RAPD Analysis of Salt-tolerant Yeasts from Contaminated Seasoned Pickled Plums and Their Growth Inhibition Using Food Additives

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Eight salt-tolerant yeasts were isolated from contaminated pickled plums which were seasoned with honey and "Umami" seasoning. They were classified into four main groups according to random amplified polymorphic DNA analysis, and three of ten kinds of food additives tested inhibited their growth. The type strains of each group were identified as Zygosaccharomyces bisporus, Pichia subpelliculosa, and two strains of Candida apicola based on the D1/D2 region sequence of the 26S rRNA gene. They were able to grow in medium containing 6% (w/v) NaCl. A number of yeasts were isolated from production lines by the swab method, but not from the salted plums used as raw materials. These results show that the production lines require washing with antimicrobial agents effective against salt-tolerant yeasts. Three commercial food additives, San-keeper 381, Sunsoft No.700P-2, and potassium sorbate inhibited the growth of Z. bisporus at 125 to 250 µg/ml. In particular, San-keeper 381 altered the morphology of this species at 125 µg/ml. C. apicola and P. subpelliculosa were inhibited by Sunsoft No.700P-2 and potassium sorbate at 250 µg/ml. These results indicate that the washing of production lines with disinfectant and the use of food additives that effectively prevent salt-tolerant yeast contamination are necessary.

Key words : Pickled plum/RAPD/Food additives.

INTRODUCTION

The production of pickled plums made with reduced salt and seasoned with "Umami" seasoning, and honey has increased with a rising consumer interest in health. Regarding food sanitation, low-salt plums containing less than 100 g/kg NaCl tend to be contaminated by salt-tolerant yeasts. The sanitary super-vision of production lines and quality control of products are always essential. Clarification of the stage of contamination in these lines is desired promptly for the prevention of microbial contamination when the pickled plums are contaminated with yeast. However, conventional methods, morphologic features, and physiological and biochemical properties give ambiguous information to distinguish the isolates and require a lot of time (Arias et al., 2002). On the other hand, the identification and typing of microbes using gene analysis techniques is becoming more common. In the food industry, these techniques are applied to detect yeasts in wine (Comi et al., 2000; Guillamon et al., 1998; Pramateftaki et al., 2000; Querol et al., 1992), cheeses (Andrighetto et al., 2000; Romano et al., 1996; Suzzi et al., 2000), sausages (Cocolin et al., 2006), carbonated orange juice (Pina et al., 2005), and other food (Foschino et al., 2004).

In general, preservatives are added to foods to prevent contamination by microorganisms. Seasoned

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pickled plums are more easily contaminated compared with salted plums. Yajima et al. (1998) reported that the growth of the genera Kloeckera, Candida, Pichia, and Debaryomyces isolated from Ume-zuke was inhibited by extracts from paprika seeds.

In this study, the salt-tolerant yeasts from seasoned pickled plums were classified into four groups by random amplified polymorphic DNA (RAPD) analysis. The RAPD method amplifies genomic DNA of isolates using short primers and facilitates strain identification, through features such as distinct DNA banding patterns (Baleiras Couto et al., 1994; Williams et al., 1990). RAPD analysis can discriminate microorganisms at both the species and subspecies levels. The representative strains in each group were identified by sequence analysis of the D1/D2 region of the 26S rRNA gene. Moreover, three commercial food additives inhibited their growth at 125 to 250 μg/ml.

**MATERIALS AND METHODS**

**Yeast isolation from pickled and raw plums**

Eight kinds of pickled plum contaminated by yeasts as well as raw plums were provided by a company manufacturing pickled plums in Wakayama Prefecture. The seasoned pickled plums contained 6 to 11% (w/v) of NaCl. Yeasts were isolated on Potato Dextrose Agar (PDA, Nissui, Tokyo, Japan) containing 6% (w/v) NaCl, pH 5.6, at 28 ºC for 96 hr. Salted plums containing 20% (w/v) NaCl and the raw plums were delivered from the company in Wakayama Prefecture and from China, respectively. They were added to Potato Dextrose Broth (PDB, Becton, Dickinson and company, Franklin Lakes, NJ, USA) and incubated at 28 ºC for 7 days. Their culture solutions were transferred into the PDB containing 3% (w/v) NaCl. Their culture broth was spread on the PDA containing 6% (w/v)NaCl, 100 μg/ml streptomycin and 60 μg/ml penicillin G. The purity of isolated colonies was confirmed by the re-smear method.

**RAPD analysis**

DNA was extracted from the contaminant yeast using Dr. GenTLE for Yeast (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Two PCR primers, Ra-1 (5'-AAGAGGCGGT-3') and Ra-2 (5'-AACGCGCAAC-3'), were used in this study. PCR amplification was performed using a My cycler (Bio-Rad Laboratories, Hercules, CA, USA) and pureTaq Ready-To-Go PCR Beads (GE Healthcare UK Ltd., Buckinghamshire, England). Each 25 μl of the reaction mixture contained 1 μl of the primer at 10 μM and 1 μl of genomic DNA at 30 ng/μl. The PCR program was as follows: a denaturing step at 94 ºC for 5 min, followed by 30 cycles of 45 sec at 94 ºC, annealing for 45 sec at 38 ºC, and extension for 45 sec at 72 ºC, followed by a final extension at 72 ºC for 10 min. The amplification products were analyzed by electrophoresis in 2.0% (w/v) agarose gels in 0.5x TAE buffer (40 mM Tris-acetate, pH 8.2; 1 mM EDTA). After electrophoresis, the gels were stained with ethidium bromide and photographed under UV light using the Gel Logic 200 system (Cosmo Bio Co., Ltd., Tokyo, Japan). The 100-bp DNA Ladder (Takara Bio) was used as a size marker.

**Sequence analysis**

The 560-bp fragment of D1/D2 domains of the 26S rRNA gene was amplified. Sequences of the forward (NL1) and reverse (NL4) primers used for the PCR were 5'-GCATATCAATAAGCGGAGGAAAAG-3' and 5'-GGTCCGTGTTCAGACGG-3', respectively. PCR mixtures were prepared with 1 μl of each primer at 10 μM and 1 μl of genomic DNA at 30 ng/μl. The PCR program was as follows: a denaturing step of 94 ºC for 5 min, followed by 30 cycles of 45 sec at 94 ºC, annealing for 45 sec at 55 ºC, and extension for 45 sec at 72 ºC, followed by a final extension at 72 ºC for 10 min. The PCR products of the extracted bands from agarose gels were purified using a QIAEX II kit (Qiagen Inc., Santa Clarita, CA, USA) and sequenced using the ABI PRISM model 3100 automatic sequencer with a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare UK Ltd.). Sequence identification was performed using BLAST of the National Center for Biotechnology Information (NCBI).

**Nucleotide sequence accession numbers**

The nucleotide sequences determined in this study have been submitted to DDBJ under the accession numbers AB375303 to AB375306.

**Screening of food additives**

Seven kinds of commercial food additives and three kinds of chemicals were screened for their ability to inhibit the growth of yeasts contaminating pickled plums: Sunsoft No.700P-2, No.750, No.760 (Taiyo Kagaku Co., Ltd., Mie, Japan), San-Keeper 381 (San-Ei Gen F.F.I., Inc., Osaka, Japan), extracts of Phyllostachys pubescens (Takey Labo Co., Ltd., Osaka, Japan), Kirayanin (Maruzen Pharmaceuticals Co., Ltd., Hiroshima, Japan), sucrose monopalmitic acid ester P1670 (Mitsubishi Chemical Co., Tokyo, Japan), potassium sorbate, cinnamic acid (Tokyo
Chemical Industry Co., Ltd., Tokyo, Japan), and 99% (v/v) ethanol. Principal components of Sunsoft No. 700P-2, No. 750, No. 760 and Kirayanin are glyceryl monocaprylate, glyceryl monolaurate, glyceryl monocaprate, and quillaja saponin, respectively. San-Keeper 381 contains 50% (w/v) polylysine. The aqueous agents were dissolved in water and sterilized by filtration with a membrane filter (0.22 μm). The non-aqueous agents were dissolved in dimethylsulfoxide and filtrated.

**Antimicrobial activity**

Three yeasts (*Candida apicola* S1, *Zygosaccharomyces bisporus* S2, and *Pichia subpelliculosa* S8) isolated from the pickled plums were used for the antimicrobial test of food additives. Antimicrobial activity was assayed using a 96-deep well microbioassay system and PDB containing 6% (w/v) NaCl. Two hundred and seventy five microliters of yeast suspensions at 10^7 cfu/ml of the medium was placed in each well of the first 96-deep well microplate (Whatman, Springfield Mill, UK). To the first-row wells of the second microplate, 1,400 μl of the medium and 160 μl of the food additive solution (13,455 μg/ml) were added, and 780 μl of the medium was added to wells of the second to eighth rows. Using a micropipette, 780 μl of the solution in the first-row wells was transferred serially into the remaining rows. To the wells of the first microplate, 725 μl of the solution in the second microplate was added and mixed. The first well of the microplate was prepared to contain 1,000 μg/ml of the food additives. The microplate was incubated at 28 °C for 72 h and 750 rpm using a Bioshaker MBR-022UP (Taitec, Saitama, Japan). Morphological changes in yeasts were observed with a microscope (OLYMPUS BX51, OLYMPUS, Tokyo, Japan).

**RESULTS AND DISCUSSION**

**RAPD analysis of yeasts isolated from pickled plums**

Eight strains (S1 to S8) were isolated from eight contaminated samples and underwent RAPD analysis with primers Ra-1 (Fig. 1-a) and Ra-2 (Fig. 1-b). The DNA fragment patterns were classified into four groups. Group Nos.1, 3, and 4 were of strains S1, S7, and S8, respectively. The DNA band pattern of strain S2 was the same as those of strains S3, S4, S5, and S6, so they were classified into Group No.2 (Table 1). Similar classification results were obtained from two independent trials of RAPD analysis using different primers.

From the 20% (w/v) NaCl salted plums, no yeast growth was observed on PDB containing 3% (w/v) NaCl, nor on the PDA plate containing 6% (w/v) NaCl. Four strains were isolated from the raw, non-salted plums using the same methods, while their DNA banding patterns on RAPD analysis differed with those of isolates from contaminated pickled plums. A number of yeasts were also detected from production lines by the swab method. These results show that contaminating yeasts might be derived from the production lines in the factory, and not from the raw, non-salted plums.

Yeasts of each group classified by RAPD analysis

![FIG. 1. RAPD analysis of yeasts isolated from pickled plums. Two primers, Ra-1 (a) and Ra-2 (b), were used. Lanes 1 to 8 are isolated yeasts. M is the molecular standard, 100-bp DNA Ladder.](image-url)
TABLE 2. Antimicrobial activity of food additives according to the micro liquid dilution method.

<table>
<thead>
<tr>
<th>Food additives</th>
<th>C. apicola S1 (µg/ml)</th>
<th>Z. bisporus S2 (µg/ml)</th>
<th>P. subpelliculosa S8 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aqueous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunsoft No.700P-2</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>San-Keeper 381</td>
<td>1,000</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>Extracts of Phyllostachys pubescens</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
<tr>
<td>99% (v/v) ethanol</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
<tr>
<td>Kirayakin</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
<tr>
<td>Potassium sorbate</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>non-aqueous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunsoft No.750</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
<tr>
<td>Sunsoft No.760</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose monopalmitic acid ester P1670</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>–</td>
<td>&gt;1,000</td>
<td>–</td>
</tr>
</tbody>
</table>

- : not tested

FIG. 2. Microscopic observation of morphological changes in Z. bisporus S2 treated with San-Keeper 381 in the micro liquid dilution test. Yeasts were incubated at 28 °C for 72 hr and 750 rpm. Control yeasts were not treated with San-Keeper 381.

were identified by the partial 26S rRNA gene sequences of the D1/D2 regions. The sequences from strains S1 and S7 selected from Group Nos. 1 and 3, and were related to the sequence of Candida apicola NRRL Y-2481 (accession No. U45703), showing 99.6% homology. Strains S2 and S8 from Group Nos. 2 and 4 were related to Zygosaccharomyces bisporus NRRL Y-12626 (U72162, 100%) and to Pichia subpelliculosa NRRL Y-1683 (U74593, 99.8%), respectively (Table 1). Genera of Candida, Pichia, and Zygosaccharomyces were typical salt-tolerant yeasts which form films at the surface of soy sauce and miso in fermentation tanks (Betts et al., 1998; Suezawa et al., 2006; Yajima et al., 1998). There have been few reports on the isolation of yeasts contaminating pickled plums, however, genera of Kloeckera, Candida, Pichia, and Debaryomyces have been isolated from Ume-zuke by Onda et al. (1997). They reported the contamination of the salted plums by salt-tolerant yeasts. We also isolated the similar genera Candida and Pichia from seasoned pickled plums. The salted or seasoned plums are thought to be contaminated by those salt-tolerant yeasts at a fairly high frequency at some factories. A disinfection process involving a numerical reduction in numbers of the salt-tolerant and film-forming yeasts is required for the stable production of seasoned pickled plums.

RAPD analysis appeared to be useful to classify contaminant yeasts from pickled plums. The combination of PCR-based techniques was needed to correctly identify the contamination sites in the production of pickled plums. For example, the combination of PCR-fingerprinting and RAPD assays was used effectively to trace the source of yeast contamination in carbonated orange juice (Pina et al., 2005). Barszczewski and Robak (2004) were also able to differentiate between brewing yeast and wild yeast isolates using RAPD and restriction fragment length polymorphism (RFLP) analysis.

Antimicrobial activity of food additives

In this study, ten kinds of commercial food additives were tested for their ability to inhibit three contaminating strains, C. apicola S1, Z. bisporus S2, and P. subpelliculosa S8. Among six kinds of aqueous compounds tested, Sunsoft No.700P-2, potassium sorbate, and San-keeper 381 inhibited their growth at 125 to 1000 µg/ml (Table 2). Especially, San-keeper 381 showed the strongest activity against Z. bisporus.
S2 among the tested samples. On the other hand, four kinds of non-aqueous food additives exhibited no growth inhibition of Z. bisporus (Table 2). The cell surface of this yeast may maintain a hydrophilic state. The cellular morphology of Z. bisporus in the presence of San-keeper 381 showed expansion at 125 µg/ml and a burst cell body at 500 µg/ml, whereas cells at 63 µg/ml maintained an elliptical shape identical to that in the control solution (Fig. 2).

Sunsoft No.700P-2, and potassium sorbate exhibited antimicrobial activity against C. apicola and P. subpelliculosa at 250 µg/ml. However, San-keeper 381 required a higher concentration for the growth inhibition of C. apicola and P. subpelliculosa than against Z. bisporus.

As stated above, contaminating yeasts showed different sensitivities to the tested food additives. San-keeper 381 contained 50% (w/v) polylysine, and was most effective against Z. bisporus. It is used in all kinds of foods, particularly in the prevention of fungal contamination of starch-containing foods. Polylsyline has the characteristic of water solubility and shows a marked thermostability and high antimicrobial effect against yeasts and bacteria (Geornaras et al., 2007; Hiraki, 2000; Shim & Sakai, 2000; Yoshida and Nagasawa, 2003). In addition, polylysine seems to be safe because it is hydrolyzed to lysine in the body (Hiraki et al., 2003). On the other hand, C. apicola and P. subpelliculosa were effectively inhibited by Sunsoft No.700P-2 and potassium sorbate. Glycerine fatty acid ester, the main component of Sunsoft No.700P-2 and potassium sorbate, has been used traditionally in many foods as a preservative.

From the above, no single food additive can inhibit the growth of all contaminant yeasts. Polysaccharide extracts from Polygonum hydropiper had a synergistic effect on the antimicrobial activity against Z. bailii, when it was combined with sorbic acid (Fujita and Kubo, 2005). The combination of food additives exhibiting different antimicrobial spectra may contribute to inhibit the growth of yeasts contaminating pickled plums. Further work is needed to select better combinations of food additives.

In this study, we revealed that three salt-tolerant yeasts, C. apicola, Z. bisporus, and P. subpelliculosa, detected from seasoned pickled plums, were the major contaminating yeasts, and that plums were contaminated with these yeasts on the production line in the factory; however, salted or non-salted plums used as the raw materials of seasoned plums were not contaminated. RAPD analysis was also useful to classify the yeasts isolated from pickled plums. Three kinds of food additives effectively inhibited their growth. Washing the production lines with disinfectant and the combined use of food additives effective against salt-tolerant yeasts may contribute to preventing seasoned, pickled plums from being contaminated by yeasts.

REFERENCES
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