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Biofilm reduction potential of micro-plasma discharged water (m-PDW) against the microbes isolated from a tofu manufacturing plant

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ABSTRACT

In this study, biofilm inactivation potential of micro-plasma discharged water (m-PDW), a commercially available water purifier known as EP purification, was evaluated. Bacterial strains were collected from the various contaminated area of tofu manufacturing industry, overall 15 different types of bacterial strains were isolated and their biofilm formation ability was determined by using crystal violet (CV) staining. Three different strains (20% of the isolated strains), namely, *Brevundimonas naejangsanensis, Delfita lacustris* and *Lactococcus lactis*, with biofilm density (OD_{590}) > 0.4, were identified as biofilm-producing strains. These three biofilm-producing strains were used to form biofilm on stainless steel (SS) coupons in a laboratory condition for four days and the biofilm containing coupons were treated with EP purification device. The inactivation obtained after 40 min of treatment was 4.8 and 4.2 log CFU/coupon for *Brevundimonas naejangsanensis* and *Delfita lacustris,* respectively, whereas no viable count was detected for *Lactococcus lactis* after the same treatment time. Biofilm reduction effects of m-PDW treatment differ according to biofilm-producing ability or strain characteristics. SEI (Secondary Electron Images) was obtained by field emission scanning electron microscope (FE-SEM) that showed m-PDW treatment has significant reduction potential on biofilm inactivation for the sample collected from tofu manufacturing industry.

1. Introduction

Tofu a cheese-like foodstuff made from boiling soymilk through coagulation with a coagulant. Protein-rich foods have traditionally been eaten in the Far East for more than 2000 years, but over the past ten years, the worldwide consumption of tofu has significantly increased (Lee et al., 2016; Prestamo & Fontecha, 2007; Rossi, Felis, Martinelli, Calcavecchia, & Torriani, 2016). Protein-rich, higher-moisture content and near-neutral pH makes tofu an exceedingly favorable medium for microorganisms to grow (Kovats, Doyle, & Tanaka, 1984). Tofu is a precooked food typically eaten without further cooking; hence, it is categorized as a potentially hazardous foodstuff and a ready-to-eat food. A number of studies have shown that tofu is a potential source of several outbreaks of foodborne illness associated with some common pathogens i.e., *E. coli*, *Salmonella* spp., *Yersinia* spp., *Listeria* spp. and *Pseudomonas* spp., (Ananchaipattana et al., 2012; Rehberger, Wilson, & Glatz, 1984; Yamamura et al., 1992). As a favorable medium for microorganisms, some spoilage bacteria can also form biofilms on tofu, which is a major cause of its short shelf-life (Asharaf, White, & Klubek, 1999).

Biofilms show strong resilience to environmental stresses, antibiotics or sanitizers. When biofilms formed on the different instruments of food plants, it can cause continuous contamination in the manufacturing process due to inherent difficulties in controlling and managing these formations, which in turn results in a reduced shelf life, spoilage and diseases (Bronowski, James, & Winstanley, 2014; Chang & Halverson, 2003; Dunne, 2002). In the U.S. market mass production and delivery of tofu requires several days to weeks before it reaches to the consumer. The vulnerability of this product to microbial decomposition suggests a need for food safety standards regarding its production and storage. Hence, to improve the overall food safety standards of tofu production, from processing to packing, it is necessary to identify the bacteria involved in biofilm formation and a suitable inactivation technique for these foodstuffs. Disinfecting of biofilm by chemical, physical or biological approaches are often unsuccessful as the microbes present inside the biofilm shows unlike characteristics

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from those in planktonic state (Stewart & Costerton, 2001; Yu, Huang, Hsieh, Huff, & Duan, 2007). Therefore, the decontamination of biofilms in an effective way is a challenge for the researcher working in this area. To develop new procedures for biofilm decontamination numerous research efforts are ongoing worldwide. Among all the existing inactivation techniques, research focuses are growing towards non-thermal physical inactivation techniques such as the plasma inactivation technique (Raso & Barbosa-Canovas, 2003).

Underwater plasma discharge is a rapid and trustworthy non-thermal plasma inactivation method that extensively used for bacterial decontamination (Attri & Choi, 2013; Attri et al., 2015; Fridman et al., 2007; Lee et al., 2016; Locke & Thagard, 2012; Sun & Qiu, 2007; Tresp, Hammer, Winter, Weltmann, & Reuter, 2013; Wu, Sun, Feng, & Fang, 2012). This technique is an eco-friendly method that has a wide application range in the area of ecological and wastewater management (Joubert et al., 2013; Locke & Thagard, 2012; Locke, Sato, Sunka, Hoffmann, & Chang, 2006) and this technique has newly been introduced in microbial decontamination research for the purposes of food safety and hygiene (Loske, Alvarez, Hernández-Galicia, Castaño-Tostado, & Prieto, 2002). Various underwater electrical discharge methods have been offered for microbial decontamination and diverse level of microbial reductions were achieved for various types of devices and discharges (Loske et al., 2002; Zuckerman, Krasik, & Felsteiner, 2002). Different types of plasma discharge produces different types of reactive species; for instance, lower pH and H_2O_2 are produced via gliding arc discharge; UV light and pressure waves are created via pulsed high-current discharge underwater; and capillary discharge produces O, H, $^{\cdot}OH$ species. Dielectric barrier discharges (DBD) generates reactive oxygen species (ROS), ozone, UV light, reactive nitrogen species (RNS) and those have robust antimicrobial properties (Fridman et al., 2008).

In the present study, micro cavity plasma technology, known as the EP purification system, developed at the University of Illinois and demonstrated as useful for the purification of air and water, was used to evaluate the biofilm reduction potential of this device. Among the strains separated and identified in samples collected from the tofu manufacturing industry, three biofilm-producing strains, namely, *Brevundimonas naejangsanensis, Delfita lacustris,* and *Lactococcus lactis*, with biofilms individually generated on SS coupon under laboratory conditions, were treated with the EP purification system (m-PDW) under water.

2. Materials and methods

2.1. Sample collection and preparation

The samples analyzed in the present study were collected from the various utensils and tools used during the tofu manufacturing process. The strains were suspended in a sterile plastic bag and cultured in Plate Count Agar (PCA, BD, Sparks, MD, USA) at 37 °C for 48 h. Bacterial colonies were collected by selecting specific spots and wiping the area using a cotton swab wetted with 1 ml of 0.85 (v/v) NaCl solution, which was shaken in 10 ml of 0.85% (v/v) sterile saline solution to produce a suspension of attached bacteria. The suspension was decimally diluted and cultured onto PCA at 37 °C for 48 h. Then, one colony from individual reference bacterium was cultured onto PCA plate for 24 h at 37 °C.

2.2. Separated strains identification

The bacterial strains with biofilm assay were identified by 16S RNA gene sequencing investigation. For PCR analysis, the primers 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3′ and 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3′ were used, which was reacted with My Cycler (BIORAD Laboratories, Hercules, CA, USA) using AccuPower® PreMix (Bioneer, Daejeon, Korea). The PCR conditions were set as: 2 min at 94 °C, 1 round; 30 s at 94 °C, 1 min at 60 °C, 1 min at 72 °C, 35 rounds; and 7 min at 72 °C. The gene sequencing of PCR products was identified using the ABI PRISM BigDye™ Terminator Cycle Sequencing Kit from Applied Biosystems (Foster City, CA, USA) and PTC-225 Peltier Thermal Cycler and ABI PRISM 3730 XL Analyzer from MJ Research (Reno, NV, USA). The homology of the 16S rRNA gene sequence was analyzed via BLASTn online database (http://www.ncbi.nlm.nih.gov/blast/), also the gene sequence of standard strains was extracted from the database of GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and compared with the gene sequences of the separated strains. In 16S rRNA gene sequencing, the length of the gene sequence was unified at approximately 1400 bp through the Bioedit database (Tom Hall Ibis Biosciences, Carlsbad, CA, USA) and united by the Clustal X program (http://www.clustal.org/clustal2/). The phylogenetic diagram was generated by the MEGA5 program (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA) with the aligned gene sequence set.

2.3. Biofilm formation assay

Biofilm Formation was investigated through CV staining of 96-well polystyrene microtiter plates (SPL). Each colony separated from the cultured sample was grown on tryptic soy broth (TSB, BD) for 15–16 h at 37 °C. The microbes from culture medium were harvested and cleaned once by centrifugation at 10,000 × g for 10 min. The cultures grown on TSB were suspended in TSB at OD_{600} 0.1–0.2 and subsequently placed in 100 ml wells and incubated at 37 °C for 72 h. Pipetting up and down method was followed to detach cell suspension from well plates and then well were rinsed twice with 150 ml sterile water. The plates were dried totally at 37 °C for 120 min. For biofilms staining, 100 ml of 1% CV solution (Sigma-Aldrich St. Louis, USA) was used at room temperature and pressure for 30 min. The granules of CV were dissolved in 100 ml of 10% acetic acid (CH_3—COOH) and 30% methanol (CH_3—OH) solution per well. A microplate reader (Infinite M200 Pro NanoQuant, Tecan, Switzerland) was used for measuring optical density at 590 nm.

2.4. Bacterial strains and preparation of inoculums

In the present study, 3 strains with biofilm formation ability, namely, *Lactococcus lactis, Brevundimonas naejangsanensis* and *Delftia lacustris*, were used among the strains separated from the samples collected from tofu manufacturing industry. One colony from individual reference bacterium was used to inoculate in TSB solution for 15–16 h at 37 °C. The cultures were centrifuged at a speed of 10,000 × g for 10 min. The supernatant was poured off, and to obtain approximately 4.0 log CFU/ml of population level the pelleted cells were suspended again in phosphate-buffered saline (PBS) and pH level was maintained within 7.4.

2.5. Biofilm development on stainless steel (SS) coupons

To attach microbes on stainless steel (304LN, Posco, Pohang-si, Republic of Korea) surface, 2 ml of cell suspension was poured in a 24-well cell culture plate (Nasco, Fort Atkinson, WI, USA) having a sterile SS coupon (1 cm × 1 cm) and incubated at 4 °C for 24 h. The SS coupons were moved out from the cell suspension with a sterile forceps. To remove unattached planktonic cells the SS coupons were rinsed with sterile distilled water by rotating the SS coupon gently in a circular motion for 5 s. To generate biofilm the coupons microbial cell were placed in a 16-well cell culture plate comprising 5 ml of TSB and incubated at 25 °C for 4 days. After day 1, the coupons were rinsed with sterile distilled water by rotating the SS coupon gently in a circular motion for 5 s. To extract biofilm into the solution, the SS coupons were shifted into 50 ml conical centrifuge tubes containing 1 g of sterile glass granules (425–600 μm diameters; Sigma-Aldrich, 3050 Spruce Street,