REMOVAL OF *PSEUDOMONAS PUTIDA* BIOFILM AND ASSOCIATED EXTRACELLULAR POLYMERIC SUBSTANCES FROM STAINLESS STEEL USING SIMULATED CLEAN-IN-PLACE SYSTEM

by

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(Under the direction of Joseph Frank)

ABSTRACT

Pseudomonas putida is a common food contact surface biofilm producer, producing high amounts of extracellular polymeric substances. The purpose of this study was to develop a method for observing *Ps. putida* biofilm and associated EPS remaining after clean-in-place treatment and determine the ability of CIP to remove EPS from stainless steel. Stainless steel coupons were soiled with 3-day *Ps. putida* biofilm growth. Samples were cleaned using a simulated CIP system. DNA staining using Hoescht 33258 and EPS staining using Lectin PNA were compared on cleaned coupons cleaned using 1.28%, 2.0%, 4.0%, and 6.0% sodium hydroxide at 66˚C for 3 min followed by a sterile water rinse, neutralizing in phosphate buffer and viewed under a epifluoerescent microscope. The effectiveness of cleaning for removing EPS was also determined using 1.5% and 2.0% sodium hydroxide at 68˚C and 70˚C. Sodium hydroxide concentration of 1.28% at 66˚C is sufficient to remove Hoescht but not Lectin binding material. A minimum of 2.5% sodium hydroxide at 66˚C was sufficient to remove Hoescht and Lectin binding material.

INDEX WORDS: Biofilm, *Pseudomonas putida*, Cleaning, Lectin, Hoescht.

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INTRODUCTION

Biofilm can be defined as living cells immobilized at a substratum. They are frequently embedded in an organic polymer matrix of microbial origin or a surface accumulation of organic matter, which is not necessarily uniform in time and space (11). Biofilms are a concern in the food industry since their substrata can be raw food material, non-food contact surfaces such as walls, floors, and drains, as well as food contact surfaces (89). Once microbial cells colonize a food contact surface, they grow and adhere, forming a multiplayer matrix embedded in adhesive polymers called exocellular polymeric substances (EPS) (60). EPS act as a 'biological glue'(12) that helps cells firmly attach to the surface and protects them from different environmental stresses such as desiccation (69), acts as nutrient entrapment (54), and restricts diffusion of molecules such as antimicrobial agents from the surrounding medium (45). EPS, along with proteins and lipids left on the surface after detachment, is referred to as microbial "footprints". This increases the subsequent ability of other bacteria to attach to the surface (66). *Pseudomonas* spp. is the most dominant genus of the psychrotrophic microflora present in milk processing equipment and on food contact surfaces (99,76,80). They are a spoilage agent of refrigerated fresh food, and have a major impact on the quality of those foods (47). They are prolific biofilm producers and produce a high amount of EPS (92). One of the most important concerns, though, for the food industry is that *Pseudomonas* spp. can act as a primary colonizer thus providing a protective barrier and the suitable environment for pathogens such as *Listeria monocytogenes* to attach (73).

Periodic cleaning can control biofilm accumulation by promoting detachment. Cleaning becomes more challenging when EPS producing microorganisms are growing on the food contact surface. Previous research on the cleanability of stainless steel demonstrated that hot alkali detergent, rinsing and sanitizer application were necessary components for adequate clean-in-place performance for biofilm removal (22). Cleaning performance increases with increasing contact time, mechanical force, and sodium hydroxide concentration. Overall cleaning efficiency increases with increased temperature (20). Detergent type is the most important clean-in-place system component in controlling biofilm accumulation (19). Previous research on the effectiveness of typical cleaning procedures demonstrated that cleaning treatments were not sufficient to penetrate and remove all biofilm or associated EPS present on the surface (79,40,62,3,13,32), suggesting that longer exposure time or greater detergent concentration may be required (97). These studies emphasize the need for improvement of typical (86) cleaning procedures in terms of removal of biofilm and especially the associated EPS.

The purpose of this study was to develop a method for observing biofilm and associated EPS remaining after CIP treatment. *Pseudomonas putida* was used to produce model biofilms to determine the ability of CIP treatments to remove biofilm and EPS from a stainless steel surface.

CHAPTER 1

LITERATURE REVIEW

BIOFILMS

Most bacteria can exist in two distinct states in natural habitats: (i) the plaktonic state, in which they function as individuals, and (ii) the sessile state, in which they attach to surfaces, form biofilms, and function as a closely integrated community (60) By definition, a biofilm consists of living cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin or a surface accumulation, which is not necessarily uniform in time and space (11).

Microbial attachment to surfaces was first described by Zobell in 1943, but the word "Biofilm" made its first appearance in scientific literature in 1970s (11).

Mechanisms of microbial attachment

Various authors, including Frank (28), Kumar and Anad (54), Marshall (60), Carpentier and Cerf (9), Hood and Zottola (43) have summarized research on the mechanisms of microbial attachment.

Biofilms occur at one of the following interfaces: (i) solid/air, (ii) inert solid/liquid, and (iii) solid nutrient/liquid interface (34). The initiation of attachment can be predicted on the basis of surface-free energies of the attachment surface and the bacterial cell, as well as the surface tension of the surrounding medium.

If the surface tension of the bacteria is less than that of the surrounding medium, the cells will more likely adhere to hydrophobic (low surface tension) surfaces. In the case where bacterial surface tension is more than that of the surrounding medium then adhesion to hydrophilic surface (high surface tension) is observed (60,28). Hydrophobic interactions have been suggested as being responsible for a range of different adherence phenomena observed in natural systems (43).

The forces involved in microbial adhesion depend on the specificity of the interaction. They include Van der Waals attractive forces, hydrogen bonding, hydrophobic interactions, ionic, dipole-dipole, and dipole-induced dipole (18).

Conditioning films

In natural systems the accumulation of molecules, often protein molecules (28), at the solid/liquid interface on surfaces forming an absorbed layer of biological macromolecules is referred to as conditioning film (54). Adsorbed proteins can either inhibit or stimulate subsequent bacterial attachment (61,43,54,28). Conditioning of surfaces with milk and milk proteins such as casein and β-lactoglobulin decreased the level of adherence of *L. monocytogenes* and *S. typhimurium* to stainless steel and Buna-N rubber (38). Stainless steel surface conditioning with skim milk decreased adherence of *S. typhimurium* and *L. monocytogenes,* but showed no effect on the degree of adherence of *P. fragi* (44). Biofilm formation by *L. monocytogenes* on polyester was completely inhibited using minimal media, whereas in the presence of hydrolyzed protein media creating a protein film, biofilm formation was observed (8).

Many species of bacteria are capable of producing polysaccharides outside the cell wall. These exopolysaccharides can either be in the form of discrete capsule or in the

form of extracellular slime (81). These exocellular polymeric substances (EPS) can be defined as substances of biological origin that participate in the formation of biofilms (81), and include organic macromolecules composed of polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds. Bacteria that produce EPS can form their own conditioning film, an example of this are microbial "footprints" (66). The term "footprints" refers to (i) adhesive molecules (polysaccharides, proteins, lipids) that bacteria leave at an interface after detachment (ii) substances produced and released by the bacteria to aid in detachment from the interface (polysaccharides, biosurfactants), and (iii) molecules produced by bacteria gliding across an interface (polysaccharides, biosurfactants). These substances remain on the surface after detachment and may increase the subsequent ability of other bacteria to attach to those polymer-covered areas.

Primary colonizers can condition the surface, thus make it suitable for other microorganisms to adhere to the surface (91,43). EPS-producing bacteria such as *Pseudomonas fragi* can enhance attachment of less adhesive species, *L. monocytogenes,* to glass cover slips under flowing conditions (73).

Attachment and biofilm formation

There are various stages leading from adhesion to the surface to biofilm formation: (i) initial colonization of the surface, (ii) growth and further adhesion of cells to form a multilayer matrix embedded in adhesive polymers, and (iii) formation of a mature biofilm (60). Bacterial adhesion at the first stage is reversible, which then becomes irreversible over time (99,9). *Pseudomonas spp.* require 4-hour attachment period for 78% of the total bacteria attached on polystyrene surface to become irreversibly attached (60). Reversible attachment is characterized by weak and low

specificity interactions between the cell and the substratum and existence of Brownian motion. In irreversible attachment, bacteria lack Brownian motion, requiring much stronger force for detachment (54).

Cell surface structures including the presence of fimbriae and capsules, and cell surface hydrophobicity affect, the attachment of bacteria (70). Fimbriae (pili) are 1-11 nm thick, threadlike cell projections anchored to the outer membrane (68). Initial attachment of *E. coli* requires the presence of fimbriae, but it does not seem to be involved in the movement of bacteria across the surface (17). Capsules are gel-like substances mostly composed of water and polysaccharides, anchored to the cell surface and completely surrounding the cell wall (28). Cell surface hydrophobicity is based on outer membrane polymers including lipopolysaccharides, lipoproteins, lipoteichoic acid, and lipomannan. The orientation of these lipids on the outer membrane determines whether the cell can interact with a hydrophilic or a hydrophobic interface, thus affecting cell surface hydrophobicity (67).

Excreted cell substances can also affect the bacterial attachment. These surface-active compounds help in the interaction of bacteria with the interfaces by conditioning them, thus allowing attachment and biofilm formation (67). Exocellular slimes are the most common of these compounds. Slimes could be the result of active cell secretion or spontaneous liberation of integral cellular components. Slimes along with capsules are the two EPS forms, which can be excreted before or after attachment, thus affecting the cell surface hydrophobicity (81). The results of a study looking at the effects of various agents on the attachment of *Ps. fragi* to stainless steel demonstrated that excreted polysaccharides were involved in the attachment (39). This was the case since

chemicals known to react with polysaccharides such as sodium periodate, cetavlon, NaOH, and concanavalin A inhibited attachment in the pretreatment assays.

Attachment of bacteria is also affected by pH, temperature, nutrient availability, nutrient concentration, flow of material and oxygen tension (54,17).

A biofilm may consist of several compartments: (i) substratum, (ii) base film, (iii) surface film, (iv) bulk liquid, and (v) gas (11). The biofilm itself contains the base and the surface film component and contains at least two phases: (i) a continuous liquid phase containing dissolved and suspended particulate materials and (ii) a series of solid components such as species of microorganisms, EPS, and inorganic particles. The bulk liquid component transports soluble (nutrients) and particulate (cells, EPS) material to the surface film while at the same time carries away waste products of metabolism and detached cells so they can be released to the liquid medium (11). The biofilm community exhibits structural as well as metabolic organization (14,15).

Advantages for cells from biofilm formation

There are several advantages to the cell of sessile growth and biofilm formation: (i) protection from the environment, (ii) increased nutrient availability and metabolic cooperation, and (iii) acquisition of new genetic traits through plasmid transfer (17).

The sessile cells are protected from various antimicrobial agents (57,29,65,52,54,83). In the presence of up to 10 μg/ml of various antibiotics including clarithromycin, *Ps. aeruginosa* biofilms did not show any decrease in cell proliferation (83). An amount of 100 μg/ml of antibiotics needed to cause a decrease in cell numbers. Biofilm formation is also associated with increased resistance of the attached cells to different sanitizers (43). Four-hour attached *L. monocytogenes* to stainless steel surface

showed resistance when exposed to 200 ppm hypochlorite (75). Adherent microcolonies of *L. monocytogenes* formed on glass slides exhibit increased resistance to quaternary ammonium and acid anionic sanitizers (29).

Within the mature biofilm structure there are many water channels. These water channels work just like a biofilm circulatory system and they represent the most effective means that the attached cells have, for exchanging nutrients within the polymer matrix as well as transferring metabolites and waste products to the bulk liquid phase (15). The water channels appear to play a role of oxygen transport in the biofilm matrix (14).

Gene transfer has been observed between biofilm populations in natural communities (17). The most likely used method for gene transfer within or between populations is conjugation, in which a self-transmissible DNA element such as plasmids are transferred.

Detachment

Detachment of cells from the attached surface can be either cell-directed or externally directed (28). The externally directed detachment can utilize application of enzymes, oxidation of attachment polymers, or physicochemical forces. The enzymatic release can employ enzymes such as cellulase and amylase to hydrolyze exocellular polymers of the biofilm matrix resulting in the detachment of microorganisms attached to the contact surface. Detachment through the oxidation of attached polymers is due to contact with strong oxidizing agents. Partial or complete removal of attached microorganisms can be obtained by applying various physicochemical forces such as surfactants, chelating divalent cations, increasing temperature and pH as well as flowing liquid or by applying mechanical shear force on the contact surface. The cell-directed

detachment that the biofilm undergoes after growth falls into two forms: (i) erosion and (ii) sloughing (91). Erosion is the continuous removal of small biofilm particles due to flow of aqueous phase at the solid/liquid interface. Sloughing is the sporadic detachment of large biofilm fragments due to changing conditions within the biofilm matrix.

BIOFILMS IN THE FOOD INDUSTRY

Biofilms in the food industry are a concern since their substratum can be raw food material, food contact and non-food contact surfaces such as walls, floors, and drains (89). This is of public health significance due to the risk of foodborne illness, when pathogenic microorganisms are present, or to unacceptable product outcomes due to spoilage and shorter shelf-life of the associated product. This is also a concern for the industry, since biofilm growth may increase the cost of production due to equipment fouling, reduce the efficiency of heat transfer, increase cleaning costs, and in some cases accelerate corrosion. In the case where pathogens are found in biofilms, the food processor's costs are even higher, and more detrimental due to the possibility of plant shut down, and legal proceedings involved (63,89). Common environmental bacteria, such as *Pseudomonas* spp., *Enterobacter* spp., and *Klebsiella* spp. (63), can attach to the surface, thus providing a possibility for product contamination, that can reduce its shelf-life, and provide an entrapment or a protection site for the subsequent attachment of pathogens (73).

The most common food contact surface material used in the food and beverage industry is stainless steel (89), due to its stability at various production temperatures, its high resistance to corrosion, and its ease of cleaning (61). Stainless steel surfaces can be classified according to surface finish. The #4 finish is most commonly used in food

applications, with the #3, #7, and 2B (standard milled or rolled) having some limited application. Surface finish has no significant influence on biofilm removal and cleanability (49), also as determined by direct microscopic observation (30). On the other hand, surface roughness has high correlation with surface cleanability. This is not surprising since surface roughness increases with surface polish defects and stainless steel crevices have been shown to serve as entrapment of both microorganisms and food residues (76,55).

Many studies have reported the attachment of spoilage microorganisms as well as pathogens to stainless steel surface (79,39,7,29,58,59,38,92,48,8,96,31,44,24,30). For example, one study showed that growth and biofilm formation of *L. monocytogenes* on stainless steel surface at moderately cold environment (10° C), maintains itself at about 10% of the total population of competitive microflora isolated from both dairy and meat plant environments (48). Another study showed the attachment capabilities of *L. monocytogenes* for attachment and biofilm growth at ambient (20° C) and cold storage temperature (10° C) on various food processing materials such as stainless steel, glass, polypropylene, and rubber at the short contact time of 20 minutes, as observed by scanning electron microscopy (58).

Many review articles have also summarized research on the microbial attachment on various food tissues including meat (lean and adipose tissue) and poultry skin (43,50,51,54,89,28). These reviews emphasize the effects of microbial attachment and the need for its control, especially through effective cleaning of both food and non-food contact surfaces, since once attached, microorganisms are difficult to remove.

CONTROL OF BIOFILMS IN THE FOOD INDUSTRY

Control of biofilm establishment and growth can be achieved on three different levels: decrease or if possible eliminate initial microbial attachment to the surface, control growth of the attached cells, or removal of the resulting biofilm and soil residues (27). An important consideration for controlling the initial microbial attachment is the design of equipment that promotes continuous product flow with no dead ends and a minimum number of protected sites, such as pits and crevices (77). Surface temperature is an important factor determining cleaning frequency, since in most food processing environments, control of water and nutrient concentration is very difficult and in some cases impossible. Removal of both biofilm and associated soil residues is possible through effective cleaning and disinfection practices (22) at specific intervals with the warm surfaces requiring more frequent cleaning.

CLEANING OF FOOD CONTACT SURFACES

Cleaning of food contact as well as environmental surfaces such as walls can be critical to the safety of the final food product (32). The time available for biofilm formation depends on both the frequency and the effectiveness of the cleaning process. This is especially applicable in food processing systems where biofilm accumulation is inevitable, thus the frequency of the cleaning is very important in the control of biofilm growth (37). Cleaning and disinfection of food contact surfaces is the most important method of controlling contamination, that has the surface as its major route (53). Effective cleaning is vital (19) because both soil and as many microorganisms as possible need to be eliminated before the application of the disinfectant (9). The interaction of these important factors: (i) sufficient time, (ii) chemical concentration, (iii) temperature, and (iv) physical action such as scrubbing or turbulent flow (26), can provide acceptable

microbial control in any cleaning system (21). For example, cells of *Ps. fragi* exhibited attachment fibrils on a stainless steel surface only when the surfaces were cleaned using lower than recommended temperature, and detergent-sanitizer concentrations (79).

The effectiveness of any cleaning system is affected by water hardness, equipment design, and soil type associated with every food contact surfaces (27). Soil remaining on the surface due to ineffective cleaning affects the attachment and retention characteristics of the surface and be a potential food source of the attached microorganisms, thus providing an indication of poor cleaning (90). Removal of soil is more effective when the soil type and soil characteristics are well characterized so the cleaning agent used is based on the particular soil solubility and ease of removal (36). Table 2.1 shows the solubility, ease of removal as well as the heating effect on soil removal. Protein is the most difficult food soil to be removed with the sugar and mineral salts being the easiest.

Cleaning of food contact surfaces gets more challenging when exopolysaccharide producing microorganisms are growing on the surface. The following section aims at giving an introduction to EPS and the difficulties in removing it from surfaces.

EPS and the cleaning challenges

Exopolysaccharides are substances of biological origin that participate in the formation of microbial aggregates. Some exopolysaccharides have been also referred to as ‘biological glue’ (12), or as glycocalyx (54). Exopolysaccharides, as already mentioned in a previous section, could be in the form of capsules, attached to the cell, or in the form of slimes, not distinctly associated with any one bacterium, secreted in the medium (81).

TABLE 2.1. Food soil characteristics

Component on surface	Solubility	Ease of removal	Change on heating
Sugar	Water soluble	Easy	Caramelization; more difficult to clean
Fat	Water insoluble, alkali soluble	Difficult	Polymerization; more difficult to clean
Protein	Water insoluble, alkali soluble, slightly acid soluble	Very difficult	Denaturation; polymerization; much more difficult to clean
Mineral salts	Water solubility varies; most are acid soluble	Easy to difficult	Generally insignificant

Modified from Hayes (1992) (36).

EPS in the biofilm matrix can have different origins: (i) active secretion by the living cells, (ii) spontaneous liberation of integral cellular components such as carbohydrates, (iii) carbohydrates released from the death and lysis of cells, and (iv) adsorption of EPS shed by microbial aggregates in areas close to the attachment site. The primary origin of most of the EPS associated with the biofilm though is from active secretion of cells. EPS synthesis by living cells is stimulated by the attachment to a solid surface. Total carbohydrate content of the attached cells was 2.5-fold greater than that of the unattached cells, and the exopolysaccharide synthesis showed a 5-fold increase after attachment (88). Metabolic stress also induces exopolysaccharide production of the marine species, *Ps. atlantica* (85).

Exopolysaccharides are composed of various monomers with glucose, galactose and mannose, being the most common (81). The main carbohydrates isolated from the EPS matrix of *Ps. fluorescens* attached to stainless steel surface*,* were mannose, glucose, and galactose (7). Uronic acids as well as acidic groups, and pyruvate are often constituents of the EPS, with the positively charged groups being rare (12). The composition of EPS though, changes during the growth cycle. The marine species *Ps. atlantica,* shows a large increase in the uronic acid composition and a decrease in galactose during the growth cycle (85). In *Pseudomonas* spp. EPS production occurred only when the culture reached the late log phase of growth (81).

The structure of the exopolysaccharides can be linear or branched with regular repeating units (12). The structure determines the physical behavior of the polysaccharides and their contribution to the biofilm processes and characteristics. Table 2.2 shows biofilm characteristics attributed to physical properties of the exopolysaccharides.

The physical properties of exopolysaccharides give many benefits to the biofilm system and its environmental protection (17). EPS plays a critical role in adsorbing organic compounds and providing the mechanism, through which the community can trap and concentrate essential nutrients and growth components (54). The EPS matrix can act as an ion exchanger, restricting diffusion of compounds from the surrounding medium such as antimicrobial agents (45), and sequestering metals, cations and toxins (17). It also contributes to protection from environmental stresses such as UV radiation, changes in pH, osmotic shock, as well as, desiccation (25), since the mucoid strains of some bacteria were much more resistant to desiccation than the corresponding nonmucoid mutants (69).

TABLE 2.2. Physical properties of exopolysaccharides and their relation to biofilms

Physical property	Relevant biofilm characteristics
Adsorption characteristics	Adsorption of cells and EPS to surfaces Formation of conditioning films
Hydration	Faster and greater EPS hydration
Ion exchange properties	Adsorption of ions to EPS matrix
Viscosity	EPS matrix viscoelastic properties Diffusion of molecules within or close to the EPS matrix
Gel formation	Cohesion and rheological properties of EPS Influence on detachment and sloughing
Polymer-polymer interactions	Synergistic effects in multi-species biofilms

Modified from Christensen (1989) (12).

Detergent classification

Detergents can be classified into four basic categories: (i) inorganic alkalis including caustic and non-caustic, (ii) inorganic and organic acids, (iii) surface active agents including anionic, non-ionic, cationic and amphoteric, and (iv) sequestering agents including both inorganic and organic agents (36).

Inorganic alkalis can be caustic or non-caustic. Some inorganic alkalis are: sodium hydroxide, sodium metasilicate, sodium carbonate, and trisodium phosphate. Sodium hydroxide (caustic soda) is one of the strongest alkalic agents. It has excellent dissolving and saponification power. An important property of sodium hydroxide is its

bacteriocidal power (36,10). The combination of sodium hydroxide (pH 10.5) at 55° C for 5 minutes with acetic acid (pH 5.4) at 55° C for 5 minutes, was the most effective treatment in the removal of 3-day *L. monocytogenes* grown on glass microscopic slides (2). The presence of sodium hydroxide solution (pH 10) inhibited the attachment of *Ps. fragi* to a stainless steel surface in the pretreatment assay, and also caused removal of the attached cells at a significant level (39). All the above properties and its low cost make sodium hydroxide the most common detergent used in the food and beverage industry. However, it is highly corrosive especially to aluminium, difficult to remove by rinsing, and irritating to skin and mucous membranes (36). These limitations can be overcome through its use in CIP (clean- in -place) systems, which have replaced manual cleaning of most equipment used to process liquid foods. Sodium metasilicate although a strong alkali with good rinsing ability and great dissolving, emulsifying, and saponification power and is non-corrosive, its low bacteriocidal power and high price make it less popular in the industry (36). Sodium carbonate (soda ash) and trisodium phosphate are both non-caustic with good dissolving and saponification power, but weak bacteriocidal power.

Inorganic acids have very weak dispersing, emulsifying, and saponification power. These properties plus their high corrosive ability limited their use in the food industry. They have some application in dairy industry since they are effective in removing mineral deposits such as milkstone (composed of protein, calcium carbonate, and other salts). Examples of inorganic acids used in the dairy industry are hydrochloric, sulphuric, and nitric acid (84,36). Organic acids such as gluconic and tartaric are milder and safer to handle, and they are commonly used in detergent formulations.

The classical example of a surface active agent is soap which is composed of sodium or potassium salts of fatty acids such as palmitic, stearic, and oleic (36). Soaps are generally being replaced by synthetic detergents (surfactants) due to their reduced effectiveness under cold-water conditions and their participation with calcium in hard water. The surfactants are good emulsifying agents, non-corrosive, non-irritating, with good wetting, and dispersing power. The largest group of the surfactants, are the anionic surface active agents, where negative charges predominate. The non-ionic agents do not dissociate in solution and the amphoteric can exist in two forms, anionic or cationic, depending on the pH of the solution. Cationic have better bacteriocidal activity than anionic or non-ionic surfactants.

Sequestering agents are usually added to the detergent formulations to prevent magnesium and calcium precipitation. The most widely used inorganic sequestering agents (chelating agents), have high solubility in liquid detergent formulations, with ethylene diamine tetraacetic acid (EDTA), being the most used, because it's cost-effective. EDTA addition to detergent formulation was found to enhance *Bacillus* biofilm removal (97).

Detergents can also be classified into four categories according to their chemistry of action as solvents, alkaline, acid, and neutral pH cleaners (10). Alkali can be heavy-duty, medium alkaline, and chlorinated alkaline. Alkaline cleaners are very good in preventing mineral scale buildup and removal of fats and oils, with heavy-duty alkalic detergents being excellent for removal of carbohydrate accumulation (84). Chlorinated alkalic readily removes proteins and carbohydrates due to its enhancement of cleaning and decolorization, so it is often used for CIP cleaning of pipes, tanks, and vats. Acid

cleaners are used for removal of mineral salts and iron buildup (33). Neutral pH cleaners are used on specialized material such as packaging systems where the acid or alkaline cleaners may cause surface deterioration. Solvents are used on surfaces having a light organic material deposit due to their ability to break down the organic matter.

Clean-in-place (CIP)

Clean-in-place systems are common in food and beverage industry. They have replaced manual cleaning of equipment such as pipelines, tanks, vats, heat exchangers, and homogenizers especially in the dairy (62,3), soft drink, and brewing industries (26,33). Information on cleaning systems such as CIP has been reviewed by various authors, including Troller (84), Hayes (36), and International Commission on Microbiological Specifications for Foods (26).

Benefits of implementing clean-in-place systems include: mechanical force generated through turbulence flow increasing cleaning effectiveness and easier soil removal, possible use of highly caustic detergents, use for equipment sites where manual scrubbing is impossible, reduced labor costs, optimum use of detergent-disinfectant solutions, faster cleaning operation, less mechanical damage to equipment, and greater safety through the automated handling of caustic alkalic and strong acid solutions (84,36).

Various researches studied the ability of clean-in-place systems to remove biofilms. Mattila et al. studied the survival of gram-negative bacteria isolated from a milking line, using milk and cream as organic challenges (62). The effectiveness of alkaline-Cl_2 and alkaline-acid clean-in-place systems were determined. Another study by Austin et al. investigated the development of biofilms, mostly consisted of gram-negative

cells, on gaskets of milk processing equipment, after clean-in-place cleaning and disinfection (3).

As already mentioned, CIP method of cleaning is very beneficial especially through its potential for using high concentrations of caustic cleaners in combination with high temperatures. As a result, concentrations of up to 1000-1500 ppm and sometimes even 3000 ppm can be used, compared to 500-900 ppm used in manual cleaning (84). The U.S. Department of Health and Human Services has specific recommendations on the combination of causticity, time, and temperature needed in order to meet sanitary requirements for cleaning and sanitizing of containers, equipment, product-contact surfaces used in the transportation, processing, handling, and storage of processed milk and milk products (86). Table 2.3 shows the suggested combination of sodium hydroxide concentration, time, and temperature needed for cleaning of containers, equipment, piping, product-contact surfaces used for the processing of milk and milk products. These specifications are jointly set by the Sanitary Standards Subcommittee of the Dairy Industry Committee, the Committee on Sanitary Procedure of the International Association of Milk, Food, and Environmental Sanitarians, the Milk Safety Branch, Center of Food Safety and Applied Nutrition, Food and Drug Administration, Public Health Service, and Department of Health and Human Services.

TABLE 2.3. U.S. Department of Health and Human Services specifications for containers and equipment cleaning for processed milk and milk products production

	Temperature, Degrees						
C	77	71	66	60	54	49	43
F	170	160	150	140	130	120	110
Time (min)	**Concentration of NaOH (percent)**						
3	0.57	0.86	1.28	1.91	2.86	4.27	6.39
5	0.43	0.64	0.96	1.43	2.16	3.22	4.80
7	0.36	0.53	0.80	1.19	1.78	2.66	3.98

Modified from U.S. Department of Health and Human Services (1999) (86).

PSEUDOMONAS SPECIES

Bergey's Manual of Determinative Bacteriology (42) describes *Pseudomonas* spp. as gram-negative aerobic rods, with most of the species and strains being psychrotrophic, showing growth at 4° C. They are characterized by fluorescent, diffusible pigments, they are oxidase positive and most of the species including *Ps. putida* are able to utilize glucose, 2-ketogluconate, valine, alanine, and arginine, as well as nitrate as their nitrogen source. They produce large amounts of exocellular polysaccharides, forming highly mucoid colonies on the agar surface (71). Most of the EPS fraction produced by *Ps. putida* exists in the noncell-associated form (slime) with a small fraction being cell associated.

Pseudomonas spp. are typically found in soil and water, and widely distributed among foods, especially vegetables, meat, poultry, dairy, and seafood products. They are the most important group of bacteria causing spoilage to refrigerated fresh foods, thus having a major impact on the quality of the foods (47). Thus *Ps. putida* biofilm growth, as is the case with any other biofilm producer, and considering its prolific nature and its "rich" EPS production, is undesirable in the food and beverage industry. Its growth leads to reduced shelf life driving the need for more effective cleaning practices. Another concern for the food industry is that *Pseudomonas* spp. biofilm provides protection and a suitable environment for pathogen attachment and growth, as in the case of *Ps. fragi* acting as a primary colonizing microorganism for the attachment of *L. monocytogenes* (73).

Pseudomonas spp. biofilm growth is a special concern in the dairy industry since it is a milk-borne species and consequently a common contaminant of dairy equipment surfaces. There are studies on the attachment, growth, and microscopic biofilm analysis of *Pseudomonas* spp. (99,76,80). Speers et al. studied the various factors which aided in the attachment of *Pseudomonas* spp. on dairy equipment surfaces, such as stainless steel surface channels, milk deposits and EPS production (76). *Pseudomonas* spp. were found to be one of the most adherent strains of psychrotrophic strains isolated from raw milk attached to stainless steel and rubber surfaces, with stainless steel being the most prone to *Pseudomonas* spp. attachment (80). Zoltai et al. (99) observed microbial attachment to milk contact surfaces using scanning electron microscopy. *Ps. fragi* attached in greater numbers compared to other microorganisms tested, exhibiting fibrous material extending from the edge of the cell to the stainless steel chip surfaces (99).

Considering the great importance and consequences of *Pseudomonas* spp. biofilm growth, many studies have been done dealing with the effect of different environmental parameters on the species biofilm accumulation (64) and the effectiveness of the cleaning practices in terms of removing the biofilm growth from food contact surfaces. Holah et al. used *Pseudomonas* spp. to determine the effectiveness of surface cleaning (41). Surface hygiene was assessed using different soiled surfaces (baked beans, eggs, fish, buttermilk) and analyzed using microscopic analysis. *Pseudomonas* spp. was used in another study to produce biofilm and exocellular polysaccharide on stainless steel for the evaluation of cleaning practices used in the food industry (93). A mixed biofilm of *Pseudomonas* and *L. monocytogenes* was used in an inactivation study testing the effectiveness of peracid sanitizers (24). Various authors, including Wirtanen et al (95) and Chumkhunthod et al (13), have published research on microbiological methods for testing the efficacy of different sanitizers against *Pseudomonas* spp. biofilms.

BIOFILM MICROSTRUCTURE STUDIES USING EPIFLUORESCENCE MICROSCOPY

Biofilm microstructure has been studied by several research groups using various microscopic methods. Epifluorescence microscopy is one of the most popular methods used in biofilm studies, since it allows the study of biofilms grown on surfaces (94). Biofilm grown on different food contact surfaces, especially stainless steel, are stained with a fluorescent dye and analyzed under an epifluorescence microscope. The most widely used fluorescent dyes used in the biofilm studies are listed in Table 2.4.

TABLE 2.4. Most commonly used fluorescent probes in biofilm microstructure studies

Name	Application
Fluorescein-5-isothiocyanate (FITC) (35)	Protein labeling (30)
Acridine orange (35)	DNA and RNA staining (39,41,13,72,98)
Fluorescein (35)	Negative staining and pH indicator
Hoescht 33258	DNA staining (24)

Epifluorescence image analysis is an informative tool for analysis of biofilms. Various applications of this method include surface hygiene studies assessing biofilm components on the associated surface like organic soil, dead and living cells and exopolysaccharides (41,98), cell morphology determination, measurement of biofilm pH, differentiation between types of microorganisms using immuno-genetic probes (94), and inactivation studies looking into the effectiveness of different sanitizers (13,24,72).

The most extensive use of epifluorescence microscopy in biofilm research is for area coverage studies. Wirtanen et al (92) used epifluorescence microscopy for directly observing the biofilm growth on stainless steel surface after staining the surfaces with acridine orange. Biofilm area coverage was converted to percentage of total area in the

aid of comparing conventional cultivation methods with results of direct microscopic surface observations (92), improving surface hygiene by detecting spoilage microorganisms attached to the stainless steel surface (93,96), and testing antimicrobial power of different disinfectants on *Pseudomonas* spp. biofilms grown on stainless steel surfaces (95).

Lectins and lectin staining

Exocellular polysaccharide staining is a challenge since there is no universal dye that can be used. In biofilm studies, various dyes have been used depending on the species of the biofilm producer and the type of microscopy used to view the stained images. An aqueous Congo red solution was used for staining the exocellular polysaccharide of gram negative bacteria isolated from a freshwater stream viewed under light microscopy (1). Ruthenium red stain, for anionic polysaccharide staining, was used in many studies using both electron (23) and fluorescence microscopy (16). Dall et al (16) ran a qualitative assay of the glycocalyx produced by *Streptococcus* using ruthenium red, cellufluor, and periodic acid-Schiff examined under a fluorescence microscope. Calcofluor is also another dye used for EPS visualization using confocal and scanning electron microscopy (78).

Even though various dyes have been used in biofilm polysaccharide studies over the years, the most promising in epifluoerescent microscopy are the lectins (82). Haugland have extensively reviewed lectins in the *Handbook of Fluorescence Probes and Research chemicals* (35). Lectins are highly specific carbohydrate-binding proteins or glycoproteins of non-immune origin that bind to specific configurations of sugar molecules, thus serving in labeling cell types or cellular components. Their activity can

be inhibited by mono, di or trisaccharide. Lectins are isolated from a variety of natural sources including seeds, plant roots, fungi, bacteria, seaweed, fish eggs, body fluids of vertebrates, and mammalian cell membranes. Many plant lectins have been characterized, but little is known about their function. Their general function is that they act as surface recognition molecules (74). Bacterial cell surface lectins play a role in the initiation of infection by mediating in adhesion to epithelial cells, in plants they aid in host-bacteria symbiosis, and in animals in uptake and differentiation of cells, and organ formation.

Lectins are available in a conjugated form as fluorescent derivatives for fluorescence microscopy use to detect cell-surface and intracellular glycoconjugates, as electron labels for electron microscopy use and as conjugated enzymes for enzyme-linked assays. Two of the most commonly used fluorescent conjugated lectins are concanavalin A and wheat germ agglutinin (35,56). Concanavalin A binds to α-mannopyranosyl and α-glucopyranosyl residues. Probably one of the best green-fluorescent dyes available is *Arachis hypogaea,* which is isolated from peanuts (35). It is a tetrameric protein with a molecular weight of about 110, 000 daltons, it is highly specific for β-galactose residues, and has an excitation/emission of 495/519 nm respectively. Its conjugated fluorescence form has superior brightness, its more photostable and less pH sensitive.

Digital Imaging and image processing and analysis

The fundamentals of digital image processing have been extensively reviewed by Baxes (6,5,4). Image processing refers to the manipulation and analysis of a two-dimensional picture using a digital computer in order to improve, correct, or analyze an image. Some of the major applications of image processing in the biological research are

for image enhancement for improving the visibility of the original image, cell analysis for counting and classification of cell structures and in analysis and classification of DNA samples (6). There are five fundamental classes of digital image processing: (i) image enhancement for improving image quality and noise reduction, (ii) image restoration for photometric and geometric correction, (iii) image analysis for object classification, (iv) image compression for motion compression, and (v) image synthesis for 3D visualization (5). Generally image analysis is used to obtain the needed information (length, width, area, optical density, and percent black and white pixels) from an image, after the appearance have been changed using image processing (46). The fundamental unit of a two dimensional image is the pixel. The number of pixels per unit area (sampling rate) has to be large enough in order for the image to preserve its information in sufficient detail (46). Each pixel of a color image has three sub-elements (red, green, blue), therefore, its difficult to fit all three colored elements into one pixel. As a result colored images have lower resolution than black and white, so a matching filter is used to capture each color in black and white (4).

One powerful tool for image analysis is Image Tool, which was developed by Wilcox and coworkers at the University of Texas Health Science Center in San Antonio (http://www.ddsdx.uthsca.edu/dig/itdesc.html). Another powerful tool is Image Pro Plus software.

CHAPTER 2

DEVELOPMENT OF EPS STAINING METHOD

Introduction

Staining biofilm and associated exocellular polymeric material (EPS) for microscopy is challenging since there is no universal stain that can be used. The most commonly used fluorescent dyes in biofilm microstructure studies are: fluorescein-5-isothiocyanate (FITC) for protein labeling (30), acridine orange for DNA and RNA binding (39,41,13,72,98), and Hoescht 33258 used for DNA staining (24). Each of these dyes causes nonspecific staining to various degrees. Fluorescent stains are sensitive to various environmental factors such as polarity of the cells and the membranes, proximity and concentration of quenching species (example O_2 and proteins), and the environmental pH, which can cause fluorophore configuration change, interfering with excitation (35).

EPS staining is different since the type of stain which is effective, depends on the species of the biofilm producer, the composition of the EPS and the specificity of the stain. In biofilm studies various dyes have been used for EPS staining. An aqueous Congo red solution was used for staining the exocellular polysaccharide of gram negative bacteria isolated from a freshwater stream viewed under light microscopy (1). Ruthenium red stain, for anionic polysaccharide staining, was used in many studies using both electron (23) and fluorescence microscopy (16). Dall et al (16) used a qualitative assay of the glycocalyx produced by *Streptococcus* using ruthenium red, cellufluor, and periodic

acid-Schiff examined under a fluorescence microscope. Calcofluor is another dye used for EPS visualization when using confocal and scanning electron microscopy (78).

Even though various dyes have been used in biofilm polysaccharide studies over the years, the most promising in epifluoerescent microscopy are the lectins (82). Haugland have extensively reviewed lectins in the *Handbook of Fluorescence Probes and Research chemicals* (35). Lectins are highly specific carbohydrate-binding proteins or glycoproteins of non-immune origin that bind to specific configurations of sugar molecules, thus serving to label cell types or cellular components. Their activity can be inhibited by mono, di or trisaccharides. Lectins are isolated from a variety of natural sources including seeds, plant roots, fungi, bacteria, seaweed, fish eggs, body fluids of vertebrates, and mammalian cell membranes. Many plant lectins have been characterized, but little is known about their function. Their general function is to act as surface recognition molecules (74). Lectins are available in a conjugated form as fluorescent derivatives for fluorescence microscopy. They are used to detect cell-surface and intracellular glycoconjugates, as electron labels for electron microscopy and to conjugate enzymes for enzyme-linked assays. Two of the most commonly used fluorescent conjugated lectins are concanavalin A and wheat germ agglutinin (35,56). Concanavalin A binds to α-mannopyranosyl and α-glucopyranosyl residues. Probably one of the best green-fluorescent dyes available is Lectin PNA from *Arachis hypogaea,* which is isolated from peanuts (35). It is a tetrameric protein with a molecular weight of about 110, 000 daltons. It is highly specific for β-galactose residues, and has an excitation/emission of 495/519 nm respectively. Its conjugated fluorescence form has superior brightness, is more photostable and less pH sensitive than the non-conjugated form.

Material and methods

Stains. To select the most appropriate stain for EPS and biofilm staining after CIP treatment, various stains were evaluated. The lectins tested were picked based on past research works stated in the previous section and their specificity for glucose, which is one of the most abundant monomers present in bacteria exopolysaccharides and therefore EPS. Hoescht and Congo red were evaluated based on their successful use in previous biofilm research studies. All stains and concentrations tested are listed in Table 3.1.

TABLE 3.1. Stain evaluation for biofilm and EPS visualization

Stain	Concentrations tested (μg/ml)
Lectin from *Erythrina Christagalli* (coral tree)	100, 150
Lectin from *Sophora Japonica* (Japanese pagoda tree)	100, 150
Wheat germ agglutinin Alexa Fluor ® 633 Conjugate	100, 150
Lectin PNA from *Arachis hypogaea* (peanut) Alexa Fluor® 488 Conjugate	10, 25, 50, 100, 150
Congo red (1)[α]	75%
Hoescht 33258 (24)[α]	50

[α] Indicates reference number

Production of biofilms. Before the biofilm cleaning a decision had to be made on the age of the biofilm to be evaluated. The objective was to obtain a high level of both biofilm and EPS so the difference in the area covered by the EPS before and after cleaning would be noticeably different and easy to detect.

After a 4-hour attachment period, biofilm was grown for 2, 3, 5, and 6 days. For each day, the stainless steel coupon was stained with both Hoescht 33258 and lectin PNA from *Arachis hypogaea* (peanut) Alexa Fluor® 488 conjugate. Ten fields per sample were captured and analyzed.

Biofilm cleaning. The combination of high temperature and sodium hydroxide concentration of cleaning treatments had the possibility to denature the DNA thus reducing binding of the DNA stain. The possible lack of DNA binding could produce false results on the cleaned surfaces. To check this possibility, a protein binding stain, fluorescein-5-isothiocyanate (FITC) (0.1mg/ml, Sigma Chemical Co., St. Louis, Mo.) was used to calculate the percent area covered.

Staining procedures. Lectin from *Erythrina chrystacalli* was tested at the concentrations of 100 and 150 μg/ml diluted with phosphate buffered saline (pH 7.5). Stained surfaces were incubated in the dark for 45 min, rinsed with deionized water, and allow to air dry before microscopic observation using a filter with an excitation wavelength of 450-490 nm, a Dichroit mirror of 500 nm (DM) and emission wavelength of 515 nm. Lectin from *Sophora japonica* and Wheat germ agglutinin were tested following the same procedure. Lectin PNA from *Arachis hypogaea required* 0.1-1.0 mM of $CaCl_2$ and $MgCl_2$ (35) for binding. Solutions of 0.1, 0.25, 0.5, and 1.0 mM of $CaCl_2$ and $MgCl_2$ were used in combination with all different lectin concentrations listed in

Table 3.1. Congo red was diluted to 75% in aqueous solution. Stained surfaces were incubated for 45 min in the dark, rinsed with deionized water and allowed to air dry before microscopic observation. Hoescht 33258 (0.05 mg/ml) stained samples were incubated in the dark for 30 min, rinsed with deionized water and allow to air dry before microscopic observation with an excitation wavelength of 330-380 nm, a Dichroit mirror of 400 nm and emission wavelength of 435-485 nm.

Results and Discussion

Lectin evaluation. Lectin PNA from *Arachis hypogaea* (peanut) Alexa Fluor® 488 Conjugate gave excellent results as shown in Table 3.2 for *Ps. putida* at the concentration of 100µg/ml, requiring 0.5 mM of $CaCl_2$ and 0.5 mM $MgCl_2$ for binding. The obtained images emitted a bright green fluorescent color that was easy to visualize.

Hoescht evaluation. Hoescht 33258 was successful (Table 3.2) for biofilm staining through DNA binding (24,30). Hoescht was found to be photostable and stable under a wide range of pH. These properties and its bright blue fluorescence color make it a useful stain for biofilm studies.

TABLE 3.2. Stain evaluation for biofilm and EPS visualization

Stain	Visualization
Lectin from *Erythrina Christagalli* (coral tree)	Poor
Lectin from *Sophora Japonica* (Japanese pagoda tree)	Poor
Wheat germ agglutinin Alexa Fluor ® 633 Conjugate	Poor

Lectin PNA from *Arachis hypogaea* (peanut) Alexa Fluor® 488 Conjugate	Excellent (100µg/ml)
Congo red (1)[α]	Poor
Hoescht 33258 (24)[α]	Excellent

[α] Indicates reference number

Production of biofilms. The percent area covered by biofilm as determined by each stain corresponding to days tested is presented in Table 3.3.

TABLE 3.3. Percent area covered of 2, 3, 5, and 6 day *Ps. putida* biofilm staining as obtained by Hoescht 33258 and Lectin PNA

Days	Stain	% area covered
2	Hoescht	18.9
2	Lectin	18.4
3	Hoescht	30.5
3	Lectin	27.6
5	Hoescht	26.7
5	Lectin	17.8
6	Hoescht	19.9
6	Lectin	15.9

Both DNA and EPS staining results indicate that the 3-day growth produced more biofilm than the other times tested.

Biofilm cleaning. Percent area covered after staining with both Hoescht 33258 and FITC was compared after cleaning using 2.0% sodium hydroxide solution at 70˚ C for 3 min. Results are shown in Table 3.4.

TABLE 3.4. Percent area covered after cleaning using 2.0% sodium hydroxide solution at 70˚ C for 3 min.

	Residual biofilm (% area covered)	Residual protein (% area covered)
Before cleaning	33.8	34.2
After cleaning	0.06	0.11

Percentage covered after cleaning and staining with Hoescht 33258 had no significant difference from the percent area covered after staining with FITC. These results provide evidence that Hoescht was able to bind to *Pseudomonas putida* biofilm after the cleaning treatment.

Conclusion

Hoescht 33258 was suitable for *Ps. putida* biofilm staining and lectin PNA from *Arachis hypogaea* was the best fluorescent stain tested for EPS staining.

Three day *Ps. putida* biofilms produced a high level of biofilm so that the difference in the percent area covered by the EPS and biofilm after cleaning was easy to detect.

The combination of high temperature and sodium hydroxide used for the cleaning treatments was not sufficient to denature the DNA, thus Hoescht was able to bind to *Ps. putida* biofilm.

CHAPTER 3

MATERIALS AND METHODS

Stainless steel coupon preparation. Stainless steel surfaces used were type 304 with #4B finish. The sheets were cut into 2 cm x 5 cm coupons. The stainless steel coupons were degreased with acetone before washing. Washing consisted of sonicating in ultrasonic bath model 550 HT (VWR, Atlanta, Ga) in 1.28% sodium hydroxide solution for 1 hour at 80˚C, rinsing in deionized water, sonicating in 15% phosphoric acid solution for 20 minutes at 80˚C, and rinsing in deionized water. Washed coupons were autoclaved submerged in deionized water.

Attachment and biofilm formation. *Pseudomonas putida* (environmental food processing plant isolate) was used for this study. The culture was stored frozen at -80˚C on cryogenic beads (Microbank®, Pro-Lab, Inc., Ontario, Canada). Before each experiment, stock cultures were activated by transferring into 10 ml of tryptic soy broth (Difco Laboratories, Detroit, Michigan) and incubating at 32˚C for 24 hours. Prior to each use, cultures were transferred twice into fresh tryptic soy broth with similar incubation. *Ps. putida* was then inoculated (0.1%) into 500 ml of 10% tryptic soy broth (3g/L) (Difco) and incubated at 32°C for 24 hours. Each stainless steel coupon was submerged into 25 mm x 150 mm test tubes containing 25 ml of the inoculated broth. The submerged coupons were incubated at 25˚C for the 4- hour attachment. Negative controls were incubated in sterile medium. After the 4-hour attachment period, coupons were

rinsed in phosphate buffer to remove nonsessile cells. After the rinse, coupons were submerged in 25 ml of 10% tryptic soy broth (3g/L) (Difco) and incubated at 25˚C for 3 days, followed by a phosphate buffer rinse and transfer to 25 ml of fresh 10% tryptic soy broth (3g/L) after 48 hours.

Cleaning apparatus. A system which applies a turbulent flow, simulating a clean-in-place procedure was used for cleaning of stainless steel coupons with biofilm as described by Frank and Chmielewski (30). The cleaning system consisted of a Buchii 461 controlled temperature water bath (Fisher Scientific, Norcross, Ga.) with a spindle model 1750 mixer (VWR, Atlanta, Ga.) with an impeller (6.35 cm in diameter). A stainless steel frame designed to hold six stainless steel coupons had a radius of 11.75 cm, as measured from the center of the water bath. Six coupons were placed around the edge of the stainless steel holding frame, being 9.5 cm apart. Coupons for treatment were placed vertically on the stainless steel holder in a bath circulating at a turbulent flow (Reynolds number 14680), in sodium hydroxide solution for a constant time of 3 min at a certain temperature. The washed stainless steel coupons were immediately rinsed in sterilized water and neutralized in a final rinse of phosphate buffer (pH 7.2) with no turbulent flow. After the cleaning, coupons were allowed to air dry for evaluation by microscopic analysis.

DNA staining. Hoescht 33258 (0.05 mg/ml, Sigma Chemical Co., St. Louis, MO) was used by submerging coupons in the stain and then incubating in the dark for 30 min at room temperature, rinsing with deionized water and allowing to air dry. Negative control surfaces were stained to obtain background fluorescence associated with the

surfaces, which were subtracted from images obtained from both positive controls and treated surfaces.

EPS staining. Lectin PNA from *Arachis hypogaea* (peanut) Alexa Fluor® 488 Conjugate (Molecular Probes, Inc., Eugene, OR) was used for polysaccharide staining. Lectin solutions of 100 μg/ml were made by dissolving the protein in an aqueous buffer at neutral pH containing 0.05 mM $CaCl_2$ and 0.05 mM $MgCl_2$ (35). Stained surfaces were incubated in the dark for 45 min, rinsed with deionized water, and allow to air dry. Positive and negative control surfaces as well as treated surfaces were stained. Negative control surfaces were stained to obtain background images, which were subtracted from images obtained before and after cleaning.

Comparison of DNA and EPS staining on cleaned coupons. The percent area covered by DNA and EPS staining was compared using coupons cleaned under different sodium hydroxide-temperature combinations. Two samples each, from the negative control (sterilized coupon with no biofilm), the positive control (3-day biofilm coupon) and the treated coupon (3-day biofilm coupon after cleaning), were stained using either DNA or EPS stains after every treatment in order to compare the two staining methods on cleaned coupons. Three replications were performed. Stainless steel surfaces subjected to the following cleaning conditions were stained either with Hoescht 33258 or Lectin PNA for comparison: 1.28% (0.32mM; pH 12.3), 2.0% (0.5mM; pH 13.2), 4.0% (1mM; pH 13.4), and 6.0% (1.5mM; pH 13.9) sodium hydroxide solution at 66˚C.

The Hoescht 33258 stained coupons were viewed under an epifluorescence microscope using a filter with an excitation wavelength of 330-380 nm, a Dichroit mirror of 400 nm (DM) and emission wavelength of 435-485nm (Nikon Eclipses E600, Nikon,

Tokyo, Japan). The lectin stained coupons were viewed using a filter with an excitation wavelength of 450-490 nm, a Dichroit mirror of 500 nm (DM) and emission wavelength of 515 nm. All coupons were viewed by using a 10x water immersion lens. The images were captured using a Magnafire camera (Optronics, Goleta, CA). Ten fields per sample were captured and processed using Image Pro Plus Imaging software (Media Cybernetics, Silver Spring, MD). Processing included image thresholding to improve contrast and reduce noise, with black pixels representing the stainless steel background and white representing the stained biofilm. The black and white images were analyzed for percent area covered by white pixels using UTHSCSA Image Tool Version 2.0 (University of Texas, Health Science Center, San Antonio, TX).

Effectiveness of cleaning for removing EPS. The effectiveness of different sodium hydroxide-temperature combinations in terms of EPS removal was evaluated by staining the cleaned stainless steel coupons with lectin PNA. The treated surfaces were subjected to the following cleaning conditions: (i) 1.28% (0.32mM), 2.0% (0.5mM), 2.5% (0.63mM), 4.0% (1mM) and 6.0% (1.5mM) sodium hydroxide solution at 66º C, (ii) 1.5% (0.38mM) and 2.0% (0.5mM) sodium hydroxide at 68º C, and (iii) 1.5% (0.38mM) and 2.0% (0.5mM) at 70º C. Each sodium hydroxide-temperature combination for treatment had three trials with each trial using duplicate coupons. The reported percent area covered was the averaged area covered of two stainless steel coupons that either belonged to the controls (positive or negative) or to the treated coupons. Percent area covered was reported for before cleaning (positive control) as well as after cleaning (treated). Percent area covered of the negative controls was subtracted from the area

covered corresponding to positive control or the treated sample. Images were captured and analyzed as described in the previous section.

Data analysis. All data were analyzed using SPSS version 10.1. ANOVA test was used for the analysis of variance. Significant difference between means was determined using Least Significant Difference (LSD) test. Significance was determined by least square means at P=0.05.

CHAPTER 4

RESULTS AND DISCUSSION

Researchers have used various methods to evaluate the effectiveness of cleaning methods in terms of biofilm and associated exocellular polymeric substances (EPS) removal, including direct surface microscopic observation (79,40,3,97), conventional cultivation by scraping bacteria from the surfaces (93,97) or swapping (32). These studies emphasized the need for improvement of current cleaning methods in terms of removal of biofilm and EPS since those conditions weren't enough for effective removal. They also emphasized the need for improved detection methods since some of those methods such as swabbing weren't so accurate resulting in underestimation of the percent area covered with biofilm and EPS (96). In this study Lectin staining and epifluorescence microscopic observation was found to be a successful method for evaluating the effectiveness of a given temperature, detergent concentration, and time combination in biofilm and EPS removal.

Comparison of DNA and EPS staining. Current cleaning conditions used in clean-in-place system were tested for their ability to remove a 3-day *Pseudomonas putida* biofilm and associated exopolysaccharide (EPS) from the stainless steel surface. Cleaning conditions used were 1.28% sodium hydroxide at 66˚C for 3 min. These conditions were evaluated by comparing DNA (Hoescht 33658) and EPS (Lectin PNA) staining viewed under epifluorescence microscopy for percent area covered. The percent area covered

material labeled by the two stains is presented in Figure 1. Results show that this treatment removes more Hoescht binding material (X=0.2) than Lectin binding material (X=7.3) (Figure 7B). This indicates that EPS is more difficult to remove than the biofilm cells. This conclusion was reached based on observation of percent area covered and statistical analysis comparing those results with the percent area covered of the corresponding controls. There was no significant difference between the area covered of the 3-day biofilm after all cleaning treatments and that of controls (no biofilm growth)(X=0.18)(Figure 11B) as shown by Hoescht staining. Area covered as observed by Lectin staining showed a significant difference ($P<0.05$) between area covered after cleaning and that of controls (X=1.1) (Figure 8B). The same differences (Figure 1) were observed by Lectin staining (X=6.3) with the corresponding controls when the sodium hydroxide concentration was increased to 2.5%. Biofilm coverage after cleaning with 2.0% sodium hydroxide determined with Lectin PNA, showed significant variation between trials (see photomicrographs, Figure 7).

Cleaning at 66˚C. Sodium hydroxide concentration was increased to 2.5%, 4.0%, and 6.0% at 66˚C for 3 min in an attempt to remove all EPS. Results (Figure 1) showed that cleaning with 2.5%, 4.0% and 6.0 % sodium hydroxide concentration was sufficient to give clean samples with no significant lectin binding material (X=0.9, X=0.8 and X=0.6, respectively) left on the surface compared to the controls (X=1.1). A representative photomicrograph is presented in Figure 8A corresponding to cleaning with 2.5% sodium hydroxide solution. Sodium hydroxide concentration greater than 2.0% was enough to remove all Hoescht and Lectin binding material. Results (Figure 1) indicate that currently recommended cleaning treatment of 1.28% sodium hydroxide at 66˚C was

enough to give a clean stainless steel surface in terms of Hoescht binding material remaining on the surface, but a concentration of a minimum of 2.5% sodium hydroxide solution at 66˚C for 3 min was necessary to give a clean surface in terms of Lectin binding material removal. These results indicated the need for determining the effectiveness of cleaning in terms of polysaccharide residue remaining on the surface through lectin staining. Higher temperatures of 68˚ C and 70˚ C were tested in order to determine cleaning effectiveness using lower sodium hydroxide concentrations at the same constant time of 3 min.

Percent area covered before and after cleaning at 66˚C. Area covered by Lectin binding material was obtained for before and after cleaning at 66˚C. Percent area of the stainless steel coupons covered with the 3-day *Pseudomonas putida* biofilm before cleaning treatments shown by lectin staining showed significant differences ($P<0.05$) between trials (Figure 2) suggesting a variation on the biofilm soiling of the coupons (see photomicrographs in Figure 6). Similar soiling variations (Figure 3) were observed between trials before cleaning treatment after Hoescht staining. A representative photomicrograph is presented in Figure 11A. This variation may be due to surface irregularities such as pits and crevices or to defects originating from the mechanical polishing process. This supports the previous conclusion of Stevens and Holah (77) that surface defects affect initial bacteria attachment.

A statistical analysis of percent area covered before and after cleaning as determined by staining with Hoescht and Lectin showed that there is no correlation between the percentages obtained for before and after cleaning. This suggests that the percent area covered before staining doesn’t affect the percent area covered after cleaning

since one is independent of the other. This is also supported through the results of lectin staining before and after cleaning (Figure 2) showing that the trial having the highest observed percentage area coverage before cleaning had also one of the lowest percent area covered after cleaning using 2.5% sodium hydroxide solution. Similar independence between percentages for before and after cleaning were observed using Hoescht staining.

Percent area covered before and after cleaning at 68ºC. Area covered by Lectin binding material was obtained for before and after cleaning at 68˚C. Area covered by lectin binding material before cleaning at 68˚C was similar between trials with the exception of one trial being significantly different ($P<0.05$) from the other 5 trials (Figure 4). Results indicated that there was a difference ($P<0.05$) between the biofilm remaining after cleaning using 1.5% sodium hydroxide solution at 68ºC ($X=1.2$) (Figure 9A) and the area covered after cleaning using 2.0% sodium hydroxide ($X=0.5$) at the same temperature. Even though there was a significant difference between a trial obtained using 1.5% sodium hydroxide solution, presented in Figure 9B, compared to the other trial at 68˚C, there was no significant difference of all trials compared to the controls. This observation shows that both 1.5% and 2.0% sodium hydroxide solution at 68ºC for 3 min was sufficient to clean the stainless steel surfaces in terms of EPS removal.

Percent area covered before and after cleaning at 70ºC. Area covered by lectin binding material was obtained for before and after cleaning at 70˚C. There were no significant differences among trials of percent area covered before cleaning observed through lectin staining (Figure 5). Both 1.5% ($X=0.9$) and 2.0% ($X=0.4$) sodium hydroxide removed lectin binding material equally well. One trial (Figure 10B) obtained

using 1.5% sodium hydroxide solution was significantly different ($P<0.05$) from the other trials (Figure 10A). Both cleaning conditions were shown to be effective in lectin binding material removal since they weren't different from the controls.

Results indicate that the recommended cleaning treatment by the US Department of Health and Human Services (87) is not sufficient to remove all Lectin binding material. This finding supports previous research of various authors. Gibson et al (32) emphasized the need for improvement of the factory cleaning and disinfection methods for the removal of factory occurring biofilms (32). Similar conclusions were drawn by Holah and Thorpe (40) as well as Austin and Bergeron (3). Increased sodium hydroxide concentration and higher temperature were effective for effective for removal of Hoescht and Lectin binding material supporting the results that possibly greater CIP NaOH concentrations may be required to remove biofilm and EPS material (97), emphasizing the importance of detergent in a cleaning treatment (19). The cleaning treatment of 1.28% sodium hydroxide at 66˚C removed more Hoescht binding material than Lectin binding material indicating that EPS is more difficult to remove than the biofilm cells contradicting with the results of Wirtanen et al (96) supporting that EPS was more easily detached than biofilm cells.

In conclusion, the currently recommended cleaning treatment of 1.28% sodium hydroxide solution at 68˚C for 3 min specified by the US Department of Health and Human Services (87) is sufficient for removal of Hoescht binding material but not for removal of Lectin binding material associated with biofilms. A sodium hydroxide concentration of greater than 2.0% is needed to remove all Lectin binding material at 66˚C. Increasing the temperature to 70˚C was sufficient to remove Lectin binding

material using 1.5% sodium hydroxide concentration which is almost doubled compared to the percentage recommended, 0.86% at 71˚C. Cleaning treatment operating at 70˚C can remove lectin binding material using 1.5% sodium hydroxide concentration.

CONCLUSIONS

Extracellular polymeric substances remain on the surface after the cleaning treatment of 1.28% sodium hydroxide solution at 68˚C for 3 min specified by the U.S. Department of Health and Human Services. This treatment was sufficient to remove Hoescht binding material still leaving lectin-binding material associated with *Ps. putida* biofilm attached to the stainless steel surface. This observation indicates that EPS associated with biofilm is more difficult to remove than the biofilm cells. A sodium hydroxide concentration of at least 2.5% at 66˚C for 3 min is sufficient to remove detectable Hoescht and Lectin binding material. Sodium hydroxide concentration as low as 1.5% at the minimum temperature of 68˚C is sufficient to remove Lectin binding material associated with the biofilm. In conclusion, stainless steel surfaces can be cleaned of biofilm and EPS using slightly higher temperature (68˚C) or sodium hydroxide concentration than specified by the Pasteurized Milk Ordinance.

REFERENCES

1. Allison, D. G., and I. W. Sutherland.1984. "A staining technique for attached bacteria and its correlation to extracellular carbohydrate production." J. Microbiol. Methods 2: 93-99.

2. Arizcun, C., C. Vasseur, and J. C. Labadie.1998. "Effect of several decontamination procedures on *Listeria monocytogenes* growing in biofilms." J. Food Prot. 61(6): 731-734.

3. Austin, J. W., and G. Bergeron.1995. "Development of bacterial biofilms in dairy processing lines." J. Dairy Sci. 62: 509-519.

4. Baxes, G. A. 1994. The digital image, 37-67. G. A. Baxes. Digital Image Processing. New York.

5. Baxes, G. A. 1994. Fundamentals of digital image processing, 13-35. G. A. Baxes. Digital Image Processing. John Wiley and Sons, Inc.,New York.

6. Baxes, G. A. 1994. What is image processing?, 1-11. G. A. Baxes. Digital Image Processing. John Wiley and Sons, Inc.,New York.

7. Beech, I. B., C. C. Gaylarde, J. J. Smith, and G. G. Geesey.1990. "Extracellular polysaccharides from *Desulfovibrio desulfuricans and Pseudomonas fluorescens* in the presence of mild and stainless steel." Appl. Microb. Biotechnol. 35: 65-71.

8. Blackman, I. C., and J. F. Frank.1996. "Growth of Listeria monocytogenes as a biofilm on various food-processing surfaces." J. Food Prot. 59(8): 827-831.

9. Carpentier, B., and O. Cerf. 1993. "Biofilms and their consequences, with particular reference to hygiene in the food industry." J. Appl. Bacteriol. 75: 499-511.

10. Carsberg, H.1996. "Selecting your sanitizers." Food Quality 2(3): 35-36,61-62.

11. Characklis, W. G., and K. C. Marshall. 1990. Biofilms: A basis for an interdisciplinary approach, 3-15. Biofilms. Wiley-Interscience,New York.

12. Christensen, B. E.1989. "The role of extracellular polysaccharides in biofilms." J. Biotechnol. 10: 181-202.

13. Chumkhunthod, P., H. Schraft, and M. W. Griffiths.1998. "Rapid monitoring method to assess efficacy of sanitizers against *Pseudomonas putida* biofilms." J. Food Prot. 61(8): 1043-1046.

14. Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James.1994. "Biofilms, the customized microniche." J. Bacteriol. 176(8): 2137-2142.

15. Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.1995. "Microbial biofilms." Annu. Rev. Microbiol. 49: 711-745.

16. Dall, L., and B. Herndon.1989. "Quantitative assay of glycocalyx produced by viridans group Streptococci that cause endocarditis." J. Clin. Microbiol. 27(9): 2039-2041.

17. Davey, M. E., and G. A. O'Toole.2000. "Microbial biofilms: from ecology to molecular genetics." Microbiol. Mol. Biol. Rev. 64(4): 847-867.

18. Denyer, S. P., G. W. Hanlon, and M. C. Davies 1993. Mechanisms of microbial adherence, 13-27. S. P. Denyer, S. P. Gorman, and M. Sussman. Microbial Biofilms: Formation and Control. Blackwell Scientific Puplications,Oxford.

19. Dunsmore, D. G.1981. "Bacteriological control of food equipment surfaces by cleaning systems.I. Detergent effects." J. Food Prot. 44(1): 15-20.

20. Dunsmore, D. G., A. Twomey, W. G. Whittlestone, and H. W. Morgan.1981. "Design and Performance of Systems for Cleaning Product-Contact Surfaces of Food Equipment: A Review." J. Food Prot. 44(3): 220-240.

21. Dunsmore, D. G., and M. A. Thomson.1981. "Bacteriological control of food equipment surfaces by cleaning systems.II.Sanitizer effects." J. Food Prot. 44(1): 21-27.

22. Dunsmore, D. G., D. A. Westwood, D. B. Jay, and M. Embling.1980. "Simulator technique for assessing the bacteriological control of food equipment surfaces by cleaning systems." J. Food Prot. 43(11): 850-855.

23. Fassel, T. A., and C. E. Edmiston.1999. "Review-Ruthenium red and the bacterial glycocalyx." Biotechnic and Histochemistry 74(4): 194-212.

24. Fatemi, P., and J. F. Frank.1999. "Inactivation of *Listeria monocytogenes/Pseudomonas* biofilms by peracid sanitizers." J. Food Prot. 62(7): 761-765.

25. Flemming, H. C., and J. Wingender.2000. "Relevance of microbial extracellular polymeric substances (EPSs) - Part II: Technical aspects." Water Science and Technology 43(6): 9-16.

26. Foods, I. C. o. M. S. f. 1980. Cleaning, disinfection, and hygiene, 232-258. Microbial Ecology of Foods. Factors Affecting the Life and Death of Microorganisms. Academic Press,New York.

27. Frank, J. F.2000. "Control of biofilms in the food and beverage industry." Industrial Biofouling Chapter 4.3: 205-224.

28. Frank, J. F.2001. "Microbial attachment to food and food contact surfaces." Adv. Food Nutr. 43: 319-370.

29. Frank, J. F., and R. A. Koffi.1990. "Surface-adherent growth of Listeria monocytogenes is associated with increased resistance to surfactant sanitizers and heat." J. Food Prot. 53(7): 550-554.

30. Frank, J. F., and R. Chmielewski.2001. "Influence of surface finish on the cleanability of stainless steel." J. Food Prot. 64(8): 1178-1182.

31. Frank, J. F., and R.A.N. Chmielewski.1997. "Effectiveness of sanitation with quaternary ammonium compound or chlorine on stainless steel and other domestic food-preparation surfaces." J. Food Prot. 60(1): 43-47.

32. Gibson, H., J. H. Taylor, K. E. Hall, and J.T. Holah.1999. "Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms." J. Appl. Microbiol. 87: 41-48.

33. Giese, J. H.1991. "Sanitation: The key to food safety and public health." Food Technol.: 74-80.

34. Gilbert, P., and D. G. Allison. 1993. Laboratory methods for biofilm production, 29-49. S. P. Denyer, S. P. Gorman, and M. Sussman. Microbial Biofilms: Formation and Control. Blackwell Scientific Publications,Oxford.

35. Haugland, R. P. 1999. Molecular probes-Handbook of fluorescent probes and research chemicals.

36. Hayes, P. R. 1992. Cleaning and disinfection: Methods, 344-387. Food Microbiology and Hygiene. Elsevier Applied Science,London.

37. Hayes, P. R. 1992. Cleaning and disinfection: practical application, 388-396. Food microbiology and hygiene. Elsevier Applied Science,London.

38. Helke, D. M., E. B. Somers, and A. C. L. Wong.1993. "Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* to stainless steel and Buna-N in the presence of milk and individual milk components." J. Food Prot. 56: 479-484.

39. Herald, P. J., and E.A. Zottola.1989. "Effect of various agents upon the attachment of *Pseudomonas fragi* to stainless steel." Journal of Food Science 54(2): 461-464.

40. Holah, J. T., and R. H. Thorpe.1990. "Cleanability in relation to bacterial retention on unsued and abraded domestic sink materials." J. Appl. Bacteriol. 69: 599-608.

41. Holah, J. T., R.P. Betts, and R.H. Thorpe.1989. "The use of epifluorescence microscopy to determine surface hygiene." International Biodeterioration 25: 147-153.

42. Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams 1994. Bergey's manual of determinative bacteriology. Williams & Wilkins, Baltimore.

43. Hood, S. K., and E. A. Zottola.1995. "Biofilms in food processing." Food Control 6(1): 9-18.

44. Hood, S. K., and E. A. Zottola.1997. "Growth media and surface conditioning Influence the adherence of *Pseudomonas fragi*, *Salmonella typhimurium*,and *Listeria monocytogenes* cells to stainless steel." J. Food Prot. 60(9): 1034-1037.

45. Hoyle, B. D., J. Jass, and J. W. Costerton.1990. "The biofilm glycocalyx as a resistant factor." J. Antimicrobial Chemother. 26: 1-6.

46. Jain, A. K. 1989. Introduction to digital image processing, 1-10. T. Kailath. Fundamentals of Digital Image Processing. Prentise-Hall Inc.,New Jersey.

47. Jay, J. M. 1996. Synopsis of common foodborne bacteria, 19-25. Modern Food Microbiology. Chapman and Hall,New York.

48. Jeong, D. K., and J. F. Frank.1994. "Growth of Listeria monocytogenes at 10 C in biofilms with microorganisms isolated from meat and dairy processing environments." J. Food Prot. 57(7): 576-586.

49. Kaufmann, O. W., T. I. Hedrick, I. J. Pflug, and C. G. Pheil.1960. "Relative cleanability of various finishes of stainless steel in a farm bulk tank." J. Dairy Sci. 43: 377-381.

50. Kim, K. Y., H. S. Lillard, J. F. Frank, and S. E. Craven.1996. "Attachment of *Salmonella typhimurium* to poultry skin as related to cell viability." J. Food Sc. 61(2): 439-441.

51. Kim, K. Y., J. F. Frank, and S. E. Craven.1996. "Three Dimensional Visualization of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy." Letters in Applied Microbiology 22: 280-282.

52. Korber, D. R., G. A. James, and J. W. Costerton.1994. "Evaluation of fleroxacin activity against etsablished *Pseudomonas fluorescens* biofilms." Appl. Environ. Microbiol. 60(5): 1663-1669.

53. Krysinski, E. P., L. J. Brown, and T. J. Marchisello.1992. "Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces." J. Food Prot. 55(4): 246-251.

54. Kumar, C. G., and S. K. Anand.1998. "Significance of microbial biofilms in food industry: a review." Int. J. Food Microbiol 42: 9-27.

55. Leclercq-Perlat, M. N., and M. Lalande.1994. "Cleanability in relation to surface chemical composition and surface finishing of some materials commonly used in food industry." J. Food Engin.23: 501-517.

56. Leriche, V., P. Sibille, and B. Carpentier.2000. "Use of an enzyme-linked lectinosorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms." Appl. Environ. Microbiol. 66(5): 1851-1856.

57. Lopes, J. A.1986. "Evaluation of dairy and food plant sanitizers against *Salmonella typhimurium* and *Listeria monocytogenes*." J. Dairy Sci. 69: 2791-2796.

58. Mafu, A. A., D. Roy, J. Goulet, and P. Magny.1990. "Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact times." J. Food Prot. 53(9): 742-746.

59. Mafu, A. A., D. Roy, J. Goulet, L. Savoie, and R. Roy.1990. "Efficiency of sanitizing agents for destroying *Listeria monocytogenes* on contaminated surfaces." J. Dairy Sci. 73: 3428-3432.

60. Marshall, K. C.1992. "Biofilms: An overview of bacterial adhesion, activity, and control at surfaces." ASM News 58(4): 202-207.

61. Masurovsky, E. B., and W. K. Jordan.1958. "Studies on the relative bacterial cleanability of milk-contact surfaces." J. Dairy Sci. 41: 1342-1358.

62. Mattila, T., M. Manninen, and A. Kylasiurola.1990. "Effect of cleaning-in-place disinfectants on wild bacterial strains isolated from a milking line." J. Dairy Sci. 57: 33-39.

63. Mattila-Sandholm, T., and G. Wirtanen.1992. "Biofilm formation in the industry." Food Rev. Int. 8(4): 573-603.

64. Molin, G., and I. Nilsson.1983. "Effect of different environmental parameters on the biofilm build-up of *Pseudomonas putida* ATCC 11172 in chemostat." European J.App.Microbiol Biotechnol 18: 114-119.

65. Mosteller.1993. "Sanitizer Efficacy Against Attached Bacteria in a Milk Biofilm." J. Food Prot. 56(1): 34-41.

66. Neu, T. R.1992. "Microbial "footprints" and the general ability of microorganisms to label interfaces." Can. J. Microbiol. 38: 1005-1008.

67. Neu, T. R.1996. "Significance of bacterial surface-active compounds in interaction of bacteria with interfaces." Microbiol. Rev. 60(1): 151-166.

68. Oliveira, D. R. 1992. Physico-chemical aspects of adhesion, 45-58. L. F. Melo, T. R. Bott, M. Fletcher, and B. Capderille. Biofilms-Science and Technology. Kluwer Academic Publishers,Dordrecht.

69. Ophir, T., and D. L. Gutnick.1994. "A role for exopolysaccharides in the protection of microorganisms from desiccation." Appl. Environ. Microbiol. 60(2): 740-745.

70. Patrick, S., and M. J. Larkin 1993. Attachment in disease, 109-131. S. P. Denyer, S. P. Gorman, and M. Sussman. Microbial Biofilms: Formation and Control. Blackwell Scientific Publications,Oxford.

71. Read, R. R., and J. W. Costerton.1987. "Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fruorescens*." Can. J. Microbiol. 33: 1080-1090.

72. Rossoni, E. M. M., and C. C. Gaylarde.1999. "Comparison of sodium hypochlorite and peracetic acis as sanitizing agents for stainless steel food processing surfaces using epifluorescence microscopy." Int. J. Food Microbiol 61(1): 81-85.

73. Sasahara, K. C., and E. A. Zottola.1993. "Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems." J. Food Prot. 56(12): 1022-1028.

74. Sharon, N., and H. Lis.1989. "Lectins as cell recognition molecules." Science 246: 227-234.

75. Shin-Ho-Lee, a. J. F. F.1991. "Inactivation of surface-adherent *Listeria monocytogenes* hypochlorite and heat." J. Food Prot. 54(1): 4-6.

76. Speers, J. G. S., A. Gilmour, T.W. Fraser, and R.D. McCall.1984. "Scanning electron microscopy of dairy equipment surfaces contaminated by two milk-borne micro-organisms." J. Appl. Bacteriol. 57: 139-145.

77. Stevens, R. A., and J. T. Holah.1993. "The Effect of wiping and spray-wash temperature on bacterial retention on abraded domestic sink surfaces." J. Appl. Bacteriol. 75: 91-94.

78. Stewart, P. S., R. Murga, R. Srinivasan, and D. DeBeer.1995. "Biofilm structural heterogeneity visualized by three microscopic methods." Wat. Res. 29(8): 2006-2009.

79. Stone, S. L., and E. A. Zottola.1985. "Effect of cleaning and sanitizing on the attachment of *Pseudomonas fragi* to stainless steel." J. Food Sci. 50: 951-956.

80. Suarez, B., C.M. Ferreiros, and M.T. Criado.1992. "Adherence of psychrotrophic bacteria to dairy equipment surfaces." J. Dairy Sci. 59: 381-388.

81. Sutherland, I. 1977. Bacterial exopolysaccharides-their nature and production, 27-96. I. Sutherland. Surface Carbohydrates of the Prokaryotic Cell. Academic Press, London.

82. Sutherland, I. W.1983. "Microbial exopolysaccharides- Their role in microbial adhesion in aqueous systems." CRC Crit. Rev. Microbiol. 10(2): 173-201.

83. Tanaka, G. M. S., H. Komatsuzawa, and M. Sugai.2000. "Effect of clarithromycin on *Pseudomonas aeruginosa* biofilms." Chemotherapy 46: 36-42.

84. Troller, J. A. 1983. Cleaning and sanitizing, 79-111. Sanitation in Food Processing. Academic Press,New York.

85. Uhlinger, D. J., and D. C. White.1983. "Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*." Appl. Environ. Microbiol. 45(1): 64-70.

86. U.S. Department of Health and Human Survices, P. H. S. F. 1993. Sanitation requirements for grade "A" pasteurized, ultra-pasteurized and aseptically processed milk and milk products-cleaning and sanitizing of containers and Equipment, 77-81. Grade "A" Pasteurized Milk Ordinance. U.S. Department of Health and Human Survices,

87. U.S. Department of Health and Human Survices, P. H. S. F. 1999. Sanitation requirements for grade "A" pasteurized, ultra-pasteurized and aseptically processed milk and milk products-cleaning and sanitizing of containers and

Equipment, 77-81. Grade "A" Pasteurized Milk Ordinance. U.S. Department of Health and Human Survices,

88. Vandevivere, P., and D. L. Kirchman.1993. "Attachment stimulates exopolysaccharide synthesis by a bacterium." Appl. Environ. Microbiol. 59(10): 3280-3286.

89. Verran, J., and M. Jones 2000. Problems of biofilms in food and beverage industry, 145-173. J. Walker, S. Surman, and J. Jass. Industrial Biofouling. John Wiley and Sons Ltd,

90. Verran, J., R. D. Boyd, K. Hall, and R.H. West. 2001. "Microbiological and chemical analyses of stainless steel and ceramics subjected to repeated soiling and cleaning treatments." J. Food Prot. 64(9): 1377-1387.

91. Wimpenny, J. W. T., S. L. Kinniment, and M. A. Scourfield 1993. The physiology and biochemistry of biofilm, 51-94. S. P. Denyer, S. P. Gorman, and M. Sussman. Microbial Biofilms: Formation and Control. Blackwell Scientific Publications,Oxford.

92. Wirtanen, G., and T. Mattila-Sandholm.1993. "Epifluorescence image analysis and cultivation of foodborne biofilm bacteria grown on stainless steel surfaces." J. Food Prot. 56(8): 678-683.

93. Wirtanen, G., and T. Mattila-Sandholm.1994. "Measurement of biofilm of *Pediococcus pentosaceus* and *Pseudomonas fragi* on stainless steel surfaces." Colloids Surf. 2: 33-39.

94. Wirtanen, G., E. Storgards, M. Saarela, S. Salo, and T. Mattila-Sandholm. 2000. Detection of biofilms in the food and beverage industry, 175-203. J. Walker, S. Surman, and J. Jass. Industrial Biofouling. John Wiley and Sons Ltd,

95. Wirtanen, G., S. Salo, I. M. Helander, and T. Mattila-Sandholm. 2001. "Microbiological methods for testing disinfectant efficiency on *Pseudomonas* biofilm." Colloids Surf. B: Biointerfaces 20: 37-50.

96. Wirtanen, G., T. Alanko, T. Mattila-Sandholm.1996. "Evaluation of epifluorescence image analysis of biofilm growth on stainless steel surfaces." Colloids Surf. B: Biointerfaces 5: 319-326.

97. Wirtanen, G., U. Husmark, and T. Nattila-Sandholm.1996. "Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems." J. Food Prot. 59(7): 727-733.

98. Zhang, T., and H. H. P. Fang.2001. "Quantification of extracellular polymeric substances in biofilms by confocal laser scanning microscopy." Biotechnol. Lett. 23: 405-409.

99. Zoltai, P. T., E.A. Zottola, and L.L. McKay.1981. "Scanning electron microscopy of microbial attachment to milk contact surfaces." J. Food Prot. 44(3): 204-208.

FIGURES

FIGURE 1. Percent area covered of stainless steel surfaces stained with Hoescht (blue bars) and Lectin PNA (red bars) after cleaning at 66º C for 3 min using 1.28%, 2.0%, 2.5%, 4.0% and 6.0% sodium hydroxide concentration. Bars with no common letters above them represent data which differ at $P < 0.05$ (LSD).

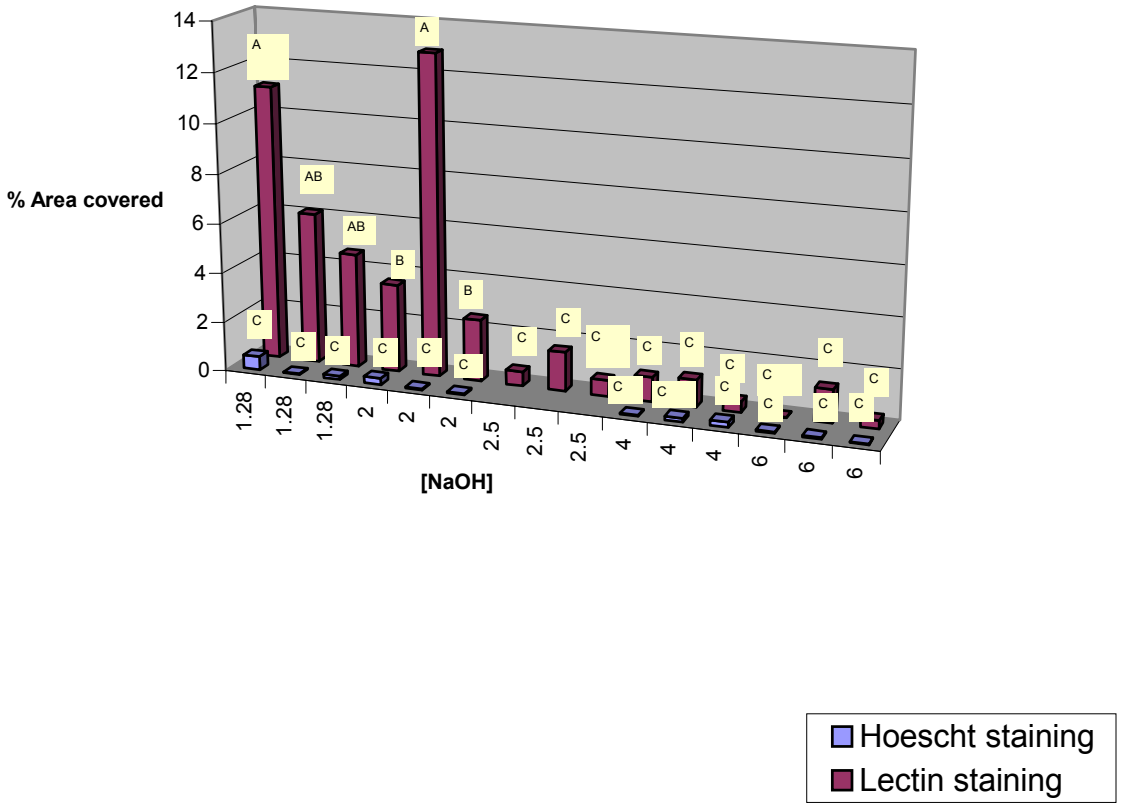

% Area covered
14
12
10
8
6
4
2
0
A
AB
AB
B
A
B
C
1.28
1.28
1.28
2
2
2
2.5
2.5
2.5
4
4
4
6
6
6
[NaOH]
Hoescht staining
Lectin staining

FIGURE 2. Percent area covered after Lectin PNA staining representing data for before cleaning treatment (blue bars) and after cleaning (red bars) at 66º C for 3 min using 1.28%, 2.0%, 2.5%, 4.0% and 6.0% sodium hydroxide. Bars with no common blue letters above them represent data before cleaning which differ at $P<0.05$ (LSD). Bars with no common red letters above them represent data after cleaning which differ at $P<0.05$ (LSD).

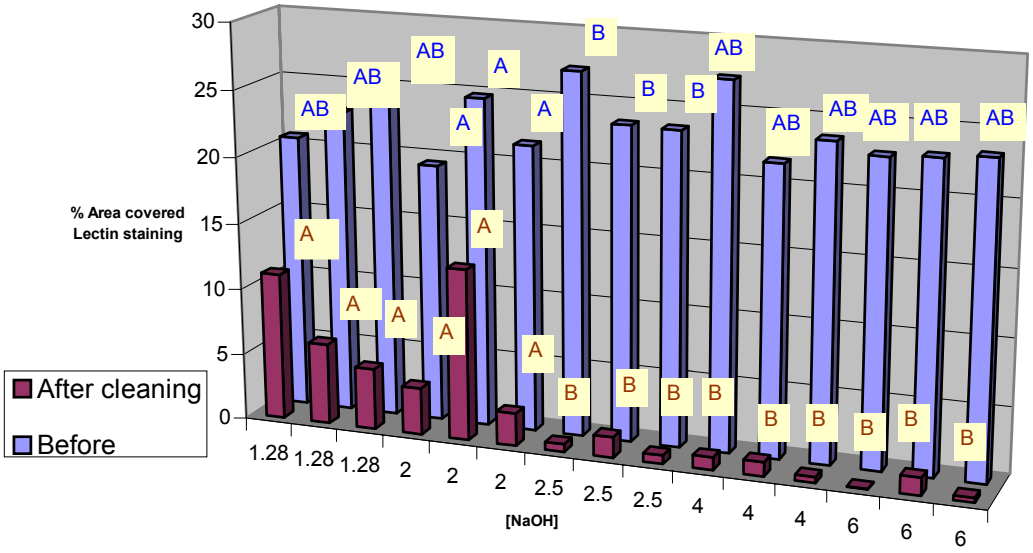

% Area covered
Lectin staining
30
25
20
15
10
5
0
After cleaning
Before
1.28
1.28
1.28
2
2
2
2.5
2.5
2.5
4
4
4
6
6
6
[NaOH]
A
AB
A
AB
A
AB
A
A
B
A
B
B
B
AB
AB
B
B
AB
AB
AB
AB

FIGURE 3. Percent area covered after Hoescht staining representing data for the 3-day *Pseudomonas putida* biofilm before any cleaning treatments. Bars with no common letters above them represent data which differ at $P<0.05$ (LSD).

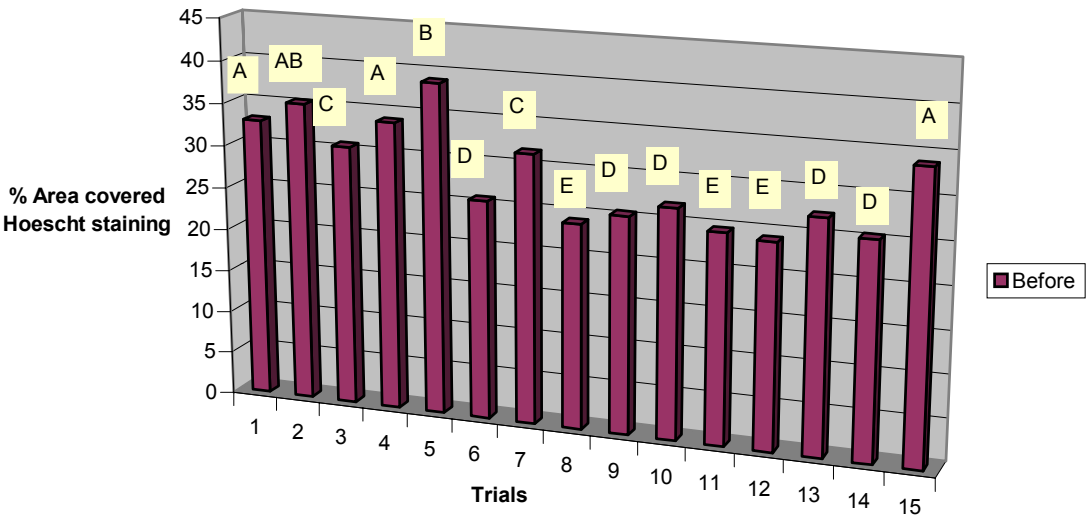

% Area covered Hoescht staining
45
40
35
30
25
20
15
10
5
0
A
AB
C
A
B
D
C
E
D
D
E
E
D
D
A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Trials
Before

FIGURE 4. Percent area covered after Lectin PNA staining representing data for before cleaning treatments (blue bars) and after cleaning treatments (red bars) using 1.5% and 2.0% sodium hydroxide at 68º C for 3 min. Bars with no common blue letters represent data before cleaning which differ at $P<0.05$ (LSD). Bars with no common red letters represent data after cleaning which differ at $P<0.05$ (LSD).

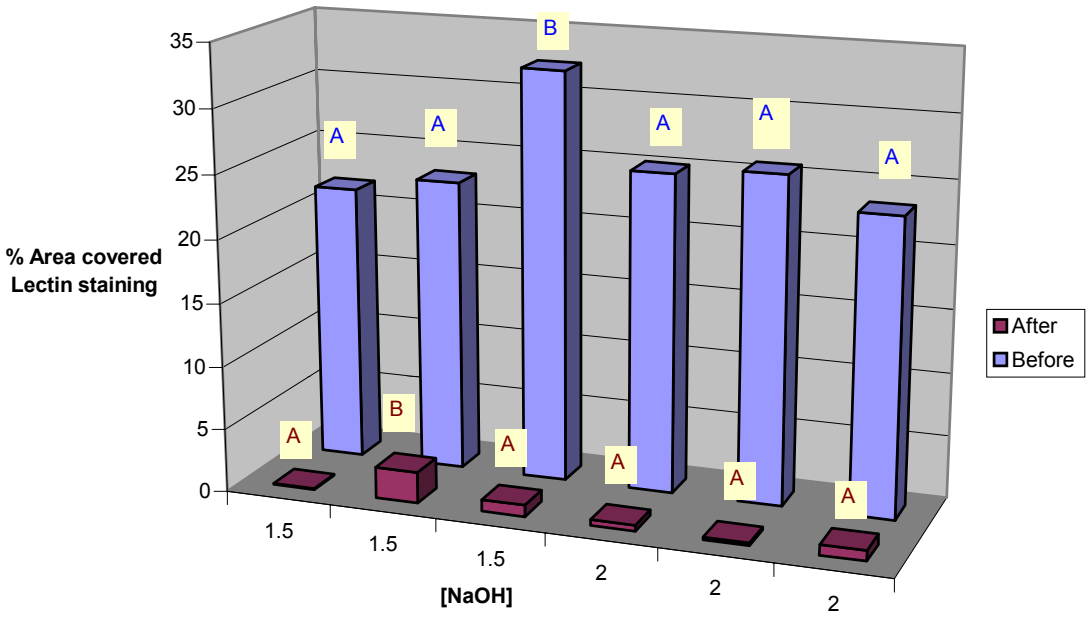

% Area covered
Lectin staining
35
30
25
20
15
10
5
0
A
B
A
A
A
A
A
A
A
A
A
B
1.5
1.5
1.5
2
2
2
[NaOH]
After
Before

FIGURE 5. Percent area covered using Lectin PNA staining representing data before cleaning (blue bars) and after cleaning (red bars) using 1.5% and 2.0% sodium hydroxide at 70º C at 3 min. Bars with no common blue letters represent data before cleaning which differ at $P<0.05$ (LSD). Bars with no common red letters represent data after cleaning which differ at $P<0.05$ (LSD).

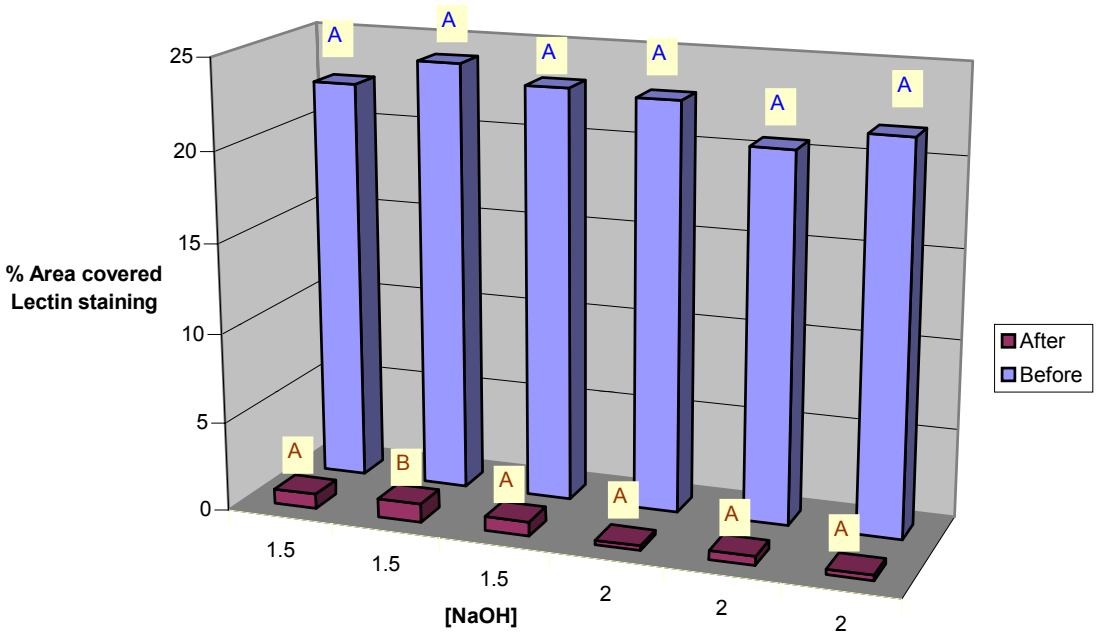
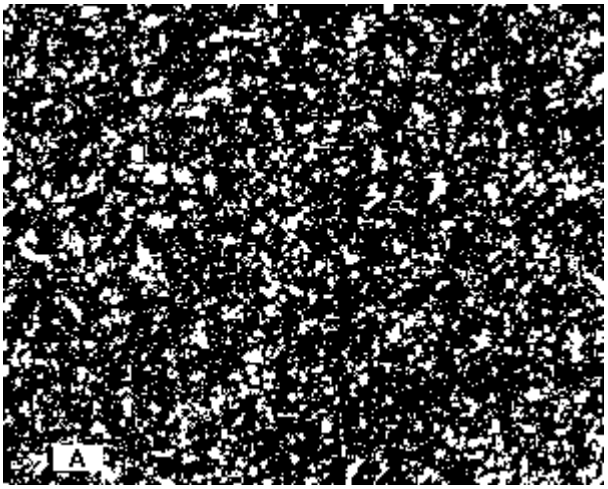

% Area covered
Lectin staining
25
20
15
10
5
0
A
A
A
A
A
A
A
B
A
A
A
A
1.5
1.5
1.5
2
2
2
[NaOH]
After
Before

FIGURE 6. Photomicrographs of *Ps. putida* biofilm on a stainless steel surface incubated for 3 days at 25˚C before cleaning, stained with Lectin PNA. Photomicrograph A represents area covered before cleaning corresponding to the statistical group labeled B. Photomicrograph B represents area covered before cleaning corresponding to the statistical group labeled AB.


A

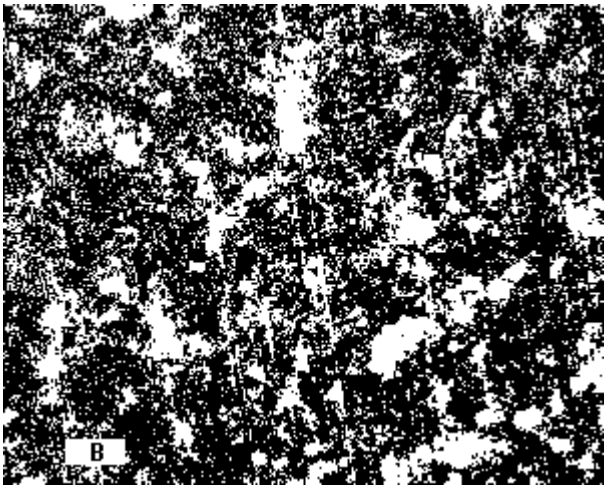
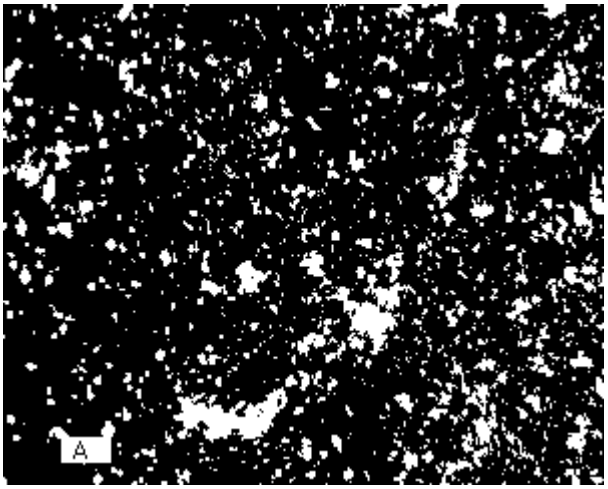
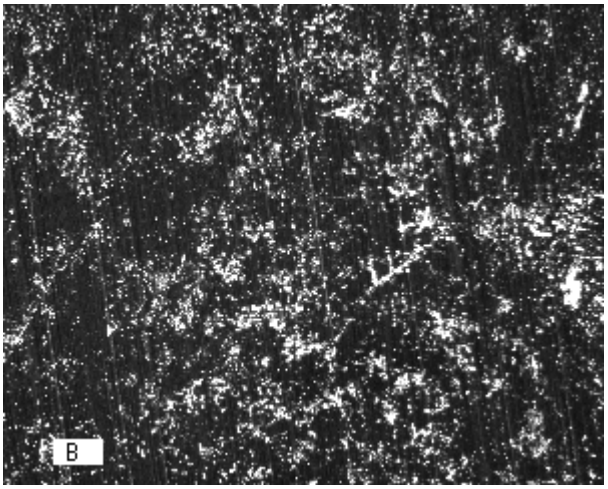

B

FIGURE 7. Photomicrographs of *Ps. putida* biofilm incubated for 3 days at 25˚C after cleaning at 66˚C stained with Lectin PNA. Photomicrograph A represents area covered after cleaning using 2.0% sodium hydroxide at 66˚C for 3 min (Figure 1 group A). Photomicrograph B represents area covered after cleaning using 1.28% sodium hydroxide at 66˚C for 3 min (Figure 1 group AB). Photomicrograph C represents area covered after cleaning using 2.0% sodium hydroxide at 66˚C for 3 min (Figure 1 group B).


A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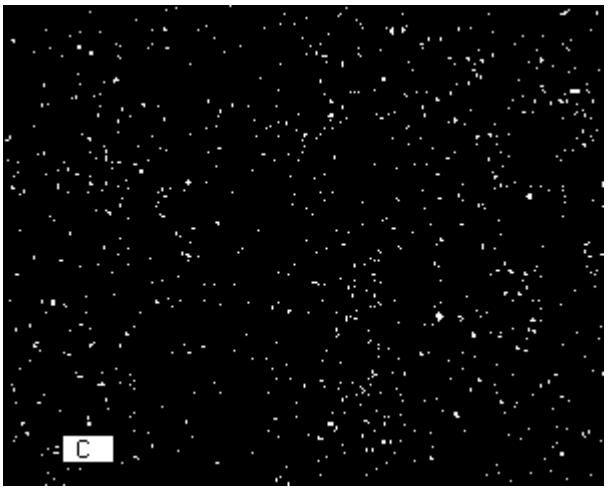
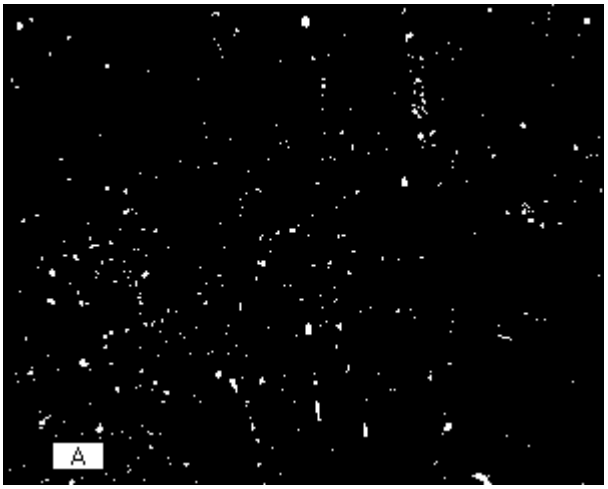

C

FIGURE 8. Photomicrograph A represents area covered by *Ps. putida* EPS after cleaning using 2.5% sodium hydroxide solution at 66˚C for 3 min stained with Lectin PNA. Photomicrograph B represents background fluorescence associated with the negative control (no biofilm growth) after Lectin PNA staining.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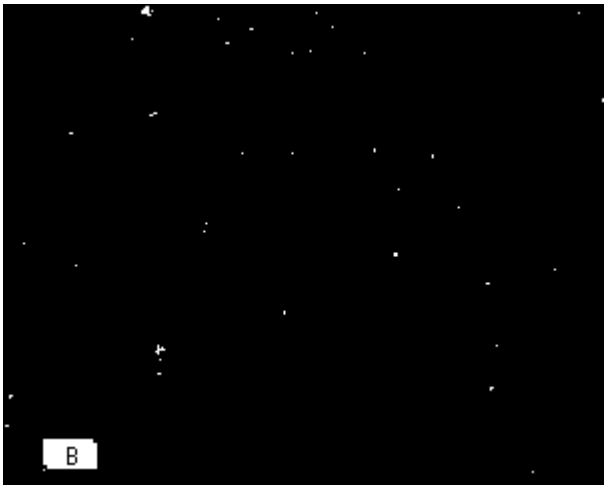
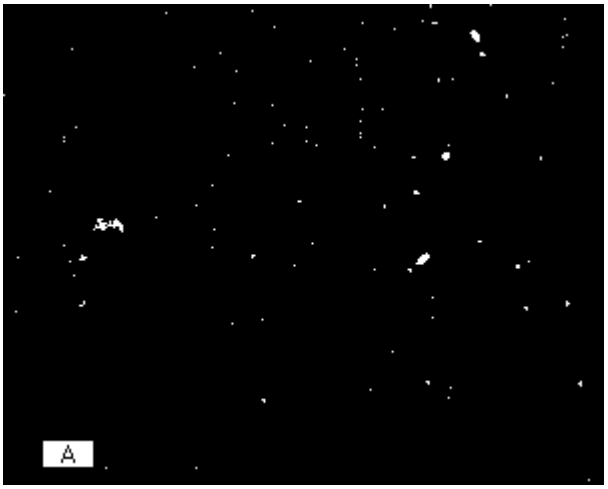

B

FIGURE 9. Photomicrographs of *Ps. putida* biofilm on a stainless steel surface incubated for 3 days at 25˚C, after cleaning using 1.5% sodium hydroxide solution at 68˚C stained with Lectin PNA. Photomicrograph A represents area covered corresponding to the statistical group labeled A (Figure 4). Photomicrograph B represents area covered corresponding to the statistical group labeled B (Figure 4).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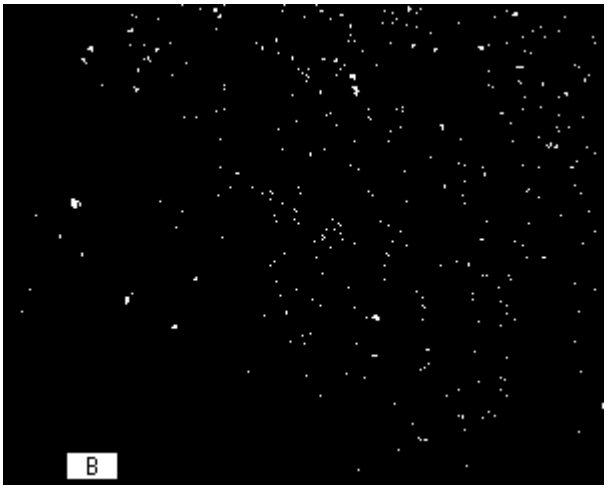
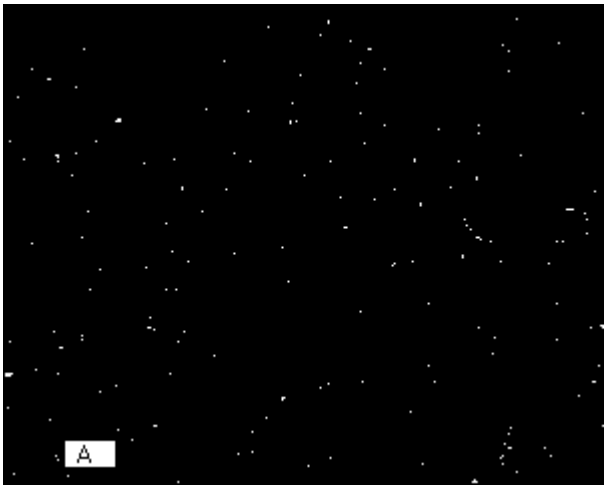

B

FIGURE 10. Photomicrographs of *Ps. putida* biofilm on a stainless steel surface incubated for 3 days at 25˚C, after cleaning using 1.5% sodium hydroxide solution at 70˚C stained with Lectin PNA. Photomicrograph A represents area covered corresponding to the statistical group labeled A (Figure 5). Photomicrograph B represents area covered corresponding to the statistical group labeled B (Figure 5).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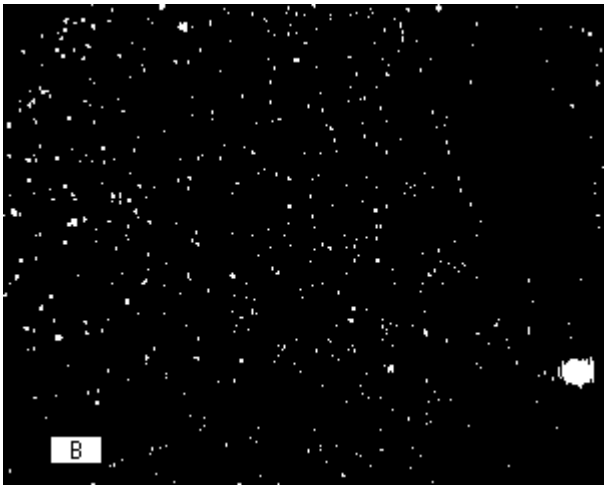
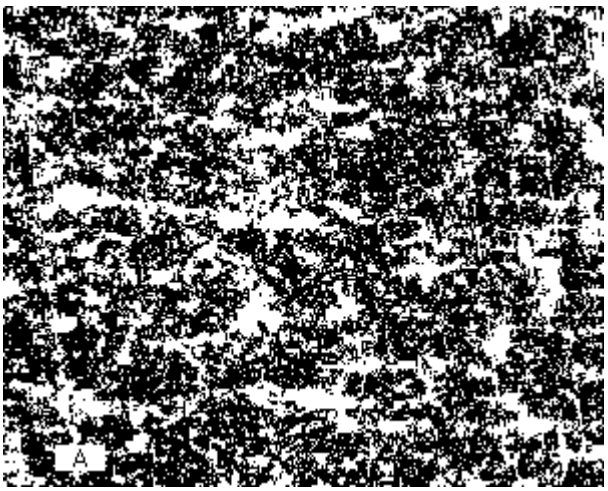

B

FIGURE 11. Photomicrographs of *Ps. putida* biofilm on a stainless steel surface incubated for 3 days at 25˚C stained with Hoescht 33258. Photomicrograph A represents area covered before cleaning corresponding to the statistical group labeled B (Figure 3). Photomicrograph B represents background fluorescence associated with the negative control (no biofilm growth).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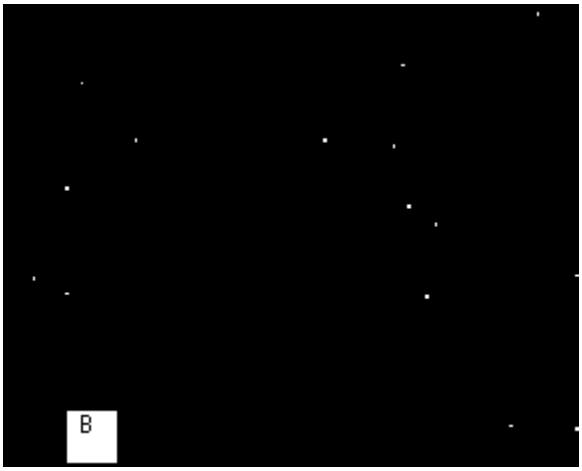
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