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Locally isolated yeasts from Malaysia: Identification, phylogenetic study and characterization

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Yeasts are a convenient platform for many applications. They have been widely used as the expression hosts. There is a need to have a new yeast expression system to contribute the molecular cloning demands. Eight yeast isolates were screened from various environment sources and identified through ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS). Full sequence of the rDNA ITS region for each isolate was BLASTed and phylogenetic study was constructed by using MEGA4. Among the isolates, isolate WB from 'ragi' (used to ferment carbohydrates) could be identified as a new species in order Saccharomycetales according to rDNA ITS region, morphology and biochemical tests. Isolate SO (from spoiled orange), RT (rotten tomato) and RG (different type of 'ragi') were identified as *Pichia* sp. Isolates R1 and R2, S4 and S5 (from the surrounding of a guava tree) were identified as *Issatchenkia* sp. and *Hanseniaspora* sp., respectively. Geneticin, 50 µg/mL, was determined to be the antibiotic marker for all isolates excepted for isolates RT and SO which used 500 µg/mL and 100 µg/mL Zeocin, respectively. Intra-extracellular proteins were screened for lipolytic activity at 30°C and 70°C. Thermostable lipase activity was detected in isolates RT and R1 with 0.6 U/mg and 0.1 U/mg, respectively. In conclusion, a new yeast-vector system for isolate WB can be developed by using phleomycin or geneticin as the drugs resistance marker. Moreover, strains RT and R1 can be investigated as a novel source of a thermostable lipase.

Key words: yeast, phylogenetic study, characterization study

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INTRODUCTION

Yeasts have played important roles in industrial development for thousands of years. Various species of yeast have been used in diverse applications such as in food industries (alcoholic fermentation), expression system, and as models for the study of higher eukaryotes. 'Ragi' is a popular traditional yeast 'cake' used as a starter in making local Malaysian food such as 'tapai' (fermented rice or cassava) and 'tempe' (soybean fermented into a soybean cake). Besides that, it has been used in baking, brewing and ethanol production (Akada, 2002). Yeasts have also been used as one of the most powerful expression system. Furthermore, like bacteria, yeasts are unicellular eukaryote which is simple to cultivate on inexpensive growth media. Unfolded and misfold protein are less risky in yeast system. Yeasts offer various advantages for heterologous genes expression. They can grow to ultrahigh cell densities (Romanos *et al.*, 1992), secrete proteins extracellularly, grow under non-selective conditions, and perform post-translational modifications such as glycosylation, disulphide bond formation, multimeric assembly and endoproteolytic cleavage (Hadfield *et al.*, 1993).

Concerned baker's yeast, *Saccharomyces cerevisiae*, huge numbers of applications and studies have been conducted particularly as a host for heterologous expression of proteins. Proteins expressed by *S. cerevisiae* are safe to humans due to its Generally Recognized as Safe (GRAS) status (Gellissen & Hollenberg, 1997). In recent years, there has been an increasing interest in using other yeast expression systems for protein production. Other widely used expression hosts include *Pichia pastoris* (Shi *et al.*, 2007), *Hansenula polymorpha* (Voronovsky *et al.*, 2009), *Yarrowia lipolytica* (Gellissen *et al.*, 2005) and *Arxula adeninivorans* (Steinborn *et al.*, 2007).

Since there is a lot of a demand for yeasts, identification and characterization of new yeast strain should be conducted to meet those needs. Yeast identification can be conducted using molecular approach and biochemical tests. Earlier classification of yeast had used lower taxonomic level by looking only at sexual state, hyphae and pseudohyphae, sugar fermentation and types of carbon and nitrogen sources compounds (Suh *et al.*, 2006). In the present study, PCR amplification of the ITS1-5.8S-ITS2 regions of the rDNA was applied for identification of locally isolated yeast (Pramateftaki *et al.*, 2000). Phylogenetic study and characterization of the yeast may help in determination of its GRAS status. Eight isolates were further characterized morphologically and biochemically on possible sources of thermostable lipases and hosts for heterologous protein expression.

MATERIALS AND METHODS

Plasmid and strains. pGEM®-T Vector was used to clone the rDNA regions. *Escherichia coli* strain JM109 was used for plasmid propagation and maintenance. The plasmid and the *E. coli* strain were purchased from Promega.

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Abbreviations: rDNA, ribosomal DNA; ITS, internal transcribed spacer; MEGA, molecular evolutionary genetics analysis; GRAS, generally recognized as safe; LB, Luria-Bertani; SDA, Sabouraud dextrose agar; YPD, yeast peptone dextrose; dH_2O, distilled water; BLAST, basic alignment search tools; DSM-DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction.

Media. Luria-Bertani broth (LB) and agar were used to grow the bacterium. Sabouraud dextrose agar (SDA) and yeast peptone dextrose (YPD) agar [1% (w/v) Yeast Extract, 2% Peptone, 2% (w/v) Dextrose, 2% (w/v) Agar) were used for yeast growth.

Isolation and identification of yeast. Thirteen isolates were collected from various locations from Malaysia for yeast isolation. Yeasts were isolated from different types of 'ragi', water, soil, spoiled orange and rotten guava (*Syzygium* sp.). Serial dilution in sterile dH_2O was prepared accordingly for each isolate. Then, 100 μL diluents were spread onto SDA and YPD agar for 2–4 days at 30°C until colonies formed. Oval, elevated, and whitish colonies were examined under light microscope. The colonies in which budding cells were seen were inoculated into 10 mL of YPD. The isolates were incubated at 30°C, 250 rpm, for 24 h prior to DNA extraction by using QIAquick® Genomic Extraction Kit (Qiagen, Germany).

PCR amplification of the ITS1-5.8S-ITS2 regions of the nuclear ribosomal gene complex was conducted for species identification. Primers for Internal Transcribed Spacer (ITS) sequence were synthesized according to fungus universal primer (Pramateftaki *et al*., 2000), Forward ITS1: 5'-GTCTCCGTTGGTGAACCAGC-3', Reverse ITS4: 5'-ATATGCTTAAGTTCAGCGGGT-3'. Amplification processes were carried out in a reaction mixture (100 μL) containing 5 μL (50–100 ng) of DNA template, 3 μL (10 pmol/μL) of each forward and reverse primers, 2 μL of 10 mM dNTP mix, 8 μL of 25 mM $MgCl_2$, 2 μL of 1 U/μL *Taq* DNA polymerase, 10 μL of 10× PCR buffer (MBI Fermentas, USA) and 67 μL of distilled water. The reaction parameters were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 5 min, and a final 5 min extension at 72°C using a gradient thermocycler (Gragdient PCR CGI-96, Rcorbett Research).

The 18S rDNA was amplified by using primers from previous study (Medlin *et al*., 1988); Forward 16S-like rDNA: 5'-AACCTGGGTTGATCCTGCCAGT-3', Reverse 16S-like rDNA: 5'-TGATCCTTCTGCAGGTTCACCTAC-3'. The reaction mixture was set as described previously. The reaction parameters were as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 63°C for 2.5 min, and 72°C for 7 min, and a final 20 min extension at 72°C. The amplicon was purified using GeneAll® Expin™ Combo GP according to the manufacturer's instructions. Then, the amplicon was cloned into pGEMT vector for sequencing. The plasmid was extracted by using GeneAll® Exprep™ Plasmid Quick. The sequencing results were analyzed using Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA4) and Basic Alignment Search Tools (BLAST) (http://www.ncbiblast.com). A cladogram tree for each isolate was constructed by using MEGA4.

Determination of antibiotic resistance. Yeast isolates were streaked on YPD agar and incubated at 30°C for 3 days. Subsequently, single colony was patched on YPD plate supplemented with different types of antibiotics at recommended concentrations. All the antibiotics were purchased from Invivogen with the exception for Zeocin, which was from Invitrogen, USA. The antibiotics and their working concentrations were as follows; Blasticidine-S (5 μg/mL), Phleomycin (10 μg/mL), Zeocin (100 μg/mL), Hygromycin B (50 μg/mL), Geneticin (500 μg/mL), and Puromycin (5 μg/mL). Second screening for antibiotic resistance was carried out using higher concentrations of Blasticidine-S (50 μg/mL), Zeocin (500 μg/mL), Phleomycin (25 μg/mL), and Puromycin (25 μg/mL). Furthermore, lower amount of Hygromycin B (25 μg/mL) and Geneticin (50 μg/mL) was used. These amounts were expected to be sufficient for the selections.

Screening for lipase activity. A single colony was inoculated into 10 mL of YPD broth and incubated at 30°C for 24 h in an incubator shaker of 250 rpm. Then, 1 mL of culture was transferred into 100 mL of YPD broth in 500 mL shake flask. The cells were cultivated for three days under the same conditions. Then, a 20 mL culture was harvested by centrifugation at $3000 \times g$ for 10 min at 4°C. The supernatant was collected and the pellet was resuspended in 10 mL of 50 mM phosphate buffer pH7. The pellet was sonicated at 30 watt with a Branson Sonifier® ultrasonic cell disruptor (DIGITAL Sonifier® UNIT Model S-250D) for 5 min. After breaking the cells, the homogenate was centrifuged at $10\,000 \times g$ for 10 min at 4°C. The clear cell lysate and culture medium were assayed for intracellular and extracellular lipase activity at 30°C and 70°C according to Kwon & Rhee (1986) method. An emulsion of olive oil (Bertoli, Italy) and 50 mM phosphate buffer pH7 (1:1) supplemented with 20 μL of 20 mM $CaCl_2$ was used as substrate. Standard curve of fatty acid released was prepared by using oleic acid. One unit of lipase activity was defined as the amount of activity releasing 1 μmole of fatty acid per minute.

Characterization of yeast WB. Single colony of isolate WB was inoculated in YPD slant agar and incubated at 30°C for 72 h and sent to DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH — the German Resource Centre for Biological Material (DSMZ) for yeast identification.

RESULTS

Identification of yeast species

Yeast isolates were collected from various environmental spots in Malaysia. SDA and YPD agar were used to screen the yeast colonies. The acidic condition with pH5.6 of SDA medium inhibits many species of bacteria, therefore promoting yeast and fungal growth. Selected colonies were observed under light microscope for budding cells, a characteristic of budding yeasts. Out of thirteen isolates, eight showed typically yeast morphology. The eight isolates were subjected to total genomic DNA extraction and the ITS region for each isolates was amplified giving 400–800 bp of PCR product (Fig. 1),

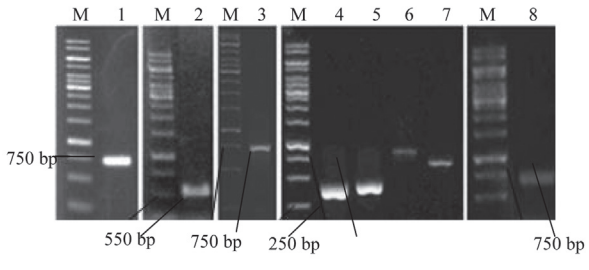

Figure 1. rDNA ITS region of locally isolated yeasts from Malaysia.
The PCR product was electrophoressed on 1% (w/v) agarose gel and stained with GelRed. M: Marker Gene Ruler 1kb DNA ladder; Lane 1: Isolate WB; Lane 2: Isolate SO; Lane 3: Isolate R1; Lane 4: Isolate S4; Lane 5: Isolate S5; Lane 6: Isolate R1; Lane 7: Isolate RT; and Lane 8: Isolate RG.

Table 1. Screening for antibiotic resistance of locally isolated yeasts.
Different concentrations of antibiotics were used. [X] represents no growth and [√] represents growth on antibiotics plates.

Isolate	Blasticidine (μg/mL)		Phleomycin (μg/mL)		Zeocin (μg/mL)		Hygromycin (μg/mL)		Geneticin (μg/mL)		Puromycin (μg/mL)		YPD control
	5	50	10	25	100	500	25	50	50	500	5	25	-
WB	√	√	√	X	√	X	√	X	X	X	√	√	√
R1	√	√	√	√	√	X	X	X	X	X	√	√	√
R2	√	√	√	√	√	√	X	X	X	X	√	√	√
RT	√	√	√	√	√	X	√	√	√	√	√	√	√
SO	√	√	√	√	X	X	X	X	√	X	√	√	√
S4	√	√	X	X	X	X	X	X	X	X	√	√	√
S5	√	√	X	X	X	X	√	√	X	X	√	√	√
RG	√	√	X	X	X	X	√	X	√	X	√	√	√

within the range suggested by Pramateftaki *et al*., (2000). Furthermore, the result shows different sizes of the ITS region which can be for species identification (Korabecna, 2007; Pramateftaki *et al*., 2000).

The amplicons were sequenced and the sequences were analyzed using BLAST at NCBI. Isolate WB shows 93% similarity to *Saccharomycodes sinensis*, isolates S4 and S5 show 96% similarity to *Issatchenkia hanoiensis*. These similarity values are below the threshold for genus and species identification, therefore they could be a new genus or species. Isolates SO, RT and RG were closely related to *Pichia guilliermondii* with 99% similarity. R1 and R2 proved that they were 99% similar to *Hanseniaspora opuntiae* and *Hanseniaspora thailandica*, respectively. R1, R2, S4 and S5 were isolated from the surrounding of a guava tree. Full sequences of the ITS regions of WB, SO, RT, RG, R1, R2, S4 and S5 have been submitted to GeneBank under the following accession numbers: JN084124, JN084128, JQ073892, JN559754, JN084125, JN084126, JN084127, JN559755.

Phylogenetic study for the isolates was conducted and the cladogram tree obtained by using MEGA4 (Tamura *et al*., 2007) is shown in Fig. 2. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 844 positions in the final dataset.

To evaluate the reliability of the inferred tree, the option of doing a bootstrap analysis is allowed. A bootstrap value is attached to each branch, and this value is a measure of confidence in this branch. The maximum value is 100. Figure 2 illustrates that isolates RG, SO and RT gave 100 bootstrap values to *Pichia guilliermondii*. Nevertheless, isolates R1 and R2 the bootstrap value was 98 to *Hanseniaspora* sp. Isolate S4 and S5 also showed 100 bootstrap values to *Issatchenkia hanoiensis*. According to this phylogenetic study, isolate WB was completely separated from other reference genera. The constructed cladogram tree showed that the phylogenetic position of WB sample is closely related to *Sd. sinensis* IFO10111^T with a bootstrap value of 99 for the ITS region therefore it was separated from any of other yeast species (Yamazaki *et al*., 2005). Further identification of isolate WB and characterization for all isolates were performed towards development as potential expression host.

Determination of antibiotic resistance

Screening for antibiotic selection markers for the isolates is shown in Table 1. Blasticidine-S and puromycin could not be used as the drug resistance markers for these particular isolates. Geneticin 50 μg/mL was found to be the antibiotic selection marker for all isolates excepted for isolates RT and SO which used 500 μg/mL and 100 μg/mL Zeocin, respectively. Hygromycin B and

Table 2. Carbon and nitrogen sources utilized by WB strain.
[+] represents growth and [–] represents no growth on tested carbon and nitrogen sources.

Anaerobic:	Glucose	+		
Aerobic :				
	Glucose	+	α-methylglycoside	–
	Galactose	+	Salicin	–
	Sorbose	–	Cellobiose	–
	Rhamnose	–	Maltose	–
	Dulcit	–	Lactose	–
	Inositol	–	Melibiose	–
	Mannitol	–	Sucrose	–
	Sorbitol	–	Trehalose	–
	Glycerol	–	Inulin	–
	Erythritol	–	Melezitose	–
	D-Arabinose	–	Raffinose	–
	L-Arabinose	–	Starch	–
	Ribose	–	Xylitol	–
	D-Xylose	–	Gluconate	–
	L-Xylose	–	2-keto-Gluconate	+
	Adonitol	–	5-keto-Gluconate	–
	Nitrate	–		

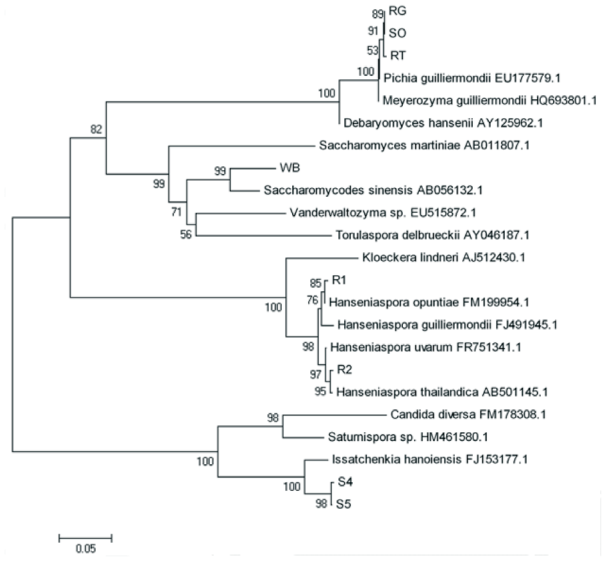

Figure 2. Phylogenetic study of locally isolated yeasts.
The cladogram tree was constructed by using MEGA4. Neighbor-Joining method with 1000 bootstrap value was used to draw the tree. Isolates WB, R1, R2, RT, RG, S4, S5, and SO were located at particular branch.

Geneticin had been used as dominant selectable markers for yeasts and prokaryotes cells. These antibiotics were used to disrupt ribosomal function and cause translational errors (Kaster *et al.*, 1984; Millerioux *et al.*, 2011). Zeocin had been used as dominant selection marker in *Pichia pastoris*. Sunga *et al.* (2008) had used different concentration of Zeocin for screening the hyper-resistance transformants. Isolates S4 and S5 were determined to be in *Issatchenkia* sp., however isolate S5 could be selected by using Hygromycin B. The *Pichia* isolates (RT, SO, and RG) had shown different pattern of selection markers. This result proved that the isolate could be a new species or strain.

Lipase activity of yeast isolates

Lipase assay was performed at two different temperatures (30°C and 70°C) for both extracellular and intracellular proteins. Screening for lipase at 30°C was conducted to identify the host that produced a lipase, whilst 70°C was used as an indicator of a thermostable lipase.

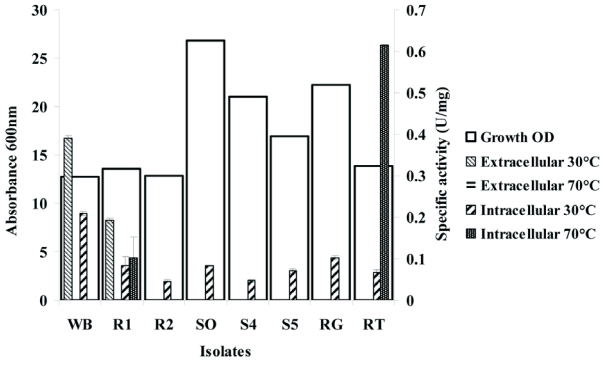

Figure 3. Screening for intra-extracellular lipase activity from local yeasts after 3 days of cultivation.
Lipase assay was performed at 30°C and 70°C. Growth OD was taken with 10× dilution in YPD medium.

As shown, no thermostable extracellular lipase activity was detected in any of the isolates after three days of cultivation (Fig. 3). Low level of intracellular lipase activity (30°C) was found in all isolates. However, isolates R1 and RT produced intracellular thermostable lipase with 0.1 U/mg and 0.6 U/mg, respectively. In this study, we tried to quantify the native lipases produced from local yeasts. *Yarrowia lipolytica* (Vakhlu & Kour, 2006) and *Candida rugosa* (Fadiloglu & Erkmen, 2002) were found to produce their own lipases. *Yarrowia lipolytica* has been used to clone heterologous genes under the control of its strong promoters (Madzak *et al.*, 2004).

Characterization of isolate WB: 18S rDNA identification

From the characterization study, isolate WB was selected for further investigation towards development as an applicable host. Isolate WB was safe to be used in many applications due to its origin from food. It showed no thermostable intracellular or extracellular lipolytic activity. Further identification through 18S rDNA was performed for confirmation. This fragment had been used by Medlin *et al.*, (1988) for 16S-like rDNA identification. The rDNA sequence was analyzed using NCBI BLAST and the result showed that 18S rDNA of yeast WB was 98% similar to *Saccharomyces dairenensis*. Since molecular approaches by using ITS and rDNA for isolate WB were divergent, further characterization study was conducted.

Characterization of isolate WB: Morphology and biochemical test

Morphological and biochemical tests on isolate WB were conducted by sending the isolate to DSMZ. The report shows that the colony morphology appeared as creme-coloured and butyrous on potato dextrose agar. The blastospores were globose-ellipsoidal with a pseudomycelium. True mycelium was totally absent from the isolate. Asci presented with one or two globose ascospores. Isolate WB did not show peculiar bipolar budding like *Saccharomyces* sp.

Effects of carbon and nitrogen sources were investigated (Table 2). Isolate WB showed a weak growth at 37°C and was totally inhibited with N-acetylglucosamine. Different kind of carbon compounds tested revealed that only glucose, galactose and 2-keto gluconate could be used as energy sources. Moreover, only glucose can support the WB growth both aerobic and anaerobically. These result proved that very limited energy sources can be used by the yeast. *Saccharomyces dairenensis* should be able to utilize Trehalose, however this isolate WB was clearly negative with this particular carbon source. According to these findings, DSMZ suggested that the WB species could be *Torulaspora delbrueckii*.

DISCUSSION

Generally, *Saccharomyces* sp. yeast can be found in 'ragi'. 'Ragi' is widely used for preparation of Malaysian food such as 'tapai', 'tempe', and bread. *Issatchenkia hanoiensis* was first discovered from the litchi fruit borer in China (Thanh *et al.*, 2003). Guava (*Syzygium* sp.) is a fruit which provides a sugar rich condition for yeast growth. *Hanseniaspora* sp. mostly can be found in various sources such as human, soil, water, fruits and sea creatures (Garc´ıa-Martos *et al.*, 1999). *Pichia guilliermondii* is known to be the same genus as *Pichia pastoris* and utilizes methanol (a toxic material) as a carbon source. From the identification result, phylogenetic study was constructed to enable

ancestral analysis of the yeast and ensure the GRAS status.

Using assimilation test, isolate WB was found to be from the genus *Torulaspora*. By 18S identification it was identified as *Saccharomyces* sp. Both these genera are grouped in the Saccharomycetaceae family. However, ITS identification proved that it belonged to *Saccharomycodes* sp. within the family of Saccharomycodaceae. Since isolate WB was identified differently from species, genus and family, using the three identification strategies, there is a need to look at the next higher taxonomic rank. However, all strategies point to the Saccharomycetales order. A brief study was reported by Suh *et al.* (2006) regarding phylogenetics of Saccharomycetales order which belonged to ascomycete yeasts. Drug resistance gene has been widely used as a marker for selection of transformants in recombinant DNA technology. It was used in industrial yeast strains because these cells are diploid or aneuploid, lack a sexual cycle, and are prototrophic. Several of drug makers could be used for yeast selections and each of these markers has their specific limitations, in defining the suitable hosts and transformation efficiency (Hashida-Okado *et al.,* 1998).

CONCLUSIONS

Yeast isolates from various sources were identified via rDNA ITS region generating four different yeast genus. Characterization studies were carried out for all the isolates. Drug resistant marker and lipolytic activity were determined for each isolate. Phylogenetic study of the isolates was conducted by using Neighbour-joining method. Since isolate WB showed lower similarity from BLAST result, it was chosen for further identification. ITS, 18S rDNA and biochemical test revealed that isolate WB could be grouped in a new family which belonged to Saccharomycetales order. New yeast expression system for this isolate WB could be developed by using phleomycin or geneticin as selection marker.

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