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Note

A Bacterial Strain with Antibacterial and Antioxidant Activities from *Tasae*, a Burmese Indigenous Alcohol Starter

Win AUNG, Yukiko TANAKA, Zhibing ZHENG, Yuka WATANABE and Fumio HASHINAGA*

Department of Biochemical Science & Technology, Faculty of Agriculture, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan

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A total of 85 microbial strains from indigenous alcohol starters of some Asian countries were screened for antibacterial and antioxidant activities. From the result of co-incubation, only one strain, M2-1 showed the antibacterial activity against all target bacteria and this strain was selected. Radical scavenging activity of ethyl acetate extract of M2-1 strain was compared with BHA using DPPH. The free radical scavenging activities were 18.0 and 29.5%, respectively, at the concentration of 100 and 200 ppm. The antioxidant activity of extract was determined according to the thiocyanate method using AAPH. The inhibition of peroxidation of extract was 22.4% at 250 ppm concentration. Antibacterial activity of extract was assayed by paper disk method using 450 μg extract per disk. The extract has broad inhibition against all the target bacteria. M2-1 strain was identified as *Bacillus* sp. on the basis of 16S rDNA sequence.

Keywords: *tasae*, an indigenous alcohol starter, antioxidant and antibacterial activities, 16S rDNA sequence.

Burma (Myanmar) is a country in Southeast Asia. In making rice wines, Burmese people are still using indigenous alcohol starters in many parts of the country (Aye and Myint, 1978). An indigenous alcohol starter may contain different kinds of microorganisms such as yeasts, molds, and even bacteria (Steinkraus, 1996). The metabolites produced by microorganisms may serve as antioxidants (Hayashi *et al.*, 1995; Ishikawa *et al.*, 1992), scavengers (Kato *et al.*, 1993), lipoxygenase inhibitors (Nihei *et al.*, 1993) and/or metal chelating agents (Kunze *et al.*, 1992). Derivatives from food constituents produced by the action of microbial enzymes have antioxidant and/or synergistic activity (Murakami *et al.*, 1984). Furthermore, microorganisms can decompose lipid peroxides and use them as nutrients (Rashid *et al.*, 1992).

Studies on the antibacterial compounds produced by bacterial flora in indigenous alcohol starter may contribute to: (1) reduce microbial contamination during the fermentation and, (2) provide new bacteriocin – like inhibitory substances with activity on associated alcohol starters as well as on food pathogens or spoilage microorganisms.

In our laboratory, we are studying antioxidant and antimicrobial activities of metabolites from yeasts isolated from indigenous alcohol starters of some Asian countries. Therefore, we collected such starters from Indonesia, China, Cambodia, Nepal, and Burma. From those source samples, a total of 85 microbial strains were isolated. Fortunately, we found only one bacterial strain that possessed antibacterial activity against target bacteria when screening tests were carried out with the co-incubation method. That strain, isolated from *tasae*, a Burmese indigenous alcohol starter, was therefore selected to study the antioxidant and antibacterial activities. The selected strain was identified on the basis of 16S rDNA gene sequence.

Abbreviations: BHA, Butylated hydroxyanisole; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; AAPH, 2,2-Azobis (2-amidinopropane) dihydrochloride

*To whom correspondence should be addressed.
E-mail: hasinaga@chem.agri.kagoshima-u.ac.jp

Materials and Methods

Isolation of microorganisms YM medium containing 0.3% yeast extract (Difco, Detroit, USA), 0.3% malt extract (Wako Pure Chemical Industries, Ltd.), 0.5% polypeptone (Wako) and 1% glucose was used as enrichment medium for isolation of microorganisms. To a 5 ml aliquot of this medium 1 g of source sample was inoculated and incubated in a shaker incubator at 30°C for 7 days. Thereafter, 2% agar streak plates with the same compositions as the above YM media were made. Colonies of distinct morphologies that were visible after 3–7 days were streaked onto new agar plates in order to obtain pure colonies and subsequently grown in 5 ml YPG medium containing 0.5% yeast extract, 1% polypeptone and 2% glucose, and incubated at 30°C for 3 days. Aliquots of 1 ml from each culture were preserved at –80°C, and grown from this stock when needed.

Target bacteria Bacterial strains of *Bacillus cereus* IFO 15305, *Bacillus subtilis* IFO 13719, *Micrococcus luteus* IFO 12708, *Staphylococcus aureus* IFO 14462, *Escherichia coli* B IFO-13168, *Escherichia coli* C IFO 13891, and *Salmonella enteritidis* IFO 3313 were used.

Co-incubation Co-incubation is a direct stab inocu-

lation with freshly grown culture into a YM agar plate seeded with a target bacterial strain and incubated at 37°C for 2 days. The clear zones formed around the stab-inoculated strains were recorded.

Optimization of medium and cultural time To optimize the cultural medium and time, the scavenging activity in YPG broth medium and NB medium containing 0.25% NaCl, 0.5% beef extract (Difco) and 1% polypeptone (pH 6.6) were compared.

One loopful of freshly grown culture on YPG slant (2% agar in YPG medium) was inoculated into 10 ml of the same YPG or NB medium and incubated at 30°C for 1 day in a shaker incubator (150 rpm). One-day-old culture was inoculated into a 300 ml Erlenmeyer flask containing 100 ml YPG or NB medium and adjusted to get the final solution of ca. 10^6CFU/ml. It was then incubated in a reciprocal shaker incubator (130 rpm) at 30°C for up to 7 days. Five milliliters of liquid culture was taken at intervals of 24 hr incubation and centrifuged at 5,000 × g for 5 min at room temperature, then 0.2 ml of supernatant was taken and the scavenging activity measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described by Terasawa *et al.* (2001). Briefly, 2 ml of 0.1 M sodium acetate solution (pH adjusted to 5.5 with acetic acid), 2 ml of ethanol (99.5%) containing 0.2 ml of sample were mixed in a test tube. One milliliter of 0.5 mM DPPH in ethanol was then added. The solution was kept at room temperature for 30 min and the absorbance at 517 nm was measured with a spectrophotometer (Shimadzu UV-160A). The radical scavenging activity (RSA %) was calculated using the equation RSA% = {(absorbance of control – absorbance of sample)/absorbance of control} × 100%.

Extraction of bacterial antioxidant and antibacterial compound Two hundred milliliters of 2-day-incubated YPG liquid culture were mixed with a 1.5 fold volume of ethyl acetate. The mixture was then shaken in a separatory funnel and the ethyl acetate layer was collected. The ethyl acetate extract was passed through Advantec No. 2 filter paper (110 mm) (Toyo Roshi, Ltd.). The solvent was removed using a vacuum rotary evaporator below 35°C. The residues were dissolved with 10 ml of 50% ethanol and kept at –80°C for 4 hr. The frozen extract solution was then lyophilized until it reached a constant weight. Lyophilized compound was dissolved in ethanol to get the concentration of 10 mg/ml.

Measurement of radical scavenging activity Radical scavenging activity of ethyl acetate extract of M2-1 strain was compared with butylated hydroxyanisole (BHA) using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described above. This time, 2 ml of ethanol containing 100 or 200 ppm of extract were mixed in a test tube instead of 0.2 ml supernatant, but the other compositions and concentrations added were not changed.

Measurement of antioxidant activity Antioxidant activity of extract of the M2-1 strain was determined according to the thiocyanate method (Shirasaka *et al.*, 1999; Terasawa *et al.*, 2001). Briefly, 0.125–1.25 mg of sample (which means 25–250 ppm in 5 ml of total volume) in 0.25 ml of ethanol was mixed with 2 ml of 1.3% linoleic acid in ethanol, 2 ml of 0.05 M phosphate buffer (pH 7), and 0.2 ml of 0.05 M 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) to accelerate oxidation and distilled water to adjust the total volume of 5 ml in a test tube, and placed in darkness at 50°C for 15 and 20 hr. After that, 0.1 ml of this solution was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of ferrous chloride (0.02 M in 3.5% HCl) which were added sequentially. After mixing for 3 min, the absorbance of the mixture was measured at 500 nm. The inhibition of peroxidation (IP%) was calculated using the equation IP% = {1–(absorbance of sample/absorbance of control)} × 100%.

Antibacterial activity assay The paper disc method was used for antibacterial activity assay. Fifteen microliters of extract solution (10 mg/ml) was impregnated into 6 mm diameter sterilized discs (Toyo Roshi, Ltd.). The discs then were left for 20 min under the blower of a clean bench and another 15 µl was impregnated twice more, so that each disc contained 450 µg of the compound. To the blank disc, the same volume of ethanol was impregnated.

A suspension containing ca. 10^6 CFU/ml of 1-day-old target bacteria grown in NB medium was prepared with 3 ml of NB soft medium containing 0.5% agar. This suspension was transferred to a petri dish containing 20 ml of NB solid medium containing 2% agar and spread evenly with a glass rod. The discs, each containing 450 µg of the test compound, and one blank disc were put on each dish and incubated in an inverted position at 37°C. The diameter of the inhibition zone was measured after 24 hr. Each experiment was repeated three times. Identification of M2-1 strain was carried out on the basis of 16S rDNA sequence.

DNA extraction, amplification, and sequencing Bacterial DNA was extracted from M2-1 strain. High molecular mass and plasmid-free DNA was prepared according to Wu *et al.* (1997) with minor modifications. PCR of pure culture was performed in a GeneAmp PCR system 7900 thermal cycler (Perkin-Elmer) using the general eubacterial primers, 27f (5-AGAGTTTGATCCTGGCTCAG-3) (Giovannoni, 1991) and 1492r (5-GGTTACCTTGTTACGACTT-3) (Lane, 1991). The PCR product was purified using a LaboPass™ purification kit (COSMO Genetech Co., Ltd., Rep. of Korea) and partly sequenced with forward primer 27f. The sequencing was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 310 analyzer (Perkin Elmer Applied Biosystems, Foster City, USA). Search for 16S rDNA was performed with a total of 639 bp from M2-1 strain of clean sequence in the GenBank database by BLAST at NCBI (www.ncbi.nlm.nih.gov).

Results and Discussion

From the 10 samples, a total of 85 isolates were picked and spread onto agar plates. Among the 85 isolates, we found that almost all the strains were yeasts, a few isolates were molds (data not shown) and only one strain was bacteria. Based on the results of co-incubation, although the bacterial strain showed a broad spectrum of inhibition against all the target bacteria, we could not find the antibacterial

activity in the rest of the yeast or mold strains. That bacterial strain was therefore selected to be tested for antioxidant and antimicrobial activities of ethyl acetate extract.

We found that the radical scavenging activity in YPG medium was higher than that of NB medium and the best incubation time for both media was 2 days as shown in Fig. 1. Thus, we used YPG medium with 2 days incubation time for the next experiments to produce ethyl acetate extract.

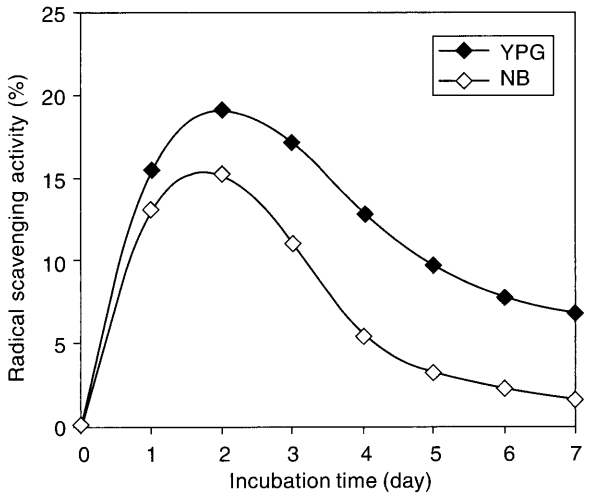

Fig. 1. Effect of medium on radical scavenging activity of M2-1 strain.

The DPPH radical is considered to be a model of a lipophilic radical. A chain reaction in lipophilic radicals was initiated by the lipid autoxidation. The scavenging effects of ethyl acetate extracts and BHA on DPPH radical were compared. According to the results shown in Fig. 2, ethyl acetate extracts had significant scavenging effects on the DPPH radical and these effects increased when the concentration increased from 100 to 200 ppm. It is suggested

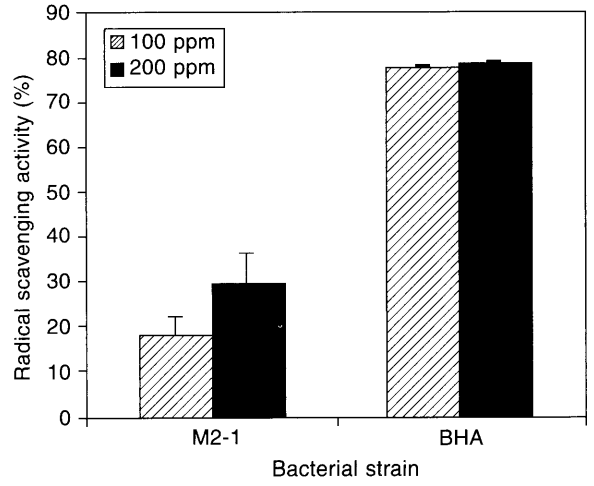

Fig. 2. Radical scavenging activity of ethyl acetate extract of M2-1 strain at 100 and 200 ppm concentrations.

that the scavenging activity of ethyl acetate extracts came from the M2-1 strain growing in the YPG. The scavenging activities of the extract from M2-1 strain were 18.0 and 29.5%, respectively, at the concentration of 100 and 200 ppm.

Rashid *et al.* (1992) reported that microorganisms might survive in an environment that contained peroxide oil. In order to examine the validity of these findings and to study the production of antioxidants in microorganisms, the extract of M2-1 strain was tested by thiocyanate method. The antioxidant effect of ethyl acetate extract from the filtrate of the M2-1 on the inhibition of peroxidation of linoleic acid after 15 hr and 20 hr incubations is shown in Fig. 3. The inhibition of peroxidation of M2-1 strain after 15 hr incubation at 250 ppm concentration was 22.4%. Although the activity was lower compared than that of BHA, this

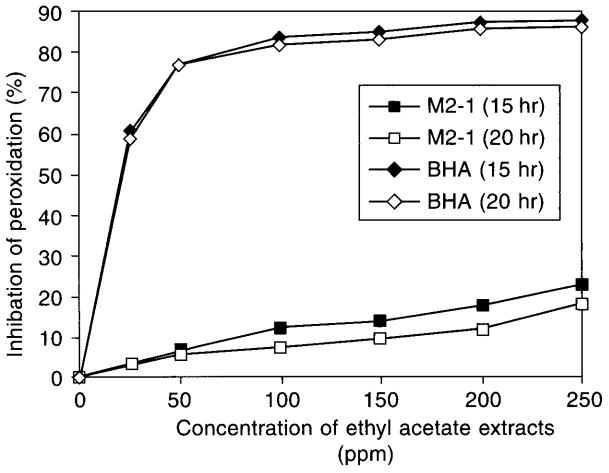

Fig. 3. Antioxidant activity of ethyl acetate extract of M2-1 strain as measured by thiocyanate method using AAPH after 15 and 20 hr incubations.

showed that the thiocyanate method with AAPH could be used to obtain quick result.

Table 1 shows that the ethyl acetate extract of M2-1 strain was effective in all target bacteria whereas the control with ethanol showed no effect (data not shown).

Table 1. Antibacterial activity of M2-1 strain.

Target bacteria	Size of effective zone (mm)
Gram-positive	
B. cereus IFO-15305	11.1
B. subtilis IFO-13719	6.7
M. luteus IFO-12708	10.3
S. aureus IFO-14462	15.9
Gram – negative	
E. coli B IFO-13168	6.5
E. coli C IFO-13891	8.0
S. enteritidis IFO-3313	16.2

IFO, Institute for Fermentation, Osaka.

In DNA extraction, Bond *et al.* (2000) have reported that the freeze-thaw method produces a greater quantity and less sheared DNA than is produced by bead-beating protocol. In this study, we confirmed that following the method

described by Wu *et al.* (1997) with freeze-thaw extraction and shorter incubation time (about 10 min) at 65°C gave good results.

The amplification of the 16S rDNA gene with the pair of primers 27f and 1492r resulted in PCR products of a nearly complete sequence (Kim *et al.*, 2000). Sequence of 639 bp from M2-1 strain was obtained with the primer 27f. In the 16S rDNA sequence analysis, this strain was shown to have a 95.8% identical sequence with a wide range of *Bacillus* species, which have the same scores. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB168006.

In conclusion, the results obtained in this study clearly demonstrate that the ethyl acetate extract of the M2-1 strain exhibited a significant antioxidant activity and broad-spectrum antibacterial activity. Further safety evaluation of ethyl acetate extract should be conducted, however. Further work is also needed on the M2-1 strain (e.g., studies with the full length of the 16S rDNA sequence, DNA-DNA hybridization) in order to resolve this deep phylogenetic relationship among the *Bacillus* species.

List of Abbreviations:

1. Butylated hydroxyanisole (BHA)
2. 1,1-Diphenyl-2-picrylhydrazyl (DPPH)
3. 2,2-Azobis (2-amidinopropane) dihydrochloride (AAPH)

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