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Minegishi H[a], Miura T[b], Yoshida Y[a], Usami R[a] and Abe F[b]

Phylogenetic analysis of pectin degrading yeasts from deep-sea environments

[a]Department of Applied Chemistry, Faculty of Engineering, Toyo University, 2100 Kujirai, Kawagoe 350-0815, Japan,
[b]Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

Corresponding author: Fumiyoshi Abe, abef@jamstec.go.jp
Phone: +81-46-867-9678, Fax: +81-46-867-9715

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Abstract Deep-sea yeast strains were isolated from mud samples collected in Sagami Bay (1,100-1,400 m) and the Japan Trench (4,500-6,500 m). All of the 46 yeast isolates were capable of growth at 24°C and atmospheric pressure, suggesting that these strains might originate neritic regions or land but survived in deep sea. Based on sequencing of 26S rRNA gene, 15 strains were classified into basidiomycetous yeasts including *Cryptococcus liquefaciens*, *Kondoa aeria*, *Rhodosporidium diobovatum*, *Rhodosporidium sphaerocarpum*, *Rhodotorula mucilaginosa*, *Rhodotorula dairenensis* and *Rhodotorula slooffiae*, and 8 strains were classified into ascomycetous yeasts including *Aureobasidium pullulans*, *Candida zeylanoides*, *Kluyveromyces nonfermentans*, *Metschnikowia bicuspidate* and *Williopsis saturnus*. Screening of the 46 isolates appeared to yield a high frequency of polygalacturonase (PGase) producers capable of degrading pectin. We suggest that deep-sea yeasts are new sources of PGase producers.

Keywords: Deep-sea yeasts, Polygalacturonase, Pectin

Introduction

Studies on yeasts in aquatic environments have focused on their application as organic pollution indicators. In general, the yeast population in oceans is lower than that in fresh water or on land[1,2]. The frequency of yeast occurrence is known to decrease with increasing ocean depth, except in sites colonized by marine invertebrates[3]. Nevertheless, new yeast species have been successfully isolated from deep-sea mud samples[3, 4], and some may be useful in industrial applications. The deep-sea is characterized by low nutrient levels, low temperature, and high hydrostatic pressure, and therefore microorganisms inhabiting such environments have developed the ability to survive under such extreme conditions. Mud samples are expected to be valuable resources for isolating extremophiles and useful enzymes that can function under extreme conditions[5,6].

Pectic compounds are polysaccharides originating in plants, with α-1,4-glycoside linkage of polymers of galacturonic acids. Bacteria and filamentous fungi produce pectin-degrading enzymes, which are essential in phytopathogenesis. Fungal polygalacturonases (PGases) have mainly been used in food industries, especially in the extraction and clarification of fruit juices. Today, the main source of PGases in industry is from the fungus *Aspergillus niger*[7]. However, commercial preparations of fungal origin contain a complex mixture of different enzymes with pectinolytic activity including endo- and exo-PGase, pectin lyase, and the undesirable pectin esterases and other nonspecific enzymes. In this sense, yeast PGases may have advantages over fungal enzymes and could offer an alternative to fungal ones for the supply of pure enzymes. Attempts have been made to isolate PGases from yeasts. The biochemical properties of the PGases were reviewed by Blanco et al.[8] The first PGase-producing yeast was *Saccharomyces fragilis* reported by Luh and Phaff[9]. A PGase produced by *Cryptococcus albidus* has been purified and biochemically characterized[10]. The biochemical characterizations of several PGases from yeasts have now been reported and these genes have been successfully expressed in *Saccharomyces cerevisiae*[11,12]. They are usually extracellular enzymes, showing differences in molecular mass, and most are glycoproteins[13,14]. In some species, the presence of

several isozymes has been demonstrated[8].

In our previous studies, two PGases designated p36 and p40 were successfully purified from deep-sea yeast strain N6 and biochemical properties of the enzymes were analyzed with respect to obtaining kinetic parameters and protein glycosylation[15]. However, the remainder of PGase producers and PGases have not been characterized. In this paper, we describe phylogenetic analysis of PGase-producing yeasts isolated from Sagami Bay and the Japan Trench.

Materials and methods

Isolation of deep-sea yeasts

The procedure for the isolation of deep-sea yeasts has been described previously[3,4,15]. Deep-sea sediment samples were obtained using core samplers installed in the submersibles *Shinkai 2000* and *Shinkai 6500* at a depth of 1,100–1,400 m in Sagami Bay and 4,500–6,500 m in the Japan Trench, respectively. Approximately 0.1 g of the sediment was directly spread on each YPD plate (1% w/v bacto yeast extract, 2% w/v bacto peptone, 2% w/v glucose) or YPP plate (1% w/v bacto yeast extract, 2% w/v bacto peptone, 1% w/v pectin) containing penicillin (50 mg/L) and streptomycin sulfate (50 mg/L). The plates were first incubated at 8°C for 2 weeks. After colonies appeared, incubation was continued at 15°C and then at 26°C, after which the colonies were collected.

DNA isolation and PCR amplification of 26S rRNA gene

For genomic DNA extraction, cells were grown on YM (0.5% bacto peptone, 0.3% bacto yeast extract, 0.3% bacto malt extract, 1% glucose) agar and suspended in 1 ml of extraction buffer (50 mM TrisHCl, 50 mM EDTA, 3% SDS, pH 8.0). The cells were disrupted by homogenization for 2 min in the presence of alumina. The resulting homogenate was extracted with an equal volume of phenol, followed by phenol-chloroform extraction. After ethanol precipitation, DNA was resuspended in 50 µl of TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 8.0).

The divergent D1/D2 domain of 26S rRNA gene was amplified with primers F63 (5'-GCATATCAATAAGCG GAGGAAAAG-3') and LR3 (5'-GGTCCGTGTTTC AAG ACGG-3')[16]. Amplification was performed for 30 cycles with annealing at 58°C for 1 min, extension at 72°C for 2 min, and denaturation at 94°C for 1 min. After PCR, the reaction mixture was treated with SAP (Amersham Biosciences, Corp., Piscataway, NJ, USA) and subjected to DNA sequencing using an ABI377 DNA sequencer**.** A phylogenetic tree was constructed using the neighbor-joining (NJ) method using Clustal X ver. 1.83 and NJ Plot[17].

The accession numbers for the D1/D2 domain of 26S rRNA gene of the deep-sea yeast strains were obtained from the DNA Data Bank of Japan (DDBJ) and are listed in legends of Fig. 1 and Fig. 2.

Identification of PGase-producing yeasts

To detect PGase production by yeasts, cells were incubated on SDP (0.67% Difco yeast nitrogen base, 1.25% polygalacturonic acid, 50 mM potassium phosphate, 0.5% agar, pH 5.5) plates at 24°C for 2 days. Then the plates were stained with 0.1% ruthenium red[18]. A deep purple halo is observed around a colony if the strain produces extracellular PGase.

Results

Phylogenetic relationships among PGase-producing deep-sea yeasts and related species

Isolation of deep-sea yeast strains was successfully performed using YPD medium containing glucose as a carbon source, although no colony was obtained using pectin-based YPP medium. Forty-six strains were isolated. Strains designated by "S" were obtained from Sagami Bay and those by "N" were obtained from the Japan Trench. The D1/D2 region of 26S rRNA gene was determined for all of the 46 strains and compared with published sequences of related species. The deep-sea yeast strains were distributed in basidiomycetous yeast genera *Cryptococcus*, *Kondoa*, *Rhodosporidium*, and *Rhodotorula* (Fig. 1), and ascomycetous yeast genera *Kluyveromyces*, *Williopsis*, *Candida*, *Metschnikowia*, and *Aureobasidium* (Fig. 2). Red yeasts such as the genera *Rhodosporidium* and *Rhodotorula* commonly occur in deep-sea mud samples[3,4]. In our preliminary observation, the deep-sea yeasts were capable of growth on poor nutrition, e.g., in 100-times diluted YPD medium. Accordingly, the high occurrence of red yeasts in deep-sea might be concerned with the ability to grow on poor nutrition.

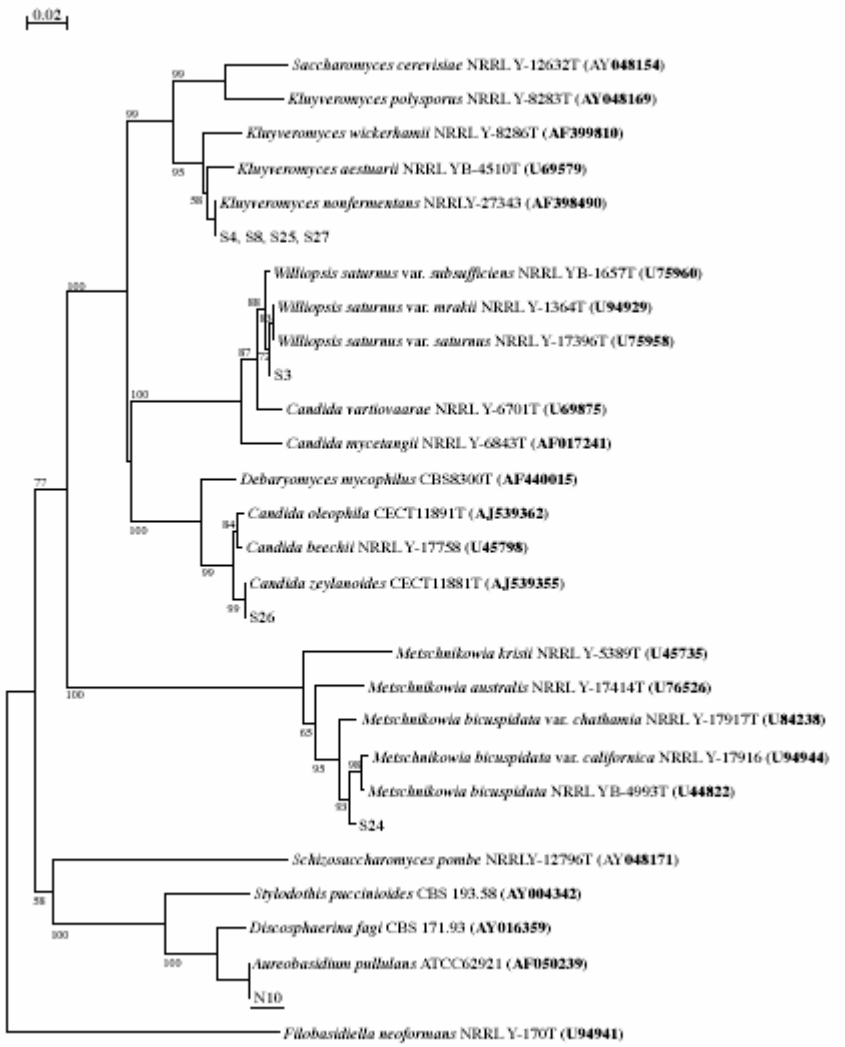

Fig. 2. Phylogenetic tree of 8 deep-sea ascomycetous yeasts and related species. The tree was constructed based on a total of 644 aligned nucleotide sites in the D1/D2 region of 26S rRNA gene. Numbers at branches are bootstrap values, derived only for the nodes supported by greater than 50% probability (100 replicates). A PGase-producing yeast-like fungus strain N10 is underlined. The accession numbers for the D1/D2 region of 26S rRNA gene are: AB217513 (S4), AB217514 (S8), AB217515 (S25), AB217516 (S27), AB217517 (S3), AB217518 (S26), AB217519 (S24) and AB217520 (N10).

All 46 yeast isolates together with some type strains were subjected to a PGase assay using ruthenium red staining to visualize degradation of PGA (Fig. 3). This yielded a high proportion of PGase-producing yeasts comprising 15 strains (Figs. 1 and 2, underlines). The PGase-producing deep-sea yeasts were classified into 3 species closely related to *Cryptococcus liquefaciens*, *Rhodosporidium diobovatum* and *Aureobasidium pullulans*. Notably, the type strains of *C. liquefaciens* NBRC0434^T and *R. diobovatum* NBRC1830^T, and a type strain of common marine yeast, *Crytptococcus albidus* IFO0378^T did not produce extracellular PGase as far as detectection using ruthenium red staining is concerned (Fig. 3). The result suggests that the production of PGase is likely to be a distinctive property of these yeast species that inhabit deep-sea environments. We noticed that a very small dilute halo was formed around the colony of *Crytptococcus liquefaciens* NBRC0434^T within 1mm (Fig. 3). This suggests that the type strain NBRC0434^T may produce a small amount of membrane bound- or cell wall associated-PGase.

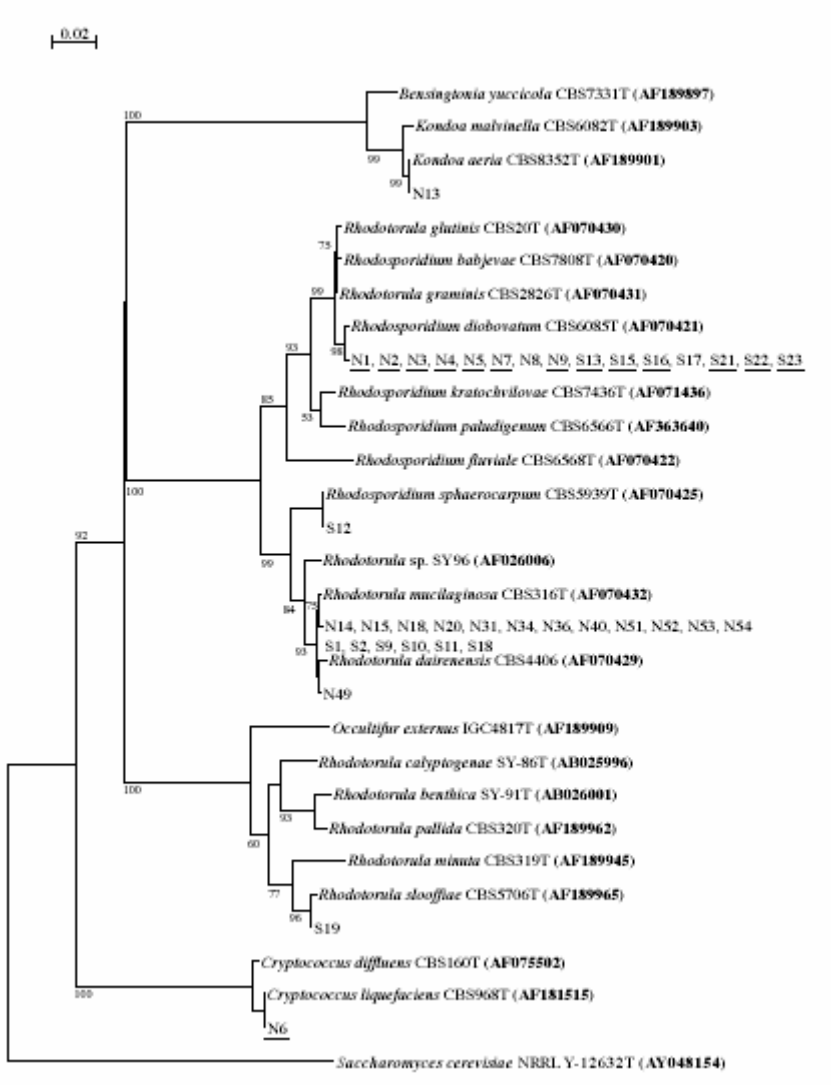

Fig. 1. Phylogenetic tree of 38 deep-sea basidiomycetous yeasts and related species. The tree was constructed based on a total of 644 aligned nucleotide sites in the D1/D2 region of 26S rRNA gene. Numbers at branches are bootstrap values, derived only for the nodes supported by greater than 50% probability (100 replicates). Fourteen PGase-producing yeasts designated by "S" or "N" are underlined. The accession numbers for the D1/D2 region of 26S rRNA gene are: AB217475 (N13), AB217476 (N1), AB217477 (N2), AB217478 (N3), AB217479 (N4), AB217480 (N5), AB217481 (N7), AB217482 (N8), AB217483 (N9), AB217484 (S13), AB217485 (S15), AB217486 (S16), AB217487 (S17), AB217488 (S21), AB217489 (S22), AB217490 (S23), AB217491 (S12), AB217492 (N14), AB217493 (N15), AB217494 (N18), AB217495 (N20), AB217496 (N31), AB217497 (N34), AB217498 (N36), AB217499 (N40), AB217500 (N51), AB217501 (N52), AB217502 (N53), AB217503 (N54), AB217504 (S1), AB217505 (S2), AB217506 (S9), AB217507 (S10), AB217508 (S11), AB217509 (S18), AB217510 (N49), AB217511 (S19) and AB217512 (N6).

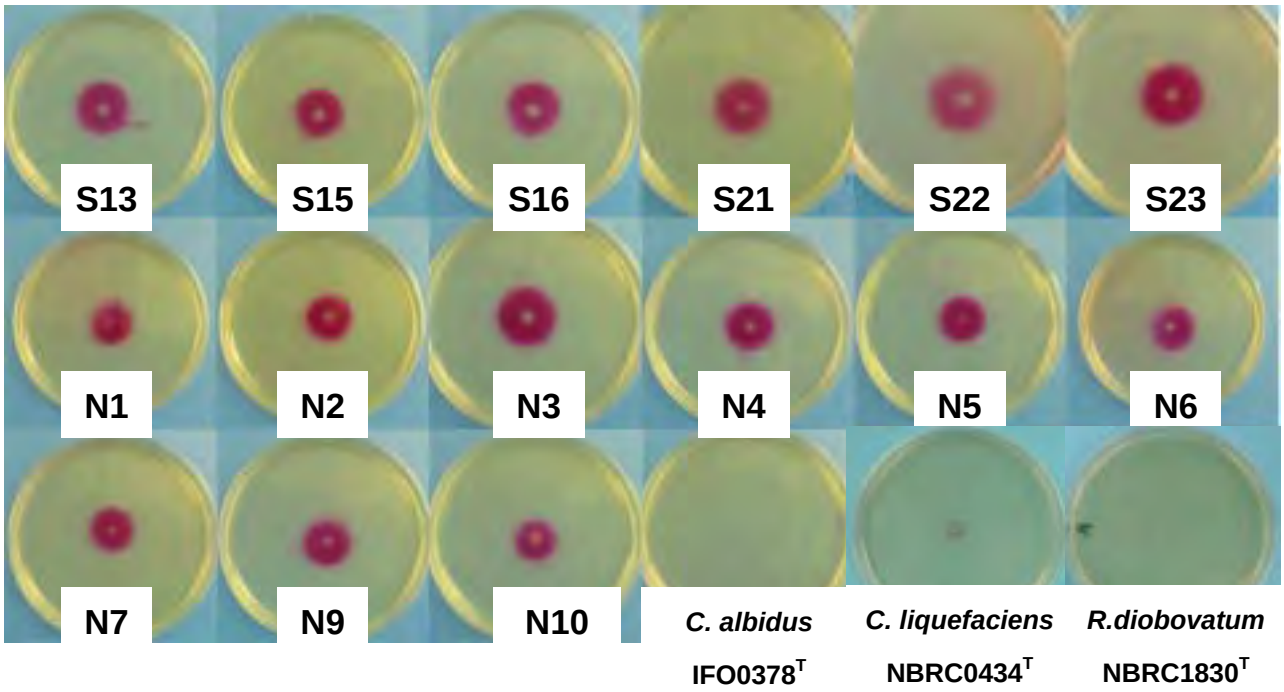

Fig. 3. Identification of PGase-producing yeasts on ruthenium red staining. Cells were incubated on SDP plates at 24°C for 2 days. A halo was visualized by staining the plate with 0.1% ruthenium red.

Discussion

Recently, the idea has emerged that PGases from a variety of yeasts could offer an alternative to fungal PGases for industrial applications, potentially providing pure and safe enzyme preparations[8]. Deep-sea sediment samples are new sources for the isolation of novel microorganisms and useful enzymes produced by the isolates. Of 46 deep-sea yeast isolates investigated, 15 strains were identified as PGase producers including *C. liquefaciens*, *R. diobovatum*, and *A. pullulans*. However, such a high occurrence of PGase-producing yeasts under deep-sea environments is still inexplicable. The deep-sea yeast isolates are capable of growth on poor nutrition (our unpublished observation). The availability of low nutrients for growth might be related to the availability of pectin, which is less favorable for growth of general fermentation yeast species.

In our previous study, two PGases designated p36 and p40 were successfully purified from *C. liquefaciens* strain N6[15], and were further characterized with respect to effects of high hydrostatic pressure and low temperature[19]. The production of PGase by *Rhodotorula* sp.[20] and *A. pullulans* ATHUM 2915[21] has been reported. It is worthwhile to analyze the phylogenetic relationships and PGases between our isolates and previously reported PGase producers. Federici reported the purification and characterization of a 41-kDa PGase from *C. albidus* var. *albidus* strain IMAT-4735[10]. Type strain of *C. albidus* IFO 0378^T did not produce PGase detectable using ruthenium red staining (Fig. 3). Therefore, production of PGase may be a distinctive property of strain IMAT-4735.

C. liquefaciens strain N6 was originally identified as a copper-tolerant yeast, and was characterized with respect to the role of superoxide dismutase[22,23]. In our preliminary result, a halo is formed on pectin-based agar containing 10 mM $CuCl_2$. Accordingly, the PGase of strain N6 might be copper tolerant. Cloning of the genes encoding both PGases is in progress. In our preliminary experiments, the supernatant from the culture of strain N6 efficiently dissolved natural orange peel. Therefore PGases of strain N6 are likely to be useful in food processing such as the clarification of juices. We suggest that deep-sea yeasts are new sources for the supply of industrially useful enzymes.

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