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Review

Significance of microbial biofilms in food industry: a review

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Abstract

Biofilms have been of considerable interest in the context of food hygiene. Of special significance is the ability of microorganisms to attach and grow on food and food-contact surfaces under favourable conditions. Biofilm formation is a dynamic process and different mechanisms are involved in their attachment and growth. Extracellular polymeric substances play an important role in the attachment and colonization of microorganisms to food-contact surfaces. Various techniques have been adopted for the proper study and understanding of biofilm attachment and control. If the microorganisms from food-contact surfaces are not completely removed, they may lead to biofilm formation and also increase the biotransfer potential. Therefore, various preventive and control strategies like hygienic plant lay-out and design of equipment, choice of materials, correct use and selection of detergents and disinfectants coupled with physical methods can be suitably applied for controlling biofilm formation on food-contact surfaces. In addition, bacteriocins and enzymes are gaining importance and have an unique potential in the food industry for the effective biocontrol and removal of biofilms. These newer biocontrol strategies are considered important for the maintenance of biofilm-free systems, for quality and safety of foods. © 1998 Elsevier Science B.V.

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1. Introduction

In nature and food systems, microorganisms get attracted to solid surfaces conditioned with nutrients, that are sufficient for their viability and growth. These microorganisms initially are deposited on the surfaces and later get attached, grow and actively multiply to form a colony of cells. In this regard, the formation of organic polymers are essential which helps in the proper colonization of microorganisms (Allison and Sutherland, 1987). These mass of cells further become large enough to entrap organic and inorganic debris, nutrients and other microorganisms leading to the formation of a microbial biofilm. The term *biofilm* refers to the biologically active matrix of cells and extracellular substances in association with a solid surface (Bakke et al., 1984). However, according to Costerton et al. (1987) a biofilm is a functional consortium of microorganisms attached to a surface and is embedded in the extracellular polymeric substances (EPS) produced by the microorganisms.

On most of the occasions where biofilms are a

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nuisance, the term microbial fouling or biofouling is generally implied. Biofouling refers to the undesirable formation of a layer of living microorganisms and their decomposition products as deposits on the surfaces in contact with liquid media. In dairy and food industry, biofouling causes serious problems such as impeding the flow of heat across the surface, increase in the fluid frictional resistance at the surface and increase in the corrosion rate at the surface leading to energy and product losses. For example, in case of heat exchangers, biofilms cause increased resistance both in liquid flow and heat transfer (Criado et al., 1994). In addition, the biofilms, including spoilage and pathogenic microflora, formed on the food surfaces like that of poultry, other meat surfaces and in processing environments also offer considerable problems of cross contamination and post-processing contamination.

2. Biofilm formation: mechanisms of microbial attachment

The concept of bacterial attachment is not very new and the early studies with buried slide method showed the attachment of soil bacteria to slide surfaces. These slide techniques were first described by Zobell (1943) which formed the first published report on biofilms. However, in the late 70s, this phenomenon was realized to be present universally in all natural environments (Costerton et al., 1978). The development of biofilms can occur on almost any surface in any environment in which viable microorganisms are present. In case of majority of microorganisms, the adhering to solid substrate, may they be animate or inanimate, living or dead and/or organic or inorganic forms an essential prerequisite to their normal life and reproduction. The formation of biofilms per se is ubiquitous in aqueous environments. The role of bacterial attachment has been very well studied in different habitats (Costerton et al., 1987; Melo et al., 1992; Zottola and Sasahara, 1994). Biofilm formation is a dynamic process and is shown to involve a series of steps.

2.1. Conditioning of a surface

The formation of a biofilm virtually occurs on any submerged surface in any environment wherein the bacteria are present. In the food processing environments, bacteria along with other organic and inorganic molecules like proteins from milk and meat gets adsorbed to the surface forming a conditioning film. These organic and inorganic molecules, and the microorganisms are transported to the surface by diffusion or in some cases by a turbulent flow of the liquid. The rate of transport and the extent of adsorption are equally important in this context (Characklis, 1981). The accumulation of molecules at the solid-liquid interface on food-contact surfaces (commonly referred to as *conditioning film*) leads to a higher concentration of nutrients compared to the fluid phase. In the food processing systems, the increased level of nutrients remaining on the food-contact surfaces acts as a conditioning film (Hood and Zottola, 1997). The nutrient transfer is also more rapid in a biofilm than for the bacterial cells in the aqueous phase. This increase in nutrient level favours biofilm formation and is also dependent on the type of the competitive culture associated with the biofilm (Jeong and Frank, 1994). The conditioning also alters the physico-chemical properties of the surface viz., surface free energy, changes in hydrophobicity and electrostatic charges (Dickson and Koohmaraie, 1989) which may also affect the subsequent sequence of microbial events.

There appears to be no evidence, however, that microorganisms always attach to a conditioned surface. In this regard, the microtopography of the food-contact surface is equally important to favour bacterial retention, particularly, if the surface consists of deep channels and crevices to trap bacteria. Scanning electron micrographs have also shown that food-borne pathogens and spoilage microorganisms accumulate as biofilms on stainless steel, aluminium, glass, Buna-N and Teflon seals and nylon materials typically found in food-processing environments (Herald and Zottola, 1988a,b; Mafu et al., 1990; Notermans et al., 1991; Blackman and Frank, 1996). The nylon and teflon surfaces are smooth and the microorganisms appear to be attached. However, stainless steel surfaces have a rough appearance due to cracks and crevices sufficient to trap bacteria (Wirtanen et al., 1996), while aluminium surfaces have larger crevices and exhibit a sponge-like appearance. Such a topography allows the escape of entrapped bacteria from the shear forces of the bulk liquid and even the mechanical methods of cleaning would be inadequate.

It is also established that adsorption of certain

proteins to surfaces play an important role in the microbial adhesion. Fletcher (1976) showed that certain proteins like albumin, gelatin, fibrinogen and pepsin inhibited the attachment of a marine pseudomonad to polystyrene. Similarly, Meadows (1971) also showed albumin to be inhibitory, while casein and gelatin favoured the process of attachment. In another study, albumin was also found to be least favourable for the adhesion of *Listeria monocytogenes* to silica surfaces (Al-Makhlafi et al., 1995). Milk and its components such as casein and β-lactoglobulin have also been found to inhibit the attachment of *Listeria monocytogenes* and *Salmonella typhimurium* (Helke et al., 1993). However, in the presence of whey proteins, an increase in attachment of several milk-associated microorganisms to stainless steel, rubber and glass surfaces was observed by Speers and Gilmour (1985).

2.2. Adhesion of cells

The second step in the formation of biofilms is the attachment of microorganisms to the conditioned surface. This process may be active or passive and depends on the bacterial motility or the transportation of the planktonic (free floating) cells by gravity, diffusion or fluid dynamic forces from the surrounding fluid phase. The physiochemical properties of the bacterial cell surface are important in determining the adhesion of cells during this initial attachment phase (van Loosdrecht et al., 1990). The bacterial adhesion is also effected by the nutrient availability in the surrounding medium and the growth stage of the bacterial cells themselves. This adhesion of cells takes place mainly in two stages: a reversible adhesion followed by an irreversible adhesion.

Initial weak interactions developed between the bacterial cells and the substratum are referred to as reversible adhesion. Various long range interaction forces influencing the reversible adhesion process are the van der Walls attraction forces, electrostatic forces and hydrophobic interactions. During this stage, bacteria still show Brownian motion and can easily be removed by the fluid shear forces e.g. merely by rinsing (Marshall et al., 1971). The irreversible attachment of cells is the next crucial step in biofilm development. The repulsive forces mainly prevent the bacterial cells in making a direct contact with the surface, however, the contact still occurs due to the production of surface appendages by the bacteria such as flagella, fimbriae, pili and the exopolysaccharide (EPS) fibrils (Jones and Isaacson, 1983; Hancock, 1991). In irreversible adhesion, various short-range forces involved include, dipole-dipole interactions, hydrogen, ionic and covalent bonding and hydrophobic interactions. The polymeric fibrils form a bridge between the bacterial cell and the substratum and this enables the irreversible association with the surface. In this process, the removal of cells requires much stronger forces such as scrubbing or scrapping (Marshall et al., 1971). Spores exhibit a greater rate of adhesion than vegetative cells to food-contact surfaces. This process is mainly facilitated by the relatively high hydrophobicity, in addition to hair-like structures on the cell surface (Rönner et al., 1990; Husmark and Rönner, 1992). On adhesion to surfaces, spores may germinate and the vegetative cells multiply and produce exopolysaccharides.

The chemostat-coupled modified Robbin's device has been proved to be a successful technique for studying biofilm adhesion and formation at controlled and low growth rates (Jass et al., 1995a). The adhesion of microorganisms to a surface can also be studied by measuring the hydrophobicity of the bacterial surfaces by different methods (Mozes and Rouxhet, 1987) viz., bacterial adherence to hydrocarbons (BATH), hydrophobic interaction chromatography (HIC) and the salt aggregation test (with ammonium sulphate). A correlation was observed between these three methods only when the microorganisms were strongly hydrophobic or hydrophilic (Mozes and Rouxhet, 1987; Sorongon et al., 1991). The variations in hydrophobicity depends on the mode of bacterial growth (Gilbert et al., 1991a) and the culture conditions (Spenceley et al., 1992). In a chemostat, as the growth rate of the culture increases, the hydrophobicity weakens (Allison et al., 1990; Gilbert et al., 1991b). The hydrophobicity can also be modified by mechanical and enzymatic treatments (Rosenberg and Kjelleberg, 1986).

The pH and temperature of the contact surface also have an influence on the degree of adhesion of microorganisms. *Pseudomonas fragi* showed maximum adhesion to stainless steel surfaces, at the pH range of 7 to 8, optimal for its cell metabolism (Stanley, 1983). Similarly, the effect of pH on the attachment of *Y. enterocolitica* and *L. monocytogenes* was also demonstrated by Herald and Zottola (1988a) and Herald and Zottola (1988b). In the

earlier study, they also observed the effect of temperature and reported that *Y. enterocolitica* adhered better to stainless steel surfaces at 21°C, rather than at 35°C or 10°C. In the case of a marine bacterium, *Deleya mariana*, maximum adhesion to a hydrophilic substratum was observed at 25°C i.e., at the optimum temperature for growth, while the adhesion was weaker at 19°C and weakest at 37°C (Shea et al., 1991).

2.3. Formation of microcolony

The irreversibly attached bacterial cells grow and divide by using the nutrients present in the conditioning film and the surrounding fluid environment. This leads to the formation of microcolonies, which enlarge and coalesce to form a layer of cells covering the surface. During this period, the attached cells also produce additional polymer (EPS) which helps in the anchorage of the cells to the surface and to stabilize the colony from the fluctuations of the environment (Characklis and Marshall, 1990). The surface microenvironment gets altered as the primary colonizers get attached, grow and divide and produce the EPS (Lappin-Scott and Costerton, 1989). In case of pseudomonads, the production of exopolysaccharide has been observed during surface attachment and the variations in the attachment among the populations may be due to different nutrient conditions (Ombaka et al., 1983; Uhlinger and White, 1983; Jass et al., 1995a).

2.4. Biofilm formation

The continuous attachment of the bacterial cells to the substratum and its subsequent growth along with associated EPS production, forms a biofilm. Multilayers of bacterial cells entrapped within the EPS-containing matrices develop within the biofilm. The biofilm formation is a fairly slow process and reaches a few millimeters thick in a matter of days depending on the culture conditions (Melo et al., 1992). The microorganisms within the biofilm are not uniformly distributed. They grow in the matrix-enclosed microcolonies interspersed within highly permeable water channels (Costerton et al., 1994a).

Composition of biofilms can be heterogeneous, due to the colonization of different microorganisms possessing different nutritional requirements. It does not necessarily exist as a uniform layer throughout the substratum surface. Further increase in the size of biofilm takes place by the deposition or attachment of other organic and inorganic solutes and particulate matter to the biofilm from the surrounding liquid phase (Melo et al., 1992).

2.4.1. Effect of interspecies microbial interactions

The interactions of various microbial populations during the initial stages of biofilm formation has a significant effect on the structure and physiology of the microbial biofilm (James et al., 1995). In natural communities, the microbial interactions observed are complex and often are of mixed type, wherein more than one type of interaction occurs between species (Bull and Slater, 1982). In addition, the initial colonizing species may potentially encourage the colonization of species which are physiologically compatible, while inhibiting the attachment of others. The biofilm formed by the microbial communities i.e., mixed species biofilms are often thicker and more stable than monospecies biofilms. In an annular reactor, the average thickness of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* monospecies biofilms were 15 and 30 μm, respectively, while a biofilm comprising of both the species was 40 μm thick (Siebel and Characklis, 1991). The EPS mainly helps in the colonization of other organisms to surfaces. It is presumed that in a mixed species biofilm, the EPS produced by one species may enhance the stability of other species within a biofilm and/or that stabilizing interactions may occur between polymers of different species (Sutherland, 1983; McEldowney and Fletcher, 1987). In one study, Sasahara and Zottola (1993) observed an extended biofilm formation by *Listeria monocytogenes* in association with a primary colonizing organism, *Pseudomonas fragi*, than with when either is grown individually.

2.5. Detachment and dispersal of biofilms

As the biofilm ages, the attached bacteria, in order to survive and colonize new niches, must be able to detach and disperse from the biofilm. The bacteria from the biofilm, mainly the daughter cells get detached individually or are sloughed off. Sloughing is a discrete process whereby periodic detachment of relatively large particles of biomass from the biofilm

occurs. This can be due to various factors such as the fluid dynamics and shear effects of the bulk fluid (Rittmann, 1989; Applegate and Bryers, 1991), presence of certain chemicals in the fluid environment or altered surface properties of the bacteria or substratum. The released bacteria may be transported to newer locations and again restart the biofilm process (Marshall, 1992).

3. Extracellular polymeric substances

After the initial contact with the surface, the microorganisms start producing thin fibers which were evidenced by scanning electron microscopic examination (Firstenberg-Eden et al., 1979). These fibers become thicker with time leading to a biofilm matrix. It is reported that within the biofilm matrix, many other organic and inorganic substances and particulate matter may get entrapped along with the microbial products and other microorganisms which join to form a consortium protected by the glycocalyx (Bryers, 1984; Marshall, 1992). Within the matrix, daughter cells may also get entrapped, which adds to the biofilm thickness. It was established by Vandevivere and Kirchman (1993) that exopolysaccharide production increased with attachment of bacteria to a solid surface and that this increase was not due to preferential attachment of a genotypic subpopulation with increased exopolysaccharide production, as reinoculation of the biofilm bacteria into liquid medium resulted in the reduction of exopolysaccharide production to the level previously found in planktonic cells. The development of an extensive extracellular matrix and growth in attached communities may also be important for the maintenance of optimum environmental conditions. Costerton et al. (1978) defined glycocalyx as the integral element of the outer membrane of the Gram negative cells and the peptidoglycan of the Gram positive cells. This is known as either slime or capsule and is composed of either fibrous polysaccharides or globular glycoproteins (Costerton et al., 1985) and in its hydrated state contains water at about 98–99% (Christensen and Characklis, 1990) or 50–95% (Flemming et al., 1992).

Terms such as glycocalyx, slime, capsule and sheath have all been often used to refer to the EPS associated with the biofilms (Geesey, 1982; Characklis and Cooksey, 1983). In case of *Pseudomonas aeruginosa*, alginate forms the major constituent of the glycocalyx and is important for the development of monospecies biofilms (Boyd and Chakrabarty, 1995). The EPS produced by the microorganisms plays an important role in initial adhesion, as well as firm anchorage of bacteria to solid surfaces (Sutherland, 1983; Marshall, 1992). It can protect the bacteria from dehydration as it can retain water several times its own mass and only slowly becomes desiccated (Roberson and Firestone, 1992; Ophir and Gutnick, 1994). For example, in *Pseudomonas aeruginosa*, the presence of acetylated uronic acids in the bacterial alginate increases its hydration capacity (Boyd and Chakrabarty, 1995). In addition, the biofilm polysaccharides are critical for the persistance and survival in hostile environments (Rinker and Kelly, 1996). It also helps in trapping and retaining the nutrients for the growth of biofilms and protecting the cells from the effects of antimicrobial agents.

4. Biofilms in food environments

The attachment of the bacteria to the food product or the product contact surfaces leads to serious hygienic problems and economic losses due to food spoilage (Holah and Kearney, 1992; Mattila-Sandholm and Wirtanen, 1992; Carpentier and Cerf, 1993). In addition to that, a number of reports have appeared on the persistance of several foodborne pathogens on food contact surfaces and many new organisms like *Listeria monocytogenes* (Farber and Peterkin, 1991), *Yersinia enterocolitica* (Kumar and Singh, 1994), *Campylobacter jejuni* (Stern and Kazmi, 1989) and *Escherichia coli* O157:H7 (Doyle and Padhye, 1988; Doyle, 1991; Dewanti and Wong, 1995) have been added to the list.

In food systems, the attachment of microorganisms leading to the formation of biofilms may be undesirable and also detrimental. The majority of data generated to date indicate the attachment of bacteria to food contact surfaces under simulated conditions. However, under suitable conditions, the formation of biofilms can occur. The attachment of foodborne bacteria to inert food contact surfaces has been the subject of a first published report (Zoltai et al., 1981).

Similarly, in the dairy industry, improperly cleaned and sanitized equipment (Czechowski, 1990; Koutzayiotis, 1992) and air-borne microflora (Schröder, 1984) are usually considered to be the major sources of contamination of milk and milk products. Cleaning-in-place (CIP) procedures are usually employed in milk processing lines (Dunsmore, 1981; Dunsmore et al., 1981). However, the limitation of CIP procedures is the accumulation of microorganisms on the equipment surfaces (Maxcy, 1964, 1969; Mattila et al., 1990) resulting in biofilm formation. The persistance of accumulated microorganisms in the form of a biofilm may cause post-processing contamination, leading to lowered shelf life of the product (Zottola, 1994). The transmission of pathogens can also result from aerosols produced during the cleaning of food-processing surfaces (Kang and Frank, 1990). If pathogens are present, then consumption of the contaminated product may pose a health risk (Dunsmore et al., 1981; Lewis and Gilmour, 1987; Koutzayiotis, 1992).

The other common sources involved in biofilm accumulation are the floors, waste water pipes, bends in pipes, rubber seals, conveyor belts, stainless steel surfaces, etc. Buna-N and Teflon seals have also been implicated as important sites for biofilm formation (Fletcher, 1985; Mafu et al., 1990; Blackman and Frank, 1996). Herald and Zottola (1988b) observed the attachment of *Listeria monocytogenes* to stainless steel and produced attachment fibrils. The pathogen also attached to glass, polypropylene and rubber (Mafu et al., 1990) and produced a sanitizer-resistant biofilm on glass, stainless steel and Buna-N rubber surfaces (Frank and Koffi, 1990; Lee and Frank, 1991; Rönner and Wong, 1993). The number of bacteria recovered from these surfaces were high and dependent on the length of exposure time. It was also found that hydrophobic interactions viz., electrostatic and exopolymer interactions were responsible for the attachment of *L. monocytogenes* to various surfaces (Mafu et al., 1991; Blackman and Frank, 1996). These bacteria may also act as a source for post-pasteurization contamination (Austin and Bergeron, 1995).

In the recent years, membrane technologies like ultrafiltration (UF) and reverse osmosis (RO) have been widely used in the dairy and food industry and in the waste water treatment processes (Golomb and Besik, 1970; Glover, 1985; Cheryan, 1986). In a RO process for waste water treatment, the development of a microbial biofilm contributes to a significant reduction in water flux and the deterioration of overall membrane performance (Ridgeway et al., 1983, Ridgeway et al., 1984). However, in the case of dairy and food processing, the UF/RO membrane systems find major application in the fractionation and concentration of liquid foods like skim milk and whey, and the clarification of beverages and fruit juices. An inherent feature of these processes is that the active membrane surface will come in contact with the feedstock. Even a small degree of adsorption causes pore blockage and as a result the filters get clogged, a phenomenon called fouling, leading to a reduction in permeate flux rate and loss in the product yields (Cheryan, 1986). This fouling of the membranes may also favour the formation of biofilms.

With regard to the food surfaces, many studies carried out by different research workers have shown the attachment of different microorganisms to poultry surfaces (Notermans and Kampelmacher, 1974; Thomas and McMeekin, 1980; Lillard, 1985, 1986, 1988) and meat (Butler et al., 1979). These organisms have not only been shown to be associated with slaughtering process but are also responsible for cross contamination of uncontaminated carcasses (Anand et al., 1989a). However, the interaction profile of different food pathogens on chicken carcass surfaces did not reveal any specific influence on the growth pattern of these organisms (Anand et al., 1994). Postmortem aging of chicken carcasses at 5°C prior to freezing also resulted in a substantial increase in these organisms and coliforms recorded a highest multiplication rate as compared to *Staphylococcus aureus* and yeast and molds (Anand et al., 1989b; Pandey et al., 1989). The attachment of *Listeria monocytogenes* (Chung et al., 1989; Dickson, 1990) and *Pseudomonas fragi* (Schwach and Zottola, 1982) to beef surfaces has also been studied. However, these studies did not clearly reflect the formation of biofilms per se with regard to these surfaces.

5. Adverse technological effects of biofilms

The formation of biofilms in drinking water distribution systems leads to decrease in water

velocity and carrying capacity, clogging of pipes, increase in energy utilization and decrease in efficiency of operations (Ridgeway and Olson, 1981; LeChevalier et al., 1987). This development is mainly due to the traces of nutrients in the water supply and even the high levels of residual chlorine may also not prevent the biofilm formation (Block, 1992; Marshall, 1992).

Biofouling in heat exchangers and cooling towers has been a major problem for many years. The bacterial attachment greatly reduces the heat transfer and operating efficiency of the processing equipment (Lehmann et al., 1992; Mattila-Sandholm and Wirtanen, 1992; Bott, 1992). In the filtration systems, biofilm formation also greatly reduces the permeability of the membranes (Flemming et al., 1992). The microbial activity in biofilms, especially by the sulphate-reducing or acid-producing bacteria causes corrosion of metal surfaces (Costerton and Lappin-Scott, 1989).

In addition, the submerged surfaces in all marine and aquatic environments are the zones of microbial biofilm accumulation. The fouling of ship hulls are mainly caused by buildup of biofilms consisting of algae (single-celled), diatoms and bacteria (Lewin, 1984). Many antifouling paints are designed to prevent such animal colonization, however, none of them prevents the formation of biofilms. The formation of biofilms increases the fluid frictional resistance and fuel consumption (Cooksey and Wigglesworth-Cooksey, 1992). This indirectly has an economic impact on the marine and naval transport and eventually the food industry.

6. Methods for the study of biofilms

The formation of biofilms on food-contact surfaces mainly causes the post-contamination of foods which may subsequently lead to food spoilage or food borne illnesses, if pathogens are present. The enumeration of biofilms helps in confirming the source and extent of contamination and the type(s) of microorganisms involved as contaminating agents. The different methods employed for sampling and enumeration of biofilms are swabbing, rinsing, agar flooding and agar contact methods. These conventional cultivating methods have been used most frequently since years as a quantitative method on both external and internal surfaces for enumeration of bacteria (Flemming and Geesey, 1991). These methods for biofilm enumeration have varied significantly. Scraping (Frank and Koffi, 1990) and vortexing (Mustapha and Liewen, 1989) can also be employed.

Different methods in microscopy, especially scanning electron microscopy of surfaces have gained considerable attention in the study of biofilms (Notermans et al., 1991; Zottola, 1991). In some of the studies, epifluorescence microscopy has also been used (Holah et al., 1988, Holah et al., 1989; Wirtanen and Mattila-Sandholm, 1993). Interference reflection microscopy, atomic force microscopy and confocal laser scanning microscopy are some other techniques that have attracted considerable interest in the study of biofilms (Ladd and Costerton, 1990; Caldwell et al., 1992; Beech, 1996; Debeer et al., 1997). Moreover, in the recent past, environmental scanning electron microscopy (ESEM) has also been widely used for biofilm enumeration (Little et al., 1991; Hodgson et al., 1995). This technique helps in visualizing samples without the need of conventional microscopic procedures like dehydration, fixation and staining. Also this method per se preserves many of the structures associated with biological samples which remain in a hydrated and viable state. A replica method using a hydrophilic polyvinyl siloxane impression material was also developed for the study of biofilms (Marrs et al., 1995). Very recently, cellular automation models have also found application for the study of biofilms (Wimpenny and Colasanti, 1997).

However, there are some limitations that arise during sampling of biofilms. Grooves, crevices, dead ends, corrosion patches, etc. are some of the areas where biofilms can grow and are hard to access. Thus, sampling of such areas becomes more difficult. Some of the bacteria in biofilms on the surfaces in food and dairy environments are subjected to various stresses such as starvation, chemicals, heat, cold and desiccation which injure the cells, rendering them non-culturable (Wong and Cerf, 1995). However, in a recent survey, viable but non-culturable *Salmonella typhimurium* subjected to chlorine treatment were successfully enumerated by employing the procedure of direct viable count (DVC) in combination with an indirect fluorescent-antibody technique (Leriche and Carpentier, 1995). Even then, a small proportion of

bacteria may also escape counting by the usual conventional culturing techniques, for which appropriate media and culture methods should be devised.

7. Increased resistance of bacteria in biofilms to antimicrobial compounds

It is well established that bacterial biofilms exhibit an increased resistance to antimicrobial treatments than the individual cells grown in suspension (Petrocci, 1983; Mustapha and Liewen, 1989; Frank and Koffi, 1990; Krysinski et al., 1992). This resistance has been widely observed and is attributed to the varied properties associated with the biofilm including; reduced diffusion, physiological changes due to reduced growth rates and the production of enzymes degrading antimicrobial substances. Further, it is difficult to establish that any single mechanism caused the resistance; rather, the combined mechanisms create the resistant populations.

A characteristic feature of microbial biofilms is the presence of an exopolysaccharide matrix embedded with the component cells as mentioned earlier. This exopolysaccharide matrix may act to various degrees as a diffusion barrier, molecular sieve and adsorbent (Boyd and Chakrabarty, 1995). The antimicrobial resistance exhibited by the biofilm is related to the 3-dimensional structure and the resistance is lost as soon as this structure is disrupted (Hoyle et al., 1992). Therefore, the production of excess amounts of exopolysaccharide by the bacteria during biofilm formation and growth may protect the innermost cells by binding with antimicrobial substances and quenching their effect as they diffuse through it (Farber et al., 1990; Hoyle et al., 1990; Stewart, 1996).

In some of the studies, it was observed that disinfectants like peracetic acid, mercuric chloride and formaldehyde have been shown to have no effect on biofilms (Carpentier and Cerf, 1993). It is also reported that *Listeria monocytogenes* attached to food-contact surfaces also exhibited increased resistance to conventional sanitizers like acid anionic sanitizers and quarternary ammonium compounds (Petrocci, 1983; Mustapha and Liewen, 1989; Frank and Koffi, 1990). The reasonable explanation for the reduced efficacy of such agents against the biofilms is the incomplete penetration of the biofilm by such reactive biocides (Huang et al., 1995) and the wide variation in the environmental conditions existing on the food-contact surfaces. The presence of abiotic particles of kaolin and calcium carbonate in biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* are also implicated for the reduced biocide efficiency (Srinivasan et al., 1995).

Furthermore, antimicrobial agents are far more effective against actively growing cells i.e., the best disinfectant for planktonic cells are not necessarily the suitable ones for biofilm cells (Holah et al., 1990). This implies that the bacteria within the biofilm exhibits a varied physiological pattern and showed nutrient and oxygen gradients across the biofilm. The cells within the biofilm were found to receive less oxygen and fewer nutrients than those cells at the biofilm surface (Brown et al., 1988). In addition, in cases of serious biofouling, thick biofilms are formed which may include many metabolically dormant and/or dead cells. This state of the bacterial cells of the biofilm may have an altered growth rate and physiology, resulting in increased resistance to antimicrobial agents (Gilbert et al., 1990; Evans et al., 1991; McFeters et al., 1995). In mixed biofilms, competition for nutrients results in nutrient deficiency, which also has a major role in the increased resistance of biofilms to antimicrobial treatments (Berg et al., 1982; Jones and Pickup, 1989). Some of the studies with food-borne bacteria have indicated that resistance against various disinfectants is more severe in older biofilms (more than 24 h) than in young ones (Anwar et al., 1990; Frank and Koffi, 1990; Lee and Frank, 1991; Wirtanen and Mattila-Sandholm, 1992).

Various researchers have demonstrated an increased resistance of bacterial biofilms towards different antibiotics (Nickel et al., 1985; Widmer et al., 1990; Anwar et al., 1992). The possible mechanism proposed for this resistance to antibiotics by the bacteria was the production of antibiotic-degrading enzymes i.e. β-lactamases. Such enzymes degrade and inactivate the antibiotics as they permeate through the cell envelope to their target sites. In adherent biofilms, many of the similar hydrolytic enzymes are produced and they become trapped and concentrated within the biofilm matrix, exhibiting enhanced protective properties. Moreover, the increased resistance by biofilms may also be due to the

changes of the molecular targets of the antibiotics (Anwar et al., 1992; Vergeres and Blaser, 1992).

8. Control and removal of biofilms

Generally, an effective cleaning and sanitation programme, when included in the process from the very beginning, will inhibit both accumulation of particulates and bacterial cells on equipment surfaces and subsequent biofilm formation (Dunsmore et al., 1981; De Goederen et al., 1989; Czechowski and Banner, 1990; European Hygienic Equipment Design Group, 1993a). However, an inappropriate cleaning strategy would lead to biofilm formation and increase the biotransfer potential (Dunsmore et al., 1981; De Goederen et al., 1989; Czechowski and Banner, 1990; Hood and Zottola, 1995). Since, removal of biofilms is a very difficult and demanding task, a complete and cost-effective cleaning procedure should be developed (Pontefract, 1991; Mattila-Sandholm and Wirtanen, 1992; Zottola and Sasahara, 1994).

The food-processing equipment design is also important to achieve better cleanability of the food-contact surface once bacterial adhesion has occurred. The mechanical treatment in cleaning proved efficient in the removal of biofilms, however, the equipment design often made this very difficult (Dunsmore et al., 1981; Lelieveld, 1985; Mosteller and Bishop, 1993). Comparative cleaning studies carried out on materials like stainless steel, glass, nylon and polyvinyl compounds showed no significant changes in the cleanability when the surfaces were new (LeClercq-Perlat and Lalande, 1994). However, with time, stainless steel exhibited better hygienic properties by resisting damage caused by the cleaning process. Even the application of sanitizers after cleaning process to eliminate residual bacteria sometimes also caused corrosion of surfaces (Dunsmore et al., 1981).

The important aspects essential in controlling biofilm formation and/or minimizing the biotransfer potential in the food-processing equipments like tanks, pipelines, joints and the accessories are good design practices. These mainly include the proper choice of equipments, materials and accessories, correct construction, process layout and process automation (Lelieveld, 1985; Mattila-Sandholm and Wirtanen, 1992; Holah, 1992). In addition, the quality and smoothness of the equipment were found to be equally important (European Hygienic Equipment Design Group, 1993b,c). The mechanical or electrolytic treatment of stainless steel also enabled the production of smooth surfaces.

The control of biofilms represents one of the most persistent challenges within food and industrial environments where the microbial communities are problematic. The biofilms in the food industry can be eliminated by adopting different strategies like physical and chemical methods. In addition, the biological means has been the newer dimension in the recent years for the biocontrol of bacterial biofilms.

8.1. Physical methods

The newer physical methods used for the control of biofilms include super-high magnetic fields (Okuno et al., 1993; Pothakamury et al., 1993), ultrasound treatment (Jeng et al., 1990; Pitt et al., 1994; Qian et al., 1997), high pulsed electrical fields on their own (Sale and Hamilton, 1967; Hamilton and Sale, 1967; Castro et al., 1993; Pothakamury et al., 1996) and in combination with organic acids (Liu et al., 1997), low electrical fields both on their own (Davis et al., 1991) and as enhancers of biocides (Blenkinsopp et al., 1992) have been currently investigated. Davis et al. (1991) reported that very low currents of 200 and 400 μA, using silver, carbon and platinum electrodes killed planktonic cells of Gram-positive and Gram-negative bacteria and *Candida albicans*. The biocidal effect was primarily attributed to iontophoresis, the generation of ions from chlorine-containing components like NaCl, $CaCl_2$ and NH_4Cl in a simple salts medium (Davis et al., 1989, 1994). In the absence of any antibiotics or biocides, the medium containing the chlorine compounds had the highest antimicrobial effects in the presence of an electrical current (Davis et al., 1992).

Recently, low electrical currents in combination with antibiotics was successfully employed for biofilm control (Costerton et al., 1994b; Jass et al., 1995b; Jass and Lappin-Scott, 1996). It was observed that the bioelectric effect generated by the combined use of antibiotics with low levels of electric current proved more effective in controlling

the biofilms. The possible mechanism established for this bioelectric effect is that the electric current drive the charged molecules and antibiotics into the cells through the biofilm matrix, thus increasing the mass transfer (Davis et al., 1992; Costerton et al., 1994b; Rajnicek et al., 1994). Even though the antibiotic moved into the cell and to the target site much more quickly in potentially lethal concentrations, it was still dependent on the rate of growth and metabolism of microorganisms for its antimicrobial activity (Taber et al., 1987; Widmer et al., 1991). This also suggested that the biofilm age and activity were the limiting factors in the antibiotic effectiveness (Jass et al., 1995b). The traditional strategy, i.e. the use of a mechanical method like brushing, obviously should not be neglected (Exner et al., 1987).

8.2. Chemical methods

It is speculated that before application of a disinfectant, it is essential to eliminate as many microorganisms as possible. However, the microorganisms become far more sensitive to disinfectants once they have been detached from the surface to which they were adhering. The mechanical or chemical breakage of the polysaccharide matrix is equally essential for successful biofilm control, as the matrix protects the microorganisms with decreased effects of detergents and sanitizers (Blenkinsopp and Costerton, 1991; Brackett, 1992; Czechowski and Banner, 1990; Wirtanen and Mattila-Sandholm, 1993, 1994). When no mechanical treatment is given, the disinfectants leave the slime intact, which may favour biofilm buildup in crevices and seams, etc. after the cleaning procedure (Pontefract, 1991; Zottola and Sasahara, 1994; Hood and Zottola, 1995). Detergents containing chelating agents like EDTA and ethylene glycol-*bis* (β-aminoethyl ether) N,N,N′,N′-tetracetic acid (EGTA) helped in removal of biofilms (Turakhia et al., 1983; Camper et al., 1985). The chelators, by binding calcium and magnesium ions, also destabilize the outer membranes of the cells (Izzat et al., 1981; Turakhia et al., 1983).

Studies have shown that some detergents are bactericidal and some disinfectants may even depolymerize EPS, thus enabling the detachment of biofilms from surfaces, e.g. oxidants such as peracetic acid (Exner et al., 1987; Holah et al., 1990), chlorine (Characklis, 1989), iodine (Cargill et al., 1992) and hydrogen peroxide (Christensen, 1989; Juven and Pierson, 1996). Monolaurin (glycerol monolaurate) was also found to be lethal to *Listeria monocytogenes* at low concentrations (Oh and Marshall, 1992, 1993a,b). Further, in a later study, it was demonstrated by Oh and Marshall (1995) that monolaurin (50 μg/ml) combined with heat treatment at 65°C for 5 min completely destroyed the biofilm formed by *L. monocytogenes*. In addition, a synergistic interaction between monolaurin and organic acids like acetic acid also caused a pronounced inhibition of *L. monocytogenes* (Oh and Marshall, 1994, Oh and Marshall, 1996). Very recently, cetylpyridinium chloride (CPC) was reported for its application in poultry processing industry as an effective agent for the reduction of attached *Salmonella* on poultry skin (Kim and Slavik, 1996; Breen et al., 1997).

The impregnation of materials with biocides have shown to play a major role in resisting the bacterial colonization for as long as the antibacterial agents are released from the surfaces. Antifoulant paints containing silver have also proved effective in controlling mixed biofilms containing *Legionella pneumophila* (Rogers et al., 1995). Food packaging materials containing antimicrobial compounds have gained practical importance in the recent years for the biocontrol of food-borne pathogenic and spoilage microorganisms on food surfaces. These antimicrobial compounds incorporated in the packaging material migrates to the food surface where microbial contamination is eliminated. The coupling of a polymer with an antifungal agent, methyl-1-butylcarbamoyl-2-benzimidazolecarbamate (Halek and Garg, 1989) and the incorporation of imazalil, an antimycotic agent into polyethylene films used for cheese packaging have been successfully employed for inhibiting surface molds (Weng and Hotchkiss, 1992). In addition, the incorporation of antimicrobial agents like anhydrides (Weng and Hotchkiss, 1993) and benzoyl chloride (Weng et al., 1997) into food packaging materials have recently been demonstrated for controlling the surface mold contamination.

8.3. Biological means

Newer strategies devised for the biocontrol of biofilm formation may be the adsorption of bioactive

compounds like bacteriocins onto food-contact surfaces for the inhibition of adhesion of bacteria. By definition, bacteriocins are proteinaceous antimicrobial compounds exhibiting bactericidal properties (Tagg et al., 1976). Nisin, a well known and most applied antimicrobial peptide has proven to be an effective inhibitor of many food pathogens and spoilage bacteria, especially sporeformers (Hurst, 1981; Ray, 1992). In 1988, nisin has been approved by the FDA as a GRAS (*Generally Recognized As Safe*) food additive for the control of *Clostridium botulinum* spores in processed cheese spreads (Food and Drug Administration, 1988). Reports also showed that surfaces adsorbed with nisin, lowered the incidence of surface contamination by *L. monocytogenes* on model food-contact surfaces (Daeschel et al., 1992; Bower et al., 1995). Application of bacteriocins have also been tried out on food packaging materials for the biocontrol of *L. monocytogenes* on meats (Ming et al., 1997). Similarly, the application of lactic cultures and their cell-free extracts have also been reported to selectively inhibit different spoilage and pathogenic microflora on the surfaces of dressed poultry (Anand et al., 1995).

Enzymes have also proved effective in cleaning the extracellular polymers which form the biofilm matrix and thus helps in removal of biofilms (Kumar, 1997; Potthoff et al., 1997). The specific enzymes required mostly vary according to the type of microflora making up the biofilm. In one study, a blend of enzyme mixture consisting of protease, α-amylase and β-glucanase was found effective in cleaning a simulated industrial biofilm formed during paper pulp manufacture (Wiatr, 1991). Workers of the Genencor International, Inc., USA have developed enzymes called endoglycosidases which deglycosylate biopolymers like glycoproteins which are widely distributed in living organisms. They employed rDNA technology to develop Endo-β-N-acetyl-glucosaminidase H (Endo H) as a cleaning agent. Endo H had a unique property to remove bacteria (Staphylococci and *E. coli*) from glass and cloth surfaces in buffer and detergent solutions (Lad, 1992). Very recently, an enzymatic preparation comprising of exopolysaccharide-degrading enzymes, particularly the colanic acid-degrading enzymes, derived from a *Streptomyces* isolate was reported for the removal and prevention of biofilm formation (van Speybroeck et al., 1996).

9. Some beneficial aspects of biofilms

Not all biofilms cause problems and in many natural environments, the maintenance of water quality is brought about by the microbial metabolism in biofilms. The bacteria present in these biofilms biodegrade many of the toxic compounds and there by minimize the buildup of pollutants, thus acting as pollutant monitors (Fuchs et al., 1996). Such systems, using mixed microbial consortia have found application in fluidized beds and trickling filters for sewage and waste water management and in water purification plants and also in waste gas treatment (Kanekar and Sarnaik, 1991, 1995; Raunkjaer et al., 1997; Pedersen et al., 1997). The organic nutrient-trapping capability of biofilms helps in reducing the organic content of the waste waters before they are released into the water streams or used for irrigation purposes. In the recent years, microbial biofilms have also received considerable attention from the viewpoint of bioremediation of various industrial effluents (Sarnaik and Kanekar, 1995; Nigam and Marchant, 1995; Nigam et al., 1996) and in the nitrification process for the treatment of high strength nitrogen fertilizer waste water (Beg et al., 1995; Cecen and Orak, 1996).

Biofilms represent a natural form of cell immobilization. The immobilized microorganisms have also been successfully employed in bioreactors to improve the productivity and stability of the fermentation processes (Demirci et al., 1993a,b; Demirci and Pometto, 1995; Pakula and Freeman, 1996). The biofilms also find application in the production of industrial products like acetic acid, ethanol and polysaccharides and in addition for metal ore leaching (Bryers, 1990; Macaskie et al., 1995). Even the gastrointestinal tract are colonized by lactic acid bacteria and *Bifidobacterium* spp. which constitute as a major part of the natural microflora (Savage, 1977; Fuller, 1989) and serves as a protective layer against the colonization of pathogenic bacteria. These organisms when present in sufficient number create a healthy equilibrium between the beneficial and potentially harmful microflora in the gut (Collins and Hardt, 1980; Anand et al., 1984; Vanbelle et al., 1989). These organisms also promote a probiotic effect when consumed through various fermented foods (Hawkins, 1993; Johannsen et al., 1993; Salminen and Tanaka, 1997).

10. Conclusions

In the past, extensive studies have been carried out on various aspects of biofilms; however, with regard to the sampling and enumeration of bacteria from dairy and food contact surfaces and environments, very little practical information has been gained. Experiments related to the attachment of the microorganisms in food processing environments must be carried out under the conditions existing in those environments. Such studies will help to understand fully the interactions between the biotic and abiotic entities in the food processing operations and to assess properly the risks posed by spoilage organisms and food borne pathogens. They are also needed for the effective analysis of the impacts of cleaning and sanitation from the microbiological viewpoint.

In view of the increased resistance of bacterial biofilms to antimicrobial treatments, new strategies should be implemented for the control of biofilms. It is found necessary to formulate new cleaning agents and disinfectants for effective removal of biofilms. Use of enzymes should be considered as a supplement to the present cleaning and disinfection agents and so should the use of bacteriocins, however, more studies should be carried out to support their effectiveness against biofilms. Each biofilm problem should be analysed in detail to determine its nature and an effective cleaning and disinfection operation defined and implemented.

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