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Genetic Diversity of Osmophilic Yeasts Isolated from Indonesian Foods with High Concentration of Sugar

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Isolation of osmophilic yeasts from a total of 70 samples consisting of jam, sweet condensed milk, honey, sweet soy sauce, and palm sugar was conducted. Sixty-eight osmophilic yeasts were isolated from strawberry jam, pineapple jam, and honey from South Sumatera. No yeast was obtained from condensed milk, honey from Sumbawa, sweet soy sauce, and palm sugar. Sequence analysis based on the ITS region showed that isolates were identified as five species belong to two genera, *Candida* and *Sterigmatomyces*. Those isolates were distributed in 5 species, *C. metapsilosis, C. etchellsii*, *C. parapsilosis, C. orthopsilosis,* and *S. halophilus. C. etchellsii* was the predominant species in South Sumatera honey, while *C. parapsilosis* group was predominant species in jams. Those species were reported as osmophilic yeasts. In both jams and honey we found *C. parapsilosis* and *C. metapsilosis*, whilst *C. orthopsilosis* was found only in pineapple jam. Phylogenetic analysis based on sequence of ITS region showed that most of the osmophilic yeasts (67 out of 68 isolates) were located in the phylum *Ascomycota* and only one isolate *Sterigmatomyces halophilus* NN38 from pineapple jam was located in the phylum *Basidiomycota*.

Key words: genetic diversity, osmophilic yeasts, food with high sugar concentration

Spoilage of food products by microorganisms results in the waste of valuable resources and causes large financial losses for the food industry that will inevitably affect the consumer. High sugar products are targets for spoilage by yeast. Various kinds of traditional high sugar foods in Indonesia such as jam, sweet condensed milk, sweet soy sauce and honey have the potential to be spoiled by yeast. Some yeasts have the ability to grow or cause spoilage in foods, especially products with low pH, generally 5.5 or lower, and other products by the presence of sugars as carbon sources. The adverse media inflicted by the low pH, low oxygen levels, and high sugar concentration of these products prevents the growth of most organisms. However, these hurdles do not inhibit the growth of many spoilage yeasts, especially osmophilic yeasts.

The reported food and beverage spoilage yeasts include a relatively large number of species representing both ascomycetes and basidiomycetes as has been published. Many yeasts were frequently described as being osmophilic, suggesting a habitat restricted to a high solute (in this case, sugar) environment. The a_w of typical concentrated syrups (more than 50% soluble solids) ranges from 0.82-0.94. Depending upon the solute and its concentration (salt, sugar, or glycerol), some yeasts are able to grow at relatively low temperatures. Many osmophilic yeasts had been identified as spoilage agents in fruit concentrates and juices (Arias *et al.* 2002; Fitzgerald *et al.* 2004) and syrup (Ancasi *et al.* 2006).

Osmophilic yeasts can create a spoilage problem in food storage, because they are able to grow under conditions of high salt or sugar content. Aside from their ability to grow in the restrictive habitat of high osmotic pressure, many yeasts are also extraordinarily resistant to common preservatives such as sulphur dioxide, sorbic acid, benzoic acid and acetic acid (Warth 1985). Yeasts can become adapted to preservatives by preexposure to low levels of these materials.

Many studies regarding these microorganisms have been carried out and indicate a desire to understand the diversity of yeasts in high sugar foods to prevent food spoilage. Morphologically and physiologically, these osmophilic yeasts were found to be extremely similar to one another, with species differentiation being based mainly on the high sugar content of media and the ability to form ascospores or pseudomycelia. In order to understand the species inter-relationships of these yeasts better, a phylogenetic study based on ITS sequence of 18S rDNA was carried out upon all strains isolated in this work. It is important to understand the characteristics of the isolated yeasts. Molecular comparisons have provided an understanding of yeast phylogeny that was not possible from analyses of morphology and physiology.

Gene-sequence determinations have also provided a rapid, accurate means for identification of individual strains to the species level. Sequencing of species-diagnostic genes represents the most accurate means for isolate identification, and several rapid identification methods using molecular probes based on these sequences have been developed and are becoming available to food microbiology laboratories. Over the last decade, different polymerase chain reaction (PCR) techniques and nuclear sequence analysis have been used for identifying many yeasts for the study of phylogeny. The ITS region of ribosomal DNA had been used for this purpose. The use of 5.8S-ITS sequence is the best tool for rapid and accurate identification of yeast. Some studies have reported that the ITS1 and ITS2 regions had higher degrees of variability and therefore higher differentiating power than D1/D2 region for closely related species (Tavanti *et al.* 2005;

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Kocsube *et al.* 2007). Partial sequencing of ITS region is convenient for the rapid characterization of yeast. Using sequences available in the GenBank database, sequences obtained could be used to compare the similarities of gene fragments among the currently recognized yeast species.

In view of these issues, the objectives of the present study were (i) to obtain isolates of osmophilic yeasts from high sugar food products, (ii) to identify their genetic diversity and to estimate the taxonomic boundaries of the osmophilic species using the ITS rDNA sequence information, and (iii) to reveal the phylogenetic relationship between osmophilic yeast isolated from many kinds of foods with high concentration of sugar in Indonesia.

MATERIALS AND METHODS

Isolation of Osmophilic Yeasts. A total of 70 samples of local high sugar food products (jams, sweet condensed milk, honey, sweet soy sauce, and palm sugar) were collected from different markets in Jakarta, Bogor, Sumbawa, and South Sumatera. Osmophilic yeasts were isolated from these samples. A procedure for isolation and preparation of osmophilic yeast was the use of potato dextrose broth (PDB) and potato dextrose agar (PDA) (Oxoid) enriched with various concentrations (10-60% w/v) of sucrose were conducted. Subsequently, the colonies which formed were isolated and purification by the quadrant streak method.

DNA Extraction and PCR Analysis. Total DNA was extracted from osmophilic yeasts by boiling method according to Sjamsuridzal and Oetari (2003). The DNA extract was amplified by PCR technique using ITS4 and ITS5 primers (White *et al.* 1990). PCR reactions were prepared in 25 µL total volume containing 9 µL DNA template; 0.5 pmol of each ITS4 and ITS5 primer; PCR buffer (10 mM Tris-Cl pH 9.0, 50 mM KCl); 200 µM (each) dATP, dGTP, dCTP, and dTTP; 1.5 mM $MgCl_2$; 2.5 unit pureTaq DNA polymerase (GE Healthcare Kit). The amplification procedure of the rDNA ITS region was carried out using a published method (White *et al.* 1990). The amplification profile consisted of 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 58°C for 30 sec, and primer extension at 68°C for 1 min; followed by post extension at 68°C for 10 min. PCR products were analyzed on 2% (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light using the Chemidoc Gel System (Biorad).

Partial Sequencing of ITS Region and Phylogenetic Analysis. DNA for sequencing was amplified using a 9600 Perkin-Elmer Cetus Thermal Cycler apparatus as described by White *et al.* (1990) with reverse primer (ITS5). DNA sequencing was performed on ABI Prism 310 DNA Sequencer with protocols supplied by the manufacturer. The sequencing results were compared with DNA sequences from GenBank database at National Center of Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program for screening sequence similarity (Altschul *et al*. 1997). Sequence alignments were performed by Clustal X1.83 program. The phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987).

RESULTS

Isolation of Osmophilic Yeasts. The isolates could not grow in media supplemented with 10, 20, and 30% (w/v) sucrose, whereas most of the isolates showed a higher cell number at 50% (w/v) sucrose, and some growth was found in the presence of 40% (w/v) sucrose and even in the presence of 60% (w/v) sucrose. The result showed that osmophilic yeast strains grew better on PDA enriched with 50% (w/v) sucrose than PDA alone. Colony size of the yeast grown in the media with lower concentration of sugar were smaller than in the media with a high sugar concentration (results not shown).

A feature of the yeast strains isolated from the high sugar foods were ascomycetous characteristics. These yeasts are osmophilic and could frequently grow under extreme conditions. Sixty eight osmophilic yeasts could be isolated from strawberry jam (a_w 0.84), pineapple jam (a_w 0.84), and honey from South Sumatera (a_w 0.72). Colonies were white to tannish white, creamy, shiny, smooth and produced a faintly acidic aromatic odor. No yeast was obtained from sweet condensed milk (a_w 0.84), sweet soy sauce (a_w 0.68), honey from Sumbawa (a_w 0.67), and palm sugar samples (a_w 0.63) (data not shown).

Analysis of the ITS Regions and Phylogenetic Analysis. Amplification of the ITS1-5.8S-ITS2 regions from the sixty eight osmophilic yeasts generated PCR products ranging in size from 400 to 500 bp. The profiles of bands representing the osmophilic yeasts were shown in Fig 1. One isolate (NN38) appeared to have a different profile from the others, so this isolate may be a different species. Phylogenetic trees of rDNA sequences were constructed based on the distance matrix methods as stated in the methodology. Sixty-seven of the sequences clustered in the phylum *Ascomycota*, however one isolate formed a different branch far away from the others and clustered within the phylum *Basidiomycota* (Fig 2). Thirteen yeast strains, representing four *Basidiomycota*-lineage and nine *Ascomycota*-lineage and their accession numbers obtained from GenBank are listed in Table 1.

Comparative analysis of the ITS region data revealed that the fifty-two strains of *Candida parapsilosis* group were highly related to one another, exhibiting sequence identity values that ranged from 99 to 100% (data not shown). Sequence analysis based on ITS region showed that 68 isolates were identified as five species belonging to two genera *Candida* and *Sterigmatomyces*. Those isolates were distributed in 5 species, i.e. *C. metapsilosis* (23), *C. etchelsii* (15), *C. parapsilosis* (25), *C. orthopsilosis* (4), and *S. halophilus* (1). *C. etchellsii* was the predominant species in South Sumatera honey and the *C. parapsilosis* group was the predominant species in strawberry jam and pineapple jam. In both jams and honey, *C. parapsilosis* and *C. metapsilosis* were found, while *C. orthopsilosis* was found only in pineapple jam (data not shown).

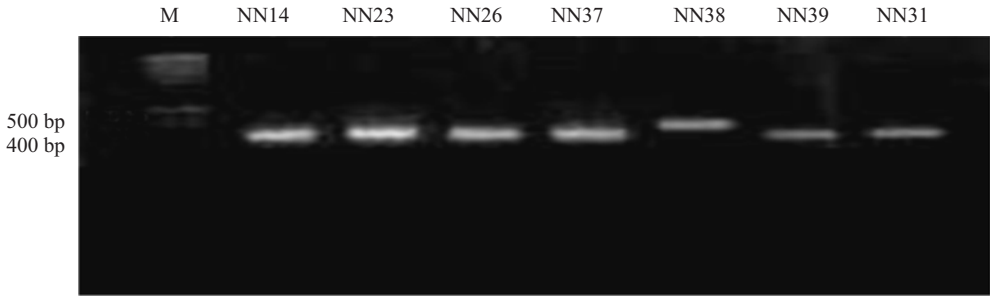

Fig 1 Gel electrophoresis images showing PCR products of yeast ITS region rDNA (about 400-500 bp in size). M, Marker; NN14, NN23, NN26, NN27, NN38, NN39, NN31, isolate from pineapple jam.

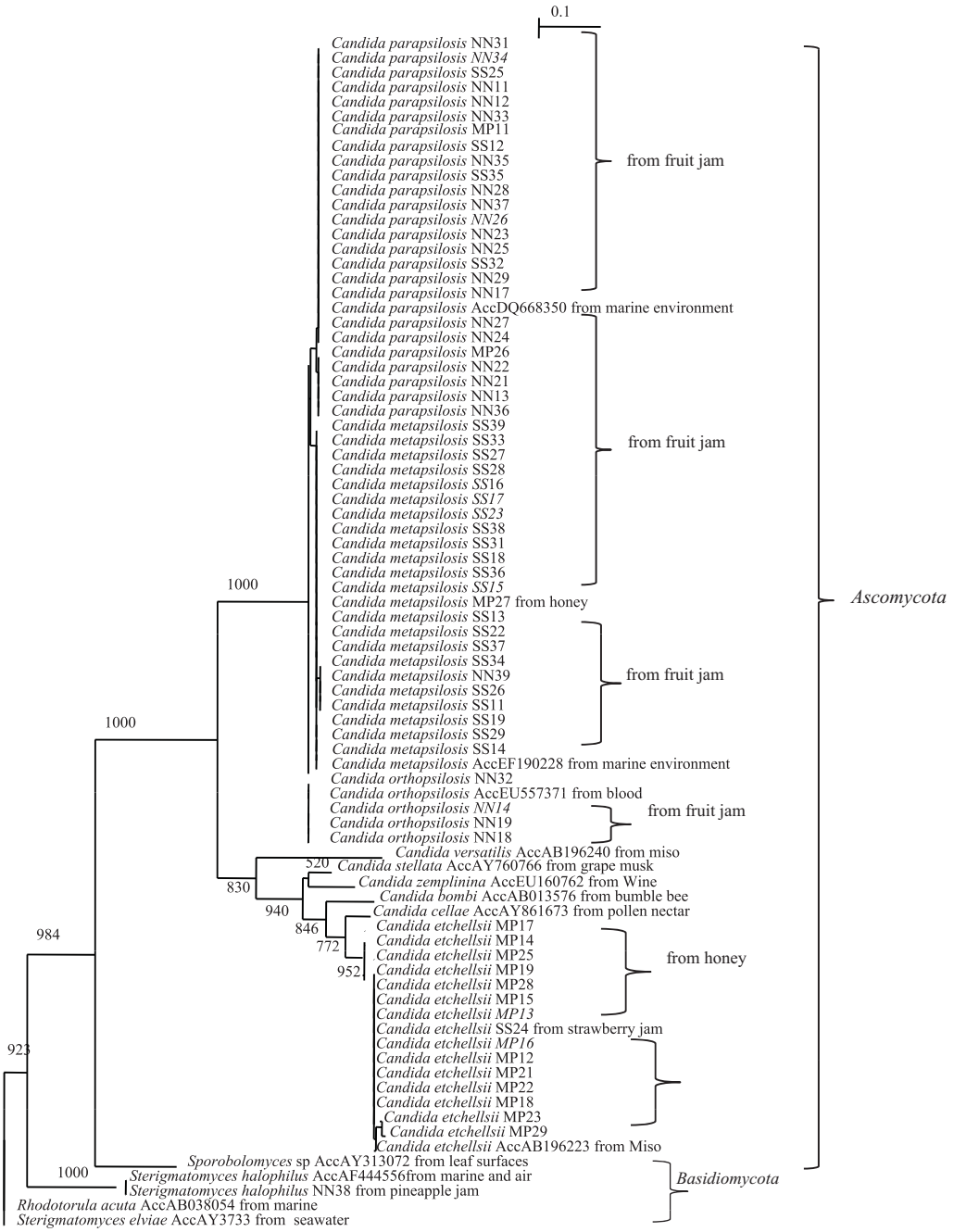

Fig 2 Dendrogram showing the phylogenetic relationship of *Candida parapsilosis and C. etchellsii* isolates from jam and honey with related yeast species based on ITS region. Bootstrap values, expressed as percentages of 1000 replications, are given at branch points (only values >50% are shown).

Table 1 Species name, source, and GenBank accession numbers of yeasts included in the phylogenetic analysis

Species name	Accesion number	Source
Candida versatillis	AB196240	Miso*
C. zemplinina	AY160762	Wine
C. stellata	AY160766	Grape musk
C. bombi	AB013576	Bumblebee
C. cellae	AY861673	Pollen nectar
C. etchellsii	AB196223	Miso*`
C. parapsilosis	DQ668350	Marine environment
C. metapsilosis	EF190228	Marine environment
C. orthopsilosis	EU557371	Blood sample
Sporobolomyces sp.	AY313072	Leaf surfaces
Rhodotorula acuta	AB038054	Marine
Sterigmatomyces elviae	AY373390	Seawater
S. halophilus	AF444556	Marine and air

* A thick fermented paste made of cooked soybeans, salt, and often rice or barley, and used especially in making soups and sauces.

DISCUSSION

Osmophilic yeasts have been isolated from Indonesian foods with high sugar concentration sugar and water activity between 0.72-0.84. Typically, the shelf-life of these products could be extended by pasteurization, evaporation, and/or the addition of chemical preservatives such as acid or benzoic. However, these preservation methods do not inhibit the growth of many spoilage-causing yeasts, especially osmophilic yeasts. A total of 68 isolates could be obtained from various samples. The results showed that osmophilic yeast strains grew better on PDA enriched with 50% (w/v) sucrose than PDA alone.

Water activity refers to the availability of water in foods or beverages and represents the amount of water that was available for microorganisms growth. A majority of food spoilage yeasts had been isolated from *food with low water activity* (foods with 65% (w/v) sucrose or 15% (w/v) NaCl or foods with a_w 0.87) and could survive on a_w 0.80-0.85 as reported by Beuchat (1983). In most cases, yeast contamination in these products occurred on the farm and they were therefore present in the fresh products.

No yeast was obtained from sweet condensed milk (SCM), even though these samples had a water activity value of 0.84. Aside of water activity, the main factor causing a lack of yeast obtained from SCM was the processing of these products. In SCM manufacturing, heat-treated milk containing sugar is taken to the evaporator, where it is concentrated by heating the mixture 93.58°C for 3 min depending on the desired outcome of viscosity, bacteriological quality, and other physico-chemical properties, then evaporated, cooled, and tinned or bottled under hygienic conditions (Beuchat 1983; Ali and Randal 2002). The method for preserving SCM using aseptic technology means contaminant osmophilic yeast could not survive in these products.

Sweet soy sauce (a_w 0.68), honey from Sumbawa (a_w 0.67) and palm sugar (a_w 0.63) have low water activity. No yeasts were obtained from these products. Some unique foods like sweet soy sauce appear to be a high moisture product, but because salt, sugars, or other ingredients bind the water content, their water activities become quite low. The presence of sugar in such a high concentration will inhibit the growth of yeast contaminants.

From sequencing results, a total of 68 strains-sequence-data were aligned together with known sequences of several species from the GenBank database. A phylogenetic tree was constructed using the NJ method as stated in the methodology, using *S. halophilus* as an outgroup. All species of *C. parapsilosis, C. orthopsilosis,* and *C. metapsilosis* were closely related and formed a single cluster (Fig 2). The species of this cluster were isolated from various sources, including honey, strawberry jam, and pineapple jam. They are widely distributed in nature and are common spoilage yeast in the food industry. Tavanti *et al*. (2005) and Andrew *et al*. (2009) had reported that all isolates within each group (*C. parapsilosis, C. metapsilosis, C. orthopsilosis*) had similar D1/D2 sequences of the large (26S) ribosomal RNA gene, and the 5.8S rDNA sequences of *C. parapsilosis* isolates from all three groups were 100% identical. Only minor differences were observed in *ITS2* sequences among the three groups, and considerable dissimilarities were found in the ITS1 sequences.

Candida etchellsii from honey and strawberry jam were in a cluster with *C. versatilis*, *C. zemplinina, C. stellata, C. cellae* (Fig 2). This cluster was an assemblage of phylogenetically diverse species with large interspecific divergences. Other yeasts used in the phylogenetic analysis were isolated from various sources, flowers and pollen nectar, wine, and grapes musk (Table 1). Many of these species are associated with insects, specifically bees, bumblebees and leaf-cutter bees, and many have been reported as the causative agent of spoilage of sugary foods, such as fruit juices and concentrates.

Many of the yeasts related to *C. parapsilosis* group have been implicated in the spoilage of foods, particularly sugary, low-a_w foods. Spoilage of high sugar commodities has been reported**.** *Candida davenportii* and *C. parapsilosis* caused fruit juice and soft drink spoilage, *C. bombicola* had been found in concentrated juice and *C. lactis-condensii* had been found in condensed milk as reported by Stratford *et al.* (2002). Spoilage by *C. lactis-condensi* was relatively uncommon and it is considered to be an opportunist spoilage yeast.

Candida stellata and *C. magnoliae* are spoilage yeasts in fruit juice concentrates and tomato sauce (Deak and Beuchat 1993). *Candida riodocensis* and *C. cellae* have been isolated from pollen-nectar and bees. *Candida parapsilosis* (Acc GM620527) had been used as fermenting yeast for xylitol production (Erisema *et al*. 2006). Among the less frequently encountered species, *C. orthopsilosis* had been isolated from fermented cocoa beans (Daniel *et al*. 2009). Ana *et al*. (2003) have isolated a new novel species from honey, pollen bee and nectar. The sequence of the D1/D2 domains of the large-subunit rDNA showed that this novel species belongs to the *Starmerella* cluster, and was most closely related to *C. etchellsii*.

Candida parapsilosis, C. metapsilosis, and *C. orthopsilosis* strains collectively formed a distinct lineage which, apart from displaying a loose association with the *C. parapsilosis* group, showed little phylogenetic affinity to any other species examined (Fig 2). Kurtzman and Robnett (1998) observed that strains showing greater than 1% difference in the ITS region were usually different species, whereas strains with zero to three nucleotide differences were either conspecific or sister species. Alignment of contiguous yeast sequences demonstrated that both single-nucleotide differences and short lengths of sequence diversity due to insertions or deletions existed in the ITS regions among the *Candida* species. A sequence similarity of 99-100% exists among *C. parapsilosis, C.metapsilosis, C. orthopsilosis,* and *C. etchellsii.*

The genus *Candida* was the largest in the number of species of the yeast genera and was present in almost every environment. Yeasts of this genus are abundantly distributed in nature. This genus is distributed across the ascomycetous yeast domain, overlapping with other genera according to phylogenetic analysis using ribosomal genes (Kurtzman and Robnett 1998). *C. parapsilosis, C. metapsilosis, C. orthopsilosis* strains collectively form a distinct lineage which apart from displaying a loose association with *C. parapsilosis* group, showed little phylogenetic affinity to any other of the species examined. The sixty-seven isolates could be divided into the *C. parapsilosis* group and the non-*C. parapsilosis* group, with the exception of one isolate which was clustered on a separate branch.

From all of data obtained in this study, we propose that Indonesian high-sugar foods contain a lot of osmophilic yeasts. Results of this analysis also indicated that most of the isolates belonged to the same clade as the isolates identified in the previous research such as isolates from miso (Suezawa *et al*. 2006), pollen and flower (Sjamsuridzal *et al*. 2010), nectar (Matthias 2004), bumblebee (Soon *et al.* 2003; Michael 2004), grape musk (Belinda *et al.* 2008), the air (Cristina *et al.* 2006), seawater (Tekolo *et al.* 2010). It is suggested that the existence of osmophilic yeasts diversity in products and manufacturing environment needs further investigation. Such knowledge will aid the development of control strategies for food spoilage.

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