Study of high protein dairy powder (MPC80) susceptibility to fouling and efficacy of micro-nano-bubble aqueous ozone in removal of *Bacillus* spp. biofilms on stainless steel surfaces

by

Gagan Gandhi

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Major Professor
Jayendra K. Amamcharla

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Abstract

Fouling and biofilm formation on stainless-steel (SS) surfaces can be sources for cross-contamination and pose a great threat to the public health and food quality. The dairy industry needs an intervention strategy focusing on technologies discouraging the biofilm attachment and developing a sustainable eco-friendly approach for biofilm removal from the dairy processing surfaces. Since fouling encourages the attachment of bacteria to the SS surfaces, it becomes important to study the ways of reducing the fouling. The bacterial attachment to the fouled SS surfaces can be prevented by modifying the SS surface properties by chemical (using coatings) or mechanical methods. On the other hand, the degree of fouling can also be reduced by using good quality raw materials. The objective-1 of the study was focused on understanding the relationship between effect of milk protein concentrate (MPC80) solubility characteristics and fouling on SS surfaces during thermal processing. The powders were stored at different temperatures (25 ºC and 40 ºC) for 2 weeks to generate powders with different dissolution characteristics. Fouling characteristics of reconstituted MPC80 powder were studied using a custom-built benchtop plate heat exchanger. Exposing the MPC80 powder to a higher temperature during storage (40 ºC) significantly decreased the solubility and increased the amount of foulant on SS coupons ($P < 0.05$). Microscopic investigations (scanning electron microscopy and laser scanning confocal microscopy) of resulting fouled layers revealed heterogeneous fouling layers of varying tomographies, consisting of lipids, proteins, and calcium.

In the second study, the efficacy of Micro- and Nano-bubble aqueous ozone (MNAO) as a disinfectant was studied in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilm from the SS surface. For the *Bacillus cereus* biofilm removal, a log reduction of only 0.68 cfu/cm^2 was

observed after the de-ionized water wash. Whereas both MNAO and cleaning-in-place (CIP) treatments significantly reduced the bacterial counts by 2.43 and 2.88 $\log_{10}$ cfu/cm^2, respectively. On the other hand, for the *Bacillus licheniformis* biofilm removal from SS surfaces, a significant log reduction observed was 1.45, 3.03, 2.92 $\log_{10}$ cfu/cm^2, respectively after de-ionzed water, MNAO, and CIP treatments. Thus, it was observed that MNAO has great potential for removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from the SS surface, and can be used in the dairy industry as an effective sanitizer/disinfectant.

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Life without thankfulness is devoid of love and passion. Hope without thankfulness is lacking in fine perception. Faith without thankfulness lacks strength and fortitude. Every virtue divorced from thankfulness is maimed and limps along the spiritual road."

-John Henry Jowett

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Dedication

I would like to dedicate this dissertation to Almighty God, my parents and my professor.

Chapter 1 - Introduction

Milk protein concentrates (MPC) are novel dairy powders used in a wide range of applications in the dairy and food industry, which include incorporation into dairy beverages, infant formulas, processed cheese, Greek-style yogurts, ice-creams, and bars (Agarwal, 2015). Dissolution behavior of high-protein dairy powders plays an important role for achieving the desired functional and nutritional characteristics of a finished food product. To serve the intended purpose, it is essential that MPC are dissolved effectively in water. However, the solubility is affected by storage temperature, time, and dissolution conditions. It has been reported that the solubility of MPCs is adversely affected by storing the powders above 40ºC (Hauser and Amamcharla; 2016). Slow dispersion of poor-soluble MPC powder have further consequences during the thermal processing of milk. During the heat processing of MPC reconstituted milk, there is a formation of undesirable milk deposits, referred to as fouling, on the processing equipment (stainless-steel). Fouling of stainless-steel surfaces is a major problem with serious implications. Milk components mainly responsible for fouling are proteins and minerals (Bansal and Chen, 2006). The thermal denaturation and aggregation of proteins (mainly β-Lactoglobulin) on the stainless-steel surfaces promotes fouling. Also, during thermal processing, minerals (mainly calcium phosphate) are precipitated on the stainless-steel surfaces.

Fouling further promotes the adhesion of bacteria on the heat transfer surfaces, resulting in biofouling (Flint et al., 2000). The development of bacterial biofilms on the heat transfer surfaces (stainless-steel) poses a great threat to the quality and safety of milk and other dairy products. It has also been reported that bacterial adhesion occurs more on the fouled surfaces (10^8cfu/cm^2) compared to unfouled surfaces compared (10^6cfu/cm^2) (Hinton et al., 2002). Due to the presence of food residues, the microorganisms are protected within the biofilm matrix on the

processing surface, and hence become resistant to antimicrobials (Simões et al., 2009). Consequently, elimination of biofilms from the food processing facilities becomes challenging. In general, the cleaning protocols in the dairy industry must ensure removal of fouled materials from the processing equipment. Whereas, sanitation protocols must ensure that the product contact surfaces are completely devoid of pathogens and substantially low in spoilage microorganisms (Khadre et al., 2001). In addition to having sound cleaning and sanitization protocols, the dairy industry must focus on developing economically and environmentally sustainable cleaning and sanitization protocols. With the passage of new regulations and ozone's effectiveness against a broad spectrum of microorganisms, the use of ozone is being considered as an environmentally friendly alternative to chlorine and other disinfecting agents. Thus, inclusion of aqueous ozone as a disinfectant offers significant opportunities for the dairy and food industry.

The purpose of this review is to describe the importance of milk derived ingredients, focusing mainly on high protein dairy powders, several heat-induced changes in milk, fouling, and its mechanism. Factors promoting the bacterial adhesion (biofouling) on stainless-steel surfaces, biofilm formation, and application of ozone in the dairy industry are also described in detail. Chapter 4 is focused on studying the effect of MPC80 susceptibility to fouling during thermal processing; whereas Chapter 5 is focused on studying the efficacy of MNAO in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from the stainless-steel surfaces.

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Chapter 2 - Literature Review

Milk and milk derived ingredients

Milk is a complex biological fluid containing many components in different states of dispersion. As per FDA (21CFR131.110), milk is defined as "the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows. Milk that is in final package form for beverage use shall have been pasteurized or ultra-pasteurized, and shall contain not less than 8 1/4 percent milk solids not fat and not less than 3 1/4 percent milkfat". However, in milk, fat is present in the form of an emulsion, protein (caseins) and some mineral matter in the colloidal state, and lactose with some minerals and soluble proteins (whey proteins/serum proteins) in the form of true solution.

Table 2.1 The composition for bovine milk (Walstra et al., 2005)

	Component (%w/w)				
	Protein	**Fat**	**Lactose**	**Water**	**Mineral**
Average	3.25	3.4	4.6	87.1	0.7
Range	2.3-4.4	2.5-5.5	3.8-5.3	85.3-88.7	0.57-0.83

As can be seen in the Table 2.1, the major component of milk is water; the remaining macro-constituents are fat, lactose, proteins, and minor components as minerals. Of the macro-constituents of milk, milk proteins are one of the most valuable components which can be readily fractionated and used in the dairy and food industry for functional, nutritional, sensory, and nutraceutical benefits (Yanjun et al. 2014; Akhavan et al., 2009). Different grades of high protein products manufactured by the dairy industry for different applications include casein and caseinates, milk protein concentrates and isolates, whey protein concentrates and isolates, and hydrolyzed proteins (Kulozik, 2009). Among the wide variety of milk protein products, MPC

and MPI are novel dairy proteins with a protein content ranging from 42% to 90% (Agarwal et al., 2015).

MPC is manufactured by ultrafiltration of skim milk by removing lactose and minerals and concentrating the retentate by evaporation and spray drying (Chandan and Kilara, 2011). The casein (as casein micelles) and whey proteins in MPC are present in the same proportion as in milk, but the protein content is varied according to concentration factor. However, MPI has a higher protein content (~90%), lower lactose; but the casein-whey protein ratio is similar as in initial milk (Rollema and Muir, 2009).The composition of different dairy-based ingredients is shown in Table 2.2.

Table 2.2 Composition for Non-Fat Dry Milk (NFDM), MPC, and WPC

Component (%)	Powder					
	NFDM	MPC70	MPC80	MPI90	WPC80	WPC90
Protein	36.0	70.0	80.0	90.0	80.0	90.0
Ash	7.7	7.0	7.0	6.0	3.0	1.0
Fat	1.0	1.5	1.5	1.5	4.0	3.0
Lactose	51.3	16.0	7.0	1.0	4.0	1.0

Composition obtained from Abd et al. (2009); Agarwal et al. (2015)

The use of MPCs is increasing in the dairy and food industry because of its wide range of applications due to its high nutritional value, excellent functional properties, and clean dairy flavor (Agarwal et al., 2015). High-protein MPCs are finding application in nutritional drinks and bars, infant formulas, Greek-style yogurts, process cheese, ice-creams, and meal replacement beverages (Agarwal, 2015). MPCs have been also been used in standardizing the cheese milk to increase their yield and also been studied in the manufacture of different chesses like Mozzarella (Harvey, 2006; Francolino et al. 2010), Cheddar cheeses (Rehman et al., 2003; Harvey, 2006) and other cheeses. MPI, due to low lactose levels and extremely high protein levels, are targeted mainly in the manufacture of nutrition supplements and medical foods (Agarwal, 2015).

Different functional properties, such as water binding capacity, viscosity, gelling, foaming/whipping, emulsification, and heat stability, are provided by incorporation of MPC and MPI in different food and beverage formulations (Ye, 2011; Huppertz and Patel, 2012). Hence, to achieve these functional properties, it becomes essential that MPC and MPI powders are dissolved effectively. Thus, maintaining the solubility of high-protein dairy powders (MPC and MPI) is regarded as a critical property by the manufacturers and its end users.

Various factors, such as composition of powder, drying conditions, storage, and dissolution conditions affect the functional characteristics of MPC/MPI. An increase in casein and minerals (calcium, magnesium, and phosphorus), which are the slowest dissolving components of MPC, negatively impacts the solubility (Mimouni et al., 2010; Sikand et al, 2011). Addition of sodium chloride, sodium citrate, or sodium phosphate before spray drying helps in improving the solubility of MPC (Schuck et al., 2007). Increasing the protein content resulted in reduced solubility of MPC powder (Hauser and Amamcharla, 2016). Fang et al. (2012) reported that increasing the inlet air temperature during the manufacture of MPC resulted in powder with poor rehydration behavior, and thus a decrease in solubility. The solubility of MPC decreased with the increase in the storage temperature (Anema et al., 2006, Fang et al., 2011; Gazi and Huppertz, 2015, Hauser and Amamcharla, 2016). It has been reported that increasing the stirring speed or the dissolution temperature results in decreased rehydration time of the powders (Jeantet et al., 2010; Richard et al., 2013).

Maintaining the solubility of high protein dairy powders has been the main focus of researchers as it will have further consequences during the thermal processing of these powders. In the dairy industry, different heat treatments are required to eliminate/minimize the risk for spoilage of milk food borne illness. The main objective of heat treatment is to inactivate all

microorganisms and most of the enzymes in milk. The two most common thermal processes used in the dairy industry are pasteurization and ultra-high temperature. During thermal processing several physical, chemical, and biological changes can occur in milk.

Fouling related heat induced changes during processing of milk

During the thermal processing of milk, several heat induced changes occur in the various major and minor components of milk, which leads to the formation of undesirable deposits, mainly consisting of proteins and minerals, on the heat transfer surfaces (fouling).

Changes in proteins

The most affected constituents on heating of milk are proteins. During pasteurization (High Temperature Short Time (HTST)) or Ultra High Temperature (UHT) heat treatment of milk, the biologically active proteins, such as immunoglobulins, lactotransferrin, lysozyme, lactoperoxidase, vitamin binding proteins, are denatured. In addition, whey proteins, mainly β-Lactoglobulin (β-Lg) and α -Lactalbumin (α-La), are subjected to thermal denaturation. On heating above about 70ºC, whey proteins participate in the sulphydryl-disulphide interchange reactions, which lead to the formation of disulphide linked complexes of β-Lg and κ-casein (Fox & McSweeney, 1998). Also, denaturation of the β-Lg is strongly correlated to the association with casein micelles and forming deposits (fouling) on the stainless-steel surfaces (Jeurnink and Visser, 1997; Dalgleish, 1990). Caseins, on the other hand, are resistant to thermal denaturation and are heat stable (140ºC for up to 20 mins). But due to the high levels of phosphorylation, caseins bind to the calcium and causes them to aggregate and precipitate, and thus affecting their heat stability. Other changes in caseins that occur during severe heat treatments include decrease in pH, dephosphorization and aggregations.

Changes in milk salts

Calcium phosphate and carbonates present in milk are affected during heating of the milk. In milk, 66 % of the Ca is present in the colloidal form (Fox & McSweeney, 1998). With the increase in temperature, the solubility of the calcium phosphate decreases and the soluble calcium phosphate precipitates onto the casein micelles. On severe heat treatment, the calcium phosphate precipitated by heat becomes insoluble, but some indigenous colloidal calcium phosphate dissolves on cooling. Calcium deposition was linked with protein deposition on the stainless-steel surfaces, with caseins being deposited in the form of micelles, colloidal calcium phosphate, and whey proteins binding calcium on aggregation. Hagsten et al., (2016) reported that heating the skim milk at UHT (137ºC for 8 s for 15 h) resulted in the precipitation and deposition of calcium phosphate on the stainless-steel surface.

Changes in lipids

Fat is present in milk in the form of fat globules with the size ranging from 1-20µm in diameter, which are stabilized by milk fat globule membrane (MFGM). Heating milk above 70ºC denatures MFGM and activate the amino acid residues which may interact with whey proteins, and at higher temperatures (>100ºC) may lead to the formation of layer of denatured whey proteins on the fat globules (Fox & McSweeney, 1998). The membrane and/or whey proteins may participate in Maillard browning at higher temperatures and might also lead to the cross linking of the protein molecules, which affects the functionality of the dairy powders by decreasing the solubility and contributing to the deposit formation (fouling).

Changes in lactose

Heat-induced changes in lactose, the principal sugar present in milk, during the processing and storage of milk and milk products include formation of lactulose, acid formation, and Maillard browning reaction. The concentration of lactulose is an index for the severity of heat treatment and is useful for differentiating between UHT and in-container sterilization. During mild heat treatment of milk, due to the loss of CO_2, an increase in the pH is observed which is offset by the precipitation of $Ca_3(PO4)_2$. But, on severe heat treatment (>100ºC), lactose is degraded to formic and lactic acids, which affects the heat stability of the milk. Also, a decrease in solubility of the powders is observed during Malliard browning reactions due to the cross-linking of protein molecules.

Thus, with the changes in the different milk components depending upon the severity of the heat treatment employed, the functional properties of the dairy powders changes, which further affects the deposits formation (fouling) during thermal processing.

Fouling on stainless-steel surfaces in plate heat exchangers

Fouling is generally referred to as "undesirable deposition of materials on heat transfer surfaces during heating" (Bansal and Chen, 2006). Fouling on heat exchangers is a major problem in the dairy industry costing billions of dollars every year. SS 316 is the most widely used material in the dairy industry for processing of milk. Unfortunately, due to its high surface energy and hydrophilic nature, SS surfaces are prone to fouling. As a result, the conditions along the walls of heat exchangers are further aggravated by the deposition of aggregated and denatured proteins along with localized precipitated mineral (Baek et al., 2010; Bansal and Chen, 2006; de Jong, 1997). Several undesirable effects caused due to fouling include:

- Decrease in heat transfer coefficient;
- Increase in the pressure drop;

- Shorten run time;
- Increase in energy consumption;
- Likelihood of biofilm formation;
- Increased environmental load and impact;
- Contamination of the processed product by loosened deposits;
- Loss in product quality. (Parkar et al., 2004, Bott, 1995; de Jong, 1997;).

The costs of fouling due to hiatus in production can be dominating compared to cost faced due to reduction in performance efficiency (Georgiadis et al. 1998). Thus, removal of these fouling deposits is a major challenge in the dairy industry as it is a significant economic and environmental burden (Barish and Goddard, 2013; de Jong, 1997)

Types of fouling

In general, on the basis of processing temperature range, dairy fouling deposits can be classified into 2 categories known as Type A and Type B (Burton, 1968; Visser and Jeurnink, 1997; Bansal and Chen, 2006; Sadeghinezhad et al., 2015).

Type A, commonly referred as protein fouling, predominates at lower temperatures in the range of 75ºC-100ºC. It is mainly observed during pasteurization process. The foulant deposits formed are white, soft, spongy, and they consist of 50-70% proteins, 30-40% minerals and 4-8% fat. β-Lactoglobulin is the major protein which constitutes the Type A foulant and governs the milk deposit formation on heat transfer surfaces (Visser et al.,1997). To remove the protein deposits, clean-in-place (CIP) systems in industries mainly employ 0.5–1.5% (w/w) NaOH solutions or formulated caustic detergents at high temperatures (70–80 °C) (Bylund, 1995). Researchers have evaluated the use of several enzymes like proteases as cleaning agents for protein deposits on stainless-steel surfaces (Boyce et al., 2010; Grasshoff, 2002). Jurado-Alameda et al., (2014) studied the use of environmental friendly cleaning agents like ozone in the removal of protein deposits. Researchers reported the high efficacy of proteases and ozone

solutions compared to highly alkaline solutions as excellent cleaners for protein deposits, thus providing environmental and economic benefits.

On the other hand, Type B, commonly referred as mineral fouling, occurs at higher temperatures in the range of 110ºC-140ºC. It is observed during UHT processing of milk. The foulant deposits formed are hard, compact, granular and gray in color, and they consist of 70-80% minerals, 15-20% proteins and 4-8%fat. The minerals present in the Type B foulant are calcium phosphate $Ca_3(PO_4)_2$ and calcium citrate $Ca_3(C_6H_5O_7)_2$ (Bansal and Chen, 2006), whereas the proteins present are mainly β-casein (50%) and α-s_1 casein (27%). Acids are used to dissolve the inorganic salts precipitates. To remove the calcium precipitates, 0.5-1.5% (w/w) nitric acid (HNO_3) is commonly employed in many dairy plants.

Mechanism of fouling

Casein and whey proteins are the two general categories of milk proteins. Caseins are heat resistant but precipitates upon acidification (Visser and Jeurnink, 1997); whereas, major whey proteins are globular and heat sensitive in nature. The two major whey proteins found in the milk are α-La and β-Lg, which constitute 50% of fouling deposits in type A fouling (Bansal and Chen, 2006); however, β-Lg is the protein that dominates heat induced fouling, and also a definite relationship has been established between the denaturation of native β-Lg and fouling of heat exchangers (Dalgelish, 1990). Thus, in order to have better understanding about the composition of different fouling deposits, it becomes imperative to have knowledge about the basic fouling mechanisms.

In general, there are three main fouling mechanisms which occur during the heat treatment of milk (Jeurnink et al., 1996):

- Denaturation and agglomeration of the whey proteins on heating above 65ºC,

- Precipitation of milk salts (directly or together with proteins) upon heating, and
- Inclusion of product fluid within the growing fouling layer.

The mechanism of fouling is explained with the help of a schematic representation shown in Figure 2.1.

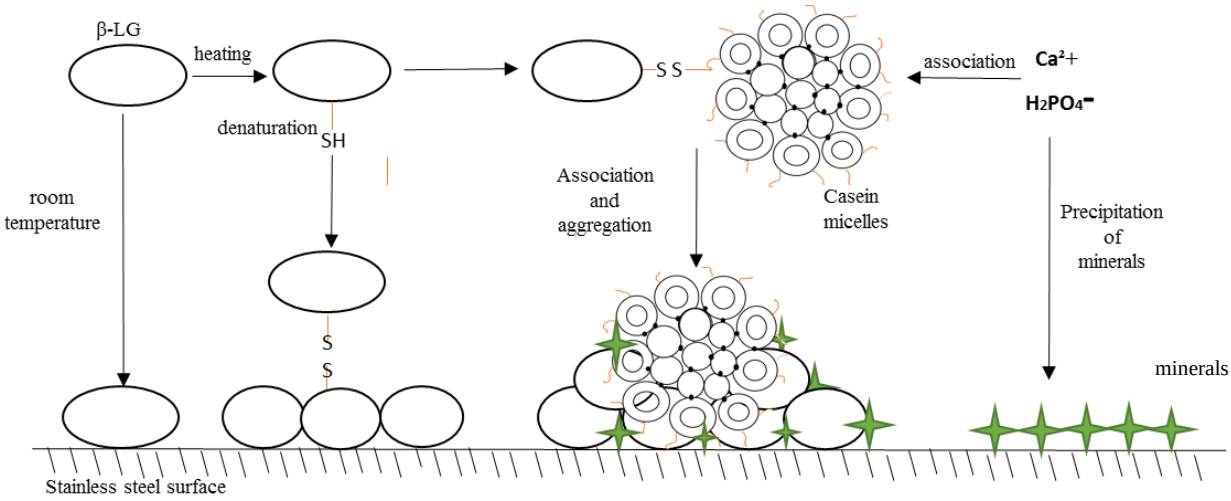

Figure 2.1 Schematic representation of mechanism of fouling during heating of milk. (Adapted from Jeurnink et al., 1996)

It can be seen that a monolayer of whey proteins adsorbs onto the stainless-steel surface at room temperature (Roscoe and Fuller, 1993). On heating the milk above 65ºC, the native whey proteins (mainly β-LG) becomes unsteady and experience the denaturation (unfolding of the structure) and the reactive sulfhydryl groups (-SH) in the core are exposed, which further aggregates on reacting with similar or other protein molecules, such as casein and α-La (Lalande and Tissier, 1984; Georgiadis and Macchietto, 2000). Different researchers have identified experimentally the involvement of denatured and aggregated proteins in the fouling formation. Delplace et al. (1994) reported the involvement of only 3.6% denatured β-Lg in the deposit formation. Changani et al. (1997) observed the fouling formation on aggregation of proteins next to heated surfaces. Chen et al. (2001) identified the involvement of both denatured and

aggregated proteins in the formation of fouling. However, the exact mechanism, whether denatured or aggregated proteins are formed first during the fouling formation on the heat transfer surfaces is not clear.

The deposition of active β-Lg and aggregates is further enhanced by calcium. Calcium influences the denaturation and aggregation of the whey proteins, stability of the casein micelles, and contributes to the fouling deposits by precipitation as calcium phosphate. The solubility of calcium phosphate decreases with the increase in temperature (Fox and McSweeney, 1998). Precipitation of calcium phosphate in the solution driven by high wall temperature, i.e large ΔT, or deposition onto casein micellar surface and/or β-Lg molecules have been reported by Visser et al. (1997). The association of the activated β-Lg and κ-caseins and its entrapment in the milk deposits have been studied. Also, the salts deposition in the form of crystals on the heat exchanger surfaces has been reported by Jeurnink and Brinkman (1994). It has been reported by Fryer and Belmar-Beiny (1991) that the first deposit layer formed is largely mineral; whereas, Visser and Jeurnink (1997) reported proteins as the first layer deposit. Though different authors have different findings, it could be stated that both whey protein aggregation/denaturation and calcium phosphate formation are to be accounted in the deposits formed during milk fouling.

With the change in conditions of milk from normal, several processes other than denaturation of whey proteins and precipitation comes into play which affect the process of fouling.

Factors affecting fouling

Several parameters such as type and quality of milk, processing of milk, hydraulic and thermal characteristics, and material and surface properties influence the fouling behavior of

milk. The factors affecting fouling can be broadly classified into following categories (Bansal and Chen, 2006):

- milk composition;
- operating conditions in heat exchangers;
- type and characteristics of heat exchangers; and
- presence of microorganisms

Milk Composition

Different components of milk and processing conditions play important roles in the formation of fouling deposits.

Role of major constituents of milk

Among the major constituents of milk, proteins play the most important role in the formation of fouling. With the change in the pH of the milk, the protein structure is affected, which greatly influences the composition of the fouling layers. It has been reported that with the decrease in pH of the heated product, an increase in the deposition of proteins in foulant deposits was observed (de Jong et al., 1998; Hege and Kessler, 1986). The increase in the protein deposits was explained by the fact of whey proteins approaching their isoelectric point and thus tending to aggregate. Also, an increase in the protein concentration of milk increased the amount of fouling during thermal processing.

The role of fat and lactose on fouling has also been studied. It has been studied that fat has no significant effect in fouling process. It has been reported by Visser et al., (1997) that during thermal treatment of milk, fat globules are associated by the whey proteins, and further the fat globules covered by whey proteins might be entrapped in the fouling deposit. Lactose contribute to the fouling deposits only at higher temperatures (due to caramelization or Maillard

reaction). Lactose, being water soluble, is scarcely found in the fouling deposits (Visser et al., 1997).

Role of minerals

Minerals play a major role in the contribution to the fouling deposits. Role of calcium concentration has been well studied in relation to heat stability and fouling. Increasing or decreasing the calcium content in milk lowers the heat stability of milk, and hence increases the amount of fouling (Jeurnink and deKruif, 1995). The mechanism for the deposition of the precipitated and aggregated mineral salts on the heat transfer surfaces due to the presence of calcium ions in milk has been described above. Due to the decrease in the solubility of calcium phosphate with heating, it precipitates as such, or deposit onto casein micellar surface and/or β-Lg, and hence increasing the amount of caseins in the deposits, suggesting an increased instability of casein micelles (de Jong et al., 1998; Visser et al., 1997). Increasing the pH of milk increases the amount of minerals in the fouling deposits, as the reduced solubility of calcium phosphate on heating results in enhanced crystallization. Thus, proteins and minerals behave differently on heating the milk with different pH (Yoon & Lund, 1989).

Role of different processing conditions

Preheating the milk decreases the amount of fouling in heat exchangers. Preheating the milk denatures the proteins and causes aggregation, and thus during the heating process, less amount of active proteins is found (Mottar and Moermans, 1988, Foster et al., 1989). It also influences the fouling behavior of milk salts, as a part of milk salts is already precipitated during the preheating process, hence less amount of milk salts is observed in the foulant deposits

It has been reported that reconstituted milk gives less amount of fouling compared to liquid milk as during the production of dairy powders, β-Lg is partly denatured, and hence less

amount of active β-Lg is present, thus decreasing the interactions with the heat transfer surfaces (Changani et al., 1997, Visser and Jeurnink, 1997).

Changani et al. (1997) reported that holding the milk for 24 hours before processing decreases the fouling; whereas, further ageing after 24 hours increases the amount of fouling. On the contrary, de Jong (1998) and Burton (1968) reported that prolonged storage of milk at refrigerated temperature (5ºC) enhances fouling due to the action of proteolytic enzymes.

Operating conditions in heat exchangers

Different operational parameters/conditions in the heat exchangers that might influence the fouling are air content, turbulence/velocity, and temperature. It has been reported that fouling is enhanced in the presence of air bubbles, acting as nuclei, formed on the heat transfer surfaces (Burton 1968, de Jong, 1997, de Jong et al., 1998). Also, mechanical forces induced by valves, expansion vessels, free-falling streams also enhance the formation of air-bubbles, which further influence the fouling (de Jong et al., 1998).

Due to increase in the turbulence and different flow patterns, induction period is reduced in the plate heat exchanger, which decreases the fouling (Belmar-Beiny et al., 1993). Changani et al. (1997) reported that with the increase in velocity, the thickness and volume of the laminar sublayer of the foulant deposit on the heat transfer surface decreases.

Temperature of milk in a heat exchanger is an important factor that affects the fouling. It has been observed by Visser & Jeurnink, (1997) that dairy fouling is diverse in structure and composition and is heavily dependent on the heating process. On heating beyond 110ºC, Type A (protein) fouling change to Type B (mineral) fouling (Burton, 1968). Surface temperature has been found to be an important factor in initiating fouling (Chen and Bala, 1998). It has been

predicted by Chen et al. (2001) that fouling can be substantially reduced by mixing caused by in-line mixers.

Type and characteristics of heat exchangers

Plate heat exchangers (PHE) are most commonly used in dairy industry due to their excellent heat-transfer performance, compactness, ease of maintenance, and cleaning compared to tubular heat exchangers. However, due to the narrow flow channels and high surface temperature, plate heat exchangers are prone to fouling and analysis of fouling becomes difficult due to their complex hydraulic and thermal characteristics (Delpace et al., 1994). Different factors such as surface charge, surface energy, presence of chromium oxide, surface roughness, and type of stainless-steel used affects the (bio)fouling on SS surfaces used in heat exchangers (Jindal et al., 2016; Barish and Goddard, 2013; Rosmaninho et al., 2008; Visser and Jeurnink, 1997). It has been reported that with the decrease in the surface energy, bacterial adhesion is reduced on the of the SS surfaces. Also, modification of the SS316 with Ni-P-PTFE blend resulted in lesser biofilm formation. Bradley and Fryer (1992) have found a reduction in the amount of fouling with the use of fluid bed heat exchanger. Another novel type of heating treatment studied by researchers is ohmic heating or direct resistance heating in which the milk has been pasterurized/sterilized using electric current (Quarini, 1995). Bansal and Chen (2006) studied and developed a mathematical model for skim milk fouling in the concentric cylinder ohmic heater and reported that less fouling should take place during ohmic heating due to lower surface temperature and heat being generated in the bulk fluid.

Presence of microorganisms

Fouling promotes the adhesion of micro-organisms on the heat transfer surfaces resulting in bio-fouling. These micro-organisms accumulate on the heat transfer surfaces, attach and

entrap in these deposits, and multiply by deriving nutrients from it (Flint et al., 2000; Bott, 1993). Hinton et al. (2002) reported that that more bacterial activity was observed to a level of 10^8 cfu/cm^2 on the fouled surface compared to unfouled surface (10^6 cfu/cm^2). Also, release of these bacteria in the process stream by the hydraulic forces contaminates the downstream. Hence, their development poses a great threat to the quality of milk and other dairy products (Yoo et al., 2005).

Biofouling/Biofilm

Biofouling, in general, is referred to as accumulation of undesirable biotic deposits on abiotic surfaces. Different authors have described biofilms differently, but the fundamental characteristics are frequently maintained. According to Characklis and Marshal (1990), a biofilm is a group of cells immobilized in a substratum and embedded in an organic polymer, whereas Heydorn et al., (2000) described biofilms as sessile microbial communities embedded in an extracellular polymeric substances (EPS) matrix and growing on the surfaces. Abdallah et al., (2014) referred to live bacteria attaching to surfaces and forming a complex structure as a biofilm. Thus, growth of biofilm requires microbes and a substrate to grow (Dunne, 2002). Development of biofilms is of great concern in the food and dairy industry (Simões et al., 2010). PHE commonly used for pasteurizing the milk in the dairy plants are fabricated using SS surfaces. The ability of bacteria to form biofilm on SS surfaces has been reported (Simões et al., 2010). As the milk is processed through PHE, the proteins (mainly β-Lg) get denatured and the minerals (calcium phosphate) are precipitated, thus causing fouling, which promotes the attachment of bacteria on the SS surfaces (Flint et al., 2000; Rosmanino and Melo, 2006). Thus, dairy biofilms can be referred to as those dominated by milk deposits, mainly of proteins and calcium phosphate, and EPS. Development of bacterial biofilms on SS surfaces poses a great

threat to the dairy processing plant (Simões et al., 2010). The implications of biofilm include product spoilage, quality and safety issues, risk to consumers, decreased production efficiency, and economic losses (Bremer et al., 2006; Gram et al., 2007).

Significance of biofilm in the dairy industry

The bacterial cells have the tendency to attach to the wet surfaces and form a complex structure called biofilms (Abdallah et al., 2014). The biofilms in the dairy industry are predominated by the bacterial EPS and milk residues (Flint et al., 2000). Biofilms have become a major concern within the dairy industry, and are recognized as sources, or potential sources, of contamination by spoilage or pathogenic microorganisms. Several pathogens have been associated with, or can produce their own biofilms, which further leads to serious hygiene problems, decreases product safety and stability, causes economic losses, and equipment impairment (Bremer et al., 2006; Gram et al., 2007).

The most commonly encountered spoilage and pathogenic bacteria genus in the dairy habitat and invading the biofilm include *Pseudomonas*, *Enterococcus*, *Lactobacillus*, *Enterobacter*, *Listeria*, *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Escherichia*, *Corneybacteria*, and *Bacillus* (Anand and Singh, 2013; Salo et al., 2006; Sharma and Anand, 2002; Waak et al., 2002). *Bacillus* spp. strains, particularly *Bacillus cereus*, which are gram positive spore forming bacteria, are implicated in food spoilage and cause food borne illness (Andersson et al., 1995; Wijman et al., 2007). In 2006, a hospital located in Japan discovered a significant significant increase in positive *B. cereus* cultures during the August, when total of 15 patients were tested positive (Sasahara et al., 2011). It has been reported that 25% of the *Bacillus* spp. have been found in dairy processing plants, and *Bacillus cereus* accounts for more than 12% of the biofilm constitutive microflora (Sharma and Anand, 2002). *Bacillus licheniformis* is also

commonly found in a wide range of dairy products (Crielly et al., 1994; Ronimus et al., 2003). Due to the ubiquitous nature of the *Bacillus* spp., they easily spread and contaminate the food production systems. Also, the spores of these species, particularly *Bacillus cereus*, are hydrophobic and resistant to stressors like heat and dehydration, which further promote its adherence to the processing equipment (Lindsay et al., 2006).

Biofilm formation

There are several mechanisms by which bacterial species come in contact with the surface, get attached to it, promote interaction, and develop into a complex structure, referred to as biofilm (Breyers and Ratner, 2004). In general, the attachment of biofilm on a surface follows a series of steps, which include (Breyer and Ratner, 2004; Karatan and Watnick, 2009; Teh et al., 2015):

- Pre-conditioning of the substratum with the macromolecules such as protein, fat and salts
- Interaction of the cells and/or spores with the surface fouled with protein, fat and salts
- Adsorption of cells and/or spores to the fouled surface;
- Desorption of the reversibly adsorbed cells and/or spores at the surface;
- Germination of spores and cell-to-cell signaling and growth, beginning to produce EPS;
- Replication of cells, forming microcolonies enclosed in EPS;
- Coalescence of microcolonies, forming complex three-dimensional aggregates of cells and secretion of polysaccharide matrix;
- Biofilm detachment and dispersal of cells and spores

Several variables affect the attachment of cells and biofilm formation, as listed in Table 2.3

Table 2.3 Variables affecting the cell attachment, formation and development of biofilm (based on Donlan,2002)

Substratum adhering properties	**Bulk fluid properties**	**Cell properties**
Texture or roughness	Flow velocity	Hydrophobicity of the cell surface
Hydrophobicity	pH and Temperature	Extracellular appendages (Fimbriae, Pili, Flagella)
Surface Energy	Cations	Extracellular polymeric substances (EPS)
Surface Charge	Presence of antimicrobials	Signaling molecules

In general, the surface properties (surface energy, surface roughness, and hydrophobicity) of the substratum, particularly stainless-steel used in dairy industries, influence the likelihood of biofouling, attachment of bacteria and growth of biofilm. Though it has been reported that bacterial attachment occurs more readily on rougher, more hydrophobic, and coated surface conditioned coated surfaces (Chae et al, 2006; Donlan, 2002; Oulahal et al., 2008; Patel et al., 2007; Simões et al., 2008). However, there have been conflicting reports regarding the effect of surface roughness on bacterial attachment and biofilm formation on stainless-steel. Some researchers have reported the increase in bacterial adhesion with increased surface roughness; while others have reported no correlation between surface roughness and cell attachment (Vanhaecke et al., 1990; Flint et al., 2000; Mitik-Dineva et al., 2009; Truong et al., 2010). An increase in the flow velocity and/or nutrient concentration is correlated to the increase in the bacterial attachment (Simões et al., 2007; Stoodley et al., 1999).

Several cell surface properties, such as hydrophobicity, presence of extracellular appendages, EPS, and cell-cell communication influence the biofilm formation and development (Allison, 2003; Donlan, 2002; Parsek and Greenberg, 2005). The hydrophobicity of the cells surface increases with the increasing non-polar nature of the microbial cell and/or the surface involved, and thus influence the bacterial adhesion (Donlan, 2002). The presence of extracellular appendages, such as flagella, pili or fimbrae, prothecae, and stalks, impart a key role in bacterial attachment. Flagella have been reported to help in transportation and initial cell-surface interactions in the biofilm formation; whereas Pili or fibrae have shown to overcome the initial electrostatic repulsion barrier between cell and surface, helps to make cells adhesive, and thus help them colonize the surfaces (Saucer and Camper, 2001; Harbron and Kent, 1988). EPS are mainly responsible for binding cells and other particulate materials, such as food residues (soil) into the biofilm, thus resulting in an increase in the biofilm's bulk and complexity (Allison, 2003; Sutherland, 2001). Also, it has been studied that cell-cell signaling (quorum-sensing) plays an important role in cell attachment or detachment from the biofilms (Daniels et al., 2004; Donlan, 2002).

Biofilm control and mitigation strategies

It is important to clean and sanitize the dairy equipment to remove the food residues and to prevent or control the attachment of unwanted bacteria to the surfaces, and hence prevent the biofilm formation (Simões et al., 2006). Proper understanding of the microorganisms involved (spore forming, non-spore forming), nature of fouling material (proteins, minerals, fat) incorporated into or cover the biofilm, and time-temperature-concentration combination of the cleaning solutions is important in developing effective cleaning and sanitation protocols. Moreover, several chemical parameters including addition of surfactants (benzalkonium

chloride, peracetic acid), wetting agents (e.g. detergent), and chelating agents (e.g. EDTA), and mechanical force (brushing, turbulent flow) also influence the effectiveness of the cleaning and sanitation process. In dairy processing plants, the typical cleaning-in-place (CIP) operation used is warm or hot cleaning solutions circulation at high velocity (Stewart & Seiberling, 1996). Alkali or surfactants are commonly used to dissolve fat and proteins, and to remove the food residues; whereas acid cleaners are used to remove the surfaces soiled with precipitated minerals (Maukonen et al., 2003; Srinivasan et al., 1995). Though the cleaning step removes almost 90% of the microorganisms from the surface, it cannot be relied to kill them. Hence, a disinfection step is required to kill these microorganisms, and thus reduce the surface populations of the viable cells after cleaning (Gram et al., 2007). The use of various disinfectants has been demonstrated in several publications to control the biofilms formed by bacteria commonly found in dairy processing plants (Simões et al., 2010). For example, a commonly used sanitizer in the food and dairy industry is chlorine. Chlorine is a germicide that is effective against most microorganisms. However, it leaves undesirable residue and may corrode many metal surfaces at high temperatures. Therefore, the dairy industry is looking for an alternative powerful sanitizing agent which is effective against a wide variety of microorganisms. For this reason, recent interest has focused on the use of ozone as an environmentally friendly alternative to chlorine.

Ozone

Ozone (O_3) is a triatomic allotrope of oxygen and is the second strongest oxidizing agent after fluorine (Guzel-Seydim et al., 2004). It was discovered in 1839 by Schonbein and was first used commercially in France to treat drinking water (Rubin, 2001). Ozone is a bluish gas with a pungent odor and a high oxidation potential of -2.07 V (Guzel Seydim et al.,2004). The main physical properties for the ozone is listed in Table 2.4. Ozone is generated by reaction of free

radical oxygen with the diatomic oxygen. The free radical oxygen molecule is produced by splitting the diatomic oxygen molecule using different methods, such as corona discharge, ultraviolet radiation, chemical, electrolytic and chemo nuclear methods (Kim et al., 1999; Khadre et al., 2001; Patil and Bourke 2012). However, ozone decomposes rapidly at the room temperature, and thus doesn't accumulate substantially without continual ozone generation (Miller et al., 1978).

Table 2.4 Physical properties of ozone (Manley and Niegowski, 1967; Guzel Seydim et al., 2004; Khadre et al., 2001)

Parameters	**Value**
Molecular weight	48
Density	2.14 kg/m^3
Boiling point	-111.9 ± 0.3ºC
Melting point	-192.5 ± 0.4ºC
Critical temperature	-12.1ºC
Critical pressure	54.6 atm
Solubility in water at 0ºC	0.640 (L/L)
Solubility in water at 15ºC	0.456 (L/L)
Solubility in water at 40ºC	0.112 (L/L)
Solubility in water at 60ºC	0.000 (L/L)

Due to its high oxidational potential, ozone is used as a disinfectant in a wide variety of applications. However, several parameters affect the disinfection ability of ozone in the liquid processing treatments.

Factors affecting the ozonation efficiency

The efficacy of ozone in the food-processing applications is affected by several factors, including temperature, pH, pressure, presence of organic impurities, flow rate, and ozone concentration (Patil and Bourke, 2012; Cullen and Norton, 2012). These factors mainly influence the solubility and reactivity of ozone. The ozone gas doesn't appreciably react with water and

therefore is physically distributed in solution (Horvath et al., 1985). Solubility of ozone in water decreases with the increase in temperature. However, with the increase in temperature, the reactivity of ozone increases (Khadre et al., 2001). Thus, for the effective disinfection efficiency, temperature during ozonation needs to be controlled carefully. It has been studied that ozone is more stable at low pH than high pH (Khadre et al., 2001; Cullen and Norton, 2012). In addition to water temperature and pH, the optimum distribution and solubility of ozone gas in water solution depends on the size and surface area of ozone gas bubbles. According to Ketzenelson et al. (1974), the optimum dissolution of ozone in water occurs when the size of bubble are between 1 and 3 mm in diameter. The smaller ozone bubbles result in larger surface area of contact, and hence increases the solubility and efficacy of ozonated water. The rate of ozone solubilization is also affected by the purity of water. Ozone is found to be dissolved faster in deionized and distilled water than tap water due to the presence of minerals (Kim, 1998). The dissolved organic or inorganic matters in the water competes with the microorganisms for the ozone, thereby decreasing the disinfection ability of ozone (Khadre et al., 2001; Cullen and Norton 2012; Patil and Bourke, 2012).

Benefits of ozone in food processing

The US Food and Drug Administration has approved ozone as an antimicrobial agent for use of direct food contact. Ozone destroys microorganisms by the progressive oxidation of vital cellular components, especially targeting the bacterial cell wall (Guzel-Seydim et al., 2004). It was hypothesized that the ozone reacts with intracellular enzymes, nucleic materials, and components of cell envelop, spore coats, or viral capsids (Khadre et al., 2001). The passage of new regulations and ozone's effectiveness against a wide variety of microorganisms makes it as a greener and more cost-effective alternative to chlorine and other disinfecting agents It has been

reported that all micro-organisms have an inherent sensitivity to ozone (Patil and Bourke 2012). Several researchers have found that bacteria are more sensitive than moulds and yeasts towards ozone, with gram-negative bacteria being more sensitive than gram positives. Also, ozone is reported to be less effective against both fungal and bacterial spores than vegetative cells (Kim et al., 1999; Moore et al., 2000; Khadre et al., 2001; Pascual et al., 2007; Cullen and Norton, 2012; Patil and Bourke, 2012). Ozone does not leave any chemical residues on the food or food contact surfaces as it decomposed to non-toxic products, thus reducing industrial costs and environmental impacts (Khadre et al., 2001, O'Donnell et al., 2012; Patil and Bourke, 2012). In addition, ozone can be generated on-site opposed to other conventional chemical disinfectants, thereby reducing the transportation and storage costs. Also, due to the consumption of limited amount of electricity, the running costs of ozonation systems are low (Pascual et al., 2007).

Application of ozone in the dairy industry

Due to the multifunctionality of ozone as a disinfectant, it is used in wide variety of applications. Studies have been reported for the use of ozone in removal of milk residues and biofilm-forming bacteria from SS surfaces. Baumann et al., (2009) investigated the individual and combined effect of ozonated water and ultrasound in the removal of *L. monocytogenes* biofilms from the SS chips. The authors have found more effectiveness in biofilm removal with the combined application of ozone and ultrasound. Dosti et al., (2005) have found significant reduction of three *Pseudomonas* spp. in biofilms on SS coupons with the application of ozone (0.6 ppm for 10 mins). Ozonated water has been reported to increase the WPC removal within 10 min compared to treatment with 0.5 % (w/w) NaOH solution (Jurado-Alameda et al., 2014). The application of ozonated cold water (10ºC) for 15 mins has been reported to remove 84% of dairy soils from the SS plates (Guzel-Seydim et al., (2000). Pressurized ozone (5-35 mg/L for 5-25

mins) has been found to preserve the skim milk by decreasing the psychrotrophs populations by more than 99% (Rojeck et al., 1995). Ozonation has been reported to completely eliminate *Listeria monocytogenes* from raw and branded milk samples (Sheelamary and Muthukumar, 2011). The foaming capacity and foaming stability of the WPI has been found to be improved with the gaseous ozone application (Segal et al., 2014). Gaseous ozone has been reported to inhibit the mould growth in several cheeses (Gibson et al., 1960; Morandi et al., 2009). Also, ozonated water (2 mg/L for 1-2 min) has been found to reduce bacterial and fungal counts in the Minas Frescal Cheese. The waste water generated in the dairy effluents during processing has been treated with the ozone and found to enhance COD solubilization (Packyam et al., 2015; Sivrioğlu and Yonar (2015). Although the use of ozone has been studied in several applications in the dairy industry, but still there is not enough research and there are several key issues which need further investigation. This literature focused on a novel approach of generation of MNAO on site and checked its efficacy in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from the stainless-steel surfaces.

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Chapter 3 - Research Objectives

This study focused on evaluating the fouling behavior of high protein dairy powder stored at different temperatures on stainless-steel surfaces Another focus of the study was to determine the efficacy of micro- and nano-bubble aqueous ozone (MNAO) as a disinfectant in biofilm removal from stainless-steel surfaces. The specific objectives are located below:

1. Effect of milk protein concentrate (MPC80) quality on susceptibility to fouling during thermal processing
2. Efficacy of micro- and nano-bubble aqueous ozone (MNAO) in the removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from stainless-steel surfaces

Chapter 4 - Effect of milk protein concentrate (MPC80) quality on susceptibility to fouling during thermal processing[1]

Abstract

This study aims to understand the effect of milk protein concentrate (MPC80) solubility on its susceptibility to initiate fouling on stainless-steel (SS) surfaces during thermal processing. In order to generate powders with different dissolution characteristics, a part of powder was stored at 25 ºC (well-soluble powder) and the remaining part was stored at 40 ºC (poor-soluble powder) for 2 weeks. Fouling characteristics of reconstituted MPC80 powder with 80 g protein per 100 g powder were studied using a custom-built benchtop plate heat exchanger. Exposing the MPC80 powder to a higher temperature during storage (40 ºC) significantly decreased the solubility and increased the amount of foulant on SS coupons ($P < 0.05$). Microscopic investigations (scanning electron microscopy and laser scanning confocal microscopy) of resulted fouled layers revealed heterogeneous fouling layers of varying tomographies consisting of lipids, proteins, and calcium. The energy dispersive X-ray spectroscopy helped in visualizing the spatial distribution of the elements (Ca, N, O, C, etc.) throughout the fouled layer. Thus, the study can be useful in understanding the fouled layer characteristics during thermal processing of MPC80 and help in designing effective cleaning protocols.

Keywords: High-protein dairy powder, Solubility, Stainless-steel, Milk-deposit

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Introduction

Fouling of stainless-steel (SS) surfaces during thermal processing of milk is a problem in the dairy industry. In the United States alone, the total costs of fouling were estimated to be $7 billion, which includes cleaning of the equipment, loss of production, additional energy consumption, and design considerations of the heat exchanger units (Müller-Steinhagen, 2000). Milk components such as proteins and minerals are responsible for the fouling of the SS surfaces (Bansal and Chen, 2006). β-lactoglobulin (β-LG) is a major whey protein which has a high heat sensitivity to denature and predominates in the fouling process (Lalande & Tissier, 1985); whereas caseins are generally resistant to thermal processing (Visser & Jeurnink, 1997). It has been determined that a definite relationship exists between the denaturation of β-LG and fouling of heat exchangers (Dalgleish, 1990). In general, heating of milk denatures or unfolds the major whey protein (β-LG), consequently exposing sulfhydryl groups. These exposed sulfhydryl's can then form disulfide bonds or cysteines with other β-LG or α-lactalbumins and caseins or other proteins depending upon the pH, forming protein aggregates and/or insoluble mineral complexes on the heat transfer surfaces (Bansal and Chen, 2006).

A study conducted by Jeurnink (1995) showed that reconstituted milk produced less fouling as compared to fresh milk. The reason can be explained by the denaturation and aggregation of serum proteins during evaporation and drying. Already denatured proteins are less active in the fouling phenomenon. However, no such information is available for dairy-based high-protein ingredients. Therefore, it is important to study the fouling behavior of high-protein dairy powders such as milk protein concentrate (MPC80). MPC is manufactured by partially removing lactose and minerals from skim milk using ultrafiltration and concentrating the retentate by evaporation and spray drying (O’ Donnell & Butler, 1999).

MPC is incorporated into a wide range of dairy beverages to improve functional, nutritional, and sensory properties. Various factors such as drying conditions, composition, storage, and dissolution conditions affect the overall functional characteristics of MPC. Decreasing the drying air outlet temperature improves its solubility because of less thermal damage (Sharma, Jana, & Chavan, 2012). Storing the high-protein powder at elevated temperatures (above 40 °C) drastically decreases its solubility (Hauser & Amamcharla, 2016a). High storage temperature of powders leads to crosslinking of the proteins at the surface of the powder, acts as a barrier to water transport, and subsequently inhibits the hydration of the MPC85 particles (Anema, Pinder, Hunter, & Hemar, 2006). As a result, with the slow dispersion rate of particles for the powders stored at higher temperature, more formation/deposition of undesirable deposits onto the heat exchanger surfaces (fouling) is expected. However, most of the fouling behavior studies have been carried out using fluid milks and less work has been done on reconstituted high-protein powders. We look to expand the understanding of high-protein powder fouling by determining the foulant characteristics of MPC80. It is also important to understand the composition and structure of a MPC80 fouled layer to minimize the fouling of processing equipment. The objective of the present study was to understand the effect of MPC80 quality, especially reconstitution characteristics, on its susceptibility to fouling on SS surfaces during thermal processing.

Materials and Methods

Experimental Design

Milk protein concentrate (MPC80) powder with 80 g protein per 100 g powder was obtained from a commercial manufacturer within the United States and divided into 2 lots. In order to create powders with different solubility characteristics, the first lot was stored at 25 °C

and the second lot at 40 °C for 2 weeks (50% relative humidity). The powders stored at 25 °C and 40 °C were referred to as “well-soluble” and “poor-soluble” powders, respectively. The solubility and dissolution characteristics of the well-soluble and poor-soluble powders were evaluated using solubility index (SI) and focused beam reflectance measurement based methods. The fouling behavior of reconstituted well- and poor-soluble MPC80 powder on SS 316L coupons were studied in a custom-built benchtop plate heat exchanger (PHE). The composition and microstructure of the fouled layer was characterized using a combination of microscopic techniques, such as scanning electron microscopy (SEM), laser scanning confocal microscopy (CLSM), energy dispersive spectroscopy (EDS), and chemical analysis. Fouling experiments were conducted in triplicate (n=3).

Evaluation of solubility and dissolution characteristics of MPC

MPC80 was reconstituted (5.263 g MPC powder in 100 g distilled water) using distilled water and the solubility of the MPC80 powders stored at 25 °C and 40 °C was evaluated as per the method described by Anema et al. (2006). A 15 mL of the MPC80 sample was taken at 30 min reconstitution and centrifuged at 700 x g for 10 min at 25 °C. The amount of insoluble material (σ) in the MPC80 was calculated as:

$$\sigma = \frac{weight\ of\ dry\ material}{weight\ of\ solution} x100\% \qquad (1)$$

The dissolution characteristics of the MPC80 powder stored at 25 °C and 40 °C were acquired using focused beam reflectance measurement as per the experimental set up described by Hauser and Amamcharla (2016b). MPC80 solution was prepared by dissolving 26.32 g of the powder in 500 g distilled water maintained at 40 °C. The dissolution behavior of the powders was characterized by the changes in the particle counts during the dissolution of a MPC80 sample for 30 min.

Experimental setup for evaluating fouling characteristics

To study the fouling behavior of reconstituted MPC80, a custom benchtop PHE was built. The designed benchtop PHE was a modification to a previous study conducted by Barish and Goddard (2013). The benchtop PHE model is shown in Figure 4.1 which includes two benchtop PHE units, water bath 1 (for preheating the sample), water bath 2 (for final heating), thermocouples, a peristaltic pump, reconstituted MPC80, and silicone tubing. For the bPHE unit, the materials were purchased from McMaster-Carr (Chicago, IL, USA). Two blocks of benchtop PHE were fabricated from high temperature multipurpose 6061 aluminum rectangular bar to the 76.2 x 60.96 x 17.78 mm3 dimensions. SS coupons (AGC Heat Transfer, Portland, OR, USA) with 25.4 x 25.4 x 0.48 mm3 dimensions were used. Inlet temperature of the reconstituted MPC80 solution in the balance tank was maintained at 40 °C using water bath 1 (Fisher Scientific, Pittsburgh, PA, USA) and stirred hot water was maintained at 90 °C in water bath 2 (Fisher Scientific, Pittsburgh, PA, USA) which served as the heat source. Temperatures of the hot water, inlet, and outlet reconstituted MPC80 solutions were monitored by thermocouples (Omega, Stamford, CT, USA). A peristaltic pump (Watson Marlow, Wilmington, MA, USA) with high temperature food-grade silicone rubber (2.38 mm ID) was used to control the reconstituted MPC80 flow through the benchtop PHE.

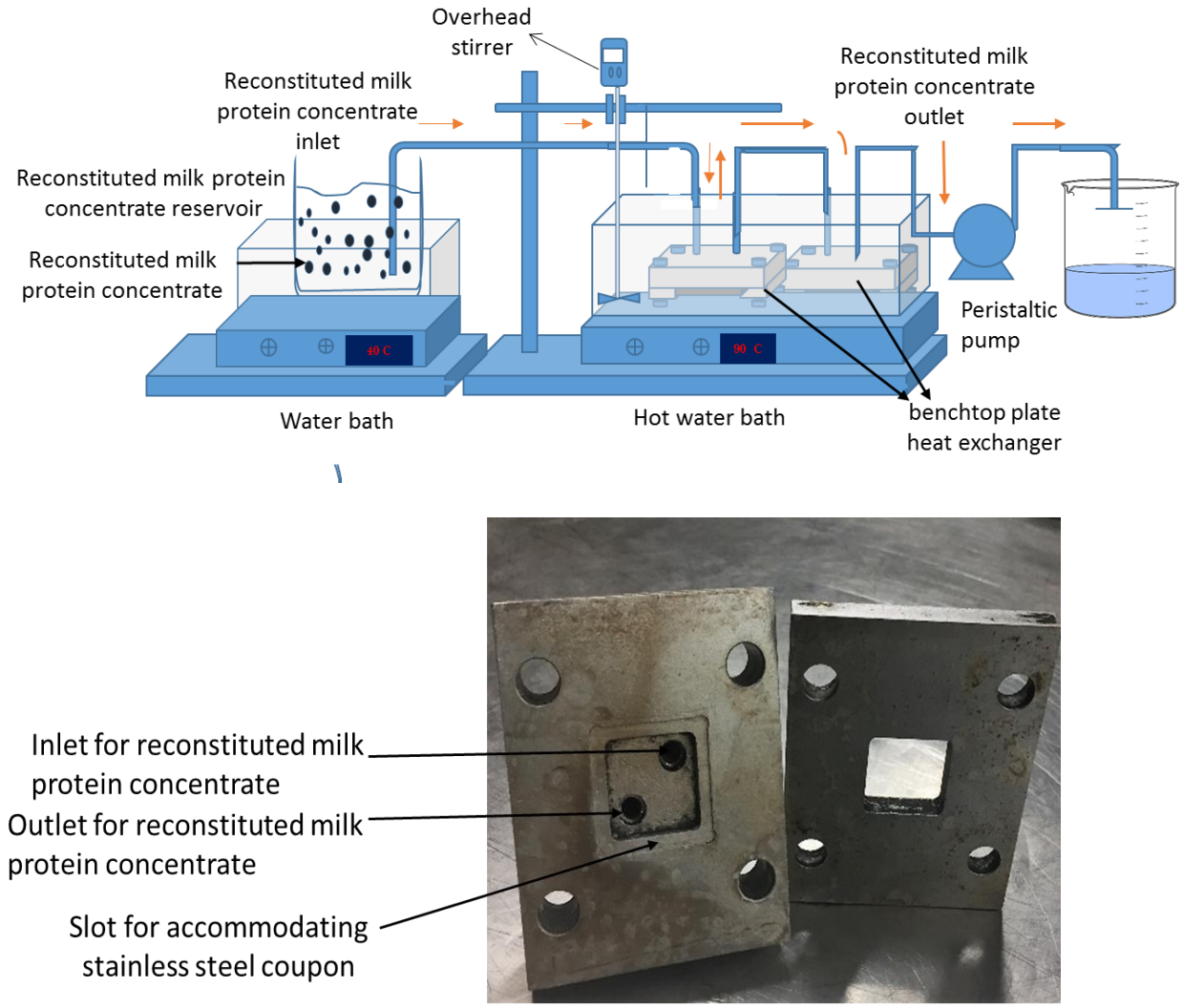


Figure 4.1 (A) Schematic for the benchtop plate heat exchanger for simulating the processing conditions of plate heat exchanger; (B) photo of the benchtop plate heat exchanger unit.

Measurement of foulant mass

Aqueous solutions of MPC80 (120 g of MPC powder in 1080 g distilled water) were prepared by stirring the MPC80 powder in distilled water (at 40 °C) with a 4-bladed overhead stirrer (Caframo, Georgian Bluffs, Ontario, Canada) at the 2500 rpm for 5 min to ensure complete dispersion of the powder. After reconstituting, each sample flowed immediately, without any rehydration time, through the benchtop PHE for 2 h at 22 mL/min flow rate. The

inlet temperature of reconstituted MPC80 was maintained at 40 °C in water bath 1 and the hot water temperature was maintained at 88 ± 2 °C in water bath 2, as shown in Figure 4.1. A 4-bladed overhead stirrer was used for maintaining uniform hot water temperature as 88–90 °C so as to mimic the hot water moving through the benchtop PHE assembly. Initially, sufficient reconstituted solution was prepared for a 1 h run, and then the solution was prepared similarly for the next 1 h run. After each 2 h run, the benchtop PHE was dissembled and weights of the coupons were taken after air drying for 15–20 min. The reconstituted MPC80 was not recirculated. Foulant weights were recorded using an analytical balance (Mettler Toledo, Columbus, OH, USA) with a measuring accuracy of 0.001 g and reported as an average of milligram of foulant per square cm of the SS 316 L coupon (mg/cm^2). Fouled coupons were stored at room temperature and a constant relative humidity (75%) for further microscopic analysis to study the composition and microstructure of fouling.

Chemical analysis of the fouled layer

Gel-electrophoresis of the fouled layer

The relative levels of various proteins, i.e. caseins and whey proteins present in each sediment layer were determined using gel-electrophoresis using the procedure described by Lalande and Tissier (1985). The foulant deposit (~ 20 mg) after scraping off the SS coupon was treated with 2 mL nitric acid (0.2 mol/L), washed with distilled water, and treated further with 3 mL fouling buffer containing 0.2 mol/L tris, 0.1 mol/L EDTA, 8 mol/L urea, and 0.03 mol/L SDS. The solution was sonicated for 30 min at 40 °C. The sonicated mixture was mixed with 2 x Laemmli loading buffer (BioRad Laboratories, Richmond, CA, USA) in 1:1 ratio and a reducing agent 2-mercaptoethanol (0.1 mol/L). The final mixture was heated at 95 °C for 5 min. Of the final prepared mixture, 10 µL was loaded on 10% precasted Tris-HCl criterion gels (BioRad

Laboratories, Richmond, CA, USA) and run at 100 V and 0.5 mA. After electrophoresis, the gels were stained in Biosafe Coomassie Blue (BioRad Laboratories, Richmond, CA, USA) and destained in the double distilled water. After destaining, the gels were scanned using a BioRad Gel Dox XR + Imager (BioRad Laboratories, Richmond, CA, USA) and the integrated intensities and the concentration of the protein bands were computed densitometrically.

Calcium measurement

The ionic calcium of the well-and poor-soluble MPC80 powder was determined using Accumet Polymer Membrane Combination Ion-Selective Electrode (Fisher Scientific, Pittsburgh, PA, USA); whereas calcium extracted from the fouled layer was analyzed using the Atomic Absorption Spectrophotometer (Perkin Elmer AAnalyst 100, Waltham, MA, USA). The fouled layer was scraped off from a SS coupons and the weight was noted. The weighed foulant was treated with 2 mL nitric acid (0.2 mol/L) (Lalande & Tissier, 1985) and ultra-sonicated (Branson 1510 Ultrasonic Cleaner, Henrico, VA, USA) for 30 min at 40 °C (Jeurnink, 1995). The sonicated sample was collected and analyzed for calcium extracted from the foulant deposits.

Characterization of fouling microstructure

Scanning electron microscopy (SEM)

SEM analysis was carried out with a Versa 3D Dual Beam (FEI Company, Hillsboro, OR, USA) using a concentric backscattered detector at 1 kV acceleration voltage for imaging. The fouled SS coupons were mounted on the carbon tape and analyzed using a concentric backscattered detector. A portion of the sample was targeted and visualized for surface microstructure of the fouling by imaging at 0 and 45° tilt. The average thickness of the fouling layer at the edge of a coupon was estimated with the help of ImageJ 1.49v (National Institute of Health, Bethesda, USA) using SEM micrographs of SS coupons tilted at 45°. Within each

sample, 3 different areas were targeted and the thickness was measured using ImageJ software. The number of craters and gas holes observed in the SEM micrographs were calculated for foulants observed by well- and poor-soluble MPC80 powder using ImageJ software by targeting a fixed area.

Confocal laser scanning electron microscopy (CLSM)

CLSM is a fluorescence based optical microscopic technique with discreet z-resolution allowing optical z-sectioning of thick samples such as fouled layers. Optical z-series were generated through full thicknesses of fouled layers. These z-series were used to generate 3-D projections of fouled layers which provided information about the depths of the fouled layer. These 3-D projections were helpful in understanding the distribution of different components such as protein, lipids, and calcium present in these fouled layers.

For CLSM analysis, fouled coupons were stained with fluorescent probes before visualizing. Calcium was stained with Calcium Green™-1 stain (Molecular probes, Thermo Fisher Scientific, Waltham, MA, USA) at the concentration of 4.4 μM for 15 min. Protein and lipids were stained using Fast green FCF (Sigma-Aldrich, St. Louis, MO, USA) and Nile red (Molecular probes, Thermo Fisher Scientific, Waltham, MA, USA) stains, respectively. Stock solutions of Fast green (5 mg dye in 5 mL water) and Nile red (100 mg dye in 500 mL acetone) were mixed in a ratio of 1:3 and 10 μL of the mixed solution was applied to the sample for 5–10 min. The stained samples were analyzed in LSM 5 Pa (Zeiss, Thornwood, NY, USA). The objective used was Plan Neofluar 10×/0.3. Multitracking was used to minimize possible bleed through and/or cross talk of the 3 fluorescence stains. Track 1 used the 488 nm Argon laser line to excite Calcium green and a 633 nm Helium-Neon laser line to excite Nile red. A primary dichroic-HFT/488/543/633 was used to excite Calcium green and Nile red in foulant, while a

secondary dichroic – NFT 635 was used to separate the emission signals of these 2 fluorescent stains. Emission signals were collected using a Band Pass (BP) 505–530 nm filter prior to Channel 2 photomultiplier tube (PMT) for detecting Calcium green (blue), while a Long Pass (LP) 650 nm filter prior to Channel 1 PMT for detecting Nile red (red). Track 2 used a 543 nm Helium-Neon laser line and a primary dichroic HFT 543 to excite Fast green FCF. The emission signal was collected with Channel 2 using a secondary dichroic NFT 635 and a BP 560–615 nm filter to detect Fast Green (green). z-series were collected through full thicknesses of foulants and used to generate 3-D projections which helped in understanding the distribution of protein, lipids and calcium in the fouled layers.

Energy dispersive spectroscopy (EDS)

EDS was used to analyze the elemental composition of select surfaces of foulant. The x-rays were emitted by atoms in the fouling layer and the elemental characteristic energy changes were recorded with an Oxford X-max 20 mm2 EDS detector. Elemental dot maps were generated and pseudo-colored maps of elements of interest (Ca, N, and O) were collected.

The EDS analysis was performed in the Versa 3D Dual Beam (FEI Company, Hillsboro, OR, USA) at an accelerating voltage of 10 kV and SEM secondary electron signals collected using an Everhart-Thornley detector (ETD). The coupons were loaded in the similar fashion as for SEM imaging, and the data was collected at 3 different areas using Oxford Instruments (Aztec Version 2.1 software). In conjunction with the atomic percentage and the weight percentage of the different elements, EDS mapping of the elements was also done.

Statistical analysis

Changes in the solubility index and foulant material with change in the storage temperature of the powders and the effect of storage temperature of the powders on the calcium

concentration extracted in the fouled layer were statistically analyzed using the PROC GLMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA).

Results and discussion

As per the certificate of analysis provided by the manufacturer, the general composition of the MPC80 on a dry weight basis was 80.8 g protein, 6.3 g lactose, 1.9 g fat, and 6.6 g ash per 100 g. In general, MPC powders are described as powders with similar proportion of proteins (caseins/whey) as in fluid milk, a low solubility index, and poor cold water solubility (Havea, 2006). The solubility and dissolution behavior of high-protein dairy powders is greatly influenced by storage temperature affecting the functional characteristics such as water binding, viscosity, emulsification, foaming and whipping, gelation, heat stability, and color/flavor development of the finished product. A significant decrease in solubility of the high-protein powders on storing at high temperatures ($P < 0.05$) further affecting the fouling behavior of the powders, and their microstructure study was investigated in the present study.

Solubility and dissolution characteristics

The solubility index of the well- and poor-soluble MPC80 powders was 93± 1% and 84 ± 2%, respectively, as shown in Table 4.1. As expected, the MPC80 stored at low temperature (25 °C, well-soluble) showed higher solubility as compared to powder stored at 40 °C (poor-soluble). Gazi and Huppertz (2015) reported that the solubility of the MPC powders is best immediately after production and it decreases as the storage time and temperatures increases. Kher, Udabage, McKinnon, McNaughton, and Augustin (2007) reported that the conformational modifications of the protein molecules during processing and storage are responsible for the loss of solubility. Storing the MPC powders at higher temperatures resulted in crosslinking networks with the neighboring proteins at the surface of the powder. These networks include interactions between

hydrophobic casein molecules and some minor whey proteins, which act as a barrier to water transport and consequently inhibit the hydration of the MPC85 particles (Anema et al., 2006; Uluko, Liu, Lv, & Zhang, 2016). Also, the reduction in the MPC solubility upon storage was attributed to the reduction in the solubility of the caseins. It has been reported by Fyfe et al. (2011) that a decrease in the solubility of MPC powders upon storage is caused in part by the thin layer of fused casein micelles on the stored powder surface. As per the study conducted by Havea (2006), with an increase in the storage temperature, the insoluble portion of MPC consisted mainly of casein and minor whey proteins, whereas the soluble portion constituted of major whey proteins are (β-LG and α-LA). However, Mimouni, Deeth, Whittaker, Gidley, and Bhandari (2010) disputed that storage-induced decrease in solubility was due to the insoluble material formed during storage, but rather was due to the changes in the rehydration kinetics. The dissolution characteristics of the powders were studied using focused beam reflectance measurement. The dissolution behavior of the powders was characterized by the changes in the particle counts during the dissolution of a MPC80 sample. Figure 4.2 shows the changes in the counts for fine (<10 μm) and large (150–300 μm) particles for both well- and poor-soluble MPC80. It was observed that fine particle counts (Figure 4.2 (A)) for both well- and poor-soluble MPC80 were similar for first 100 s, but then the rate of increase in fine counts was higher for well-soluble MPC80 as compared to poor-soluble MPC80. On the other hand, large particle counts (Figure 4.2 (B)) increased for first 300 s for both well- and poor-soluble MPC80. The increase in the large particle counts can be attributed to the initial aggregation of the powder particles. However, subsequently the large particle counts started to decrease more rapidly for well-soluble MPC80 than poor-soluble MPC80, which indicated the slow disintegration of larger particles into smaller for MPC80 stored at 40 °C when compared to the powder stored at 25 °C.

The slow dispersion rate of particles for MPC80 stored at 40 °C indicated a reduction in the solubility and the effect of storage temperature on the changes in the solubility index was observed to be significant ($P < 0.05$). Similar results were obtained in a study by Hauser and Amamcharla (2016b), where reduction in fine particle counts was observed for the MPC stored at higher temperature. The decrease in the dissolution rate with the increase in storage temperature and time was also reported by Fang, Selomulya, Ainsworth, Palmer, and Chen (2011). Thus, storing the powders at different temperatures helped us to achieve powders with different solubility characteristics to further study their effect on fouling characteristics.

Table 4.1 Average solubility (%), foulant mass (mg/cm^2), and calcium (%) in the fouled layer observed by milk protein concentrate (MPC80) powder stored at different temperatures.

Temperature (°C)	**Solubility*(%)**	**Foulant mass*(mg/cm^2)**	**Calcium*(g per 100 g) in fouled layer**
25	93 ± 1^a	14 ± 3^a	0.7 ± 0.4^a
40	84 ± 2^b	26 ± 5^b	0.5 ± 0.2^a

*Means of the 3 replicates, means with the same letter within a column are not significantly different ($P<0.05$)

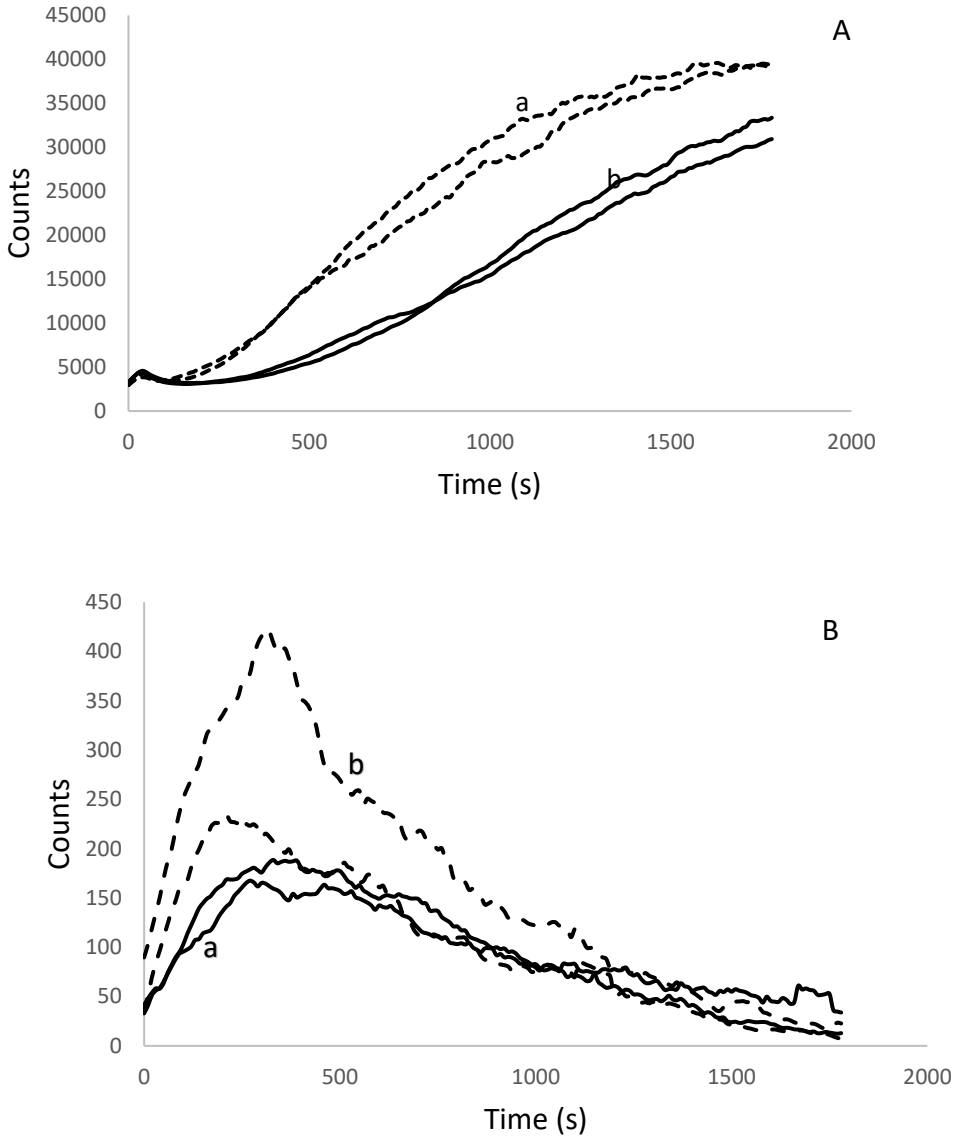


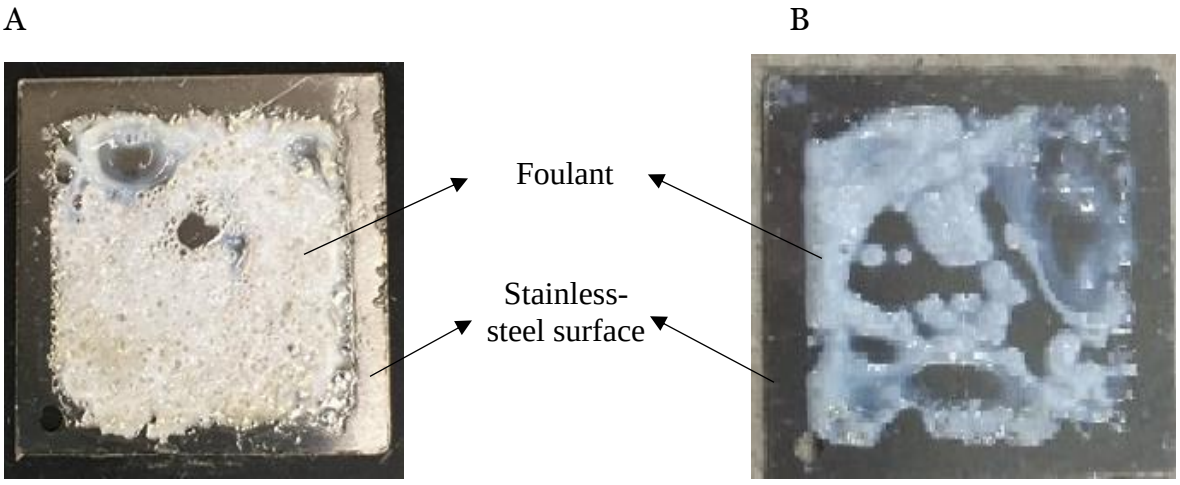
Figure 4.2 Changes in fine (<10 μm) (A), and large (150–300 μm) (B) counts obtained from data collected with the focused beam reflectance measurement for powders: (a) well-soluble milk protein concentrate (MPC80); (b) poor-soluble milk protein concentrate (MPC80) with a dissolution temperature of 40 °C.

Foulant mass

The photographs of fouling deposits obtained for poor- and well-soluble powder are shown in Figure 4.3 A and B, respectively. On general inspection of the fouled SS coupons, it was observed that the deposit (foulant) formed by the poor-soluble powder was hard and compact, but the deposit formed by well-soluble powder was soft and spongy. The average hot

water, inlet, and outlet temperature of the reconstituted MPC80 during the experimental run was recorded as 90 °C, 40 °C, and 71.9 °C, respectively. However, the outlet temperature of the reconstituted MPC80 solution dropped to 69 °C towards the last 15 min of completion of the experiment and was difficult to maintain at 71.9 °C, especially for the poor-soluble powder. The amount of fouling material accumulated over the SS coupons during the thermal processing of reconstituted MPC80 powder stored at different temperatures is reported in Table 4.1. The average foulant mass observed for the poor-soluble and well-soluble MPC 80 powder was 26 ± 5 mg/cm^2 and 14 ± 3 mg/cm^2, respectively. The increase in storage temperature of powders from 25 °C to 40 °C significantly increased the amount of fouling on SS coupons ($P < 0.05$). The increase in the foulant observed for powders stored at high temperature could be attributed to a decrease in the solubility of the powder with the increase in storage time. In a similar study conducted by Huang and Goddard (2015), it was reported that average fouling material accumulated over the SS coupons for the thermal processing of raw whole milk was 6.80 ± 0.48 mg/cm^2. Jeurnink (1995) reported that amount of foulant generated by reconstituted skim milk was much less than fresh skim milk. The decrease in foulant generated by reconstituted skim milk was attributed to the partial denaturation of whey proteins during manufacture (evaporation and drying) of skim milk powders, and already denatured and aggregated proteins were less active in fouling reaction. On the other hand, high amount of foulant observed for MPC powders stored at different temperatures compared to raw whole milk can be explained by high amount of protein in MPCs. More protein-protein interactions are expected to take place in MPC than in raw milk (Singh, 2007) which might have resulted in high degree of fouling deposits. Hence, foulants generated from the powder with different solubilities were further studied for their composition and microstructure.

Figure 4.3 Representative Photographs of (2.54 cm × 2.54 cm) fouled stainless-steel coupon by milk protein cocncentartae (MPC80) powder stored at different temperature: (A) 40 °C; (B) 25 °C.



Chemical analysis of the fouled layer

Gel-electrophoresis profile of the foulant

The gel-electrophoresis profile of the proteins extracted from the fouled layers of the well- and poor-soluble powder with densitometric analysis is shown in Figure 4.4. Since the amount of foulant generated was not sufficient enough to measure its protein content, the relative intensities of different proteins were not compared between the foulants generated by well- and poor-soluble powder. Rather, relative intensities of different proteins per g of the foulant were compared within the same powder sample. The lane 1 and lane 2 represents the different polypeptides identified in the foulant produced by well-soluble powder and poor-soluble powder, respectively. Four major bands were detected in the fouled layers produced by the powders stored at different temperatures. For the well-soluble powder, on comparing the relative intensities, the relative percentage of intensities for casein and major whey proteins in the foulant were observed as 81% and 19%, respectively. On the other hand, for the foulant generated by poor-soluble powder, the relative percentage of intensities for casein and major whey proteins in the foulant were observed as 62% and 38%, respectively. It can be observed in the foulants by

both well- and poor-soluble powder that the dominant protein present was casein. The ratio of serum protein to casein in the deposit formed from the pilot-plant-scale plate heat exchanger using skim milk was reported in a study by Jeurnink (1995) as 3.0; which was in contrast to the ratio observed in the present study for MPC80. The large contribution of caseins than whey proteins in the deposit formed from both well- and poor-soluble MPC80 powder could be explained by the adsorption of casein in the foams at the air-liquid interface due to a low Reynolds number. On the other hand, in the pilot-plant-scale PHE, due to a high Reynolds number, the air bubbles formed during heating were small due to high operational pressures and entrained with the flow from SS coupons.

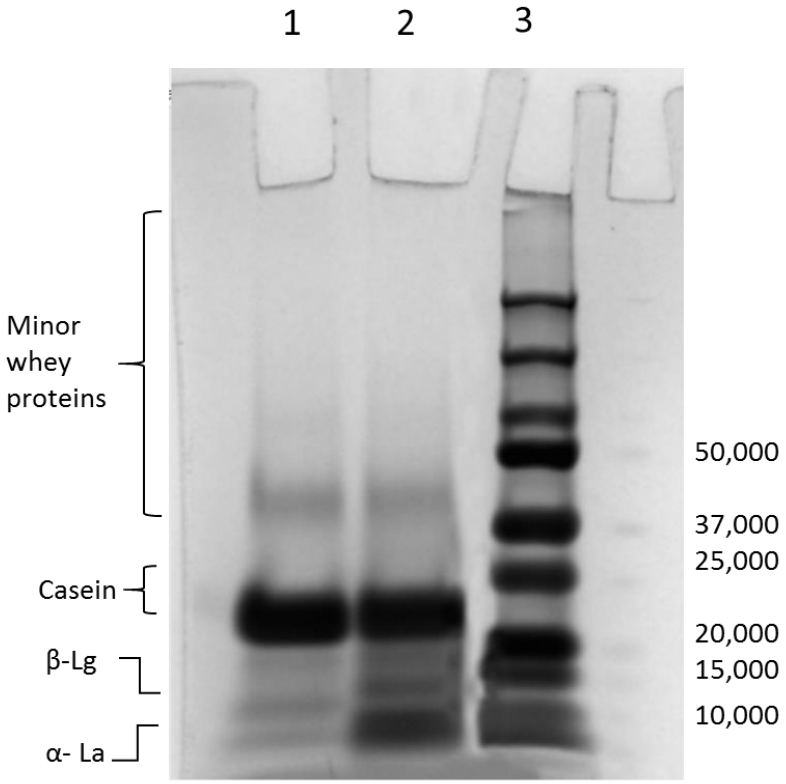


Figure 4.4 Gel-Electrophoresis patterns of different protein bands for: (1) foulant generated by milk protein concentrate (MPC80) stored at 25 °C; (2) foulant generated by milk protein concentrate (MPC80) stored at 40 °C; (3) protein standard.

Calcium measurement

The ionic calcium activities of the well- and poor-soluble dispersions were observed as 3.2 mmol/L and 3.4 mmol/L, respectively. It is to be noted that the calcium ion electrode measures the total calcium ion activity, not the calcium ion concentrations in a given solution. Crowley et al. (2014) reported that the total calcium ion activity increased with protein content in reconstituted MPC and calcium ion activity of 1.14 mmol/L for MPC80 reconstituted to 3.5 g protein per 100 g solution at pH 6.79. Also, Crowley et al. (2014) concluded that the heat stability of MPC suspensions was adversely affected by the increased calcium ion activity. On the other hand, addition of lactose to reconstituted MPC80 did not significantly affect the heat stability. In the present study, a higher calcium ion activity led to lower heat stability and consequently, caused an increased fouling in the poor-soluble dispersion. Whereas, the average percentage of the calcium content extracted from the foulant of well- and poor-soluble powder was $0.7 \pm 0.4\%$ and $0.5 \pm 0.2\%$, respectively. There was no significant difference observed among the means of the calcium content extracted (P-value > 0.05). The role of calcium concentration has been well studied in relation to heat stability and fouling. It has been reported by Jeurnink and Dekruif (1995) that the change in the calcium concentration in the milk affected the mineral content in the deposit and also led to an increase in the deposition of proteins. Deposition of calcium was linked with protein deposition, where micellar casein and serum proteins bind calcium on aggregation. In the milk, 66% of the Ca is present in the colloidal form and with the increase in the temperature, the solubility of the colloidal calcium phosphate decreases (Fox & McSweeney, 1998).

Microstructures of high-protein dairy fouling

Representative scanning electron micrographs display the surface microstructure of the foulants from well-soluble (Figure 4.5 A and B) and poor-soluble (Figures 4.6 A, B, 4.7 A, and 4.7 B) MPC80. The tomography of fouling surfaces was characterized by changes in surface height similar to the topography of rolling hills, with small circulate depressions (termed "craters") within the fouled layer thickness as shown in Figures. 4.5 A, 4.6 A and 4.6 B. In addition, micrographs revealed that the craters were more numerous in the fouled layer generated by poor-soluble MPC80 compared to well-soluble MPC80. Using ImageJ software, the number of craters within an area of 0.02 cm^2 of SEM micrographs for the fouled layer produced by well- and poor-soluble powders were measured and were found to be 6 and 10, respectively. Similarly, gas holes within the fouled layer were also observed (Figures 4.5 A, 4.6 A and 4 6 B). These holes were more numerous in poor-soluble MPC80 fouled layer as compared to well-soluble MPC80 fouled layer. The gas holes were also counted as craters and were found to be 2 and 5 for the foulants generated by well- and poor-soluble MPC80 powder, respectively. The more gas holes observed in the fouled layer by poor-soluble MPC80 can be correlated to the reduced solubility and poor dissolution characteristics of the poor-soluble MPC80 powder. In a study by Mistry and Hassan (1991), smooth surface and dents observed in the SEM analysis of the high-protein milk powders were suggested to act as a barrier to water entry during resolution. Furthermore, high cross-linkages are observed on the protein surfaces at higher storage temperatures, which might have generated more gas holes in the fouling film by poor-soluble MPC80 than fouling film by well-soluble MPC80 by restraining the movement of water within the core of the powder particles. The thickness of the fouled layers for well- and poor-soluble MPC80 powder was observed using SEM by tilting the SS coupons at 45° (Figure 4.7). Also, the

topography of the fouled layer generated by both well- and poor-soluble MPC80 was observed to be non-uniform and smooth (Figures.4.5 A, B, 4.6 A and 4.6B). The average thickness of the fouled layer for well- and poor-soluble MPC80 was calculated to be 170 ± 73 μm and 262 ± 32 μm, respectively. The average thickness was found to be not significant ($P > 0.05$). But, the average thicknesses observed in this study for fouled layers of well- and poor-soluble MPC80 powder were within ranges (100–300 μm) reported for UHT processing of the skim milk at high temperature (Hagsten et al., 2016)

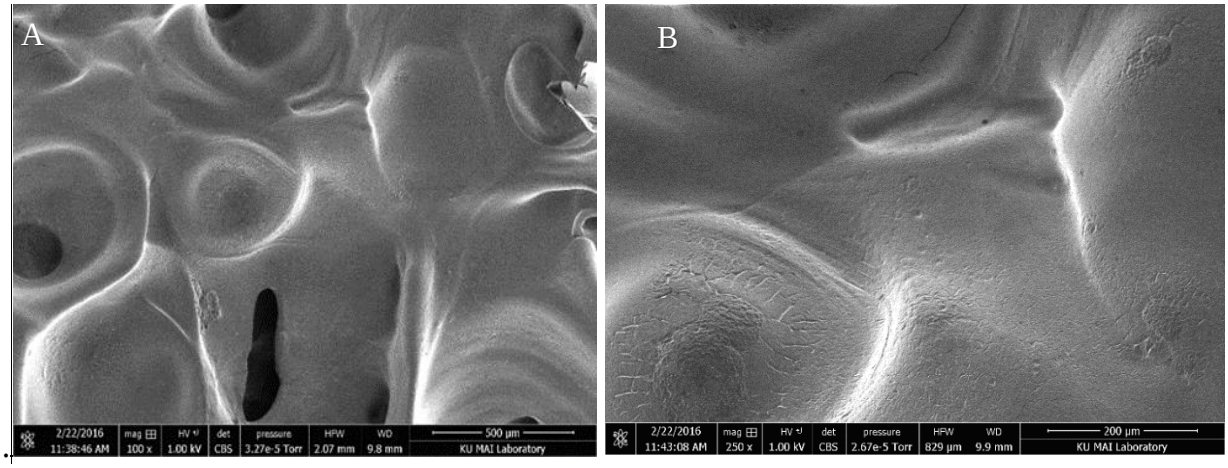


Figure 4.5 Representative Scanning Electron Microscopy micrographs of stainless-steel coupons showing the surface microstructure of the fouled layer for well-soluble milk protein concentrate (MPC80): (A) lower magnification; (B) higher magnification**.**

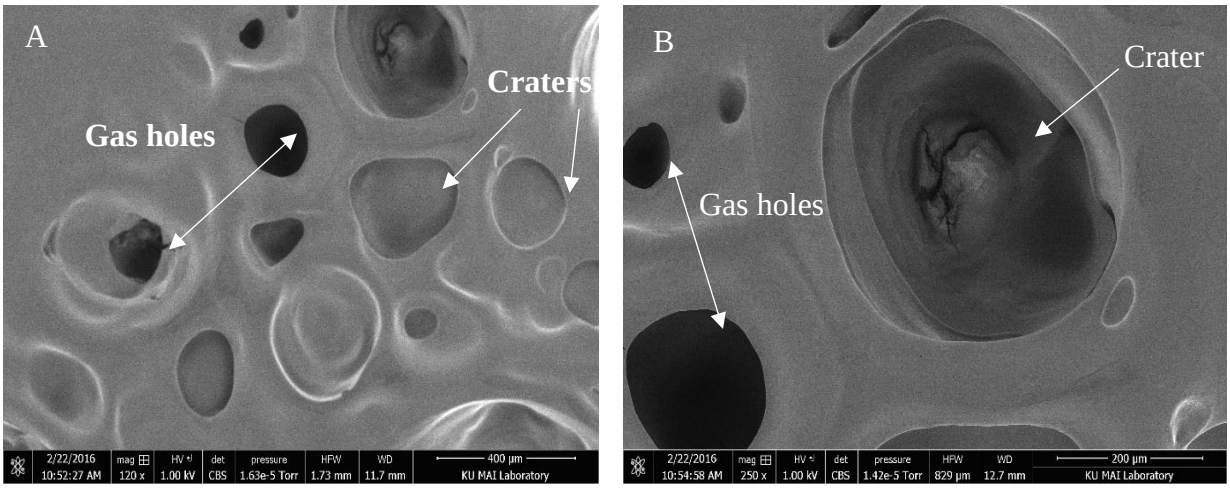


Figure 4.6 Representative Scanning Electron Microscopy micrographs of stainless-steel coupons showing the surface microstructure of the fouled layer for poor-soluble milk protein concentrate MPC80: (A) lower magnification; (B) higher magnification.

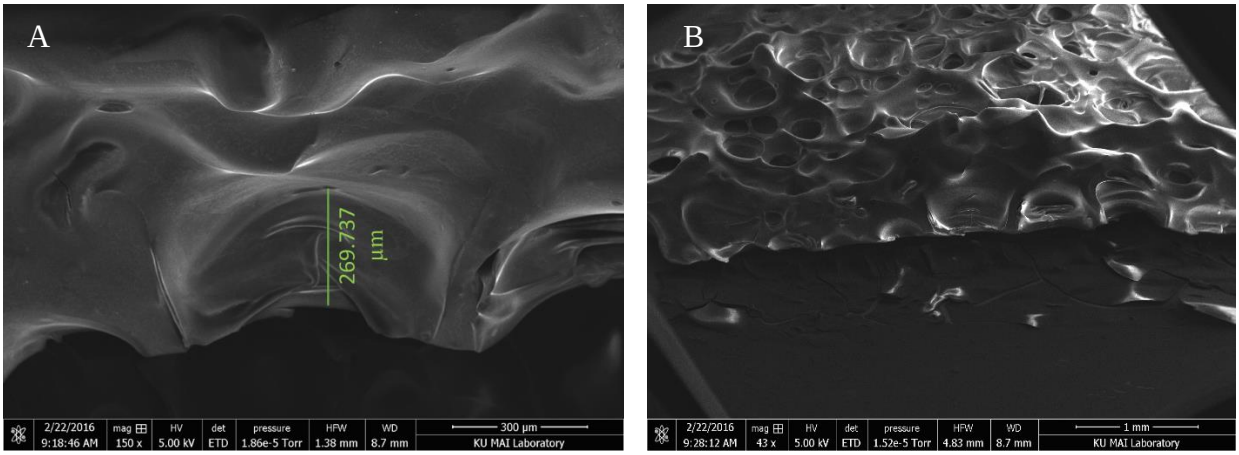
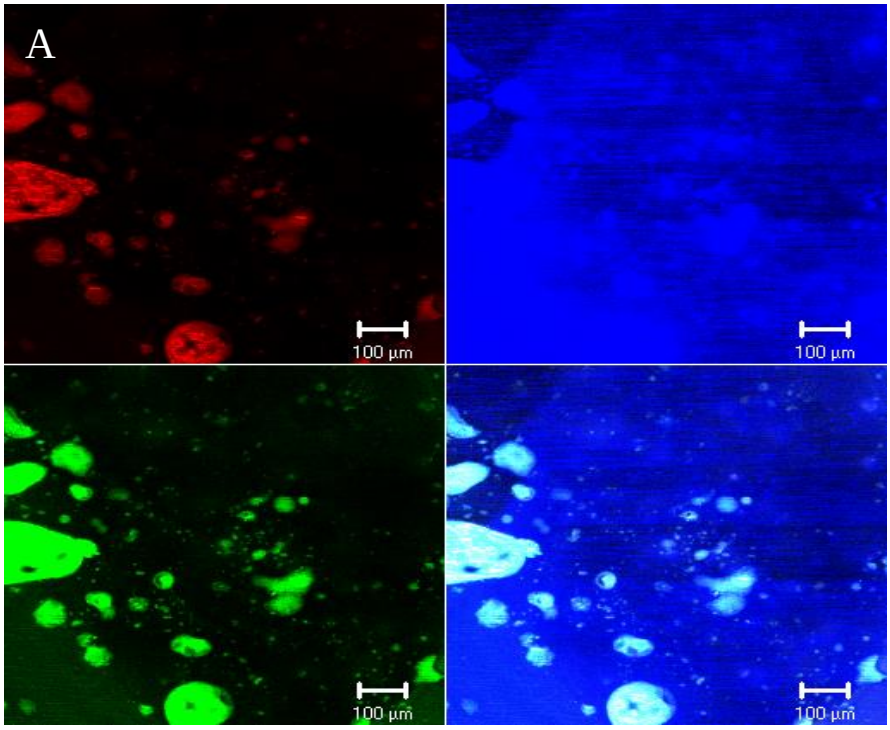
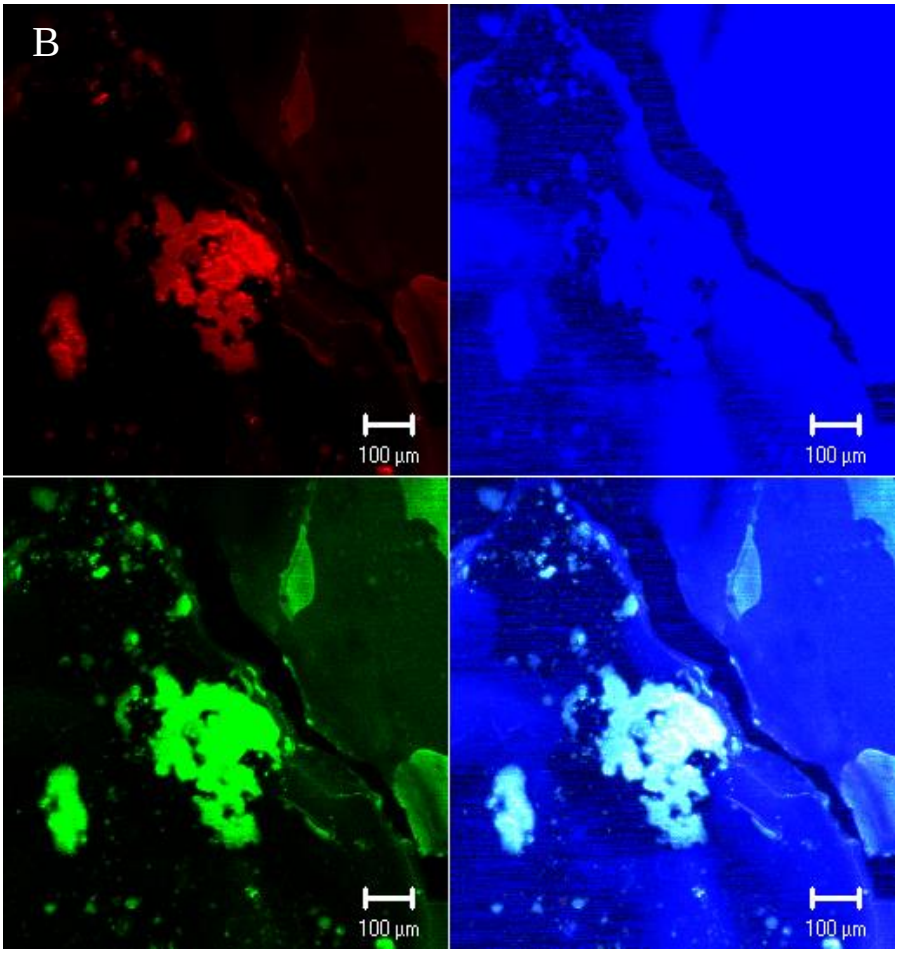


Figure 4.7 Representative Scanning Electron Microscopy micrographs of stainless-steel coupons tilted 45° showing the thickness of the fouled layer for poor-soluble milk protein concentrate (MPC80): (A) At high magnification; (B) At low magnification**.**

CLSM 3-D projections of a z-series observed through the microstructure of the fouled layer are shown in Figure.4.8 A, B, and 4.8 C. The lipids were stained in red, calcium in blue, and protein in green. It can be visualized from the images that foulant was present as cluster assemblies for well-soluble MPC80, whereas crater-like structures were seen in foulants for poor-soluble MPC80. On the same SS coupon, 2 different areas with relatively higher and lower foulants were targeted and the CLSM images were taken (Figure.4.8 A and B). Optical sectioning through the fouled layers of the 2 areas revealed the uniform calcium distribution, with discrete lipids and proteins distribution. Craters like structures observed for the foulants for the well- and poor-soluble MPC80 with larger holes for foulants for poor-soluble MPC80 observed by CLSM analysis correlates to the similar structure seen by SEM. The information about distribution of lipids, calcium, and proteins was provided by CLSM, whereas tomographic and spatial distribution of elements was provided by SEM and EDS.


A
100 µm
100 µm
100 µm
100 µm


B
100 µm
100 µm
100 µm
100 µm

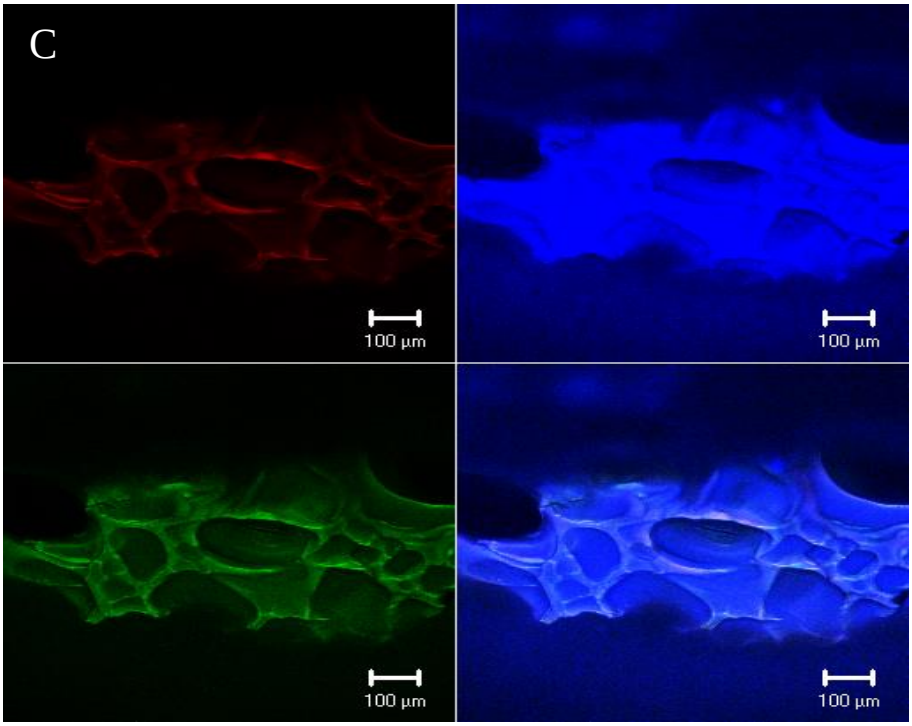


Figure 4.8 Representative Confocal Laser Scanning Microscopy micrographs of stainless-steel coupons showing the 3-D projection of a z-series through the microstructure of the fouled layer: (A) well-soluble milk protein concentrate (MPC80) targeted at area with less foulant; (B) well-soluble milk protein concentrate (MPC80) targeted at area with more foulant; (C) poor-soluble milk protein concentrate (MPC80). The lipids are stained in red, calcium in blue, and protein in green. The image is composed of several confocal images in the z direction and compiled to one image.

For well-soluble MPC80, a z-series consisting of 0.9 mm^2 with 9 optical slices taken at an interval of 6.9 µm showed the mean thickness/depth as 54.9 µm. For the 3 replicates, mean pixel intensity for lipids, calcium, and proteins of a projection (Figure.4.8 A) was observed to be 14 ± 5, 193 ± 26 and 34 ± 8, respectively. On the other hand, for poor-soluble MPC80, a z-series consisting of 0.9 mm2 with 12 optical slices taken at an interval of 6.9 µm showed the mean thickness/depth as 75.5 µm. Mean pixel intensity for lipids, calcium and proteins of a projection (Figure.4.8 B) was observed for 3 replicates to be 27 ± 23, 186 ± 60, and 38 ± 34, respectively. These pixel intensities indicated relatively higher concentrations of lipids and proteins and lower calcium concentrations in poor-soluble MPC80 as compared to well-soluble MPC80.

Fluorescence/pixel intensities of the 3 dyes used to indicate lipids, calcium, and proteins are not a direct measure of concentrations of those 3 components in samples; however, they can be used to indicate relative amount of these 3 components between samples. The CLSM images also showed that the fouled layer was non-uniform throughout the coupons and that calcium was distributed all over, which can be seen in Figure.4.8 A, B and 4.8C. The lipids were discretely and non-uniformly distributed. The structures observed for the MPC80 fouled layers were non-uniform and smooth; whereas rough structures were observed for the fouled layers generated by UHT processing of skim milk in the study conducted by Hagsten et al. (2016). The reason can be attributed to the presence of high mineral composition, mainly calcium phosphate, which imparted crystallinity to the foulant generated during UHT processing.

In general, the relative high intensities were observed for the calcium as compared to proteins and lipids. The reason might be attributed to the use of Calcium Green™-1 over Calcium Green™-2 because of its higher affinity for calcium, making it particularly suited to measuring relatively low levels or baseline levels of calcium in foulant.

Both SEM and CLSM images have shown that fouled layers formed by poor-soluble MPC80 was thicker than fouled layers by well-soluble MPC80. Also, similar kinds of structures were observed by both analytical techniques which confirms the uniform distribution of Ca and non-uniform foulant thickness.

EDS analysis (Figure.4.9) revealed the spatial distribution of the elements present in the fouled layer. The atomic percent of the different elements in the fouled layers of well- and poor-soluble MPC80 (Table 4.2) showed that C, N, O and Ca were the main elements constituting the fouling. The percentage of C was the most, followed by O, N and Ca. Also, a small percentage of S and P were reported. The presence of C and O supports the presence of lipids, whereas the C

and N presence supports the presence of proteins in the fouled layer found by CLSM. The presence of a small amount of S might indicates the presence of S-containing amino acids. Also, the mapping of the elements showed that an indicator of potential protein, N, was present close to the substrate, whereas Ca was distributed all over the fouled layer.

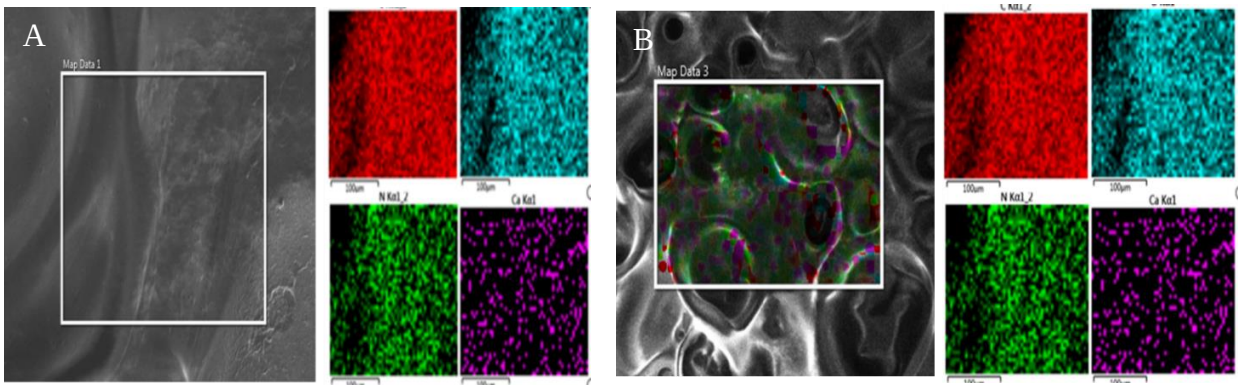


Figure 4.9 Representative Energy Dispersive Spectroscopy images of the microstructure showing the dot mapping of the elements distributed in the foulant and its energy spectrum: (A) well-soluble MPC80; (B) poor-soluble MPC80.

Table 4.2 Average weight (%) of the different elements observed by Energy Dispersive Spectroscopy analysis for the fouled layer on the SS coupons for well- and poor-soluble milk protein concentrate (MPC80) powder. (n = 2).

Elements	Well-soluble* powder	Poor-soluble* powder
	Wt%	Wt%
C	56.8 ± 0.8	54.3 ± 4.8
N	13.1 ± 0.01	17.7 ± 1.1
O	27.8 ± 0.5	27.6 ± 3.3
P	0.6 ± 0.4	-
S	0.3 ± 0.2	-
Ca	1.5 ± 0.2	0.5 ± 0.3

*Means of the 2 replicates

Thus, microscopic analysis of the fouled layers formed by well-and poor-soluble MPC80 powder helped in better explaining the structure with the spatial distribution of the various components and elements within the foulant.

Conclusions

The well-soluble MPC80 powder was found to be more soluble with better dissolution characteristics than poor-soluble MPC80 powder. The poor solubility resulted in high amounts of fouling. The foulant generated by poor-soluble powder was ~ 84% more than well-soluble powder. Both SEM and CLSM images have revealed the heterogeneity of the fouled layer with non-uniform thickness, discrete lipids and proteins distribution, and uniform calcium distribution. Thus, the study will be helpful in understanding the structure of fouling layer and in optimizing processing conditions and in designing effective cleaning strategies based on product composition. For the cleaning protocol to be effective, it should penetrate into the fouled layer, and for that the nature of the foulant (proteinaceous or mineral-rich) needs proper understanding.

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Chapter 5 - Efficacy of micro- and nano-bubble aqueous ozone (MNAO) in the removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from stainless-steel surfaces

Abstract

The study aims to evaluate the effectiveness of micro- and nano-bubble aqueous ozone (MNAO) in the removal of the *Bacillus cereus* and *Bacillus licheniformis* biofilms from the stainless-steel (SS) surfaces. The MNAO was generated using the MNAO generator supplied by Clean Core Technologies (Omaha, NE). *Bacillus cereus* and *Bacillus licheniformis* biofilms developed on SS coupons were treated with de-ionized water, cleaning-in-place (CIP), MNAO, and combined CIP+MNAO. For the *Bacillus cereus* biofilm removal, a log reduction of only 0.68 cfu/cm^2 was observed after the de-ionized water wash. Whereas, both MNAO and CIP treatments significantly reduced (P>0.05) the bacterial counts by 2.43 and 2.88 $\log_{10}$ cfu/cm^2, respectively. After the combined MNAO and CIP treatment, the *Bacillus cereus* counts were reduced significantly (P>0.05) by 2.88 $\log_{10}$ cfu/cm^2. On the other hand, for the *Bacillus licheniformis* biofilm removal from SS surfaces, a significant log reduction observed was 1.45, 3.03, 2.92, 4.27 $\log_{10}$ cfu/cm^2, respectively after de-ionzed water, MNAO, CIP, and combined CIP+MNAO treatment. Thus, it was observed that MNAO and combined CIP+MNAO has a great potential in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from the SS surface, and can be used in the dairy industry as an effective sanitizer/disinfectant. The microscopic structures for the SS coupons for the biofilm for both *Bacillus cereus* and *Bacillus licheniformis*, before and after the cleaning treatments were observed under scanning electron microscopy (SEM).

Keywords: Biofilm, micro-nano aqueous ozone, stainless-steel

Introduction

The dairy industry needs an efficient biofilm control strategy to meet legal as well as self-imposed safety standards. Biofilm formation in the dairy industry constitutes serious economic consequences, product quality, and safety concerns. A major concern with the finished product contamination is the possibility of presence of pathogens. The bacteria commonly associated with biofilms found in the dairy environment include *Pseudomonas*, *Enterococcus*, *Lactobacillus*, *Enterobacter*, *Listeria*, *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Escherichia*, *Corneybacteria*, and *Bacillus* (Anand and Singh, 2013; Salo et al., 2006; Sharma and Anand, 2002; Waak et al., 2002). Of the various spoilage and pathogenic bacteria associated with dairy biofilms, some of the bacteria are capable of attaching to the surfaces, while others are found to be housed within the biofilm matrix or environment. It has been reported in a study that 25% of the *Bacillus* spp. have been reported in the dairy environment constituting the biofilm. Also, *Bacillus cereus,* pathogenic microorganism, has been reported to be to account for more than 12% of the biofilms constitutive microflora. (Sharma and Anand, 2002). Due to its ubiquitous nature and resistance to stressors like heat and dehydration, *Bacillus cereus* has been implicated in many foodborne illnesses. *Bacillus cereus* is one of the major aerobic spore-formers incriminated in a large number of food poisoning outbreaks from milk and products. According to a report by the World Health Organization *B. cereus* is responsible for approximately 5-10 out of 100 foodborne diseases (McGuiggan et al., 2002). In a study by Rückert et al., (2004), it has been reported that three species of bacilli, namely *B. licheniformis, G. stearothermophilus and Anoxybacillus flavithermus*, constituted 92% of total bacterial

population in milk powder collected from 18 different countries and screened by a RAPD based survey. In 1988, It has been reported that 36 people became ill from consuming milkshake at a fast food restaurant in Ontario. It was found that the product contained more than 100,000 *Bacillus cereus* per gram which caused the illness. It has been reported that six patients experienced bloody diarrhea and three died, giving the *Bacillus cereus* outbreak a mortality rate of 6.8% (Ceuppens et al., 2013). Production of diarrheagenic and emetic toxins takes place even in micro aerobic conditions but may not relate to symptoms of food spoilage, thereby creating more vulnerability to the consumers (Foltys and Kirchnerová, 2006).

Bacterial cells tend to attach to the surfaces, particularly stainless-steel (SS), live, and form complex biofilm (Abdallah et al., 2014; Sasidharan et al., 2011). Release of a part of bacteria from the matured biofilm by the hydraulic forces can contaminate the downstream milk. Hence, the biofilm development poses a great threat to the quality and safety of milk and other dairy products (Yoo et al., 2005). Previous research focused on biofilms has revealed that the bacteria embedded in biofilms are more resistant than their counterparts. It is considered that bacterial cells in biofilms are difficult to eradicate due to resistance to sanitizers and washing treatments, which can lead to economic losses due to food and equipment deterioration (Römling and Balsalobre, 2012; Cos et al., 2010; Bremer et al., 2006).

Thus, robust cleaning and sanitation protocols must be followed to ensure that processing equipment is completely free of fouled material, pathogens, and substantially low in spoilage microorganisms. Chlorine has been widely used as a cost effective and easy to implement sanitizer in the dairy industry (Gibson et al., 1999). It is effective against most microorganisms; however, it leaves an undesirable residue and may corrode many metal surfaces at high temperatures. Also, chlorine can form potentially carcinogenic trihalomethanes and haloacetic

acids by oxidizing organic matter in water under certain conditions, (Jang et al., 2006). Hence, ozone was considered as an eco-friendly alternative to chlorine.

Ozone is a strong oxidizing and a potent bactericidal agent used in diverse applications (Guezl-Seydim et al., 2004). It has been found to be effective against a wide spectrum of microorganisms. However, the solubility, reactivity, and stability of ozone is influenced by several factors such as pH, temperature, relative humidity, and organic impurities (Cullen and Norton, 2012; Khadre et al., 2001). The ozone gas doesn't appreciably react with water and is physically distributed in solution. The solubility of ozone in water depends upon the size and surface area of ozone gas bubbles. Ketzenelson et al. (1974) reported that the optimum dissolution of ozone in water occurs with the bubble size ranging between 1 and 3 mm in diameter. But with the smaller diameter and larger contact surface area, the nano-bubbles were speculated to be for longer in suspension. Hence, the deionized water with micro- and nano-bubbles will increase the overall effectiveness of ozone and penetrate deeper into biofilms. It has been reported by Henderson et al. (2016) that the maximum ozone half-life was achieved with pH 4.0 solution at 10°C, with an average half-life of 478 min. So, de-ionized water (at pH 4.0 and 10°C) injected with micro- and nano-bubbles was used to evaluate biofilm removal from the SS surfaces. Nano-bubbles are not only small but have distinctive properties which have been found to have longer suspension in solution due to its high gas solubility into liquid with an increase in internal pressure, and negatively charged surface area (Ebina, et al., 2013).

Thus, as a novel approach, nano-micro sized ozone bubbles in water were studied in the present study to evaluate its efficacy in the biofilm removal from SS surfaces. The objective of the present study was to evaluate the efficacy of MNAO in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilm from the SS surfaces. The efficacy was evaluated by comparing

the effectiveness of MNAO treatment compared to other cleaning treatments which included de-ionized water and cleaning-in-place (CIP).

Materials and Methods

Experimental Design

To study the efficacy of micro- and nano-bubble aqueous ozone (MNAO) in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from SS coupons, biofilm was grown over the SS coupons by individual *Bacillus* species by incubating at 37ºC for 72h. After the biofilm growth, the SS coupons were treated with de-ionized water, CIP, MNAO (pH 4.0, Temperature 10ºC), and combined CIP and MNAO treatment. The viable counts of the biofilms for the *Bacillus cereus* and *Bacillus licheniformis* biofilms were observed, before and after the cleaning treatments. The microstructures of the biofilm were observed under the scanning electron microscope. For each trial, 2 coupons were used for each treatment. One coupon was used for the enumeration of counts, while the other was used for the microscopic analysis. All trials were performed in triplicate for both *Bacillus cereus* and *Bacillus licheniformis* biofilm, for each cleaning treatments (n=3).

Maintenance and growth of bacterial cultures

Two aerobic spore forming bacteria, namely *Bacillus cereus* (ATCC 10987) and *Bacillus licheniformis* (ATCC 6634) were used for the biofilm formation-related studies. The reference stains for the *Bacillus* cultures used in the study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The bacterial stock cultures were maintained on plastic beads in cryogenic vials at -80ºC. Vegetative cells of the respective cultures were prepared by transferring the culture from the cryo-vials in to Luria-Bertani (LB) Broth (15 mL) and stored at

37ºC overnight. After overnight incubation, the vegetative cells were stored at 4ºC until further used. The working culture was prepared by spiking the activated culture to the growth medium (a loopful of culture to the 10-mL autoclaved non-fat dry milk (NFDM) and stored at 37ºC for overnight for further study of biofilm formation on SS coupons.

Cleaning of SS coupons

Stainless-steel (SS316) coupons (AGC Heat Transfer, Portland, OR) with 25.4 x 25.4 x 0.48 mm^3 dimensions were used for the study. The coupons were initially washed with de-ionized water, followed by cleaning with a detergent, rinsed with de-ionized water, sprayed with 70% ethanol, rinsed again with de-ionized water, and were then sterilized by autoclaving at 121ºC for 10 minutes. After cleaning, the coupons were air-dried for 15 minutes under the laminar air-flow within the hood before immersing in the working media for the biofilm development.

Development of biofilm on SS coupons

The biofilm was developed on the SS coupons as per the method described by Jindal et al., (2016). The dried coupons were immersed in 24 mL autoclaved 11% (w/w) Total Solids (TS) NFDM in a petri dish. Overnight grown cultures of both *Bacillus cereus* and *Bacillus licheniformis* were spiked to the level of ~7 $\log_{10}$ cfu/mL separately to the petri dishes containing autoclaved milk and SS coupons. Then, the petri dishes with the culture and the milk were incubated at 37ºC for 72h. To provide fresh nutrients and to remove the metabolic toxins produced, the growth medium (autoclaved 11% (w/w) TS NFDM) was replaced after every 24h. The growth medium with the *Bacillus* toxins in the incubated petri-plates was removed and then replaced with the fresh growth medium without disturbing the biofilm growing on the stainless-steel coupons. The experiments were performed in triplicate for the respective bacterial cultures.

Enumeration of viable bacterial cells

After 72h of incubation, the coupons were taken out of the petri dishes with the sterile tweezers and washed with 0.1% peptone water to remove the loosely adhered cells. The coupons were then swabbed to the target area (1x1 inch stainless-steel coupon covered with biofilm) with the help of Quick swabs (3M, St. Paul, MN). Swab tip was pressed and twisted against the wall of 9 mL swab tube (0.1% peptone), and vortexed to facilitate the recovery of all bacterial cells. The contents in the tube were then mixed and serially diluted in sterile 0.1% peptone. The bacteria were enumerated on the Mannitol Egg Yolk Polymyxin (MYP) Agar (Oxoid, Thermo Scientific, UK) using spread plate technique (Downes and Ito, 2001). The plates were incubated at 37ºC for 24-48h. Counts within 25-250 cfu range were considered for calculations and colony forming units were reported as $\log_{10}$ cfu/cm^2 (Wehr and Frank, 2004).

Biofilm susceptibility to different cleaning treatments

Biofilm of *Bacillus cereus* and *Bacillus licheniformis* were developed on the SS coupons as described above. Each coupon containing biofilm formed by respective *Bacillus* species was treated separately with de-ionized water, CIP treatment, MNAO, both CIP treatment and MNAO treatment together, which are explained below.

Treatment of biofilm with de-ionized water

The biofilms produced on SS coupons by both *Bacillus cereus* and *Bacillus licheniformis* were treated separately with de-ionized water at 10ºC for 6 mins. The de-ionized water was made to flow in recirculation mode through the benchtop PHE. The benchtop PHE was fabricated from a high temperature multipurpose 6061 aluminum rectangular bar (McMaster-Carr, Chicago, IL, USA) to the 76.2 x 60.96 x 17.78 mm^3 dimensions and contained the biofilm formed SS coupons, as shown in Figure 5.1. After treatment with de-ionized water, the treated SS coupons

were taken out of the benchtop PHE assembly, and counts for the viable cells on the treated SS coupons were enumerated as per the method described above.

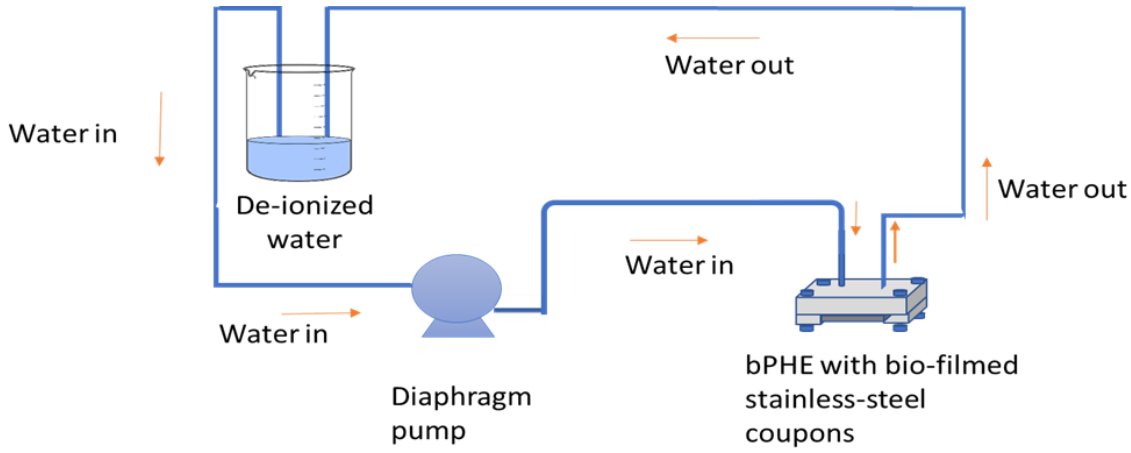

Figure 5.1 Schematic representation of the treatment of respective *Bacillus* spp biofilm with the de-ionized water recirculated for 6 mins through the benchtop PHE accommodating stainless-steel coupon with biofilm grown on it.

Treatment of biofilm with CIP

Cleaning in place (CIP) treatment was performed to remove the biofilms produced by both *Bacillus cereus* and *Bacillus licheniformis* on the SS surfaces. The biofilm formed SS coupons were placed in a benchtop PHE and the following CIP procedure was carried out: (1) 2 min de-ionized water at 50ºC; (2) 6.5 min 1% (w/v) PCC Alkali (Ecolab, St. Paul, MN) at 70ºC; (3) 2.5 min de-ionized water at 50ºC. The CIP solutions were made to flow through the benchtop PHE in the recirculation mode. The treated coupons were swabbed in the same way as described above and the aerobic plate counts were enumerated on MYP agar plates.

Treatment of biofilm with MNAO

The aqueous MNAO was generated in-house using the aqueous ozone generator system supplied by Clean Core Technologies Inc. (Omaha, NE). The ozone generation set-up shown in Figure 5.2. included an oxygen concentrator supplying oxygen to six ozone generators, injector that combined gaseous ozone with water, a reactor for breaking larger ozone bubbles into micro

sized bubbles, ozone and oxidation-reduction potential (ORP) sensor for sensing dissolved aqueous ozone concentration, MNAO reservoir, and a diaphragm pump for the recirculation of water through the assembly.

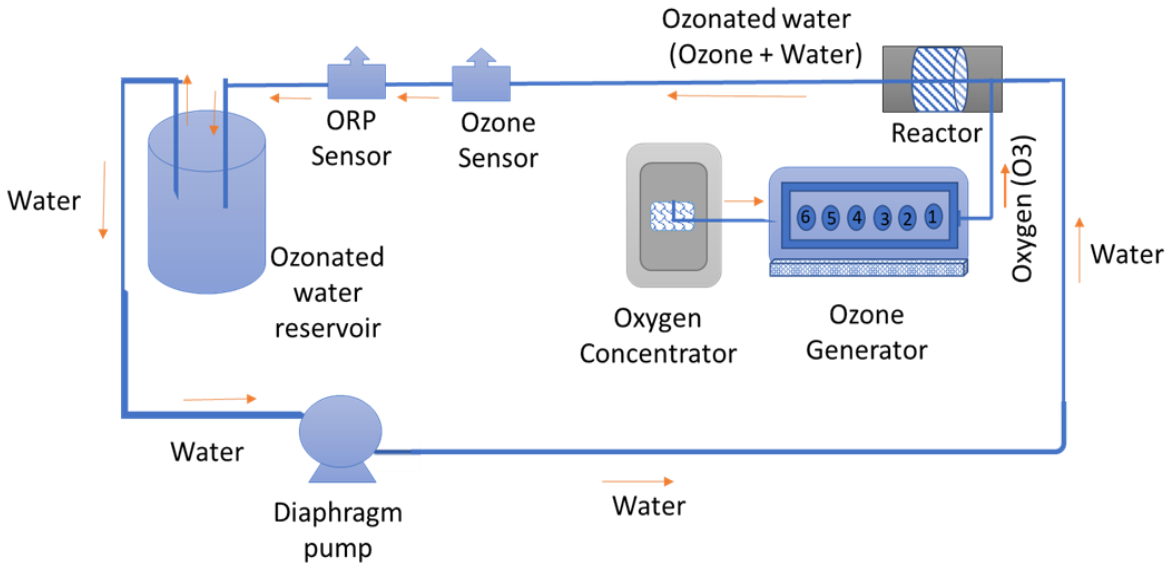

Figure 5.2 Schematic representation of the aqueous MNAO generation using ozone generator. The representation shows the flow of aqueous ozone through the closed system. The ozone produced was mixed with the water inside the reactor and the aqueous MNAO was generated was stored inside the MNAO reservoir.

In a study by Henderson et al., (2017) it was found that maximum half-life of ozone was observed at a pH 4.0 and temperature 10ºC. So, the de-ionized water with pH 4.0 and temperature 10ºC was used for the generation of aqueous MNAO, which was used further in the removal of biofilms from the SS coupons. After the concentration of ozone reached 14 ppm, the reservoir with the MNAO was removed and the benchtop PHE accommodating the biofilm formed coupon was included within the set-up as shown in Figure 5.3. The MNAO was pumped in the recirculation mode through the benchtop PHE assembly for 6 mins removing the biofilm. After treatment, the treated SS coupons were taken out of the benchtop PHE assembly, and enumerated for the counts for the viable cells as per the method described above.

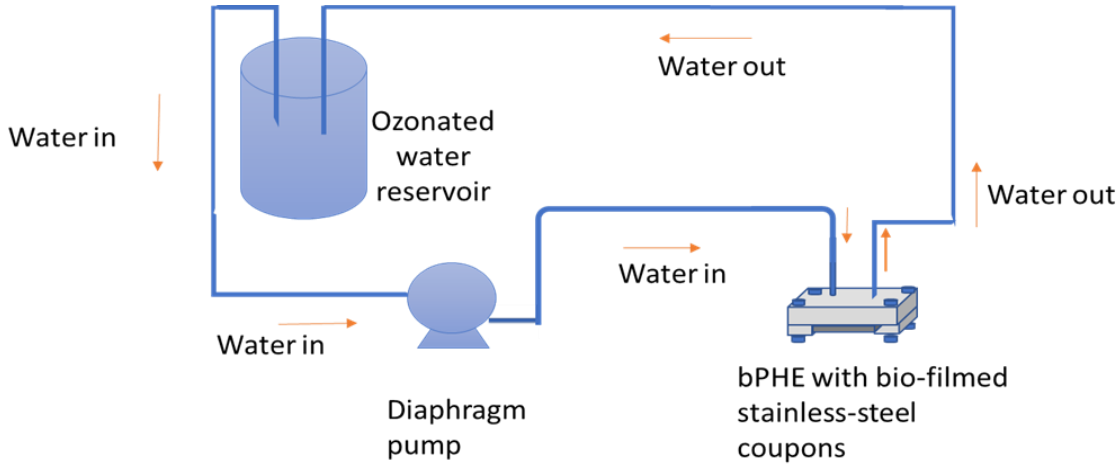

Figure 5.3 Schematic representation of the treatment of respective *Bacillus* spp biofilm with the MNAO recirculated for 6 mins through the benchtop PHE accommodating stainless-steel coupon with biofilm grown on it.

Treatment of biofilm with CIP and MNAO

To see the combined treatment effect, biofilm generated coupons were treated with combined CIP and MNAO treatment, with CIP followed by MNAO, for both *Bacillus cereus* and *Bacillus licheniformis* separately. After treatment, the treated SS coupons were taken out of the benchtop PHE assembly, and enumerated for the counts for the viable cells as per the method described above.

Microscopic visualization of control and treated coupons

Scanning electron microscopy (SEM) was used to study the microstructures of the matured biofilm for both *Bacillus cereus* and *Bacillus licheniformis*, before and after cleaning the SS coupons. The electron micrographs were obtained using a scanning electron microscope (Hitachi S-3500N, Hitachi America Ltd., Tarrytown, NY). The samples were fixed with Trumps fixative agent for 2 h and then washed with 0.1% peptone and air-dried overnight under a laminar air-flow hood. The dried coupons were then sputter coated with Au-Pd for 10 mins. After coating, the samples were mounted on the carbon tape and observed under microscope at 5

kV accelerating voltage at different magnifications. All the experiments were performed in triplicate for each bacterial species.

Statistical Analysis

All the trials for each of the bacterial species were repeated thrice. The bacterial counts were calculated for the mean values and standard error for each of the treatments. For the statistical analysis, half of the detection limit (0.15 $\log_{10}$ cfu/cm^2) were used as for the treatments with colonies on the lowest dilution. Means were compared using Tukey multiple comparison test using the SAS 9.3 software (SAS Institute Inc., Cary, NC) with least significance difference as $P<0.05$.

Results and discussion

Biofilm removal from the stainless-steel surfaces using different washing treatments (de-ionized water, CIP, MNAO, and combined CIP and MNAO) was evaluated by the viable counts in the biofilms of *Bacillus cereus* and *Bacillus licheniformis* on the SS coupons, before and after the treatments. The different cleaning treatments were compared to see their effectiveness in biofilm removal. The microstructures of the biofilms were also studied for the biofilms, before and after the treatments, using scanning electron microscopy and the findings are described below.

Comparison of different cleaning treatments in the biofilm removal

As can be seen in Table 5.1., the average bacterial counts for the *Bacillus cereus* rods after 72h incubation were observed as 2.95 ± 0.21 $\log_{10}$ cfu/cm^2. The detection limit for the microbial enumeration was 0.15 $\log_{10}$ cfu/cm^2. After the de-ionized water treatment, the average bacterial counts observed were 2.27 ± 0.44 $\log_{10}$ cfu/cm^2. The de-ionized water wash reduced the *Bacillus cereus* bacterial counts by 0.68 ± 0.23 $\log_{10}$ cfu/cm^2, which was not significant

(P>0.05). The average bacterial counts observed after CIP treatment were 0.51 ± 0.76 log_{10} cfu/cm^2, which were reduced significantly by 2.43 ± 0.84 log_{10} cfu/cm^2 (P>0.05). Anand and Singh (2013) reported that the regular CIP treatment achieved <2 log reduction in the biofilm embedded microorganisms. But it was observed that after MNAO treatment and combined treatment of CIP and MNAO, the *Bacillus cereus* counts were less than the detection limit. So, a significant reduction of ≥2.88 ± 0.22 log_{10} cfu/cm^2 was observed with the MNAO treatment (P<0.05). In another study by Broadwater et al. (1973), it was reported that there was >2 log_{10} cfu/mL reduction of *B. cereus* bacterium on treatment with 0.12 ppm aqueous ozone for 5 mins at 28ºC. The destruction of target microorganisms by ozonation can be explained by the oxidation of the cellular components (Guzel-Seydim et al., 2004).

Table 5.1 Viable counts reported in biofilms of *Bacillus cereus* before and after different cleaning treatments (control, de-ionized water, cleaning in-place (CIP), micro- and nano-bubble aqueous ozone (MNAO)) formed on SS surface.

	Type of treatment				
Description	Initial population	De-ionized water	CIP	MNAO	CIP+MNAO
Average count* (log_{10} cfu/cm^2)	2.95 ± 0.21[a]	2.27 ± 0.44[a]	0.51 ± 0.76[b]	<0.15[b]	<0.15[b]
Average reduction count* (log_{10} cfu/cm^2)	-	0.68 ± 0.23[a]	2.43 ± 0.84[b]	≥2.88 ± 0.22[b]	≥2.88 ± 0.22[b]

[a-b] Values with different lowercase superscript letters within a row are significantly different at P-value < 0.05.
* Mean ± SD

On the other hand, as shown in Table 5.2., for the *Bacillus licheniformis* biofilm, the average bacterial counts observed on the SS surfaces were 4.34 ± 0.38 log_{10} cfu/cm^2. In another study by Jindal et. al (2016), the viable counts in the biofilm of *Bacillus licheniformis* on SS

surface were reported as 5.13 ± 0.02 $\log_{10}$ cfu/cm^2. A slight increase can be explained by the different incubation temperature (50ºC). After the de-ionized water treatment, the average bacterial counts observed for the *Bacillus licheniformis* biofilm were 2.89 ± 0.50 $\log_{10}$ cfu/cm^2, which were reduced significantly by 1.45 ± 0.28 $\log_{10}$ cfu/cm^2 ($P<0.05$). The CIP treatment average log reduction of the viable counts in biofilm of the *Bacillus licheniformis* was 3.03 ± 0.12 $\log_{10}$ cfu/cm^2; whereas the MNAO treatment caused the average log reduction of the viable counts in biofilm of the *Bacillus licheniformis* by 2.92 ± 0.73 $\log_{10}$ cfu/cm^2. With both the treatments, the counts were reduced significantly ($P<0.05$). After the combined CIP and MNAO treatment, the *Bacillus licheniformis* counts observed were less than the detection limit. A substantial bacterial log reduction of 4.27 ± 0.38 $\log_{10}$ cfu/cm^2 and 2.92 ± 0.73 $\log_{10}$ cfu/cm^2 were observed by the combined CIP+MNAO treatment and MNAO treatment, respectively. The reduction of *Bacillus spp*. populations by the MNAO treatment agree with experiments by Greene et al. (1993) where ozonated water effectively inactivated milk spoilage bacteria adhered to the biofilm on stainless-steel. Dosti et al. (2005) reported ~3-4 $\log_{10}$ cfu/in^2 reductions in the *Pseudomonas* biofilm on the stainless-steel coupons during UHT processing on application of nano-ozonated phosphate buffered saline (0.6 ppm for 10 mins). In a study by Guzel-Seydim et al. (2000), it has been reported that there was 84% removal of dairy soil from the metal plates on treatment of nano-ozonated cold water (10 ºC) for 15 mins. The reduction of bacterial counts with the ozone treatment can be explained by the ozone's mode of action by attack on the bacterial membrane and glycoproteins and/or glycolipids (Guzel-Seydim et al., 2004).

It can be inferred from the findings that the *Bacillus cereus* bacterial detachment from the stainless-steel surface were greatly influenced by the MNAO treatment. Thus, highlighting the

importance of MNAO and its effectiveness in the removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms.

Table 5.2 Viable counts reported in biofilms of *Bacillus licheniformis* before and after different cleaning treatments (control, de-ionized water, cleaning in-place (CIP), micro- and nano-bubble aqueous ozone (MNAO)) formed on SS surface**.**

	Type of treatment				
Description	Initial population	De-ionized water	CIP	MNAO	CIP+MNAO
Average count* (log_{10} cfu/cm^2)	4.34 ± 0.38^a	2.89 ± 0.50^b	1.31 ± 0.27^c	1.42 ± 0.37^c	$<0.15^d$
Average reduction count* (log_{10} cfu/cm^2)	-	1.45 ± 0.28^b	3.03 ± 0.12^c	2.92 ± 0.73^c	$\geq 4.27 \pm 0.38^d$

[a-d] Values with different lowercase superscript letters within a row are significantly different at P-value < 0.05.
* Mean ± SD

The above results were further supported by the microscopic visualization of the biofilm on SS coupons, before and after the cleaning treatments.

Microscopic visualization of biofilm

Scanning electron microscopic images were obtained to examine the microstructures of the biofilm of *Bacillus cereus* (Figure 5.4) and *Bacillus licheniformis* (Figure 5.5) on the SS coupons, before and after the cleaning treatments. It was observed that the *Bacillus cereus* rods before any cleaning treatment were densely populated and attached to the SS surface. After the de-ionized water wash, the density of the *Bacillus cereus* rods reduced compared to no treatment. The micrograph of the SS coupon with the biofilm washed with the de-ionized water observed correlated to the counts observed. After the CIP treatment, the biofilm was detached mostly; but

still small fraction of biofilm was observed in the micrographs. In a study by Faille et al. (2014), the authors also found a small fraction of *Bacillus* biofilm remaining after the mild CIP treatment. Though the *Bacillus cereus* counts observed after the aqueous MNAO treatment were less than the detection limit, but a residual amount of *Bacillus cereus* biofilm rods were observed in the micrographs.

Similarly, a densely populated *Bacillus licheniformis* biofilm can be seen in the micrographs (Figure 5.5) after the 72h period of incubation. The biofilm was found to be attached to the SS surface as a close-knit structure. However, the de-ionized water treatment reduced the biofilm load and less biofilm was attached to the SS coupon, as can be seen in Figure 5.5 (B). This further supports the viable counts results after the de-ionized water wash. The CIP treatment substantially decreased the biofilm attached to the stainless-steel surface, but still the *Bacillus licheniformis* biofilm were found adhered, which indicates resistance to complete detachment of *Bacillus licheniformis* biofilm by the CIP treatment. With the MNAO treatment, the biofilm was also removed appreciably as seen in Figure 5.5 (D). But, no biofilm was observed attached to the SS coupons after the combined CIP and MNAO treatment.

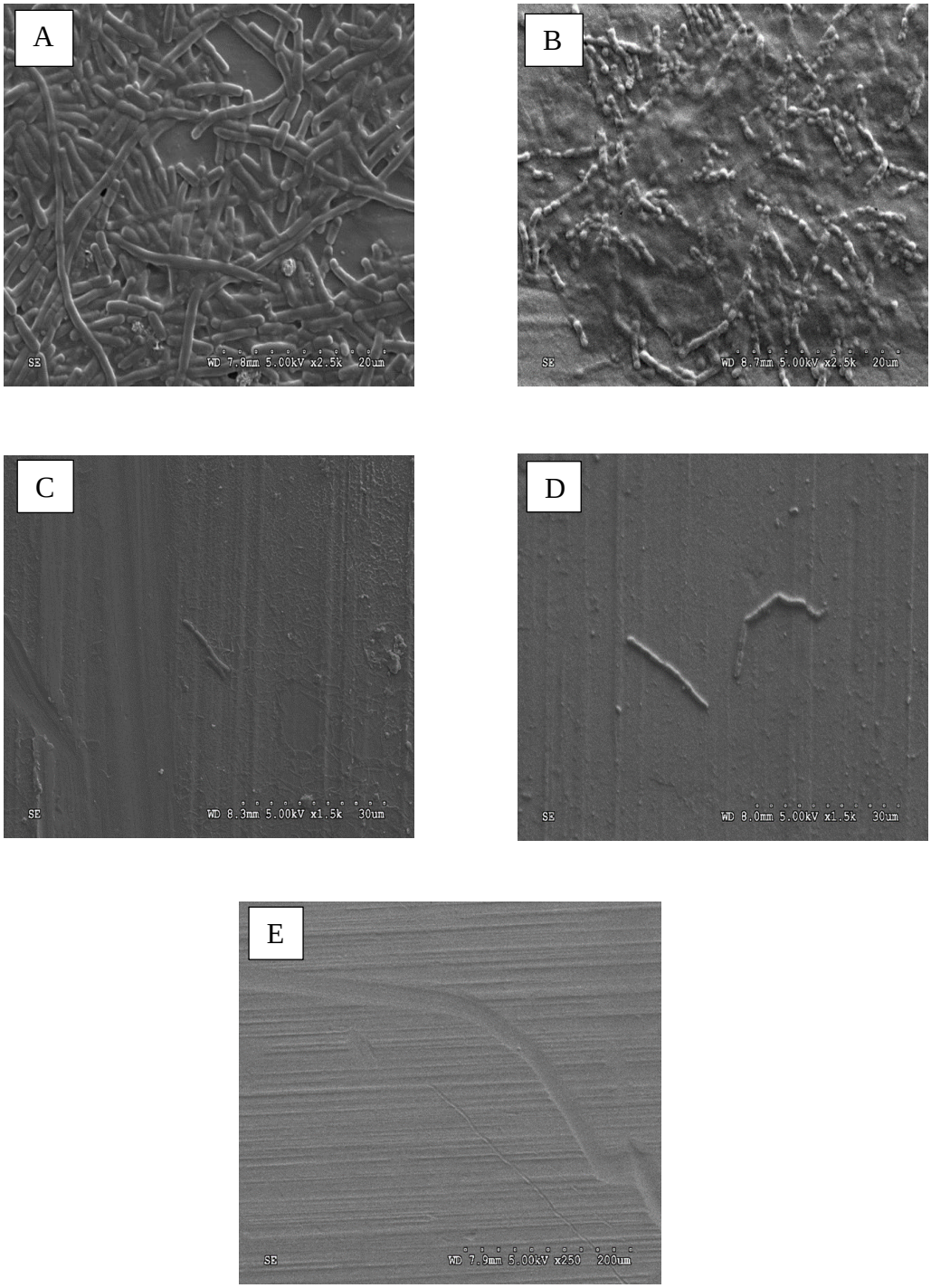

Figure 5.4 Scanning electron micrographs of SS coupons showing attachment of biofilm of *Bacillus cereus* rods before and after cleaning treatments (A) control; (B) de-ionized water; (C) cleaning-in-place (CIP); (D) micro- and nano-bubble aqueous ozone (MNAO); (E) cleaning-in-place (CIP) and micro- and nano-bubble aqueous ozone (MNAO)

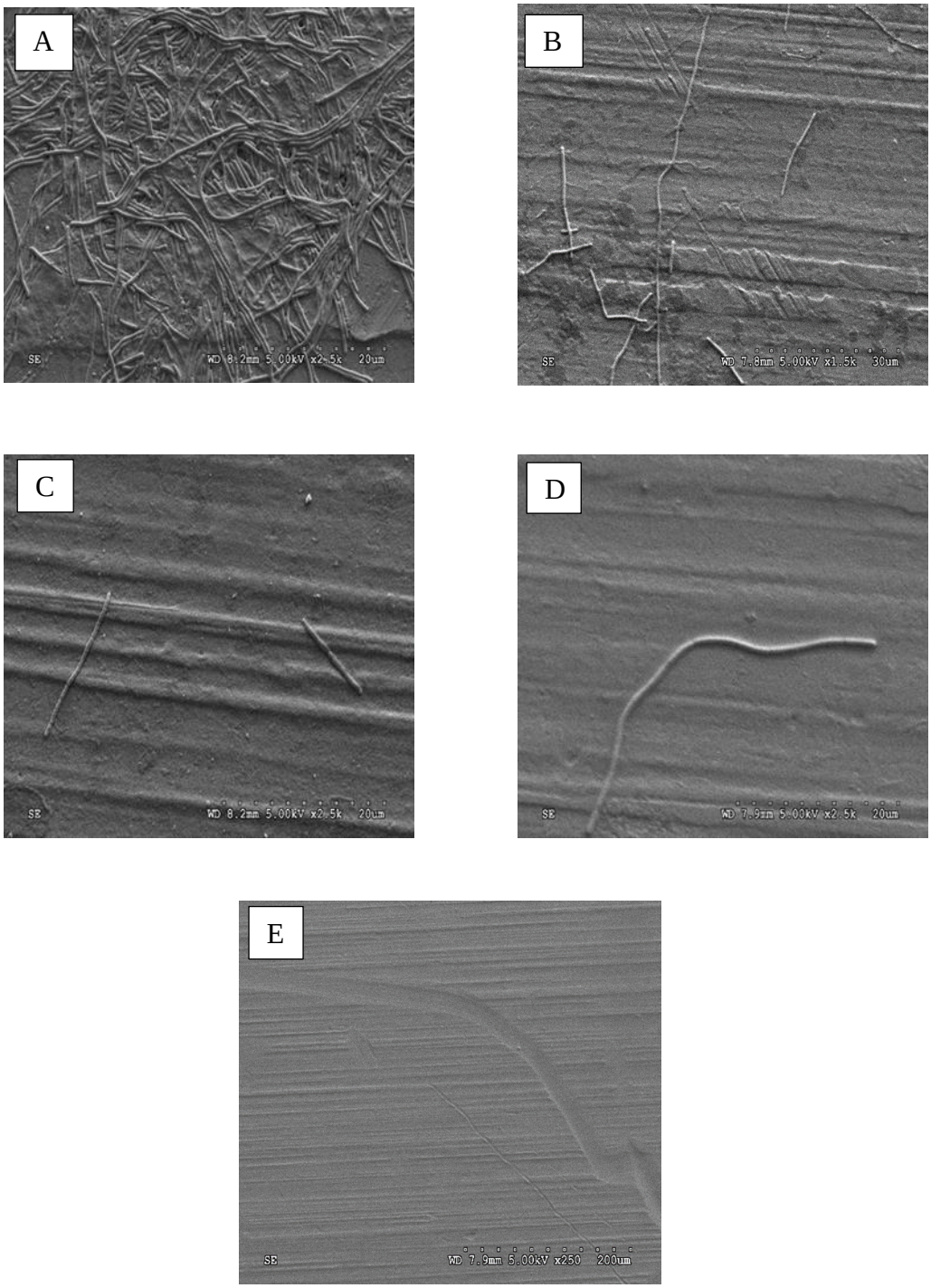

Figure 5.5 Scanning electron micrographs of SS coupons showing attachment of biofilm of *Bacillus licheniformis* rods before and after cleaning treatments (A) control; (B) de-ionized water; (C) cleaning-in-place (CIP); (D) micro- and nano-bubble aqueous ozone (MNAO); (E) cleaning-in-place (CIP) and micro- and nano-bubble aqueous ozone (MNAO)

Conclusions

It can be concluded based on the findings of the present research that the MNAO treatment has great potential in removal of *Bacillus cereus* and *Bacillus licheniformis* biofilms from the SS coupons. The application of MNAO as a disinfectant alone or in combination with the regular CIP protocol followed in dairy industry, can be of great significance in reducing the bacterial loads. This could further benefit the dairy industry by reducing the cost for cleaning, increasing the production, and contributing to the environmental sustainability.

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Chapter 6 - Conclusions

Fouling of stainless-steel surfaces is a major problem in the dairy and food industry, causing severe economic losses. The fouled surfaces are further found to be contaminated by several microorganisms, leading to biofilm formation. The adherent bacteria embedded in a biofilm can have detrimental effects, including food spoilage, illness, and economic losses. The dairy industry needs a two-stage intervention strategy to address biofilms on the plate heat exchangers, which include biofilm prevention and biofilm removal strategy.

In order to prevent the formation of biofilm on the stainless-steel surfaces, it is important to prevent fouling, as fouling promotes effective biofilm formation. Proper understanding of the foulant formation is essential to design effective reduction protocols. Fouling can be reduced either by modifying the stainless-steel surfaces using specialty coatings and/or by using good quality product.

The use of high-protein dairy powders is increasing in the dairy and food industry to improve the functional, nutritional, and sensory properties of a product. The dissolution characteristics of the high-protein powders are important to achieve the desired characteristics in the end product. Storage temperature of high-protein ingredients affects their functional properties, which have additional consequences during thermal processing. Storing the MPC80 powders at a higher temperature (40ºC) resulted in decreased solubility. Further, it was found that the poor-soluble MPC80 powder resulted in a higher amount of milk deposit (foulant) on the stainless-steel surfaces during thermal processing. Hence, it can be concluded that increasing the storage temperature decreases the solubility of MPC80, which accounts for the high amount of foulant. Further, microstructures of the foulant formed by the MPC80 powders during thermal processing, observed under the SEM, CLSM and EDS analysis, revealed its non-uniform and

smooth structure on stainless-steel. Craters and bubbles are observed in higher frequencies in foulant generated by poor-quality MPC80 powder (stored at 40ºC) than good-quality MPC80 powder. Thus, the study can be helpful in understanding the fouled layer characteristics and designing the effective cleaning protocols.

Successful biofilm removal in a sustainable manner is also important for the dairy industry. The proposed research evaluates the use of ozonated water to remove biofilms on plate heat exchanger plates, as well as dairy membrane systems. So as a novel cleaning approach, nano-micro aqueous ozone (NMAO) was studied as a disinfectant to remove the biofilm of *Bacillus cereus* and *Bacillus licheniformis* on stainless-steel coupons. It was found that nano-micro aqueous ozone (NMAO) was effective in significantly removing the *Bacillus cereus* and *Bacillus licheniformis* biofilm from stainless-steel surfaces. Thus, NMAO has a great potential in removing the *Bacillus* spp. biofilms from the stainless-steel surfaces in the dairy and food industry from the food contact surfaces.