Isolation and Identification of Ethanol Tolerant Yeast Strains

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ABSTRACT

The aim of the present study was to isolate a potential yeast strains and characterized them with respect to their ethanol tolerant. Two yeast strains were isolated from two different soil locations of Menoufia governornate, Egypt. The two isolates were identified at the genus level by colony and cell morphology and biochemical characteristics and at the species level by 26S rRNA gene sequencing. An attempt has been made to check the viability of yeast cells under different concentrations of ethanol. Ethanol tolerance of each strain was determined by allowing the two yeast strains to grow in liquid YEPD (Yeast Extract Peptone Dextrose) medium having different concentrations of ethanol. The results showed the ability of the two isolates to grow in medium having up to 12% ethanol. Molecular taxonomy and phylogeny revealed that the two isolates showed 99% similarity to Candida parapsilosis, they named Candida parapsilosis Can 1 and Candida parapsilosis Can 2.

Key words: yeast, Ethanol tolerant, 26S rRNA sequencing

Introduction

Yeasts are the safest and most effective microorganisms for fermenting sugars to ethanol and traditionally have been used in industry to ferment glucose based agricultural products to ethanol (Ho et al., 1998). Yeast is ubiquitous in the environment, but is most frequently isolated from soil rich samples. Some yeast strains are found in association with soil and insects. In assessing a yeast strain for industrial use, specific physiological properties are required (Ekunsanni and Odunfa, 1990). Ethanol tolerance, sugar tolerance and invertase activities are some of the important properties for use in industrial ethanol production (Jiménez and Benitez, 1988). Yeast has also been isolated from many fermenting sources including fermenting cassava tubers (Osho et al., 2010). Many research workers found yeast in large numbers in a wide variety of natural habitats as different as leaves, flowers, sweet fruits, tree exudates, grains, roots fleshy fungi, insects, dung and soil (Miller et al., 1962). Recently, they have been used in the production of bio fuels, a potentially important alternative energy source. Renewable energy is one of the most efficient ways to achieve sustainable development. Increasing its share in the world matrix will help prolong the existence of fossil fuel reserves, address the threats posed by climate change, and enable better security of the energy supply on a global scale (Tikka et al., 2013). Successful fermentations to produce ethanol using yeast require tolerance to high concentrations of both glucose and ethanol. These cellular characteristics are important because of high gravity fermentations, which are common in the ethanol industry, give rise to high sugar concentrations, at the beginning of the process, and high ethanol concentration at the end of the fermentation. Candida parapsilosis is an important microorganism in bioindustry and its tolerance to ethanol is one of the main characteristics to decide whether it can be used as biofermentation resources (Tikka et al., 2013).

The yeasts are a phylogenetically diverse group of unicellular fungi. Although morphological and biochemical phenotypes continue to be the main criteria used for the identification of an unknown isolate (Kreger-van Rij, 1984 and Barnett et al., 1990). The phenotypic characters used in yeast systematic have more recently been combined with molecular based criteria. Determining DNA relatedness using reassociation studies has been of exceptional value in yeast systematics. More recently, phylogenetic relationships amongst yeasts have been studied by comparing the sequences of the rRNA genes. The principal advantage of this method is that, since ribosomes share a common evolutionary origin in all organisms, it permits the comparison of both closely and distantly related species (Kurtzman, 1992).

Traditional method of classification and identification of fungi has relied upon microscopic features, colony characteristics on artificial media and biochemical reactions (Sutton and Cundell, 2004). Recently, there have been worldwide interest on molecular techniques (Abu Bakar et al., 2010), amplification and sequencing of target regions within the ribosomal DNA gene complex has emerged as a useful adjunctive tool for the identification of fungi and does not depend on mold sporulation for identification (Buzina et al., 2001; Iwen et al., 2002; Rakeman et al., 2005 and Schwarz et al., 2006). The internal transcribed spacer (ITS) regions 1 and 2 located between the highly conserved small (18S) and large (28S) ribosomal subunit genes in the rRNA operon are known to have sufficient sequence variability to allow identification to the species level for many fungi (White et al., 1990; Buzina et al., 2001; Iwen et al., 2002; Brandt et al., 2005; Rakeman et al., 2005 and Schwarz et al., 2006).

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In the present study, we are reporting the isolation and identification of ethanol tolerant yeast strains with the help of partial 26S rRNA sequence analysis and determining its phylogenetic relationship.

**Materials:**

**Strains:**

*Candida* strains were isolated from cultivated soils of Menoufia governorate, Egypt and identified by morphological and biochemical characterization according to Bergey’s Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The method described by Tavanti *et al.* (2005) have been used to identify and differentiate this species.

**Media:**

**Complete medium:**

It is the same medium of Cox and Bevan (1962) which used for growth and maintenance of yeast strains. The ingredients of this medium are; 0.3% yeast extract, 0.5% peptone, 2% glucose and 2% agar and the cultures were incubated at 30 °C for 24 h.

**Methods:**

**Identification and Screening of yeast isolates for ethanol tolerance:**

Two yeasts were isolated from different soil locations after their purification on Yeast Extract Peptone Dextrose Agar (YEPDA). Simple staining were performed for 24 h-cultures and observed with microscope for their morphological characters such as shape, size and budding. The obtained isolates were given specific names for further experimentation and easy recognition.

Ethanol tolerance of each strain was studied by allowing the yeast to grow in liquid YEPD having different concentrations of ethanol i.e., 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 12% 13%, 14%, and 15% (Osho *et al.*, 2010).

**DNA extraction:**

Genomic DNA was extracted from pure yeast cultures, grew in YEPD media at 30°C for 24 hr. DNA was isolated from the two strains by following the method for devised by Sambrook and Russel (2001). The crude lysate (freshly prepared) was subjected to 26Sr RNA PCR partial amplification.

**rDNA isolation and sequencing:**

Partial sequences approximately 800bp of the isolates 26S rDNA were obtained using a strategy based on Boekhout *et al.* (1994). A divergent 5’ domain of the gene was amplified using primers NL 1 (5’-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5’ GGTCCGTGTTCAGCACGG). PCRs were run for 25 cycles: denaturation at 94° C for 1 min, annealing at 50° C for 2 min, and extension at 74° C for 1.5 min. An initial 3 min denaturation at 94° C and a final 5min extension at 74° C were used. Amplified products were isolated with a silica matrix (Gene clean 11 Kit; BiolOI). The purified rDNA genes were sequenced using the external primers NL1 and NL4. Sequencing was performed using dyedideoxy sequencing chemistry. Phylogenetic relationships were determined according to Swofford (1993).

**Phylogenetic analysis:**

The 26S rDNA sequences obtained from PCR partial amplification, after adding to publically available yeast 26SrRNA sequences, were integrated to the data base with the automatic alignment tool. Phylogenetic tree was generated by performing distance matrix analysis using neighbor joining method. The data base was compared with the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/; NCBI, Bethesda, MD, USA) (Altschul *et al.*, 1997; Benson *et al.*, 1999).
**Results And Discussion**

*Isolation and Screening of ethanol tolerant yeast strains:*

Two yeast strains were isolated from two different soil locations. The isolated yeasts were purified by repeated sub-culturing of single colonies on the YEPA medium after incubation at 30°C for 24 h. The isolates were identified based on the colony morphology and microscopic observation. The two isolates exhibited smooth surfaces with circular margins. The colour of the colonies was white creamy. The cells were found to be of various shapes such as round and oval (Fig 1). The isolates were named Can1 and Can2.

![Fig 1: Yeast colonies (A and C) and cells (B and D) of Can1 and Can 2 isolates, respectively](image)

**Table 1:** Carbon source utilized by yeast isolates.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Isolate</th>
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<tbody>
<tr>
<td></td>
<td>Can 1</td>
</tr>
<tr>
<td>Glucose</td>
<td>+++</td>
</tr>
<tr>
<td>Sucrose</td>
<td>++</td>
</tr>
<tr>
<td>Fructose</td>
<td>+++</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>++</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Peptone</td>
<td>+++</td>
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</table>

Growth: + = weak; ++ = moderate; +++ = very good.

**Ethanol tolerant:**

The tolerance levels of the two strains were determined (Table 2). The two strains were able to grow and tolerate up to 12% ethanol. Results showed also that Can 1 strain was more ethanol tolerant than Can 2 where it could grow to 0.032 cell density at OD$_{595}$ compared with to 0.023 cell density of Can 2. Miguel *et al.* (2013) screened several yeast strains for ethanol tolerance, among them the *Candida parapsilosis* could only grow at 4% ethanol. The two isolates in this study therefore represent a distinguished ethanol tolerant strains among *Candida parapsilosis*.

Ethanol has two major effects on yeast; decreases the rates of growth and cell viability.
Table 2: Cell density* of the yeast isolates at various level of ethanol concentrations.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ethanol concentrations</th>
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<tbody>
<tr>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>Can1</td>
<td>1.432</td>
</tr>
<tr>
<td>Can2</td>
<td>1.333</td>
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*Growth was measured by culture absorbance at 595nm

PCR amplification of 26S rDNA:

PCR targeting the 26S rRNA has been used extensively to study eukaryote diversity and allows identification of eukaryotes as well as the prediction of phylogenetic relationships (Pace, 1996, 1997 and 1999). Nucleic acids extracted from yeast colonies were subjected to primer designation to amplify a region of the 26S rRNA gene and the two isolates produced the expected size (ca. 800 bp as shown in Figure (2)). This result does not indicate that the tested yeast belong to specific genus or a particular species, because the primer pair used was a universal yeast primer designed to classify related yeast according to sequencing results (Jennifer et al., 2004).

26S rDNA gene sequence similarity and phylogenetic analysis:

Molecular identification of the isolated strains was carried out based on 26S rDNA sequence analysis. The partial sequence of 26S rRNA obtained from yeast strains were aligned with the available 26S rRNA sequences in Gen Bank data base. The 26S rRNA partial sequence of yeast isolates are presents in Figures (3 and 5). As a result, a phylogenetic tree was mapped using the neighbor joining method, and is shown in figures 4, 5 and 6. Phylogenetic analysis using the 26S rDNA sequences indicated that Can1 and Can2 belonged to the genus Candida and according to blast results, they were identified as Candida parapsilosis strains and named Candida parapsilosis Can 1 and Candida parapsilosis Can 2.

![Fig. 2: PCR product profiles of the two isolated strains](image)

![Fig. 3: Partial 26S ribosomal RNA gene sequence of Candida parapsilosis Can 1 strain.](image)
**Fig. 4:** Phylogenetic tree based on partial 26S rDNA sequence of *Candida parapsilosis* Can 1 strain.

CTCCGTCTTTTCGATGCCACATCCTAGGCCCCGAAGCCGAGTCTCAGTCTAAAGCTGGCAGTATCGACAAAGACTATAACACACTACCGAAGCAGTGCCACATTTCTTTGCACTTATCCTACCGCTCAAACGTATGCTGGCCCGGTAAACTGTAGAGGCCACCCCCGAGAGAGTAACATACAAAATACCAAGTCTGATCTCAAGCCCTTCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAGTGTTTTTTATCTTCACATCTGATCTGCTAGTCTGCTCCTGAGGAAACCTTTACATAGTTCTGGGCACATCTCATCGACGGGATTCCATCCCTGTGACGTTCTGTTCCAAGAAACATAGACGAGCCAGACCCAAGATACCTTCTTCAAATTACACATCGGACACTGAAAGTGCCAGATTTCAAATTTGAGCTTTTGCCGCTTCACTCGCCGCTACTAAGGCAATCCCTGTTGGTTTCTTTTCCTCCGCTATTGGAAAATGCAAATT

**Fig. 5:** Partial 26S ribosomal RNA gene sequence of *Candida parapsilosis* Can 2 strain.

**Fig. 6:** Phylogenetic tree based on partial 26S rDNA sequence of *Candida parapsilosis* Can 2 strain.
The 26S rRNA gene sequence is generally a highly conserved region, particularly in yeasts (Iwen et al., 2002). Thus, it is foreseeable that 26S rRNA (D1/D2) gene sequence may not be an optimal marker for differentiating closely related species.

Since the description of *C. orthopsilosis* and *C. metapsilosis* in 2005 (Tavanti et al., 2005), several methods have been proposed to identify and differentiate these species from *C. parapsilosis* sensu stricto. Tavanti et al. (2005) proposed PCR amplification of the SADH gene followed by restriction digestion with *Bam*I and used amplification fragment length polymorphism (AFLP) for identification and genotyping of about 400 isolates previously classified as *C. parapsilosis*. AFLP was shown to identify *C. orthopsilosis* (Tavanti et al., 2007) and *C. metapsilosis* (Hensgens et al., 2009) to the species level, and to be an efficient genotyping tool as well, delineating intraspecific genetic relatedness. Campa et al. (2008) developed an oligonucleotide microarray based on the arrayed-primer extension technique to simultaneously identify pathogenic fungi. They designed probes complementary to the ITS1 and ITS2 region rapidly and robustly distinguished the three species. Finally, Asadzadeh (2008) reported the correlation of RFLP genotyping with ITS sequence, RAPD and multilocus sequencing for differentiating closely related species.

In conclusion, in this research we isolated and characterized two ethanol tolerant yeast strains which could tolerate up to 12% ethanol. Morphological, biochemical and molecular analysis were used for identification of the two strains and they named them *Candida parapsilosis* Can 1 and *Candida parapsilosis* Can 2.

References


Borman, A.M., C. J. Linton, D. Oliver, M.D. Palmer, A. Szekely, F.C. Odds and E.M. Johnson, 2009. Pyrosequencing analysis of 20 nucleotides of the ITS2 region rapidly and robustly distinguished the three species. Finally, Asadzadeh et al. (2009) introduced primers derived from unique sequences within the ITS1–5.8S rDNA–ITS2 region for the purpose of differentiating the three species. All these methods have the advantages of a sequencing-based approach, but are time consuming and labour intensive, and some are expensive.

In conclusion, in this research we isolated and characterized two ethanol tolerant yeast strains which could tolerate up to 12% ethanol. Morphological, biochemical and molecular analysis were used for identification of the two strains and they named them *Candida parapsilosis* Can 1 and *Candida parapsilosis* Can 2.


