Effect of microbial sanitizers for reducing biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* on stainless steel by cultivation with UHT milk

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Abstract Biofilm is a serious issue in the dairy factory due to it increases the opportunity for microbial contamination. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the bacteria capable to construct the biofilm on materials and equipments. Therefore, the bacterial growth and efficiency of sanitizing agents to solve the problems were evaluated. These bacteria grew well in UHT milk when they were cultivated at 37 °C, especially *S. aureus*. The exponential growth phase and biofilm on stainless steel were discovered by short contact time at 2 h. The mature stage of biofilm cycle was found at 4 h during bacteria growth and it was continuously constructed until 48 h. The 10, 24, and 48 h-old biofilm adhering on stainless steel were established with oxisan and chlorine used as microbial sanitizers. The 4% of sanitizing agents was the efficiency concentration to reduce biofilm on stainless steel up to 82% when these bacteria grew in UHT milk.

Keywords Biofilm · *Staphylococcus aureus* · *Pseudomonas aeruginosa* · Oxisan · Chlorine

Introduction

In dairy industrials, almost equipments are made from stainless steel such as pipes, milk cans and milk cooling tanks. Blended pipes are the critical point that accumulating microorganisms, especially bacteria produce biofilm lead to food contamination and spoilage. Raw milk from bovine mastitis and milk contamination during transport is the initial cause to obtain bacteria into dairy products and producing processes. Pipes leaking is another factors allowing bacteria into the producing systems of product when low liquid pressure in pipes potentially harmful contaminants into dairy products (Dewangan et al., 2015). Since the European and American legislation has strict regulations concerning materials coming into contact with foods and milk processing necessitates hygienic equipment material resistant to corrosion in alkaline and acidic conditions, the dairy industry has employed stainless steel in almost all segments of the dairy chain (Marchand et al., 2012). Biofilms are not only a potential source of microbial contamination, but can also increase corrosion rate, decrease heat transfer and increase fluid frictional resistance (Kumar and Anand, 1998; Marchand et al., 2012). This structure is a matrix of polysaccharides secreted by contaminated bacteria strains by greatly adheres on stainless steel surface, especially conning the scratch. Biofilm structure can protect the bacteria from sanitizing agents causing they will survive and multiply under that structure. Thus, reducing biofilm constructed from bacteria in dairy is the important processes to decrease milk contamination causing spoilage.

Staphylococcus aureus is one of bacteria strains causing milk spoilage and food borne diseases in human (Gutiérrez et al., 2012). This bacterium causes bovine mastitis and one of the most cost intensive diseases in the dairy industry

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(Dufour et al., 2012). In addition, enterotoxigenic *S. aureus* strain contains the potentially to make food borne intoxications in human transmitted by consuming the contaminated dairy products (Hennekinne et al., 2012; Jørgensen et al., 2005; Ostyn et al., 2010; Schmid et al., 2009). Moreover, withstand pasteurization in thermal processes and resistant against human gastrointestinal proteases are found in *S. aureus* (Balaban and Rasooly, 2000).

Pseudomonas spp. is among the bacteria most frequently isolated from general surfaces in the food industry and it produces multispecies biofilm on the wall of milk cooling tanks and pipelines prior to heat processing. Due to the production of flagellum as a motility mediator, *P. aeruginosa* can form cell monolayers on abiotic and biotic surfaces at least 4 h after contact (Marchand et al., 2012; Shirliff et al., 2002).

Clean-in-place (CIP) is a method to clean the interior surfaces of pipes, vessels, process equipments, filters and associated fittings, without disassembly for industries as followed dairy, beverage, brewing, processed food, pharmaceutical and cosmetics require high levels of hygiene. The benefit of CIP to industries is faster, less labor intensive, more repeatable and less of a chemical exposure risk to people. Elevating the temperature of a cleaning solution increases its dirt removal efficiency. Concentration of cleaning agents and long contacting time will clean a dirty surface better than a diluted solution with short prior. Strong acid and base chemicals following hot water is the processes found in the dairy industrials (Kitis, 2004; Marquis et al., 1995; Russell, 2003). Oxisan is a commercial sanitizer found the strong chemical as its compositions. While, chlorine chemical is widely used to sanitize whole fruits and vegetables as well as fresh-cut produce on a commercial scale but has limited application at the food service or consumer level (Beuchat et al., 1998).

Reducing biofilm constructed from bacteria in dairy is the important processes to decrease milk contamination with bacteria causing milk spoilage. Therefore, this study was aimed to investigate the cycle of *S. aureus* and *P. aeruginosa* biofilm constructed on stainless steel surface in culture medium and UHT milk. Moreover, the effectiveness of oxisan and chlorine as sanitizers was also examined the ability to reduce the bacteria biofilm adhering on stainless steel surface under different conditions. The appropriate concentration of the sanitizers and biofilm eliminating time could be applied in CIP process.

Materials and methods

Bacterial strain and inoculums preparation

Bacterial strains *S. aureus* and *P. aeruginosa* were taken from department of Applied Biology, Faculty of Science and Liberal Art, Rajamangala University of Technology Isan. *S. aureus* and *P. aeruginosa* were cultivated with Nutrient agar and King's B medium (Proteose peptone 20 g/L; Dipotassium hydrogen phosphate 1.5 g/L; Magnesium sulphate heptahydrate 1.5 g/L; Glycerol 15 mL; Agar 20 g/L), respectively. Their characteristics (colony and cell morphology) were re-checked as following bacteria on culture plate, Gram staining and investigation under light microscopy. The colony morphology of *S. aureus* on Nutrient agar showed the golden yellow circular colony and exhibited Gram positive cocci cells under microscopy at 100 $\times$, whereas *P. aeruginosa* explored the greenish yellow colony on King's B medium and Gram negative rod cells. Bacterial inoculums were cultivated in Nutrient broth with shaking at 150 rpm, 37 °C for 18–24 h. The concentration of bacteria inoculums were adjusted the turbidity to 0.5 (10^9 CFU/mL) by using a spectrophotometer at OD 600 nm (Biesta-Peters et al., 2010).

Preparation of stainless steel

Stainless steel grade 316 was chosen for this experiment. It was cut into a single square piece consist a size 10 $\times$ 15 mm. All stainless steel pieces were immersed in acetone solution for 1 h following washed with distill water and finally soaked in 70% ethanol. Sterilization of stainless steel was done by autoclaving at 121 °C, 15 psi for 15 min and oven dried at 80 °C for 2 h before using in the experiment (Rossoni and Gaylarde, 2000).

Bacteria biofilm construction on stainless steel using culture medium

S. aureus and *P. aeruginosa* inoculums were transferred to Nutrient broth contained 3 pieces of stainless steel with 3 replicates. The bacteria with stainless steel were incubated together by shaking at 50 rpm, 37 °C for 0–48 h. Population of bacteria and quantity of adhered biofilm on each stainless steel pieces were established at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h after inoculation. Each stainless steel pieces was measured the adhered biofilm following washed with sterile 0.85% w/v normal saline solution (0.85% NSS) to remove the planktonic bacteria and then soaked in crystal violet solution (Crystal violet 2 g/L; 95% Ethanol 20 mL; Ammonia citrate 0.8%; Distill water 80 mL) for 5 min and finally rinsed again with distill water. Stained biofilm on

stainless steel surface was detached by 5 mL of acetic acid solution for 5 min. The biofilm solution was measured the concentration of purple color by using a spectrophotometer at OD 590 nm. The bacteria population in each time of sampling was done with serial dilutions in 0.85% NSS and then the colony of them on Nutrient agar were counted after incubation at 37 °C for 18 h.

Construction of bacteria biofilm on stainless steel using UHT milk

Commercial ultra heat-treated (UHT) milk was selected for the experiment. Prepared stainless steel pieces were placed into the flask containing UHT milk. Then, the 10% v/v bacteria inoculums were transferred to UHT milk flasks. The conditions of experiment were compared the temperatures to construct biofilm from bacteria on stainless steel surface at 4 and 37 °C by shaking at 50 rpm. The population of bacteria and their biofilm amount were evaluated by detecting with above methods at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h after inoculation.

Efficiency of sanitizers

The 100% concentration of oxisan (Hydrogen peroxide 25% v/v; Peracetic acid 5% v/v; Acetic acid 15% v/v; Distill water 55 ml) and chlorine ($C_5H_14NO.Cl$) solutions were prepared at our laboratory by following commercial compositions and diluted to 0.3, 1, and 4% v/v before using. The stainless steels incubated with *S. aureus* and *P. aeruginosa* in different conditions for 10, 24, and 48 h were collected and then they were soaked in each sanitizer with various concentrations for 0, 5, 30, and 60 min at room temperature (25–30 °C) and finally rinsed with distill water. The remaining biofilm quantity on stainless steels after cleaning with sanitizing agents were stained with crystal violet solution for 5 min following rinsed in distill water and finally detracted the stained biofilm on stainless steel surface to solution with 5 mL of acetic acid for 5 min. The concentration of biofilm in acetic acid solution after staining was measured with a spectrophotometer at OD 590 nm. The efficiency of sanitizers was compared the biofilm reducing percentages.

Statistical analysis

Each experiment was performed two times. Three stainless steel pieces were tested for each treatment in each replicate experiment. Data were analyzed using the One Way ANOVA.

Results and discussions

Bacteria growth in culture medium and UHT milk

The inoculums of *S. aureus* and *P. aeruginosa* were initially adjusted the bacterial cell concentration to 10^9 CFU/mL in Nutrient broth. Then, the 10% v/v inoculums was transferred to fresh culture medium remaining bacteria 10^4 CFU/mL at 0 h after inoculation. Both bacteria strains could grow in culture medium and UHT milk by showing the exponential of growth phase at 2 h after incubation (Figs. 1, 2). The result state that Nutrient both and UHT milk as culture medium could enhance growth of bacteria population. Therefore, UHT milk had an opportunity to contaminate and support growth of these bacteria during production process and package leaking. Normally, the food poisoning bacteria showed the optimum temperature for growth as 37 °C although they could multiply quite quickly. The temperature must be kept below 5 °C to prevent their growth. Bacteria could rapidly increase in UHT milk when it was stored at 37 °C, whereas they slowly multiplied at 4 °C. The bacteria population was initial stable after 8 h until 48 h without decreasing in their number during growth in UHT milk as shown in Fig. 1A. Mesophilic microorganisms, including *S. aureus*, showed a lack of growth at temperatures of 4–6 °C, but it still continuously developed their population under these temperatures (Valero et al., 2009). This result stated that the contamination of experimented bacteria strains in dairy product stored at 4 and 37 °C could transmit food pathogenic bacteria to consumers. In comparison to other psychrotrophic bacteria, *P. aeruginosa* demonstrated the characterization by a short generation time less than 4 h as shown in Fig. 1B, which implies that contamination with just single microbial cells could lead to increase their numbers greater than 10^6 CFU/mL in UHT milk after 8 days of storage at temperatures of 4 °C. Such foods include meat, gravy, sea food, poultry, dairy produce and cooked rice. Dried foods such as dried egg or milk powder did not support bacterial growth and so had a long storage life. Whereas, given an appropriate ambient temperature, adequate moisture and nutrient some bacteria strains could rapidly divide in duplicate every 10–20 min after contamination (Barbano et al., 2006).

Bacteria biofilm construction

Biofilm construction was initially determined in exponential phase of bacteria growth at 2 h after incubation. This time which they rapidly constructed biofilm with high amount on stainless steel (Fig. 2A, B). The bacteria population continuously increased according to biofilm that

Fig. 1 Growth of *S. aureus* (A) and *P. aeruginosa* (B) on stainless steel detected at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h

they secreted the exopolysaccharide (EPS) as the important composition to create the matrix support biofilm structure. The matrix of biofilm was composed with EPS, proteins and extracellular DNA and was responsible for biofilm maturation that was the result of an organized community construction (Barbano et al., 2006). Crystal violet solution as dye could be absorbed in EPS structure of biofilm. This mechanism indicated that the intensive of purple color from crystal violet staining detected with spectrophotometer exhibited the EPS amount secreted by bacteria to construct biofilm adhering on stainless steel surface. The volume of biofilm in experiment was stable during 2–6 h after incubation. This prior was the mature stage of biofilm construction contained large amount of bacteria cell under that structure. For this reason, the mature biofilm was weak structure making easily to broke and release partial of bacteria out to environment as free cell or planktonic bacterial. Dispersion stage as one step in biofilm cycles making *S. aureus* and *P. aeruginosa* were released from the matrix structure after mature stages found at 8 h of incubation. Planktonic bacterial could attach new area on stainless steel surface returning to first step of cycles to proliferate the cell monomer before development to mature

stage again. Biofilms of *P. aeruginosa* and *S. aureus* were formed from individual planktonic cells in a complex and presumably highly regulated developmental process with various signals, including the nutritional status of the environment (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Rahimi, 2013). *S. aureus* strains were known to be frequently resistant to antibiotic treatment due to their capacity to produce an EPS barrier and because of their location within microabscesses, which limit the action of drugs and sanitizing reagents (Lund and Ormerod, 1995). More importantly, in dairy equipment biofilms, the development was also very rapid by 8–12 h (Anand et al., 2014; Scott et al., 2007) with numbers of up to 10^6 bacteria cell per cm^2 being recorded in the generation section of a pasteurizer after 12 h of operation (Anand et al., 2014; Bouman et al., 1982).

Currently, the predominant Gram-negative microorganisms limiting the shelf life of UHT processed fluid milk at 4 °C were *Pseudomonas* spp., especially *P. fragi*, *P. lundensis*, *P. fluorescens* and *P. aeruginosa* organisms. *P. aeruginosa* could grow to high numbers and form biofilm during refrigerated storage. Many of them produce heat-

Fig. 2 Biofilm construction of *S. aureus* (A) and *P. aeruginosa* (B) on stainless steel detected at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h

stable extracellular lipases, proteases, and lecithinases that contribute to milk spoilage (Marchand et al., 2012).

Efficiency of sanitizers to reduce bacteria biofilm on stainless steel using UHT milk

Oxisan and chlorine chemical were explored the efficiency after improving the biofilm construction on stainless steel in culture medium and UHT milk. Oxisan was formulated to sanitize surfaces in contact with food, reservoirs, CIP, evaporators, fillers, aseptic equipment and pasteurizers found in dairies, wineries, breweries, and food, beverage and meat processing and packaging plants (Van Houdt and Michiels, 2010). For treatment of working surfaces was applied at the concentration 0.3–0.5% solution to circulate at 5–40 °C and keep in contact for at least 60 min (Hallam et al., 2001). No reducing percentages of *S. aureus* and *P. aeruginosa* biofilm on stainless steel were observed in

control treatments that did not clean with any sanitizers. Soaking the 10 h-old *S. aureus* biofilm adhering on material with 4% of oxisan for 30 min exhibited 76% reducing. Likewise, this concentration of sanitizing agent displayed a high efficiency to reduce the 10, 24, and 48 h-old biofilm constructions when they were soaked in a long time for 60 min as shown 68–72% (Fig. 3). This result state that biofilm constructed from the first stage of *S. aureus* growth not over 10 h was a period for bacteria beginning to form microcolony under biofilm structure on material after dispersion stages to free cell in liquids culture. In contrast, microcolony developing to large biofilm contained high *S. aureus* population difficult to destroy with low concentration of oxisan and short time to contact. Chlorine was appropriately decreased 24 h-old *S. aureus* biofilm after construction that they showed 57–82% reducing (Fig. 4). Soaking constructed biofilm on stainless steel with 0.3, 1.0, and 4.0% for 0, 5 and 30 min exhibited no significant

Fig. 3 Efficiency of oxisan sanitizer and reducing percentages of *S. aureus* biofilm compared with control treatments by cultivation in UHT milk at 37 °C for 10, 24, and 48 h after inoculation. The alphabets indicate significant statistic values

Fig. 4 Efficiency of chlorine sanitizer and reducing percentages of *S. aureus* biofilm compared with control treatments by cultivation in UHT milk at 37 °C for 10, 24, and 48 h after inoculation. The alphabets indicate significant statistic values

reduction of biofilm. In the other hand, cleaning the 24 h-old biofilm on material with 4% chlorine for 60 min significantly explored the reduction up to 82%. Moreover, the maintenance of a free chlorine residual of 0.05 mg/L was able to prevent biofilm formation on new material such as stainless steel, plastic pipes during an 18-month investigation period. No evident relationship between the potential of biofilm to develop and the type of pipe material such as glass, cement, MDPE, PVC and stainless steel when chlorine concentrations were greater than 0.3 mg/L. When chlorine concentrations were less than 0.3 mg/L the biofilm potential was in the order glass, cement, PVC and stainless steel; however, these differences were small in comparison with the impact of disinfection (Hallam et al., 2001;

Ndiongue et al., 2005). Traditionally, chlorine used in form sodium hypochlorite based sanitizers had been used, however, a wide variety of sanitizers including quaternary ammonium compounds, anionic acids, iodophores, and chlorine-based compounds were currently in use or being evaluated for use in CIP systems. The selection of detergents and disinfectants in the dairy industry depended on the efficacy, safety, and risibility of the agent and whether it is corrosive or affects the sensory values of the processed products (Marchand et al., 2012).

In case of *P. aeruginosa* biofilm, stainless steels cleaned with various concentration of oxisan solution greatly exhibited the biofilm reducing percentage, especially 10 h-old biofilm (Fig. 5). Time to contact the sanitizing agent of

Fig. 5 Efficiency of oxisan sanitizer and reducing percentages of *P. aeruginosa* biofilm compared with control treatments by cultivation in UHT milk at 37 °C for 10, 24 and 48 h after inoculation. The alphabets indicate significant statistic values

materials for reducing biofilm had less importance than the phase of *P. aeruginosa* biofilm. Low concentrations and short time to contact with sanitizers were found 28–38% reducing. The 24 h-old biofilm were significantly reduced biofilm quantity by 68–75% with different concentrations of chlorine (Fig. 6). Cleaning with 3 levels of concentrations for 60 min, especially, exhibited the highest percentages of reducing by 76%. The increasing numbers of microorganisms also supported the production of greater amounts of EPS. The researchers also found an increasing of *Pseudomonas* biofilm resistance to chlorine with increasing age of the biofilm (Sommer et al., 1999). The

316L-grade stainless steel had been developed for a fouling cell assembly that may be placed in dairy pipes and silos (Marchand et al., 2012). Such assemblies enable monitoring of biofilm development without removal of the processing equipment out of the plant and could be utilized to generate objective data on the effectiveness of cleaning procedures (Janknecht and Melo, 2003; Marchand et al., 2012).

S. aureus and *P. aeruginosa* exhibited the exponential phase of bacteria growth at 2 h after inoculation. They could construct the biofilm on stainless steel surface during their growth and the mature structure was also found in

Fig. 6 Efficiency of chlorine sanitizer and reducing percentages of *P. aeruginosa* biofilm compared with control treatments by cultivation in UHT milk at 37 °C for 10, 24 and 48 h after inoculation. The alphabets indicate significant statistic values

short contact time after 2 h of incubation. The 4% concentration of oxisan and chlorine solution displayed a highly reduce the *S. aureus* biofilm amount on stainless steel up to 76 and 82%, respectively, whereas *P. aeruginosa* biofilm exhibited 68 and 75%, respectively. The 24 h-old biofilm, especially, clearly demonstrated biofilm decreasing and could be destroyed by 4% of sanitizing agents.

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