both strains as well as standard yeast strains at pH 4 and 5, but 1.30 ± 0.04 and 1.25 ± 0.05 %v/v ethanol was recorded in *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42, respectively, at pH 3. This test will provide information to use yeast strains in fermentation systems where bacterial contamination is the main threat. The low pH and NaCl concentration will help to minimize the contamination risk.

4. Discussion

Thirty flowers were processed to isolate 65 yeasts. All yeast isolates were subjected to anaerobic fermentation with 5% glucose in Durham tubes. Only 12 isolates were selected on the basis of CO_2 release in Durham tubes from *Tagetes*, *Abelia*, *Torenia*, *Zinnia*, and *Melampodium paludosum* flowers. Scheffers (1987) argued that the anaerobic

Table 5. Day-wise estimation of bioethanol (%) in acid hydrolysate of rice by different yeast strains.

Days post incubation	Yeast isolates					
	K7	Y31	Y34	Y37	Y38	Y42
Day 1	0.90 ± 0.06	0.70 ± 0.01 ^{b}	0.90 ± 0.01 ^{a}	0.70 ± 0.03 ^{b}	0.80 ± 0.05 ^{ab}	0.90 ± 0.04 ^{a}
Day 2	1.95 ± 0.05	1.55 ± 0.03 ^{b}	1.80 ± 0.05 ^{c}	1.75 ± 0.03 ^{c}	1.75 ± 0.03 ^{c}	1.85 ± 0.06 ^{ac}
Day 3	1.80 ± 0.06	1.80 ± 0.05 ^{a}	1.80 ± 0.05 ^{a}	1.50 ± 0.06 ^{b}	1.70 ± 0.03 ^{a}	1.90 ± 0.04 ^{ac}
Day 4	1.75 ± 0.01	1.70 ± 0.03 ^{a}	1.75 ± 0.03 ^{a}	1.70 ± 0.01 ^{a}	1.65 ± 0.03 ^{a}	2.20 ± 0.21 ^{b}
Day 5	1.50 ± 0.04	1.65 ± 0.05 ^{b}	1.70 ± 0.06 ^{b}	1.70 ± 0.03 ^{b}	1.65 ± 0.03 ^{b}	2.10 ± 0.06 ^{c}
Day 6	1.25 ± 0.03	1.65 ± 0.05 ^{b}	1.65 ± 0.04 ^{b}	1.60 ± 0.02 ^{b}	1.50 ± 0.05 ^{b}	2.05 ± 0.10 ^{c}
Day 7	1.10 ± 0.06	1.55 ± 0.04 ^{b}	1.55 ± 0.03 ^{bc}	1.40 ± 0.03 ^{bc}	1.40 ± 0.02 ^{c}	1.85 ± 0.09 ^{d}
Day 8	0.80 ± 0.06	1.35 ± 0.03 ^{b}	1.45 ± 0.03 ^{b}	1.30 ± 0.02 ^{bc}	1.20 ± 0.02 ^{c}	1.60 ± 0.04 ^{d}
Day 9	0.50 ± 0.02	1.10 ± 0.06 ^{b}	1.20 ± 0.02 ^{bd}	1.00 ± 0.05 ^{bc}	0.95 ± 0.03 ^{c}	1.30 ± 0.03 ^{cd}
Day 10	0.30 ± 0.04	0.90 ± 0.01 ^{b}	0.95 ± 0.03 ^{be}	0.80 ± 0.02 ^{c}	0.70 ± 0.02 ^{d}	1.00 ± 0.02 ^{e}

All values represent means of three replicates ± SEM. Two values within a row not sharing a common letter differ significantly from standard and experimental strains. Values are significantly different at $P \leq 0.5$ in single-factor analysis of variance. K7: *Saccharomyces cerevisiae* K7(standard strain).

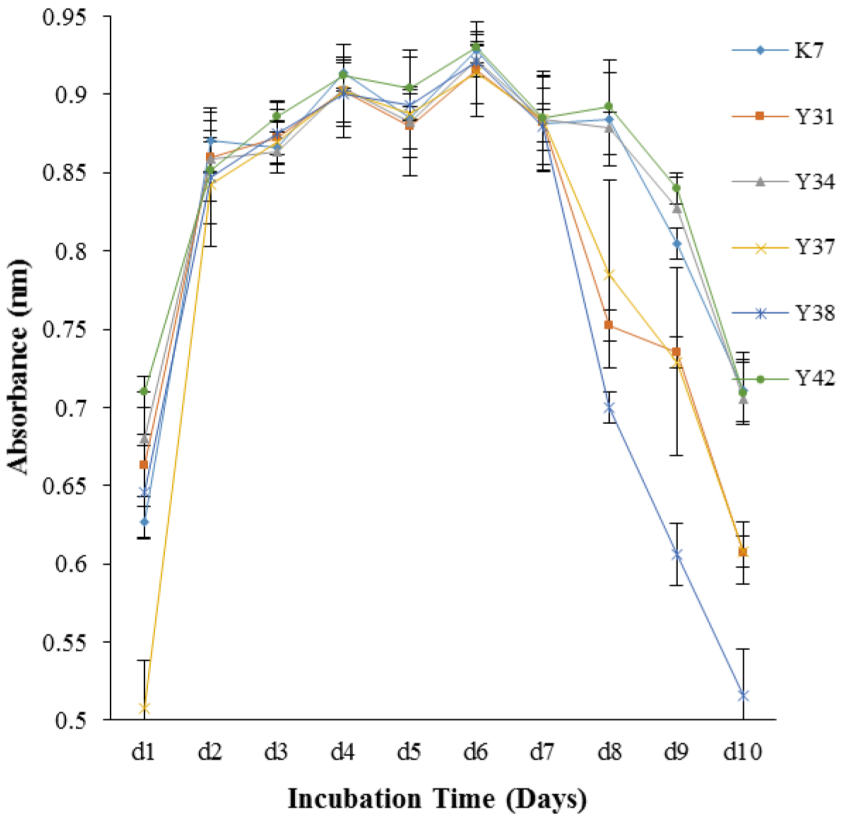

Figure 3. Growth pattern of different yeast strains in hydrolysate of rice. Bars represent SEM.

liberation of CO_2 into Durham tubes is not very accurate for detecting slowly fermenting yeast species. Fruit and flowers are considered as easily available raw materials and natural sources for isolation of ethanol-producing yeasts. Yeasts mostly use sugars as a substrate and are supposed to be associated with the flower's nectar, fruits, and vegetables naturally (Tournas, 2005; Li, 2008; Tsegaye, 2016). Manwar et al. (2013) reported yeasts from flowers of

Table 6. Percent bioethanol production by yeast strains in fermentation medium with 1% NaCl and 5% glucose at different pH levels.

Yeast strains	pH	Day 1	Day 3	Day 5	Day 7	Day 9
K7	3	1.85 ± 0.03[a]	1.90 ± 0.17	1.25 ± 0.03	0.65 ± 0.05	0.00 ± 0.00
	4	1.20 ± 0.12[b]	1.70 ± 0.17	1.25 ± 0.03	0.60 ± 0.06	0.00 ± 0.00
	5	1.70 ± 0.06[a]	1.80 ± 0.21	1.15 ± 0.09	0.50 ± 0.06	0.00 ± 0.00
Y34	3	1.35 ± 0.03[a]	1.75 ± 0.14	1.75 ± 0.08[a]	1.55 ± 0.03[a]	1.30 ± 0.04[a]
	4	2.15 ± 0.09[b]	1.85 ± 0.20	1.35 ± 0.06[b]	0.75 ± 0.06[b]	0.00 ± 0.00[b]
	5	2.20 ± 0.12[b]	2.20 ± 0.15	1.55 ± 0.03[a]	0.65 ± 0.03[b]	0.00 ± 0.00[b]
Y42	3	2.05 ± 0.14	2.00 ± 0.06	1.90 ± 0.06[a]	1.75 ± 0.07[a]	1.25 ± 0.05[a]
	4	2.15 ± 0.03	1.95 ± 0.08	1.75 ± 0.14[a]	0.80 ± 0.06[b]	0.00 ± 0.00[b]
	5	2.20 ± 0.12	1.95 ± 0.06	1.15 ± 0.09[b]	0.75 ± 0.04[b]	0.00 ± 0.00[b]

All values represent means of three replicates ± SEM. Two values within a column not sharing a common letter differ significantly for other pH levels against each strain. Values are significantly different at $P \leq 0.5$ in single-factor analysis of variance. K7: *Saccharomyces cerevisiae* K7(standard strain).

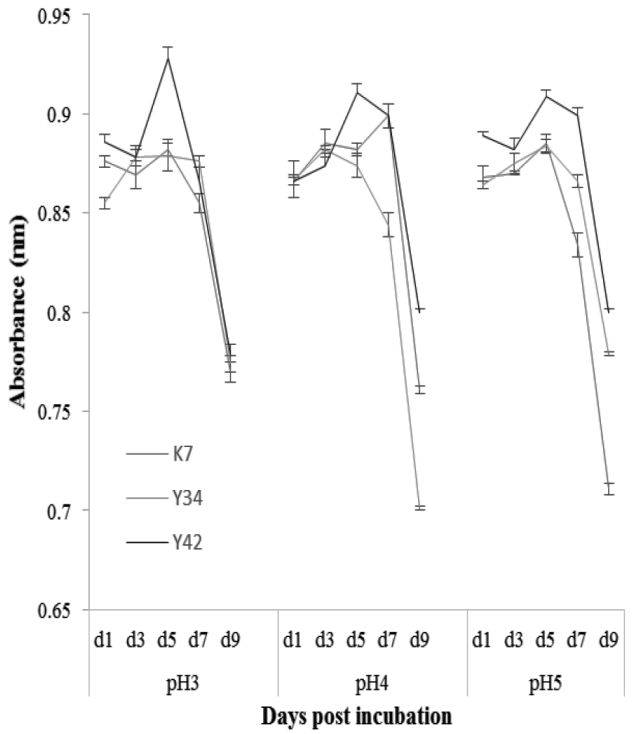

Figure 4. Growth pattern of yeast strains in fermentation medium with 1% NaCl and 5% glucose at different pH levels. Bars represent SEM.

Woodfordia fruticosa while Stringini et al. (2008) reported them from leaves and fruit of banana, cacao, papaya, sugar cane extract, soil, and plant wastes. Yeasts usually utilize monosaccharides for their growth and a few of these monosaccharides can be fermented into ethanol. Ethanol can be produced by hexose, such as glucose, mannose, galactose, and pentose, including xylose and arabinose by means of microorganisms (Mosier et al., 2005; Hisamatsu et al., 2006). Glucose is considered the best substrate for both growth and ethanol production by fermentation.

For evaluation of ethanol production, 12 selected isolates were processed in a synthetic mineral medium with 5% glucose. All yeast strains produced a good percentage of ethanol with 0.8 g/L $MgSO_4$ and 0.3 g/L $CaCl_2$. $MgSO_4$ and $CaCl_2$ are essential components for yeast growth. In this experiment, a slight increase of cell growth was noted at the high concentration (0.8 g/L) of Mg^{2+} ions in the medium. A lowered concentration (0.5 g/L) of these ions in the medium inhibited the rate of cell growth to some extent. A similar tendency was observed by Walker et al. (1996) and Duszkiewicz-Reinhard et al. (2005) for the growth and metabolism of *Saccharomyces cerevisiae* cells applied at a dose of 13–496 µmol/L (corresponding to 0.003–0.012 g Mg^{2+}/L), while Mandels et al. (1999) investigated the highest growth and the cellulase activity with metal cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+} in *Trichoderma viride* QM6a.

Yeast cells require certain macro- and microelements for growth, metabolism, and cell stability. Magnesium and calcium are macroelements. Magnesium constitutes 0.3% of the cell dry weight and acts as an enzyme activator (especially for all synthetases, phosphatases, and kinases) and a stress suppressor, and it helps to control cell division, growth, and size (Rees and Stewart, 1997; Briggs, 2004; Walker, 2004). It counteracts the toxic effects of Cu, Co, Cd, and Al. Magnesium has been reported to regulate metabolic enzymes of the fermentative pathway (via pyruvate decarboxylase) or the respiratory pathway (via pyruvate dehydrogenase) and the switching between respiratory and fermentative processes (Rees and Stewart, 1997; Walker, 2004; Walker et al., 2006; Udeh and Kgatla, 2013). Meanwhile, calcium is involved in regulating amylase activity and phosphate precipitation and also plays a protective role for cell membranes (Walker, 2004; Trofimova et al., 2010).

Fermentative yeast has a high demand for Mg due to glycolytic enzyme activity and free intracellular available Mg may not be sufficient to fulfill the requirement. Moreover, the interaction of Mg and Ca is antagonistic. Calcium affects the uptake and bioavailability of magnesium. Calcium inhibited many transphosphorylases of glycolysis that were stimulated by Mg (Walker, 2004). Industrial fermentations may be manipulated by supplementing yeast media with magnesium salts, especially $MgSO_4$. Thus, the adjustment of the Mg/Ca ratio in yeast fermentation media will lead to improve alcohol production because the cellular demand for Mg and Ca is not met by industrial yeast media (Walker et al., 1996). Okon and Nwabueze (2009) evaluated the maximum ethanol yield (12.53% v/v) with a 2:1 Mg/Ca ratio along with a combination of Zn while Slininger et al. (2006) reported the impact of magnesium to improve biomass and ethanol production with xylose.

The pH of the fermentation medium decreased from 6 to 4.5–4.75 within 7 days, as was observed by Yu et al. (2004). According to Silva et al. (2001), yeast is capable of maintaining a relatively stable pH that helps to inactivate toxic compounds in the hydrolysate. Ethanol production assays were considered to be more suitable determinants of sugar fermentation by yeasts (Walker, 1998).

On the basis of fermentation of glucose and ethanol production, 5 yeast strains were selected and characterized on a molecular basis. These strains belonged to the genera *Metschnikowia* and *Meyerozyma*. Lachance et al. (2001) also reported two *Metschnikowia* species isolated from flowers, nectars and pollinators.

Those 5 yeast isolates were subjected to fermentation of reducing sugars in rice hydrolysate after dilute acid treatment. Growth patterns of yeasts and ethanol production were evaluated up to 10 days. Significant ethanol, i.e. 1.80 ± 0.05 on days 2 and 3 and 2.20 ± 0.21 %v/v on day 4, was produced by *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42, while 1.95 ± 0.05 %v/v was recorded by *Saccharomyces cerevisiae* K7 from 5% glucose present in rice hydrolysate. Isono et al. (2012) reported 10 *Issatchenkia orientalis* strains producing ethanol between 0.5% and 5.3% (v/v) in the medium containing 50 g/L Na_2SO_4 at pH 3.0 while no ethanol was detected by the *S. cerevisiae* K7 strain under the same conditions. However, the MRF-121 strain produced 1.3%–5.3% (v/v) ethanol under the combined stress of a low pH between 2 and 3 and a high salt concentration of 50 g/L Na_2SO_4. Gohal and Duan (2012) reported ethanol yields of 11.23 ± 0.08 to 12.09 ± 0.07 %v/v by hydrolyzing 25% dry solids of Indian broken rice (68.45% starch) using granular starch hydrolyzing enzyme in 72 h. Van Hanh and Kim (2009) optimized the ethanol yield to 16.8% from 15.1% (v/v) via simultaneous saccharification and fermentation (SSF) of low-value rice wine cake (RWC) without cooking within 90 h. SSF of RWC containing 23.03% starch was carried out by the raw-starch-digesting enzyme of *Rhizopus* sp. and *Saccharomyces cerevisiae* KV25. The ethanol yield (2.2 ± 0.21 %v/v) in the present investigation was obtained from the wet weight of rice (170 g) containing 5% glucose contents through fermentation of yeast and can be optimized by adjusting different growth conditions and using waste fruit substrates.

Logarithmic growth was observed up to day 2, followed by a stationary phase. The rice hydrolysate fermentation assay was conducted at pH 3, 4, and 5 with 1% NaCl with *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42. Both strains showed good ethanol yield at pH 3. Palmqvist and Hahn-Hagerdal (2000) supposed that the cell growth of microbes depends on pH and a low pH provides a large concentration of dissociated weak ions. This test will provide information to use yeast strains in fermentation systems where bacterial contamination is the

main threat. Sodium chloride concentration and low pH will support the minimization of the contamination risk.

In conclusion, 5 yeast strains were isolated and characterized on the molecular level based on screening and ethanol production. Maximum ethanol concentrations were produced by *Metschnikowia cibodasensis* Y34 and *Meyerozyma caribica* Y42 and could be used at the industrial level for bioethanol production in future. Rice, being rich in carbohydrates, has the ability to be hydrolyzed by acid and then fermented into ethanol. Unmarketable and broken rice during processing provides a good source for ethanol production. Now these yeast strains are going to be used to ferment hydrolysates of fruit wastes.

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References

Agrawal M, Pradeep S, Chandraraj K, Gummadi SN (2005). Hydrolysis of starch by amylase from *Bacillus* sp. KCA102: a statistical approach. Process Biochem 40: 2499-2507.

Agu RC, Amadife AE, Ude CM, Onyia A, Ogu EO, Okafor M, Ezejiofor E (1997). Combined heat treatment and acid hydrolysis of cassava grate waste (CGW) biomass for ethanol production. Waste Manage 17: 91-96.

Arisa Y (2005). Alcoholic fermentation from food leftover by salt and acid tolerant yeast. MSc, Mie University, Tsu, Japan.

Bai FW, Anderson WA, Moo-Young M (2008). Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv 26: 89-105.

Behera S, Ray RC, Mohanty RC (2010). Comparative study of bioethanol production from mahula (*Madhuca latifolia* L.) flowers by immobilized cells of *Saccharomyces cerevisiae* and *Zymomonas mobilis* in calcium alginate beads. J Sci Ind Res 69: 472-475.

Bhadra B, Rao SR, Singh PK, Sarkar PK, Shivaji S (2008). Yeasts and yeast-like fungi associated with the bark of trees: diversity of yeasts and identification of yeasts producing extracellular endoxylanases. Curr Microbiol 56: 489-494.

Bonciu C, Tabacaru C, Bahrim G (2010). Yeasts isolation and selection for bioethanol Production from inulin hydrolysates. Innov Romanian Food Biotechnol 6: 29-34.

Briggs DE, Boulton CA, Brookes PA, Stevens R (2004). Brewing Science and Practice. Cambridge, UK: Woodhead.

Demirkan ES, Mikami B, Adachi M, Higasa T, S. Utsumi S (2005). α-Amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*. Process Biochem 40: 2629-2636.

Duszkiewicz-Reinhard W, Gniewosz M, Blazejak S, Bankowsk A (2005). Studies into *Saccharomyces cerevisiae* baker's yeast capacity for binding magnesium under batch conditions. Pol J Food Nut Sci 14: 249-255.

Ghassem T, Sadat DA, Kulkarni DK (2012). Optimization of yeast for ethanol production. International Journal of Research in Ayurveda and Pharmacy 3: 95-97.

Gohel V, Duan G (2012). No-cook process for ethanol production using Indian broken rice and pearl millet. International Journal of Microbiology 2012: 680232.

Hisamatsu M, Furubayashi T, Karita S, Mishima T, Isono N (2006). Isolation and identification of a novel yeast fermenting ethanol under acidic condition. J Appl Glycosci 53: 111-113.

Ibeto CN, Ofoefule AU, Agbo KE (2011). A global overview of biomass potentials for bioethanol production: a renewable alternative fuel. Trends in Applied Sciences Research 6: 410-425.

Isono N, Hayakawa H, Usami A, Mishima T, Hisamatsu M (2012). A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions. J Biosci Bioeng 113: 76-78.

Kleih U, Ravi SB, Rao BD, Yoganand B (2007). Industrial utilization of sorghum in India. SAT eJournal 3: 1-37.

Lachance MA, Starmer WT, Rosa CA, Bowles JM, Barker JSF, Janzen DH (2001). Biogeography of the yeasts of ephemeral flowers and their insects. FEMS Yeast Res 1: 1-8.

Li H, Veenendaal E, Shukor NA, Cobbinah JR, Leifert C (2008). Yeast populations on the tropical timber tree species *Milicia excels*. Lett Appl Microbiol 21: 322-326.

Limtong S, Sringiewa C, Yongmanitchai W (2007). Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresource Technol 98: 3367-3374.

Mandels M, Reese ET (1999). Fungal cellulases and the microbial decomposition of cellulosic fabric. J Ind Microbiol Biotechnol 22: 225-240.

Manwar JV, Mahadik KR, Sathiyanarayanan L, Paradkar AR, Patil SV (2013). Comparative antioxidant potential of *Withania somnifera* based herbal formulation prepared by traditional and non-traditional fermentation processes. Integr Med Res 2: 56-61.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Biores Technol 96: 673-686.

Okon AA, Nwabueze TU (2009). Simultaneous effect of divalent cation in hydrolysed cassava starch medium used by immobilized yeast for ethanol production. Afr J Food Sci 3: 217-222.

Omemu AM, Akpan I, Bankole M, Teniola OD (2005). Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. Afr J Biotechnol 4: 19-25.

Palmqvist E, Hahn-Hagerdal B (2000). Fermentation of lignocellulosic hydrolysates I: Inhibition and detoxification. Biores Technol 74: 17-24.

Pan YC, Lee WC (2005). Production of high-purity isomalto-oligosaccharides syrup by the enzymatic conversion of transglucosidase and fermentation of yeast cells. Biotechnol Bioeng 30: 797-804.

Patle S, Lal B (2008). Investigation of the potential of agro-industrial material as low cost substrate for ethanol production by using *Candida tropicalis* and *Zymomonas mobilis*. Biomass Bioenergy 32: 596-602.

Rees EMR, Stewart GG (1997). The effects of increased magnesium and calcium concentrations on yeast fermentation performance in high gravity wort. J Inst Brew 103: 287-291.

Roble ND, Ogbonna JC, Tanaka H 2003). A novel circulating loop bioreactor with cells immobilized in loofa (*Luffa cylindrica*) sponge for the bioconversion of raw cassava starch to ethanol. Appl Microbiol Biotech 60: 671-678.

Scheffers WA (1987). Alcoholic fermentation. Stud Mycol 30: 321-332.

Silva CJSM, Roberto IC (2001). Improvement of xylitol production by *Candida uilliermondii* FTI 20037 previously adapted to rice straw hemicellulosic hydrolysate. Lett Appl Microbiol 32: 248-252.

Siripattanakul S, Ratanapongleka K, Sangthean P, Yoottachana K, Pimwongnok K (2010). Fermented rice noodle wastewater treatment and ethanol production potential using entrapped yeast cells. Water Practice Technol 5: 1-7.

Siripattanakul-Ratpukdi S (2012). Ethanol production potential from fermented rice noodle wastewater treatment using entrapped yeast cell sequencing batch reactor. Appl Water Sci 2: 47-53.

Stringini M, Comitini F, Taccari M, Ciani M (2008). Yeast diversity in crop-growing environments in Cameroon. Int J Food Microbiol 127: 184-189.

Sumari D, Hosea KMM, Magingo FSS (2010). Genetic characterization of osmotolerant fermentative *Saccharomyces* yeasts from Tanzania suitable for industrial very high gravity fermentation. Afr J Microbiol Res 4: 1064-1070.

Tan HT, Lee KT, Mohamed AR (2010). Second-generation bioethanol (SGB) from Malaysian palm empty fruit bunch: energy and energy analyses. Bioresource Technol 101: 5719-5727.

Tikka C, Osuru HP, Atluri N, Raghavulu PVC, Yellapu NK, Mannur IS, Prasad UV, Aluru S, Varma KN, Bhaskar M (2013). Isolation and characterization of ethanol tolerant yeast strains. Bioinformation 9: 421-425.

Tournas VH (2005). Moulds and yeasts in fresh and minimally processed vegetable and sprouts. Int J Food Microbiol 99: 71-77.

Trofimova Y, Walker GM, Rapoport A (2010). Anhydrobiosis in yeast: influence of calcium and magnesium ions on yeast resistance to dehydration-rehydration. FEMS Microbiol Lett 308: 55-61.

Tsegaye Z (2016). Isolation, identification and characterization of ethanol tolerant yeast species from fruits for production of bioethanol. Int J Mod Chem Appl Sci 3: 437-443.

Udeh HO, Kgatla TE (2013). Role of magnesium ions on yeast performance during very high gravity fermentation. J Brew Dist 4: 19-45.

Van Hanh V, Kim K (2009). Ethanol production from rice winery waste - rice wine cake by simultaneous saccharification and fermentation without cooking. J Microbiol Biotechnol 19: 1161-1168.

Verna G, Nigam P, Singh D, Chaudhary K (2000). Bioconversion of starch to ethanol in a single-step process by coculture of amylolytic yeast and *Saccharomyces cerevisiae*. Bioresource Technol 72: 261-266.

Walker GM (1998). Yeast Physiology and Biotechnology. Chichester, UK: John Wiley & Sons.

Walker GM (2004). Metals in yeast fermentation processes. Adv Appl Microbiol 54: 197-230.

Walker GM, Birch RM, Chandrasena G, Maynard AI (1996). Magnesium, calcium and fermentative metabolism in industrial yeast. J Am Soc Brewing Chem 54: 13-18.

Walker GM, De Nicola R, Anthony S, Learmonth R (2006). Yeast-metal interactions: impact on brewing and distilling fermentations. In: Institute of Brewing & Distilling Asia Pacific Section 2006 Convention; 19–24 March 2006; Hobart, Australia.

Walker GM, Maynard IA (1996). Magnesium-limited growth of *Saccharomyces cerevisiae*. Enzyme Microbe Technol 18: 455-456.

Ward OP, Singh A (2002). Bioethanol technology: developments and perspectives. Adv Appl Microbiol 51: 53-80.

Yu Z, Zhang H (2004). Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with *Saccharomyces cerevisiae*. Bioresource Technol 93: 199-204.