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BIOFILMS IN DAIRY PLANTS

This bulletin includes technical information based on latest development on products, systems, techniques etc. reported in journals, companies' leaflets and books and based on studies and experience. The technical information on different issues is on different areas of plant operation. It is hoped that the information contained herein will be useful to readers.

The theme of information in this issue is **Biofilms in Dairy Plants**. It may be understood that the information given here is by no means complete.

In this issue:

1. INTRODUCTION

The cases of food borne disease and food poisoning are becoming more and more common throughout the world. Although some of these can be attributed to poor quality assurance and sanitization procedures, contamination problems occur despite the application of normal preventive measures and cleaning / sanitation regimes. An important reservoir of microbial contamination that has received relatively little attention is the microbial biofilm.

'Biofilm' is a convenient term to designate microorganisms adhering to and growing on wet surfaces, and acquiring in a matter of hours, resistance to adverse environmental conditions[1]**.** Biofilms are micro-colonies of bacteria closely associated with an inert surface attached by a matrix of complex exopolysaccharides, excreted by the organisms attached to the solid surface, in which other debris, including nutrients and microorganisms, may be trapped[2]. Bacteria, both pathogenic and non-pathogenic, are incorporated into a biofilm during its formation[3]. The biofilm will eventually become a tough plastic that often can be removed only by scraping.

In general, biofilms can be of beneficial type or undesirable type.

Examples of desirable biofilms are those in natural environment where the resident bacteria biodegrade many toxic compounds, engineered biofilms in sewage and wastewater management systems to degrade or transform chemicals in sewage or wastewater, and immobilized microorganisms that are increasingly being utilized in many bio-technical industries to improve productivity and stability of fermentation[4].

Microbial activity in biofilms is associated with corrosion of metallic surfaces. Biofilms in heat exchangers and cooling towers reduce heat transfer and efficiency of the equipment. Biofilms in water pipelines reduce the carrying capacity of pipes and increase the risk of public health hazards due to possible presence of pathogenic bacteria. Biofilms on teeth, gums and implanted devices in the body can lead to dental diseases and infections[4].

Biofilms on food contact surfaces in dairies may result in several defects in products[5]. This issue of Technews provides information on biofilms on food contact surfaces in dairy plants including measures to control them.

2. CONCERNS WITH BIOFILMS

Biofilm accumulation on food contact surfaces in any food environment is a major concern. Biofilms can act as a major source of contamination in dairy processing from milk production to packaging for final consumption. Their presence in dairies may result in various problems of bacteriological origin as listed below[4-7]:

- cross-contamination;
- decreased shelf life of products;
- product defects such as package bloating;
- off-flavours in products;
- appearance and textural defects in products;
- if pathogens are involved, transmission of diseases;
- mechanical blockages;
- increased resistance to heat transfer; and
- deterioration (e.g., corrosion) of components of metallic or polymeric equipment.

Biofilms containing non-starter lactic acid bacteria, especially *Lactobacillus curvatus* strain JBL2126, have been implicated in the calcium lactate white haze defect in cheese during ripening[8].

Possibility of biofilm formation and increased resistance and virulence of *L. monocytogenes* [1] in biofilm raises concerns with respect to disease transmission.

Biofilms on the surfaces of heat exchangers can be found in the cooling sections after the heat treatment. They can act as sources of recontamination to products, especially by heat resistant streptococci[7]. The same applies to membrane units, and to centrifuges operated at mid-range temperatures of 45-50^{o}C. Reports indicate that milk powder, despite low levels of microorganisms in the raw milk, can have high microbial contents due to contamination from biofilms formed due to the milk pretreatment step, including preheating by thermization and fat separation in a milk separator. Likewise, ultra-filtration or micro-filtration units and intermediate storage or filter buffer tanks with temperatures comparable to those mentioned above might be a source of contamination if not monitored carefully or not operated at lower temperatures.

In addition to the direct problems discussed above, several adaptive advantages have been ascribed to bacterial life within a biofilm[9], making their presence and control more troublesome for a dairyman. Specific examples are:

- The bacterial cells in a biofilm that have endured different types of stresses may enter a state known as ‘viable but non-culturable’. The bacteria in such state would not be detected easily[4].
- Surface-attached bacteria display increased resistance to adverse conditions, including antibiotics, disinfectants and heat[3,4,9]. Chlorine, iodophors and most quaternary ammonium compounds are ineffective in removing them[6]. While biocides have been shown to be 100% effective in destruction of cells suspended in solution, they do not effectively destroy cells attached to surfaces, unless mechanical action is employed[3].

- The efficacy of disinfectants on bacteria in biofilms varies depending upon the type of surface[4] and therefore, different approaches to control biofilms on different surfaces may be required.
- Biofilms afford a protection that allows for extended bacterial longevity[3].
- Biofilms bacteria at the gasket-metal interface in dairies can spread over other locations at each occasion when there is a differential of metal and gasket expansion caused by heating or cooling, mostly when gasket material ages and becomes less elastic[1].

3. FORMATION OF BIOFILMS

In nature, most bacteria do not exist as planktonic (suspended) cells. Rather, they exist as attached to a surface[3] resulting into formation of biofilms. Biofilm formation is a dynamic stepwise process as described below:

First, some inorganic or organic molecules, such as milk deposits or fouling, are adsorbed to a surface. This creates a conditioning layer, or bacterial primer, that increases the ability of bacteria to attach to that surface. However, microorganisms can form biofilms independently by forming glycocalyx or adhering to the clean surfaces by means of extra-cellular proteins[7].

Once a conditioning layer is formed, bacterial cells are adsorbed in two stages: reversible adhesion followed by irreversible adhesion. Reversible adhesion involves weak interaction between the bacterial cells and the substratum, and the bacterial cells can be removed easily, e.g. by rinsing. A fraction of reversibly adhered cells become irreversibly adsorbed to the surface requiring much stronger action, such as scrubbing, for removal[4]. Within 8 hours of initial adherence, 91 percent of bacteria are irreversibly attached[5].

Bacterial cells that are irreversibly adhered can grow using nutrients in the conditioning film and from the fluid in environment. Micro-colonies are formed and extracellular polymeric substances are produced. Other organic and inorganic solutes and particulate matter from the fluid phase become entrapped. Such deposits are favourable even for non-adhesive microorganisms[7]. Biofilms may build up in processing units such as milking equipment, heat exchangers, evaporators, centrifuges, stainless steel tubes or membrane filters.

Due to shear stress, presence of certain chemicals in the fluid environment, or altered surface properties of the bacteria or substratum, some of bacteria in the biofilm are detached either individually or sloughed off[4]. The released bacteria may be transported to other locations and start biofilm formation process again and/or contaminate the products passing subsequently over the surface.

4. ACCUMULATION OF BIOFILMS ON FOOD CONTACT SURFACES IN DAIRY EQUIPMENT

While the general mechanism for formation of biofilms in dairy environment remains as described above, the development of biofilms in dairy plants is affected by the following factors[1,3,4,7,9,10]:

- **Type and growth stage of microorganism:** Viable bacteria on the biofilm on gaskets in dairy industry have been reported to be mainly Gram positive strains. The genera most frequently reported are *Bacillus, Micrococcus, and Staphylococcus* while *Pseudomonas* is reported to be in minority. Heat resistant microorganisms like *Streptococcus thermophilus* can adhere to pasteurized milk section of a pasteurized or a heat exchanger. Psychotrophic microorganisms like *Pseudomonas* species as well as *Listeria monocytogenes* can produce extra-cellular polymeric material and thus provide the preconditions for biofilm-formation during refrigeration in raw milk cooling tanks, especially during extended storage. *Bacillus* spores have enhanced attachment capability on hydrophobic surfaces compared to vegetative cells.

- **Type of product being processed:** Deposition of casein and β-lactoglobulin has been found to inhibit attachment of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel. On the other hand, an increase in attachment of several milk-associated microorganisms to stainless steel, rubber, and glass surfaces in the presence of whey proteins has been reported.

Poor raw milk quality, with the presence of heat-resistant streptococci, favours biofilm formation.

- **Type of surface / equipment:** In general, increased surface hydrophobicity enhances bacterial attachment. Stainless steel is an example of hydrophobic surface. Biofilm formation is a problem on equipment that is difficult to clean and dry, for example pipes, tanks or equipment with inaccessible corners. The accumulation of organic matter in a liquid on surfaces of equipment in spite of cleaning and disinfection represent the “conditioning film". Especially rubber seals, conveyor belts and teflon seals are excellent sites for biofilm formation. The inner surface of rubber teats of milking machines is another potential location where bacteria can be sheltered and develop into biofilms. In addition surfaces that are corroded or otherwise have pits and cracks where soil and bacteria can collect represent potential sites for biofilm formation. Dead ends, joints and valves are all susceptible to biofilm accumulation. Biofilms developed on gaskets in dairy processing lines have been shown to be mainly located at the metal gasket interface.

• **Operation parameters:** Processing factors that increase bacterial attachment to surfaces include high or low pH extremes, high contact surface temperature and high temperature difference between heating medium and product. These situations result in denaturation of proteins, facilitating the formation of a conditioning layer. Further, low fluid flow rates over a biofilm allow increased nutrient contact time. Extended operating times in continuously operated equipment favour biofilm formation and contamination of product with bacteria.

Other factors related to processing conditions, that favour biofilm formation are: heat pretreatment below pasteurization conditions leading to a thermal selection of more heat stable bacteria; re-circulation of already processed milk through the pasteurizer; elevated temperatures (45-50^{o}C) in extended sections or after pasteurizer and insufficient cleaning.

Experience has shown that very high degrees of heat recovery of up to 97% in pasteurizers under certain circumstances leads to an enhanced biofilm formation, especially when the raw milk quality is compromised(7).

Cleaning and sanitation regimes: Cleaning is the most important stage for minimizing microbial colonization and to avoid biofilms on industrial dairy processing equipment. It is of vital importance to remove as many microorganisms as possible before applying a suitable disinfectant like peracetic acid and chlorinated alkalis.

5. SAMPLING AND DETECTION OF BIOFILMS

Detection of biofilms is necessary to initiate appropriate control measures to effectively control undesirable biofilm formation. Acquisition of representative samples from surfaces is particularly important to detect biofilms. Monitoring techniques that are specific for biofilms in dairy operations have been reviewed[4]. The table below provides the possible applications and disadvantages of the routinely used techniques.

Table : Routine methods to detect biofilm

Method and its application	**Disadvantages**
Agar flooding method: Assessment of the internal surface of pieces of equipment (tubing, valves, pumps etc.), and of cans, bottles, etc.	The count of colony forming units can under- estimate, sometimes grossly, the actual number of bacteria.
Agar contact method: Applicable to flat or slightly bent surfaces. Contact films are supple and can accommodate surfaces of various shapes.	The proportion of bacteria detached from biofilm-covered surface is unknown. It is highly dependent on the past history of soiling-cleaning-disinfection cycles. Unless caution is taken to apply agar to the sampled surface with constant pressure and time, reproducibility of sampling can be questionable. Number of colonies that can be counted is limited to a few tens.
Swab method: Applicable to any surface, flat or curved, horizontal, vertical or sloped, which can be reached with hand held sticks or tweezers about 10-15 cm length, and which is larger than a few millimeters.	The proportion of bacteria detached from biofilm-covered surface is unknown. It is highly dependent on the past history of soiling-cleaning-disinfection cycles. Reproducibility is variable, being highly operator, day, and time-of-day dependent.
Rinse solution method: Assessment of internal surface contamination of containers.	The proportion of bacteria detached from biofilm-covered surface is unknown. It is highly dependent on the past history of soiling-cleaning-disinfection cycles.

In the above methods, the proportion of injured and non-culturable bacteria is related to the past history of the biofilm, and is unknown. New methods for overcoming these limitations have been proposed. These employ different approaches as given below:

- *In-situ* enumeration of bacteria. Examples: impedance measurement technique, direct epifluorescence technique or direct viable count technique.

- Recovery methods. Examples: mechanical action such as brushing or ultrasound application, use of chelating agents such as ethylene-diamine-tetra-acetate (EDTA) or use of selected enzymes.

- Adenosine tri-phosphate (ATP) bioluminescence measurement of swabbed bacteria. This is a recent and rapid technique and is the most effective means for biofilm detection(3). The limitation is its cost and the fact that it does not differentiate between the bacterial and non-bacterial organic biomass(10).

The presence of biofilm may be manifested in sporadic colony counts that are observed on the plates of swabs taken from environmental and food contact surfaces. Therefore, a high total plate count or “marginal/reject” ATP bioluminescence value one day and low total plate count coupled with an “accept” ATP bioluminescence value the next day often indicates the presence of biofilms(3).

6. CONTROL OF BIOFILMS

Approaches to control biofilms include:

- Minimizing biofilm formation.
- Removing/inactivating bacteria in the already formed biofilms.

A. Minimizing biofilm formation: The best way of controlling biofilms is to prevent their development[6]. This is possible as follows:

- Use of cleanable equipment, and adequate and frequent hygiene operations: These are the current means to achieve a low level contamination on food processing surfaces[1].
- Effective cleaning and sanitation, which combines physical and chemical methods within the program: This will often prevent the accumulation of food product residues and bacterial cells on equipment surfaces[6]. If pasteurizer is operated for extended periods, it should be cleaned intermediately, preferably every 8 hours of operation.
- Timely replacement of worn out or aged gaskets (that lack appropriate elasticity): This is likely to reduce the probability of spreading of the biofilms from one place to other.
- Avoiding high temperatures (above 80°C) when dealing with products rich in whey proteins: Whey proteins get denatured and adhere to surfaces at high temperatures, which can facilitate biofilm formation on equipment surfaces[6].
- Maintaining cleaned and sanitized food contact surfaces in dry state when not in use: Biofilms are known to develop on wet surfaces.
- Use of acid cleaners to remove inorganic soil or material such as rust: Inorganic soil aids in biofilm formation.
- Avoiding use of high-pressure jets for cleaning from a distance greater than 25 cm from the surface: It, otherwise, results into increase in formation of aerosols and spreads bacteria over a larger area.

Some new ideas that are under investigation to improve surface hygiene and reduce biofilm formation are as follows[1,10]:

- Surface modification
  - Incorporation of biocides in a surface
  - Adsorption of an antimicrobial agent on a surface: Nisin adsorbed on the silica surfaces has been shown to slightly decrease adhesion on *Listeria monocytogenes* accompanied with a poor physiological state of the adhering bacteria.
  - Incorporation of catalysts into the surface: Catalysts (copper and cobalt phtalocyanine) that result into formation of oxygen radicals, superoxides and hydroxyl radicals from peroxides and persulfates at the biofilm-substratum interface have been shown to drastically increase antimicrobial efficiency of peroxides and persulfates.
  - Reducing surface roughness by electro-polishing stainless steel surfaces.

- Improved methods of disinfection by exploiting the synergistic effect of different molecules: It has been reported that cleaning-in-place in presence of chelating agent EDTA improves efficiency of inactivation of *Bacillus cereus, Bacillus thuringensis and Bacillus subtilis* on stainless steel surfaces(11).
- New disinfection processes: Pulsed beams have been shown to eliminate microbial contamination from surfaces but are suitable only for small surfaces such as part of an equipment requiring decontamination at critical points.
- Bio-control: Possibility of maintaining natural competition that would inhibit biofilm formation by pathogenic bacteria on dairy surfaces is being explored. Use of specifically cultivated bacteriophages (viruses that attack bacteria) against resistant strains of *L. monocytogenes* has also been suggested.

B. Removing / inactivating bacteria in the already formed biofilms: Some residual contamination may still remain after routine cleaning and disinfection of surfaces. Moreover, routinely used sanitizers like chlorine, iodophors and quaternary ammonium compounds have been found to be ineffective in removal of biofilms[6]. Therefore, formation/accumulation of biofilms remains a possibility in dairy plant environments. Under such circumstances, removal of biofilms or inactivation of bacteria in biofilms should be resorted to as follows:

- Cleaning by brushing, scrubbing and scraping surfaces: Once bacteria are released from the protection of a biofilm, they are much less resistant to subsequent application of sanitizers[6]. Any chemical treatment combined with mechanical action will remove biofilms more efficiently. Therefore, circulating the water in clean-out-of-place (COP) tanks, use of floor scrubbers, and cleaning potential surfaces with brushes or scrapers are highly recommended. However, care should be taken because some brushes and scrapers may be abrasive and leave scratches on stainless steel surfaces, further promoting biofilm formation. In such situations, a strict, routine sanitation protocol is recommended[3]. Use of soft water increases effectiveness of cleaning chemicals.

  An example protocol for COP for equipment parts is as follows: First, place parts to be cleaned in a tank with water containing chlorinated alkaline detergent (0.3 percent, pH 11-12) and circulating at 71°C. Keep for 20 minutes. Second, rinse with potable water and place parts in circulating water with phosphoric acid (0.6 percent) at 71°C for 20 minutes. Then, rinse with potable water and place parts in chlorine solution (0.2 percent) for 15 minutes. Finally, rinse with potable water. This regime is most effective for detaching biofilms and has been found to be extremely effective on biofilms containing organisms such as *Staphylococcus aureus*[3].

  If extended production runs are performed, cleaning and sanitation routines must be exceedingly rigorous in order to remove mature and recalcitrant biofilms[3].

- Use of sodium hypochlorite: Exposure to sodium hypochlorite concentrations of 100 ppm heated at 65°C for 5 minutes or to 72°C for 1 minute can inactivate *L. monocytogenes* biofilms on stainless steel surfaces. With chlorine compounds, pH is an important variable. High pH promotes detachment of mature films and low pH favours disinfection of thin films[(9)].
- Use of 2-methyl-5-chloro-2-methyl isothiazolone: At a concentration of 10 ppm, it can successfully control *L. monocytogenes* associated with conveyor systems in a dairy processing and packaging operations[(9)].
- Use of ozone: Ozone has been shown to be promising for disinfecting the surfaces that come in contact with milk. It is prepared on-site by passing dry oxygen or air through high voltage corona arc discharge electrodes[(9)].
- Use of electrical fields in presence of antimicrobials: Use of low levels of electrical fields (5 V / cm; 15 μA / cm^2) through biofilms in presence of antibiotics and industrial biocides enhances inactivation of bacteria in biofilms[(9)].
- Use of SU727 Trippel: Produced by Suomen Unilever, it contains anionic active tensides, organic complex formers, alkali, and hypochlorite at a working pH of 12.5. This product has been proven to remove 90% of the bacterial load contained within biofilms along with the extra-cellular polysaccharide matrix[(3)].
- Use of peroxides and peroxide containing sanitizers: These have been found to be highly effective in removal of biofilms. Their reaction rate is very rapid (1-2 minutes) and they are relatively non-corrosive[(6)].
- Use of hydrogen peroxide and peracetic acid: A stock solution of 23% hydrogen peroxide and 4% peracetic acid, used at a working concentration of 1-2%, with a contact time of 5 minutes at 25°C has been found to effectively reduce the survival of *Pseudomonas, Escherichia coli, Salmonella, Bacillus, Staphyloccus and Listeria.* Similarly, a 50% and 0.05% concentration of hydrogen peroxide and peracetic acid,

respectively, mixed to a working concentration of 1-2% and used with a contact time of 5 minutes at 25°C was shown to be effective in reducing survival of the above organisms contained within a biofilm[3].

- It has been demonstrated that it is useful to combine chemical and physical treatments to control *Listeria monocytogenes* biofilms in food industries. *Listeria monocytogenes* in biofilms can be destroyed by using 50 ppm of monolaurin at 65°C for 5 minutes[12].

The Best Defence: The best defence against biofilms in the food production facility is a good offence developed as part of the effective cleaning and sanitation program. Since some bacteria within a biofilm may be pathogenic, strategies to prevent the formation of a biofilm on food contact surfaces are of high priority. However, removal of biofilms from food contact surfaces should be carried out at a predetermined frequency by paying attention to formulations and concentrations of cleaning and sanitizing agents, temperature, time of exposure and mechanical activity. Cleaning and sanitizing regimes that incorporate steps to remove biofilms will result in a more sanitary processing environment and a safer product that has a longer shelf-life.

7. CONCLUSIONS

Biofilms on dairy equipment provide favourable ecological environment for microorganisms which can grow to high cell densities and so increase the risk of contaminating or spoiling products. An important factor in formation of biofilms is formation of deposits of protein on plant surfaces, whereby an ideal environment for biofilms is created, even for non-adhesive cells.

It should be emphasized that the focus should be continuously directed towards good hygiene practices along the entire line of milk production, milk storage prior to processing and milk processing in order to prevent hygienic problems through excessive biofilm formation. Preventive and control strategies such as good qualities of raw milk, hygienic plant layout and design of equipment, choice of materials, correct processing methods and parameters, and correct use of detergents and disinfectants can be suitably applied for controlling biofilm formation on milk-contact surfaces.

A high degree of mechanical action, either through the flow velocity or through an effective high-pressure spray, is an important aid to the action of an alkaline and acidic cleaning step.

Selection of suitable methods for removal of biofilms is important. Cleaning with enzyme-containing agents targeting glycocalyx materials or proteins mediating between adhering cells and surfaces increase the reliability of cleaning procedures in difficult situations.

In regular situations, a final sanitizing step following an alkaline/acidic cleaning procedure using hydrogen peroxide solutions and/or a hot water sterilization to inactivate potentially remaining bacteria adhering to plant surfaces is normally sufficient to re-establish hygienic conditions.

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