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Comparison of Methods for Identification of Yeasts Isolated during Spontaneous Fermentation of Freshly Crushed Pineapple Juices

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ABSTRACT

The aim of this study was to evaluate the performances of methods such as sequencing of the internal transcribed spacer (ITS) and 26S (D1/D2) regions of ribosomal DNA, RFLP analysis of the ITS region and commercial biochemical test kit for the identification of the yeasts isolated during spontaneous fermentation of fresh crushed pineapple juice. The experiments were conducted in Thailand and Australia. The yeast isolates in Thailand were identified by sequencing the ITS and 26S (D1/D2) regions of ribosomal DNA and RFLP analysis of the ITS region. The yeast isolates in Australia were identified by sequencing analysis of the two DNA regions and commercial biochemical test kit. The identification results conducted in both countries were relatively similar. Mainly, the yeast isolates could be identified by the use of 26S rDNA in combination with ITS sequencing analysis. In Thailand, approximately 80% of the yeast isolates identified by sequencing analysis of the two regions gave similar identities and included *Rhodotolula mucilaginosa*, *Issatchenkia orientalis*, *Hanseniaspora uvarum*, *Hanseniaspora opuntiae*, *Pichia guilliermondii*, *Aureobasidium pullulans*, *Saccharomycodes ludwigii*, *Candida tropicalis*, *Pichia fermentans*, *Zygosaccharomyces bailii*, *Candida stellata* and *Erythrobasidium hasegawianum*. In Australia, 86% of the yeast isolates gave similar identifications by the sequencing analysis of the two regions and included *P. guilliermondii*, *Pichia membranifaciens*, *P. fermentans*, *H. uvarum*, *H. opuntiae*, *I. orientalis*, *Candida* sp., *Yarrowia lipolytica*, *Tremella globispora*, *R. mucilaginosa* and *A. pullulans*.

Keywords: Biochemical test kit, Fruit juices, ITS-RFLP, rDNA sequencing, Yeast diversity.

INTRODUCTION

The indigenous yeasts have been reported to be wild yeasts producing fermented grape juice of unique flavors and exceptional quality when used for traditional grape juice fermentation (Fleet *et al.*, 2002). To commence the development of fermented pineapple juice making technology, the fundamental knowledge of the indigenous yeast in pineapple juice fermentation requires investigation. These yeasts could be selected and developed as species specific starter cultures for pineapple juice fermentation. However, in studying the indigenous yeasts associated in pineapple ecosystems, the most difficult task is species identification. Identification of yeast isolates is traditionally based on the determination of morphological characteristics as well as physiological and biochemical properties. Consequently, the traditional cultural approach to yeast identification is labor-intensive and time consuming (Giudici and Pulvirenti, 2002). Several alternative

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approaches are now being used for yeast identification instead of conventional methods. Biochemical test kits are widely spread for yeast identification. However, it has been reported that these systems are not appropriate for identification of environmental isolates (Ramani *et al.*, 1998). Hence, molecular methods based on DNA analysis is another approach, which are now popular for identification of yeasts because they could be used to rapidly identify yeasts to genus and species level. The workload is minimal and, usually, reliable data can be obtained within 1-2 days (Kurtzman and Fell, 1998; Barnett *et al.*, 2000; Fernandez-Espinar *et al.*, 2006). Of the various molecular approaches available, RFLP analysis of the ITS region of rDNA, sequencing of the D1/D2 domain of the 26S rDNA and sequencing of the ITS region of rDNA are finding most application (Kurtzman and Robnett, 1998). ITS-RFLP analysis was reported to be the most convenient method for yeast identification because of its speed (10 hr), simplicity, and cheaper cost (Esteve-Zarzoso *et al.*, 1999; Granchi *et al.*, 1999; Chen *et al.*, 2000; Fernandez-Espinar *et al.*, 2000). For DNA sequencing analysis, previous literatures reported that the reproducibility of this method had been found to be very high (Lee *et al.*, 1992; Parker *et al.*, 1995; Demeter *et al.*, 1998).

This study aimed at the comparison of three different methods and a commercial biochemical test kit for identification of yeast species isolated during spontaneous fresh crushed pineapple juice fermentation in Thailand and Australia. The two molecular methods, sequence of ITS and 26S (D1/D2) regions of rDNA were used as the main methods for examination of yeast isolates from both countries to test the reproducibility of the methods. RFLP analysis of the ITS region of rDNA was an additional method used to determine the yeasts isolated in Thailand. The commercial ID 32 C system was used instead of RFLP analysis for examination of yeasts isolated in Australia.

MATERIALS AND METHODS

Isolation of Yeasts from Spontaneous Fermentations

The study of yeasts in spontaneous fermentation of pineapple juice was conducted in Thailand and Australia. The pineapple samples "Smooth cayanne" (*Ananas comosus* (L.) Merr.) were collected from the field cultivated without chemical applications located in Prachuapkirikhun Province and from a market located in Bangkok, Thailand**.** The pineapple samples in Australia were collected from the market located in Kingsford and Cronulla, Sydney, Australia. Whole pineapple fruits at harvesting stage (three fruits in each experiment) were freshly crushed in sterile stomacher bag. Crushed juices (500 ml) were poured and collected in 1,000 ml sterile Erlenmeyer flasks. These steps were handled under aseptic condition. The juices were incubated at $25^{o}C$ for 6 days. Yeasts were routinely isolated everyday during the fermentation. The fermented pineapple juice was serially diluted in 0.1% peptone water. The yeasts in each dilution were isolated and enumerated by spread inoculation of 0.1 ml onto plates of Malt Extract Agar (MEA) (Oxoid, England) and incubation at $25^{o}C$ for 2-4 days. Yeast colonies were counted. Representative colonies of the different yeasts were purified by restreaking on MEA and then maintained and subcultured on the same medium until identification. Yeast isolates from pineapple samples of Thailand were identified by sequencing the internal transcribed spacer (ITS) and 26S (D1/D2) regions of ribosomal DNA and RFLP analysis of the ITS region. The yeast isolates from pineapple samples of Australia were identified by sequencing analysis of the two DNA regions and API ID 32 C system.

DNA Extraction from Yeasts

Yeasts were grown in 5 ml of Malt Extract Broth (MEB) (Oxoid, England), at $25^{o}C$ and

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200 rpm for 24 hours, or other periods as specified. The culture (1.0 ml) was transferred to a 1.5 ml cryogenic tube and centrifuged at 16,000×g for 2 minutes at 4°C to sediment the yeast cells. Cell pellets were stored at –20°C until extraction of DNA for analysis. DNA was extracted from pellets of yeast cells according to procedures described by Cocolin *et al.* (2000). Cells were re-suspended in 200 µl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA) and homogenized with 0.3 g of glass beads (0.5 mm in diameter) in a bead beater at 6000 rpm for 1 min in the presence of 200 µl of phenol/chloroform/isoamyl alcohol (50:48:2). TE buffer (200 µl) (10 mM Tris, 1 mM EDTA, pH 7.6) was mixed with the disrupted cells and the suspension was centrifuged at 16,000×g for 10 minutes at 4°C. The supernatant was collected and 2.5 volumes of absolute ethanol were added to precipitate the DNA, which was precipitated by centrifuging at 16,000×g and 4°C for 10 minutes, washed with 70% ethanol and then re-suspended in 50 µl of TE buffer.

DNA Amplification and Primers

The D1/D2 domain of the 26S rDNA was amplified by PCR with the forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Cocolin *et al.*, 2000). Amplification was done in a standard reaction mixture containing 10 mM Tris HCl (pH 8.3), 20 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 0.2 mM, 1.25 IU of *i-Taq*™ DNA Polymerase (Intron Biotechnology, Korea), each primer at a concentration of 0.2 µM and the DNA template at a final concentration of 10 ng. The PCR reaction was run with an initial step at 95°C for 5 minutes, and 30 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for 2 minutes, extension at 72°C for 2 minutes, with a final extension at 72°C for 7 minutes.

The ITS region of yeast rDNA was amplified by PCR with the forward primer ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GE-3') described by White *et al.* (1990). The conditions of this reaction were the same as previously described, except that the concentration of $MgCl_2$ was increased to 2.25 mM. The PCR reactions were run with the initial step of 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 2 minutes, annealing at 56°C for 2 minutes, extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes (Esteve-Zarzoso *et al.*, 1999). Reagents for the PCR were *i-Taq*™ DNA Polymerase (Intron Biotechnology, Korea), and the primers were obtained from Bio Basic Inc. (Canada). The reaction was conducted in a DNA Engine (MJ research PTC–200, Korea). In Australia, reagents for the PCR were ABI Amplitaq Gold Polymerase (Applied Biosystems, AUS.), and the primers obtained from Sigma Genosys (Sigma-Aldrich, USA.). The reaction was conducted in GeneAmp (Applied Biosystems, AUS.).

ITS-RFLP Analysis

The PCR products of the ITS region of yeast rDNA (5 µl) amplified as previously described were digested for 90 minutes at 37°C with 5 U of restriction enzyme in 15 µ l reaction volumes, using the manufacturer's instructions and conditions. The restriction enzymes used were *Cfo* I (Promega, USA.), *Hae* III (Toyobo, Japan) and *Hinf* I (Toyabo, Japan). RFLP products were analyzed by horizontal agarose gel 2.5% (w/v) electrophoresis, using a 100 bp EZ Load™ Molecular Rulers (Bio-Rad, USA.) as the size standard. Electrophoresis was performed with an Electrophoresis Power Supply EPS 301 (Amersham Pharmacia Biotech, Sweden) at 110V for 1 hour. After electrophoresis, the gels were stained with ethidium bromide and photographed under transluminated UV light. The RFLP profiles were scanned and the size

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of restriction fragments was measured using a computer program (Carnoy 2.0, Lab of Plant Systematics, Belgium). The restriction fragment profiles were compared with the data of Guillamón *et al.* (1998), Esteve-Zarzoso *et al.* (1999), Granchi *et al.* (1999), Sabate *et al.* (2002) and Heras-Vazquez *et al.* (2003).

Sequencing Analysis and Yeast Identification

The PCR products from amplification of 26S rDNA and ITS of yeast isolates from Thailand were sent to a commercial sequencing facility (Macrogen, Souel, Korea). In Australia, The PCR products from amplification of 26S rDNA and ITS were cleaned with QIAquick PCR Purification Kit (Qiagen, USA.) and, then, labeled with dye terminators using the ABI Big Dye terminator Version 3.1 Kits (Applied Biosystems, AUS.). The labeled PCR products were purified by ethanol precipitation and sent to a commercial sequencing facility (The Ramaciotti Centre, UNSW, AUS). DNA base sequences were analyzed by comparison with the GenBank databases of the National Center for Biotechnology Information (NCBI). Searches in GenBank with Blast program were performed to determine the closest known relative of partial 26S rDNA and ITS sequences (Altschul *et al.*, 1997).

Biochemical Test Kit

Yeasts were grown in 5 ml of Malt Extract Broth (Oxoid, Basingstoke, England) at 25°C and 200 rpm for 24 hours. The yeast inoculums were subjected to API ID 32 C system (Biomerieux, France) following the manufacturer's instructions. The yeast isolates were identified by the API ID 32 C Analytical Profile Index (Ramani *et al.*, 1998).

RESULTS

In this study, yeasts associated with spontaneous fermentation of freshly crushed pineapple juices were isolated. Total yeast isolates from the fermentation systems were primarily screened and grouped based on their colony morphological properties. Then, 20% of total yeast isolates of each group was selected as representative and identified.

Identification of Yeast Isolates from Thailand by Three Molecular Methods

Based on morphological identification, 36 yeast isolates were obtained and identified by three molecular analyses. The results are shown in Table 1. Colony isolates that were mucoid to butyrous and had pink to red color (Profile 1, Table 1) were identified by sequencing of D1/D2 region of 26S rDNA. The sequence gave 100% identity with the database sequence of *R. mucilaginosa*. The DNA sequence of this yeast also gave 97% identity with *R. mucilaginosa* when identified by sequence of the ITS region. For the RFLP analysis, DNA pattern of this yeast was not identical to any previous RFLP profiles reported. The other yeast isolates that gave the same identification by the sequencing of two regions were *I. orientalis*, *H. uvarum*, *H. opuntiae*, *C. tropicalis*, *P. fermentans*, *Z. bailii*, *C. stellata* and *E. hasegawianum* (Profiles 3 and 4, 5, 6, 10, 11, 12, 13, and 14, respectively, Table 1). When identified by RFLP, these isolates did not correspond to both sequencing analyses. Colony isolates that were fluid, mucoid to butyrous, and had pink to red color gave a 26S rDNA sequence that did not match with any database sequences. However, their ITS sequence gave 99% identity with *Rhodosporidium toluloides* (anamorph *Rhodotorula glutinis*) (Profile 2, Table 1). For RFLP analysis, its RFLP profile matched with the database profile of *R. glutinis*. Colony isolates that were butyrous and had white to cream color gave a sequence of 26S rDNA, with 100%

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Table 1. Identification of yeasts isolated from spontaneous fermentation of freshly crushed pineapple juice in Thailand.

Profile	Sequencing of D1/D2 region of 26S rDNA	Accession number (NCBI)	Percent identity	Sequencing of ITS region	Accession number (NCBI)	Percent identity	Restriction fragments results[a]			Closest relative of Restriction fragments	No. of isolates
							Cfo I	*Hae* III	*Hinf* I		
1	*R. mucilaginosa*	DQ132885	100	*R. mucilaginosa*	AF444614	97	320+240	425+215	340+225	No matching	1
2	Unidentified			*Rh. toluloides*	AB073266	99	300+230+100	450+230+150	180+150	*R. glutinis*	1
3	*I. orientalis*	DQ466533	100	*I. orientalis*	AY939808	98	200 +180+50	400	250+150	*Candida intermedia var intermedia*	1
4	*I. orientalis*	AY601160	98	*I. orientalis*	AY939796	85	230	400+200+ 150+100	270+200+150	*Candida montana*	2
5	*H. uvarum*	AY305681	100	*H. uvarum*	AM160628	100	720	580	450+320	*H. uvarum*	3
6	*H. opuntiae*	DQ872866	100	*H. opuntiae*	DQ872855	100	320	850	400+190	No matching	3
7	*C. fermentati*	AY187283	100	*P. guilliermondii*	DQ088676	100	320+290	400	450+380	*P. guilliermondii*	7
8	*A. pullulans*	AB304735	98	*A. pullulans*	EF197817	98	200+100	450+150	300+200	*A. pullulans*	3
9	*S'codes ludwigii*	U73601	98	*S'codes ludwigii*	AB056133	87	350	750	450+280+150	*S'codes ludwigii*	1
10	*C. tropicalis*	AY601158	100	*C. tropicalis*	AY939810	96	270	450	300	*Candida albican*	2
11	*P. fermentans*	EF554827	99	*P. fermentans*	AF411062	99	200+100	320+100	250+230+70	*Candida sorbata*	1
12	*Z. bailii*	U72161	99	*Z. bailii*	X84640	99	320+300+280	750	400+250+180	*H. guilliermondii*	2
13	*C. stellata*	AJ966340	98	*C. stellata*	AY160766	97	230	500+250	250+250	No matching	1
14	*E. hasegawianum*	AY560616	99	*E. hasegawianum*	AF444522	90	680	650+200	450+350	No matching	1
15	*C. nivariensis*	AY627307	99	*Candida sp.*	AY787833	88	350	600	450+300	*Zygosaccharo-myces mellis*	5
16	Unidentified			*Cryptococcus* sp.	AF444396	98	250	270+100	250+230	No matching	2

[a] Values refer to the number of base pairs per fragment.

identity with the database sequence of *Candida fermentati* (synonyms *Candida guilliermondii*; anamorph *P. guilliermondii*) (Profile 7, Table 1), which was identical to the ITS sequencing analysis (100% identity with *P. guilliermondii*). The RFLP profile of this isolate also matched with the database profile of *P. guilliermondii*. The yeast isolates that had similar identification by three methods were *A. pullulans* and *S'codes ludwigii*. (Profiles 8 and 9, respectively, Table 1). Two yeast isolates, with colonies that were butyrous and white to cream color, gave ITS sequences with 98% identity to *Cryptococcus* sp., but their 26S rDNA sequence did not match with 26S rDNA database sequences. Their RFLP profile also did not match any profile reported.

Identification of Yeast Isolates from Australia by Two Molecular Methods and Biochemical Test Kit

Through morphological identification, 21 yeast isolates in Australia were selected and identified by D1/D2 and ITS analysis and API ID 32 C system. The results are shown in Table 2. Colony isolates that were butyrous and had white to cream color, when sequenced by the D1/D2 region of 26S rDNA, gave 99% identity with the database sequence of *P. guilliermondii* (Profiles 1 and 2, Table 2). Its ITS sequence also gave 97% identity with *P. guilliermondii*. By the ID 32 C system, it gave 88.3% identity with *Candida famata* (Profile 1, Table 2). The yeast isolates identified by two sequencing analyses giving identical results were *P. fermentans*, *I. orientalis*, *Candida* sp., *Y. lipolytica*, *T. globispora*, *R. mucilaginosa* and *A. pullulans* (Profiles 5 and 6, 13, 14-15, 16, 17, 18, and 19, respectively, Table 2). The isolates with butyrous, white to cream color colonies, when identified by sequencing of 26S rDNA, gave 99% identity with the database sequence of *P. guilliermondii* (Profile 2, Table 2). This isolate also gave 97% identity with *P. guilliermondii* when identified by the ITS sequencing. For the ID 32 C system, it gave 89.4% identity with *C. guilliermondii* (anamorph *P. guilliermondii*), which corresponded to the identification result of both sequencing analysis. Yeast isolates that gave similar identities by two molecular methods and ID 32 C system were *P. membranifaciens* (anamorph *Candida valida*), *H. uvarum* (anamorph *Kloeckera apiculata*) and *I. orientalis* (anamorph *Candida krusei*) (Profiles 3 and 4, 8 to 11, and 12, respectively, Table 2). Two yeast isolates with butyrous and white to cream color colonies gave identification results totally different by the three methods. By ITS sequencing analysis, they gave 92-98% identity to *Saccharomycetales* sp., whereas 26S rDNA sequencing gave 97-98% identity to *Candida edaphicus*. Their biochemical test gave 94.8% identity to *Geotrichum capitatum* and 99.2% identity to *Candida glabata* (Profiles 20 and 21, respectively, Table 2).

DISCUSSION

From the results of the identification of yeast isolates from Thailand by three molecular methods, it was found that 14% of 36 yeast isolates could be identified by all the three methods, giving similar identification results. These were *H. uvarum, P. guilliermondii, A. pullulans,* and *S'codes ludwigii*. Seventy-eight percent of all isolates gave similar identifications by sequencing analysis of two DNA regions. These were *R. mucilaginosa*, *I. orientalis*, *H. uvarum*, *H. opuntiae*, *P. guilliermondii*, *A. pullulans*, *S'codes ludwigii*, *C. tropicalis*, *P. fermentans*, *Z. bailii*, *C. stellata* and *E. hasegawianum*. Three percent of yeast isolates identified by ITS sequencing and RFLP analysis gave similar identification results, including *Rh. toluloides* (anamorph *R. glutinis*). However, fourteen percent of yeast isolates gave totally different identification results by the three methods. Based on the percent of identification results mentioned above, identification of yeast

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Table 2. Identification of yeasts isolated from spontaneous fermentation of freshly crushed pineapple juice in Australia.

Profile	Sequencing of D1/D2 region of 26S rDNA	Accession number (NCBI)	Percent identity	Sequencing of ITS region	Accession number (NCBI)	Percent identity	API ID 32 C	Percent identity	No. of isolates
1	*P. guilliermondii*	EU833236	99	*P. guilliermondii*	EU568993	97	*C. famata*	88.3	1
2	*P. guilliermondii*	AB260128	99	*P. guilliermondii*	EU568969	97	*C. guilliermondii*	89.4	1
3	*P. membranifaciens*	AY529509	99	*P. membranifaciens*	AB193175	98	*C. valida*	99.7	1
4	*P. membranifaciens*	AY529507	98	*P. membranifaciens*	AF411062	96	*C. valida*	99.7	1
5	*P. fermentans*	EF554827	99	*P. fermentans*	EF061132	92	*Candida rugosa*	99.8	1
6	*P. fermentans*	EF554827	99	*P. fermentans*	FJ231463	90	*C. krusei*	97.8	1
7	*P. fermentans*	EF554827	98	*Pichia* sp.	AB286072	85	*Cryptococcus humicola*	99.2	1
8	*H. uvarum*	EU359819	100	*H. uvarum*	DQ872856	94	*K. apis/apiculata*	99.9	1
9	*H. uvarum*	EF139133	98	*H. opuntiae*	DQ872855	95	*K. apis/apiculata*	99.9	1
10	*H. uvarum*	EF139133	100	*H. opuntiae*	DQ872855	94	*K. apis/apiculata*	99.9	1
11	*H. uvarum*	EF139133	98	*H. opuntiae*	DQ872855	98	*K. apis/apiculata*	99.9	1
12	*I. orientalis*	FJ515259	98	*I. orientalis*	EU315767	95	*C. krusei*	87.9	1
13	*I. orientalis*	AY305675	98	*I. orientalis*	AB467299	95	*Cr. humicola*	99.2	1
14	*Candida* sp.	EF460674	100	*Candida* sp.	EF612207	91	*Cr. humicola*	86.6	1
15	*Candida* sp.	EF460674	99	*Candida* sp.	EF612207	84	*C. rugosa*	99.9	1
16	*Y. lipolytica*	EU327113	98	*Y. lipolytica*	FJ515197	98	*Cr. humicola*	86.6	1
17	*Tr. globispora*	EF551317	99	*Tr. globispora*	AF444432	90	*C. guilliermondii*	91.0	1
18	*R. mucilaginosa*	FJ515267	100	*R. mucilaginosa*	AB193175	98	*Cr. humicola*	98.9	1
19	*A. pullulans*	FJ515253	98	*A. pullulans*	FJ515198	98	*C. famata*	-[a]	1
20	*C. edaphicus*	AB247371	98	*Saccharomycetales* sp.	AF411062	92	*G. capitatum*	94.8	1
21	*C. edaphicus*	AB247371	97	*Saccharomycetales* sp.	EF060939	98	*C. glabrata*	99.2	1

[a] No percent identity.

isolates was best achieved by D1/D2 sequencing analysis in combination with ITS sequencing analysis. This could be a potential methodology for identification of yeast isolates from ecosystem of the spontaneous fermentation. When the yeast isolates were identified by RFLP analysis, identification results frequently differed from those obtained by sequencing analysis and many results also did not match the database. It seems that the RFLP had no potential for identification of these unknown yeasts due to the lack of diversity of information in the ITS-RFLP databases. These limitations have been stated by Esteve-Zarzoso *et al.* (1999), Heras-Vazquez *et al.* (2003) and Clemente-Jimenez *et al.* (2004), and should be resolved as the profiles of more yeast are determined and information is deposited in the databases.

Because of the limitations of the RFLP analysis mentioned above, the ID 32 C system was used instead of RFLP analysis to identify the yeast isolates in Australia. Based on the comparison of sequencing analysis of the two DNA regions and ID 32 C system, 14% out of the 21 yeast isolates gave similar identities by all the three methods. These were yeasts *P. membranifaciens* (anamorph *C. valida*), *H. uvarum* (anamorph *K. apiculata*) (Profile 8) and *I. orientalis* (anamorph *C. krusei*). Eighty-six percent of all isolates identified by sequencing analysis of the two DNA regions displayed similar identification results, including *P. guilliermondii*, *P. fermentans*, *H. uvarum* (Profile 8), *I. orientalis*, *Candida* sp., *Y. lipolytica*, *T. globispora*, *R. mucilaginosa* and *A. pullulans*. However, fourteen percent of yeast isolates identified by the three methods gave totally different identification results. These observations were also consistent with the results of yeast isolates from Thailand by the three molecular methods, which confirmed that the use of 26S rDNA in combination with ITS sequencing analysis could be a proper methodology for identification of yeast isolated from naturally fermented juice ecosystems. By ID 32 C system, yeast identification gave data that was frequently different from the two sequencing methods. As found in this study, two isolates of Profiles 1 and 2 (Table 2) identified by two sequences gave similar identity corresponding to *P. guilliermondii*. When these yeasts were identified by ID 32 C system, the identity of the Profile 2 was similar to the sequencing analysis methods of the two regions, but Profile 1 did not give consistent results with Profile 2. It could be due to the error of interpretation of the growth in the ID 32 C wells. Partial or weak growth may result in an alternative profile. As mentioned in the introduction, biochemical test kits are widely adopted for rapid and inexpensive yeast identification, particularly in commercial environments (food industry, pharmacy, patient isolates), where mainly genus is important to take the proper precautions against contaminants. However, it has been reported that these systems are not appropriate for identification of environmental isolates. In addition, it has been reported that the identity result in terms of % identity system varied depending upon the geographic origin of the yeast isolates (Ramani *et al.*, 1998). Consequently; the kit might not be suitable for the identification of unknown yeast isolates from environmental isolates like spontaneous fermentation.

Based on identification results of yeast isolates from both countries, more than 75% of the yeast isolates were identified by using 26S rDNA in combination with ITS sequencing analysis giving similar identities. The yeast isolates which could be identified by sequencing analysis of two regions were *R. mucilaginosa*, *I. orientalis*, *H. uvarum*, *H. opuntiae*, *C. tropicalis*, *P. fermentans*, *Z. bailii*, *C. stellata, E. hasegawianum, P. guilliermondii*, *I. orientalis*, *Candida* sp., *Y. lipolytica*, *T. globispora*, *R. mucilaginosa*, *S'codes ludwigii,* and *A. pullulans*. With the exception of some yeast isolates from Australia, the sequences of isolates in the group of *H. uvarum* and *H. opuntiae,* when identified by 26S rDNA analysis, gave 98-100% identity with *H. uvarum* (Profiles 8 to

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11, Table 2). When identified by ITS sequencing analysis, their sequences gave 94-98% identity with *H. opuntiae* (Profiles 9 to 11, Table 2). These identification results were relatively different from the identification conducted in Thailand. According to the results in Table 1, when six isolates (Profiles 5 and 6) with morphological characteristics similar to *H. uvarum* were examined, it was found that DNA sequence of both regions of three isolates (Profile 5) gave 100% identity with *H. uvarum* and their RFLP profiles were also identical to *H. uvarum.* However, in the case of the other three isolates (Profile 6), the DNA sequences of both ITS and 26S rDNA regions gave 100% identity with *H. opuntiae* and their RFLP profile did not match to any profiles reported. There are some reports about the identification of these two yeasts, demonstrating that the sequencing of the ITS region could be an accurate identification of *H. opuntiae* and could have sufficient potential for distinguishing of *H. opuntiae* from *H. uvarum* (Cadez *et al.*, 2003). In addition, the report of Cadez *et al.* (2003) also mentioned that RFLP analysis of ITS region could be an alternative rapid method for distinguishing *H. opuntiae* form the other close species. However, the RFLP profile of ITS region of *H. opuntiae* with three endonucleases (Cfo I, Hae III and Hinf I) has not been reported in any previous research. Therefore, RFLP profile of *H. opuntiae* (Profile 6, Table 1) reported in the present study could be used as database for further research. According to the results obtained from this study, to identify *H. opuntiae* and *H. uvarum,* the sequencing of the ITS region in combination with RFLP analysis could be a more appropriate and reliable methodology.

Through these identification methodologies as evaluated in this study, yeasts associated with spontaneous fermentation of freshly crushed pineapple juices could be identified and new knowledge has been added to the field. It was found that the main yeast species associated with spontaneous fermentation from both countries were noticeably similar. *H. uvarum* and *P. guilliermondii* were the main species similarly isolated from the natural fermentation systems of freshly crushed pineapple juice in all samples from Thailand and Australia. Their populations increased from the initial approximately 5 to 8 log CFU ml^{-1} through to the end of fermentation. Ethanol generated in the system of these natural fermentations was varied between 1-4 % (v/v) (data not shown). The other yeast species found in spontaneously fermented pineapple juices in a few occasions were *I. orientalis*, *Candida* sp., *Y. lipolytica*, *T. globispora*, *Z. bailii*, *P. fermentans*, *C. tropicalis*, *C. stellata*, *E. hasegawianum* and *S'codes ludwigii*. Interestingly, *Saccharomyces* yeasts, in particular *Saccharomyces cerevisiae* which is a significant yeast normally associated with the spontaneous fermentation, were not observed in this study. This observation is relatively different form the other reports. Many reports have demonstrated that, in the spontaneous fermentation systems of the fruit juices, the yeast species and strain present in these systems were normally diverse. In the spontaneous system of grape juices, the diverse species of *Hanseniaspora*, *Pichia*, *Saccharomyces,* and many other non-*Saccharomyces* yeasts are found in spontaneous fermentation (Heard and Fleet, 1985; Pretorius, 2000; Fleet *et al.,* 2002, and Fleet, 2003). A similar community of yeasts associated with spontaneous fermentation of cider was also reported (Morrissey *et al.*, 2004; Coton *et al.*, 2006). Interestingly, in the spontaneously fermenting oranges and orange juice, the acidic profile of the juice was similar to pineapple juice (Singleton and Gortner, 1965; Li-ying *et al.*, 2008) and the large number of *S. cerevisiae* and non-*Saccharomyces* species, such as *H. uvarum*, *C. tropicalis*, *Clavispora lusitaniae* and *Trichosporon asahii,* were presented as the main species (Heras-Vazquez *et al.*, 2003). However, in the spontaneous fermentation of freshly crushed pineapple juice, only two main yeasts were found: *H. uvarum* and *P.*

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guilliermondii. In addition, the results obtained from the study conducted in Thailand and Australia revealed that climatic or regional factor were not associated with the occurrences of *H. uvarum* and *P. guilliermondii* in the spontaneous pineapple juice fermentations. Thus, from a microbiological perspective, only broad conclusions can be drawn about influences of freshly crushed pineapple juice properties. This is an interesting observation that requires further investigation to determine the factor associated with the presence of these main yeasts in the spontaneous pineapple juice fermentation. Besides, more detailed, systematic investigation of yeasts associated with pineapple fruit ecology and spontaneous pineapple juices fermentation are needed to resolve some inconsistent information in the literature.

CONCLUSIONS

Molecular methods could facilitate identification of yeasts isolates. However, since there is insufficient information in databases of biochemical test, RFLP and even sequence to rely on any one method for the identification of yeast isolates, at least two independent approaches should be used. In this study, the sequencing analysis of two regions (ITS and 26S rDNA) were robust and gave reproducible data. Therefore, the use of 26S rDNA in combination with ITS sequencing analysis could be used as the main methods for the identification of yeast isolates during spontaneous fermentation. Also, this methodology could be potentially adopted for species identification of yeast isolates from other natural ecosystems.

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REFERENCES

1. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Res.*, **25:** 3389-3402.
2. Barnett, J.A., Payne, R.W. and Yarrow, D. 2000. *Yeasts: Characteristics and Identification*. 3rd Edition. Cambridge University Press, Cambridge, PP. 23-38.
3. Cadez, N., Poot, G. A., Raspor, P. and Smith, M. Th. 2003. *Hanseniaspora meyeri* sp. nov., *Hanseniaspora clermontiae* sp. nov., *Hanseniaspora lachancei* sp. nov. and *Hanseniaspora opuntiae* sp. nov., Novel Apiculate Yeast Species. *Int. J. Syst. Evol. Microbiol.*, **53:** 1671–1680.
4. Chen, C., Eisner, J. D., Kattar, M., Rassoulian-Barrett, L., Lafe, K., Yarfitz, S. L., Limaye, A. P. and Cookson, B. T. 2000. Identification of Medically Important Yeasts Using PCR-based Detection of DNA Sequenced Polymorphisms in the Internal Transcribed Spacer 2 Region of the rRNA Genes. *J. Clin. Microbiol.*, **38:** 2302-2310.
5. Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martinez-Rodriquez, S., Heras-Vazquez, F. J. L. and Rodriguez-Vico, F. 2004. Molecular Characterization and Oenological Properties of Wine Yeasts Isolated during Spontaneous Fermentation of Six Varieties of Grape Must. *Food Microbiol.*, **21:** 149-155.
6. Cocolin, L., Bisson, D. A. and Mills, D. A. 2000. Direct Profiling of the Dynamics in Wine Fermentations. *FEMS Microbiol. Lett.*, **189:** 81-87.
7. Coton, E., Coton, M., Levert, D., Casaregola, S. and Sohier, D. 2006. Yeast

[Downloaded from jast.modares.ac.ir on 2024-04-20]
[DOR: 20.1001.1.16807073.2013.15.7.10.9]

Ecology in French Cider and Black Olive Natural Fermentations. *Int. J. Food Microbiol.*, **108:** 130-135.

8. Demeter, L., D'Aquila, R., Weislow, O., Lorenzo, E., Erice, A., Fitzgibbon, J., Shafer, R., Richman, D., Howard, T. M., Zhao, Y., Fisher, E., Huang, D., Mayers, D., Sylvester, S., Arens, M., Sannerud, K., Rasheed, S., Johnson, V., Kuritzkes, D., Reichelderfer, P. and Japour, A. 1998. Interlaboratory Concordance of DNA Sequence Analysis to Detect Reverse Transcriptase Mutations in *HIV-1* Proviral DNA. *J. Virol. Methods*, **75:** 93-104.
9. Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. 1999. Identification of Yeasts by RFLP analysis of the 5.8S rRNA Gene and the Two Ribosomal Internal Transcribed Spacers. *Int. J. Syst. Bacteriol.*, **49:** 329-337.
10. Fernandez-Espinar, M. T., Esteve-Zarzoso, B., Querol, A. and Barrio, E. 2000. RFLP Analysis of the Ribosomal Internal Transcribed Spacers and the 5·8S rRNA Gene Region of the Genus *Saccharomyces*: A Fast Method for Species Identification and the Differentiation of Flor Yeasts. *Antonie van Leeuwenhoek*, **78:** 87-97.
11. Fernandez-Espinar, M. T., Martorell, R., De Llanos, R. and Querol, A. 2006. Molecular Methods to Identify and Characterize Yeasts in Foods and Beverages. In: *"Yeasts in Foods and Beverages*", (Eds.): Querol, A. and Fleet, G. H.. Springer, Berlin, PP. 55-82.
12. Fleet, G. H. 2003. Yeast Interactions and Wine Flavour. *Int. J. Food Microbiol.*, **86:** 11 - 22.
13. Fleet, G. H., Prakitchaiwattana, C., Beh, A. L. and Heard, G. 2002. The Yeast Ecology in Wine Grapes. In: *"Biodiversity and Biotechnology of Wine Yeasts*", (Ed.): Ciani, M.. Research Signpost, Kerala, PP. 1-17.
14. Granchi, L., Bosco, M. and Vicenzini, M. 1999. Rapid Detection and Quantification of Yeast Species during Spontaneous Wine Fermentation by PCR-RFLP Analysis of the rDNA ITS Region. *J. Appl. Microbiol.*, **87:** 949-956.
15. Guillamón, J. M., Sabaté, J., Barrio, E., Cano, J. and Querol, A. 1998. Rapid Identification of Wine Yeasts Species Based on RFLP Analysis of the Ribosomal Internal Transcribed Spacer (ITS) Region. *Arch. Microbiol.*, **169:** 387-392.
16. Giudici, P. and Pulvirenti, A. 2002. Molecular Methods for Identification of Wine Yeasts. *In: "Biodiversity and Biotechnology of Wine Yeasts*", (Ed.): Ciani, M.. Research Signpost, Kerala, PP. 35-52.
17. Heard, G. M. and Fleet, G. H. 1985. Growth of Natural Yeast Flora during the Fermentation of Inoculated Wines. *Appl. Environ. Microbiol.*, **50:** 727-728.
18. Heras-Vázquez, F. J., Mingorance-Cazorla, L., Clemente-Jiménez, J. M. and Rodríguez-Vico, F. 2003. Identification of Yeast Species from Orange Fruit and Juice by RFLP and Sequence Analysis of the 5.8S rRNA Gene and Internal Transcribed Spacers. *FEMS Yeast Res.*, **3:** 3-9.
19. Kurtzman, C. P. and Fell, J. W. 1998. *The Yeasts: A Taxonomic Study*. 4th Edition, Elsevier Science B.V., Amsterdam, PP. 77-100.
20. Kurtzman, C. P. and Robnett, C. J. 1998. Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences. *Antonie van Leeuwenhoek*, **73:** 331-371.
21. Lee, L. G., Connell, C. R., Woo, S. L., Cheng, R. D., McArdle, B. F., Fuller, C. W., Halloran, N. D. and Wilson, R. K. 1992. DNA Sequencing with Dye-labeled Terminators and T7 DNA Polymerase: Effect of Dyes and dNTPs on Incorporation of Dye Terminators and Probability Analysis of Termination Fragments. *Nucleic Acids Res.*, **20:** 2471-2483.
22. Li-ying, N., Ji-hong, W., Xiao-jun, L., Fang, C., Zheng-fu, W., Guang-hua, Z. and Xiaosong, H. 2008. Physicochemical Characteristics of Orange Juice Samples from Seven Cultivars. *Agric. Sci. China*, **7:** 41-47.
23. Morrissey, W. F., Davenport, B., Querol, A. and Dobson, A. D. W. 2004. The Role of Indigenous Yeasts in Traditional Irish Cider Fermentations. *J. Appl. Microbiol.*, **97:** 647-655.
24. Parker, L. T., Deng, Q., Zakeri, H., Carlson, C., Nickerson, D. A. and Kwok, P. Y. 1995. Peak Height Variations in Automated Sequencing of PCR Products Using Taq Dye-terminator Chemistry. *BioTechniques*, **19:** 116-121.
25. Pretorius, I. S. 2000. Tailoring Wine Yeast for the New Millennium: Novel Approaches

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[DOR: 20.1001.1.16807073.2013.15.7.10.9]

to the Ancient Art If Winemaking. *Yeast*, **16:** 675-729.
26. Ramani, R., Gromadzki, S., Pincus, D. H., Salkin, I. F. and Chaturvedi, V. 1998. Efficacy of API 20C and ID 32C Systems for Identification of Common and Rare Clinical Yeast Isolates. *J. Clin. Microbiol.*, **36:** 3396-3398.
27. Sabate, J., Cano, J., Esteve-Zarzoso, B. and Guillamon, J. M. 2002. Isolation and Identification of Yeasts Associated with Vineyard and Winery by RFLP Analysis Ribosomal Genes. *Microbiol. Res.*, **157:** 1-8.
28. Singleton, V. L. and Gortner, W. A. 1965. Chemical and Physical Development of the Pineapple Fruit. II. Carbohydrate and Acid Constituents. *J. Food Sci.*, **30:** 19-23.
29. White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: "*PCR Protocols: A Guide to Methods and Applications*", (Eds.): Innis, M. A., Gelfalnd, D. H., Sninsky, J. J. and White, T. T.. Academic Press, San Diego, PP. 315-320.

مقایسه روش های شناسایی مخمر های جدا شده از تخمیر خود به خودی آب آناناس تازه له شده

ا. چانپرانسارتوسوک، چ. پراکیتچایواتانا، و ر. سانگواندکو

چکیده

هدف این پژوهش ارزیابی عمل روش هایی مانند توالی یابی جداکننده رونویسی شده داخلی (ITS) و(26S (D1/D2 ناحیه ریبوزیم دی.ان.ا، و روش تحلیل RFLP از ناحیه ITS و کار برد جعبه آزمون بیو شیمیایی تجارتی برای شناسایی مخمر های جدا شده از آب آناناس تازه له شده در حین تخمیر خود به خودی بود. آزمایش ها در تایلند و استرالیا انجام شد. شناسایی جدایه های مخمر ها در تایلند با روش های توالی یابی ITS و(26S (D1/D2 ناحیه ریبوزیم دی.ان.ا، و روش تحلیل RFLP از ناحیه ITS انجام شد. جدایه های مخمر ها در استرالیا با توالی یابی دو ناحیه دی.ان.ا و کاربرد جعبه آزمون تجارتی شناسایی شدند. نتایج شناسایی انجام شده در دو کشور نسبتا مشابه بود. جدایه های مخمر ها عمدتا با کار برد 26S rDNA همراه با تحلیل توالی یابی ITS شناسایی شدند. در تایلند، تقریبا ۸۰ درصد جدایه های مخمر با توالی یابی دو ناحیه مزبور هویت های مشابه نشان دادند. این جدایه ها مشتمل بودند بر *Rhodotolula mucilaginosa*, *Issatchenkia orientalis*, *Hanseniaspora uvarum*, *Hanseniaspora opuntiae*, *Pichia guilliermondii*, *Aureobasidium pullulans*, *Saccharomycodes ludwigii*, *Candida tropicalis*, *Pichia fermentans*, *Zygosaccharomyces bailii*, *Candida stellata* و *Erythrobasidium hasegawianum*. در استرالیا، ۸۶ درصد جدایه ها با استفاده از تحلیل توالی یابی دو ناحیه مزبور شناسایی مشابه داشتند و شامل موارد زیر بودند *P. guilliermondii*, *Pichia membranifaciens*, *P. fermentans*, *H. uvarum*, *H. opuntiae*, *I. orientalis*, *Candida* sp., *Yarrowia lipolytica*, *Tremella globispora*, *R. mucilaginosa* و *A. pullulans*.

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