REVIEW ARTICLE

Biofilms and the food industry

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Abstract

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In the past, interest in biofilms was limited to research related to water distribution systems, waste water treatment and dental plaques. Biofilm has become a more popular research topic in many other areas in recent years including food safety. Biofilm formation can compromise the sanitation of food surfaces and environmental surfaces by spreading detached organisms to other areas of processing plants. Unfortunately, these detached organisms are not similar to normal microorganisms suspended in an aquatic environment but are more resistant to several stresses or microbial inactivation including some food preservation methods. Microstructures of biofilms as revealed by different types of microscopic techniques showed that biofilms are highly complex and consist of many symbiotic organisms, some of which are human pathogens. This article reviewed the process of biofilm formation, the significance of biofilms on food or food contact surfaces, their ability to protect foodborne pathogens from environmental stresses and recent methods for the study of biofilms on food contact surfaces.

Key words : biofilm, biofouling

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บทคัดย่อ

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ความสนใจเกี่ยวกับแผ่นชีวะในอดีตจำกัดอยู่ในการวิจัยเกี่ยวกับระบบการทำน้ำเพื่อสารธาณูปโภค การกำจัดและบำบัดน้ำเสีย และคาบจุลินทรีย์ที่เกาะบนฟัน แต่ในช่วงไม่กี่ปีที่ผ่านมาแผ่นชีวะเริ่มได้รับความสนใจจากนักวิจัยหลายแขนงรวมถึงด้านความปลอดภัยในอาหาร แผ่นชีวะเป็นแหล่งแพร่เชื้อจุลินทรีย์ไปสู่ส่วนต่าง ๆ ในโรงงานแปรรูปอาหาร เชื้อจุลินทรีย์ที่หลุดออกมาจากแผ่นชีวะมีความแตกต่างไปจากจุลินทรีย์ทั่วไป คือสามารถมีชีวิตในสภาพแวดล้อมที่ไม่เหมาะสมและทนต่อวิธีการฆ่าเชื้อหลายแบบได้ดี การศึกษาโครงสร้างระดับจุลภาคของแผ่นชีวะด้วยกล้องจุลทรรศน์หลายแบบได้แสดงให้เห็นความซับซ้อนของแผ่นชีวะและพบว่าแผ่นชีวะประกอบด้วยจุลินทรีย์ที่สามารถพึ่งพาอาศัยกันได้ โดยจุลินทรีย์บางชนิดสามารถทำให้เกิดโรคได้อีกด้วย บทความนี้ได้แสดงเนื้อหาเกี่ยวกับกระบวนการเกิดขึ้นแผ่นชีวะ ความสำคัญของแผ่นชีวะในระบบของอาหารและวัสดุที่สัมผัสอาหาร ความสามารถในการช่วยป้องกันจุลินทรีย์เชื้อโรคจากสภาพแวดล้อมที่ไม่เหมาะสม และวิธีการศึกษาแผ่นชีวะบนวัสดุสัมผัสอาหาร

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Definition and significance of biofilm in the food industry

Biofilms, or in more conventional terms biofouling and slime layers, are matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces (Costerton *et al*., 1995). The biofilm community exhibits primitive homeostasis, a primitive circulatory system, genetic material exchange, and metabolic cooperation (Costerton *et al*., 1995, Costerton *et al*., 1994, Lappin-Scott *et al*., 1992). The presence of biofilms is highly prevalent and difficult to completely eliminate. Biofilms can exist on all types of surfaces in food plants ranging from plastic, glass, metal, wood, and food products. Buna-n rubber, a gasket material commonly used in food processing environment had bacteriostatic effect on several foodborne pathogens, especially, *Listeria monocytogenes* (Ronner and Wong, 1993). Biofilms harbor and protect pathogens and spoilaging microorganisms thus compromising sanitation standards of food processing plants. Moreover, microbial biofilms on surfaces result in equipment damage, product contamination, energy losses and medical infections. It is believed that biofilm formation is part of the survival strategies of microorganisms in adverse environments. Inactivation of bacteria by conventional methods such as use of antibiotics and disinfectants are often ineffective against biofilm bacteria. New biofilm control strategies derived from a better understanding of how bacteria attach, grow and detach are urgently needed by the food industry. The presence of biofilm or attached cells on foods and food contact surfaces often adversely impacts food safety, especially in minimally processed foods and raw foods (Frank, 2001). Elimination of attached pathogen and spoilaging microorganisms on minimally processed produces can be difficult because of the hydrophobic cutin, diverse surface morphologies, and abrasions in the epidermis of fruits and vegetables (Burnett and Beuchat, 2001). Several groups of researchers at the University of Georgia have extensively studied the attachment of pathogens on produces. Results from a study of *Escherichia coli* O157:H7 attachment on apples showed that the organism could attach to internal core structures or within tissues of apples and damaged tissues and discontinuities in waxy cuticle on apple surface harbored the bacterial attachment (Burnett *et al*., 2000). This corresponds with the result of another study (Takeuchi and

Frank, 2000) which reported the preference of *E. coli* O157:H7 to attach to the cut edges of iceburg lettuce. Different species of microorganisms may possess diverse ability to attach or form biofilm on different surfaces. In a study that compared attachment of *Listeria monocytogenes, E. coli* O157:H7, and *Pseudomonas fluorescens* on iceburg lettuce showed that *L. monocytogenes* and *E. coli* O157:H7 attached preferentially to cut edges while *P. fluorescens* attached to the intact surfaces (Takeuchi *et al*., 2000).

Biofilm formation

Biofilm is a dynamic and complex environment. In general, biofilm formation involves attachment, colonization, and growth of microorganisms (Forsythe, 2000) as shown in Figure 1. Different nutrients in aquatic environments absorbed on surfaces to form conditioning film with different physicochemical properties. Physicochemical properties of a surface determine how bacteria attach. Biofilm forms when bacteria adhere to conditioned surfaces in aquatic environments and excrete an exopolysaccharide (EPS), glue-like substance that can anchor them to all kinds of material - such as metals, plastics, soil particles, medical implant materials, and tissue. A biofilm can be formed by a single bacterial species. In nature, many species of bacteria, fungi, algae, protozoa, and debris form biofilms.

In aquatic systems, bacterial cells attach to surfaces for several reasons. Adhesion to surfaces of bacterial cells is an essential strategy for survival. When biofilms formed in a natural aquatic system, each biofilm member has been naturally selected by its cell surface properties including the presence of capsules, fimbriae, and cell surface hydrophobicity (Frank, 2001). Cell surface hydrophobicity is based on compounds associated with the outer membrane including lipopolysaccharides, lipoproteins, lipoteichoic acid, and lipomannan. Orientation of these compounds on the outer membrane determines cell surface hydrophobicity (Neu, 1996). Most gram negative bacteria have long polysaccharide regions of their lipopolysaccharide exposed, resulting in a hydrophilic surface while gram positive bacteria have the lipid portion of lipoteichoic acid extending outward from the cell, resulting in a hydrophobic surface (Frank, 2001). Some bacteria can excrete substances including EPS, before or after attachment, which affect hydrophobicity of the cell surface. Excretion of polysaccharide slimes before attachment may enhance or inhibit the attachment process depending on the hydrophobicity of cell surface after the excretion. Emulsan, excreted by

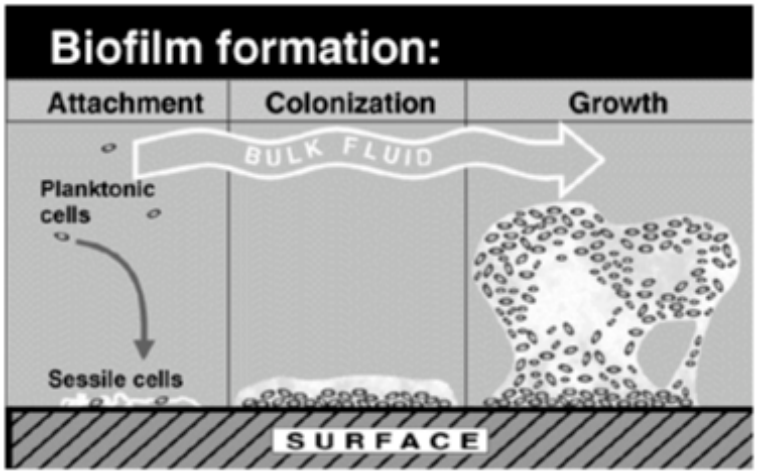

Figure 1. Biofilm formation

Acetobacter calcoaceticus, changes hydrophobic surfaces to hydrophilic resulting in inhibitory effect on hydrophobic cell attachment (Rosenberg *et al*., 1983). Attachment of bacteria to a surface is also affected by nutrient availability, nutrient concentration, pH, temperature, electrolyte concentration, and the flow of material (Lappin-Scott *et al*., 1992).

Biofilms may increase in size over time but not all constituent members in biofilm actively grow. Biofilms can vary in thickness from a monocell layer to several centimeters thick depending on the biofilm producers and growth conditions. Detachment from the surface can benefit the bacteria since they can move on to a new growth niche and establish a new biofilm. Factors affecting biofilm detachment include the biofilm thickness, fluid shear stress, nutrient availability, and fluid velocity (Lappin-Scott *et al*., 1992).

Stress responses of cells in biofilm growth mode

In food processing environments, bacteria in both biofilm and suspended forms encounter stresses such as dehydration, high heat, low temperature, and antimicrobial agents. Biofilm bacteria can be physically and morphologically different from their planktonic counterparts. Biofilm bacteria are up to 500 times more resistant to antimicrobial agents (Costerton *et al*., 1995). *Campylobacter jejuni* is a thermophilic and microaerophilic enteric pathogen associated with poultry. Biofilm is believed to be a source of *C. jejuni* in poultry house water systems since they can protect constituent microorganisms from environmental stress. In a biofilm inactivation experiment (Trachoo and Frank, 2002), quaternary ammonia compounds, peracetic acid, and mixture of peracetic acid and peroctanoic acid could not eliminate *C. jejuni* embedded in binary species biofilms after treatment at 50 and 200 ppm for 45 sec. Chlorine was the most effective sanitizer that completely inactivated *C. jejuni* in biofilms after treatment at 50 ppm for 45 sec. In another experiment, results from direct viable count and cultural method indicated that biofilms enhanced the survival of *C. jejuni* during incubation at 12 and 23ºC over a 7-day period (Trachoo *et al*., 2002).

Biofilm bacteria often have a slow growth rate. Studies suggested that the slow growth rate is not associated with nutrient limitation but with a general stress response initiated by biofilm growth (Brown and Barker, 1999, Hengge-Aronis, 1996). High cell density induces the production of RpoS protein, or alternative σ factor, which is usually expressed only in stationary state of laboratory-grown cells of various organisms (Liu *et al*., 2000). By using RT-PCR, *rpoS* mRNA was detected in *P. aeruginosa* biofilm from patients with chronic infections (Liu *et al*., 2000). *E. coli* cells that lack *rpoS* are unable to form a normal biofilm (Adams and McLean, 1999).

Advantages of biofilm growth mode

Advantages of biofilm growth mode are: 1) Protection from antimicrobial agents; 2) Increased availability of nutrients for growth; 3) Increased binding of water molecules, reducing the possibility of dehydration; 4) Proximity to progeny and other bacteria, facilitating plasmid transfer (Lappin-Scott *et al*., 1992).

Biofilm formation has been repeatedly demonstrated to protect the biofilm cells from many antimicrobial agents including antibiotics, biocides, and host defense mechanisms (Anwar and Costerton, 1990; Blenkinsopp *et al*., 1992; Frank and Koffi, 1990; Korber *et al*., 1994; Mosteller and Bishop, 1993). LeChevallier and coworkers (1988) studied factors promoting survival of bacteria in chlorinated water supplies and found that age of biofilm, bacterial encapsulation and previous growth conditions (growth medium and temperature) affected the resistance of biofilm to chlorination. EPS is thought to act as a physical barrier responsible for failure of antimicrobial agents to penetrate the biofilms. As revealed by a confocal scanning laser microscopy (CSLM) and Fourier transmission infrared spectroscopy (FTIR), antibiotics can penetrate biofilms. However, antibiotics cannot penetrate effectively to reach biofilm cells that are packed in clumps and microcolonies (Lappin-Scott *et al*., 1992). De Beer and coworkers

(1994) measured transient chlorine concentration profiles in biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* during chlorine disinfection by using a chlorine detecting microelectrode. Like antibiotics, chlorine penetrated into the biofilms with the concentrations measured in biofilms of 20% or less of the concentration in the bulk fluid. These authors concluded that penetration depth of chlorine into the biofilm and rate of penetration varied depending on the location, reflecting heterogeneity in the distribution of biomass and in local hydrodynamics. This result indicates the existence and effect of microcolonies on penetration of antimicrobial agents. In summary, the EPS matrix is not an impenetrable barrier to diffusion of antimicrobial agents, but EPS does lower the rate of antimicrobial agent penetration. Other mechanisms must take place to enhance biofilm cell survival.

Another benefit for biofilm cells present in biofilms is nutrient availability. In aquatic systems, nutrient availability is limited especially in drinking water distribution systems. It has been known that reduction of nutrients in raw water reduces the occurrence of biofilm formation in water distribution systems. Volk *et al*. (1999) suggested three measures for biofilm control in water distribution systems: reduction of nutrient level, corrosion prevention, and use of disinfectants. The polyanionic nature of EPS matrix allows it to serve as an ion exchange column concentrating nutrients from the surrounding fluid.

In some ecosystems such as riverbeds, streams, ponds, and soils, the availability of water may be seasonal and therefore limited. EPS binds water molecules within the biofilm and, thus, is highly hydrated. This reduces the effect of desiccation to which the planktonic cells are subjected (Lappin-Scott *et al*., 1992). Doyle and Roman (1982) demonstrated that *C. jejuni* is sensitive to dryness. It is possible that *C. jejuni* by being present in biofilms can survive the desiccation during dry seasons.

In nature, biofilms consist of mixed species. An exchange of metabolites among species can occur. Toxin removal by some biofilm cells in biofilms was studied by Stewart and coworkers (2000) who measured the penetration of hydrogen peroxide into biofilms formed by wild type and catalase-deficient *P. aeruginosa* strains using microelectrodes. Hydrogen peroxide (flow rate, 50 mM/h) was unable to penetrate or kill wild-type biofilms but did penetrate and partially kill biofilms formed by catalase-deficient *P. aeruginosa*. This suggests that catalase produced by wild-type strains protect biofilm cells in biofilm by decomposing hydrogen peroxide. This implies that wild type *P. aeruginosa* biofilm could potentially protect biofilm cells of other species against hydrogen peroxide.

Plasmid transfer in biofilms may occur at a high rate due to the close proximity of cells. This is beneficial to the cells since the transferred DNA may carry useful capabilities that enhance survival. Christensen *et al*. (1998) demonstrated that plasmid transfers occurred in a biofilm consisting of three species, *P. putida, Acinetobacter* sp., and an unidentified isolate.

Biofilm studies on food contact surfaces

Several research groups have studied biofilm microstructures. Data obtained from these studies reveals adaptive strategies and significance of biofilms. This section includes a brief review on biofilm microstructure studies and some novel findings.

Epifluorescence microscope can be used in the study of biofilm microstructure. Biofilm grown on surfaces such as plastic, stainless steel or rubber is usually stained with a fluorescent dye (Table 1) and viewed under an epifluorescence microscope. In a binary species biofilm study (Trachoo *et al*., 2002), *C. jejuni* was inoculated into a biofilm isolated from the water system in a chicken house. Samples were stained using two different fluorescent probes for each organism and observed under an epifluorescence microscope using two excitation wavelengths. Two different images (*C. jejuni* and the background biofilm) were captured with appropriate wavelengths. The images were then combined to construct a new image that simultaneously showed both organisms as shown in Figure 2.

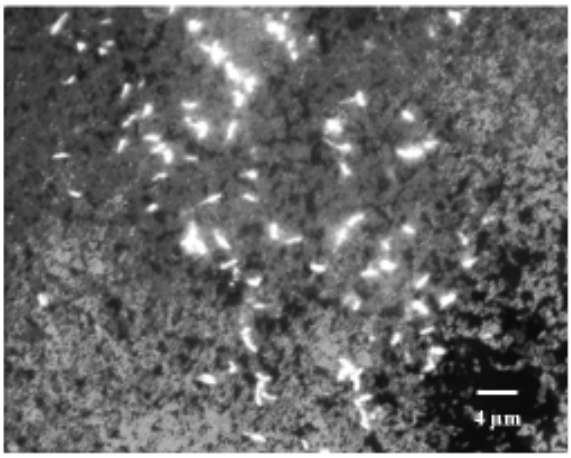

Figure 2. *Campylobacter jejuni* (bright cells) in biofilm (darker background) isolated from the water system in a chicken farm (Trachoo *et al.*, 2002)

The applications of this method include determination of viable cells, biofilm cell arrangement, microcolony formation, cell morphology, biofilm pH, and chemicals distribution in biofilm (Caldwell *et al*., 1993). This information is essential to understanding how some pathogens

Table 1. Common fluorescent probes used in biofilm studies

Name	Excitation/Emission	Applications
FITC	490/520	Binds to proteins, may be conjugated to antibodies, lectins, dextrans, ficols, etc.
TRITC	541/572	Same as FITC
RITC	570/595	Same as FITC
Acridine orange	490/530	Stains DNA and RNA
Hoescht 33258	362/470	Stains DNA
Nile red	450/530	Stains neutral lipids and phospholipids
Propidium iodide	530/615	RNA, DNA intercalating agent, viability assay
Fluorescein diacetate	495/520	Indicates esterase activity
Fluorescein	490/520	Negative staining, pH indicator
Carboxyl-SNARFL-2	Acid 485 or 514/546 Base 547/630	Dual emission pH indicator
NCECF	500/530 or 620	pH indicator
5- and 6-carboxy fluorescein	594/529	pH indicator
Fluo-3	506/526	Calcium indicator

Modified from Caldwell *et al.* (1993)

survive conditions in foods and food processing plants. Improved plant design, intervention and prevention programs can thus be developed. Different dyes serve different types of application. A wide selection of commercially available fluorescent probes is available from Molecular Probes, an Oregon based company (http://www.probes.com).

Wirtanen and coworkers (1996) used computer software and an epifluorescence microscope to determine area coverage of biofilms from various organisms. Biofilms were grown on stainless steel coupons. The coupons were then rinsed and stained with acridine orange for 2 min. An image system for the detection of viable but nonculturable (VBNC) *C. jejuni* was reported by Rowe and coworkers (1998). Their vital staining method is based on the ability of viable cells to uptake non-fluorescent dye and intracellularly hydrolyze the dye. The hydrolyzed dye becomes fluorescent. Cells exhibited fluorescence of vital stain and in the coccoid category were regarded as VBNC. Image analysis software was used to set criteria to differentiate rods from coccoid cells.

CSLM has several advantages over the epifluorescence microscope. CSLM is an improved version of epifluorescence microscope that creates a thin (about 0.3 μm) plane-of-focus in which out-of-focus light has been blocked, conventionally by optical barriers but now also by the physics of light absorption as equipped in multiphoton microscope (Sheppard and Shotton, 1997). Optical sectioning or XZ scanning can also be obtained. These optical sections can then be stacked by computer software to generate a three-dimensional reconstructed image (Palmer and Sternberg, 1999). Silyn-Robert and Lewis (1997) used CSLM to determine the coverage and thickness of biofilms. They used computer software (ImageTool) to analyze each optical sectioning captured by CSLM. Biofilm coverage is calculated as percent black pixels by the software. Since each image is collected at a specific height, it is possible to relate biofilm coverage in an image to height at which it was captured. Another application of CSLM was presented by Vroom and coworkers (1999). They used CSLM to detect pH gradients in biofilms. The interior pH of biofilms was measured by fluorescence lifetime imaging technique using fluorescein as a pH indicator. Bacteria cells can be visualized under fluorescence microscopes without using fluorescent probes through the use of plasmid encoding for green fluorescent protein (GFP). Transformed *E. coli* O157:H7 was used in studies that observed its attachment on produces (Burnett *et al.*, 2000, Takeuchi and Frank, 2001). In addition, viability can be determined by staining the transformed cells with membrane impermeable fluorescent dye (Takeuchi and Frank, 2001).

Biofilm microstructure study may involve digital image processing and analysis using computer software. Image processing is applied to an image to change its appearance through the use of electronic filters, difference imaging, pseudocolor, and deconvolution while image analysis is used to obtain information from an image. This information includes length, width, area, perimeter, optical density, and percent black and white pixels.

Confocal Assistant, a Windows™ based software developed by Todd Clark Brelji is now widely used as a tool for image processing and analysis to some extent (ftp://ftp.genetics.bio-rad.com/public/confocal/cas). This software was particularly designed to work with captured images from BioRad™ CSLM systems. Powerful image processing/analysis software for Macintosh called NIH Image developed by National Institutes of Health (http://rsb.info.nih.gov/nih-image) is now available for Windows™ based machines. This new software, namely Scion Image developed by Scion Cooperation (http://www.scioncorp.com) can reconstruct optical sections into a three-dimensional image. It also handles TIFF file format while the Confocal Assistant does not. Leica CSLM system made by a German based company, Leica (http://www.leica-microsystems.com), usually generates optical sections in TIFF format. Another powerful image analysis software is ImageTool, 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). This software allows plug-in addition and users can write a script for an automated operation.

Summary

The presence of biofilms is common in food processing plants. Biofilms harbor and protect pathogens and spoilaging microorganisms. Moreover, microbial biofilms on surfaces result in equipment damage, product contamination, energy losses and medical infections. It is believed that biofilm formation is part of survival strategies of microorganisms in adverse environments. Advantages of biofilm growth mode are protection from antimicrobial agents, increased availability of nutrients and plasmid transfer, and increased binding of water molecules, therefore reducing the possibility of dehydration (Lappin-Scott *et al*., 1992). Biofilm microstructures can be studied using epifluorescence microscope and CSLM with the help of computer software for image processing and analysis. Recent microstructure studies of biofilm on foods, food contacts and environmental surfaces revealed information that is essential to understanding how some pathogens survive conditions in foods and food processing plants.

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