High Catalase Production by *Rhizobium radiobacter* Strain 2-1

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To promote the application of catalase for treating wastewater containing hydrogen peroxide, bacteria exhibiting high catalase activity were screened. A bacterium, designated strain 2-1, with high catalase activity was isolated from the wastewater of a beverage factory that uses hydrogen peroxide. Strain 2-1 was identified as *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*) on the basis of both phenotypic and genotypic characterizations. Although some strains of *R. radiobacter* are known plant pathogens, polymerase chain reaction (PCR) analysis showed that strain 2-1 has no phytopathogenic factor. Compared with a type strain of *R. radiobacter*, the specific catalase activity of strain 2-1 was approximately 1000-fold. Moreover, Strain 2-1 grew faster and exhibited considerably higher catalase activity than other microorganisms that have been used for industrial catalase production. Strain 2-1 is harmless to humans and the environment and produces catalase efficiently, suggesting that strain 2-1 is a good resource for the mass production of catalase for the treatment of hydrogen peroxide-containing wastewater.

[**Key words:** catalase, hydrogen peroxide, *Rhizobium*, *Agrobacterium*]

Hydrogen peroxide, a strong oxidant, is widely used as a washing reagent for silicon wafers in semiconductor factories and as a bleaching and sterilization reagent in food and beverage industries. Because hydrogen peroxide is decomposed into nontoxic compounds (water and oxygen), it has also been used as a substitute for chlorides to prevent environmental pollution (1). Hydrogen peroxide production was approximately 220,000 t in 2006; this is twice that of 20 years ago (2). Reducing agents such as sodium hydrogen sulfite are often used to decompose hydrogen peroxide after use in most factories, but sodium hydrogen sulfite is also toxic itself and might damage activated sludge, which is used in subsequent wastewater treatment steps.

With increasing hydrogen peroxide use, a safe decomposition method is required. The enzymatic decomposition of hydrogen peroxide using catalase is safer than chemical decomposition methods. Many fungi or bacteria have been reported as sources of catalase. However, these microorganisms are unable to efficiently produce catalase (3, 4). In this study, we screened bacteria that produce large amounts of catalase. The catalase of strain 2-1 isolated was stable at high temperatures. Therefore, this isolate is a suitable resource of catalase and the catalase of this isolate can be used for industrial applications.

MATERIALS AND METHODS

Screening of bacteria with high catalase activity   Various soil and water samples were used for bacterium isolation. Each soil (0.2 g) or water sample (0.2 ml) was added to 10 ml of basal medium containing 10 g of polypeptone (Nihon Pharmaceuticals, Tokyo), 5 g of yeast extract (Difco Laboratories, Detroit, MI, USA), and 10 g of NaCl in 1 *l* of deionized water. To select bacteria exhibiting catalase activity, $0.5 \ g \cdot l^{-1}$ hydrogen peroxide was added to the basal medium. Cultures were grown at 30°C in a reciprocal shaker (130 oscillations per minute) for 24 h, after which the culture broth was spread on basal medium plates containing $15 \ g \cdot l^{-1}$ agar. The different colonies that grew on the plates were isolated and incubated in the basal medium at 30°C on a reciprocal shaker (130 oscillations per minute) for 24 h. The culture broths ware disrupted by ultrasonic treatment (Sonifier 450D; Branson, Danbury, CT, USA). Sonication was performed under the following conditions: tip diameter, 3 mm; output, 180 W; pulse for 2 s with 80% duty cycle; and total time, 30 s. The cell suspensions were assayed for their catalase activities.

Identification of isolate   Physiological characterization of the isolate was carried out according to Bergey's manual (5). The isolate was also identified using the BIOLOG system (Biolog, Hayward, CA, USA), which is a database of known strains based on the differential utilization pattern of 95 organic test substrates. In addition, sequence analysis of the first 469 bases of its 16S rDNA was also carried out by Techno Suruga (Shizuoka) using a MicroSeq 500 16S rDNA Bacterial Identification Kit (Applied Biosystems, Foster City, CA, USA). Homology searches were conducted using BLAST on the DDBJ (DNA Data Bank of Japan). This sequence data have been submitted to the DDBJ databases under accession no. AB377521.

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Plasmid analysis for detecting phytopathogenicity *Agrobacterium* strains harboring Ti (tumor-inducing) or Ri (root-inducing) plasmids cause plant disease (6). To detect these plasmids, PCR analysis was carried out (7). The primer set VCF3/VCR3 was used to amplify the 414-bp products of almost all phytopathogenic *Agrobacterium* strains harboring Ti or Ri plasmids. The sequence of VCF3 was 5′-GGCGGGCGYGCYGAAAGRAARACYT-3′ and that of VCR3 was 5′-AAGAACGYGGNATGTTGCATCTYAC-3′. A colony that was cultured on a basal medium plate was suspended in sterile water, and this suspension was used as the template. PCR amplification of the target sequence was performed in a total volume of 10 µl of the following mixture: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1.0 pmol of each primer, 1.0 µl of bacterial suspension as template, and 1.0 U of Ex *Taq* polymerase (Takara Bio, Shiga). PCR was performed with a DNA thermal cycler MP (Takara) using the following protocol: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62.5°C for 30 s, and extension at 72°C for 40 s. After amplification, the total reaction mixture (10 µl) was separated by electrophoresis with a 1.5% agarose gel in TAE buffer, and the fragment was visualized by ethidium bromide staining under UV radiation. Strains of *Rhizobium radiobacter* (formerly known as *Agrobacterium tumefaciens*) MAFF301276 (as positive control) and NBRC13532^T (as negative control) were used.

Preparation of cell extracts The cells were grown in a 1 *l* Erlenmeyer flask containing 200 ml of the basal medium at 30°C on a rotary shaker (130 rpm) until early stationary phase. The cells were harvested by centrifugation (10,000×*g* for 10 min) and washed once with 50 mM Tris–HCl (pH 8.0). Then, the cells were resuspended in 40 ml of 50 mM Tris–HCl (pH 8.0) buffer containing 1 mM EDTA and disrupted by single passage through a French pressure cell (SLM-Aminco, NY, USA) at a pressure of 20,000 psi at 4°C. The cell debris was removed by centrifugation (15,000×*g* for 15 min), and the supernatant was used. For comparison, cell-free extracts of *R. radiobacter* MAFF301276, *Escherichia coli* (laboratory strain), *Micrococcus luteus* (*lysodeikticus*) NBRC3333^T, and *Pseudomonas fluorescens* NBRC15840 were also prepared under the same conditions.

Catalase activity and protein assays Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the disappearance of hydrogen peroxide (8), using a spectrophotometer (U-3300; Hitachi, Tokyo). The ε at 240 nm for hydrogen peroxide was assumed to be 43.6 M^{-1}·cm^{-1} (9). The standard reaction mixture for the assay contained 50 mM sodium phosphate buffer (pH 7.0), 30 mM hydrogen peroxide, and 6 µl of cell-free extract in a total volume of 1.0 ml. The amount of enzyme that decomposed 1 µmol of hydrogen peroxide per minute was defined as 1 U of activity. The protein content in the cell-free extract was measured using the BCA protein assay kit (Sigma-Aldrich, St. Louis, MO, USA) with bovine serum albumin as a standard. Each treatment was carried out in triplicate, and the catalase activities were assayed each time. Means and standard deviations for these replicates are shown.

Effects of temperature and pH on catalase activity The effect of temperature on the stability of catalase was examined after incubation of the cell extract at various temperatures for 15 min in 50 mM sodium phosphate buffer (pH 7.0). The optimum temperature was determined by assaying the catalase activity at different temperatures. Means and standard deviations for 3 determinations are shown.

The effect of pH on the stability of catalase was examined after incubation of the cell extract at 30°C for 30 min in the following 50 mM buffers: sodium citrate (pH 3–6), sodium phosphate (pH 6–8), Tris–HCl (pH 8–9), and glycine–NaOH (pH 9–11). The optimum pH was determined by assaying the catalase activity at different pH ranges. Means and standard deviations for 3 determina-
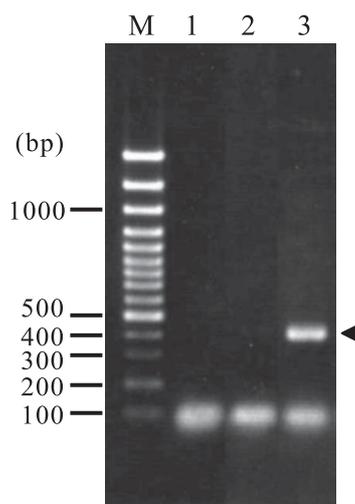

FIG. 1. PCR products that are 414 bp in length were amplified from Ti or Ri plasmids using the universal primer set (VCF3/VCR3). Lanes: M, marker DNA Ladder (BioLabs); 1, nonpathogenic *Rhizobium radiobacter* NBRC13532^T (as negative control); 2, strain 2-1; 3, pathogenic *R. radiobacter* MAFF301276 (as positive control).

tions are shown.

RESULTS

Screening for bacteria exhibiting high catalase activity From 80 soil and water samples, 71 bacterial strains that could grow in the presence of hydrogen peroxide were isolated, and these were tested for catalase activity. Among them, 1 strain, designated as strain 2-1, grew well and showed the highest catalase activity. Strain 2-1 was obtained from the wastewater of a beverage factory that uses hydrogen peroxide and was used for further study. Strain 2-1 was deposited in the International Patent Organism Depository (Tsukuba) as strain FRRM P-20348.

Identification of the isolate Strain 2-1 was a motile, rod-shaped, gram-negative, aerobic, oxidase-positive, and non-sporing bacterium. The BIOLOG system identified strain 2-1 as *R. radiobacter* (formerly known as *A. tumefaciens*/ *radiobacter*) with 100% probability. The homology search results for first 469 bases of the 16S rDNA gene of strain 2-1 also showed that this strain is closely related to *R. radiobacter* (99% sequence similarity).

Previously, *A. tumefaciens* and *R. radiobacter* were differentiated on the basis of the presence of disease-inducing plasmids (6); recently, changes in the nomenclature and taxonomic classification of *A. tumefaciens* have been proposed by Young *et al.* (10, 11), such that *A. tumefaciens* is now classified as *R. radiobacter*.

PCR analysis for detecting phytopathogenicity Phytopathogenic *R. radiobacter* MAFF301276 (as positive control) showed a clear band of 414 bp, and both the non-phytopathogenic *R. radiobacter* (NBRC13532^T) and strain 2-1 showed no band of 414 bp (Fig. 1). Furthermore, the ability of strain 2-1 to cause plant disease was tested using tomato (12). Only the positive control produced crown galls on the stalk inoculated with the bacterial suspension (data

TABLE 1. Catalase activities in cell extracts of strain 2-1 and other catalase producers

Strain or species	Time[a] (h)	Activity (U·ml^{-1})	Protein (mg·ml^{-1})	Specific activity (U·mg^{-1})
Strain 2-1	20	85176±2418	2.8±0.1	30420±1083
M. luteus	34	34601±2385	3.2±0.2	10938±427
P. fluorescens	14	825±67	6.7±0.1	123±10
R. radiobacter (*A. tumefaciens*)	24	95±34	2.5±0.1	37±13
E. coli	8	77±40	3.4±0.1	23±13

Each cell was suspended in the buffer of 1/5 amount of the culture broth.
[a] Cultivation time until early stationary phase.

not shown). From these results, it was concluded that strain 2-1 is nonphytopathogenic.

Time course of the growth of and enzyme production by strain 2-1   The time courses of the growth of and enzyme production by strain 2-1 in the basal medium are shown in Fig. 2. The stationary phase was attained after 18–22 h of cultivation. Catalase activity showed a rapid increase at the late log phase and was maximum after 16 h of cultivation. At that time, catalase activity reached 18,000 U·ml^{-1}.

Comparative study of the catalase activity between strain 2-1 and other catalase producers   The catalase of strain 2-1 was produced intracellularly because only 0.4% of the total activity was determined in the culture broth (data not shown). Catalase activities in the cell-free extracts of strain 2-1 and other typical catalase producers were compared (Table 1). Each strain was incubated until the stationary phase. All strains attained the stationary phase within 24 h, except *M. luteus* that required 34 h of incubation. The specific activity of the catalase of strain 2-1 was approximately 30,000 U·mg protein^{-1} that was about 3-fold that of the catalase of *M. luteus*, which is known to exhibit high activity (National Agriculture and Food Reseach Organization, Report on industrial enzyme, http://nfri.naro.affrc.go.jp/yakudachi/koso/4_shishitu4_1.index.html [2007] [in Japanese]; 13).

Effect of temperature and pH on enzyme activity
The catalase activity of strain 2-1 was stable over a wide temperature range of 30–60°C. The optimum temperature was 20–40°C. Moreover, the catalase activity was stable at pH 5–11 and optimum pH was 6–11. In terms of the effect of pH, the catalase derived from strain 2-1 showed high stability over a wide range of pH from 5 to 11 (Fig. 4B).

The activity of the catalase derived from strain 2-1 remained at approximately 30% after incubation for 15 min at 65°C. On the other hand, that of catalase from *M. luteus* was only 2% under the same conditions (Fig. 3A).

DISCUSSION

In this study, we isolated a bacterium designated strain 2-1 that produces a large amount of catalase from wastewater containing hydrogen peroxide. We anticipated that strain 2-1 acquired the capability to efficiently produce catalase in order to grow in wastewater. This strain could grow in culture broth containing high concentration of hydrogen peroxide, at least 5% (w/v) in which usual bacteria cannot grow, indicating that strain 2-1 to endure peroxide is particularly high (data not shown). In this condition, bubbles are generated immediately after inoculation into hydrogen per-
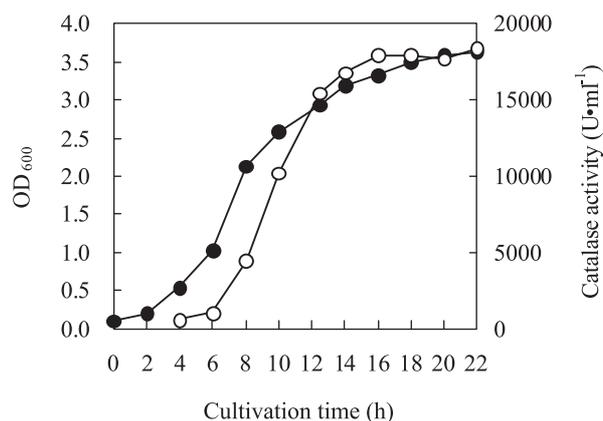

FIG. 2. Time courses of the cell growth (closed circles) and enzyme activity (open circles) of strain 2-1 at 30°C. Cell pellets harvested by centrifugation (10,000×*g* for 10 min) at each culture time were suspended in 50 mM Tris–HCl (pH 8.0). These suspensions were subjected to sonication and were used for enzyme assays. The catalase activity was converted into the volume of the culture broth.

oxide-containing culture broth, we concluded that strain 2-1 can grow by decomposing hydrogen peroxide in the culture medium.

Strain 2-1 produced a large amount of catalase in a short duration (30,420 U·mg^{-1} in 20 h). This yield is considerably greater than that of other known catalase-producing microorganisms such as a strain of *Thermoascus aurantiacus* (5100 U·mg^{-1} in 10 d) (3), a strain of *Aspergillus niger* (9 U·mg^{-1} in 72 h) (14), a strain of *Halobacterium halobium* (61 U·mg^{-1} in 18 h) (15), and a strain of *Vibrio rumoiensis* (4092 U·mg^{-1} in 48 h) (16). In addition, we optimized the components of culture medium for strain 2-1; as a result, the yield of catalase increased to fivefold than that of culture in a basic medium (data not shown), and because this optimized culture condition was economical, this culture process can be scaled up to the industrial level.

Usual bacterial catalases are heat labile, and their activity decreases on preservation. The catalase derived from strain 2-1 was more stable at high temperatures than that of *M. luteus*, which is widely used for industrial catalase production (National Agriculture and Food Reseach Organization, Report on industrial enzyme, http://nfri.naro.affrc.go.jp/yakudachi/koso/4_shishitu4_1.index.html [2007] [in Japanese]; 13). Additionally, the activity of the catalase derived from strain 2-1 is stable for 6 months at 50°C (data not shown). Strain 2-1 catalase is expected to remain active for long periods while it circulates as a commodity, and it can be used for wastewater treatment under high temperature conditions.
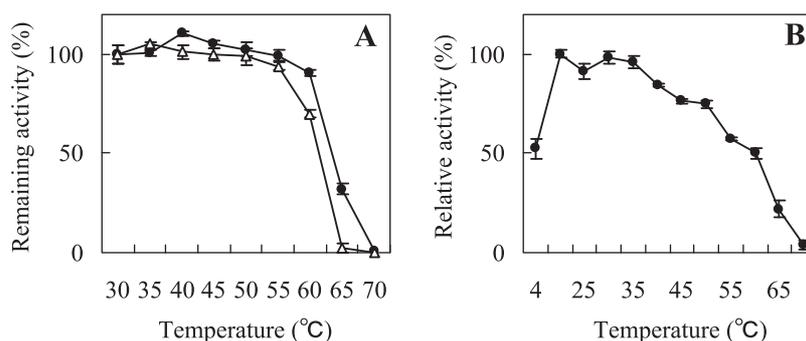
FIG. 3. Effect of temperature on the stability (A) and activity (B) of catalase. Symbols: closed circles, strain 2-1; open triangles, *Micrococcus luteus* NBRC3333ᵀ.
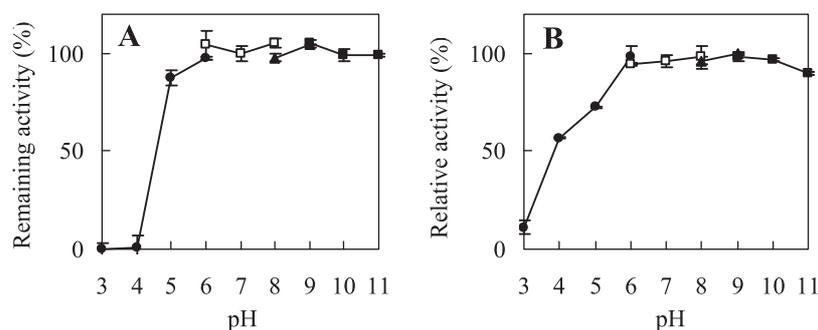

FIG. 4. Effect of pH on the stability (A) and activity (B) of catalase. Symbols: closed circles, citrate buffer; open squares, phosphate buffer; closed triangles, Tris–HCl buffer; closed squares, glycine–NaOH buffer.

Strain 2-1 was identified as *R. radiobacter* (*A. tumefaciens/radiobacter*). The specific catalase activity of strain 2-1 was found to be approximately 1000-fold that of the type strain of *R. radiobacter*. This is the first report demonstrating the high catalase activity of *Rhizobium* (*Agrobacterium*). Some strains of *R. radiobacter* interact with plants and cause desease, and catalase is known to play an important role in combating hydrogen peroxide toxicity produced by the host plants in response to pathogenic infection (17). Strain 2-1 is considered to possess a ability to combat hydrogen peroxide produced by the host plants and thus to survive on the surface of the plants for long periods. However, this strain showed no phytopathogenicity because of the absence of disease-inducing plasmid. The plasmid of the type strain used as a positive control in this study was lost easily during preservation; therefore, the plasmid of strain 2-1 might also have been lost during growth in wastewater.

In conclusion, strain 2-1 produces catalase more efficiently than any other reported bacteria, and it is harmless to humans and the environment. Moreover, the catalase derived from strain 2-1 is stable in heat. Thus strain 2-1 has the necessary elements for industrial use, it is a good resource for the mass production of catalase. The optimum culture and catalase extraction conditions for strain 2-1 have been established in our laboratory, and the scale-up of the culture process is in progress.

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