CHARACTERIZATION OF MARINE YEASTS ISOLATED FROM DIFFERENT SUBSTRATES COLLECTED IN CALATAGAN, BATANGAS

CARLY SIMON P. RAMIREZ[1], CONNEL O. GO[1], SOPHIA ANGELICA S. HERNANDEZ[1], HANAH I. RUIZ[1], MAUREEN B. SABIT[1] AND THOMAS EDISON E. DELA CRUZ[1,2]

[1] Department of Biological Sciences, College of Science, and
[2] Research Center for Natural Sciences,
University of Santo Tomas, España 1015 Manila, Philippines
Corresponding author email: *tedelacruz@mnl.ust.edu.ph*

ABSTRACT

Marine yeasts can be isolated from various substrata. Their potential industrial application merits a thorough study of these microorganisms. Thus, our research study aimed to isolate and characterize marine yeasts from several substrata collected from Calatagan, Batangas. Marine yeasts were isolated using enrichment culture of seawater, marine sediments, and living and decaying seagrasses and seaweeds on Glucose-Yeast Extract-Peptone broth (GYPS) supplemented with 33 g/L marine salts. Following incubation at room temperature, a total of 13 yeast strains were isolated. Most of the marine yeasts were obtained from either living or decaying seagrasses. Growth on GYP Broth with and without salt showed that six out of the 13 isolates were able to grow in the presence or absence of marine salts. Characterization of the isolated marine yeasts was done using the conventional morphological and biochemical methods and modern molecular techniques. All marine yeasts have cream-colored, circular and convex colonies with entire margin. The cells were spherical with a size range of 2.5 – 12.5 µm. They utilized galactose, maltose, sucrose, trehalose, xylose, sorbitol, N-acetyl-glucosamine and 2-keto-gluconate. Only one isolate (PCL08-LG04) utilized glycerol, arabinose, cellobiose, and raffinose. Comparison of the API 20C Aux profiles with the API database indicated that the isolated marine yeasts have affinity to the genus *Candida*. Gene sequence analysis of their ITS 1 and 2 regions of nuclear rDNA identified the isolates as *Candida tropicalis* and *Pichia carribica.*

KEYWORDS: marine yeasts, enrichment culture, polyphasic taxonomy, gene sequence analysis

INTRODUCTION

Marine fungi are those that grow and/or sporulate in estuarine or marine habitats (Kohlmeyer, 1974). At present, a total of 530 species belonging to 321

genera are considered marine (Jones *et al*., 2009). Included in the list are at least 171 species of marine yeasts (Kohlmeyer & Kohlmeyer, 1979). A majority of these marine yeasts was isolated from seawater, often collected near shores or along intertidal zones (Roth *et al*., 1962). Among the commonly isolated marine yeasts are species of *Rhodotorula, Candida, Trichosporon, Debaryomyces* and *Cryptococcus* (Roth *et al*., 1962). Though known as terrestrial yeast genera, these species were able to adapt in environment with high salinity (Butinar *et al*., 2005). Yeasts also account for the majority of the fungal diversity in deep sea environments (Bass *et al.*, 2007), with *Cryptococcus curvatus* as the dominant species (Takishita *et al.*, 2006). Seagrasses, seaweeds and marine sediments also harbored marine yeasts (Kutty & Philip, 2008). For example, Raghukumar *et al.* (1992) isolated 171 and 106 yeasts from the algae *Ulva fasciata* and *Centroceras clavulatum*, respectively. Kutty & Philip (2008) also described species of *Candida, Cryptococcus, Debaryomyces* and *Rhodotorula* from seawater, marine deposits and seaweeds. In Asia, Wang *et al*. (2007) isolated a species of *Candida* from the China Eastern Sea while Sheng *et al*. (2007) isolated a species of *Cryptococcus* from the South China Sea.

Interest on marine yeasts increased recently mainly due to the potential of marine fungi as sources of enzymes with industrial applications and biologically active natural products (Liberra & Lindequist, 1995; König & Wright, 1996). For example, *Pichia guilliermondii* produced inulinase, which hydrolyzes inulin into fructose (Yu *et al*., 2008) while *Kodamaea ohmeri* produced phytase which catalyses the release of phosphate from phytate (Li *et al*., 2008). Another marine yeast, *Aureobasidium pullulans*, also produced phytases. Phytases can be used in manufacturing detergents, in leather and food processing, in silver recovery and waste treatment, and for medical, feed and chemical industrial purposes (Chi *et al*., 2006). *A. pullulans* was also capable of producing siderophore with antimicrobial activities against pathogenic *Vibrio anguillarum*, *V. parahaemolyticus* and *Bacillus subtilis* (Wang *et al*., 2009). Wang *et al*. (2006) also showed biological activity of the marine yeast *Pichia anomala* against the pathogenic yeast *Portunus trituberculatus.* In spite of this economic importance, very few studies explore marine yeasts in the Philippines. Thus, our research study aims to isolate and characterize marine yeasts from various substrata collected in Calatagan, Batangas. Identification was done by conventional morphocultural and biochemical tests and modern molecular method.

MATERIALS AND METHODS

Collection of Substrates

Substrates for the enrichment culture of marine yeasts were collected from Brgy. Dos, Calatagan, Batangas on June, 2008. Collected seawater and marine sediments were placed in pre-sterilized bottles. Decaying seagrasses (*Enhalus*

acoroides) and seaweeds (*Kappaphycus* sp.) were also collected along the shores and placed immediately in zip-locked plastic bags. Healthy and living seagrasses and seaweeds were also collected along intertidal zones and placed in zip-locked plastic bags. All collected samples were then stored inside a cooler box, transported to the laboratory and processed within 24 h of collection. Herbaria were also prepared for the collected seagrasses and seaweeds and were sent to the University of Santo Tomas – Research Center for the Natural Sciences Herbarium for identification and verification.

Enrichment Culture and Isolation of Marine Yeasts

For the enrichment culture of marine yeasts, seagrasses and seaweed samples were initially washed with sterile artificial seawater and cut into 5 mm^2 explants. The explants (five explants per tube) were then inoculated onto 25 x 200 mm culture tubes (three tubes per sample) pre-filled with 10 ml Glucose-Yeast Extract-Peptone medium (GYPS: glucose, 20g/L; yeast extract, 1 g/L; peptone, 5 g/L) supplemented with 33g/L marine salts (Biomix Seawater Formula, BioResearch) and antibiotics: 300 mg/L tetracycline (Sigma) and 180 mg/L streptomycin (Sigma) in order to prevent the growth of bacteria. For the seawater and marine sediments, one milliliter of seawater and one gram of soil samples were inoculated on the enrichment culture tubes pre-filled with 10 ml GYPS in triplicates. All culture tubes were incubated at room temperature for 24 – 48 h and observed for turbidity.

Following incubation, enrichment culture tubes exhibiting growth/turbidity were then streaked on freshly prepared GYPS agar plates and incubated at room temperature for 24 – 48 h. Subsequent subculture was done on freshly prepared GYPS agar plates until pure cultures were obtained.

Characterization and Identification of Isolated Marine Yeasts

For morphocultural characterization, isolated marine yeasts were initially cultured on GYPS agar plates without antibiotics. Inoculated culture plates were then incubated at room temperature for 24 – 48 h. Following incubation, colony descriptions, e.g. color, surface appearance, margin and elevation, were determined. For cell morphology, isolated colonies for each of the marine yeast isolates were stained with crystal violet for 1 min. The stained cells were then observed and measured under the microscope (400-1,000x, Olympus CX31) to determine cell shape and sizes.

To determine the ability of our isolated yeasts to grow in marine habitats, selected strains were initially grown on GYPS agar plates at room temperature for 48 h. Following incubation, a loopful of yeast colonies were then suspended in sterile artificial seawater (ASW) and the cell suspension adjusted to a

concentration equivalent to 0.5 MacFarland Standard. One hundred microliter of the adjusted cell suspension was inoculated on culture vials pre-filled with 30 ml GYP broth (in triplicates) with or without 33 g/L marine salts. All culture vials were incubated at room temperature for 48 h. Cell biomass was then harvested by filtration with pre-weighed filter paper and oven-dried for one week at 75 ^{o}C or until the weight remained constant. Dried cell biomass was then determined. Statistical analysis (One Way ANOVA) was done using SigmaStat 3.1 (Systat Software Inc., USA) to determine their significant differences.

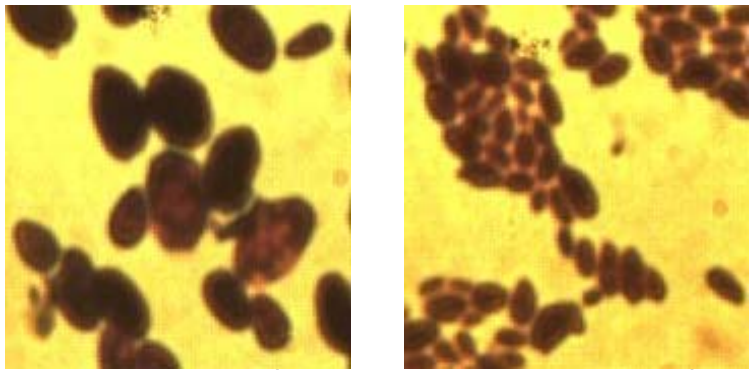
The isolated marine yeasts were also tested to determine their ability to utilize various carbon sources. Isolates were initially inoculated on GYPS agar as previously described. Then, cell suspension was prepared with sterile ASW and adjusted to 1 McFarland Standard. Then, one hundred microliters of the adjusted cell suspension were added to API C Medium (bioMerieux, Inc., France). The cupules of the API 20C Aux were inoculated with the prepared inoculum. The API set-up was then incubated at room temperature for 48 - 72 h. After incubation, a cupule more turbid than the control indicated a positive reaction and was recorded on the API result sheet. Results obtained were compared and analyzed with the database using the API Web to obtain preliminary identification of the yeast.

To confirm the identity of the isolated marine yeasts, genomic DNA of selected strains were then extracted and sequenced. Initially, selected marine yeast strains were grown on GYPS broth at room temperature for 48 h. Following incubation, the culture tubes were transferred onto sterile Falcon Tubes and centrifuged for 5 min at 10,000 rpm. The harvested cells were then macerated in liquid nitrogen and the genomic DNA extracted using the DNeasy® Plant MiniKit (Qiagen GmbH, Germany). Extracted DNA was stored at -20^{o}C until use in gene sequence analysis. PCR amplification of the nuclear DNA was done as previously described (Turner *et al.,* 1997; Kullnig-Gradinger *et al.,* 2002). The extracted genomic DNA was PCR-amplified with the fungal specific primer combinations for the internal transcribed spacers 1 and 2 (ITS 1 and 2) regions including the flanking 5.8S rRNA gene: SR6R (5'-AAG TAG AAG TCG TAA CAA GG - 3') and LR1 (5' - GGT TGG TTT CTT TTC CT - 3'). The PCR amplification parameters were as follows: initial denaturation (94 ^{o}C, 1 min), 30 cycles (94 ^{o}C, 1 min; 50 ^{o}C, 1 min, 72 ^{o}C, 90 sec) and final extension (72 ^{o}C, 7 min). The PCR products were then purified with the commercially available QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany), and the PCR products (10 µl) loaded on 1 % agarose gel (300ml) mixed with 6 µl ethidium bromide in TAE buffer. Gel electrophoresis was set up at 80 V for 80 min and the DNA bands were visualized with UV. The PCR products were then sent for outdoor sequencing (Macrogen Inc., Seoul, Korea). Unaligned sequences were then aligned with BioEdit Sequence Alignment Editor. Forward aligned sequences were used in a BLAST search (NCBI

BLAST) in order to find similarities with other sequences of fungi. Sequences obtained from the related sequences were then sent to Dr. Irina S. Druzhinina, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria for alignment and construction of a dendogram. The relationship of the isolated marine yeasts with the sequences found in NCBI GenBank was determined based on the dendogram.

RESULTS

A total of thirteen marine yeast strains were isolated from marine sediments, seawater and living and decaying seaweeds (*Kappaphycus* sp.) and seagrasses (*Enhalus acoroides*). Three isolates were obtained from sediments and one strain was isolated from seawater. One yeast strain was isolated from both living and decaying seaweed. Two strains were obtained from living seagrasses while five strains were isolated from decaying seagrasses. All isolates appeared as cream-colored, circular and convex colonies with entire margin on agar (Fig. 1.0). The cells were spherical in shape with a size range of 2.5 – 12.5 μm.



A **B**

Figure 1.0. Large, spherical cells of marine yeasts: (A) PCL08-DG02, 7.5 - 10μm in diameter and (B) PCL08-LG04, 2.5 - 12.5 μm in diameter.

Representative six strains of marine yeasts (PCL08-DG02, PCL08-DA03, PCL08-LG04, PCL08-SD02, PCL08-SD03 and PCL08-SW04) were then tested for their growth in the absence or presence of marine salts. These six strains were chosen from each of the collected substrates. Results showed the ability of all tested marine yeasts to grow equally well in the absence or presence of 33 g/L marine salts, the concentration equivalent to natural seawater (data not shown). This shows that the isolated yeasts were capable of growing in saline environments as facultative marine organisms.

All thirteen isolated marine yeasts were also tested for their carbon source utilization using the API 20C Aux. The isolated marine yeasts utilized different sugars as C sources: (1) hexose/monosaccharide: glucose, galactose and xylose, (2) disaccharide: maltose, sucrose and trehalose, and (3) sugar alcohol: sorbitol. N-acetyl-glucosamine (amide/monosaccharide) and 2-keto-gluconate were also utilized by the isolated marine yeasts. Only one yeast isolate (PCL08-LG04) utilized glycerol (sugar alcohol), arabinose (monosaccharide), cellobiose (disaccharide) and raffinose (trisaccharide) as sole carbon sources. Comparison of the isolated marine yeasts with the API database identified all of the isolates except one (PCL08-LG04) as *Candida tropicalis*. These strains of *C. tropicalis* were isolated mainly from sediments, seawater, seagrasses, and seaweeds. One isolate from a living seagrass was identified only up to the genus level as *Candida* sp.

To confirm further the identity of the isolated marine yeasts, genomic DNA was then extracted for two species, one representative (PCL08-DG02) for all yeast isolates characterized as *Candida tropicalis* and an isolate characterized as *Candida* sp., to find its closest taxonomic neighbor. The internal transcribed spacer region (ITS 1 and 2) with the flanking 5.8S genes showed that the yeasts belong to the genus *Candida*. The sequences of the two marine yeast isolates, PCL08-DG02 and PCL08-LG04, were compared with other sequences found in the NCBI GenBank. Blast searches identified PCL08-DG02 as *Candida tropicalis* and PCL08-LG04 as *Pichia caribbica* with 88-94% and 92% homologies, respectively (Fig. 2.0).

DISCUSSION

Marine yeasts can be isolated from numerous substrates including seawater, sediments, algae and seagrasses, fishes, crustaceans, and even lower invertebrates (Roth *et al*., 1962). In our research study, thirteen marine yeast strains were isolated from four substrata: seawater (1), marine sediments (3), seaweeds (2) and seagrasses (7) (Table 1). Raghukumar (2008) reported that marine fungi play a significant role in the degradation of macrophytes, including seaweeds and seagrasses. Thus, this could explain the isolation of yeasts from these substrates. Seawater and sediments also yielded yeast isolates. Roth *et al*. (1962) noted that ocean waters contain yeasts, though it had lower numbers as compared to other substrates, e.g. seagrass flats and sediments.

Morphocultural characterization of the isolated marine yeasts showed cream-colored, circular and convex colonies with large, spherical cells with sizes ranging from 2.5-12.5 µm (Fig. 1.0). Most of the isolated marine yeasts grew equally well in the presence or absence of marine salt (data not shown). The growth of our yeast isolates on medium with marine salts indicated their adaptation and ability to grow in marine habitats. For biochemical

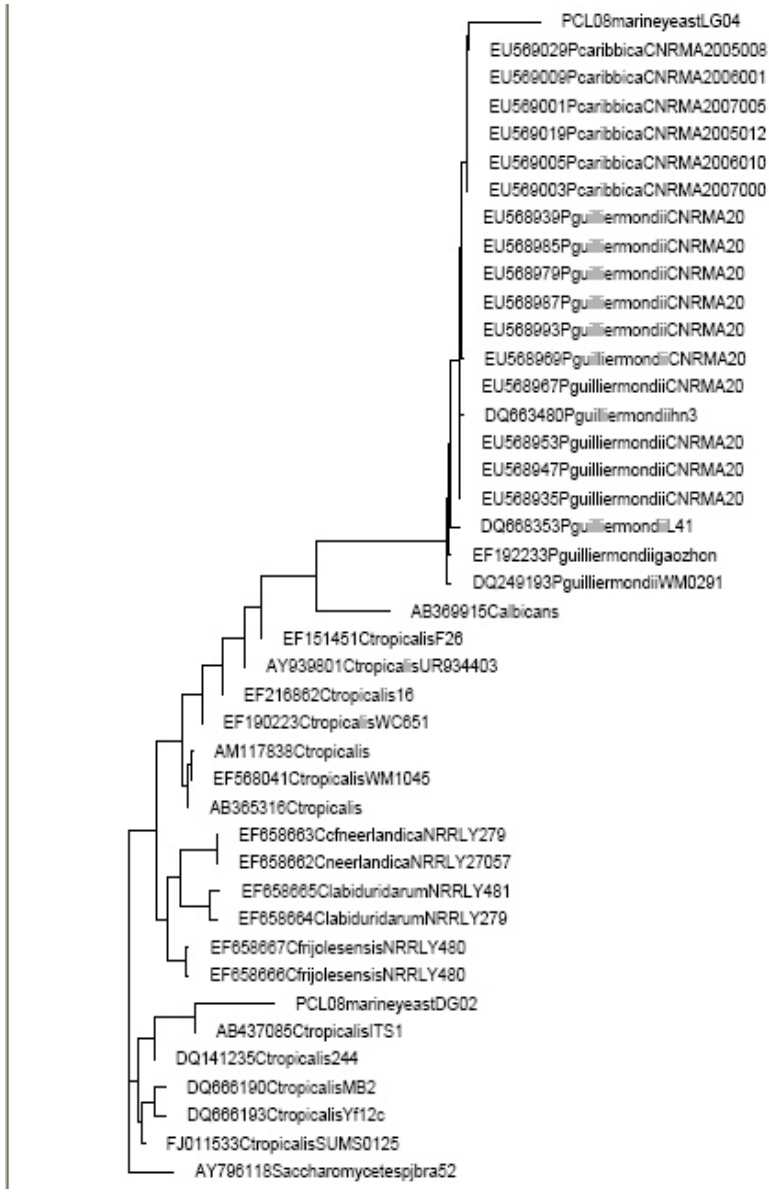


Figure 2.0. Dendrogram showing the isolated marine yeasts and their related species as inferred from their ITS 1 and 2 gene sequence analysis.

characterization, we tested the ability of the isolates to utilize various carbon sources using the API 20C Aux. Results suggest that the isolated marine yeasts were able to utilize various carbon sources, e.g. glucose, galactose, maltose, sucrose, trehalose, xylose, sorbitol, N-acetyl-glucosamine, 2-keto-gluconate, glycerol, arabinose, cellobiose, and raffinose, but failed to grow on xylitol, inositol, and lactose (Table 1). Sheperd & Sullivan (1975) also showed that *Candida* species were able to utilize various carbon sources for growth. The utilization of these carbon sources affects the morphology of yeasts. Comparison of their profiles with the API 20C Aux database identified our yeasts isolates as *Candida* spp. Twelve yeasts strains were identified further as *Candida tropicalis,* though with a low discrimination rate of 70.2%. *C. tropicalis* was reported as facultative marine yeast that is common in seawater near grass flats and in marine areas subjected to recreational bathing (Roth *et al*., 1962). Our collection site, Barangay Dos in Calatagan, Batangas, is near residential areas and harbors many seagrasses and seaweeds along its coastal waters. On the other hand, one isolate could be identified only as a *Candida* sp. (PCL08-LG04). Due to the low discrimination rate using the API 20C Aux, we chose to confirm the identity of our yeasts isolates with modern molecular methods. The use of molecular methods can provide a more rapid and accurate technique in identifying cryptic species and may be used to confirm identification based on morphological and physiological methods. In our research study, the ITS region of two representative strains were then sequenced and compared with the sequences found in the GenBank database. Comparison with the database identified one of the isolates as *Candida tropicalis* (PCL08-DG02) (Fig. 2.0). This identification is in agreement with the identification based on their API profile. Interestingly, the yeast strains initially identified as *Candida* sp. with the API database clustered well with *Pichia caribbica* (PCL08-LG04).

CONCLUSION

In conclusion, our research study characterized marine yeasts on various substrates from Calatagan, Batangas using a polyphasic approach. The conventional morphocultural methods and biochemical tests (API 20C Aux) identified the yeasts as *Candida* spp. Sequence analysis further confirmed our isolates as *C. tropicalis*, in agreement with the identification based on their biochemical characteristics. One isolate, initially identified as *Candida* sp. using the API 20C Aux profile, was later identified as closely related to *Pichia carribica.* The combined application of morphocultural, biochemical and molecular methods proved useful in the identification of marine yeasts.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Irina S. Druzhinina from the Institute of Chemical Engineering, Vienna University of Technology (Austria) for her

assistance in the DNA sequence analysis and to Assoc. Prof. Rosie S. Madulid for the identification of our seagrass and seaweed samples.

LITERATURE CITED

Bass D, Howe A, Brown N, Barton H, Demidova M, Harlan M, Li L, Sanders H, Watkinson SC, Willcock S, Richards TA. 2007. Yeast forms dominate fungal diversity in the deep oceans. *Proceedings of the Royal Society B* 274: 3069-3077.

Butinar L, Santos S, Spencer-Martins I, Oren A, Gunde-Cimerman N. 2005. Yeast diversity in hypersaline habitats. *FEMS Microbiology Letters* 244: 229-234.

Chi Z, Gao L, Gong F, Li H, Liu Z, Ma C, Wang X, 2006. Marine Yeasts and Their Applications in Mariculture. *Journal of Ocean University of China* 5 (3): 251-256.

Jones EBG, Sakayaroj J, Suetrong S, Somrithipol S, Pang KL. 2009. Classification of marine Ascomycota, anamorphic taxa and Basidiomycota. *Fungal Diversity* 35: 1-187.

Kohlmeyer J. 1974. On the definition and taxonomy of higher marine fungi. *Veroffentliche Institut Meeresforschung Bremerhaven Supplement* 5: 263-286.

Kohlmeyer J, Kohlmeyer E. 1979. *Marine Mycology: The Higher Fungi*. Academic Press, New York.

König GM, Wright AD, 1996. Marine natural products research: current directions and future potentials. *Planta Medica* 62: 193-211.

Kullnig-Gradiner, Szakacs G, Kubicek CP. 2002. Phylogeny and evolution of the fungal genus *Trichoderma* – a multigene approach. *Mycological Research* 106(7): 757-767.

Kutty SN, Philip R. 2008. Marine Yeast – a review. *Yeast* 25(7): 465-483

Li XY, Liu ZQ, Chi ZM. 2008. Production of Phytase by a Marine Yeast Kodamaea ohmeri BG3 in an oats medium: Optimization by Response Surface Methodology. *Bioresource Technology* 99: 6386-6390.

Liberra K, Lindequist U, 1995. Marine Fungi- a prolific resource of biologically active natural products? *Pharmazie* 50: 583-588.

Raghukumar C. 2008. Marine fungal biotechnology: an ecological perspective. *Fungal Diversity* 31: 19-35.

Raghukumar C, Nagarkar S, Raghukumar S. 1992. Association of thraustochytrids and fungi with living marine algae. *Mycological Research* 96: 542-546.

Roth FJ, Ahearn DG, Fell JW, Meyers SP, Meyer SA. 1962. Ecology and Taxonomy of Yeasts Isolated from Various Marine Substrates. *Limnology and Oceanography*. 7: 178-185.

Sheng J, Chi Z, Li J, Gao L, Gong F. 2007. Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. *Process Biochemistry* 42: 805-811.

Sheperd MG, Sullivan PA. 1975. The Production and Growth Characteristics of Yeast and Mycelial Forms of *Candida albicans* in Continuous Culture. *Journal of General Microbiology* 93: 361-370

Takishita K, Tsuchiya M, Reimer JD, Maruyama, T. 2006. Molecular evidence demonstrating the basidiomycetous fungus *Cryptococcus curvatus* is the dominant microbial eukaryotic in sediment at the Kuroshima Knoll methane seep. *Extremophiles* 10: 165–169.

Turner D, Kovacs W, Kuhls K, Lieckfeldt E, Peter B, Arisan-Atac I, Strauss J, Samuels GJ, Boerner T, Kubicek Cp. 1997. Biography and phenotypic variation in *Trichoderma* sect. *Longibrachiatum* and associated *Hypocrea* species. *Mycological Research* 101(4): 449-459.

Wang WL, Chi ZM, Chi Z, Li J, Wang XH. 2009. Siderophore production by the marine-derived *Aureobasidium pullulans* and its antimicrobial activity. *Bioresource Technology* 100: 2639-2641.

Wang L, Chi Z, Wang X, Ju L, Chi Z, Guo N. 2007. Isolation and characterization of *Candida membranifaciens* subs. *flavinogenie* W14-3, a novel riboflavin-producing marine yeast. *Microbiological Research* 163: 255-266.

Wang X, Chi Z, Yue L, Li J, Li M, Wu L. 2006. A marine killer yeast against the pathogenic yeast strain in crab (*Portunus trituberculatus*) and an optimization of the toxin production. *Microbiological Research* 162: 77-85.

Yu X, Guo N, Chi Z, Gong F, Sheng J, Chi Z. 2008. Inulinase overproduction by a mutant of the marine yeast *Pichia guilliermondii* using surface response methodology, inulin hydrolysis. *Biochemical Engineering Journal* 43: 266-271.

Strain	Substrata	Carbon Sources [a, b]															
		GLU	GLY	2KG	ARA	XYL	ADO	GAL	SOR	MDG	NAG	CEL	MAL	SAC	TRE	MLZ	RAF
PCLO8-SD01	Sediment	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-SD02	Sediment	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-SD03	Sediment	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+
PCLO8-SW04	Seawater	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-LA01	Living Seaweed	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-DA03	Decaying Seaweed	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-LG01	Living Seagrass	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-LG04	Living Seagrass	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PCLO8-DG01	Decaying Seagrass	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-DG02	Decaying Seagrass	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-DG02N	Decaying Seagrass	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-DG03	Decaying Seagrass	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-
PCLO8-DG04	Decaying Seagrass	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-

Table 1.0. Utilization of various carbon sources by the marine yeasts isolated from Calatagan, Batangas.

[a] GLU: glucose
GLY: glycogen
2KG: 2-keto-gluconate
ARA: arabinose
XYL: xylose
ADO: adonitol
GAL: galactose
SOR: sorbitol
MDG: methyl-D-glucopyranoside
NAG: N-acetyl-glucosamine
CEL: cellobiose
MAL: maltose
SAC: sucrose
TRE: trehalose
MLZ: melezitose
RAF: raffinose

[b] All 13 isolates failed to utilize xylitol, inositol and lactose.