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Morphological and Molecular Tools for Identification of *Saccharomyces Boulardii* Isolated from Active Dry Yeast

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Abstract **– Isolation and identification of Saccharomyces boulardii from a product "High Active Dry Yeast" were the aim of this study. Yeast colonies were isolated and purified thrice by repetitive streaking on the YPD agar medium supplemented with 250 µg/ ml of Chloramphenicol and 30 µg/ml of Ampicillin. The morphological identification was investigated regarding the shapes, color and morphology characteristics. S. boulardii clustered in a circular shape, whitish cream color, elliptical morphology, and formed pseudohyphae. Genomic DNA (gDNA) was extracted from S. boulardii by using the TIANamp Yeast DNA Kit and the universal primers were used for species identification. Identification of yeast genetically processed by using the primer pair NL1 and NL4 to amplify and sequence the 26S rDNA gene D1/ D2 domain. The internal transcribed spacer was amplified and sequenced by using ITS1 and ITS4 for fungus identification, besides using the primer pair of NS1 and NS6. Discrimination of yeast from bacteria processed by using the primer pair 7F, 1540R, and another primer pair 27F, and 1492R. PCR technique could approve that the S. boulardii was the only frequent strain in the active dry yeast. The photographed pattern by UV transilluminator types the target DNA band to S. boulardii that was achieved by amplifying and sequencing the 26S rDNA region. The amplified band (740 bp) and the resulted sequence length of 26S rDNA gene (585 bp) assigned the isolate to S. boulardii.**

Keywords **– Yeast, PCR Technique, 26S rDNA Gene, Morphological Characteristics.**

I. Introduction

Saccharomyces boulardii has been isolated from lychee fruit localized in Indochina in the 1950s, and since became a commercial product in Africa, South America, and Europe [1]. *S. boulardii* is considered as one of the most eukaryotes studies in the field of molecular biology. The taxonomic characterization of *S. boulardii* is controversial, but it was initially typed as a distinct species of the hemiascomycete genus *Saccharomyces* [2]. Literary, the genotyping analysis of *S. boulardii* is argumentative [3], [4]. Other studies have been assigned the *S. boulardii* as a subtype of *S. cerevisiae* [4], [5], [6]. Genetically, amplification of specific sequences via PCR technique for typing of yeast genomes has been used on a large scale. Frequently, proceeding the identification assays mole--cularly for the yeast is depending on the variability of the ribosomal genes 18S and 26S rDNA [7], [8]. These genes have been described by their capability to exhibit the high interspecific variability and the low interspecific poly--morphism [8]. In some of the studies, the Internal transcribed spacer ITS regions were used as tools to discriminate the yeast genealogically from fungus since it is far greater interspecific variations than genes of the 26S and 18S rDNA [7], [9]. Identification of *S. boulardii* in a routine manner, referring to the phenotypic traits in such of sugar assimilation, enzyme activity is not significant tools [2]. However, the phenotypic tools might support the definitive identification [6]. In this study, the molecular tools were set up for *S. boulardii* identification isolated from High Active Dry Yeast. As well, analysis of genomic DNA pattern was applied. The morphological identification was also performed to define the classical characteristics of *S. boulardii*.

II. Materials and Methods

A. *Isolation of Saccharomyces Boulardii*

Isolation technique was implemented with few modifications according to a previous protocol [10]. One gram was picked from a product "High Active Dry Yeast" contained *Saccharomyces boulardii* (Reflor; Biocodex, Cedex, France), and then diluted into 10 ml of saline NaCl (8.5 g L^{-1}). The mixture was inoculated into broth medium of Yeast extract peptone dextrose YPD (5 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose) at concentration of 1:5 (*v/v*), and then incubated at shaker incubator under conditions of 200 rpm/ 37 °C for 24 h. Sequence of the serial dilutions was prepared successively from 10^1 to 10^7, and then 100 µl of each dilution was plated onto the YPD agar medium supplemented with 250 µg/ ml of Chloram--phenicol [11] and 30 µg/ml of Ampicillin [12] to inhibit the potential growth of bacteria, and then the plates were incubated aerobically at 37 ± 1 °C for 24 h. The yeast colonies were purified thrice by repetitive streaking on the same selective medium and picked from plates at the logarithmic growth phase for sub-culturing onto YPD slants that were maintained at 2 – 4 °C for short term storage. The colonies were maintained into YPD broth supplemented with 15% (*v/v*) of glycerol at -80 °C for long term storage.

B. *Morphological Identification Procedure*

Identification of the *S. boulardii* colonies was distingui-

-shed according to a previous protocol [13].

C. *Genomic DNA Extraction*

Genomic DNA (gDNA) was extracted from *S. boulardii* by using the TIANamp Yeast DNA Kit. *S. boulardii* was activated by inoculation a one colony into 10 ml of YPD broth, and then incubated at the shaker incubator under conditions of 200 rpm at 30 °C for 12 h. UV Spectro--photometer was used to detect the transmittance and absorbance of yeast suspension at OD 600 value of 1.0, equivalent to 1-2 × 10^7 colony forming units per mL (CFU mL^{-1}). YPD cultures (1 ml) were centrifuged at 12000 rpm for 1 min/ 20 °C, supernatant was discarded, and then the cell pellet was washed using saline NaCl (8.5 g L^{-1}). Sorbitol buffer (600 µl) was added to the pellet, and then Lyticase was added at an enzyme concentration of 50 U Lyticase per ml^{-1}. Mixture was stirred by using vortex to digest the cell wall, followed by incubation at 30 °C for 30 min, and then centrifuged under conditions of 4000 rpm for 10 min at 20 °C. Supernatant was removed, sediment was supplemented with buffer GA (200 µl), and then stirred for dissolving the protein. Proteinase K solution (20 µl) was added alongside with buffer GB (220 µl), and then mixed well by inversion both downwards and upwards. The mixture was incubated at 70 °C/10 min for degradation the protein wall surrounding the genomic DNA, and then centrifuged under conditions of 12000 rpm for 1 min/ 20 °C to remove the water drops inner wall of the lid. Ethanol (220 µl) was added to the mixture, and then mixed by inversion both downwards and upwards. Approximately, 700 µl of the resulting mixture was transferred to a spin column CB3 (spin column into collection tube). The mixture was centrifuged for 30 sec/12000 rpm, the aqueous phase was discarded, and the spin column CB3 was putted back into the collection tube. Buffer GD (500 µl) was added, mixed by inversion, and then centrifuged for 30 sec/12000 rpm. The aqueous phase was discarded, and the spin column CB3 was putted back into the collection tube. Buffer PW (600 µl) was added, mixed by inversion, and then centrifuged for 30 sec/12000 rpm. The aqueous phase was discarded, and the adsorption spin column CB3 was putted back into the collection tube. Treating of mixture with buffer PW was repeated 9 times, and then centrifuged for 2 min/12000 rpm. The aqueous phase was discarded, and the spin column CB3 was placed at room temperature for 5 min to dispose of remaining buffers in absorbent material entirely. The spin column CB3 was transferred to a clean centrifuge tube, and then added a 50 µl of buffer TE onto the middle of adsorbed film. The spin column CB3/ centrifuge tube was placed at room temperature for 2 min, and then centrifuged under conditions of 12000 rpm for 2 min at 20 °C. The yield of genomic DNA was collected in the centrifuge tube, and then maintained at -20 °C till further analysis.

D. *PCR Amplification and Sequencing Analysis*

Table 1, displays the universal primers used for species identification. The 26S rDNA gene D1/D2 domain was amplified and sequenced for yeast identification [14]. The Internal transcribed spacer ITS was amplified and sequenced for fungus identification [15]. Likewise, 18S rDNA gene was amplified and sequenced for fungus identification. The 16S rDNA gene was amplified and sequenced for bacterial identification.

Polymerase chain reaction PCRs were performed in a volume of 50 µl template DNA (20 ng/ µl), 20 pmol of each primer, 1 µl of deoxynucleoside triphosphate dNTP (2.5 mM, D0056 brand, Sangon Biotech., Shanghai, Co., Ltd., China), and 20 µl of DyNAzyme EXT DNA polymerase in the incubation buffer provided by the enzyme manufacturer. Amplification was circulated by using PCR amplifier (2720 thermal cycler brand, Applied Biosystems, resources) under conditions of 94 °C for 4 min (initial denaturation program), 94 °C for 45 s, 30 cycle of 55 °C for 45 s, and 72 °C for 1 min, followed by 72 °C for 10 min (Repair & extension program). PCR reactions were analyzed by using electrophoresis (DYY-5 brand, Beijing Liuyi Biotech., Co., Ltd., China). Electrophoresis conditions were set up on a 1% (*wt/vol*) of agarose gel, 150 V, 100 Ma, 20 min for observation, detected by ethidium bromide staining, and then photographed using UV transilluminator (FR980 brand, Shanghai Furi Science & Technology Co., Ltd.). Brand sizes were determined by using a standard molecular size marker (500 bp DNA Ladder Mix marker; Cat., No. SM0337, Sangon Biotech., Shanghai, Co., Ltd., China).

Table 1. Universal Primers used for the Molecular Identification.

Type	Name	Sequence 5'→3'	Amplified sequence	PCR length/bp
Bacteria	7F 1540R	CAGAGTTTGATCCTGGCT AGGAGGTGATCCAGCCGCA	16S rDNA	about 1500 bp
	27F 1492R	AGTTTGATCMTGGCTCAG GGTTACCTTGTTACGACTT	16S rDNA	about 1500 bp
Fungus	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Internal transcribed spacer	about 600 bp
	NS1 NS6	GTAGTCATATGCTTGTCTC GCATCACAGACCTGTTATTGCCTC	18S rDNA	about 1300 bp
Yeast	NL1 NL4	GCATATCAATAAGCGGAGGAAAAG GGTCCGTGTTTCAAGACGG	26S rDNA D1/D2 region	about 500 bp

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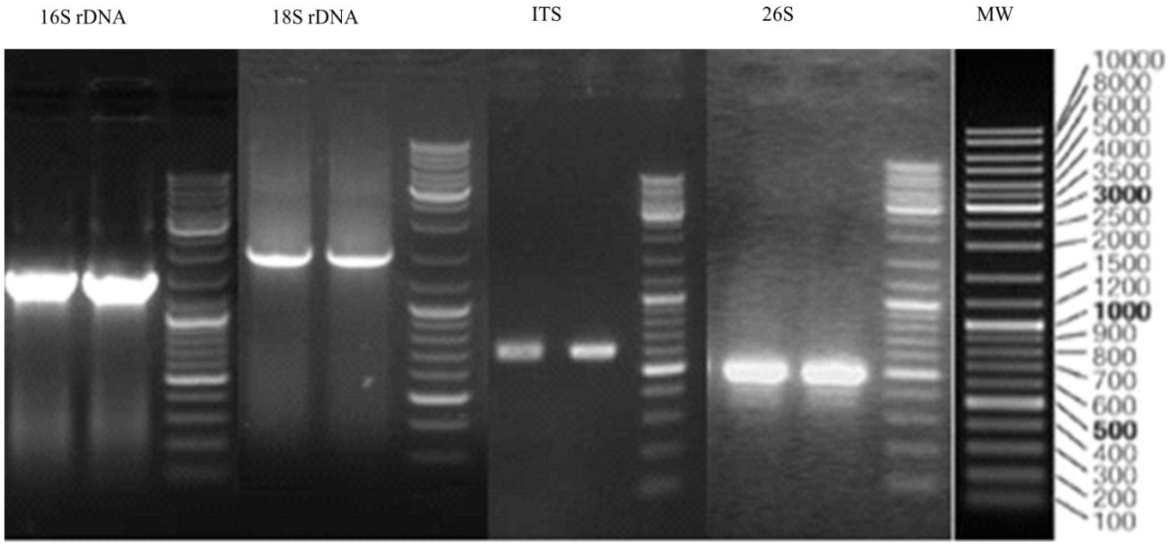

Fig. 2. Electrophoresis, DNA Ladder Mix marker.

III. Results and Discussions

A. *Morphological Assay*

Isolation of *S. boulardii* from probable bacterial contamination was submitted through YPD medium supplemented with Chloramphenicol and Ampicillin. *S. boulardii* is thermophilic yeast and can grow favorably under temperature condition of 37 °C that was examined herein and reported in previous study [5]. *S. boulardii* colonies exhibited biomass formation due to presence of sugar in the YPD medium that is accepted with other finding [12]. Colonies grew invasively into YPD medium that might be attributed to carbon limitation leading to a developmental switch and penetrate the surface of an agar medium [16]. The invasively grow of *S. boulardii* caused also by the absence of NaCl of YPD medium that would exert osmotic stress toward yeast colonies [17] . Discrimination of *S. boulardii* was obvious through its morphological features that assign the colonies to the *S. boulardii*. The data obtained is given in Fig. 1, where the isolated *S. boulardii* could be clustered obviously. Morphologically, *S. boulardii* clustered in a circular shape, whitish cream color, elliptical morphology, and formed pseudohyphae.

The morphological characterizations of *S. boulardii* showed rough and dispersed colonies that are accepted with other reports [16]. *S. boulardii* has been considered as a strain of *S. cerevisiae*, sharing >99% genomic relatedness [18], [19]. Therefore, this result is compatible with findings of another study that stated the criteria of *S. cerevisiae var. boulardii* identification [20]. None of the *S. boulardii* strain formed any spores upon repeated testing at 37 °C, thus it has been considered as non-toxic yeast [21].

B. *Genomic DNA (gDNA) Identification*

Fig. 2 illustrates the different markers used to identify the target DNA band of isolated yeast. The UV transilluminator was photographed the amplified pattern obtained from the genomic DNA of isolated yeast as shown in Fig. 3. The amplified band (740 bp) type the isolated yeast to *S. boulardii* that has been reported in another study [15].

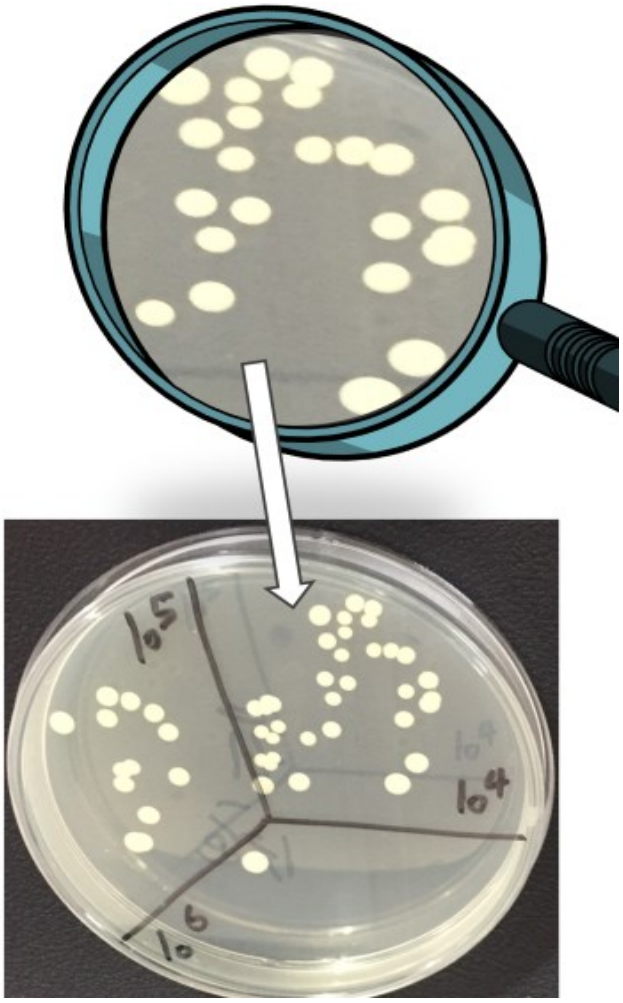

Fig. 1. The morphological features of *S. boulardii* colonies on YPD agar medium.

This might be an effective tool to identify *S. boulardii* belongs to the isolate. The partial sequence of *S. boulardii* isolate was given in Fig. 4. The resulted sequence length of 26S rDNA gene was present by 585 bp and assigned the isolate to *S. boulardii* (see Table 2).

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Table 2. Identities of DNA band obtained from the PCR product electrophoresis gel.

Isolate source	Sequence length[a] (bp)	Closest relative	Identity[b] %	Accession no.
High Active Dry Yeast	585	*S. boulardii*	100	KU059757.1

[a] Size (base pairs bp) of the D1/D2 domains of 26S rDNA genes of the isolate that were amplified with NL1 and NL4 primers.
[b] Identical nucleotides percentage in the resulted sequence of D1/D2 domains of 26S rDNA genes.

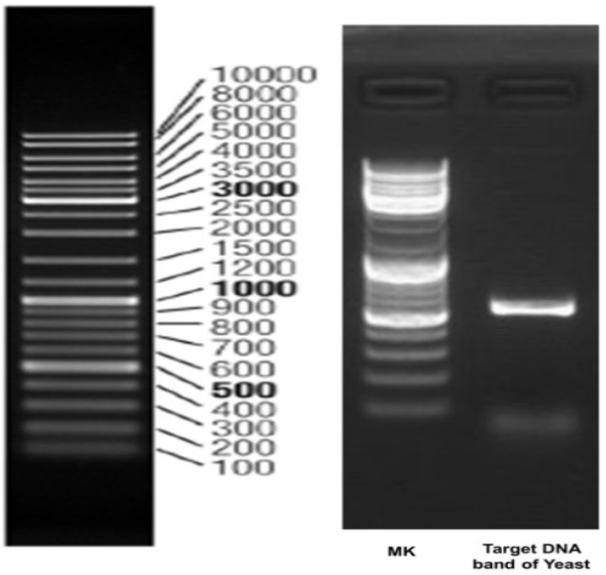

Fig. 3. The electrophoretogram of target DNA band for the isolated yeast.

Identification of 16S rDNA sequence is presented in the ribosome database; this is the link, http:// rdp.cme.msu.edu /index.jsp. 18S rDNA, ITS, and 26S were identified via BLAST searches of the NCBI GenBank data library (http: // www.docin.com/ p-545514843.html). Due to the high interspecific polymorphism that shows by the rDNA regions, several studies reported these regions to distinguish the yeast species [15], [22]. *S. boulardii* represented the yeast community in the used product depending on the genomic DNA sequence. Thereupon, one strain was isolated, namely, *S. boulardii* was detected as the only proportion in the yeast product. Our findings are confirmed by sequencing the 16 rDNA from the isolated yeast via PCR assay. The intergenic regions of the rDNA were used in several studies for yeast identification [23]. The amplified sequence of internal transcribed spacer ITS1 and ITS4 have submitted also in this study to separate the ribosomal gene, thereby achieving the intraspecies variability. Another study confirms our results in sequencing the intergenic regions to type the phylogenetic of eukaryotes in such as *S. boulardii* [24]. The resulted DNA band of isolated yeast approved the identification of the *S. boulardii* based on its capability to control the aneuploidy. In this situation, the chromosome IX trisomy was genetically labeled for *S. boulardii* [25]. The Chromosome IX is described by its aneuploidy that characterizes *S. boulardii* by the sporulation deficiency. In addition, the sequence divergence of gDNA may cause a sporulation deficiency for *S. boulardii*. On the other hand, the genomic DNA of *S. boulardii* distinguishes by genes that associated with stress responses, growth induction, and proteolysis system. Other technique in such of microsatellite DNA polymorphism was suggested to map the gDNA fragments of *S. boulardii*, besides hybridization assay [6], [26]. This study covered the amplification and sequence of the specific rDNA region, discriminating of *S. boulardii* in the yeast community of isolate.

Fig. 4. The partial sequence of gDNA of the isolated yeast.

IV. Conclusions

The molecular technique processed in this study proved the typing of isolated yeast to be *S. boulardii*. Subjecting the *S. boulardii* was recognized through the 26S rDNA D1/D2 region that might be recommended as specific useful tools. *S. boulardii* is one of the most studied eukaryotes in the fields of cell biology. Yeast could invade YPD medium associated with elliptical and pseudohyphal morphology. The budding action associates with reproduction of the *S. boulardii* cells, resulting in rough

colonies. Due to the short generation period, *S. boulardii* cultured easily under conditions of 37 °C in YPD medium. Eventually, using various universal primers to identify *S. boulardii* from bacteria and fungus endorsed the documenting of *S. boulardii* in the community of isolated yeast.

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