A New Isolation and Evaluation Method for Marine-Derived Yeast spp. with Potential Applications in Industrial Biotechnology

Abdelrahman Saleh Zaky^{1,2,3}, Darren Greetham¹, Edward J. Louis⁴, Greg A. Tucker¹, and Chenyu Du^{1,2*}

¹Division of Food Sciences, School of Biosciences, University of Nottingham, Nottingham LE12 5RD, UK

²School of Applied Sciences, University of Huddersfield, Huddersfield HD1 3DH, UK

³Department of Microbiology, Faculty of Agriculture, Cairo University, Giza 12613, Egypt

⁴Centre for Genetic Architecture of Complex Traits, Department of Genetics, University of Leicester, Leicester LE1 7RH, UK

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*Corresponding author
Phone: +44 1484 472 378;
E-mail: c.du@hud.ac.uk

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Yeasts that are present in marine environments have evolved to survive hostile environments that are characterized by high exogenous salt content, high concentrations of inhibitory compounds, and low soluble carbon and nitrogen levels. Therefore, yeasts isolated from marine environments could have interesting characteristics for industrial applications. However, the application of marine yeast in research or industry is currently very limited owing to the lack of a suitable isolation method. Current methods for isolation suffer from fungal interference and/or low number of yeast isolates. In this paper, an efficient and non-laborious isolation method has been developed and successfully isolated large numbers of yeasts without bacterial or fungal growth. The new method includes a three-cycle enrichment step followed by an isolation step and a confirmation step. Using this method, 116 marine yeast strains were isolated from 14 marine samples collected in the UK, Egypt, and the USA. These strains were further evaluated for the utilization of fermentable sugars (glucose, xylose, mannitol, and galactose) using a phenotypic microarray assay. Seventeen strains with higher sugar utilization capacity than the reference terrestrial yeast *Saccharomyces cerevisiae* NCYC 2592 were selected for identification by sequencing of the ITS and D1/D2 domains. These strains belonged to six species: *S. cerevisiae*, *Candida tropicalis*, *Candida viswanathii*, *Wickerhamomyces anomalus*, *Candida glabrata*, and *Pichia kudriavzevii*. The ability of these strains for improved sugar utilization using seawater-based media was confirmed and, therefore, they could potentially be utilized in fermentations using marine biomass in seawater media, particularly for the production of bioethanol and other biochemical products.

Keywords: Marine yeast, phenotypic microarray, identification, screening, fermentation, seawater

Introduction

Yeasts are eukaryotic unicellular microorganisms belonging to the fungal kingdom. Currently, there are around 1,500 described species of yeasts, which represent about 1% of the total fungal species existing in nature [19, 20]. Yeasts, such as *S. cerevisiae*, have been successfully exploited in various industries such as ethanol production, bakery, wine making, and brewing. In order to satisfy the growing demand for a fermentation-based economy, researchers

have been trying to isolate novel yeast strains with promising properties, such as high fermentation capability, high stress tolerance, and producing novel products [4, 47]. The vast majority of existing yeasts have been isolated from terrestrial resources such as sugar refineries, breweries, wineries, bakeries, and beet and cane molasses, as well as from various fruits and vegetables.

The marine environment, which accounts for 71% of the planet's surface area, has not been widely explored for yeast isolation. Recently, marine yeasts have been identified

as a potential source for producing valuable compounds such as biofuels, amino acids, proteins, vitamins, polysaccharides, fatty acids, phospholipids, and enzymes [6, 35]. These products have been shown to have potential for commercial exploitation. Research on marine yeasts has highlighted several promising features over terrestrial yeast strains, *e.g.* higher tolerance to extreme environments and higher productivity [28, 47]. Urano *et al.* [38] suggested that yeasts that inhabited a marine environment would have already developed a mechanism to tolerate high osmotic stress. However, utilization of marine yeasts for commercial purposes has not been widely established. This is often due to a lack of intensive research on marine yeast and the limited availability of marine yeast isolates [6, 46, 47].

Torula sp. and *Mycoderma* sp. were the first yeasts isolated from the marine environment in 1894 by Bernhard Fischer [23]. Since then, various other marine yeasts have been isolated from different marine sources, including seawater, sea sand [15, 39], seaweeds [36], fish, and different marine animals [3]. According to a recent report, the number of marine yeast species that have been classified and described has reached 213 species [13].

The development of an efficient and reliable isolation method for marine yeast will advance their employment of by introducing new species and strains with desired properties for research projects and industry. In general, the development of a marine yeast isolation method involves three aspects: (i) specify a sample collection technique, (ii) design a yeast isolation medium, and (iii) propose a strain isolation protocol. The main challenges in marine yeast isolation are the relatively low yeast population in the marine samples [9, 23] and the presence of molds [8]. To maximize the chance of obtaining a larger number of yeast strains from the marine samples, isolation was conducted within 2 days of sampling in most of the reported marine yeast isolation methods [1, 23]. The growth of bacteria contained in the marine samples can be inhibited by the addition of antibiotics [9]. However, growth of filamentous fungi could not be prevented, as there is no medium that is yeast specific and does not support the growth of filamentous fungi. Therefore, several strategies have been proposed in the isolation protocol development stage to minimize filamentous fungi growth, including reducing the incubation period [7, 15], reducing the amount of the sample to be used in the strain isolation [7], or reducing the incubation temperature [8, 26]. As a consequence of reducing the presence of filamentous fungi, yeast growth was also negatively affected. Therefore, the possibility of isolating a marine yeast was diminished.

The current methods for marine yeast isolation have several limitations, including (i) requirement of recent collected samples, (ii) utilizing growth media that encourage mold growth, and (iii) producing low numbers of yeast isolates. Therefore, the main objective of this study was to develop an efficient method for marine yeast isolation, including the design of new enrichment and isolation media and the construction of a new isolation protocol. In particular, the new method enables the usage of an aging marine sample for the potential isolation of marine yeasts. Furthermore, we designed a framework for screening large numbers of yeast isolates using a microarray technique, which was applied to 116 selected isolates. Seventeen isolates that showed high sugar utilization properties were identified by sequencing the ITS and D1/D2 domains and using the YT Micro plate technique (Biolog).

Materials and Methods

Marine Samples

Fourteen marine samples were obtained from different locations in the UK, Egypt, and the USA (Table 1). All samples were collected near the shore and were taken from a depth of a maximum of 1 m using 1 L sterilized plastic bottles with screw caps to prevent leaking and contamination. Samples were then transported immediately to the laboratory or sent by a courier to the laboratory. When samples were received at the laboratory, they were stored immediately in a fridge at 4°C until isolation was performed. The isolation was performed within 2 weeks of sampling.

Seawater

The seawater used in this paper for media preparation and dilutions was collected from the North Sea near Whitby, UK with salinity of about 33 g/l. The seawater was filtered using Whatman Glass microfiber filters (pore size, 1.2 µm) and autoclaved at 121°C for 15 min, and then stored at 4°C until required.

Microorganisms

Besides isolated marine yeasts, a terrestrial strain *S. cerevisiae* (NCYC 2592; <http://www.ncyc.co.uk>) was used in this study as a reference strain. It was maintained on yeast peptone dextrose (YPD) agar slopes containing (w/v) 1% yeast extract, 2% peptone, 2% glucose, and 2% agar at pH 6 ± 0.2. Propagation was carried out aerobically using YPD broth medium (as above, no agar) in an orbital shaker (150 rpm) at 30°C.

Pure marine isolates were propagated using modified YM medium, containing (w/v) 2% dextrose, 0.3% malt extract, 0.3% yeast extract, and 0.5% peptone dissolved in natural seawater (designated as SW-YM broth in this paper). The medium was adjusted to pH 5.0 before autoclaving at 121°C for 15 min. All marine yeast isolates were maintained on SW-YM agar slopes,

Table 1. Distribution of new marine yeast isolates.

	Sample type	Locations	Numbers of isolates using different substrates					Total Isolates
			Glucose	Xylose	Galactose	Starch	Cellulose	
A	Seawater	Alexandria, Egypt	4	3	3	N/A	N/A	10
B	Seawater	Swiss, Egypt	3	2	2	N/A	N/A	7
C	Seawater	Ras Sedr, Egypt	7	0	2	3	N/A	12
D	Seawater	Ismailia, Egypt	4	2	2	2	N/A	10
E	Seawater	Wales 1, UK	1	0	2	N/A	N/A	3
F	Seawater	Wales 2, UK	3	2	2	N/A	3	10
G	Seawater	Plymouth1, UK	4	3	2	3	N/A	12
H	Seawater	Plymouth2, UK	4	2	2	N/A	1	9
I	Sea sand	Alexandria, Egypt	3	0	3	N/A	N/A	6
J	Seaweed	Plymouth3, UK	3	1	3	N/A	6	13
K	Rotten seaweed	Plymouth4, UK	2	1	2	N/A	N/A	5
L	Seawater	Whitby, UK	3	0	0	N/A	N/A	3
M	Seawater	New York, USA	2	3	2	1	N/A	8
N	Seawater	San Diego, USA	3	2	2	1	N/A	8
Total			46	21	29	10	10	116

containing the above-mentioned SW-YM broth with the addition of 2% (w/v) agar. Glycerol stock of each marine isolate was prepared using 1:1 glycerol and yeast broth that was cultured in SW-YM broth for 48 h. The glycerol stocks were stored at -80°C .

List of Microbiological Media Used in This Study

SW-YM broth (w/v): 2% dextrose, 0.3% malt extract, 0.3% yeast extract, and 0.5% peptone.

SW-YM agar (w/v): 2% dextrose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, and 2% agar.

YM medium (w/v): 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% agar [43].

Sabouraud's Dextrose Agar (SDA) (Oxoid, UK): 65 g of commercial medium was dissolved in 1 L of water to reach the following concentrations: 4% glucose, 1% mycological peptone, and 1.5% agar (w/v), with a final pH of 5.6.

Zaky's enrichment medium (w/v): 3% glucose, 3% xylose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 0.1% $(\text{NH}_4)_2\text{SO}_4$, and 0.025% KH_2PO_4 .

Zaky's isolation broth medium (w/v): 6% glucose or alternative carbohydrate source (*i.e.*, xylose, galactose, starch, or cellulose), 0.3% yeast extract, 0.5% peptone, 0.1% $(\text{NH}_4)_2\text{SO}_4$, and 0.025% KH_2PO_4 .

Zaky's isolation agar medium (w/v): 6% glucose or alternative carbohydrate source (*i.e.*, xylose, galactose, starch, or cellulose), 0.3% yeast extract, 0.5% peptone, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.025% KH_2PO_4 , and 2% agar.

All components were dissolved in seawater or deionized water and adjusted to the required pH using either NaOH or HCl (1N), and then autoclaved at 121°C for 15 min.

Marine Yeast Isolation Methods

Three marine yeast isolation methods were used in this study.

Isolation method 1: Kutty method [22]. Isolation of marine yeasts was carried out by filtration and pour plate using the method described by Kutty [22] with some modifications. First, 30 ml of seawater was filtered through nitrocellulose filters (pore size $0.45\ \mu\text{m}$) using an autoclaved filter apparatus (Fisher). For solid marine samples (sea sand or seaweed), 10 g of each sample was suspended and vortexed in 30 ml of sterilized seawater. Then, the seawater containing the solid marine sample was filtered through nitrocellulose filters (pore size $0.45\ \mu\text{m}$) using an autoclaved filter apparatus (Fisher). The filters were then placed face up on an empty petri dish followed by the addition of YM medium (using seawater and supplemented with chloramphenicol (200 mg/l) after autoclaving) and incubated at room temperature $23 \pm 1^{\circ}\text{C}$ for 14 days.

Isolation method 2: Dinesh method [7]. Isolation of marine yeast was carried out by serial dilution and pour plate using the method described by Dinesh *et al.* [7] with some modifications. First, 1 ml of seawater was serially diluted with sterilized seawater to reach a dilution factor range of 10^{-1} to 10^{-6} . Then, 1 ml of the original sample or the diluted sample was transferred to an empty petri dish, followed by pouring of melted and cooled SDA, prepared using 50% seawater. For solid samples, 1 g of the sample was suspended and vortexed in 10 ml of sterilized seawater. Then, it was serially diluted with sterilized seawater to reach a dilution factor range from 10^{-1} to 10^{-6} . After that, 1 ml from each dilution was transferred into an empty petri dish, followed by pouring of melted and cooled SDA prepared using 50% seawater, and then

Fig. 1. Schematic diagram of the new marine yeast isolation method proposed in this study.

incubation at 35°C for 48 h.

Isolation method 3: the new method developed in this study.

The new method developed in this study involves three steps (Fig. 1). The first step was three cycles of enrichment, including a Primary Enrichment cycle, a Scale-Up Enrichment cycle, and a Differential Enrichment cycle. In the second step, the isolation of yeasts from the final enriched culture was carried out using the pour-plate technique. The final step was conducted using the streak-plate technique, followed by microscopic examination of selected colonies to confirm the purity of the new isolates.

In the Primary Enrichment cycle, 100 ml of seawater samples was transferred into a 500 ml conical flask containing 100 ml of 2× Zaky's enrichment medium. For solid samples, 20 g of the sample was transferred into a 500 ml conical flask containing 100 ml of

Zaky's enrichment medium. Then, the flasks were incubated in a shaking incubator (180 rpm) at 30°C for 48 h.

In the Scale-Up Enrichment cycle, 20 ml of culture from the Primary Enrichment cycle was transferred into a 500 ml conical flask containing 180 ml of Zaky's enrichment medium. Then, the flasks were incubated in a shaking incubator (180 rpm) at 30°C for 48 h.

In the Differential Enrichment cycle, 10 ml of culture from the Scale-up Enrichment cycle was transferred into a 250 ml conical flask contained 90 ml of Zaky's isolation broth medium. Then, the flasks were incubated in a shaking incubator (180 rpm) at 30°C for 48 h.

All media used in this step were prepared using seawater and adjusted to pH 5.0 using HCl (1N). A mixture of antibiotics

(penicillin-G 500 mg/l and streptomycin sulfate 500 mg/l) was added to all enrichment media after autoclaving. Microscopic inspection was carried out after each enrichment cycle to monitor the type of growth after the period of cultivation.

In the second step (isolation step), a 10-fold serial dilution was carried out from the Differential Enrichment cycle, and 1 ml of diluted broth (with a dilution factor of 10^{-4} to 10^{-7}) was transferred into a petri dish and then poured with 10 ml of Zaky's isolation agar medium. The plates were then incubated for 48 h at 30°C.

In the third step (confirmation step), selected single colonies from the isolation step were streaked on fresh SW-YM agar plates and incubated for 48 h at 30°C, followed by microscopic examination of smear slides stained with methylene blue.

Screening and Metabolism Evaluation Using Phenotypic Microarray (PM) Assay

PM assays were carried out using sterilized empty Biolog 96-well plates based on the method developed by Greetham *et al.* [11] with minor modifications. Each well in the plate was supplied with a mixture of growth medium (30 µl) and buffered cell suspension (90 µl). Stock solutions of (40% (w/v)) of each sugar (glucose, mannitol, xylose, and galactose) and (28.7% (w/v)) of yeast nitrogen base (YNB) were prepared individually in seawater and then filter sterilized. Sugars were prepared from the stock solutions to give a 6% (w/v) final concentration per well and 0.67% (w/v) of YNB in the well's final volume (120 µl). Hence, 18 µl of stock solution's sugar, 2.8 µl of YNB stock solution, 9 µl of seawater, and 0.2 µl of dye D (Biolog, Hayward, CA, USA) were aliquoted to individual wells in the Biolog 96-well plate. Marine isolates and the reference strain were prepared for inoculation into the PM assay plates as follows: A fresh culture (48 h) on SW-YM agar slope was prepared from stock cultures stored at 4°C. A small amount of yeast growth was transferred into test tubes (20 × 100 mm) that contained 10 ml of sterile seawater and transmittance was adjusted to 62% ($\sim 5 \times 10^6$ cells/ml) (turbidimeter; Biolog). Then, 1 ml of the cell suspension was added to 5 ml of IFY buffer (Biolog) to obtain the cell suspension for the inoculum. Next, 90 µl of the above cell suspension was inoculated into each well of the Biolog plate. Semi-anaerobic conditions were created by placing the plates inside PM gas bags (Biolog) and vacuum sealing them using DMC 260PD Vacuum Packaging Machine. Inoculated plates were then incubated at 30°C in the Omnilog plate reader (Biolog) and the reading was recorded every 15 min for 24 h. By the end of the run, the signal data were compiled and exported from the Biolog software using Microsoft Excel. Only the isolate that had a final Biolog reading (redox signal intensity) of 20 or over was counted. All experiments were performed in triplicates, and the mean signal values are presented.

PCR-Based Method (D1/D2 and ITS Primers) for Yeast Identification

Identification of selected marine isolates was carried out based on the sequence of the D1/D2 region of the large 26S rDNA

subunit using primers NL-1 (5'-GCATATCAATAAGCGGAG GAAAAG-3') and NL-4 (5'-GGTCCGTGTTCAAGACGG-3'). Identification was also performed using the internal transcribed spacer (ITS) region (5.8S rRNA) using primers ITS1 (5'-TCC GTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATG-3') as described by White *et al.* [41] and Mitchell *et al.* [25].

For the PCR master mix for PCR amplification, each reaction contained 8 µl of 10× PCR buffer, 1 unit of *Taq* DNA polymerase, 25 pmol of each forward and reverse primers, 100 µM of each deoxynucleoside triphosphate, and enough distilled water up to a total reaction volume of 50 µl. A small amount of yeast growth from a culture (24–48 h) was picked using a micropipette tip and suspended in 50 µl of deionized water and then incubated for 10 min at 95°C. Then, 4 µl of the preheated yeast suspension was transferred into the PCR tubes as a DNA template. The tubes were then placed in a thermocycler (TECHNE TC-5, UK), using the following settings: initial denaturation at 98°C for 30 sec, followed by 35 cycles of 98°C for 15 sec, 52°C (for D1/D2 primers) or 54°C (for ITS primers) for 45 sec, and 72°C for 90 sec, with a final extension step of 72°C for 5 min. The PCR products were detected by electrophoresis separation using 1% agarose gel containing 4 µl of ethidium bromide in TBE buffer (0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA, pH 8.3). A PCR cleaning kit (Sigma-Aldrich, UK) was used for purification before sequencing. The sequences obtained were aligned using BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST>) for comparison with currently available sequences.

The ITS and D1/D2 phylogenetic comparisons were done with the core sequences shared by all PCR clones; that is, any long sequences were trimmed so that all bases were included in the multiple sequence alignments. Sequences that were too short were not included. The alignments were made with DNASTar Megalign, and the cladograms of the phylogenetic relationships were constructed with ClustalW embedded within Megalign. Bootstrap analysis was done using 1,000 generated trees.

YT MicroPlate (Biolog System) for Yeast Identification

Biolog YT MicroPlate (Biolog) was used to identify 17 of the marine isolates and the reference strain. YT MicroPlate contained pre-prepared substrates for 94 different tests in a ready-to-use 96 Biolog microplate with two reference wells. Fresh cultures (48 h) were prepared on malt extract agar slopes using the working culture stocks that were stored at 4°C. For each strain, a small amount of yeast broth was aseptically transferred into a test tube (20 × 100 mm), which contained sterile deionized water. Then, the test tube was placed on a Biolog turbidimeter to reach a transmittance of 48%. Using a multichannel micropipette, one YT MicroPlate was used for each strain by loading 100 µl of the cell suspension into each well. Plates were incubated at 26°C and the reads were taken after 24, 48, and 72 h at a wavelength of 450 nm for oxidation tests (the upper 3 rows in the plate) and a wavelength of 590 nm for assimilation tests (the other 5 rows in the plate) using a TECAN (Infinite 200 PRO) plate reader. Reads

were generated and manually converted to values (positive, negative, and weak). All reads below 0.1 were considered negative, reads between 0.1 and 0.3 were considered weak reaction, and any reads above 0.3 were considered positive. The results were sent to Technopath for identification using the MicroLog manual microbial ID system.

Results

Development of a Methodology for the Isolation of Yeasts from a Marine Environment

In this study, two methods that were developed for isolation of marine yeast from newly collected samples, the Kutty method [22] and Dinesh method [7], were used for the isolation of yeasts from the 14 marine samples we collected (Table 1). As our samples were approximately 2 weeks old, no yeast colonies were obtained using either method. Instead, mold colonies were obtained from 10 of the 14 samples using Kutty's method (data not shown).

Based on these results, a new marine yeast isolation method was required to isolate yeast from marine environments. The new methodology involved three steps: enrichment, isolation, and confirmation. By the end of the enrichment step, yeast cell numbers reached 10^5 – 10^8 cells/ml, while mold and bacterial cell growth was inhibited. An inspection after each enrichment cycle revealed that the number of fungi-like cells decreased between cycles (Fig. 2A).

Three cell culture cycles (2 days each) were designed to reduce filamentous fungal strains, as yeast cells are expected to grow faster than fungal strains in a liquid medium with high sugar concentration. For the third enrichment cycle, different carbon sources were used to screen for yeast strains with potentially desirable sugar utilization phenotypes.

Following three cycles of enrichment, large numbers of yeast colonies were obtained with no obvious bacterial or fungal colonies observed on the agar plates of the isolation step. The morphologies of each colony, such as color (white, cream, yellowish, red), shape (circular, oval, irregular, spindle, star, triangle), size (small, medium, large), surface (smooth, fluffy, rough, dry), and elevation (flat, raised, convex) were recorded. Colonies that were present on the same plate and had the same appearance were considered to be the same strain.

Table 2 shows the yeast viable count (YVC) of the isolates from various samples. When glucose was used as the carbon source in the third cycle of enrichment and isolation

Fig. 2. Images of microorganisms obtained at different stages of isolation.

(A) Microscopic images (1,000×) of the cultures after each enrichment cycle. a. i: primary enrichment cycle; a. ii: scale-up enrichment cycle; a. iii: differential enrichment cycle. The strains were stained with methylene blue. (B) Microscopic images (1,250×) after the confirmation step for six newly isolated marine yeasts that represent different species: b.i, *P. kudriavzevii*; b.ii, *C. glabrata*; b.iii, *W. anomalus*; b.iv, *C. viswanathii*; b.v, *C. tropicalis*; and b.vi, *S. cerevisiae*. Polymorphic yeast cells appear in b.iv, *C. viswanathii*.

media, the YVC ranged from 24.7 to 220 million CFU/ml. A similar YVC range was obtained when galactose was used as the carbon source in the final cycle of enrichment and isolation media (Table 2). In comparison with glucose and galactose, using xylose as the carbon source resulted in a significant reduction of YVC in almost all samples, indicating glucose and galactose were relatively preferred substrates for marine yeasts. Starch was also used as a sole carbon source in the last step of enrichment. Isolates were obtained in five samples, and the YVC ranged from 0.25 to 2.0 million CFU/ml. When cellulose was used, isolates were detected in three samples (F, H, and J), and YVCs were 2.0, 0.55, and 0.5 million CFU/ml, respectively (Table 2).

Table 2. Yeast viable count of the isolates for 14 marine samples using Zaky's isolation method.

Isolation media	Glucose	Xylose	Galactose	Starch	Cellulose
Samples	CFU/ml (million ^a)				
A	206.67 ± 20.82	32.67 ± 4.04	230.00 ± 20.00	N/A	N/A
B	220.00 ± 20.00	5.53 ± 0.45	45.33 ± 4.51	N/A	N/A
C	49.00 ± 3.61	N/A	25.67 ± 6.03	0.75 ± 0.05	N/A
D	24.67 ± 5.03	14.33 ± 4.04	86.33 ± 4.04	1.60 ± 0.28	N/A
E	42.67 ± 5.03	1.77 ± 0.64	17.33 ± 5.03	N/A	N/A
F	129.00 ± 5.57	7.97 ± 2.28	83.67 ± 7.77	N/A	2.00 ± 0.46
G	24.33 ± 4.51	12.33 ± 2.52	68.33 ± 3.51	1.80 ± 0.15	N/A
H	91.67 ± 3.51	34.67 ± 5.03	20.67 ± 3.06	N/A	0.50 ± 0.13
I	73.33 ± 6.66	25.33 ± 4.51	38.67 ± 9.16	N/A	N/A
J	38.33 ± 5.69	39.33 ± 5.51	61.67 ± 7.77	N/A	0.55 ± 0.05
K	27.00 ± 4.58	14.67 ± 3.51	N/A	N/A	N/A
L	122.67 ± 7.37	30.33 ± 6.11	84.67 ± 6.11	N/A	N/A
M	106.33 ± 12.10	4.37 ± 0.78	33.67 ± 6.51	0.75 ± 0.10	N/A
N	169.33 ± 14.36	31.00 ± 15.10	33.33 ± 7.64	0.25 ± 0.13	N/A

^a Average of 3 replicates; ± standard deviation.

In the third enrichment step, the purity of the 116 selected colonies was confirmed by streaking them on a second agar plate. Those colonies were selected to represent different sample sources, different isolation media, and different colony morphology. Microscopic images showed that some of the isolates were polymorphologically yeast-like cells (Fig. 2B).

Determination of Yeast Metabolic Output on Monomeric Fermentable Sugars Using a Phenotypic Microarray Assay

The ability of the 116 marine yeast isolates to utilize glucose, mannitol, xylose, and galactose in seawater-based media was assessed using a PM assay. The terrestrial yeast *S. cerevisiae* NCYC 2592 was included in this study as a reference strain. *S. cerevisiae* NCYC2592 had been well defined previously for sugar utilization and tolerance to the presence of inhibitory compounds [29, 45].

Results revealed that 96 out of the 116 marine isolates were able to utilize glucose in a seawater-based medium, and 11 of the marine isolates utilized a higher amount of glucose than the reference strain. Thirty-eight out of the 116 marine isolates were able to utilize mannitol, with 26 of them showing better utilization than the reference strain. Thirty-nine out of the 116 marine isolates were able to utilize galactose with three of them showing a better utilization of galactose than the reference strain. Twenty marine strains were able to utilize xylose, whereas the reference strain was not able to (Fig. 3).

Genetic Identification of Marine Yeast Isolates and Phenotypic Characterization

Based on the results of the PM assay, 17 strains (Table 3) with interesting sugar utilization abilities were selected for identification using D1/D2 and ITS sequencing. The length of PCR sequences of all isolates ranged between 553 and 677 nucleotides with D1/D2 primers, and 418 and 839 nucleotides with ITS primers, which was sufficient for identification. All sequences obtained were blasted against sequences in GenBank, EMBL, DDBJ, and PDB (<http://blast.ncbi.nlm.nih.gov/>). Out of the 17 isolates, nine isolates belonged to the genus *Candida* as follows: *C. tropicalis* (4 isolates), *C. viswanathii* (4 isolates), and *C. glabrata* (1 isolate). The rest of the isolates were *S. cerevisiae* (4 isolates), *W. anomalus* (3 isolates), and *P. kudriavzevii* (1 isolate) (Table 3). The ID% for all identified isolates was at least 99%.

In Fig. 4, the phylogram of the D1/D2 sequences (Fig. 4A) and the ITS sequences (Fig. 4B) based on a ClustalW alignment are shown. Bootstrap support is shown as a percent age out of 1,000 trees. The phylogenetic relationships of both sequence sets are essentially the same, and are consistent with the known phylogenies of the species based on larger sequence comparisons.

When Biolog YT MicroPlate and the Biolog database were used for the identification of the isolates, the results differed from those obtained using the PCR-based method (Table 3). No identification was obtained for five isolates: S10, S62, S69, S83, and S84. Out of the 12 identified isolates,

Fig. 3. Metabolic output of 116 marine yeast isolates and the reference strain *S. cerevisiae* NCYC2592 (Ref.) on 4 monomeric fermentable sugars using a phenotypic microarray assay.

(A) Glucose 6% in Seawater; (B) Mannitol 6% in Seawater; (C) Xylose 6% in Seawater; (D) Galactose 6% in Seawater.

six isolates matched the genetic identification at the genus level, but none matched at the species level. The identified isolates of *C. viswanathii* strains S1, S7, and S8 were identified as *Hyphopichia burtonii*, *Rhodotorula acheniorum*, and *Candida parapsilosis*, respectively, according to Biolog YT MicroPlate. *C. tropicalis* strains S45, S57, and S68 were identified as *Candida albicans*. *S. cerevisiae* isolates S117 and S118 were identified as *Saccharomyces boulardii*, and S71 was identified as *Kluyveromyces marxianus*. *W. anomalus* strains S80, S115, and S116 were identified as *Endomyces fibuligera*, *Pichia onychis*, and *Pichia subpelliculosa*, respectively.

The genetic identification using ITS or D1/D2 primers

for marine yeasts gives reliable identification up to the species level but cannot distinguish between strains. Hence, a phenotyping microarray identification method (YT Plates) was applied. Table 4 shows the oxidation ability of the yeast isolates on 35 different substrates. Differences in substrate utilization were recorded within the isolates of the same species. For example, *C. viswanathii* isolate S8 was able to oxidize mannitol, but isolate S1 was not able to oxidize mannitol, whereas isolates S7 and S10 recorded weak oxidation. For *S. cerevisiae* isolates, there was a difference in oxidation results between the reference strain (NCYC 2592) and marine-derived yeasts. However, there

Table 3. Identification outcome of 17 marine yeast isolates using ITS and D1/D2 primers and YT MicroPlate.

Isolate source	Sample	Strain	Genetic identification (ITS & D1/D2 primers)		Biochemical identification (TY Micro Plates)	
			Genus	Species	Genus	Species
Medit. Sea. Alexandria, Egypt	A	S 1	<i>Candida</i>	<i>viswanathii</i>	<i>Hyphopichia</i>	<i>burtonii</i> C
		S 7	<i>Candida</i>	<i>viswanathii</i>	<i>Rhodotorula</i>	<i>acheniorum</i>
		S 8	<i>Candida</i>	<i>viswanathii</i>	<i>Candida</i>	<i>parapsilosis</i> B
		S 10	<i>Candida</i>	<i>viswanathii</i>	N/A	N/A
Red Sea. Ismailia, Egypt	D	S 45	<i>Candida</i>	<i>tropicalis</i>	<i>Candida</i>	<i>albicans</i>
Irish Sea.	E	S 57	<i>Candida</i>	<i>tropicalis</i>	<i>Candida</i>	<i>albicans</i>
Wales, U.K.	F	S 62	<i>Candida</i>	<i>tropicalis</i>	N/A	N/A
		S 68	<i>Candida</i>	<i>tropicalis</i>	<i>Candida</i>	<i>albicans</i>
English Channel. Plymouth, U.K.	G	S 69	<i>Saccharomyces</i>	<i>cerevisiae</i>	N/A	N/A
		S 71	<i>Saccharomyces</i>	<i>cerevisiae</i>	<i>Kluyveromyces</i>	<i>marxianus</i>
		S 80	<i>Wickerhamomyces</i>	<i>anomalus</i>	<i>Endomyces</i>	<i>fibuligera</i>
	H	S 83	<i>Pichia</i>	<i>kudriavzevii</i>	N/A	N/A
		S 84	<i>Candida</i>	<i>glabrata</i>	N/A	N/A
	J	S 115	<i>Wickerhamomyces</i>	<i>anomalus</i>	<i>Pichia</i>	<i>onychis</i>
		S 116	<i>Wickerhamomyces</i>	<i>anomalus</i>	<i>Pichia</i>	<i>subpelliculosa</i>
	K	S 117	<i>Saccharomyces</i>	<i>cerevisiae</i>	<i>Saccharomyces</i>	<i>boulardii</i>
		S 118	<i>Saccharomyces</i>	<i>cerevisiae</i>	<i>Saccharomyces</i>	<i>boulardii</i>

were also differences in the oxidation results among the marine yeast, indicating that we possessed different strains of *S. cerevisiae*. In general, results suggested that all isolates could utilize glucose and inulin. Sucrose could be utilized by all strains except *C. glabrata* and *P. kudriavzevii*. Table 5 shows the differences in the assimilation capability of these yeasts on 65 different substrates. Similar to the results obtained from the oxidation tests, the assimilation ability of the yeast isolates varied within the same species.

Identification results obtained from both methods suggested that similar species of marine yeasts and terrestrial yeast are genetically similar but differ phenotypically. This would explain the marine environmental impact on the yeast, as most marine yeasts have a terrestrial origin and reached a marine environment through wind, rivers, or human activity.

Discussion

The samples used for marine yeast isolation in the current study were collected from coastal water within a 1 m depth. Near-shore was chosen as a location for sampling in this study because it was expected to contain a larger number of yeasts compared with off-shore sites [1, 23] and

to have a higher possibility of obtaining marine yeasts with potential characteristics for industrial application. Most reported marine yeast isolation methods were carried out using fresh samples (*e.g.*, usually within 2 days of sampling), to avoid the reduction of yeast cell number in the sample [8, 39]. This limited the potential of finding novel yeast strains from marine samples out of the local area as well as studies requiring samples from diverse marine habitats. The new isolation protocol reported in this study successfully overcame this limiting factor and maintained a high number and variety of yeast strains. It has been reported that filamentous fungi are present in high numbers in samples taken near the shore [8]. This was confirmed by the results obtained in this study, since filamentous fungal colonies were observed from 10 out of 14 marine samples when Kutty's method was used. Kutty's method utilizes Wickerham's yeast medium, which is a rich medium suitable for mold growth as well as for yeasts. There was no yeast, bacterial, or fungal growth when Dinesh's method was used for isolation. This was probably due to the small volume of the samples (1 ml) recommended by the Dinesh method as well as the samples being old. It was reported that near-shore seawater samples only contain 10–10,000 yeast cells per liter [8, 23]. Therefore,

Fig. 4. Phylogenetic tree of the new marine yeasts. The phylogram was calculated from the divergence in large-subunit region D1/D2 (A) and ITS region (B). Branch lengths are proportional to nucleotide differences, as indicated on the marker bar. Numbers given on branches are the frequencies (expressed as percentages) with which a branch appeared in 100 bootstrap replicates. Frequencies under 50% are not shown.

1 ml of the sample may not contain any yeast cells for isolation. Table 6 compares the three isolation methods used in this study.

The fact that our new isolation method generated a large number of pure yeast colonies from relatively old samples was mainly because of the three enrichment cycles. Generally, filamentous fungi propagate slower than yeasts. The incubation period in fermentations using filamentous fungi is generally 3–7 days; while using yeasts it is normally 1–2 days [5, 27]. In the new marine yeast isolation method, the subculturing time was selected to be 2 days so that molds did not have enough time to increase their number. Thus, when the enriched culture was used to inoculate the next enrichment cycle, the number of molds was reduced. Throughout the three cycles of enrichment step, the reproduction of filamentous fungi was prevented,

while a rapid yeast growth was maintained.

In our new isolation method, 30°C was used for the cell growth, which is relatively higher than the normal temperature of seawater. This was due to the following two considerations: (i) a relatively higher temperature like 30°C is preferable for industrial application. A high temperature would lead to faster fermentation rates and therefore higher productivity; (ii) it has been demonstrated that although marine yeast strains' usual temperature is relatively low, their optimum growth temperature could be higher. Kutty [22] studied the effect of temperature on the growth of marine yeasts obtained from slope sediments of the Arabian Sea at different depths (up to 100 m), where the temperatures range at 6–16°C, and concluded that the maximum growth was 30°C in almost all isolates.

Our new method for marine yeast isolation was easy

Table 4. Comparison of oxidation tests (using YT plates for yeast identification) of 17 marine yeast isolates and the terrestrial *S. cerevisiae* NCYC 2592.

	Yeast strains																	
	<i>S. cerevisiae</i>					<i>C. tropicalis</i>				<i>C. viswanathii</i>				<i>W. anomalus</i>		<i>C. glabrata</i>	<i>P. kudriavzevii</i>	
Oxidation test (substrate)	Ref	S69	S71	S117	S118	S45	S57	S62	S68	S1	S7	S8	S10	S80	S115	S116	S 84	S 83
D-Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-
N-Acetyl-D-glucosamine	-	-	-	-	-	w	+	w	w	-	w	+	w	-	-	-	-	-
Acetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-
α-D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Formic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	-	-	+	+	+	+	+	+	+	+	+	+	+	w	+	w	-
D-Galactose	+	+	+	+	+	+	w	w	w	+	w	+	+	-	w	w	w	-
Propionic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltotriose	-	-	-	-	-	w	+	w	w	+	+	+	+	+	+	+	w	-
D-Psicose	-	-	w	-	w	-	w	-	-	-	-	w	w	-	w	-	w	-
Succinic acid	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	w	w	-	-	w	w	w	w	+	w	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-
Succinic acid mono-methyl ester	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	w	w	+	+	w	-
L-Aspartic acid	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-
Palatinose	-	+	+	-	-	+	+	+	+	-	w	w	w	w	+	w	w	-
Mannitol	-	-	-	-	-	+	+	w	+	-	w	+	w	-	+	-	w	-
L-Glutamic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-
D-Raffinose	w	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	w	-
D-Sorbitol	-	-	-	-	-	+	w	w	w	w	w	+	w	-	w	-	w	-
L-Proline	-	-	-	-	-	-	-	w	-	w	w	+	w	-	w	-	-	-
Stachyose	w	-	w	w	w	-	-	-	-	-	-	-	-	-	w	-	w	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-	w	-
D-Gluconic acid	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-
Dextrin	-	-	w	-	w	w	w	w	-	w	w	w	w	w	w	-	w	-
D-Trehalose	-	-	-	w	-	+	+	+	+	-	-	w	-	-	w	-	+	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	w	-
Inulin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Turanose	+	-	-	+	+	+	+	+	+	w	w	+	+	+	+	+	-	-
Tween 80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

“+” means positive response, “-” means negative response, and “w” means weak positive response.

when applied to either liquid or solid marine samples. This method took 8–10 days to obtain a large number of pure

yeast colonies. It was successfully applied to samples up to 2 weeks old, so fresh samples are not necessary. This

Table 5. Comparison of assimilation tests (using YT plates for yeast identification) of 17 marine yeast isolates and the terrestrial *S. cerevisiae* NCYC 2592.

	Yeast Strains																	
	<i>S. cerevisiae</i>					<i>C. tropicalis</i>				<i>C. viswanathii</i>				<i>W. anomalus</i>		<i>C. glabrata</i>	<i>P. kudriavzevii</i>	
Assimilation test (substrate)	Ref	S69	S71	S117	S118	S45	S57	S62	S68	S1	S7	S8	S10	S80	S115	S116	S84	S83
D-Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-
N-Acetyl-D-glucosamine	-	-	-	-	-	+	+	+	+	w	w	+	w	-	-	-	-	-
Maltitol	-	-	-	-	-	w	w	w	w	-	-	w	-	w	w	w	-	-
D-Xylose	-	-	-	-	-	-	-	-	-	-	w	-	w	-	-	-	-	-
Fumaric acid	-	-	-	-	-	w	w	w	w	w	w	w	w	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-
D-Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
D-Mannitol	-	-	-	-	-	w	w	+	+	-	w	w	w	-	w	w	-	-
Succinic acid + D-xylose	-	-	-	-	-	-	-	-	-	w	w	w	w	-	w	w	-	w
L-Malic acid	-	-	-	-	-	w	w	w	w	w	w	w	w	-	-	-	-	-
Maltose	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
α-D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	w	-	w	w	-	-	w	-	-	w	-	-	-
N-Acetyl-L-glutamic acid + D-xylose	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-
Succinic acid mono-methyl ester	-	-	-	-	-	w	-	-	-	-	-	+	-	-	-	-	-	-
Maltotriose	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-
D-Galactose	+	+	+	+	+	+	w	w	w	w	w	w	w	-	w	w	-	-
Adonitol	-	-	-	-	-	w	w	w	w	-	w	w	w	-	w	-	-	-
Quinic acid + D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-
Bromosuccinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	w	w	w	w	w	w	w	-	w	w	w	-	-
D- Psicose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-
D-Glucuronic acid D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Glutamic acid	-	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dextrin + D-xylose	-	-	-	-	-	w	-	-	-	-	w	w	w	-	-	w	-	-
γ-Aminobutyric acid	-	-	-	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-
Palatinose	-	+	+	-	-	+	+	+	+	w	-	+	+	w	w	w	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-
α-D-Lactose + D-xylose	-	-	-	-	-	-	-	-	-	-	w	-	w	-	w	-	-	-
α-Ketoglutamic acid	-	-	-	-	-	w	w	w	w	-	-	-	-	-	-	-	-	-
D-Raffinose	w	w	w	w	w	-	-	-	-	-	-	-	-	w	+	+	w	-

Table 5. Continued.

	Yeast Strains																	
	<i>S. cerevisiae</i>					<i>C. tropicalis</i>				<i>C. viswanathii</i>				<i>W. anomalus</i>		<i>C. glabrata</i>	<i>P. kudriavzevii</i>	
Assimilation test (substrate) ²	Ref	S69	S71	S117	S118	S45	S57	S62	S68	S1	S7	S8	S10	S80	S115	S116	S 84	S 83
α -Methyl-D-glucoside	-	-	-	-	-	w	w	w	w	w	-	w	w	w	w	w	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Melibiose + D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Keto-D-gluconic acid	-	-	-	-	-	w	w	+	w	w	+	+	+	-	-	-	-	-
Stachyose	w	-	-	w	w	-	-	-	-	-	-	-	-	-	-	w	-	-
β -Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	w	-	-
Tween 8-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Galactose + D-xylose	+	+	+	+	+	+	w	w	w	w	w	w	w	-	w	w	+	-
D-Gluconic acid	-	-	-	-	-	w	-	w	w	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m-Inositol + D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
Dextrin	-	-	-	-	-	-	-	-	-	-	w	w	w	-	-	-	-	-
D-Trehalose	-	-	-	-	-	+	+	+	+	-	w	w	-	-	w	-	+	-
Arbutin	-	-	-	-	-	-	-	-	-	-	-	-	-	w	+	+	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propanediol + D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-
Inulin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Turanose	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Salicin	-	-	-	-	-	-	w	-	-	-	-	-	-	w	w	w	-	-
D-Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoin + D-xylose	-	-	-	-	-	-	-	-	-	w	-	-	w	-	w	-	-	-

methodology allows for the isolation of yeasts that are present at very low numbers in the original sample.

Screening and Metabolism Evaluation Using Phenotypic Microarray Assay

Conversion of monomeric sugars into commercially valuable products is a desirable trait for any yeast strain for potential industrial application. In this study, PM assays have been used to screen for novel marine-derived yeasts with high capability for the utilization of the monomeric sugars, including glucose, xylose, galactose, and mannitol, in a seawater-based medium. *S. cerevisiae* NCYC 2592, a strain that has high metabolism capacity and high tolerance to a wide range of inhibitors presented in the lignocellulosic hydrolysate [29, 44], was also used in the PM assays as a control to evaluate the isolated marine yeast for their metabolism capacity. The results showed that

S. cerevisiae NCYC 2592 grew well in the seawater-based medium. However, 11 marine isolates have been identified that performed better than *S. cerevisiae* NCYC 2592 in terms of glucose utilization in seawater-based media. This may be due to the fact that the marine yeasts acquired high tolerance to salts and other inhibitors, which exist in seawater; therefore, their growth was not disturbed by the presence of high level of salts in the medium. As expected, the xylose utilization experiments revealed that the reference strain did not grow [40, 44]. However, many of our marine isolates showed good xylose utilization and they could be potential candidates for bioethanol fermentations using xylose as the substrate.

Isolating yeast strains with the capacity for utilizing mannitol is of particular interest. Mannitol is considered as the most abundant sugar alcohol in nature [2] and has been highlighted as a potential bulk marine sugar [33] as it is

Table 6. Comparison of the three marine yeast isolation methods used in this study.

	Zaky's method (developed in this study)	Kutty's method ^a	Dinesh's method ^b
Sample preparation	Enrichment 3 cycles (48 h each)	Filtration filters (0.45 µm)	-
Media ^c	Zaky's media (enrichment, isolation broth, isolation agar)	Wickerham's medium	Sabouraud's Dextrose Agar
Antibiotic	Penicillin G + streptomycin sulfate (500 mg/l each)	Chloramphenicol (200 mg/l)	-
pH	5.0	7.0	5.6
Incubation temp.	30°C	18 ± 2°C	35°C
Total time	10 days	16 days	4 days
Sample size	10 g or 100 ml	30 ml	1 g
Sample	Up to 2-week-old samples	New samples only	New samples only
Number of isolates	Very high	Low	Very low
Specificity	Specific to yeasts	Specific to fungi	Not specific
Applicability	Suitable for both liquid and solid marine samples	Fresh liquid samples	Fresh solid samples

^aThis method was carried out based on the procedure suggested by Kutty [22].

^bThis method was carried out based on the procedure suggested by Dinesh *et al.* [7].

^cDetailed medium composition is reported in Materials and Methods.

found in different types of marine algae, such as brown and golden algae, and red algae [14]. PM assay results revealed that *S. cerevisiae* NCYC 2592 cannot utilize mannitol. Many marine yeasts isolated in the current study had high mannitol utilization, especially isolate S45, which showed the highest mannitol consumption; this strain may have potential to be used as an industrial strain for converting mannitol to value-added products. It was reported that mannitol is rapidly metabolized by bacteria in marine habitats [17]. White *et al.* [42] suggested that several herbivorous fish cannot assimilate mannitol directly but it can be utilized as an indirect nutrient via fermentation in the hindgut. It has been reported that mannitol plays an osmoregulatory role in algae that experiences significant changes in salinity [2, 14]. Therefore, we suggest that marine yeast that are able to ferment mannitol are of particular importance as candidates for marine fermentation (fermentations that use marine yeast, marine biomass, and seawater), not only because mannitol is widely available in marine biomass but also because mannitol is expected to weaken the inhibitory effects of seawater's salinity.

Galactose is another sugar that is present in marine biomass substrates, such as red seaweed [18]. Isolate S45 showed the highest utilization level among the marine isolates and was higher than the reference strain. This makes it a potential candidate for bioethanol production from marine substrates such as seaweed. Galactose

utilization is of particular interest as most yeast galactose utilization is suppressed by the presence of glucose [30], and research may lead to identification of marine yeast that are capable of efficient utilization of both glucose and galactose simultaneously.

Biomass hydrolysates are characterized by a complex mixture of sugars. Efficient conversion of these sugars into end-metabolites has been highlighted as a desirable phenotype. PM assays revealed that marine-derived yeasts have good overall sugar utilization capabilities, and several marine yeast isolates have the capacity for utilizing all four of these sugars.

Fresh water is traditionally used in fermentations. However, many parts of the world struggle to produce enough potable water for human consumption. There are only limited studies using seawater for fermentation [24]. PM assays revealed that the sugar utilization by marine yeasts was almost identical after 24 h of incubation in experiments using seawater and using fresh water. These results indicated that the presence of salt was not deleterious to sugar utilization by marine yeasts.

Genetic Identification of Marine Yeast Isolates and Phenotypic Characterization

New isolates of marine yeasts were identified using the same procedures as used for terrestrial yeasts, as no specific procedure for marine yeast identification has yet been reported. Genetic identification methods (ITS and

D1/D2) successfully identified our marine-derived yeasts and that proves they have terrestrial origin (as they share the same genotype). This finding supported previous reports that the majority of marine yeasts, including those isolated from deep-sea regions, are not indigenous [3, 16]. We found that genus *Candida* was more common in the marine environment compared with other yeast genera, which agrees with several previous studies [9, 34]. *S. cerevisiae*, *C. tropicalis*, *W. anomalus*, and *C. glabrata* were previously isolated from different marine habitats [12, 28]. *C. viswanathii* was recently isolated from deep-sea hydrothermal animals [3]. *P. kudriavzevii* has not previously been isolated from a marine environment.

In this study, the identification of the 17 isolates using YT MicroPlate did not match the genetic identification (Table 3). This could be due to the fact that the Biolog database was based on substrate utilization by terrestrial yeast strains only. YT Biolog is commercially available and is a mature method for yeast identification [31, 32]. It was reported that 49 out of 72 yeasts that have been isolated from foods and beverages were correctly identified using the Biolog system [32]. However, in order to obtain a valid identification, a large database is needed. Marine yeasts have inherited new characteristics for living in a marine habitat and so have altered phenotypes. This means that available biochemical-based identification methods such as YT Biolog plates maybe not be suitable for identification of marine yeasts using the terrestrial yeast-based database. Foschino *et al.* [10] reported that MicroPlate YT Biolog failed to identify yeast isolates from sourdough samples probably because their database was based on clinical yeast isolates. If an intensive database could be built based on substrate utilization in marine yeasts, the YT Biolog plate could be used for marine yeast identification as well.

In general, identification using sequence data has been shown to be more robust than using phenotypic data. However, it has been useful in highlighting the metabolic requirements of marine microorganisms with interesting industrial capabilities [37]. The advancement of the D1/D2 and ITS database in GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) allows laboratories around the world to easily and accurately identify more yeast species. Furthermore, phylogenetic analysis of the gene sequences is leading to a major modification of yeast systematics that will result in redefinition of almost all genera [21]. It is worth mentioning that the PM screening assay revealed that several marine yeast isolates had the capacity for utilizing xylose or mannitol (Fig. 3). However, use of YT MicroPlate revealed no utilization of these compounds (Tables 4 and

5). This inconsistency could be due to the differences of the culturing conditions, including the concentrations of the tested sugars, the incubation temperature, and the inoculation level. In addition, the PM assay used liquid media whereas the YT MicroPlate used solid media.

In conclusion, the limited availability of marine yeast strains is one of the key challenges that restricts the development of a marine-yeast-based industry. This work developed an efficient and selective method for marine yeast isolation. Using this method, 116 marine yeast isolates were selected from 14 marine samples collected from different regions around the world. These isolates were then screened for their sugar utilization ability. Seventeen strains representing the best utilizer for each sugar were subjected to identification using the PCR-based method. These 17 strains belonged to six different species, including *P. kudriavzevii*, which has not previously been reported as a marine yeast. Phenotypic microarray assay using YT Biolog MicroPlate was found to be a useful technique for strain discrimination but not for identification of marine yeasts.

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