Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–4, 2017

www.elsevier.com/locate/jbiosc

Isolation of aquatic yeasts with the ability to neutralize acidic media, from an extremely acidic river near Japan's Kusatsu-Shirane Volcano

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Received 8 July 2016; accepted 6 February 2017
Available online xxx

The Yukawa River is an extremely acidic river whose waters on the east foot of the Kusatu-Shirane Volcano (in Gunma Prefecture, Japan) contain sulfate ions. Here we isolated many acid-tolerant yeasts from the Yukawa River, and some of them neutralized an acidic R2A medium containing casamino acid. *Candida fluviatilis* strain CeA16 had the strongest acid tolerance and neutralizing activity against the acidic medium. To clarify these phenomena, we performed neutralization tests with strain CeA16 using casamino acid, a mixture of amino acids, and 17 single amino acid solutions adjusted to pH 3.0, respectively. Strain CeA16 neutralized not only acidic casamino acid and the mixture of amino acids but also some of the acidic single amino acid solutions. Seven amino acids were strongly decomposed by strain CeA16 and simultaneously released ammonium ions. These results suggest strain CeA16 is a potential yeast as a new tool to neutralize acidic environments.

[**Key words:** Acid-tolerant yeast; Acidic river; Acid tolerance; *Candida fluviatilis*; Neutralization]

The various extreme environments on Earth include physical environments (e.g., temperature, radiation, pressure extremes) and geochemical environments (desiccation, salinity, pH, oxygen species and redox potential environments) in which various organisms have adapted and thrived (1). For example, the biodiversity and ecology of eukaryotic organisms living in extreme acidic environments near volcanos have been studied (2). Yeast diversity has been extensively investigated (3). Several types of yeast have been isolated from these extreme acidic environments to date; e.g., *Rhodotorula glutinis* (4), *Candida maltosa* (5), *Cryptococcus tepidarius* (6). In *Saccharomyces cerevisiae*, yeast genes involved in responses to acid stress have been studied (7–9). It has also been reported that acidification of the external medium during yeast growth caused the activation of the plasma membrane ATPase (10). Additionally, several studies on mechanisms underlying the prevention of cell damage from acid stress in various acidic environments and proteomic analyses of such stress have been performed for bacteria related to food and the human body (11,12). However, to our knowledge, there are no reports on the mechanisms of acid resistance in environmental acid-tolerant yeasts.

Mount Kusatu-Shirane is an active volcano whose soil contains an abundance of sulfides (13). The Yukawa River, whose water at the east foot of Mt. Kusatu-Shirane contains sulfate ions, is a strongly acidic river (pH 2–3). The Yukawa River water originates from the Kusatsu Hot Spring area and flows downward to the Shinaki Dam. In order to remove damage due to the river's acidity for the purpose of river conservancy and to provide water for agricultural, industrial and other uses, the Yukawa River water is neutralized by adding milk of lime (i.e., calcium carbonate) from the Kusatsu neutralization plant (14). The Yukawa River thus has an unusual aquatic environment composed of both the natural aquatic environment and artificial elements.

In this study, we isolated many acid-tolerant yeasts from both upstream and downstream of the Kusatsu neutralization plant in the Yukawa River. Interestingly, some of these isolated acid-tolerant yeasts possessed a neutralizing ability against an acidic medium. We discuss the mechanisms and the optimum conditions for the neutralization of acidic media by these novel yeasts.

MATERIALS AND METHODS

Collection of environmental samples In June 2010, we collected water samples from the Yukawa River in Agatsuma-gun, Gunma Prefecture, Japan. The samples were transported to the laboratory in sterile bottles in contact with ice. The water temperature was measured at each of the two sampling stations, and the pH value of each sample was measured in our laboratory.

Medium cultures For the medium cultures, we used an R2A medium consisting of: 0.1% yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), 0.1% proteose peptone (Becton Dickinson), 0.1% casamino acid (Nihon Pharmaceutical, Tokyo), 0.1% D(+)-glucose, 0.1% soluble starch (Kokusan Chemicals, Tokyo), 0.06% sodium pyruvate, 0.03% K_2HPO_4, and 0.005% $MgSO_4 \cdot 7H_2O$. We prepared the solid medium (plate) by adding 1.2% gellan gum (Kanto Chemical, Tokyo) at pH 3.0 or agar (Kokusan Chemicals) at pH 7.0 to the R2A medium. The YDC medium consisted of: 1.0% yeast extract (Becton Dickinson), 2.0% D(+)-glucose, and 2.0% casamino acid (Nihon Pharmaceutical).

Isolation of bacteria and yeast strains For the bacterial and yeast isolation, we used both R2A(i) medium (R2A plate medium adjusted to pH 3.0 with sulfuric acid) and R2A(ii) medium (R2A plate medium without any addition). The water samples were concentrated 100-fold using an acid-resistant 0.2-μm PTFE

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http://dx.doi.org/10.1016/j.jbiosc.2017.02.005

membrane-filter (H020A047A, Advantec, Tokyo). The enriched water samples were appropriately diluted when needed, and a 100-μl portion of each preparation was spread on an R2A medium. After several days' incubation at 10°C and 25°C, colonies growing on the plate were picked up.

With the colonies thus obtained, we carried out the yeast isolation procedure. R2A(iii) (R2A plate medium adjusted to pH 2.0, 1.5, and 1.0 with sulfuric acid) was prepared to select acid-tolerant yeasts from the isolates. Using R2A(iv) (R2A plate medium at pH 3.0 with the addition of 0.02% bromocresol purple), we separated the yeasts capable of the neutralization of acid from the acid-tolerant yeasts by observing the change of medium color from yellow to purple.

Neutralization of casamino acid or amino acid solution at pH 3.0 After strain CeA16 was precultured at 25°C for 1 day in YDC medium at pH 3.0 with shaking, the yeast pellet obtained by centrifugation at 3,000 rpm was washed three times with saline adjusted to pH 3.0 with sulfuric acid. The washed cell pellet was added to 10 mL of each solution: 0.5% (w/v) casamino acid, a 0.8–2.2 mM 17 amino acids mixture (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe, Lys), or 20 mM single amino acid adjusted to pH 3.0 with sulfuric acid, and it was then incubated at 25°C for 3 days with shaking.

Identification of the yeasts by an ITS1 sequence analysis The isolated yeasts were identified by an ITS1 sequence analysis for efficiently identifying yeast belonging to the genus *Candida* (15). To amplify the internal transcribed spacer 1 (ITS1) region, we performed a polymerase chain reaction (PCR) with the yeast-chromosomal DNA extracted by bead-beating, using the universal primers ITS1F (5′-GTAACAAGGT(T/C)TCCGT-3′) and ITS1R (5′-CGTTCTTCATCGATG-3′) and Premix Ex Taq (Takara Bio, Shiga, Japan). After the purification of the amplified DNA fragments by a MinElute PCR Purification Kit (Qiagen, Venlo, The Netherlands), we determined their nucleotide sequence with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). We analyzed the similarities among the ITS1 sequences by using the BLAST program (16).

Amino acid analysis and measurement of the ammonium ion and pH values The amounts of amino acids were measured by the detection of the phenylthiocarbamyl (PTC)-amino acids (17). The following procedure for the PTC derivatization was performed. Aliquots of 20 μl of ethanol/water/triethylamine (2/2/1, v/v/v) were added to each vacuum-dried sample and then vacuum-dried again. For the PTC derivatization of amino acids, aliquots of 20 μl of ethanol/water/triethylamine/phenylisothiocyanate (PITC) (7/1/1/1, v/v/v/v) were added to each sample and left standing at room temperature for 20 min. After vacuum drying, 1.0 mL of PTC-derivatized amino acid mobile phase A (Wako Pure Chemical Industries, Osaka, Japan) was added to each sample for the high-performance liquid chromatography (HPLC) sample preparation of the PTC-derivatized amino acid samples. The PTC-derivatized amino acid samples were analyzed using a Prominence HPLC system (Shimadzu, Kyoto, Japan) through a Wakosil-PTC column (200 × 4.0 mm; Wako) at 40°C at 1.0 mL min^{-1} using an amino acid mixture standard solution, Type H (Wako) as a standard.

The analytical conditions for the HPLC were as follows: mobile phase A, PTC-amino acid mobile phase A; mobile phase B, PTC-amino acid mobile phase B (Wako); flow rate, 1.0 mL min^{-1}; gradient of mobile phase B, 0–70% from 0 to 15.00 min (linear), 75–100% from 15.00 to 15.01 min; injection volume, 15 μl; and UV detection, 254 nm.

The amounts of ammonium ion were measured by the enzymatic method with F-kit ammonium (JK International, Tokyo). The pH value was measured using a LAQUA pH meter (F-72, Horiba, Kyoto, Japan).

Nucleotide sequence accession number We have submitted the nucleotide sequences to DNA Data Bank of Japan (DDBJ) under the accession numbers LC133464, LC133465, LC133466, LC133467, LC133463, LC133468 and LC133469.

RESULTS

Isolation of yeast strains possessing the ability to neutralize an acidic medium With the 94 isolates and 230 isolates obtained from upstream and downstream of the neutralization plant in the Yukawa River using R2A(i) or (ii) medium, we performed a screening to identify the microorganisms that could neutralize acid, using the R2A(iv) medium at pH 3.0. As a result, 10 isolates from upstream and 22 isolates from downstream of the neutralization plant in the Yukawa River changed the color of medium containing a pH indicator from yellow to purple after 7 days' cultivation. Therefore, we selected these 32 isolates as the yeasts capable of the neutralization of acid. Then, among these 32 strains, five (CeA14, CeG17, EeB28, EeC21, GeC45) and two strains (AeA6, CeA16) capable of growing on R2A(iii) medium at pH 1.5 and 1.0, respectively, were identified on the basis of the DNA sequence analysis included complete sequence of their ITS1 in this study (Table 1). Six of these seven isolates showed identical sequence with *Candida fluviatilis* type strain and the other showed identical sequence with *Candida palmioleophila* type strain. In particular, out of two strains grown at pH 1.0, strain CeA16 of *C. fluviatilis* showed faster growth than strain AeA6. We therefore focused on strain CeA16 to clarify the mechanism underlying the acid neutralization in this study. Strain CeA16 grew well at 20°–25°C and showed a strong ability to neutralize acid (data not shown).

TABLE 1. Identification of the isolates by an ITS1 sequence analysis.

Strain	Organism (BLAST result)	Sequence identity[a]	GenBank accession no.
(i) Sampling site upstream of neutralization plant (27.9°C, pH 1.9)			
AeA6	*Candida fluviatilis*	100% (189/189)	LC133464
EeB28	*Candida palmioleophila*	100% (191/191)	LC133465
EeC21	*Candida fluviatilis*	100% (189/189)	LC133466
(ii) Sampling site downstream of neutralization plant (27.8°C, pH 4.9)			
CeA14	*Candida fluviatilis*	100% (189/189)	LC133467
CeA16	*Candida fluviatilis*	100% (189/189)	LC133463
CeG17	*Candida fluviatilis*	100% (189/189)	LC133468
GeC45	*Candida fluviatilis*	100% (189/189)	LC133469

[a] Percentage of identity (number of identical sequence /aligned sequence shared with type strain). Type strain: *C. fluviatilis* ATCC 38621^T (GenBank accession no. HQ652068, NCBI RefSeq accession no. NR_111589), *C. palmioleophila* CBS 7418^T (GenBank accession no. KY102283, NCBI RefSeq accession no. NR_077076).

Neutralization of amino acid solutions by the isolated yeast strain To clarify the mechanism underlying the acid neutralization, we performed a neutralization test with strain CeA16 using casamino acid, which is a component of R2A medium, adjusted to pH 3.0 with sulfuric acid. Strain CeA16 elevated the pH from 3.0 to

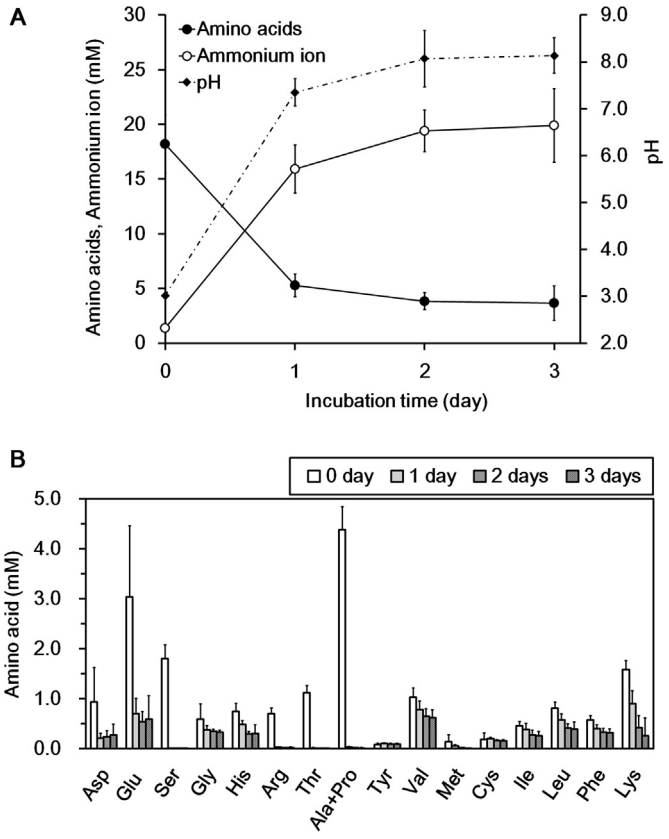

FIG. 1. Neutralization of casamino acid solution by *C. fluviatilis* strain CeA16. Cultivation was carried out for 3 days at 25°C under casamino acid solution. The initial pH of the medium was adjusted to pH 3.0 with sulfuric acid before autoclaving. (A) The measurements of the pH values and the amounts of ammonium ion and amino acids over time. (B) The measurements of the amount of each amino acid over time. Each value is the mean ± SD (n = 3).