Controlling Biofilm Development on Ultrafiltration and Reverse Osmosis Membranes Used in Dairy Plants

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ABSTRACT

This study aimed to develop improved cleaning strategies for controlling biofilms on the surfaces of membranes used in dairy ultrafiltration (UF) and reverse osmosis (RO) plants.

Eleven UF / RO membrane modules from 7 different New Zealand dairy membrane processing plants were received after typical cleaning-in-place (CIP) procedures. Microorganisms were isolated from both the retentate and permeate sides of these membrane surfaces and from the liquids collected from a UF membrane plant. Also some foulants scraped from a RO membrane were tested. The routine CIP currently used in the dairy plants was not adequate to completely remove organic material, including microbial cells, proteins and carbohydrates from the membrane surfaces. These residues may influence the surface characteristics and interactions between microorganisms and membranes and thus affect biofilm formation. Thirteen isolates including both bacteria and yeast were identified using biochemical techniques. *Klebsiella oxytoca* were isolated from 3 different membrane plant sites. This is, so far as we know, the first report of *K. oxytoca* being isolated from dairy membrane surfaces. The ability of the 13 strains to attach to negatively charged polystyrene surfaces was tested using a microtitre plate assay. Three *K. oxytoca* strains demonstrated higher ability to adhere than the other strains, suggesting that these strains might play an important role in developing biofilms on dairy membrane surfaces. Two *K. oxytoca* strains (*K.* B006 from plant A, UF and *K.* TR002 from plant C, RO) that performed best in the microtitre screening assay with respect to attachment capabilities were chosen for the remainder of the study.

The cell surface hydrophobicity of all isolates was determined using the microbial adhesion to hydrocarbon assay (MATH) and the cell surface charge was determined by measuring the surface zeta potential. These two characteristics did not show a clear relationship with the adherence of the isolated strains. However, it was found that bacterial attachment was enhanced in the presence of whey or mixed strains.

A commercial biofilm reactor CBR 90 was modified for developing biofilms on membranes and investigating strategies for biofilm removal. Biofilms of single and dual *K. oxytoca* strains were developed under a continuous flow of whey. The saturated biofilm was approximately 8 $\log_{10}$ CFU cm^{-2}. The results of our study suggested that the whey protein concentration, membrane type including membrane material (polyethersulfone (PES) and polyvinylidene fluoride (PVDF)), membrane age (used and new), bacterial strain and the interactions between different microorganisms are all significant factors for biofilm development on membrane surfaces.

Three enzymatic cleaners and four sanitisers, including sodium hypochlorite (pH 6.5, 200 ppm free available chlorine (FAC)), Perform® (peracetic acid/hydrogen peroxide, 2% v/v), ozonated water (pH 7.0, 0.5 ppm free available ozone (FAO)) and anolyte of MIOX® electrolysed water (EW) (pH 6.8, 120 ppm FAC) were tested for their efficacies in killing culturable cells from biofilms formed by single or dual *K. oxytoca* strains on used PES membrane surfaces. With no sanitation applied, two of three enzymatic cleaners performed better than sodium hypochlorite (pH 10.8-11, 200 ppm FAC) commonly used for CIP of UF membranes in the dairy industry. The four sanitisers were used to treat the membranes after a CIP wash regime. The results indicated that if a dairy processor were to use a standard CIP on membrane systems, then a further flush with MIOX® EW anolyte would reduce residual attached microbial populations further. In addition, using protease followed by a sanitation (sodium hypochlorite, Perform® or anolyte of MIOX® EW) produced the best clean based on a greater than 2 log reduction in residual cells and left no culturable and viable cells at a detection limit of 0.1 $\log_{10}$ CFU cm^{-2}.

Keywords**:** biofilm, dairy, ultrafiltration, reverse osmosis, membrane, *Klebsiella*, attachment, surface hydrophobicity, surface charge, CIP, electrolysed water, enzymatic cleaner

LIST OF PUBLICATIONS

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2. Tang, X., Flint, S.H., Brooks, J.D., Bennett, R.J., & Morton, R.H. (2009). Biofilm growth of individual and dual strains of *Klebsiella oxytoca* from the dairy industry on ultrafiltration membranes. *Journal of Industrial Microbiology and Biotechnology, 36*(12), 1491-1497.

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ABBREVIATIONS

Atomic Force Microscopy	AFM
Attenuated Total Reflection – Fourier Transform Infrared spectroscopy	ATR-FTIR
Autoinducer-2	AI-2
Bovine Albumin	BA
Bovine Serum Ablumin	BSA
Cellulose Acetate	CA
Cholerae Autoinducer 1	CAI-1
Clean-in place	CIP
Commercial Biofilm Reactor	CBR
Confocal Laser Scanning Microscopy	CLSM
3, 5-dinitrosalicylic acid	DNS
Electrolysed Water	EW
Extracellular Polymeric Substances	EPS
Free Available Chlorine	FAC
Free Available Ozone	FAO
Glycomacropeptides	GMP
Hydrophobic Interaction Chromotography	HIC
Microbial Adhesion to Hydrocarbon Assay	MATH
Microfiltration	MF
Milk Permeate	MP
Milk Protein Concentrate	MPC
Molecular Weight Cut-Off	MWCO
N-acylhomoserine Lactones	AHLs

Nanofiltration	NF
N-nonanoyl-cyclopentylamide	C_9-CPA
New Zealand's Biotech	NZBio
NEW Zealand Institute of Food Science and Technology	NZIFST
New Zealand Microbiological Society	NZMS
New Zealand Society for Biochemistry and Molecular Biology	NZSBMB
Optical Density	OD
Peracetic Acid	PAA
Phosphate Buffer Saline	PBS
Polyamide	PA
Polyethersulfone	PES
Polymerase Chain Reaction	PCR
Polysulphone	PS
Polyvinylidence Fluoride	PVDF
Reverse Osmosis	RO
Scanning Electron Microscopy	SEM
Skim Milk Agar	SMA
Standard Deviation	SD
Standard Plate Count Agar	SPCA
Thin Film Composites	TFC
Trans-membrane Pressure	TMP
Tryptocase Soy Broth	TSB
Ultrafiltration	UF
Whey Permeate	WP
Whey Protein Concentrate	WPC

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Chapter 1

INTRODUCTION

This thesis describes a programme of research based at Massey University, Palmerston North, New Zealand, to develop methods to control the growth of biofilms on the surfaces of synthetic membranes used for ultrafiltration (UF) and reverse osmosis (RO).

Biofilm is a community of microorganisms attached to a surface, producing extracellular polymeric substances (EPS) and interacting with each other. It can form on any surface in any environment where the bacteria are present. Biofilms in many food processing plants have been studied, however, the surfaces colonised are typically stainless steel, aluminium, glass, Buna-N, Teflon and nylon seals, rather than membranes used for filtration. Studies that have examined biofilms on membrane surfaces have been in wastewater environments rather than food manufacturing plant.

In the dairy processing environment, protein (milk or whey protein) with other organic or inorganic molecules can form a conditioning layer on manufacturing plant surfaces. These conditioning layers alter the physico-chemical properties of the surface, including the surface free energy, hydrophobicity and electrostatic charge, with subsequent effects on the adhesion of different microorganisms. The colonisation of a surface by one species is also found to influence the attachment of other species to the same surface. Once the mature biofilm forms, it is difficult to remove using normal cleaning procedures.

UF and RO membranes are growing in use in the dairy industry. The spiral-wound configuration is most commonly used in membrane applications, as it is competitively priced. However, because of the close spacing of the membrane leaves, these membranes are susceptible to fouling. The large surface area of the membranes provides ample support for the development of biofilms. Previous studies have focused on protein fouling of membranes. With the increase in use of this technology, there is a growing awareness of the limitations imposed by biofilm growth on membrane surfaces. Biofilm growth on membranes has two effects on dairy manufacture. Firstly, when

biofilm is present on the membrane surface, colloidal solids and insoluble precipitates can adhere to the biofilm and form a physical barrier that reduces the membrane flux (volumetric flow rate per unit area) which results in a reduction of the operating run time of manufacturing plants. Secondly, the constant release of microorganisms from the biofilm increases the cell numbers in the liquid phase and thus has a high probability of contaminating the product stream.

There are many possible sources of bacteria that may contaminate membrane plants. These include feed solutions, diafiltration water, the environment of the manufacturing plant, and liquids (e.g. water) used for clean-in-place operations (CIP). However, there is no information on the microbial composition of biofilms in dairy membrane processing. Residual bacteria following CIP procedures may also act as a nidus for subsequent biofilm development.

This project was initiated by the New Zealand dairy industry to investigate biofilm development in dairy membrane processing plants and recommend strategies for biofilm control in this environment. The objectives of this study included defining the microbial populations in the biofilms on the membrane surfaces, developing a fundamental understanding of adhesion of microorganisms and the relationship between the adhesion and the cell surface characteristics, investigating the factors influencing biofilm growth and examining methods for improving CIP strategies used in dairy membrane plants in terms of removing biofilm.

Specific materials and methods have been described in each chapter. Chapters 4 – 6 have been peer-reviewed as published papers.

Chapter 2

BIOFILMS ON ULTRAFILTRATION AND REVERSE OSMOSIS MEMBRANES IN DAIRY PLANT – LITERATURE REVIEW

2.1 INTRODUCTION

Filtration is a process for separating two or more substances, based on differences in their physical size and shape, by allowing liquid to pass through a porous barrier. Membrane filtration technology was adopted early by the dairy industry (D'Souza & Mawson, 2005), and UF and RO membranes have been widely used in the dairy industry (Kumar & Anand, 1998). However, biofilms, which can form an undesirable layer of living microorganisms and their decomposition products on the membrane surfaces limit the application of membrane technologies (Kumar & Anand, 1998).

The following is a critical review of recent literature about UF and RO membranes and biofilms. The review includes the fundamental working principles and functions of these two membrane types in the dairy plants, the principles of biofilm development and its relationship to the characteristics of microorganisms and membranes, techniques that can be used in biofilm research and the latest improvements in control.

2.2 UF AND RO MEMBRANES

UF and RO membranes are both semi-permeable membranes that have many tiny pores. Depending on the size of the pores, smaller molecules can pass through the membrane and larger molecules are retained. The feed stock will generally be split into two streams; materials that pass through the membrane are called permeates, those that are retained by the membrane are called retentates (Bird, 1996).

2.2.1 UF membrane

UF is widely used in the dairy industry (Daufin et al., 2001). The pore size used for UF membranes (10^{-2} - 10^{-1} μm) is larger than that used for RO membranes (10^{-4} - 10^{-3} μm)

(Peinemann et al., 2010) (Fig. 2.1), allowing the protein and fat to be retained, while permitting the water, lactose and ash to pass through (Goff, 1995). The applications of UF membranes in the dairy industry include the manufacture of whey protein concentrates (WPCs) and milk protein concentrates (MPCs), milk standardisation before cheese manufacture, liquid milk concentration for market milk product and clarification of cheese brine (Bird, 1996).

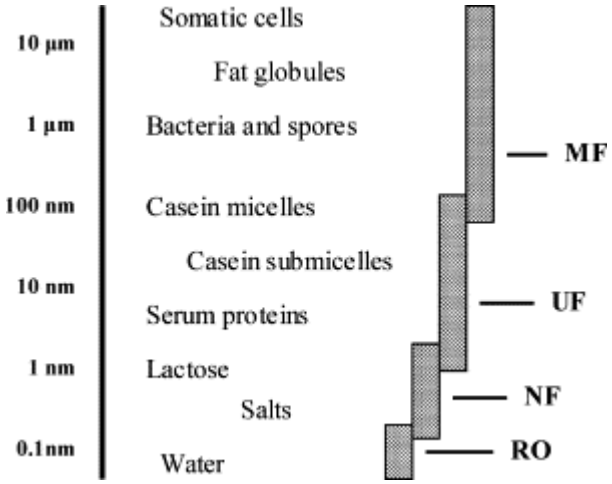


Figure 2.1: Permeability of membranes in dairy manufacturing. MF: microfiltration, UF: ultrafiltration, NF: nanofiltration, RO: reverse osmosis. (From Brans et al., 2004; used with permission from Elsevier.)

2.2.2 RO membrane

RO is a high pressure membrane separation process that operates at between 25-40 bar (Bird, 1996; Hiddink et al., 1980) and allows only water to pass through the membrane (Fig. 2.1). The applications of RO membranes in the dairy industry are concentration of UF permeates for lactose manufacture, milk standardisation, lactose fermentation; recovery of proteins and lactose from casein whey wash waters, recovery of clean-in-place (CIP) water from UF and concentration of whey prior to transportation (Bird, 1996).

2.3 CROSS-FLOW AND BIOFOULING

Membrane filtration in the dairy industry is almost exclusively operated in a cross-flow mode (Fig. 2.2), especially for the more difficult feeds (Pearce, 2008). The circulation in cross-flow filtration is parallel to the membrane (Anon, 2007). The consistent turbulent flow (Anon, 2007) creates the shearing effect of the fluid as it passes over the membrane to remove any particles that may have accumulated at the surface of the membrane (Caridis & Papathanasiou, 1997). This helps to maintain a relatively steady flux through the membrane.

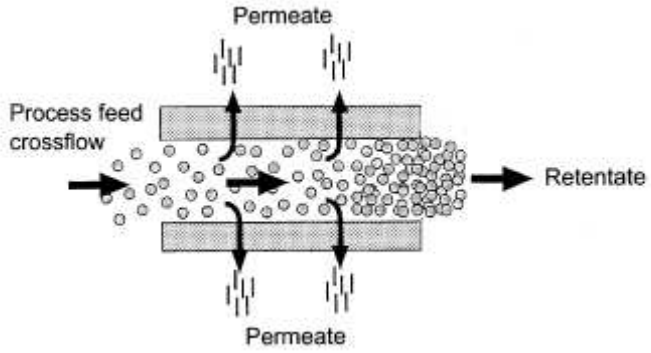


Figure 2.2: Cross-flow filtration. (From Caridis & Papathanasiou, 1997; used with permission from Springer.)

Cross-flow filtration is a pressure-driven process and is profoundly influenced by the applied pressure differential between retentate and permeate (Caridis & Papathanasiou, 1997). During the filtration of protein solutions (e.g. whey suspension), increased trans-membrane pressure (TMP) results in accumulation of a stronger fouling layer on the membrane surface (Karasu et al., 2009). This pre-conditioning layer will influence the subsequent biofilm formation, which is described in section 2.5.3. Results from Karasu et al. (2009) on a modeling study on UF of whey determined that higher feed flow rate caused a larger volume of particles to be removed from the fouling layer. Therefore, very high cross-flow velocities may be necessary to control fouling (Pearce, 2008).

2.4 MEMBRANE CONFIGURATION AND MATERIALS

A spiral-wound configuration (Fig. 2.3) is most commonly used in membrane applications today (Ridgway et al., 1983) owing to its high membrane surface area to volume ratio and the convenience in replacing and purchasing (Bodalo-Santoyo et al., 2004). However, this configuration has extreme susceptibility to fouling, owing to the close spacing of the membrane leaves (Cartwright, 2003). In spiral-wound membrane module (Fig. 2.3), feed is separated by membrane layers. Retentates are collected from the side of the layers, and permeates enter the central tube through permeate collection holes. Other configurations include plate and frame, tubular and hollow fiber (Maubois, 1980).

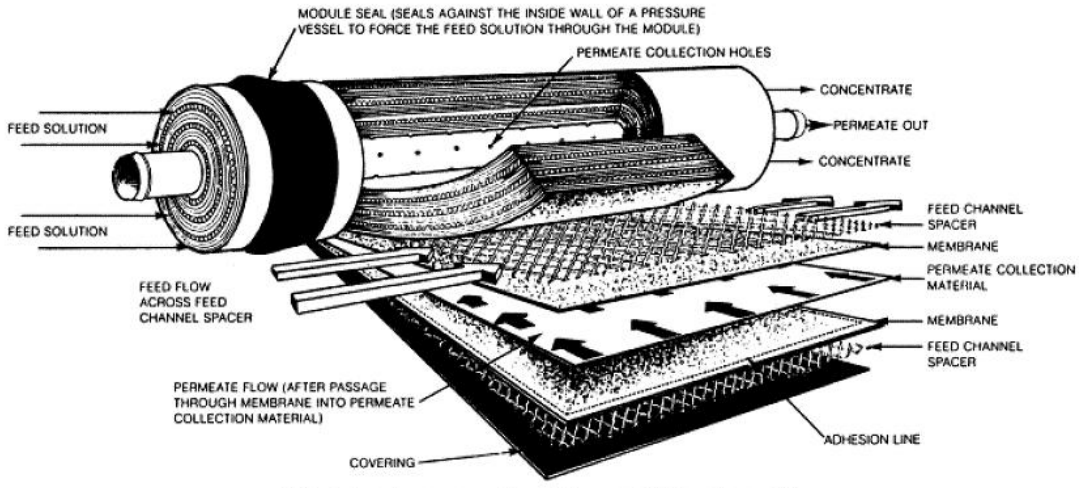


Figure 2.3: Spiral-wound configuration of filtration membranes. (From Ridgway et al., 1983; used with permission from American Society for Microbiology.)

The major materials for spiral-wound membranes in the dairy industry are typically polyethersulphone (PES) and polysulphone (PS) (D'Souza & Mawson, 2005; Pearce, 2007a). PES membranes have good strength and high permeability, and the properties of PES can be modified through a polymer blend (Pearce, 2008). Membranes are usually modified to have a hydrophilic surface because of the advantages of being easily wetted and resisting fouling (Pearce, 2007b). Polyvinylidene fluoride (PVDF) became another polymer used for membranes in the 1990s (Pearce, 2008). Both PES and PVDF are now important materials for the membrane market (Pearce, 2007b). PVDF is

stronger and more flexible than PES, and excellent in chemical resistance (Boributha et al., 2009). Thus, PVDF membrane tends to have longer life (Pearce, 2007b). However, since the hydrophobic surface of PVDF membrane is difficult to modify (Fontananova et al., 2006), it is more susceptible to fouling than others (Lozier et al., 2006; Pearce, 2007b).

2.5 BIOFILM DEVELOPMENT

2.5.1 Concerns regarding biofilm in dairy manufacturing plants

Generally bacteria prefer to grow on an available surface rather than in the surrounding aqueous phase (Katsikogianni & Missirlis, 2004). Biofilm can develop on any surface exposed to an aqueous environment (Flint et al., 1997a). In the dairy and food industries, serious problems caused by biofilms include interfering with the flow of heat across the surface (Criado et al., 1994), increases in the fluid frictional resistance (Kumar & Anand, 1998) and the corrosion rate at the surface (Liu et al., 2007). In addition, microorganisms growing in biofilms are more difficult to eliminate than free floating bacterial cells (Flint et al., 1997a), and thus cross contamination and post-processing contamination may occur once biofilms have become established in a manufacturing plant (Kumar & Anand, 1998) leading to reduced product shelf life (Zottola, 1994). Such microbial contamination is the major cause of poor quality dairy products (Flint et al., 1997a). The use of membranes has been significantly limited by the problem of fouling, as a small degree of adsorption causes membrane pore blockage (Cheryan & Mehaia, 1986). Biofilm fouling may be favoured by the fouling of the membrane (Kumar & Anand, 1998) that will eventually block the membrane pores preventing further manufacture (Flint et al., 1997a). A mature biofilm on the membrane surface can also change the distribution of filtration and surface properties of the filter (Cogan & Chellam, 2008).

2.5.2 Mechanism of biofilm formation

Biofilm formation is initiated by the attachment of microorganisms to surfaces and the development of biofilm starts when the attached microorganisms grow (Ivnitsky et al., 2005) (Fig.2.4). The formation of mature biofilms is thought to be the result of early

surface colonization by some microorganisms that change the surface properties and facilitate attachment and growth of others (Dang & Lovell, 2000; Jefferson, 2004). Initial adhesion of microorganisms to the surfaces is essential for biofilm formation (Dang & Lovell, 2000). Given adequate nutrients, time and suitable temperature, the initial sessile microbial population can eventually form a confluent lawn of bacteria on the membrane surface (Ridgway et al., 1999). The initiation of biofilm formation is influenced by a multitude of factors, including conditioning film (Lewandowski & Beyenal, 2003), van der Waals and electrostatic interactions (Vadillo-Rodriguez et al., 2005), surface characteristics of microorganisms and substratum (Palmer et al., 2007) and quorum sensing (Cogan & Chellam, 2008). In addition, biofilm formation on membranes is also affected by factors such as the condition of feedstock (e.g., pH, ionic strength and divalent cation concentrations), fluid flow and interaction between foulants (Lee et al., 2010b).

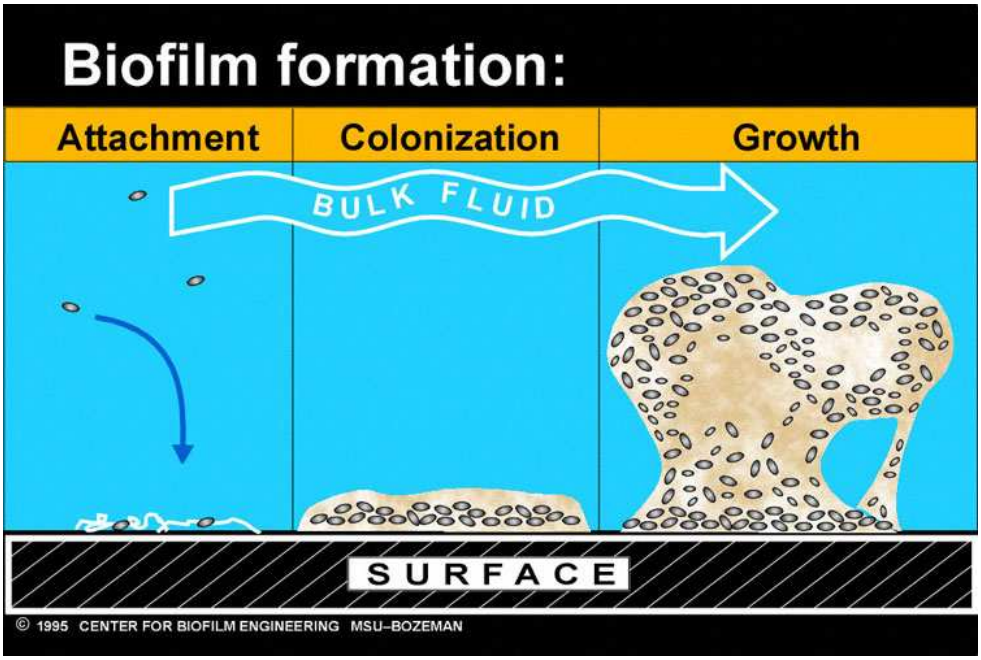


Figure 2.4: Biofilm formation: Attachment, colonization and growth. (Used with permission. Copyright held by the Center for Biofilm Engineering at Montana State University, Bozeman, MT, USA.)

2.5.3 Conditioning and biofilm formation

A conditioning film is the accumulation of organic molecules (e.g., milk proteins) covering a solid surface (Palmer et al., 2007; Zottola, 1994), and leads to a higher

concentration of nutrients at the surface compared with the liquid phase (Palmer et al., 2007). This favours the growth of microorganisms as the concentrations of nutrients needed for the growth of microorganisms in the biofilm are much higher than in the fluid (Bryers, 1987). The increase in biofilm formation also depends on the type of the competing microorganisms associated with the biofilm (Kumar & Anand, 1998).

A protein conditioning film was believed to be an essential pre-requisite for biofilm formation in a dairy processing environment (Kirtley & Mcguire, 1989). Kirtley and Mcguire (1989) explained that the conditioning film of proteins may either establish a dynamic equilibrium with the bulk fluid, resulting in no more adsorption, or be followed by denaturation to an irreversibly adsorbed species leading to further deposition and biofilm development. However, other studies showed high numbers of microorganisms attach to solid surfaces submerged in dilute casein, lactose, and non-casein environments (Meadows, 1971; Speers & Gilmour, 1985) and some researchers found casein and β-lactoglobulin reduced attachment of *Listeria monocytogenes* and *Salmonella* Typhimurium to stainless steel (Helke et al., 1993). In addition, studies found that the presence of albumin, gelatin and fibrinogen inhibited attachment of a marine *Pseudomonas* to polystyrene (Fletcher, 1976). Most recent studies showed that the attachment of *Klebsiella* was significantly discouraged where surfaces were coated with a fish muscle α-tropomyosin (Vejborg & Klemm, 2008).

Almost all microorganisms adhered to surfaces secrete exo-polymeric substances (EPS) (Ramsey & Whiteley, 2004) that are mainly carbohydrates (Sutherland & Kennedy, 1996), proteins, lipids, small quantities of nucleic acids and a variety of humic substances (Lee et al., 2010b; Liu & Fang, 2002; Nielsen et al., 1996). EPS are responsible for the membrane fouling (Jacquement et al., 2005) by irreversibly binding to the membranes (Davies et al., 1998). It enhances the survival and robustness of the biofilm microorganisms by forming a chemically reactive diffusion transport barrier with bacterial cells (Goldman et al., 2009), impeding convective flow and slowing the penetration of biocide into the biofilm (Ivnitsky et al., 2005). The EPS matrix also reinforces cellular bonding to surfaces and stabilizes the biofilm, thereby reducing its susceptibility to sloughing by hydrodynamic shear (Ivnitsky et al., 2005). The bacteria and EPS can even change the surface composition of membranes (Khan et al., 2010), which probably results in a shorter membrane life. By using an individual-based model,

Kreft and Wimpenny (2001) found that EPS production dramatically changes the biofilm structure. They observed that the density of cells at the bottom of the biofilm was very low when the rate of EPS production was high, owing to more energy being consumed on EPS synthesis than they could gain at the low oxygen tensions in the depth of the biofilm (Kreft & Wimpenny, 2001). Pang et al. (2005) found that *Sphingomonas sp.* EPS on RO membranes tended to be more closely associated with the single cell or small cell clusters than with larger colonies and the EPS matrix was detected in areas free of biofilm cells, suggesting that the EPS matrix was quite extensive.

2.5.4 Characteristics of microorganisms and biofilm formation

Following the establishment of the conditioning film, microorganisms attach to the conditioned surface (Kumar & Anand, 1998). The adhesion of microorganisms has two phases. The first phase involves physicochemical interactions between bacteria and surface (Katsikogianni & Missirlis, 2004) and the adhesion is believed to be affected by hydrophobicity (Araujo et al., 2010) and electrical charge (Palmer et al., 2007). The second phase is molecular and cellular interactions between bacteria and surface, when bacteria firmly attach to a surface by the selective-bridging functional part of their surface polymeric structures including capsules, fimbriae, pili and slime (Katsikogianni & Missirlis, 2004), mediated by cell motility (Pang et al., 2005) and quorum sensing (Kim et al., 2009). Subsequently, mature biofilm forms through cell-cell interactions and cells aggregating on the surface (O'Toole et al., 2000).

2.5.4.1 Cell surface hydrophobicity

Upon contact with the substratum, short-range attachment forces, such as hydrophobic interactions, can mediate bacterial adhesion. Previous studies showed that the hydrophobic interaction played a key role in bacterial adhesion on an RO membrane surface (Ghayeni et al., 1998; Pang et al., 2005; Ridgway et al., 1985). Cell surface hydrophobicity also affects bacterial adhesion to different types of substrata (Gilbert et al., 1991). For example, the attachment of a hydrophobic strain of *Pseudomonas aeruginosa* to glass, copper, stainless steel, and silicon surfaces was more effective than a strain of *Pseudomonas fluorescens* with lower cell surface hydrophobicity (Mueller et

al., 1992). Ridgway et al. (1985), demonstrated that a hydrophobic *Mycobacterium* strain isolated from a biofouled RO membrane attached more strongly to cellulose acetate (CA) membranes than did a hydrophilic strain of *Escherichia coli*. However, other studies found that there was no relationship between cell surface hydrophobicity of 12 thermophilic *Streptococci* strains and their attachment to stainless steel (Flint et al., 1997b). The reason may be that the difference of cell surface hydrophobicity of different bacteria is due to the difference of cell surface molecules such as proteins and lipids (Palmer et al., 2007), while the surface composition of bacteria changes in response to the environment, therefore, there is no clear trend in cell adhesion based mainly on hydrophobicity effects (Araujo et al., 2010).

2.5.4.2 Cell surface charge

Bacterial cells generally have negative surface charge in aqueous suspensions with neutral pH (Rijnaarts et al., 1999). The bacterial surface charge differs according to species and is also influenced by factors such as growth medium, pH, the ionic strength of the suspending buffer, bacterial age, and bacterial surface structure (Katsikogianni & Missirlis, 2004). Studies showed that a *Dermacoccus sp.* strain having more negative surface charge generated more biofilms on an RO membrane surface than a *Sphingomonas sp.* strain with lower negative charge (Pang et al., 2005). Palmer et al. (2007) reviewed the relationship between cell surface charge and attachment on other substrata including meat surface, cantaloupe rind and stainless steel, however, the correlation is still not always clear.

2.5.4.3 Cell motility

The process of biofilm formation is a developmental process mediated by a combination of adhesion mechanisms and bacterial motility including swimming, twitching and swarming (Pang et al. 2005). Twitching motility is a form of surface translocation dependent on type IV pili and is demonstrated to be necessary for the formation of microcolonies within the biofilm (Harshey, 2003; O'Toole & Kolter, 1998). By extending and retracting their pili, bacteria can push or pull themselves across a surface (Bradley, 1980). Swimming and swarming motilities depend on flagella (Henrichsen, 1972). Swimming on a surface takes place when the fluid film is sufficiently thick and

the micro-morphological pattern is unorganized (Rashid & Kornberg, 2000). When the fluid layer on a surface is relatively thin, the swimming bacteria become elongated and hyperflagellated and move in a coordinated manner known as “swarming” (Rashid & Kornberg, 2000). Flagella and type-IV pili were found to play an important role in the early stages in biofilm development by *Pseudomonas aeruginosa* (O'Toole et al., 2000; Wall & Kaiser, 1999). It was observed that before attachment *P. aeruginosa* swims along the surface as if it is scanning for an appropriate location for initial contact. After forming a monolayer, *P. aeruginosa* continues to move along the surface with other bacteria using twitching motility instead of swimming motility (O'Toole et al., 2000). Twitching motility is a community behavior, as it occurs only when microorganisms are in contact with other cells (Semmler et al., 1999). Cell swarming motility is faster than other forms of surface motility (Harshey, 2003), which suggests bacterial strains with high swarming motility can possibly colonize membrane surfaces rapidly after initial attachment (Pang et al., 2005). However, by using a gene replacement method, Huber et al. (2001) found that swarming motility of *Burkholderia cepacia* H111 was not essential for biofilm formation on abiotic surfaces.

2.5.4.4 Quorum sensing

Recent molecular studies showed that quorum sensing was important for biofilm formation on membranes (Kim et al., 2009; Paul et al., 2009; Yeon et al., 2009). Quorum sensing refers to a cell-cell communication system (Choudhary & Schmidt-Dannert, 2010) which is a density-dependent regulation of gene expression in microorganisms (Tomlin et al., 2005). The sense mechanism is based on the production, secretion, and detection of small signal molecules, whose concentration correlates to the abundance of secreting microorganisms in the vicinity (Choudhary & Schmidt-Dannert, 2010). A coordinated change in the gene-expression profiles of communicating microorganisms occurs when the signal concentration reaches a threshold (Fuqua et al., 2001).

Quorum sensing can occur within a single bacterial species and between multiple species, mediated by signals for species-specific and interspecies communication respectively (Xiong & Liu, 2010). Species-specific quorum sensing is regulated by oligopeptides in Gram-positive bacteria and by *N*-acylhomoserine lactones (AHLs) in

Gram-negative bacteria (March & Bentley, 2004; Xiong & Liu, 2010). Autoinducer-2 (AI-2) is a universal signal recognised by both Gram-positive and Gram-negative bacteria (Miller & Bassler, 2001). Cholerae autoinducer 1 (CAI-1), produced by some *Vibrio* species was reported as a second type of interspecies autoinducer (Henke & Bassler, 2004).

Quorum sensing molecules are believed to be required for biofilm formation (Kuchma & O'Toole, 2000). Mutation of the *lasI* in the quorum sensing systems of *Pseudomonas aeruginosa* appear to affect the later stages of biofilm formation by forming a much thinner biofilm than the biofilm formed by the wild-type (Davies et al., 1998). The *cepIR* was required in attachment of *Burkholderia cepacia* to inert surfaces and the formation of mature biofilm structures (Huber et al., 2001). LuxS-dependent signal might play an important role in the biofilm formation of *Streptococcus mutans* (Merritt et al., 2003).

Quorum sensing can also affect bacterial motility (Morohoshi et al., 2007) which is related to biofilm formation (Pang et al., 2005). Morohoshi et al. (2007) found that *N*-nonanoyl-cyclopentylamide (C_9-CPA) was able to inhibit the swarming motility and biofilm formation of *Serratia marcescens* AS-1. Swarming motility of *B. cepacia* H111 is regulated by quorum sensing, possibly through the control of biosurfactant production (Huber et al., 2001). The *qseC* mutant (VS138) reduces flagella production and motility of *Escherichia coli* O157:H7 (Sperandio et al., 2002).

2.5.5 Membrane surface characteristics and biofilm formation

The surface properties of membranes are believed to be important in biofilm formation (Pasmore et al., 2001). Bacterial attachment is regulated by the physico-chemical nature of both the bacterial cell and the polymer membrane surface (Ridgway, 1991). In addition to the physico-chemical properties of membranes, the surface roughness, hydrophobicity and charge will also affect biofilm formation (Herzberg et al., 2009).

2.5.5.1 Surface roughness

Membrane roughness refers to the steepness, evenness and topology of peaks and

valleys on the membrane surface (Lee et al., 2010b). Membrane surface roughness is an important surface property affecting biofilm formation (Characklis, 1990b; Elimelech et al., 1997; Vrijenhoek et al., 2001). Surface roughness affects the development of younger biofilms more than mature biofilms (Pang et al., 2005). Pasmore et al. (2001) concluded that bacterial attachment was affected by surface roughness through two primary ways. Firstly, the roughness disrupts fluid flow by creating surface areas of low shear, where the forces that might remove attached bacteria are significantly reduced. Secondly, the increased roughness increases surface area that makes more room available for cells to attach and provides locations where cells can attach, since rough surfaces have contours and valleys (Pasmore et al., 2001). They also observed an increase in biofilm with *P. aeruginosa* on a rougher UF membrane surface (Pasmore et al., 2001). Similarly, it was found that the degree of roughness had a strong linear relationship with the maximum adhered cell concentration of *P. aeruginosa* PAO1 on nanofiltration (NF) membranes (Myint et al., 2010).

Pang et al. (2005) observed that both roughness and depression areas were increased when membranes were in a hydrated form when they analysed the surface morphology of dry and hydrated RO membranes made up of CA, polyamide (PA), and thin film composites (TFC) using atomic force microscopy (AFM) combined with scanning electron microscopy (SEM). They also compared the roughness of those three types of membranes and concluded that the CA membrane had the lowest roughness, while the PA membrane had the largest depression areas (18888 nm^2 for dry membrane and 33416 nm^2 for hydrated membrane). Microorganism entrapment is relatively easy in the depression areas, and therefore, PA membrane is more likely to promote biofilm formation (Pang et al., 2005). Similar observations were also reported by Campbell and co-workers (Campbell et al., 1999), who studied the attachment of *Mycobacterium sp*. onto PA and CA membranes in batch assays.

2.5.5.2 Surface hydrophobicity

The hydrophobicity of inanimate substrata influences the strength and kinetics of microbial adhesion and early biofouling (Ridgway et al., 1999). It has been proposed that a hydrophobic substratum attracts bacteria with hydrophobic surface and a hydrophilic substratum attracts bacteria with hydrophilic surface (An & Friedman, 1998;

Katsikogianni & Missirlis, 2004). It was found that a NF membrane, which is relatively hydrophilic, has a higher potential for biofouling by hydrophilic bacteria than a hydrophobic UF membrane (Lee et al., 2010a). Pasmore et al. (2001) found that biofilm initiation by a *P. aeruginosa* strain increased as a UF membrane surface became more hydrophobic. Similarly, Lee et al. (2010b) observed that the adhered cell concentration of *P. aeruginosa* PAO1 increased proportionally to the RO membrane hydrophobicity.

2.5.5.3 Surface charge

Most polymer membranes possess some degree of surface charge due to trace quantities of free carboxylate or sulfonate groups (Ridgway et al., 1999). The charge of the substrate surface can affect the attractive and repulsive forces between the bacterial cells and substrate (Pasmore et al., 2001). Charge attraction was even suggested as having a stronger effect than hydrophobicity on fouling (Koo et al., 2002). Under physiologically relevant pH values (~7), RO membranes tend to be negatively charged (Elimelech et al., 1997; Vrijenhoek et al., 2001). Negative membrane surface charge can reduce fouling due to electrostatic repulsion of negatively charged bacterial surfaces (Her et al., 2000). However, other studies observed that the ability to recover the performance upon washing was higher for membranes with chemically neutral surfaces than for charged membranes (Kochkodan et al., 2006; Pasmore et al., 2002).

2.5.6 Other factors

Biofilm formation is an extremely complicated process that is affected by various factors. In addition to the factors described above, biofilm formation is also influenced by the environmental parameters, such as the flow conditions, the level of nutrients, the concentration of electrolytes and the pH value (Lee et al., 2010b).

Flow rate is considered a dominant factor that strongly influences bacterial adhesion (Isberg & Barnes, 2002) and biofilm structure (Stoodley et al., 1999b). Higher shear rates result in higher detachment forces that decrease the number of adhered cells (Katsikogianni & Missirlis, 2004). However, studies show that a high flow rate will not prevent bacterial attachment nor completely remove existing biofilm (Dreeszen, 2003), but it will make the biofilm denser and thinner (Chang et al., 1991). This may due to the

lower growth yield obtained when the shear rate is increased (Katsikogianni & Missirlis, 2004).

Bacteria require certain nutrients for growth and multiplication. Limiting the nutrients will limit bacterial growth. However, biofilm will reach a certain equilibrium thickness depending on both shear force and available nutrient levels (Dreeszen, 2003). For instance, Ivnitsky et al. (2005) observed a bacterial count of approx. 10^7 CFU/cm^2 in biofilm on a NF membrane surface regardless of the feed applied.

Ionic strength and pH influence bacterial attachment by changing surface characteristics of both the bacteria and the materials, resulting in changing interactions between bacteria and surfaces (Katsikogianni & Missirlis, 2004). Bunt et al. (1993) found that pH and ionic strength influenced the cell surface hydrophobicity and charge. Highest adhesion to hydrophobic surfaces was found at pH in the range of the isoelectric point when bacteria are uncharged (Bunt et al., 1993). Increasing solution pH in a range (pH 3 – 9) higher than their isoelectric points (pH 3 – 4) resulted in an increased negative surface charge of the PA membranes, and an increased rejection through electrostatic repulsion (Bellona & Drewes, 2005). The chemicals adsorbed to the membrane surface are responsible for most of the changes in surface properties (Pasmore et al., 2001). Studies have shown that positively charged ions such as sodium, calcium, magnesium and cationic surfactants can bind to the negatively charged membrane surface resulting in a reduced negative surface charge (Bellona & Drewes, 2005).

2.6 TECHNIQUES FOR STUDYING BIOFILM ON MEMBRANES

2.6.1 Isolation and identification

Biofilm microorganisms are normally scraped using a sterile scalpel, or swabbed from the biofilm growing surface, and transferred onto agar plates for multiplication, identification or selection (Dautle et al., 2003; Flemming et al., 2007). Piao et al. (2006) used sonication at 80 W for 2 min in an ice-water bath to dislodge bacteria in biofilms on a membrane surface. In addition to the classic microbiological methods, such as biochemical characterisation using kits such as the BBL CRYSTAL or API identification systems (Dautle et al., 2003; Tang et al., 2009a), the identification of

microorganisms from biofilm is often based on molecular techniques, such as 16S rRNA gene polymerase chain reaction (PCR) cloning and sequencing (Kwon et al., 2002; Liaqat & Sabri, 2009; Piao et al., 2006).

2.6.2 Characterisation of microorganisms

2.6.2.1 Microtitre plate assay

A microtitre plate assay is widely used for detecting the propensity of bacteria to stick to surfaces (Li et al., 2003; Pitt & Ross, 2003). The microorganisms that have the ability to form biofilms attach and grow on the surfaces of wells of microtitre plates. By using stains such as crystal violet, the microorganisms adhering on the surfaces can be stained, with the amount of stain being retained by the biofilm representing the amount of biofilm present.

2.6.2.2 Cell surface hydrophobicity and charge

Cell hydrophobicity can be determined by using the microorganism adhesion to hydrocarbon (MATH) test, the loss in absorbance in the aqueous phase relative to the initial absorbance value being taken to represent the amount of cells adhering to hydrocarbons, e.g. hexadecane and xylene (Rosenberg et al., 1980). However, if the cell surface has an extremely high affinity to water, it will be difficult to obtain change of absorbance (Pang et al., 2005). It has been suggested that the MATH test should be measured at pH values where the zeta-potential of the test organism and/or hydrocarbon are near zero to reduce the potential interference of electrostatic interactions (van der Mei et al., 1995). Alternative tests can be used to determine cell hydrophobicity including hydrophobic interaction chromatography (HIC) (Palmer et al., 2007) and water contact angle measurements (van der Mei et al., 1998). Cell surface charge can be determined by measuring zeta potential (Pang et al., 2005).

2.6.2.3 Cell motility

Twitching motility is a special kind of bacterial surface translocation that may lead to the production of spreading zones on solid surfaces (Henrichsen, 1983). For testing the

twitching motility, Pang et al. (2005) stab-inoculated the bacterial strains to the underlying Petri dish of 1% (w/v) agar plates using sterile toothpicks. After incubation, the agar was removed and unattached cells were rinsed off gently in a stream of ultrapure water. The zone of twitching motility was then visualised by staining the attached cells with 1% crystal violet (Pang et al., 2005).

Swimming motility can be assessed qualitatively by examining the circular hazy zone formed by the bacterial cells migrating away from the point of stab-inoculation within the 0.3% (w/v) agar using sterile toothpicks (Pang et al., 2005). The swim plates should be wrapped to prevent dehydration (Rashid & Kornberg, 2000).

Swarming motility can be determined based on movement of bacterial growth on the surface of the plate away from the point of inoculation (Pang et al., 2005). For testing the swarming motility, Pang et al. (2005) point-inoculated 1 µl of liquid culture onto the surface of 0.5% (w/v) agar. Rashid & Komberg (2000) found that swarming efficiency could be improved when cells were inoculated onto swam plates from previously incubated swim agar plates.

2.6.3 Characterisation of membrane surface

2.6.3.1 Membrane surface hydrophobicity

Solid surface hydrophobicity can be determined by measuring contact angles (Yasuda et al., 1994). However, membranes cannot be air dried, as is required for measuring contact angle between water and the surface, without introducing significant surface artifacts, such as shrinkage and cracking (Ridgway et al., 1999). Therefore, membrane surface hydrophobicity is usually determined by measuring the contact angle between an air bubble of defined volume and the membrane surface immersed in a temperature controlled bath, known as captive (air) bubble method (Zhang et al., 1989).

2.6.3.2 Membrane surface charge

Membrane surface charge can be determined by the uranyl cation-binding assay (Ridgway et al., 1999), and the zeta potential can also be measured by using a streaming

potential analyzer (Pang et al., 2005).

2.6.3.3 Membrane surface topography

AFM can be used to investigate membrane surface topography (Ridgway et al., 1999). It can also be used for measurements of electrostatic forces for a number of systems, including surfactants, bacteria and cell adhesion proteoglycans (Frank & Belfort, 2003).

2.6.4 Biofilm structure

SEM and confocal laser scanning microscopy (CLSM) are widely used for visualizing and investigating biofilm structure. A membrane sample carrying biofilm can be fixed and dyed with suitable stains for reading under the CLSM, or examined under the SEM without dying (Camargo et al., 2005), when the parameters of biovolume and substratum coverage can be analyzed (Pang et al., 2005).

2.6.5 Models and bioreactors for biofilm study

Molecular modeling techniques are proposed for exploring and delineating some of the theoretical mechanisms underlying primary bacterial adhesion to synthetic membrane materials. Such techniques may provide information on the structures and conformations of the adhesive biopolymers and the membrane materials, and their dynamic interactions in different chemical environments. However, accurate modeling needs proper software tools (Flemming, 2003).

A recent biofilm project used a continuous flow model (Pang et al., 2005) as in Figure 2.5, to investigate biofilms on membrane surfaces. However, using this model, only one sample can be obtained for each run. This model examines only flow parallel to the membrane and not through the pores. Laminar or turbulent flow in glass flow cell biofilm reactor can be achieved by adjusting flow velocity (Stoodley et al., 1999a).

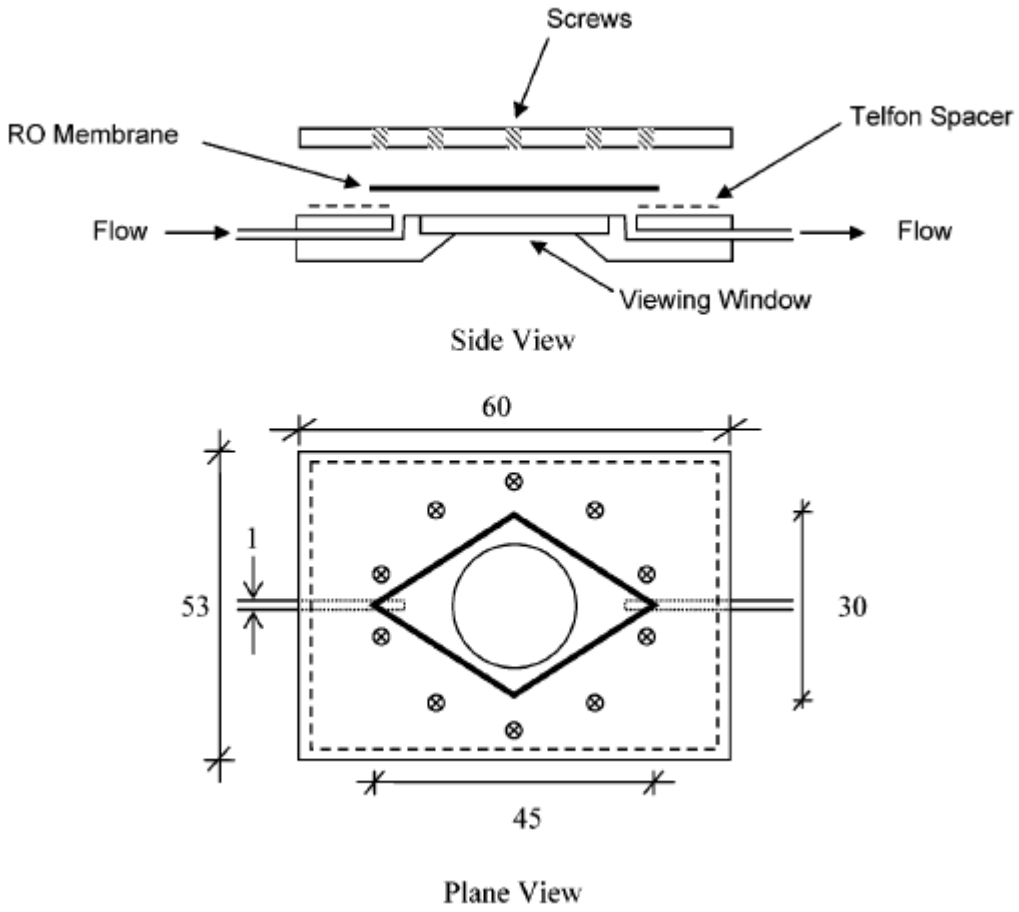


Figure 2.5: Schematic representation of the flow cell used in monitoring biofilm development. The channel depth is set by the thickness of the Telfon spacer (1mm). All dimensions are given in mm. (From Pang et al. 2005; used with permission from American Chemical Society.)

A CBR 90 biofilm reactor (BioSurface Technologies, Bozeman, USA) (Fig. 2.6) that can generate up to 24 coupon samples was tested by Goeres et al. (2005). However, the target coupon surface material is polystyrene, which results in difficulties for comparison with membrane surface materials.

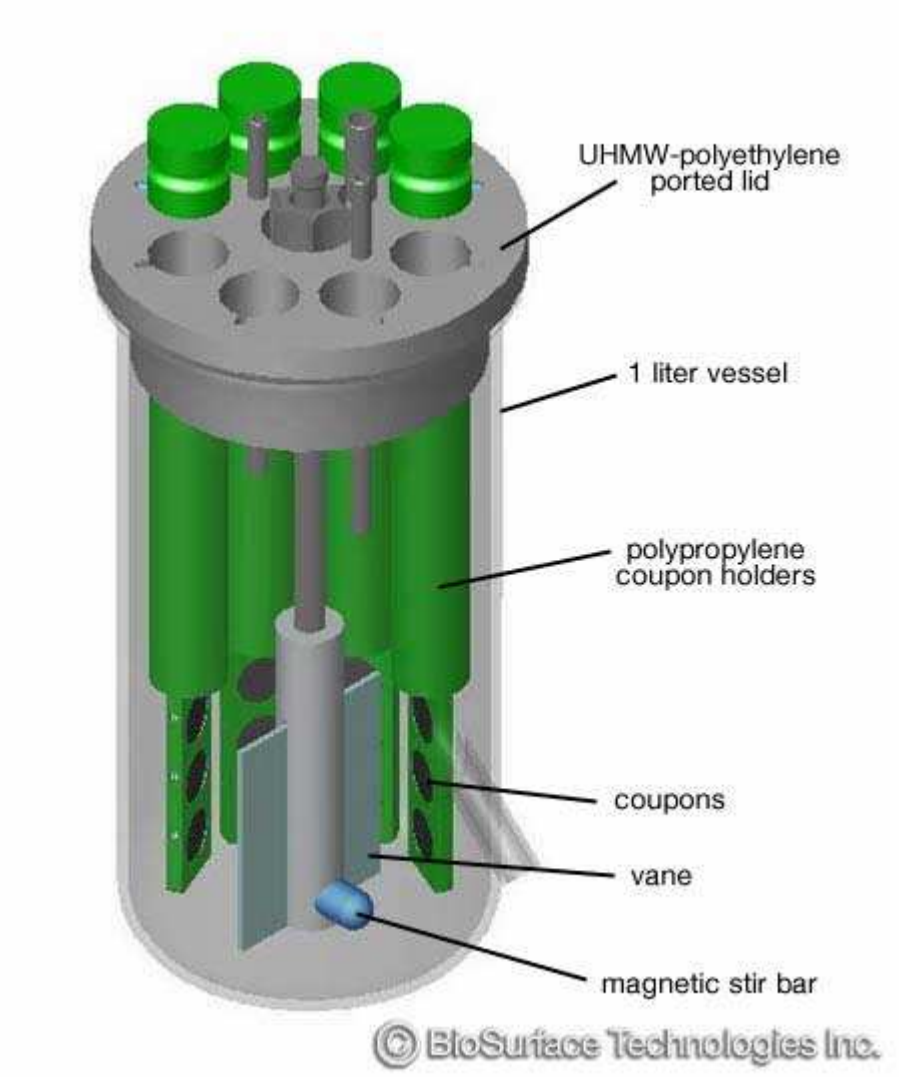


Figure 2.6: The CBR 90 biofilm reactor.

(From http://cu.imt.net/~mitbst/CDC_Specs.html; used with permission from BioSurface Technologies Inc.)

2.7 CONTROL OF BIOFILM ON MEMBRANES

Biofilms on membrane surfaces have been physically, chemically and biologically treated in order to find efficient cleaning strategies. However, membrane materials are sensitive, therefore, control of biofilm on membranes needs particular caution in both the efficiency of the cleaning strategies and the effects on membranes (McDonoug & Hargrove, 1972). It was found that preventing the initial attachment of bacteria on a membrane surface was more important than killing bacteria that had already attached (Liu et al., 2010). Therefore, membrane engineering was used to modify membrane surfaces in terms of reducing or preventing bacterial adhesion (Liu et al., 2010; Yang et al., 2009).

2.7.1 Physical methods

Currently, physical control for membrane biofouling includes reducing the concentration of solids in the liquid flow into the membrane module, applying a tangential surface shear force and backwashing the membrane module (Chang et al., 2002). However, it was suggested that a high tangential shear force may result in the development of a structurally strong biofilm that could resist shear force (Percival et al., 1999).

2.7.2 Chemical methods

Biocides decrease bacterial levels but are less efficient on biofilms than planktonic (free floating) cells and do not penetrate the biofilm matrix on the surface (Simoes et al., 2006). The food debris and other residues that may contain microorganisms may actually behave as a surface for colonization (Simoes et al., 2006). Sanitation of membrane plants should therefore be carried out following an effective cleaning (D'Souza & Mawson, 2005).

Hypochlorites and hydrogen peroxide have been widely used as effective disinfectants (D'Souza & Mawson, 2005). Chlorine dioxide is less effective than many other chlorine based sanitisers (e.g. dichloroisocyanurate) (Bohner & Bradley, 1992). Hypochlorites working as membrane-swelling agents assist in flushing out material lodged within the

membrane pores (Cheryan, 1998), however, they often decrease membrane life and should be used with caution (D'Souza & Mawson, 2005).

Peracetic acid which is a mixture of acetic acid and hydrogen peroxide in an aqueous solution can pass through RO membranes, enabling sanitation of the permeate side, and has good rinsability (Krack, 1995). Also, peracetic acid-based disinfectants generally do not lead to resistance (Bore & Langsrud, 2005). Therefore, they are expected to perform with a consistent efficiency in killing bacteria.

Quaternary ammonium compounds and iodophore-based products cannot be used on membranes, as they are often adsorbed onto the membrane surface, causing flux decline and irreversible damage to the membrane (Krack, 1995). Sodium metabisulphite can be used on more sensitive membranes; nevertheless, it requires long contact time (Krack, 1995). Prolonged exposure to chemicals (e.g. hypochlorite) may damage the membrane structure (Begoin et al., 2006; Causserand et al., 2006).

Ozone is believed a more powerful sanitiser than chlorine (Flint et al., 1997a). It was observed that although it had an antibacterial effect on planktonic *Enterococcus faecalis* cells, ozone had little effect on its biofilms (Hems et al., 2005). However, ozonation significantly increased the disinfection capacity of a membrane plant in Amsterdam (van der Hoek et al., 2000).

Surfactants and surfactants/biocide combinations have been widely used to treat biofilms. It was found that the combination of sodium dodecyl sulfate with urea was the most effective surfactant combination for cleaning RO membranes (Whittaker et al., 1984). A surfactant (Teepol) has been suggested that can increase the negative cell wall charge of *Burkholderia sp.* JS150 and reduce the biofilm accumulation on membrane surfaces (Splendiani et al., 2006). Humic substances and surfactants adsorbed to the membrane can also influence membrane surface charge (Childress & Deshmukh, 1998).

The anolyte of electrolysed water produced by the electrolysis of sodium chloride solution using elctrolysis apparatus was found to be an alternative way of controlling biofilms (Thantsha & Cloete, 2006). The anodic solution has high levels of dissolved oxygen and available chlorine in a form of hypochlorous acid with strong potential for

sterilization (Mahmoud, 2007). Compared with the biocides, electrolysed water is less toxic, less volatile, easier to handle and compatible with other water treatment chemicals (Lenonov, 1997).

2.7.3 Biological methods

Enzymatic cleaning agents can be used to treat fouling on membranes and they are less aggressive to the membranes than many chemical cleaners (D'Souza & Mawson, 2005). Compared with the traditional cleaning method using alkali, it was found that enzymatic cleaning by protease had a much better performance in terms of removing biofilms from an UF membrane for wastewater treatment (Poele & van der Graaf, 2005).

Bacteriophage can infect the host bacteria by rapid replication of virions to lyse the host cells or by incorporation into the genome of host cells (Xiong & Liu, 2010). The advantage of bacteriophage is that it can continuously infect/multiplicate as long as the host is present and grows (Goldman et al., 2009). It has been reported that bacteriophage can reduce microbial attachment to UF membrane surface by an average of 40% (Goldman et al., 2009). However, bacteriophage tends to be rather host-specific and so cocktails of phage would be required for reliable microbial control.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) extracted from vanilla beans was reported to inhibit the short-and long-chain AHL-mediated quorum sensing system (Xiong & Liu, 2010), and is able to prevent RO membrane from biofouling by *Aeromonas hydrophila* (Ponnusamy et al., 2009).

2.7.4 Membrane modification

Membrane modification for reducing or preventing bacterial adhesion includes photochemical modification, plasma treatment, the radiation-induced grafting of monomers and the photo-induced polymerization of different monomers (Kochkodan et al., 2006).

It was found that membranes (PS, PES and regenerated cellulose) deposited with TiO_2 particles under black UV-irradiation at 365 nm had a strong photobactericidal effect and

resulted in 1.7-2.3 times higher water fluxes compared with those for control membranes (Kochkodan et al., 2008).

To reduce the hydrophobic interaction between bacteria and membranes, PES and PS membranes were modified with three hydrophilic monomers using UV-assisted graft polymerization (Kaeselev et al., 2001).

PES membrane surfaces can be modified by argon plasma treatment followed by polyacrylic acid grafting in a vapor phase, allowing the membrane surfaces to become permanently hydrophilic (Wavhal & Fisher, 2002). This modified membrane is also easier to clean and requires little caustic to recover permeation flux (Wavhal & Fisher, 2002).

Yang et al. (2009) observed good performance of an RO membrane used for seawater desalination, when coated with nanosilver. They treated either membrane surfaces or membrane spacers and found that almost no multiplication of cells was detected on the membrane when the membrane spacer was nanosilver-coated (Yang et al., 2009).

2.8 CONCLUSIONS

Biofilm formation is a major impediment to the use of filtration membranes in cross-flow processes in dairy plants. Membrane cleaning strategies require improvements for effective control of biofilms.

The main effects of biofilm on membranes are: (1) reduction of membrane flux and productivity, (2) the biodegradation of the membrane material, (3) an increase in power consumption for raising operation pressure, (4) increase in the cost of cleaning and even consequent replacement of membrane modules.

The initiation of biofilm formation on membrane surfaces not only depends on the physical and chemical characteristics of membranes, but also on the characteristics of early adhering bacteria and the operating conditions inside the membrane system. A suitable laboratory scale biofilm reactor must be developed that can closely mimic the conditions in the dairy membrane plant to enable further study of the factors (e.g.

membrane material, strains, feed, flow rate, pH and temperature) affecting biofilm formation and membrane cleaning.

Dairy manufacturers have been focusing on the control of biofilm formed by *Pseudomonas* species and food borne pathogens (Flint et al., 1997a). Detailed studies of biofilm on membranes need an understanding of the microbial community that exists in membrane plants. For example, if membranes are predominantly colonized by mixed species biofilms, this will have an impact on the ability to clean. The biofilm developed by mixed cultures is more complicated than that of pure cultures. A map showing where control should be focused can be generated only when the mechanisms of biofilm formation by the true biofilm formers are explored. This requires setting up a microbe library for specific membrane plants before further study can be carried out.

The control of biofilm on membranes in the dairy industry has been dependant upon frequent CIP with chemicals, enzymes or disinfectants/sanitizers commonly used in cleaning systems in food manufacturing plants. However, a study of improved control strategies should focus on both the membranes (e.g. selection of membrane materials with modifications) for lowering bacterial attachment and improvements in membrane cleaning methods for eliminating the biofilm and preventing re-growth.

Chapter 3

ISOLATION AND IDENTIFICATION OF MICRO-ORAGANISMS AND THE MEASURMENT OF PROTEIN AND CARBOHYDRATE ON MEMBRANE SURFACES

3.1 INTRODUCTION

One of the limitations in the use of UF and RO membranes is fouling during filtration, including the biofouling by microorganisms. Biofilm development on membranes reduces filtration efficiency and eventually results in the need for replacement. In addition, there is a potential for biofilms to release bacteria and contaminate the final products made by membrane processing.

Biofilm is usually made up of layers of assorted microbial populations, mostly bacteria, held together in a sticky matrix of EPS (Wingender et al., 1999). The formation of biofilm is initiated by the attachment of microorganisms. Therefore, to study the particular biofilm on particular membrane samples, it is important to investigate the population existing in the biofilm.

Because of the high price of the membrane modules used in the dairy manufacturing plant, it is generally not possible to obtain the membrane samples until the membranes are due for replacement. The decision to replace membranes may be triggered by membrane leakage, unrecoverable fouling or any other damage. Whether the membrane will be replaced is most often determined after cleaning. Routine CIP in dairy plants may not remove all microbial cells (Flint et al., 1997a) and these cells may allow more rapid recolonisation of the plant (Marshall, 1992). Therefore, in this trial the focus was on microorganisms that can survive membrane cleaning. We assumed that the presence of microorganisms on cleaned membrane surfaces is a good indication of their resilience in this environment and their potential to form biofilms.

The surface of membrane samples from the dairy manufacturing plants may have a conditioning film of proteins or carbohydrates that provide the surface for microbial

attachment. Such a conditioning film may change the surface properties (Dickson & Koohmaraie, 1989) and enhance biofilm formation, as milk components on the surfaces had a high protective effect on bacteria (Mattila et al., 1990).

The objective of this study was to set up a culture library of the isolates from UF and RO membrane plants and quantify the protein and carbohydrate residues on the surfaces of the membrane samples.

3.2 MATERIALS AND METHODS

3.2.1 Source of samples

The spiral-wound UF and RO membranes were obtained from dairy plants in New Zealand. All membranes had been in routine use in manufacturing plants processing milk, whey or whey permeate. Membrane plants operated under turbulent flow at pH 4.6–6.2. Specific details of the shear rate and flux were not provided, though all dairy product manufacturers aim to operate the plants according to the guidelines obtained from membrane manufacturers. Membranes had been cleaned, using the standard caustic based clean-in-place (CIP) system in the plant, before being removed, sealed in plastic bags to retain moisture and sent by courier to our research laboratory. The details are described in Table 3.1.

Table 3.1: Details of membrane samples from New Zealand dairy manufacturing plants.

Manufacturing Plant	Sample Details
A	Polyethersulphone (PES) RO membrane used for processing whey at 10-12ºC
B	Four PES UF membranes used for whey processing under temperatures of 10-12ºC and sometimes 55ºC. Four different stages of the plant were labeled as 1-4.
C	PES RO membrane used for processing casein whey permeates at 10-12°C
D	Two PES RO membranes used for the milk permeate treatment at 10-12ºC. Loop 1 was the first stage of the membrane processing, while loop 4 was the last stage.
E	PES RO membrane from a pilot plant used for processing milk protein concentrate at 10-12°C
F	PES UF membrane used for whey processing at 55°C
G	PES RO membrane used for whey processing at 55°C

Additionally, some liquid samples, including feed solution, diafiltration water, retentate and permeate from the first and last stages of the plant, were taken from a UF membrane plant processing whey at 10-12ºC in manufacturing plant A and kept on ice for transfer to the laboratory.

Some foulant (14.8 g), which was considered to contain biofilm, was scraped from a membrane surface area of approximately 100 cm^2 in plant C before CIP and forwarded to the laboratory.

3.2.2 Isolation and identification

To obtain microbial isolates from the surfaces of PES spiral wound UF and RO membrane samples, the membrane cartridges were cut into sections (30 cm in length) using a sanitized band saw. Small pieces (2 cm × 4 cm) of membrane were cut from the unrolled membrane sections using sterile scissors and observed under the microscope to record the general appearance of fouled zones on the membrane surface. In order to examine the total (viable and non-viable) microbial content of the deposits on the membrane more thoroughly, solid deposits were removed using sterile swabs and transferred to microscope slides for Gram staining and observation. As the microflora on the membranes had survived cleaning, we assumed that much of the population was firmly attached to the membrane and therefore difficult to remove. To isolate these firmly attached cells, membrane samples were incubated on skim milk agar (SMA) plates (Merck, Germany) by placing either the permeate side or the retentate side directly onto the SMA. For each membrane sample, at least 30 plates were incubated at three different temperatures (25ºC, 30ºC, 37ºC and 52ºC). After incubation, the predominant colony types were streaked onto SMA for subsequent identification using the API culture identification system (BioMerieux, Durham, NC, USA).

3.2.3 Quantification of membrane surface protein and carbohydrates

The solid deposit was scraped from one PES RO membrane after CIP from plant C (Table 3.1) using sterile swabs and weighed. The membrane surface area sampled was approximately 100 cm^2. The protein and carbohydrate composition of 1 g of this solid deposit (after CIP) and of the 14.8 g foulants obtained from the same membrane plant (before CIP) (Section 3.2.1) was determined using the Bradford assay (Bradford, 1976) and 3,5-dinitrosalicylic acid (DNS) assay (Bernfeld, 1955) respectively. The standard curve for measuring membrane surface protein was obtained using bovine serum albumin (BSA) (Sigma). The standard curve for measuring membrane surface carbohydrate was obtained using glucose.

3.3 RESULTS

3.3.1 Examination of the membranes and isolation of micro-organisms

Large amounts of solid material were visible macroscopically on the membrane surface (Fig. 3.1).

Figure 3.1: Surfaces of a piece of UF membrane after being CIP treated.

On many membrane surfaces few bacteria were observed microscopically, suggesting that either these membranes were clean or the contamination remaining after cleaning was too low to be detected. However, the scrapings from one RO membrane showed both yeast (*Blastoschizomyces capitatus*) and bacteria (*Pseudomonas fluorescens, Klebsiella oxytoca* and *Bacillus licheniformis)* (Fig. 3.2).

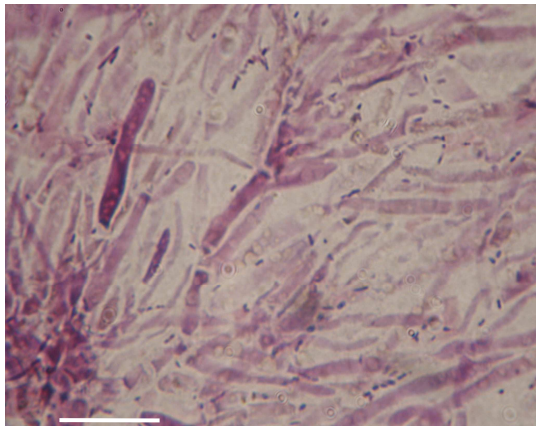


Figure 3.2: Gram stain images of the microorganisms on a PES RO membrane photographed using a light microscope. This image showed the mixture of the yeast (*Blastoschizomyces capitatus*) and bacteria found in the biofilm on membrane.

Strains isolated from permeate and retentate sides of each membrane were different (Fig. 3.3).

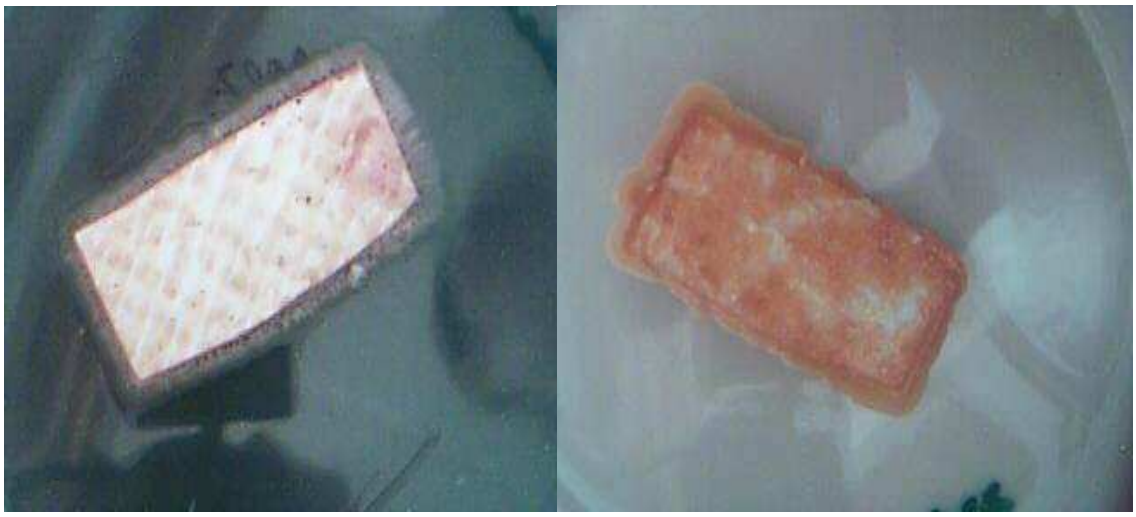


Figure 3.3: Different species grew on the retentate side and permeate side of the same membrane. (Left: on retentate side; Right: on permeate side)

Seven of the 13 isolated strains were Gram-negative micro-organisms (Table 3.2). No culture was successfully recovered at 52ºC.

Table 3.2: Strains isolated from the dairy membrane plants. (WP = Whey Permeate; MP = Milk Permeate)

Strain	Species	Dairy Plant	Type of plant (Feed)	Permeate / Retentate Side of Membrane
WL001	*Chryseobacterium indologenes*	A	UF (whey)	Retentate Side
WL004	*Bacillus firmus*	A	UF (Whey)	Retentate Side
WL008	*Lactococcus lactis* ssp *cremoris*	A	UF (Whey)	Retentate Side
B001	*Klebsiella oxytoca*	A	UF (Whey)	Permeate side
B003	*Cronobacter sakazakii*	A	UF (Whey)	Permeate Side
B006	*Klebsiella oxytoca*	A	UF (Whey)	Permeate Side
WA001	*Lactobacillus*	B	UF (Whey)	Permeate Side
WA002	*Bacillus licheniformis*	B	UF (Whey)	Retentate Side
TR001	*Pseudomonas fluorescens*	C	RO (WP)	Retentate side
TR002	*Klebsiella oxytoca*	C	RO (WP)	Retentate Side
TR004	*Bacillus licheniformis*	C	RO (WP)	Retentate Side
H1	*Blastoschizomyces capitatus*	C	RO (WP)	Retentate Side
EL4019	*Klebsiella oxytoca*	D	RO (MP)	Retentate Side

3.3.2 Membrane surface protein and carbohydrates

The standard curves for measuring membrane surface protein and carbohydrates are shown in Figure 3.4 & 3.5.

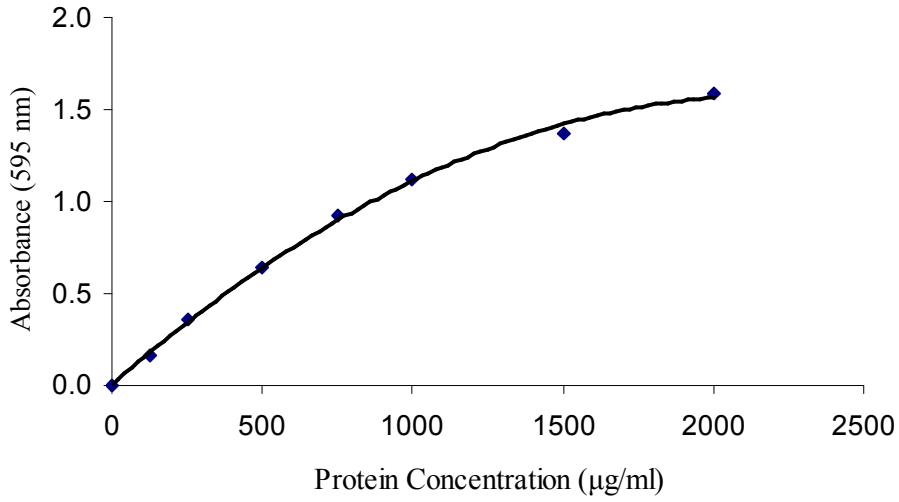


Figure 3.4: Standard curve for Bovine Serum (Bradford Assay). (R^2=0.9993)

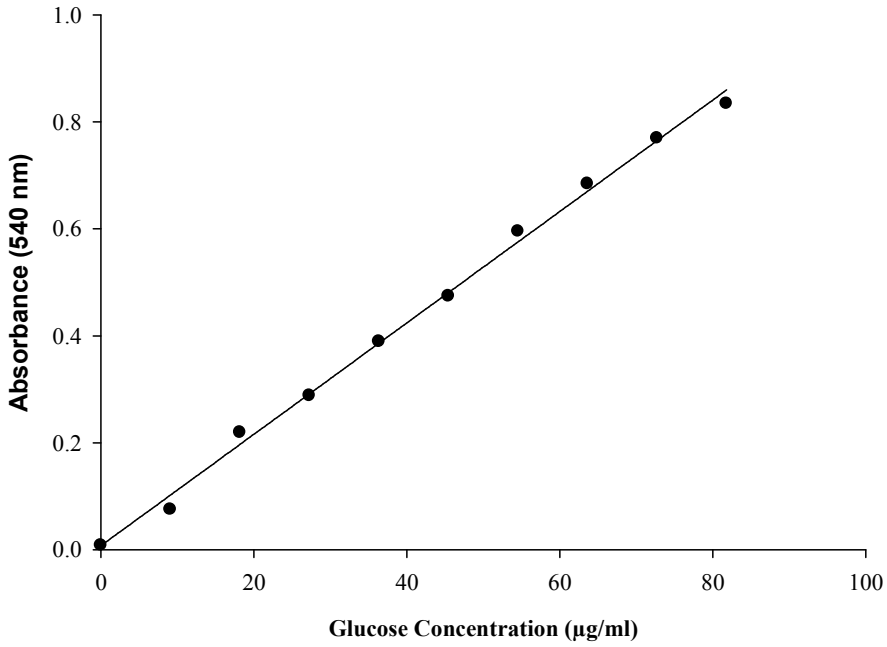


Figure 3.5: Standard curve for glucose (DNS Assay). (R^2=0.9966)

The amounts of protein and carbohydrate deposits on membranes from Plant C were measured before and after CIP. The total solid deposits on a membrane surface area of 100 cm^2 were 14.8 g before CIP and 2.1 g after CIP. The typical CIP removed about 86% solids from the membrane surfaces. The results in Table 3.3 show very low protein and carbohydrate levels in the foulant, both before and after CIP. There was an obvious decrease in both organic residues after CIP treatment. The CIP removed 95.8% protein and 89.2% carbohydrates in terms of unit area.

Table 3.3: Protein and carbohydrate content of solid material from a PES RO membrane before and after CIP treatment. (Results are shown as means of dry weights per cm^2 that were taken from triplicates.)

Membrane (Plant C)	**Protein ($mg \cdot cm^{-2}$)**	**Carbohydrate ($mg \cdot cm^{-2}$)**
Before CIP	4.55	0.83
After CIP	0.19	0.09

3.4 DISCUSSION

The predominant genera in raw milk are Gram-positive bacteria (Lewis & Gilmour, 1987). Whey normally contains predominantly Gram-positive organisms from the starter population (lactic acid bacteria) (Friedrich & Lenke, 2006) or thermo-resistant species such as spore-forming *Bacillus* species (Schreiber, 2001). However, the predominant isolates recovered from the surfaces of our membrane samples taken from dairy manufacturing plants were Gram-negative bacteria. Therefore, the high proportion of Gram-negative isolates, especially coliforms, found in this study indicates that the most likely source of contamination may be poor water quality or insufficient plant CIP, which accumulates microorganisms on the equipment surfaces, resulting in biofilm formation (Kumar & Anand, 1998). The isolates obtained from the permeate side may be the result of the leakages of the membrane modules, such as damage of membrane sheets and permeate collection tubes resulting in opportunities for bacteria to contact the

permeate side of membranes. Cultures on the retentate side and permeate side of the UF membrane samples were different (Table 3.2). This may due to the different surface characteristics of membrane permeate and retentate sides and nutrient levels (Bryers, 1987; Kumar & Anand, 1998; Pasmore et al., 2001; Ridgway, 1991). Also the backwash cleaning process (Goldman et al., 2009) may bring contamination from the collection side. There were no isolates recovered from the membranes from plants operating hot (55ºC) processes. The bacteria existing in the biofilm in those hot membrane plants were probably non-culturable, as it was found that some of the bacteria in biofilm on the surfaces in dairy environments are subjected to various stresses such as starvation, chemicals, heat, cold and desiccation which injure the cells, rendering them non-culturable (Wong & Cerf, 1995). Our sampling method used in this study has a limitation in that it is only able to detect culturable microorganisms in the biofilm. The isolates were identified using biochemical methods. However, the results could be confirmed using some biomolecular techniques (i.e. PCR and sequencing).

Cronobacter sakazakii is a common environmental contaminant (Lehner & Stephan, 2004) and therefore it is not too surprising that it was found together with other Gram-negative micro-organisms. The presence of this organism is a concern in infant formula(Lehner & Stephan, 2004), but none of the manufacturing plants we studied produced infant formula. Manufacturers of infant formula need to know more about the ecological niche of this micro-organism and how it enters dairy processes.

The isolation of the *Klebsiella* species from more than one membrane modules suggests that they are likely components of a biofilm rather than accidental contaminants entering during sampling. Previously, *K. oxytoca* has been found in milk products in Jordan (El-Sukhon, 2003). Tondo et al. (2004) in Brazil identified a *K. oxytoca* strain from raw milk producing heat stable protease. Mattila et al. (1990) isolated *Klebsiella spp.* from a milking line in a Finland dairy plant. Sharma and Anand (2002) found that *Klebsiella spp.* were predominant Gram-negative isolates in the biofilms from post-pasteurization lines in an experimental dairy plant in India. Both their and our current studies showed the presence of *Klebsiella spp.* after CIP (Mattila et al., 1990; Sharma & Anand, 2002). However, this is so far as we know the first report of *K. oxytoca* strains being isolated from dairy UF and RO membrane plants. They were probably introduced

into the membrane plants through diafiltration water and CIP liquids. Therefore, a study on these *K. oxytoca* strains is of importance.

No attempt was made to examine anaerobic bacteria from UF membrane surfaces. Previous study showed that dairy manufacturing membrane contamination is primarily due to aerobic microflora (Bore & Langsrud, 2005; Flint et al., 1997a), therefore, the isolation of anaerobic microorganisms was not expected to represent a significant population in the dairy biofilms.

The low levels of protein and carbohydrate on the membrane from Plant C possibly reflected the way these membranes were used. That membrane module was previously used for processing whey permeate in which there was little protein and most of the lactose would have passed through the membrane, so that the amount remaining on the membrane surface was low.

Although the current CIP had removed much of the organic material from the membrane surface, still some remained. Residual organic material may contribute to biofilm formation, by enhancing bacterial attachment and protecting microbial cells from cleaning. We assume that there will be much more organic residues left on the surfaces of membranes used to process milk or whey rather than whey permeate. The successful isolation of microorganisms from the membranes after CIP indicates that the current CIPs were not efficient in terms of biofilm removal, leaving a seed for further biofilm development.

Since biofilm formation is initiated by the cell attachment to the surface, the ability of these strains to attach was investigated in subsequent experiments. Whey medium containing protein and carbohydrates was used for studying the cell attachment and cell surface characteristics that are important in the initiation of biofilm.

3.5 CONCLUSIONS

A culture library with thirteen identified strains was prepared from isolates obtained from biofilms on UF and RO membranes. The results supported the hypothesis that a variety of different microorganisms is associated with dairy UF and RO membranes

after cleaning, indicating several possible sources of contamination. The routine CIP currently used in the dairy plants is not adequate to completely remove organic material, including microbial cells, from membrane surfaces. The residues of proteins and carbohydrates remaining on the membranes after CIP might influence surface characteristics of microorganisms and membranes and thus affect the biofilm formation. This is the first report of *K. oxytoca* being isolated from dairy UF and RO membrane plants. Others have also reported *Klebsiella spp.* in dairy products (El-Sukhon, 2003; Tondo et al., 2004) or dairy processing lines (Mattila et al., 1990; Sharma & Anand, 2002). These references indicate that *K. oxytoca* strains did not turn up by chance and our isolation is representative. *Klebsiella* strains were found on membranes from three different manufacturing plant sites, suggesting that a study on these strains is of importance.

Chapter 4

CELL SURFACE CHARACTERISTICS AND ADHESION

4.1 INTRODUCTION

A biofilm is initiated by the attachment of microorganisms to a surface and developed when the attached microorganisms start growing (Ivnitsky et al., 2005). The formation of mature biofilms is thought to be the result of early surface colonization by some microorganisms that change the surface properties and facilitate attachment and growth of others (Dang & Lovell, 2000; Jefferson, 2004). Initial adhesion of microorganisms to surfaces is essential for biofilm formation (Dang & Lovell, 2000). Given adequate nutrients, time and suitable temperature, the initial sessile microbial population can eventually form a confluent lawn of bacteria on the membrane surface (Ridgway et al., 1999).

Hydrophobicity and charge of the microbial cell surface are considered to be important factors in the determination of adherence of bacteria to surfaces (Klotz, 1990; Krepsky et al., 2003; Kumar & Anand, 1998; Vacheethasanee et al., 1998). It is commonly observed that cell surface hydrophobicity can affect bacterial adhesion to different types of substratum (Gilbert et al., 1991).

A hydrophobic strain of *Pseudomonas aeruginosa* attached to glass, copper, stainless steel and silicon surfaces more successfully than did a strain of *Pseudomonas fluorescens* with lower cell surface hydrophobicity (Mueller et al., 1992). The hydrophobicity of inanimate substrata also influences the strength and kinetics of microbial adhesion and early biofouling (Ridgway et al., 1999). If the cell surface charge is large, the electrostatic interactions with the substratum will also be significant (Pang et al., 2005). It was observed that at physiologically relevant pH values (~7), PA membranes tend to be negatively charged (Elimelech et al., 1997; Vrijenhoek et al., 2001). Thus, strains having more negative charges, such as *Microbacterium sp.* and *Sphingomonas sp.,* would experience greater repulsion than those strains with lower negative charges, such as *Dermacoccus sp.* and *Rhodopseudomonas sp*. (Pang et al., 2005), which would adhere more easily onto PA membranes.

Physicochemical forces, comprising hydrophobicity and charge, are the only nonspecific interactions between cell and surface (Smith et al., 1998) that are involved in cell attachment (Marshall, 1991). In order to reduce potential for proteins and bacteria to attach to membrane surfaces, commercial membrane filtration systems are modified to have a hydrophilic, negatively charged surface (Chen & Belfort, 1999; Lin-Ho & Espinoza-Gomez, 2001). Therefore, our model test system to screen isolates for adhesion to membrane surfaces utilized hydrophilic tissue culture microtitre plates. However, in the dairy industry, membranes with increased hydrophilicity will always be more hydrophobic than the aqueous solutions being treated by membrane filtration. The rationale behind generating negative surface charges on membranes is to prevent negatively charged colloidal particles, such as micro-organisms, adhering to the surface by increasing the repulsion between those microorganisms and the membrane surfaces.

The objective of this part of study was to investigate how the attachment of micro-organisms isolated from dairy plant membranes was influenced by their surface characteristics. The hypothesis was that the cell surface hydrophobicity and charge would be the dominant factors for initiating cell attachment. The ability of cell attaching was investigated using a microtitre assay plate as a model surface. The effect of cell hydrophobicity and charge, medium including whey and its components (α-lactalbumin, β-lactoglobulin, glycomacropeptides (GMP) and bovine albumin (BA)) and interactions between species were investigated to determine key factors involved in microbial attachment to dairy membranes.

4.2 MATERIALS AND METHODS

4.2.1 Source of strains

All the isolated and identified strains listed in Table 3.2 (Section 3.3.1) were used for this study.

4.2.2 Preparation of inocula

Pure cultures of the microbial isolates were grown on SMA for 24 h (bacteria) or 48 h (yeast) at 25ºC or 30ºC according to the temperature at which they were isolated and

then inoculated into sterile trypticase soy broth (TSB) (BD, Fort Richard Laboratories, Auckland, New Zealand) and incubated for 24 h. After incubation, strains were harvested by centrifugation at 2500 g for 10 min and then resuspended in sterile phosphate buffer saline (PBS) (pH 6.5), whey permeate (pH 6.5) or rennet whey (pH 6.5) for the microtitre plate assay, cell surface hydrophobicity and cell surface zeta potential measurements. Whey and whey permeate were obtained from Fonterra Co-operative Group Ltd, Auckland, New Zealand. The rennet whey has a pH of 6.5, and the pH of whey permeate was adjusted using 1 M NaOH to pH 6.5. The optical density (OD) of the culture was adjusted to 1.0 at 600 nm using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech Science & Technology Ltd, Cambridge, UK). The number of bacterial cells after adjustment was around 10^7~10^8 CFU ml^{-1}. For the experiments with combinations of two strains, these were prepared from equal proportions of the two cell suspensions at the same $OD_{600\ nm}$ reading of 1.0.

4.2.3 Microtitre plate assay

Sterile 96-well polystyrene tissue culture microtitre plates (Becton Dickinson Labware, NI, USA) surface treated by the manufacturer so as to be hydrophilic were used in a standard microtitre plate assay (Djordjevic et al., 2002) to screen for potential microbial attachment to membrane surfaces. Aliquots (200 µl) of each cell suspension were dispensed into three wells of the microtitre plate. Each plate also contained three wells with 200 µl of PBS, whey permeate or whey as controls.

A preliminary screening trial had established that 4 h gave the highest attachment for some strains, ensuring that cells firmly adhered to the surface. The plates were left at ambient temperature for 4 h without agitation. The attachment of cells to the microtitre plate after standing for 4 h in PBS was used as a reference to compare attachment in whey and whey permeate with the same exposure time.

Attachment of 3 individual strains (*Klebsiella* TR002, B001 and B006) in the presence of individual components of whey was tested at concentrations that reflect the composition of whey: 13% α-lactalbumin, 48% β-lactoglobulin, 18% GMP and BA (0.2 g l^{-1}) (Sigma Chemical Co., St. Louis, MO, USA). The pH of the four components was adjusted to 6.5 – the same as the pH of the whey used in the earlier trial.

To determine the attachment of single strains and the effect of microbial interactions on attachment, both single strains and combinations of strains (*Pseudomonas* TR001 mixed with *Klebsiella* TR002, B001 or B006) were used as inocula in different experiments. The volume ratio of the mixed strains was 1:1.

After incubation, the media were removed and the wells washed four times with sterile distilled water. The plates were then inverted and blotted on paper towels and allowed to dry in air for 30 min. Aliquots (200 µl) of crystal violet solution (0.05 %) were added to each well and allowed to incubate at ambient temperature for 15min. Then the crystal violet solution was removed and the wells washed four times with sterile distilled water to remove unbound dye.

Aliquots (200 µl) of 95 % ethanol were added to each well and the plates were allowed to stand for 2-5 min to release the dye from the cells. Optical density of the crystal violet solution was measured at 595 nm using the ELx808 Ultra Microplate Reader (Bio-Tek Instruments, Inc., VT, USA). An $OD_{595\ nm} > 0.1$ was taken as positive for attachment in the microtitre plate assay.

4.2.4 Attachment to the membrane

Polysulfone membranes with a diameter of 47 mm and a pore size of 0.45 µm (Pall Corporation, Alphatech Systems Ltd, Auckland, New Zealand) were cut into eight pieces using sterile blades, placed in distilled water and autoclaved for 15 min at 121ºC. The detecting area of each membrane slice was 4.34 cm^2. This membrane is specially manufactured for filters that can be autoclaved.

Each piece of the membrane was placed into a 25 ml sterilized glass bottle and immersed in 1 ml of inoculated PBS with $OD_{600\ nm}$ of 1.0. They were incubated for 4 h at room temperature without shaking. After incubation, the membranes were washed twice with sterile distilled water. Then 3 ml of sterile distilled water and 4 sterile glass beads with diameter of 5 mm were added. The cells attached to the membranes were extracted by vortex mixing for 1 min and the numbers of cells released were measured by plating serial dilutions on SMA, followed by incubation at the growing temperatures of the strains (e.g. *Klebsiella* B001 at 25ºC, *Klebsiella* B006 at 37ºC and *Bacillus*

WL004 at 25ºC). This modified method of removing attached microorganisms refers to the validated method for removing biofouling layer in an ultra pure water system, where shaking instead of vortex mixing was used (Schaule et al., 2000). The vortex mixing time was validated in this study by testing culturable cells after vortex mixing for 0.5, 1 and 2 min.

4.2.5 Microbial adhesion to hydrocarbon assay

In order to understand how the cell surface hydrophobicity affects the attachment of our strains, cells were incubated in PBS, whey and whey permeate for 4 h at room temperature and the cell surface hydrophobicity and attachment were measured. After incubation, samples were prepared as before (Section 4.2.2), with $OD_{600\ nm}$ of 1.0. Microbial cell surface hydrophobicity was determined using a modification of the MATH test (Rosenberg et al., 1980). Xylene (M&B, Dagenham, UK) was used as the hydrophobic target for cells to attach (Flint et al., 1997b). Samples of each strain suspension (3 ml) were added to 3 ml of xylene, mixed briefly on a vortex mixer, incubated at 30ºC for 10 min and then mixed vigorously with a vortex mixer at ambient temperature for 2 min. The absorbance of the aqueous phase was measured at 600 nm after standing at ambient temperature for 20 min to allow phase separation. Each test was performed in triplicate and the results expressed as mean and standard deviation. The hydrophobicity was calculated using the percentage hydrophobicity formula (Flint et al., 1997b):

$$Hydrophobicity\ (\%) = \frac{Ab_{600nm}(before\ \ xylene) - Ab_{600nm}(after\ \ xylene)}{Ab_{600nm}(before\ \ xylene)} \times 100\%$$

In the MATH test, the loss in absorbance in the aqueous phase relative to the initial absorbance value is taken to represent the amount of cells adhering to xylene and this is a reflection of the hydrophobicity of the cell surface. The cell surface hydrophobicity measured in PBS (pH 6.5) was considered to be the base line and compared with attachment in whey permeate.

4.2.6 Zeta potential

Cell surface zeta potential was measured in PBS and whey permeate using the Malvern Zetasizer Nano NS (Malvern Instruments Ltd, Worcestershire, UK) (Denyer et al., 1993). Readings were made in triplicate. The zeta potential measurement relies on light scattering. If whey proteins bind to the surface of the micro-organisms, they will influence the zeta potential. Therefore, the cell surface zeta potential was not measured in whey.

4.2.7 Statistical analysis

In all analyses, triplicate tests were performed under identical conditions and the results expressed as mean and standard deviation. Regression, including stepwise regression, was processed using Minitab software (Release 15; Minitab Inc., State College, PA, USA) to assess the impacts of cell surface hydrophobicity and surface charge on the ability of cells to attach.

4.3 RESULTS

4.3.1 Attachment of strains suspended in different media

Generally, five strains (TR001, TR002, WL001, B001 and B006) showed ability to attach (Fig. 4.1). In PBS, all three *Klebsiella* strains (TR002, B001 and B006) attached to the microtitre plate wells, while the other 10 strains did not attach. All three strains that attached in the presence of PBS showed increased attachment in whey and whey permeate. Two other strains (*Pseudomonas* TR001 and *Chryseobacterium* WL001) did not attach in PBS, but did attach in the presence of whey and whey permeate (Fig. 4.1).

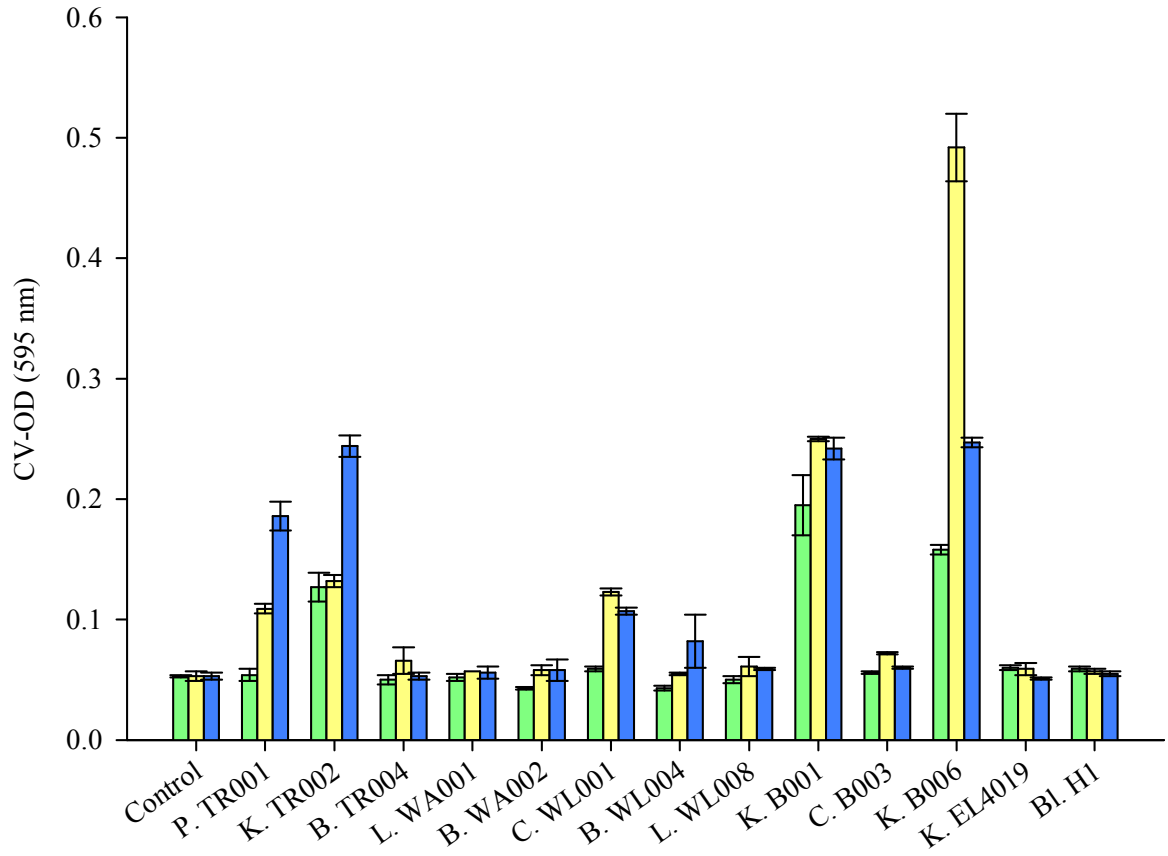


Figure 4.1: The attachment expressed as CV-OD at 595 nm of strains to microtitre plates in different media in 4 h: PBS (■), whey permeate (■) and whey (■). (Results expressed as mean and standard deviation, which were from triplicates.)

4.3.2 Attachment of mixed strains

When *P.* TR001 was mixed with *K.* TR002, B001 and B006, attachment, as indicated by the crystal violet $OD_{595\ nm}$, was greater than that seen for the individual strains, suggesting a synergistic effect (Fig. 4.2).

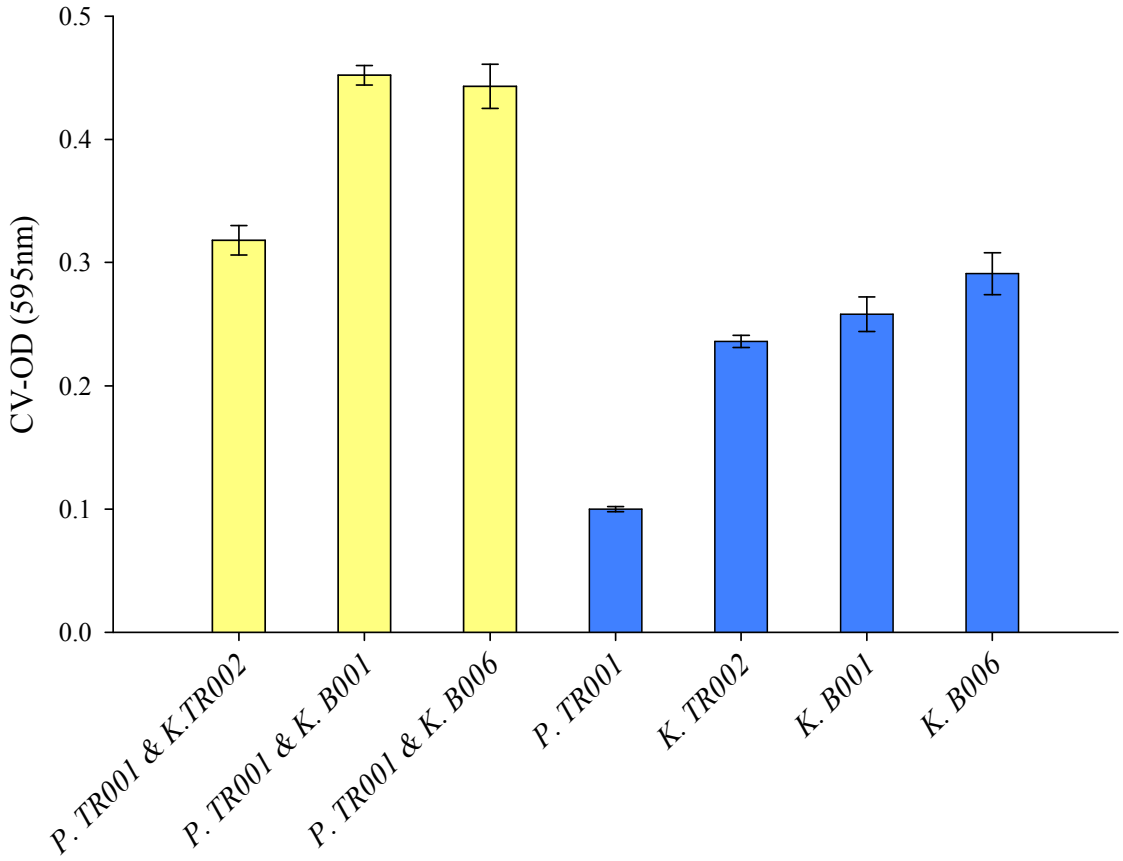


Figure 4.2: The attachment expressed as CV-OD at 595 nm of mixed strains to microtitre plates in PBS (pH 6.5) after incubation for 4 h. Means were taken from triplicates. The first three bars are *P.* TR001 mixed with one of *K.* TR002, B001 and B006. The mixing ratio was 1:1 in volume. The striped bars demonstrate the attachment of pure individual strains.

4.3.3 Attachment to the membranes and validation of method

The vortex mixing time was validated by measuring the plate counts of removed cells at different times (0.5 min, 1 min and 2 min). The maximum plate counts were achieved with 1 min vortex mixing (Table 4.1). The attachment of *K.* B001 and B006 and *Bacillus* WL004, representing two strains that attached (CV-OD $_{595\ nm}$ > 0.1) and one strain that did not attach (CV-OD $_{595\ nm}$ < 0.1) in the microtitre plate assay (Fig. 4.1), were chosen to verify the use of the microtitre plate assay as a screening model for attachment to the polysulfone membranes. The numbers of cells that attached to the polysulfone membrane are shown in Table 4.1. *K.* B001 and B006 that attached to the polystyrene microtitre plates showed high abilities to attach to the polysulfone membranes, with densities of > 7 $\log_{10}$ CFU cm^{-2}. *B.* WL004 that did not attach to the polystyrene microtitre plates showed poor ability to attach to the polysulfone membranes, with a density of < 5 $\log_{10}$ CFU cm^{-2}. Thus, the microtitre plate assay was efficacious to be used as a screening model for attachment to the polysulfone membrane.

Table 4.1: Plate counts ($\log_{10}$ CFU cm^{-2}) of the cells attached to the polysulfone membranes (Means and standard errors were taken from duplicates.)

Vortex Mixing Time / Strains	0.5 min	1 min	2 min
K. B001	6.59 ± 0.15	7.81 ± 0.02	7.78 ± 0.06
K. B006	6.34 ± 0.37	7.18 ± 0.09	7.18 ± 0.11
B. WL004	3.55 ± 0.09	4.99 ± 0.29	4.86 ± 0.18

4.3.4 Attachment in presence of components of whey

Previous results showed that the attachment of three strains (*K.* TR002, B001 and B006) was increased in the presence of whey and whey permeate (Fig. 4.1). In order to analyse further which component in whey played the major role in attachment, attachment of these three *Klebsiella* strains was measured in each of four whey components using the microtitre plate assay.

None of the four whey components (α-lactalbumin, β-lactoglobulin, BA and GMP), when used individually, showed a significant increase of attachment relative to PBS of the three strains that had shown greater attachment in the presence of whey (Fig. 4.3).

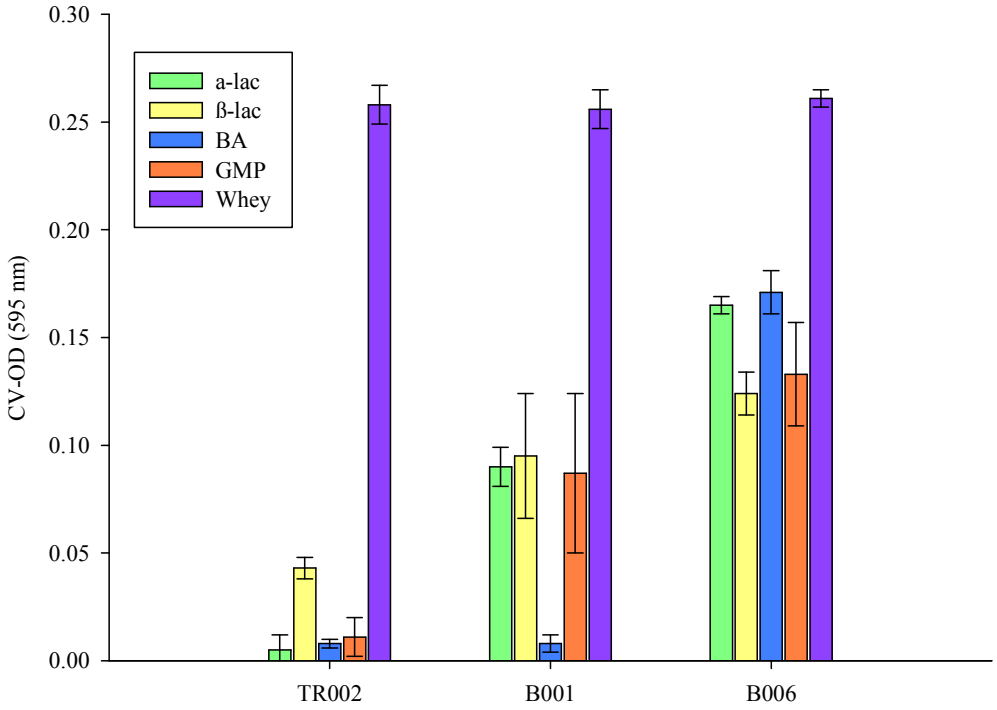


Figure 4.3: Attachments to microtitre plates expressed as CV-OD at 595 nm of 3 *Klebsiella* strains (TR002, B001 and B006) in whey and in its 4 individual components (Means and standard deviations from triplicate measurements.)

4.3.5 Cell surface hydrophobicity

The cell surface hydrophobicity in PBS was generally higher than that in whey but lower than that in whey permeate (Fig. 4.4). Two strains, *Lactobacillus* WA001 and *Chryseobacterium* WL001, were extremely hydrophobic in PBS, with their hydrophobicity greater than 94%. *Klebsiella* B006 demonstrated the lowest hydrophobicity (2.4%) in whey permeate. Its hydrophobicity increased in PBS and whey, but was still less than 16%. Three strains (*Klebsiella* TR002, *Klebsiella* B001 and *Cronobacter* B003) showed very high hydrophobicity (> 70%) in whey permeate but rather low hydrophobicity in PBS (< 35%) and in whey (< 25%).

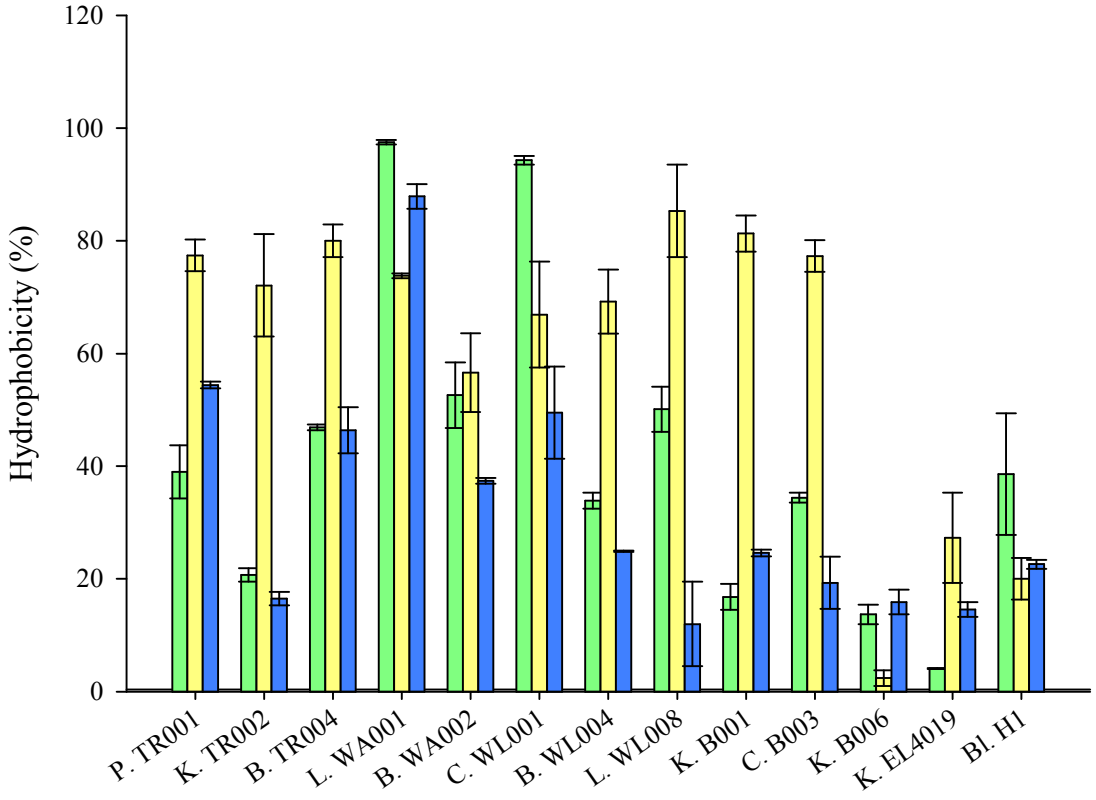


Figure 4.4: Cell surface hydrophobicity in different media (pH 6.5): PBS (■), whey permeate (■) and whey (■). (Means and standard deviations from triplicate measurements.)

4.3.6 Cell surface charge

Cell surface charge was determined by measuring cell surface zeta potential. All strains indicated zeta potentials less than -20 mV in PBS (pH 6.5) and whey permeate (pH 6.5) (Fig. 4.5). In whey permeate, the maximum zeta potential was less than -15 mV, and the zeta potential of 7 strains (TR002, TR004, WA001, WA002, WL008, B001 and B006) became less negative than in PBS (Fig. 4.5). However, the other strains such as *P.* TR001 and *C.* WL001 had slightly more negative charges in whey than in PBS (Fig. 4.5).

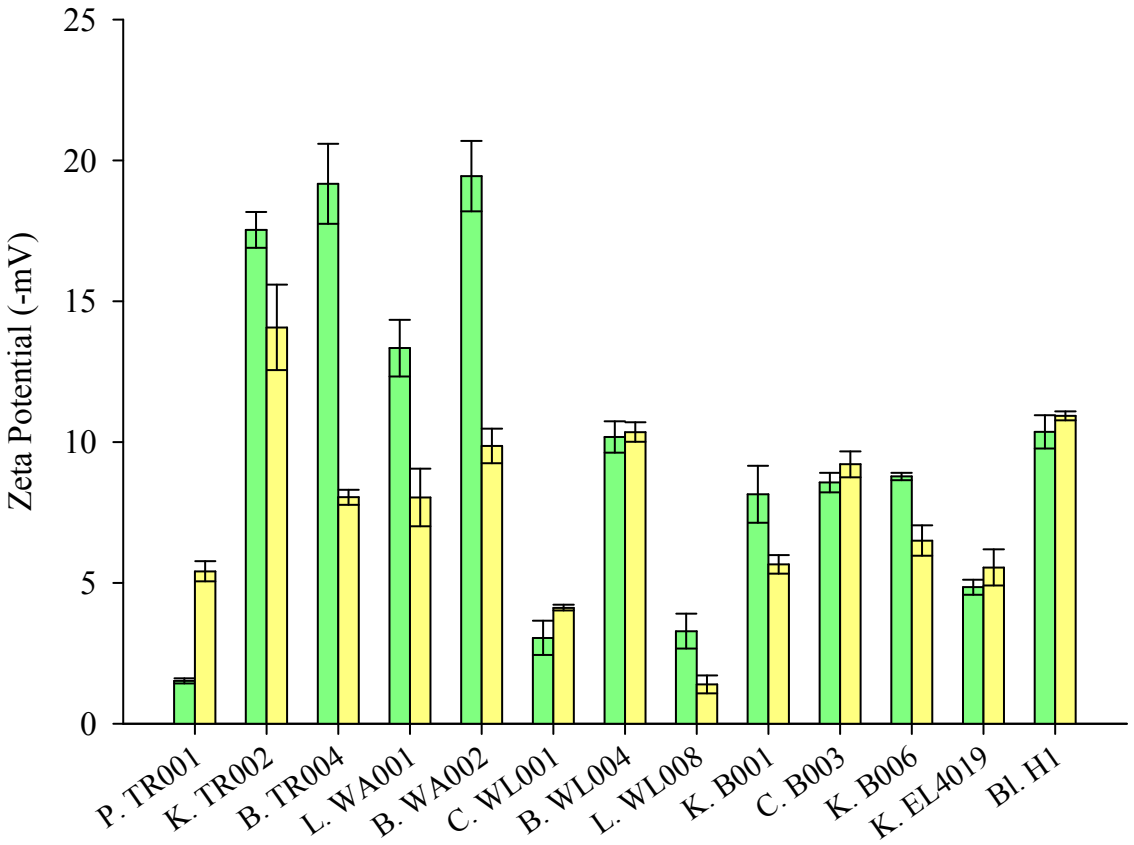


Figure 4.5: Cell surface charge in PBS pH 6.5 (■) and whey permeate pH 6.5 (■). (Results expressed as mean and standard deviation, which were from triplicates)

K. TR002 was highly negatively charged in both media (Fig. 4.5). This suggested that electrostatic interactions with the substratum would be more significant for TR002 than for other strains that had low negative charges such as TR001, WL001 and WL008.

4.3.7 Impact of cell surface hydrophobicity and charge on attachment

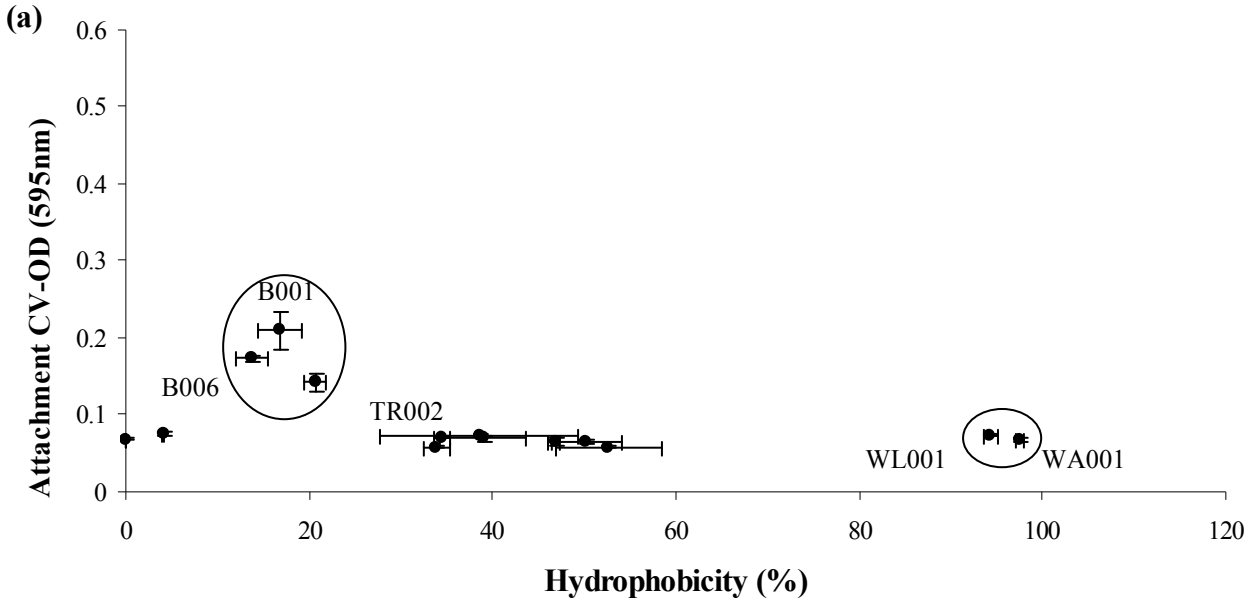
Regression analysis of the relationships between cell surface hydrophobicity, cell surface charge and ability to attach was performed using Minitab software. However, the attachment data for strains TR001, TR002, WL001, B001 and B006 with observed ability to attach to microtitre plates showed no clear regression ($R^2 \leq 74.6$ %).

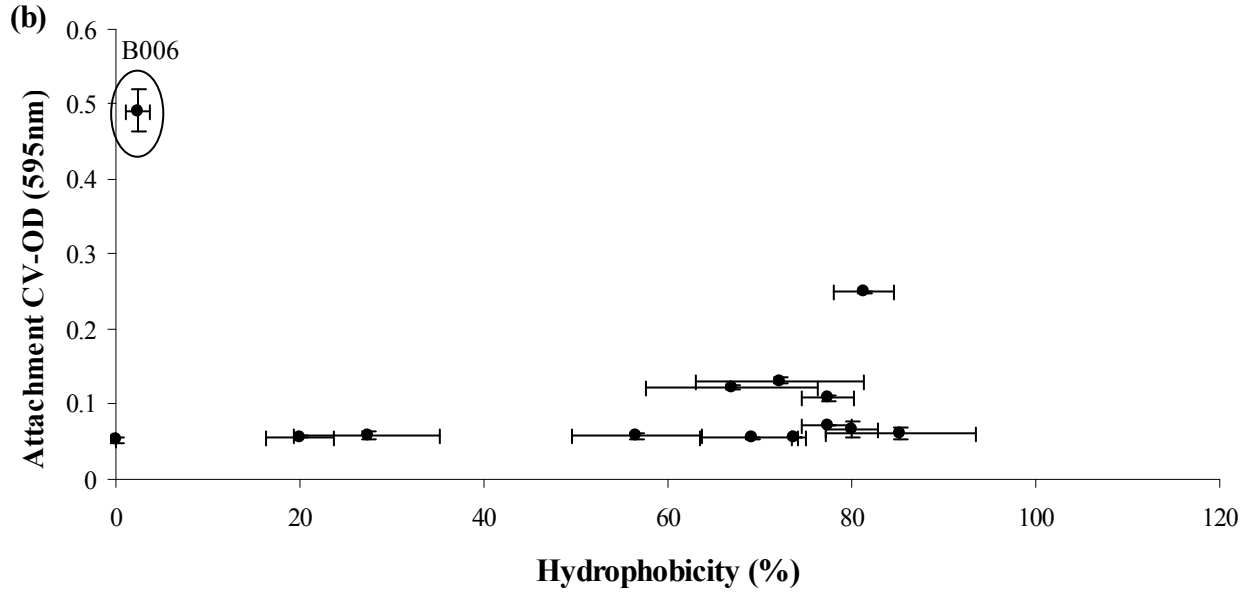
4.3.7.1 Impact of cell surface hydrophobicity

Three *Klebsiella* strains (B006, B001 and TR002) with low hydrophobicity (13.7%, 16.8% and 20.7%) showed high attachment in PBS (Fig. 4.6a). However, the other strains with hydrophobicity in the range from 4.1% to 97.5% did not attach. The two strains (*Chryseobacterium* WL001 and *Lactobacillus* WA001) with extremely high hydrophobicity (94.3% and 97.5%) did not attach (Fig. 4.6a).

Experiments in whey permeate showed little correlation between attachment and hydrophobicity of the cells. Figure 4.6b shows a slight trend towards increased attachment with increasing hydrophobicity, the exception being strain B006, with a hydrophobicity of 2.4%, demonstrating the highest rate of attachment.

In whey, three *Klebsiella* strains (B006, B001 and TR002) that had low hydrophobicity showed high attachment, and *Lactobacillus* WA001 that had the highest hydrophobicity did not attach (Fig. 4.6c).


(a)
Attachment CV-OD (595nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
B001
B006
TR002
WL001
WA001
0
20
40
60
80
100
120
Hydrophobicity (%)


(b)
Attachment CV-OD (595nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
B006
0
20
40
60
80
100
120
Hydrophobicity (%)

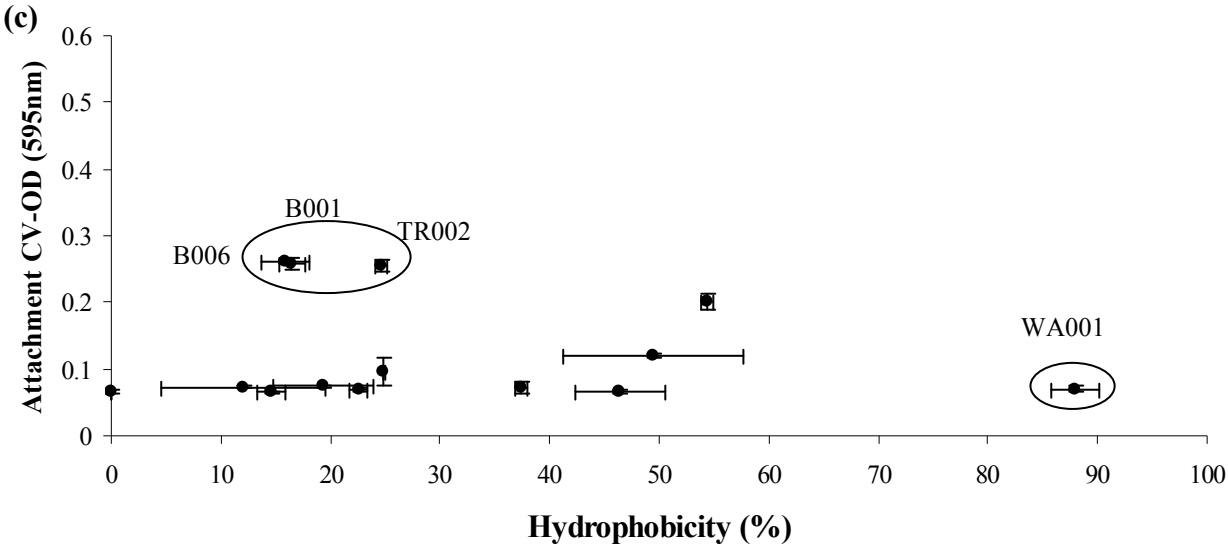


Figure 4.6: Cell surface hydrophobicity and attachment to microtitre plates. **(a)** in PBS (pH 6.5) **(b)** in whey permeate (pH 6.5) **(c)** in whey (pH 6.5) (Means and standard deviations of hydrophobicity were calculated from duplicates, and those of attachment were from triplicates.)

Overall, the ability of the isolates to attach showed no clear relationship with their surface hydrophobicity.

4.3.7.2 Impact of cell surface charge

The bacterial surface zeta potential and their ability to attach to microtitre plates were plotted in Figure 4.7.

In PBS (Fig. 4.7a), it was observed that some strains with low negative zeta potential did not attach well, e.g. strain *P.* TR001 and *C.* WL001, while some strains with medium (*K.* B001 and B006) or higher (*K.* TR002) negative zeta potentials showed high attachments.

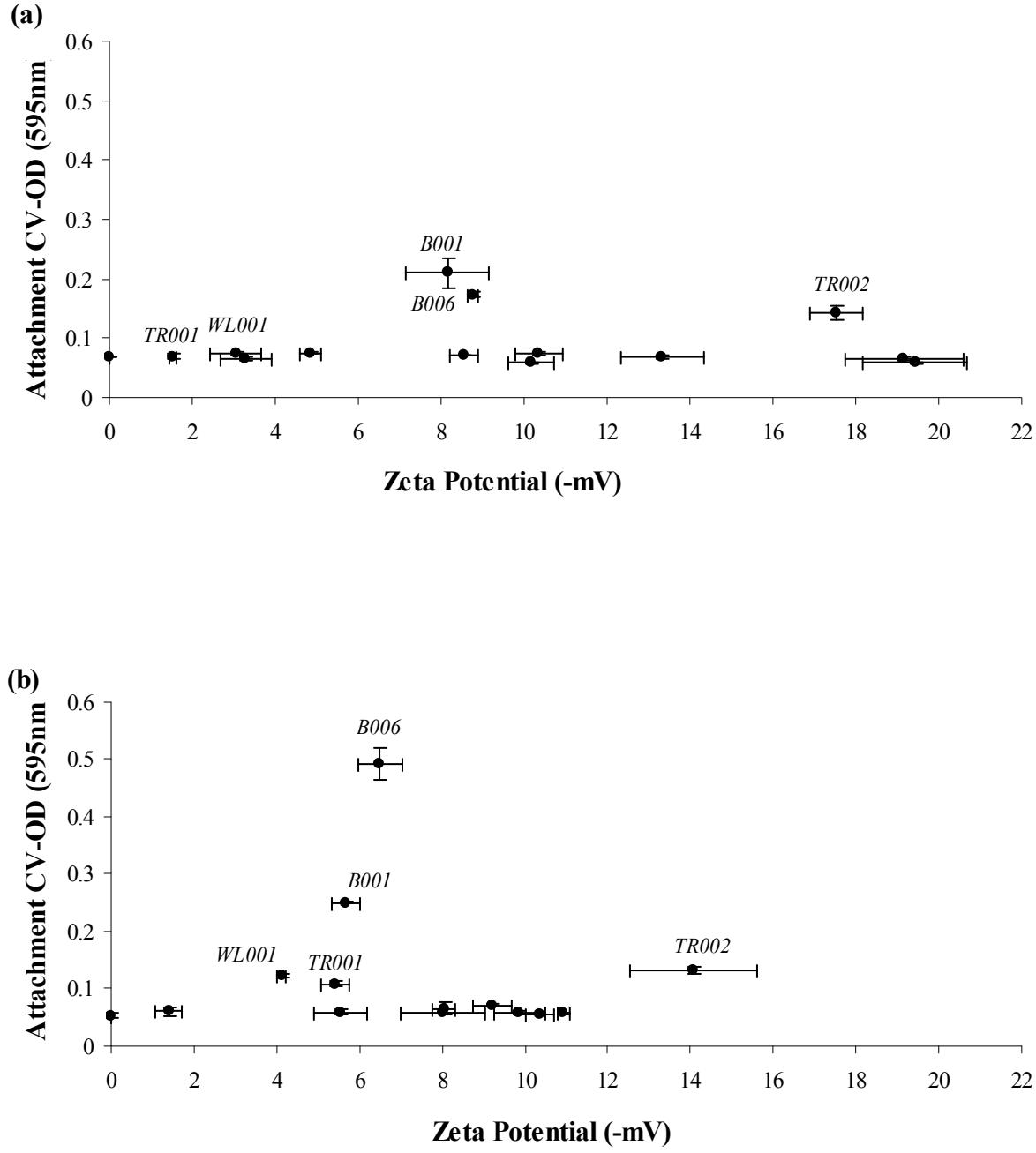


Figure 4.7: Cell surface charge and attachment to microtitre plates. **(a)** in PBS (pH 6.5) **(b)** in whey permeate (pH 6.5) (Means and standard deviations were calculated from triplicates.)

In whey permeate (Fig. 4.7b), some strains with medium (*C.* WL001, *P.* TR001, *K.* B001 and *K.* B006) or higher (*K.* TR002) negative zeta potentials showed better attachments than the other strains with medium or even lower negative zeta potentials.

The ability of strain *K.* B001 to attach increased in whey permeate while its negative surface charge reduced a little compared with those in PBS (Fig. 4.7). The same but more obvious results were observed for strain *K.* B006 (Fig 4.7). However, for strain *P.* TR001, attachment increased in whey permeate when its negative surface charge increased compared with those in PBS (Fig 4.7). The attachment of *K.* TR002 did not change, while its surface charge decreased from that measured in PBS to that in whey permeate (Fig. 4.7).

Overall, the ability of the isolates to attach showed no clear relationship with their surface charge.

4.4 DISCUSSION

In our study, the three *Klebsiella* strains (TR002, B001 and B006) that readily attached to our model microtitre plate system and membrane surfaces were isolated from two different manufacturing plants. The other *Klebsiella* strain (EL4019) with poor ability to attach originated from a third manufacturing plant. There is no indication that the isolates with the greatest attachment were specific to any manufacturing plant.

The microtitre plate assay is a standard assay used to screen micro-organisms for their ability to attach and form biofilm (Djordjevic et al., 2002). It showed that the three strains with a high ability to attach were Gram-negative bacteria (*K.* TR002, B001 and B006). Gram-negative bacteria are known to be prolific biofilm formers and this may be due, in part, to their ability to produce polysaccharide slime, associated with the formation of a true biofilm.

The attachment of various strains measured using the microtitre plate assay compared with attachment to polysulfone membrane verified the use of the microtitre plate assay as a tool to screen for the attachment to polysulfone membrane surfaces. Vortex mixing rather than sonication (which was used to remove cells in the mature biofilm from the

surfaces in the later study, Chapter 5 & 6) was used to remove the attached cells from the membrane surfaces, as the bonds between cells attached within 4 h and the surfaces were believed weaker than the mature biofilm. Validation of vortex mixing showed that 1 min was sufficient to take off the maximum number of cells attached on the membrane surfaces.

The increase in the attachment of two mixed strains (*P.* TR001 with *K.* TR002 / B001 / B006) compared with the attachment of each individual strain, indicates an interaction between these strains in the initiation of a biofilm. Biofilms in many environments are multi-species rather than single species (Kawarai et al., 2007; Macleod & Stickler, 2007). It is well known that *Pseudomonas* are often primary colonizing organisms of surfaces and have been shown to enhance the attachment of others to surfaces (Zottola, 1994) and that co-existence with *Klebsiella* has previously been documented (Stewart et al., 1997). Ten of our thirteen strains showed no ability to attach from pure culture, which suggests that either the majority of isolates did not form biofilm but were trapped in the accumulation of protein and biofilm on the membranes, or the required conditions were not present in our experiments (e.g. combination with other micro-organisms or specific environmental conditions required for attachment).

Different media including PBS, whey and whey permeate, all at pH 6.5, were used in the present study. The results in PBS were considered as a base line, while the results in casein whey and whey permeate were taken as reflecting the situation in a dairy environment. Whey and whey permeate were found to increase the attachment of most of the strains. Therefore, further details on the effects of whey components were investigated using three *Klebsiella* strains in the microtitre plate assay. Four whey components: α-lactalbumin, β-lactoglobulin, GMP and BA were used. These experiments did not show which component played a major role in increasing attachment. It can be concluded that all components of whey may enhance bacterial attachment.

Cell surface characteristics, especially hydrophobicity and charge, are generally believed to be the dominant factors that influence the ability of cells to attach (Gilbert et al., 1991; Kumar & Anand, 1998; Mueller et al., 1992). To understand how the attachment of our strains was affected by their surface hydrophobicity and charge, these

characteristics were measured and correlated with observed attachment. Whey influenced the hydrophobicity of the cell surface, but the degree of change in hydrophobicity varied between different strains. It appeared that the responses to whey permeate or whey was a function of the individual strains. All the strains were negatively charged in PBS and whey permeate (pH 6.5). Cell surface charge was less negative in whey permeate, possibly due to the interaction of ions in whey permeate with the cell surface (Seale et al., 2008). This enhanced the attachment of the strains to the plate surface, since the plates were also negatively charged (Becton Dickinson Labware, USA). However, regression of attachment on these two factors failed to show a significant relationship. It suggests that some other factors may be of equal or greater importance in the attachment of our strains, such as chemicals (Pasmore et al., 2001) and proteins (Kirtley & Mcguire, 1989) in the medium, ionic strength, pH and temperature (Bunt et al., 1993; Kumar & Anand, 1998; Parkar et al., 2003), exo-polymeric substances (EPS) secreted by bacteria (Jacquement et al., 2005), cell motility (Pang et al., 2005) and bacterial quorum sensing (Kim et al., 2009; Paul et al., 2009; Yeon et al., 2009). Other studies have also found that the cell surface hydrophobicity or charge did not play a dominant role in determining the extent of attachment (Flint et al., 1997b; Jameson et al., 1995; Vacheethasanee et al., 1998).

The MATH assay used in this study is a screening tool widely used to compare the hydrophobicity of different bacterial isolates. It does have limitations as reported (Busscher et al., 1995), and a kinetic MATH assay may provide more accurate results (van der Mei & Busscher, 2001).

4.5 CONCLUSIONS

Studies on the initial adhesion to the surface are essential in any programme aimed at biofilm elimination (Dang & Lovell, 2000). The results of our study showed that the attachment of the different isolates was highly variable. *K. oxytoca* B001, B006 and TR002 showed greater ability to attach than other strains, suggesting their importance in biofilm development on membranes. Mixed strains of *Klebsiella* and *Pseudomonas* showed increased attachment, indicating multi-species interactions.

The results rejected our hypothesis. Cell surface hydrophobicity and charge were not the dominant factors influencing the ability of our strains to attach, while their attachment was generally enhanced in the presence of whey. However, none of the individual whey components demonstrated the ability to increase the attachment.

Chapter 5

GROWTH OF BIOFILM ON MEMBRANES

5.1 INTRODUCTION

The limitation on membrane usage is the frequent cleaning required for removal of foulant, including solute adhesion and microbial fouling (Chang et al., 2002; Ivnitsky et al., 2007; Ridgway et al., 1999; Susanto & Ulbricht, 2007). Biofilms on membrane surfaces have been studied in water processing environments (Ivnitsky et al., 2005; Speth et al., 1998) but not in dairy manufacturing.

In the dairy industry, UF is used most frequently to concentrate or separate whey components (Maubois, 1980). In membrane processing of rennet whey, fouling by protein and calcium-phosphate precipitation can be reduced and prevented by adjusting pH to around 6.0 (Hiddink et al., 1980). Biofilm growth is affected by the substrate concentration (Komlos et al., 2005). From our previous study, we also found that whey influenced the attachment of our isolated strains. Therefore, by choosing whey as the medium and using different concentrations of whey protein, the relationship between biofilm growth and whey protein concentration can be investigated.

The composition of whey at different stages of a multistage UF membrane plant in a typical New Zealand dairy industry factory is shown in Fig. 5.1. Whey media were artificially made according to the compositions at select stages - 1, 8 and 14 - within the UF module, to represent the beginning, middle and final concentrations in a UF membrane plant.

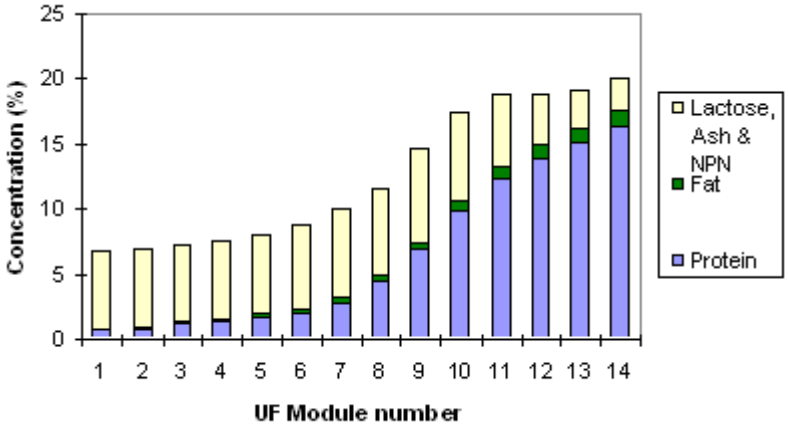


Figure 5.1: The typical concentrations of whey components from the beginning (module 1) stage to the final (module 14) stage of the UF membrane plant in the dairy manufacturing industry. (NPN = Non-protein nitrogen) (Ferreira et al., 2006)

Some *in-vitro* continuous systems that can be used to investigate biofilm growth in a laboratory include rotating disc reactors, the modified Robbins device, the annular reactor, and specifically designed commercial laboratory reactor systems such as the commercial biofilm reactor (CBR) 90 (BioSurface Technologies, Bozeman, USA) (Goeres et al., 2005). The CBR 90 reactor system is a biofilm reactor containing 24 removable polycarbonate coupons that allows controllable shear rate, continuous flow and temperature control (Donlan et al., 2002). This system has been used for monitoring biofilm formation and characterising biofilm structure (Donlan et al., 2004) and for statistical assessment of biofilm growth (Goeres et al., 2005). In our study, the CBR 90 reactor was modified to enable membranes to be fixed into the coupon holders. However, it still has limitations in that not all the parameters in the membrane processing plant are able to be introduced into the CBR 90 reactor. Those parameters include the permeability through the membrane, membrane spacer, and the exact same flow rate and oxygen consumption in the plants.

The objective of this study was to investigate how the biofilm growth of single and dual *K. oxytoca* strains is affected by the membrane type (PES and PVDF), membrane age (new and used) and whey protein concentration. Our hypothesis was that all these factors were significant for biofilm growth on membranes.

5.2 MATERIALS AND METHODS

5.2.1 Sources of strains

Two *K. oxytoca* strains were previously isolated from two different dairy membrane manufacturing plant sites (Chapter 3) and both of them had high ability to attach (Chapter 4). *K.* B006 was isolated from a liquid sample taken from a UF membrane plant processing whey at dairy manufacturing plant A, and *K.* TR002 was isolated from a biofilm sample scraped from a RO membrane plant processing whey at dairy manufacturing plant C (Table 3.2, Section 3.3.1).

5.2.2 Preparation of medium

Whey medium was prepared by mixing 1%, 5% and 20% of whey protein concentrate powder (WPC 80 containing 80% whey protein, (Fonterra Co-operative Group Ltd, Auckland, New Zealand)) with sterilized lactose (Fonterra Co-operative Group Ltd, Auckland, New Zealand) and artificial whey permeate, which was prepared by mixing the following minerals in deionised water to make 1 L (pH 6.0-6.1) (52.7 ml 2 mol l^{-1} KOH (BDH, Poole, England), 24.29 g $C_6H_5O_7Na_3 \cdot 2H_2O$ (Trisodium citrate dihydrate) (Merck KGaA, Darmstadt, Germany), 4.99 g $C_6H_5O_7K_3 \cdot 2H_2O$ (Tripotassium citrate dihydrate) (UNIVAR, Auckland, New Zealand), 3.67 g $CaCl_2.2H_2O$ (Biolab, Clayton, Australia), 5.85 g $MgCl_2.6H_2O$ (J.T.Baker, Phillipsburg, Mexico), 23.36 g KH_2PO_4 (Merck KGaA, Darmstadt, Germany) and 17.1 ml 3 mol l^{-1} H_2SO_4 (Biolab, Clayton, Australia)) (Kauter, 2003). To mimic the composition of lactose and minerals in three different stages of UF, lactose and minerals were added in appropriate concentrations to make the final approximate composition. At the beginning of UF the product composition is 1% whey protein, 6% lactose and 6% minerals (Fig. 5.1). The middle stage contains 5% whey protein, 6.1% lactose and 6.1% minerals, and the final concentrated stage contains 20% whey protein, 2.4% lactose and 2.4% minerals (Fig. 5.1). The final pH of the prepared whey medium was around 5.8-6.0 that is the operation pH in the dairy membrane manufacturing plants and a compromise between the fouling effects of protein and those of calcium phosphate (Cheryan, 1998). The re-constituted whey powder was not sterilized because nothing in the powder grew at 25ºC.

5.2.3 Preparation of inocula

Pure cultures of *K. oxytoca* were grown on SMA at 30ºC for 24 h and then a large loopful of colony was inoculated into 10 ml whey and incubated for 24 h. This was diluted in whey to reach a density of 10^6~ 10^7 CFU ml^{-1}, confirmed by agar plate counting.

5.2.4 Description of the CBR 90 and the target membrane surface

The CBR 90 reactor was described by Goeres et al. (2005). For our study, the polycarbonate disk coupons were covered with UF membrane by clipping a 13.0 mm × 13.0 mm membrane square into a hole on the rod with a disk coupon. New coupons were made 1 mm diameter smaller than the original coupons to enable the membrane to fit into the coupon holder with the polycarbonate disks, and so that the actual surface exposed and available for biofilm growth was still 1.27 cm^2.

Three types of the UF membranes were used, including new PES membrane flat sheets (10,000 Molecular weight cut-off (MWCO)) (Synder Filtration, Vacaville, CA, USA), new PVDF membrane flat sheets (800,000 MWCO) (Synder Filtration, Vacaville, CA, USA) and used spiral-wound PES membranes from a New Zealand dairy manufacturing membrane plant processing cheese whey. The used PES membrane was cut into several small rolls using a band saw sterilised by 95% ethanol, and stored in a 4ºC cold room. Before each experiment, a piece of membrane sheet was cut using sterile scalpel blades and the membrane samples were cut to provide a surface area of 1.27 cm^2, to fit in the CBR 90 reactor (Biosurface Technologies, Bozeman, USA). The membranes were supported in the holders by standard polycarbonate coupons. This necessity introduced a limitation on the experiment, in that the membrane was positioned with one side against an impermeable surface. This configuration is not the same as found in the plant, where there is a constant flux of products through the membrane in addition to the cross flow. However, this approach allowed easier evaluation of different sanitisers under comparable conditions. The stirring speed was set up to be 180 rpm, so that the resulting flow corresponded to a Reynold's number of approximately 1850 and fell in the turbulent flow region (Buckingham-Meyer et al. 2007). It is also a turbulent flow in the commercial dairy membrane system.

5.2.5 Biofilm development

The CBR 90 was used to grow biofilms in order to determine the biofilm growth rate and biofilm densities of the two strains grown individually and in combination, for three concentrations of whey medium and for three different types of membrane. The whole system is demonstrated in Fig. 5.2. Membrane pieces were clipped onto the rods of the CBR 90. CIP (Section 5.2.6) was completed before pumping 330 ml medium into the reactor from a supply stored at 4ºC. The medium in the reactor was then heated to 25ºC. Although these *Klebsiella* strains isolated from our cold membrane plants (10 – 12ºC) were able to grow at low temperature, their cell doubling time tested in 20% whey at 10ºC (*K.* B006 8.50 h, *K.* TR002 6.59 h) was much longer than that at 25ºC (*K.* B006 1.15 h, *K.* TR002 1.23 h). Therefore, 25ºC was a practical temperature for laboratory experiments.

Experiments with no inoculum injected were run as negative controls. In order to grow biofilms of individual strains, 1 ml of inoculum was injected into the reactor using a sterile syringe. One milliliter of each inoculum was used for growing biofilms of dual strains. To allow the microorganisms to attach to the membrane surface, the reactor was run for 1 h at 25°C with a rotating speed of 180 rpm (Reynold's number was approximately 1850 (Buckingham-Meyer et al., 2007)) before medium was continuously pumped through at 5.5 ± 0.5 ml min^{-1}. The flow rate was set using a graduated cylinder and stopwatch, based on the calculated planktonic growth data obtained from the batch experiments, to ensure that the hydraulic retention time was less than the shorter cell doubling time (Komlos et al., 2005) of the two strains. Membrane samples were taken after 24 h incubation when the density of the culturable cells in the biofilm became to > 10^6 CFU cm^{-2} and rinsed in a sterile glass bottle containing 15 ml sterilized RO water for 1 min. Then they were transferred into 10 ml sterilized peptone water (Merck KGaA, Darmstadt, Germany) with four glass balls (d = 5 mm) and treated for 2 min in a sonicator water bath (Soniclean Pty Ltd., Thebarton, SA, Australia) to remove biofilm from the membrane surface and disrupt biofilm clumps. The peptone containing biofilm cells was then diluted in peptone in serial 10-fold dilutions and surface-plated (0.1 ml) onto standard plate count agar (SPCA) (Merck KGaA, Darmstadt, Germany). The treatment with a sonicator water bath has also been used by others (Schaule et al., 2000). Three different times (1, 2 and 3 min) for sonication were

tested for selecting the best sonication time that is able to provide the maximum removal and survival of cells from the biofilms on membranes.

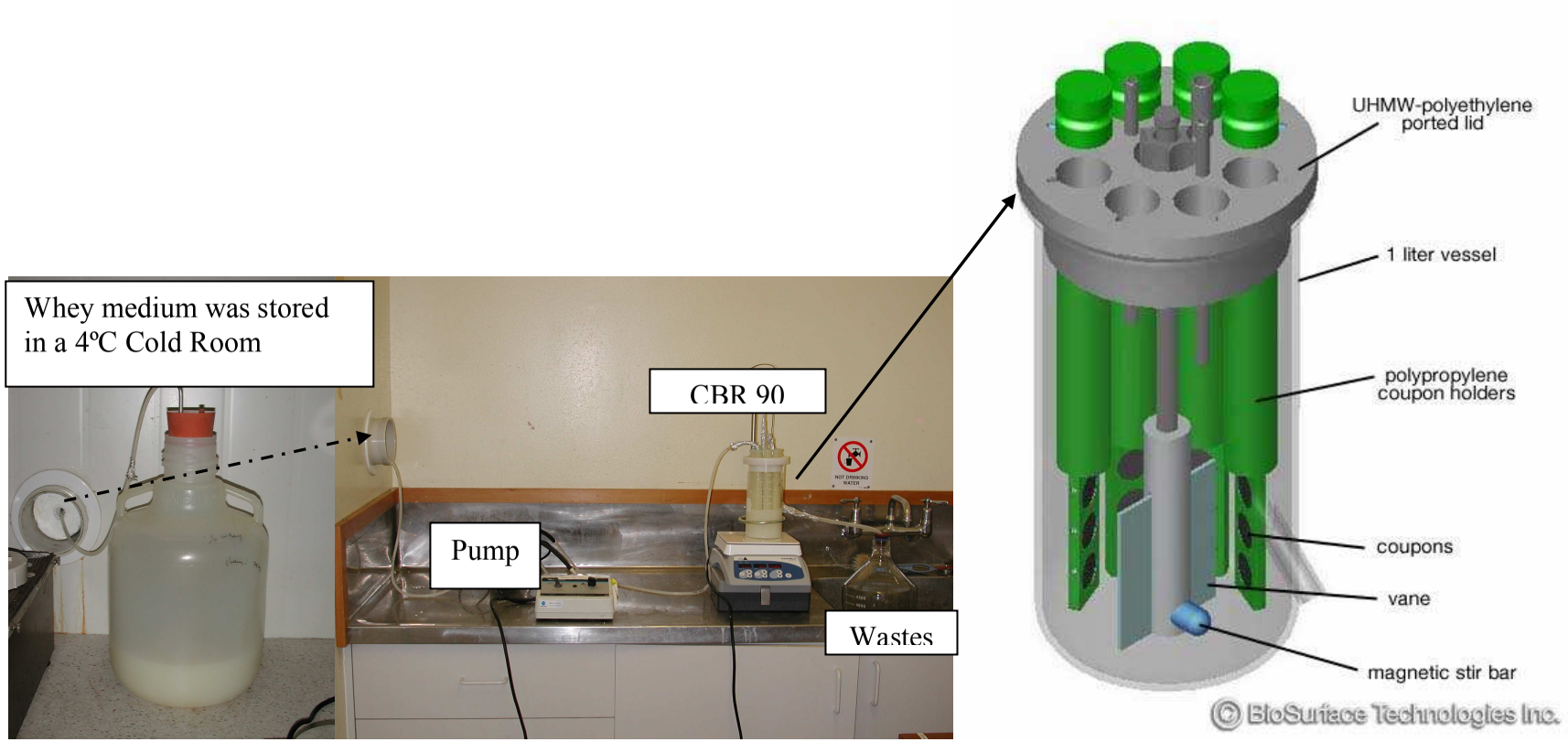


Figure 5.2: The whole laboratory scale biofilm growth system.

(The image of CBR 90 biofilm reactor was obtained from http://cu.imt.net/~mitbst/CDC_Specs.html and used with permission from BioSurface Technologies Inc.)

5.2.6 CIP procedures

The membranes were inserted into the rods and cleaned in the CBR 90, according to the procedures (Table 5.1 & 5.2) provided by the membrane manufacturer and the dairy manufacturing plant, before pumping the medium into the reactor. Reflux® 7C (Appendix II) was obtained from Orica New Zealand Ltd, Auckland, New Zealand.

Table 5.1: CIP procedure for new membranes, obtained from the membrane supplier (Synder Filtration, Vacaville, CA, USA).

Step	Chemicals	Time (min)	Temp (°C)	pH
Water Pre-flush	-	60	50	-
Alkaline Recirculation	50% w/w NaOH + Reflux® 7C (0.25-0.50 ml. L^{-1} of water)	20	50	10-11
Water Flush	-	20	50	-

Table 5.2: CIP procedure for used membranes, obtained from a New Zealand dairy manufacturing plant. The free available chlorine (FAC) was determined using a standard sodium thiosulfate titration.

Step	Chemicals	Time (min)	Temp (°C)	pH target
Water Pre-flush	-	10	50	-
Acid Recirculation	80% w/w H_3PO_4	30	50	~3
Water Flush	-	20	50	-
Alkali Recirculation	50% w/w NaOH + Reflux® 7C (0.25 ml. L^{-1} of water)	20	50	10-11
Water flush	-	20	50	-
Alkali + Sodium Hypochlorite Recirculation	50% w/w NaOH + NaOCl	20	50	10-11 (200 ppm FAC)
Water Flush	-	10	50	-

5.2.7 Experimental design

A full factorial design was used for testing three factors (Table 5.3). The full experimental design is shown in Appendix I. This involved completing $3^3 = 27$ experiments plus some extra experiments as controls. The results were based on four randomly sampled coupons in each experiment.

Table 5.3: Factors in the experimental design.

Factors	Levels		
	L1	**L2**	**L3**
Strains	*K.* B006	*K.* TR002	Mixture (1:1 ratio)
Whey Concentration	1 %	5 %	20 %
Membrane Type	New PES	New PVDF	Used PES

5.2.8 Scanning Electron Microscopy (SEM)

Biofilm structures were imaged using an FEI quanta 200 scanning electron microscope (FEI Electron Optics, Eindhoven, Netherlands). Membrane samples were cut to 4 mm × 4 mm using sterile blades. Then they were fixed with 3% glutaraldehyde and 2% formaldehyde in 0·1 molar phosphate buffer pH 7.2 for 24h at room temperature. The fixed samples were washed through buffer 3 times, dehydrated through a graded series of ethanol solutions (25%-100%), and critical point dried using liquid CO_2. Dried samples were mounted on to aluminum specimen support stubs using conductive silver paint and then sputter coated with gold. Images were taken using 20 KV accelerating voltage in the high vacuum mode.

5.2.9 Statistical analysis

All statistical calculations were performed on the log density values. Each mean and standard deviation of log density came from four identical tested membrane samples. The ANOVA in Minitab software (Release 15; Minitab Inc., State College, PA, USA) was used to analyse the variance of factors affecting biofilm development. These included whey concentrations, membrane types and strains.

5.3 RESULTS

5.3.1 Biofilm Growth

Samples of new and used membranes, prepared for this study by cleaning using standard procedures, without any inoculum showed no biofilm growth over 24 h in the CBR reactor. The biofilm growth on membranes following inoculation under various experimental conditions is summarized in Table 5.4. The average density of *K. oxytoca* biofilm in terms of culturable plate counts on membrane surfaces was between 4.9 – 7.99 $\log_{10}$ CFU cm^{-2}.

Table 5.4: Biofilm log density of two strains and their combination in whey on UF membranes after 24 h incubation. (Each mean and repeatability standard deviation (SD) was calculated from 4 membrane samples.)

Whey Concentration		**1%**			**5%**			**20%**		
Stain	**Biofilm Density (log_{10} CFU cm^{-2})**	**New PES**	**New PVDF**	**Used PES**	**New PES**	**New PVDF**	**Used PES**	**New PES**	**New PVDF**	**Used PES**
***K.* B006**	Mean Density	5.17	5.92	6.88	6.28	6.30	7.64	6.87	6.81	7.55
	SD	0.21	0.18	0.05	0.09	0.11	0.11	0.04	0.45	0.15
***K.* TR002**	Mean Density	5.15	5.62	6.17	4.90	5.59	7.82	5.92	6.03	7.99
	SD	0.19	0.12	0.03	0.09	0.05	0.03	0.06	0.16	0.15
Mixture	Mean Density	6.91	7.07	6.23	6.75	6.66	7.64	7.18	7.07	7.98
	SD	0.12	0.11	0.10	0.04	0.15	0.14	0.10	0.10	0.12

5.3.2 Validation of time for sonication

It was observed that the maximum number of removal and survival cells from biofilms on membranes was after ultrasonic treatment for 2 min (Table 5.5).

Table 5.5: Validation of time for sonication by comparing detectable biofilm densities (log_{10} CFU cm^{-2}) based on plate counts. Used PES membrane samples for testing were obtained after 24 h incubation in 1% whey. (Each mean and standard deviation was calculated from 8 membrane samples.)

Sonication Time / Strains	1 min	2 min	3 min
K. B006	5.36 ± 0.16	6.90 ± 0.08	6.21 ± 0.06
K. TR002	5.02 ± 0.27	6.13 ± 0.09	6.05 ± 0.23
Mixture	5.28 ± 0.19	6.55 ± 0.11	6.18 ± 0.14

5.3.3 Impact of whey protein concentration, membrane type and strains

The impact of the three single factors (whey protein concentration, membrane type and strain type) and their two-factor interactions on *K. oxytoca* biofilm growth were analysed using the ANOVA of Minitab statistical software (Table 5.6). All the single factors and their two-factor interactions showed significant effects on biofilm growth.

Table 5.6: ANOVA data of main and interaction effects of strains, whey concentration and membrane type on biofilm growth.

Source	DF	MS	F	P
Whey Concentration	2	7.6328	86.11	< 0.001
Membrane Type	2	14.6463	165.24	< 0.001
Strains	2	7.6554	86.37	< 0.001
Interaction of Whey Concentration & Membrane Type	4	1.5668	17.68	< 0.001
Interaction of Whey Concentration & Strains	4	0.2305	2.60	0.041
Interaction of Membrane Type & Strains	4	2.2472	25.35	< 0.001
Error	89	0.0886		
Total	107			

The biofilm density increased with increasing whey concentration. The used PES membrane supported more biofilm than the new membranes. Biofilm grew slightly better on new PVDF membranes than on new PES membranes. *K.* B006 grew better biofilm than *K.* TR002. The mixture of the two strains showed a biofilm growth with higher density than either single strain. (Fig. 5.3)

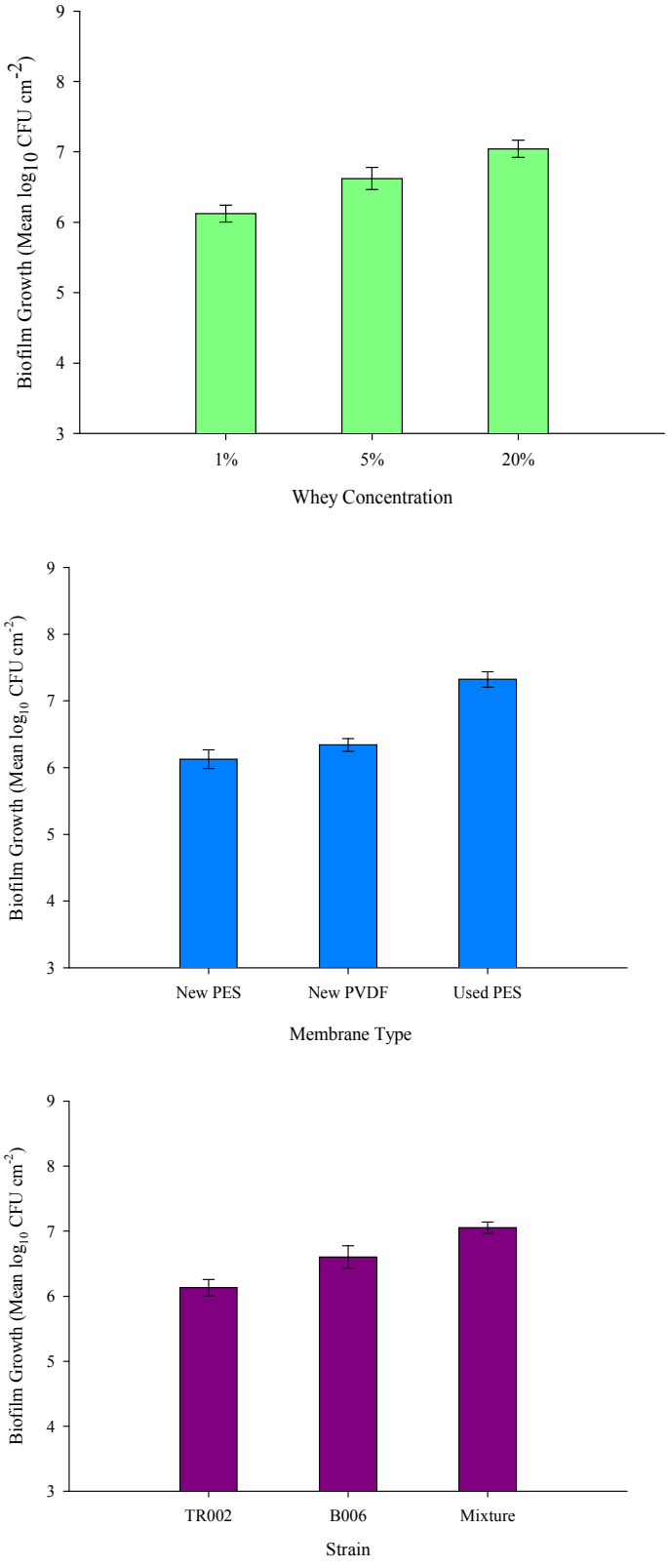


Figure 5.3: Main effects of single factors on biofilm growth. (Each mean and the standard deviation of the mean were calculated from 36 membrane samples.)

The ANOVA analysis showed that all the two-factor interactions had significant effects on biofilm growth. The biofilm log densities on the used membranes were generally higher than those on the new membranes, no matter what strains were used (Fig. 5.4). On the new membranes, the dual strains showed much higher biofilm density than the single strain, and B006 produced more biofilm than TR002 (Fig. 5.4). However, on used PES membranes, there was little difference between single and dual strains (Fig. 5.4). This indicated that those biofilms on the used membranes were saturated with an approximate density of $10^7 - 10^8$ CFU cm^{-2}.

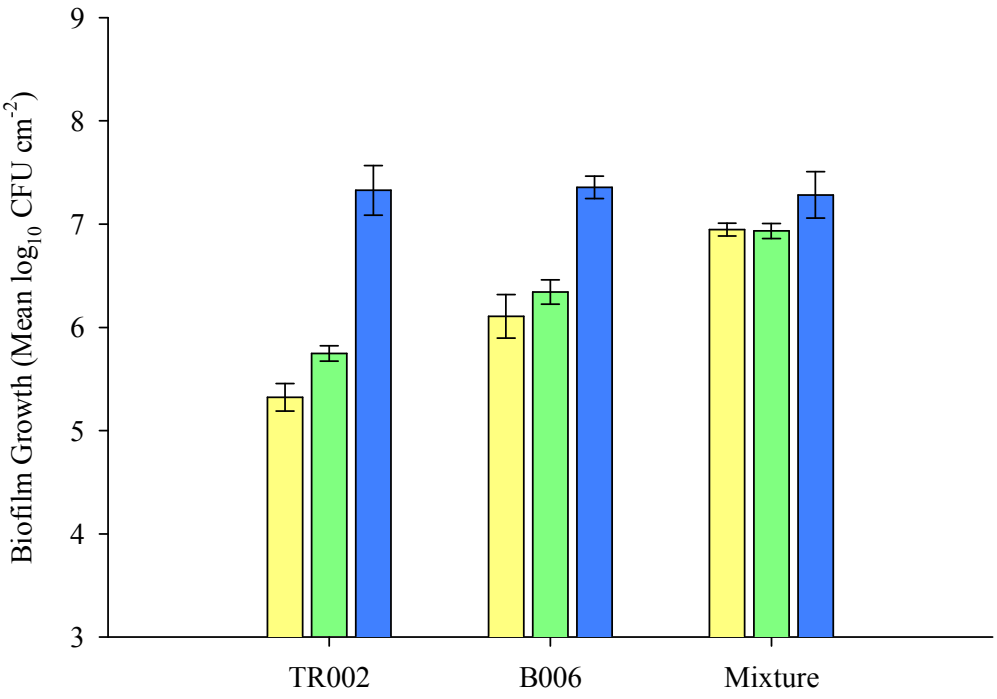


Figure 5.4: The effect of two-factor interaction of membrane type and strains on biofilm growth. Membrane types: New PES (■), New PVDF (■) and Used PES (■) (Each mean and the standard deviation of the mean were calculated from 12 membrane samples.)

The biofilm formed by the inoculation of either pure strain or mixture showed increased growth with the increased whey protein concentration (Fig. 5.5). The dual strains produced higher biofilm density than single strains in all three whey protein concentrations (Fig. 5.5).

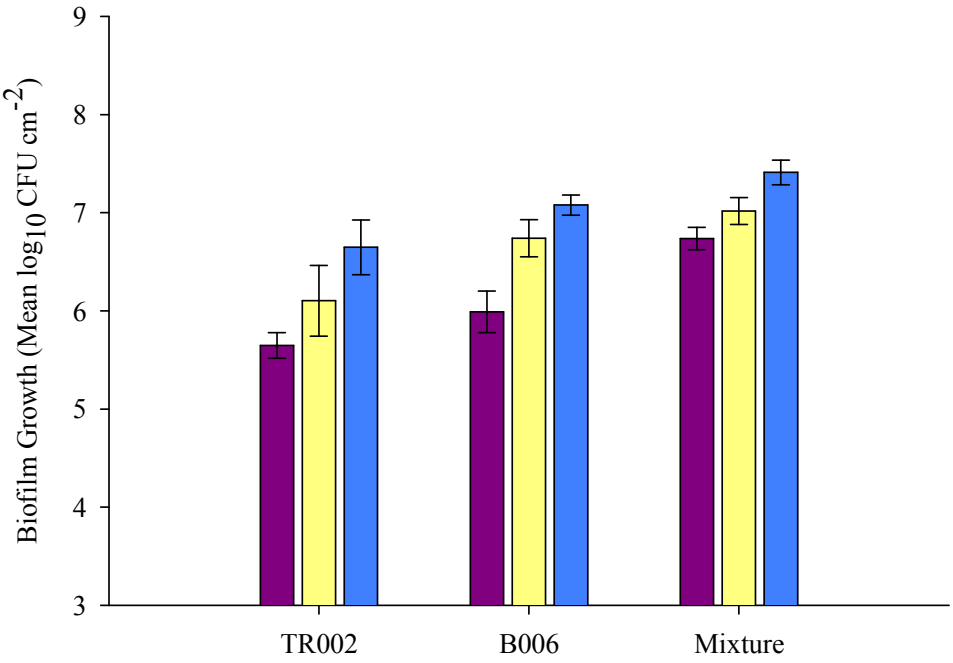


Figure 5.5: The effect of two-factor interaction of whey protein concentrations and strains on biofilm growth. Whey protein concentrations: 1% (■), 5% (■) and 20% (■) (Each mean and the standard deviation of the mean were calculated from 12 membrane samples.)

In 5% and 20% whey, the biofilm on used membranes was significantly denser than those on new membranes, while there was not much difference between the biofilm density on new PES and new PVDF membranes (Fig. 5.6).

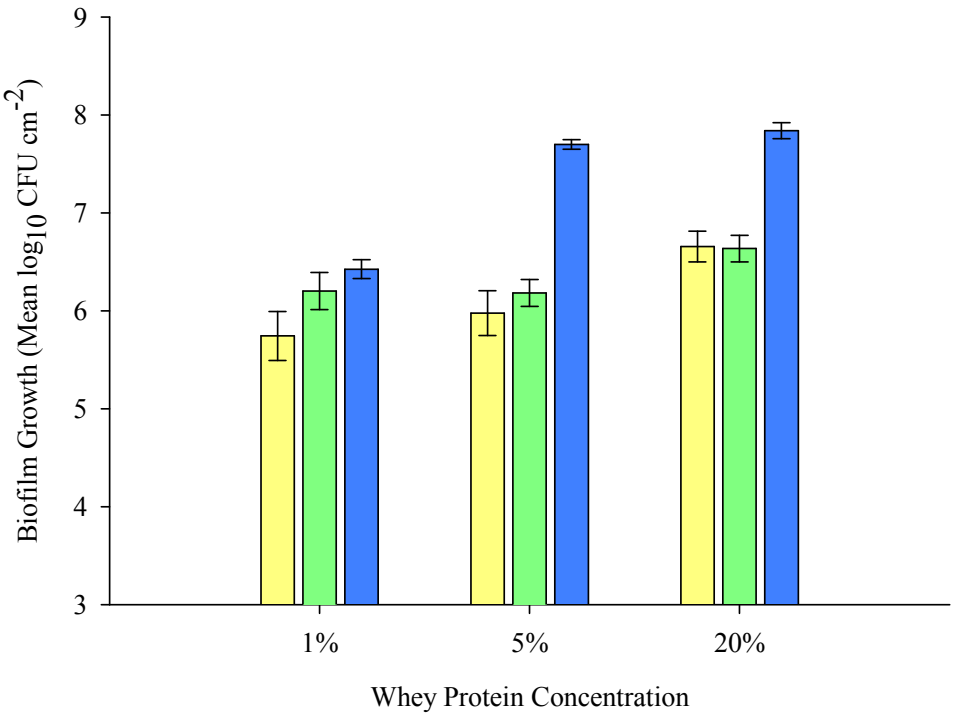


Figure 5.6: The effect of two-factor interaction of membrane type and whey protein concentrations on biofilm growth. Membrane types: New PES (■), New PVDF (■) and Used PES (■). (Each mean and the standard deviation of the mean were calculated from 12 membrane samples.)

In a summary, the biofilm density reached the highest when the reactor was inoculated with dual *K. oxytoca* strains in 20% whey protein medium and used PES membranes were fitted into the holders.

5.3.4 Scanning electron microscopy

The plate counts showed that the used membranes tended to have high biofilm densities. The images from SEM also confirmed that there were areas of used PES membrane that were highly colonized with microorganisms (Fig. 5.7).

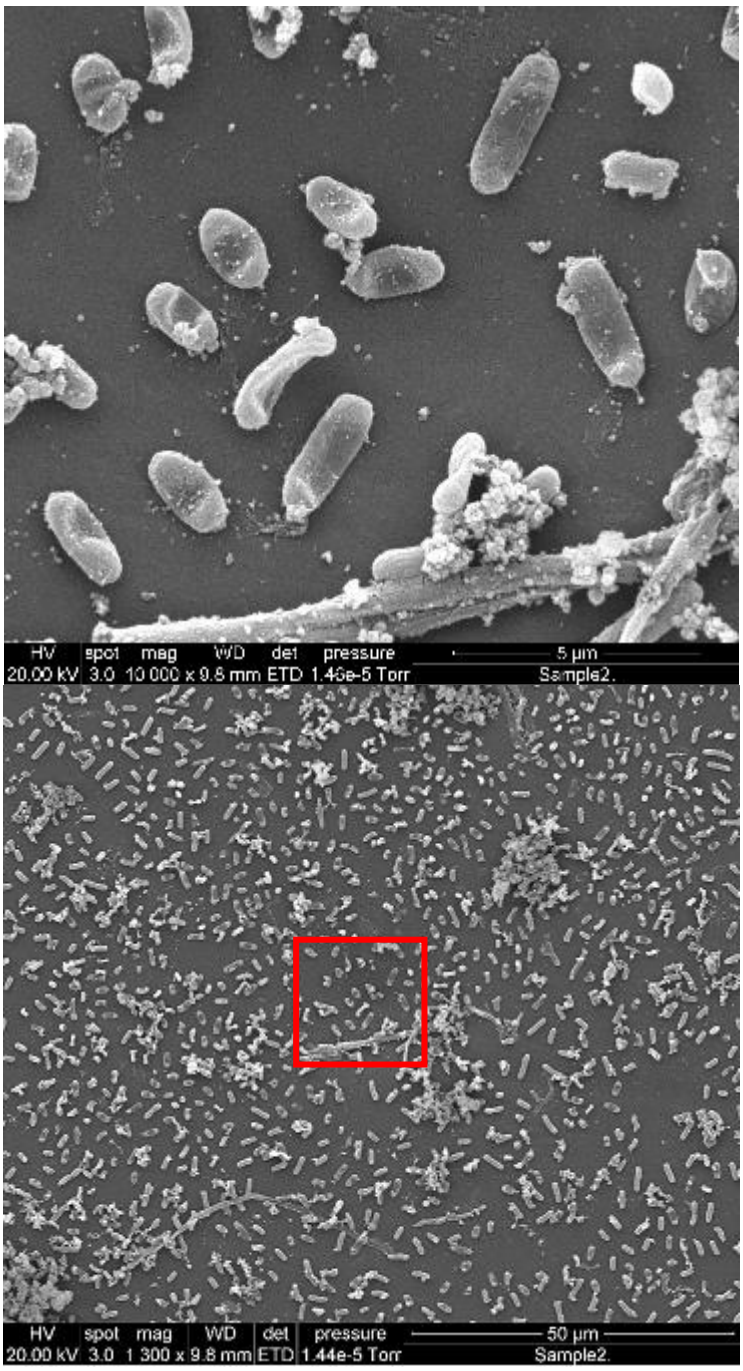


Figure 5.7: SE Micrographs of biofilm of *K. oxytoca* B006 on used PES membranes after 24 h incubation with 5 % whey. (The top is the higher magnification image of the rectangular area in the bottom image.)

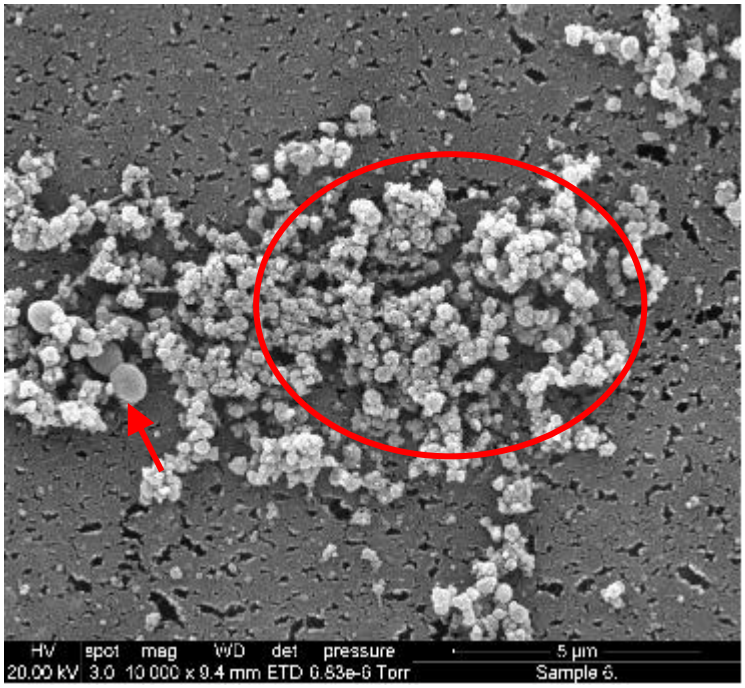


Figure 5.8: SE Micrograph of biofilm of *K* TR002 on a new PVDF membrane after 24 h incubation with 5 % whey. (Microorganisms are indicated by the arrow and the circled position shows the qualitatively observed foulant which is probably the whey proteins.)

Comparing Fig. 5.7 (PES) and Fig. 5.8 (PVDF), it was found that the two membrane surface structures appeared to be different. The PVDF structure looked more open, probably due to its large MWCO (800,000) or an artifact of preparation. Nevertheless, the biofilms on new PVDF membrane were generally less dense, compared with used PES membrane, as indicated in Table 5.4. Interestingly, on new PVDF membrane, the amount of qualitatively observed foulant was much higher than on used PES membrane (Fig. 5.7 & encircled in 5.8).

5.4 DISCUSSION

This study aimed to evaluate which factors influence biofilm growth on UF membrane surfaces, using a laboratory scale system. The CBR 90 system was confirmed to be an effective system for growing biofilms on membrane samples. As far as we are aware, this is the first time that the CBR 90 system has been used for studies on membrane

systems, although the system has been used to study biofilm growth on a variety of surfaces (Goeres et al., 2005).

The two *Klebsiella* strains were isolated from two different UF membrane plants processing whey, therefore whey was chosen as the nutrient medium for these trials. To mimic conditions in the manufacturing plants, the whey medium was made artificially by mixing minerals, lactose and WPC 80 in the proportions to reflect concentrations expected during whey processing. Thus, the three whey concentrations used are representative of three stages (start, middle and end) in the UF membrane plant processing whey. This method of preparing the whey medium ensured consistency throughout the study and avoided variation that would be expected with different batches of whey.

All three factors (whey protein concentration, strain and membrane type) were shown to be significant factors by themselves. Whey protein concentration played an important role in biofilm development of the two strains. The higher the whey protein concentration, the more biofilm was formed (Fig 5.3). This indicates that *K. oxytoca* growth was limited by the medium concentration. Some other studies also found that *K. oxytoca* reached higher biofilm density in higher substrate concentration (Komlos et al., 2005). The dual strains always reached high biofilm density on any membrane surface, but single strains, especially *K.* TR002, reached much higher biofilm density on used membranes than on new membranes (Fig. 5.4).

The two strains of *Klebsiella* really only differed in abilities to form biofilm on new PES membranes. *K.* TR002, which was isolated from PES UF membrane surfaces, showed less ability to grow as a biofilm than *K.* B006, which was isolated from the liquid in a UF membrane plant. This corresponds to our previous study, in which *K.* B006 had a greater ability to attach to surfaces than *K.* TR002 (Chapter 4).

On new membranes, the mixture of strains showed higher biofilm density then the single strains for each whey protein concentration. However, on the used membranes, the biofilm density of single strains and the mixture were not significantly different. Note that the maximum biofilm density in our experiments was about 7-8 $\log_{10}$ CFU cm^{-2}. Ivnitsky et al. (2005) reported a bacterial count in biofilm on a nanofiltration

membrane of approx. 7 $\log_{10}$ CFU cm^{-2} regardless of the feed applied. This might indicate that the biofilm growth reached a steady state (Characklis, 1990a), due to a balance between the available nutrients and the shear forces over the membrane surface, or other limiting conditions (Melo et al., 1992).

The biofilms formed on used membranes were significantly denser (mean = 7.32 $\log_{10}$ CFU cm^{-2}) than those on new membranes (mean = 6.23 $\log_{10}$ CFU cm^{-2}). We speculate that the most likely cause is that repeated cleaning of the used membranes has modified the physicochemical properties of the surface and enhanced the bacterial adhesion.

Our observations may be explained by the presence of organic material, especially protein, on the membrane surface. Proteins may either enhance or block microbial attachment (An & Friedman, 1998). These influences may depend upon whether the proteins are in a denatured or native form. In the first case, in our trials with used PES membranes, we presumed that organic material not completely removed by standard cleaning had been denatured during the CIP process and might have acted as a conditioning film, easily binding or trapping bacteria on the surface. Such conditioning layers composed of denatured proteins may have enhanced the bacterial adhesion.

In the other case, the probable fresh whey proteins, as observed on the SEM (Fig. 5.8), were seen to foul the new PVDF membrane surface but not the used PES membrane surface (Fig. 5.7). The lower microbial biofilm density observed on the new PVDF membrane (Fig. 5.8) might be explained by the attachment of fresh whey proteins to the surface of new PVDF membranes more readily than to PES membrane and by these fresh, native proteins blocking the attachment of bacteria. We assume that the used PES membrane surface properties may have been altered by frequent chemical cleaning, and the changed membrane surface may not favor the attachment of fresh whey proteins. This protein blocking of microbial attachment has been seen by others (Bernbom et al., 2006). It has also been suggested that fresh proteins present in the liquid inhibit bacterial adhesion (Brokke et al., 1991; Fletcher, 1976). In addition, the footprint left by the old biofilms after CIP might enhance the cell attachment to the surfaces. It is clear that the behaviour of biofilms on new and used membranes merits further investigation.

5.5 CONCLUSIONS

With the continuous flowing CBR 90 system, we aimed to create a similar environment to that experienced in a UF plant processing whey, with the exception that product was not actually passing through the membrane. The whey composition, temperature and turbulence were all representative of what could be expected in a whey processing plant.

The results of our study supported our hypothesis and suggested that the whey protein concentration, membrane type including membrane material and age, types of strain and the interactions between different microorganisms are all important factors for biofilm development on UF membrane surfaces. Both strains formed good biofilms, although *K.* B006 formed a denser biofilm than *K.* TR002. This corresponded to our previous study on the attachment of these organisms, where *K.* B006 attached in greater numbers than *K.* TR002. The dual strains produced a higher biofilm density than single strains on the new membranes. Biofilm density tended to increase with increased whey protein concentration. The saturated biofilm was approximately 8 $\log_{10}$ CFU cm^{-2}. PES membranes appeared to support biofilm growth less readily than did PVDF membranes and therefore may be the preferred material for UF membranes to reduce problems with microbial colonization. Used membranes were more susceptible to colonisation with biofilm than were new membranes. Therefore, selecting a membrane type and monitoring membrane age will help manage biofilm development during UF.

Chapter 6

REMOVAL OF BIOFILMS FROM MEMBRANES

6.1 INTRODUCTION

Fouling is a serious problem in the application of membrane technology (Cabero et al., 1999; Marshall et al., 1993; Nilsson, 1988). Dairy components, such as proteins, fats and minerals, are considered to be the key membrane fouling particles. To maintain the permeability and the selectivity of the membranes, regular chemical cleaning is required every 18 – 24 h. Many studies related to membrane cleaning have been done in the past 10 years (Arguello et al., 2002; Cabero et al., 1999; Gillham et al., 2000; Rabiller-Baudry et al., 2008). However, most of them have focused on removal of protein foulant (Arguello et al., 2002; Cabero et al., 1999; Gillham et al., 2000; Rabiller-Baudry et al., 2008), while the removal of biofilm on the membranes has been rarely studied. Biofilms growing on the membranes have been reported to be a problem resulting in membrane blockage, product contamination and reduction of membrane life due to the microbial action on the membrane material (Bodalo-Santoyo et al., 2004; Lim & Bai, 2003; Ridgeway & Flemming, 1996).

A typical dairy CIP process consists of an alkaline or acid wash followed by a sodium hypochlorite wash at pH 11-12 (200 ppm). The alkaline treatment solubilises proteins, fats and carbohydrates, while the acid dissolves minerals. Sodium hypochlorite is widely used as a disinfectant. However, when it is used at alkaline pH, it is not considered a true sanitiser, as this pH limits the amount of hypochlorous acid produced (Estrela et al., 2002). Treatment with hypochlorous acid reportedly damages polyamide RO membranes (Gabelich et al., 2005). PES membranes were found to be unstable in solutions containing chlorine (Begoin et al., 2006). However, after analysing the molecular mass of PES, it has been found that there is no reaction between PES and hypochlorite at pH 6.9 – 11.5 (Wienk et al., 1995).

Enzymes (proteases and lipases) are often selected as complementary cleaning agents when simple chemicals (alkali and acid) are not enough for cleaning and recovering the

membrane capacity. However, most of the studies using enzyme cleaners focused on removing protein foulant, but did not evaluate the microbial component i.e. biofilms (Arguello et al., 2002).

Although many cleaners have some ability to disinfect, control of biofilms always requires detergent cleaning followed by the use of a sanitiser (Zottola & Sasahara, 1994). There is a wide choice of sanitisers available for use in the food processing industries.

Peracetic acid (PAA) is a sanitiser with high oxidising potential sometimes used in dairy plants and it is effective against bacteria, fungi and spores (Loukili et al., 2006). It is not inactivated by catalase or peroxidase.

Ozone has been used for many years in European countries. The main use is to disinfect drinking water (Guzel-Seydim et al., 2004). Greene et al. (1993) reported that both ozonated water and chlorine have equivalent decontamination efficacies. However, the necessary contact time is likely to be less than when using chlorine, as ozone is a more powerful oxidizer than chlorine (Greene et al., 1993). Most recent studies have found that ozonated water was effective in inactivating biofilms of *Pseudomonas fluorescens* on glass slides (Tachikawa et al., 2009).

Anolyte of electrolysed water (EW) composed of ClO_2, ClO^-, H_2O_2, HO_2^-, NaOH, O_2, O_3, HClO, Cl_2 and $^{\cdot}OH$ (Thantsha & Cloete, 2006) is an alternative treatment for controlling biofilms (Cloete & Maluleke, 2005), which contains positively charged oxidant solution (Thantsha & Cloete, 2006). The advantages of using EW are that it is easy to produce, is stable if stored in a sealed container (Len et al., 2002) and it does not require a high temperature for operation. The production of EW anolyte and its antimicrobial activity on different foods has been reviewed by others (Mahmoud, 2007), where the efficacy of using EW anolyte as sanitiser for food is reported. Researchers suggested using electrolysis for sanitising water for final rinsing of vegetables (Ongeng et al., 2006). It was found that slightly acidic electrolysed water from the anode was effective for inactivating the *Salmonella enteritidis* (Cao et al., 2009).

The performance of those cleaners and sanitisers described above in terms of removing and killing biofilms on membranes is unknown. The objective of this study was to

investigate the efficacy of selected cleaners and sanitisers in removing and killing biofilms comprising single and dual *Klebsiella* strains on used PES UF membranes. Our hypothesis was that the enzymatic cleaner would be more efficient than the chemical cleaners and the EW anolyte containing a mixer of oxidants would be more efficient than other sanitizers used for this trial. Also it was supposed that the current CIP would be improved by adding an extra step of sanitizing.

6.2 MATERIALS AND METHODS

6.2.1 Sources of strains

The two *K. oxytoca* strains (B006 and TR002) used in this study were the same strains as used in Chapter 4.

6.2.2 Preparation of medium

5% whey protein medium was prepared (Section 5.2.2).

6.2.3 Preparation of inocula

Inocula of *K.* B006 and TR002 were prepared using the same method as described in Section 5.2.3.

6.2.4 Membranes

Spiral-wound PES membranes provided by a New Zealand dairy manufacturing membrane plant processing cheese whey were used in this study. The methods for preparing membrane coupons to fit them into a CBR 90 biofilm reactor (BioSurface Technologies, Bozeman, USA) and testing with a typical CIP before biofilm development were the same as described in Section 5.2.4.

6.2.5 Biofilm development

The method for developing *K. oxytoca* biofilms in this study was the same as described

in Section 5.2.5.

6.2.6 Cleaners and sanitisers

After generating the biofilm on the membrane surfaces, different CIP treatments using different cleaners were tested, followed by treatment with a selection of sanitisers. The standard CIP procedure (Table 6.1) was as used by the New Zealand dairy industry and was considered as a control.

Table 6.1: Standard CIP for dairy membrane processing plants. (The sixth step of using sodium hypochlorite at high pH was considered as the control. Compositions of Reflux® chemicals are given in Appendix II.)

	Step	Chemicals	Time (min)	Temp (°C)	pH target
1	Water Pre-flush	-	10	50	-
2	Alkaline Recirculation	Reflux® B615	30	50	10.8 - 11.0
3	Water Flush	-	20	50	-
4	Acid Recirculation	Reflux® R400	25	50	1.8 - 2.0
5	Water flush	-	20	50	-
6	Alkali + Sodium Hypochlorite Recirculation	Reflux® B615 + Reflux® S800	30	50	10.8 - 11.0 (200 ppm FAC)
7	Water Flush	-	20	50	-

The FAC was determined using a standard sodium thiosulfate titration (Willson, 1935). Cleaning solution (500 ml) was re-circulated through the reactor containing membrane samples at a rate of 198 ml min^{-1}.

The cleaners listed in Table 6.2 were used to take the place of the "Alkali + Hypochlorite" step (step 6) in the standard CIP (Table 6.1). Reflux® chemicals

(Appendix II) and enzymes including QuatroZyme® and Perform® (Appendix II) were obtained from Orica New Zealand Ltd, Auckland, New Zealand.

Table 6.2: Cleaners used to compare with the control (Sodium hypochlorite at pH 10.8-11).

Chemicals	Dose (v/v)	Temp (ºC)	pH	Exposure time (min)
Reflux® E2001 (Protease & Lipase)	0.2%	48	8.5-9.5	45
Reflux® E1000 (Protease)	0.2%	48	9.0-10.0	45
QuatroZyme® (Lipase, Protease, Cellulase, Amylase)	0.3%	48	7.0-8.0	30

Sanitisers (Table 6.3) were used as an additional step in the CIP and followed step 7, the water rinse.

Table 6.3: Sanitisers used following the CIP.

Chemicals	Dose	Temp	Exposure time (min)
Sodium Hypochlorite (Reflux® S800) pH 6.5	200 ppm FAC	30ºC	20
Perform®	2% v/v	25ºC	20
MIOX® EW Anolyte pH 6.8 (1 day old)	120 ppm FAC	Room Temperature	10
Ozonated Water pH 7.0	0.5 ppm FAO	Room Temperature	10

EW was generated by a laboratory scale mixed oxidants brine pump system (MIOX® BPS) (MIOX Corporation, New Mexico, USA) using 1% NaCl solution at 5 A and 12 V. The mixed oxidant solution composed of ClO_2, ClO^-, H_2O_2, HO_2^-, NaOH, O_2, O_3, HClO, Cl_2 and $^{\cdot}OH$ (Thantsha & Cloete, 2006) from the anolyte was stored in a sterile container in a 4ºC cold room for 1 day before use to ensure that the efficacy of anolyte was the same for each experiment. The recommended storage life of EW is 48 h (MIOX Corporation, New Mexico, USA). The pH was adjusted to 6.8 and the measured FAC was 120 ppm. The ozone was generated by the ozone generator (VT-2A Model, EnvirOzone, Napier, New Zealand) and pumped into the autoclaved RO water, then the ozonated water containing 0.5 ppm free available ozone (FAO) as previously used by Greene et al. (1993) was pumped into the reactor after the pH was adjusted to 7.0. The free active ozone in the ozonated water was calculated from the ozone pumping speed

and pumping time.

6.2.7 Sanitiser screening test

After the CIP steps (steps 1-7 in Table 6.1 with the 6th step replaced by the cleaners listed in Table 6.2), coupon holders with 3 membrane samples on each were removed from the CBR 90 reactor and placed into a 200 ml beaker containing 150 ml sanitiser and sanitised as described in Table 6.3 with stirring at 180 rpm. The standard CIP followed by sanitising was the control. After sanitising, each membrane sample was inserted into a 25 ml glass bottle containing 10 ml Dey/Engley (D/E) neutralizing solution (Difco^{TM}, Sparks, MD) (Engley & Dey, 1970; Sutton et al., 1991) (see composition in Appendix II) and incubated at room temperature for 10 min to neutralize the sanitisers. Membrane samples with no sanitiser treatment were considered as the control. To estimate the numbers of viable and culturable cells left on the membrane surfaces, the membrane samples were sonicated for 2 min in 10 ml sterile peptone water with glass beads and the liquid was then centrifuged for 10 min at 2500 × g. Eight milliliters of the supernatant fluid was discarded and the pellet was resuspended to obtain a final 2 ml sample. The centrifugation method was tested to verify the recovery of cells. Serial 10-fold dilutions were prepared in sterile peptone water. Tempered SPCA was inoculated with 2 ml samples, pour plated and incubated at 30°C for 3 days before the colonies were counted.

6.2.8 Validation of centrifugation for recovering cells

A 10 ml inoculum of *K.* B006 after 24 h incubation was plate counted and the results were considered as the control. Another 10 ml of the same inoculum was centrifuged. Eight milliliters of the supernatant fluid was discarded and the pellet was resuspended in sterile peptone water to obtain a final 10 ml sample. This resuspension was plate counted and the results were compared with the control.

6.2.9 Statistical analysis

Plate counts for each membrane sample were converted to $\log_{10}$ values. Each mean and standard deviation was calculated from the counts of three identical tested membrane

samples. All the data were analysed using the general linear model of ANOVA test in Minitab software (Release 15; Minitab Inc., State College, PA, USA) at the 95% confidence level.

6.3 RESULTS

6.3.1 Validation of centrifugation for recovering cells

The mean log density of the *K.* B006 control without being centrifuged was 7.61 ± 0.14 $\log_{10}$ CFU ml^{-1}. The mean log density of the *K.* B006 after being centrifuged and resuspended was 7.50 ± 0.14 $\log_{10}$ CFU ml^{-1}. The recovery of using centrifugation was 99% ($p = 0.019$). All the means and standard deviations were calculated from 9 samples.

6.3.2 The efficacy of standard CIP

Both this and earlier studies (Chapter 5) using the CBR 90 biofilm reactor generated consistent biofilms of *K. oxytoca* with a log density of approximately 7.42 ± 0.30 $\log_{10}$ CFU cm^{-2} on membrane surfaces. The results showed that a standard CIP allowed a culturable count of 1.91 ± 0.42 $\log_{10}$ CFU cm^{-2} for biofilm formed by a single *K. oxytoca* strain and 2.19 ± 0.20 $\log_{10}$ CFU cm^{-2} for biofilm formed by dual *K. oxytoca* strains to remain on the membrane surface. (Means and standard deviations were taken from 72 membrane coupon samples.)

6.3.3 The efficacy of cleaners

The efficacy of different cleaners used in the CBR 90 in reducing counts of culturable bacteria on membrane surfaces is shown in Table 6.4. The variance data from ANOVA are shown in Table 6.5. Both the strain inoculated and cleaner significantly affected the removal of biofilms. The inoculation of single or dual strains significantly ($p < 0.001$) affected cleaning efficiency. All the cleaners were more effective on biofilms of a single strain, than on those composed of the dual strains, when no sanitiser was applied. The effectiveness of different cleaners in reducing culturable bacterial numbers also differed significantly ($p = 0.005$). The control clean removed 70-80% of culturable cells from the membrane surfaces. QuatroZyme® containing a mixture of enzymes performed the

best among all the cleaners, but still left at least 1.2 $\log_{10}$ CFU cm^{-2} culturable cells on the membrane surfaces. Reflux® E1000 (Protease) was less effective than the control in removing the bacteria from the membrane surface.

Table 6.4: The efficacy of different cleaners in reducing the culturable cells in *K. oxytoca* biofilms on membrane surfaces. Initial concentration was 7.42 ± 0.30 $\log_{10}$ CFU cm^{-2}. (Comparisons were between the 6th step of standard CIP in Table 6.1 and three enzymatic cleaners substituted for alkaline hypochlorite. Means and standard deviations were taken from triplicates.)

Cleaners	**Single strain *K.* B006**	**Dual strains *K.* B006 & TR002**
	Reduction ($\log_{10}$ CFU cm^{-2})	**Reduction ($\log_{10}$ CFU cm^{-2})**
Alkali + Hypochlorite 200 ppm FAC (Control)	6.01 ± 0.54	5.23 ± 0.13
Reflux® E2001 (Protease & Lipase)	6.02 ± 0.31	5.31 ± 0.36
Reflux® E1000 (Protease)	5.17 ± 0.14	4.98 ± 0.12
QuatroZyme®	6.15 ± 0.22	5.31 ± 0.23

Table 6.5: Analysis of variance for culturable cell reductions in *K. oxytoca* biofilms cleaned by different cleaners in Table 6.4.

Source	DF	MS	F	P
Cleaner Type	3	0.5462	5.97	0.005
Strain	1	2.3814	26.02	< 0.001
Error	19	0.0915		
Total	23			

6.3.4 The efficacy of sanitisers

The efficacy of different sanitisers (applied in beakers with stirring at 180 rpm) in reducing counts of culturable bacteria from residual biofilm following CIP with different cleaners is shown in Table 6.6. The variance data from ANOVA is shown in Table 6.7. The effectiveness of sanitising was significantly affected by selection of individual sanitiser ($p < 0.001$), individual cleaner ($p < 0.001$), the combination of cleaner and sanitiser ($p < 0.001$) and the combination of cleaner and the strain ($p < 0.001$), but not significantly affected by the inoculation of pure or dual strains ($p = 0.176$) or the combination of sanitiser and the inoculum ($p = 0.218$).

MIOX® EW anolyte (120 ppm FAC, pH 6.8) gave the highest or equal highest log reduction in all experiments (Table 6.6). In most cases, counts were below the lower detection limit of 0.1 $\log_{10}$ CFU cm^{-2}. Ozonated water produced the lowest log reduction recorded around 0.27 $\log_{10}$ CFU cm^{-2} (Table 6.6).

Table 6.6: Reduction of culturable cells in *K. oxytoca* biofilms on cleaned membrane surfaces by different sanitisers. Initial concentration was 7.42 ± 0.30 $\log_{10}$ CFU cm^{-2}. (Means and standard deviations were taken from triplicates.)

	Single strain (*K.* B006)				**Dual strains (*K.* B006 & TR002)**			
	Reduction ($\log_{10}$ CFU cm^{-2})				**Reduction ($\log_{10}$ CFU cm^{-2})**			
Cleaner / **Sanitiser**	Alkali + Sodium Hypochlorite 200 ppm FAC (Control)	Reflux® E2001 (Protease & Lipase)	Reflux® E1000 (Protease)	QuatroZyme®	Alkali + Sodium Hypochlorite 200 ppm FAC (Control)	Reflux® E2001 (Protease & Lipase)	Reflux® E1000 (Protease)	QuatroZyme®
Sodium Hypochlorite 200 ppm FAC pH 6.5	0.61 ± 0.55	1.86 ± 0.28	1.85 ± 0.03*	1.45 ± 0.27	0.63 ± 0.16	0.64 ± 0.26	2.19 ± 0.11*	2.06 ± 0.18*
Perform® 2% v/v	0.51 ± 0.62	1.74 ± 0.49	1.85 ± 0.03*	1.39 ± 0.31	0.57 ± 0.14	0.95 ± 0.28	2.19 ± 0.11*	2.06 ± 0.18*
MIOX EW Anolyte 120 ppm FAC pH 6.8	1.21 ± 0.51*	1.92 ± 0.07*	1.85 ± 0.03*	1.55 ± 0.20*	1.76 ± 0.47	1.87 ± 0.55	2.19 ± 0.11*	2.06 ± 0.18*
Ozonated Water 0.5 ppm FAO pH 7.0	0.59 ± 0.66	0.41 ± 0.24	0.27 ± 0.07	0.56 ± 0.25	0.33 ± 0.04	0.46 ± 0.22	0.78 ± 0.23	0.40 ± 0.27

* indicates the case that all the detectable culturable cells were killed. The results were obtained from pour plate counting at a detection limit of 0.1 $\log_{10}$ CFU cm^{-2}.

Table 6.7: Analysis of variance for culturable cell reductions in *K. oxytoca* biofilms removed by different sanitisers in Table 6.6.

Source	**DF**	**MS**	**F**	**P**
Sanitiser Type	3	7.6244	65.69	< 0.001
Cleaner Type	3	3.3290	28.68	< 0.001
Strain	1	0.2166	1.87	0.176
Combination of Sanitiser and Cleaner	9	0.5798	5.00	< 0.001
Combination of Sanitiser and Strain	3	0.1757	1.51	0.218
Combination of Cleaner and Strain	3	1.0747	9.26	< 0.001
Error	73	0.1161		
Total	95			

The means of measurements were plotted in Figure 6.1. MIOX® EW anolyte (120 ppm FAC, pH 6.8) was the most effective sanitiser in reducing culturable cell numbers, regardless of any cleaner used. Ozonated water was the weakest sanitiser tested. Sodium hypochlorite and Perform® resulted in very similar log reductions to the MIOX® EW anolyte when used after treatment with Reflux® E1000 (Protease). The use of MIOX® EW anolyte (120 ppm FAC, pH 6.8) after standard CIP reduced more culturable cells compared with the standard CIP. When MIOX® EW anolyte (120 ppm FAC, pH 6.8) was used in combination with Reflux® E1000 (Protease), even greater culturable cell reduction was achieved.

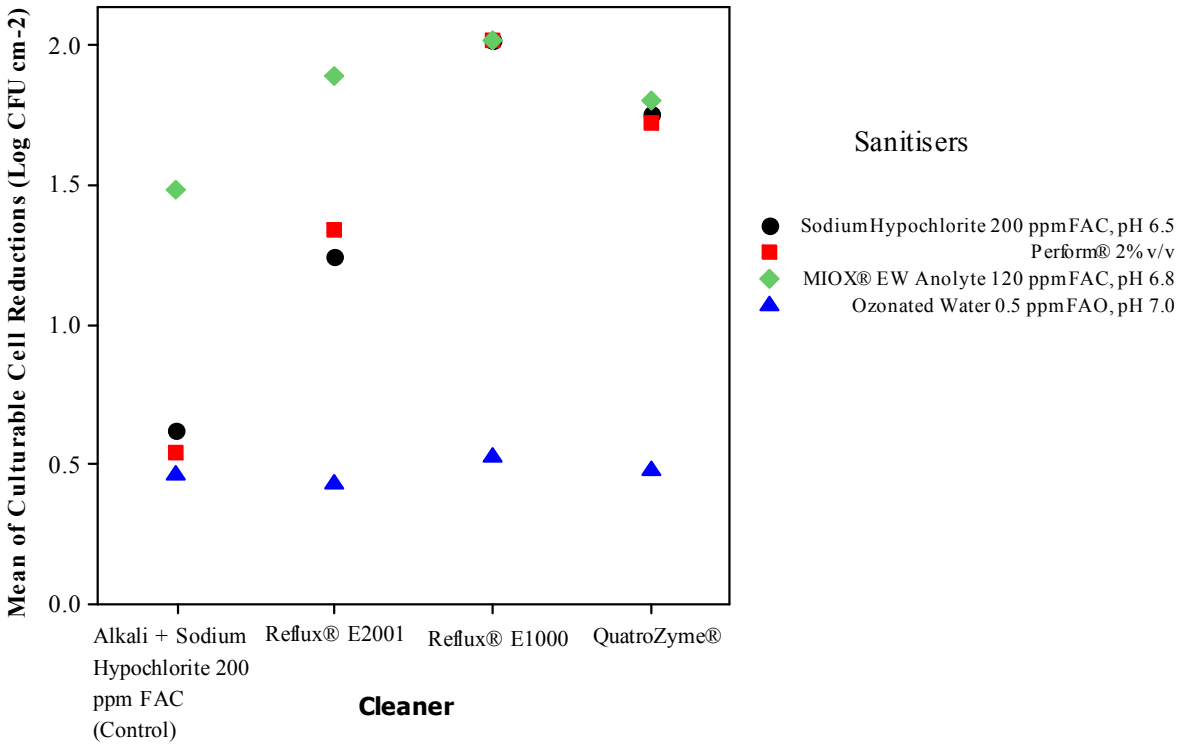


Figure 6.1: The efficacies of cleaners and sanitisers on controlling *K. oxytoca* biofilms on used PES membranes (Data were analysed using ANOVA in Minitab software (Release 15; Minitab Inc., State College, PA, USA). Three data symbols on the column of Reflux® E1000 overlap, because the means are the same number.)

6.4 DISCUSSION

This study investigated the efficacy of current CIP used in the dairy industry and compared different cleaners and sanitisers in controlling the biofilm formed by single or dual strains of *K. oxytoca* on used PES UF membrane surfaces.

A typical CIP procedure involving alkali, acid and sodium hypochlorite at alkaline pH was chosen as the cleaning control, because this CIP procedure is widely used in the dairy industry. Unfortunately, the CIP control in the CBR 90 did not completely remove the biofilm, perhaps as a result of imperfect cleaning between the membrane sample and the holder in the reactor. The mounting of the membrane sample also affects the efficiency of cleaning, as has been pointed out previously (Section 5.2.4). In some ways, this experimental deficiency mimics the situation in a plant, where rubber seals become cracked and harbour biofilms. The number of remaining culturable cells (1.9 – 2.19 $\log_{10}$ CFU cm^{-2}) after standard CIP on a small membrane surface area (1.27 cm^2) used in our experiments would be a concern when multiplied to reflect the total area of an industrial scale plant. There is evidence elsewhere that biofilms may protect bacterial cells against CIP chemicals and that culturable bacterial cells can remain attached to dairy manufacturing surfaces following a CIP (Austin & Bergeron, 1995; Flint et al., 1999). The cells remaining on the surface will enable rapid regeneration of biofilm once suitable conditions are restored during the processing of dairy liquids.

Biofilm formed by single or dual strains behaved differently during cleaning, and the combination of the inoculum and the selection of cleaner brought significant differences during sanitising. This might be explained by a difference in the structure of the biofilm formed by a single strain compared with dual strains, or some mutual interaction between the two strains, such as increased polysaccharide production, that resists removal by cleaners. During the sanitation phase, the sanitisers might be expected to kill viable attached cells and thus reduce the viable cell count, rather than removing the biofilms. Choosing the right cleaner for the removal of biofilm formed by specific strains might loosen or destroy the biofilm structure and would enhance the sanitising operation.

An enzyme mix has been found to reduce the number of viable cells significantly in the biofilm formed by *Lactobacillus brevis* (Walker et al., 2007). In our studies, cleaning with QuatroZyme®, containing a mixture of enzymes, performed better than other enzyme cleaners. However, more than 17% of the original cells remained culturable after the CIP with QuatroZyme®.

The limitation of using plate counting for assessing cell numbers is that this method may not recover all the viable cells, as only culturable cells are countable. Therefore, it is possible that viable but non-culturable cells may persist in the different treatments. The significance of such non-culturable cells in an industrial plant is not known. However, failure to remove all extracellular polymeric compounds and other organic material accumulated in the biofilm is thought to contribute to rapid recolonisation of the surface (Hem & Efraimsen, 2001).

Our laboratory trials differed from the industrial scale in the amount of cleaning agent used per unit area of membrane surface, even though the concentrations used for our laboratory experiments were the same as the industrially applied values. The volume of cleaning solution used in dairy membrane plants is 4 – 5 L m^{-2} (Krack, 1995) while in our laboratory reactor systems, the amount of cleaning solution was more than 82 L m^{-2} membrane. This was mainly due to the operating volume of the CBR 90 reactor (330 ml) and a membrane sample with a small surface area (total 30.48 cm^2). Thus the main differences between the laboratory and industrial scale cleaning regime were the relative volumes of water for flushing, and reagents for cleaning or sanitising. This suggests that using the same cleaners or sanitisers in an industrial scale membrane plant might result in higher residual culturable bacterial counts than those achieved in our trials.

Ozonated water appeared to be the weakest sanitiser among those used in this study. This may be because ozonated water application must be strictly controlled, such as being used freshly made in a completely closed unit; otherwise the ozone will transform into oxygen and lose the disinfectant activity (Guzel-Seydim et al., 2004). The effectiveness of ozone in terms of killing microorganisms is affected by ozone concentration, strains, temperature and pH (Jarroll, 1999). Any residual organic materials would also react with ozone, inactivating it quickly (Zottola & Sasahara, 1994).

6.5 CONCLUSIONS

Our study supported our hypothesis and demonstrated that the use of sanitisers following a CIP procedure improved the reduction of culturable bacterial cells on the membrane surfaces. The most effective sanitiser from our studies was the MIOX® EW anolyte (120 ppm FAC, pH 6.8) when compared with the control CIP clean. Sodium hypochlorite and Perform® functioned equally well when combined with Reflux® E1000 (Protease). This study would indicate that if a dairy processor were to use a standard CIP (such as the control) on membrane systems, then a further flush with MIOX® EW anolyte would reduce residual attached microbial populations further. In addition, using protease followed by a sanitation (sodium hypochlorite, Perform® or anolyte of MIOX® EW) produced the best clean based on a greater than 2 log reduction in residual cells and left no culturable and viable cells at a detection limit of 0.1 $\log_{10}$ CFU cm^{-2}.

The active disinfectant agent in the EW is believed to be hypochlorous acid (Len et al., 2002). However, our results showed that the effectiveness of the MIOX® EW anolyte with 120ppm FAC (pH 6.8) in reducing culturable bacteria was equal to or greater than sodium hypochlorite with 200ppm FAC (pH 6.5). This indicates that there might be something else (e.g. other oxidants), beside hypochlorous acid, killing the culturable cells.

The presence of chlorine and the low pH are the main concerns in the application of anolyte of EW on membranes. This aspect of membrane sensitivity requires investigation to determine the effects of using the anolyte of EW at different pH values on the life of PES membranes.

Chapter 7

FINAL DISCUSSION

Biofilm formation on filtration membranes, like that on other solid surfaces, is initiated by bacterial attachment (Ivnitsky et al., 2005). In dairy plants, once bacteria attach, they grow and multiply at the expense of nutrients in the feed solution or on the membrane surfaces, forming a biofouling layer which reduces permeate fluxes, damages the membrane and is more difficult to eliminate than free living cells (Flint et al., 1997a; Ivnitsky et al., 2005). Although biofilms in dairy plants have been widely recognised (Flint et al., 1997a; Kirtley & Mcguire, 1989; Zottola & Sasahara, 1994), little is known about the microbial community of biofilms on membranes, the factors involved in their forming biofilm in a dairy environment and the most efficient membrane cleaning strategy.

In studying biofilms, samples can be taken either by removing material directly from surfaces or by removing parts of the system carrying the biofilm layers (Schaule et al., 2000). The whole UF and RO membrane modules were transferred to our laboratory before they were cut into small pieces using a sterilized band saw. As replacement of membranes is very expensive, only membranes with unrecoverable membrane flux after CIP were available to us. Therefore, the limitation of using these CIP treated membrane samples is that the surface properties of both membranes and microorganisms in the biofilm and the wild biofilm structures may have already been altered or damaged by chemicals, enzymes and mechanical shocks involved in the CIP process.

In this study, microorganisms were isolated from both UF and RO membrane modules forwarded from 7 different dairy membrane plant sites in New Zealand. The limitation of the isolation method used in this study is that it can recover only the viable and culturable microorganisms. The non-culturable bacteria can be detected using PCR and 16S rRNA sequencing (Flint et al., 1997a), however, the bias against this method is that mixed microbial populations containing cells in different physiological states may not be amplified representatively unless cells are completely lysed (Silva & Batt, 1995). Among 13 identified strains, *K. oxytoca* was the most common species and existed in 3

different plant sites involving both UF and RO membrane modules. Others have also reported *Klebsiella spp.* in dairy products (El-Sukhon, 2003; Tondo et al., 2004) or dairy processing lines (Mattila et al., 1990; Sharma & Anand, 2002). These references indicate that *K. oxytoca* strains did not appear by chance and our isolation is representative.

Bacterial adhesion, which is believed to initiate biofilm formation (Ivnitsky et al., 2005), was the first factor investigated in this project and the 13 identified strains were then divided into two groups with strong or weak adhesion according to the results from a microtitre plate assay. Three *K. oxytoca* (Gram-negative bacteria) strains were found to have a greater ability to attach to microtitre plates than the other identified strains. Some other studies have also found that Gram-negative bacteria adhered more readily than Gram-positive ones (Criado et al., 1994; Speers & Gilmour, 1985).

Whey media, including whey permeate and whey, were found to enhance the attachment of our strains. This might due to the salts, lactose or proteins in whey. Firstly, the higher nutrients level in whey medium than in PBS might permit bacterial growth and thus higher attachment numbers, as maximum bacterial adhesion was found to occur at the optimum condition for growth (Shea et al., 1991). Secondly, if these molecules adhered to the substratum, they could provide a conditioning film with higher levels of nutrients than in the liquid phase, which might then encourage more cells to attach (Kumar & Anand, 1998).

Subsequently, cell surface hydrophobicity and charge were investigated, as these two surface properties were thought to be important for bacterial adhesion (Ghayeni et al., 1998; Pang et al., 2005). All the identified strains were found to have hydrophobic and negatively charged surfaces. It was expected that microorganisms with high surface hydrophobicity and low negative charge would present better attachment. However, the ability of our strains to attach showed no clear relationship with their surface hydrophobicity and charge. We concluded that the hydrophobicity and charge were not the predominant factors but might work together with other factors (e.g. medium composition, cell surface structure, cell motility and quorum sensing) that influence the adhesion of our strains.

The MATH assay was used for testing cell surface hydrophobicity. The results from this assay are affected by two factors. One is the electrostatic interactions between strain and hydrocarbons (van der Mei & Busscher, 2001), as hydrocarbon droplets in aqueous suspensions are negatively charged (Medrzycka, 1991). To reduce the interference of electrophoretic mobility, the MATH test should be conducted at pH values where the zeta-potential of the test organism and/or hydrocarbon are near zero (van der Mei et al., 1995). The other factor is the vortex mixing time applied for allowing bacteria to adhere to hydrocarbons (van der Mei & Busscher, 2001). The MATH assay measured the adhesion after a certain vortex mixing time. It was observed that different strains showed various levels of adhesion to the hydrocarbon at a given vortex mixing time. Therefore, use of a kinetic mode of MATH analysis, by measuring bacterial adhesion to hydrocarbon as a function of the vortex mixing time, is necessary. In the kinetic MATH assay, linear-regression analysis is carried out to derive an initial microbial removal rate by the hydrocarbon as a measure of hydrophobicity (van der Mei & Busscher, 2001).

The effect of three factors - membrane type, whey protein concentration and microbial strain - on biofilm formation on membrane surfaces were investigated using a CBR 90 biofilm reactor. The membrane types examined were new PES, used PES and new PVDF. PES was the membrane type in our sampled membrane modules. PVDF is another dominant material and was recommended by our membrane supplier (Synder Filtration, Vacaville, CA, USA) for comparison with PES. Used PES was compared with new membranes. *K.* B006 and TR002 were used as the inocula. The reasons are: (1) these two *K. oxytoca* strains showed high ability to attach in the microtitre plate assay, (2) they were isolated from two different membrane plant types (B006 – UF; TR002 – RO) and two different plant sites (B006 – plant A; TR002 – plant C), indicating that they might have different properties and high risk potentials. Whey was selected as the medium, because B006 and TR002 strains were isolated from the membrane plants processing whey and whey permeate.

With the successful modification of the CBR 90 biofilm reactor, membrane coupons could be placed onto the reactor coupon holders. The advantages of using this biofilm reactor are: (1) up to 24 coupon samples can be obtained for each run, while some other models (e.g. flow cell) can produce only one sample. This makes the subsequent analysis and comparison of results easier and faster than in other laboratory systems.

(2) Turbulent flow can be achieved by adjusting the stirrer rotation speed. (3) It generated consistent results that have been demonstrated by this project (Tang et al., 2009b) and other studies (Goeres et al., 2005). However, there are also two limitations of using this reactor. Firstly, there is no true filtration through the membrane coupons, thus the physical (e.g. force and velocity) and chemical (e.g. concentration) environmental factors close to the membrane coupon surfaces may be different from those in the spiral-wound industrial module. Secondly, the spacer that is used for separating membrane layers in the spiral-wound module was not able to be introduced into the CBR 90 biofilm reactor. It has been observed that the spacer was a major problem in biofouling (Cornelissen et al., 2007) and the biomass structure on membranes without spacers will be different from those with feed spacers (Vrouwenveldera et al., 2010).

The three individual factors listed above (membrane type, whey protein concentration and microbial strain) were found to have significant effects on biofilm development in terms of viable cells on membrane surfaces. The sizes, in terms of MWCO, of these two types of new membrane sheets were different. The MWCO for new PES membrane was 10,000, while for new PVDF membrane was 800,000. Such large MWCO differences may bring differences in membrane surface morphology or topology and in biofilm development. However, the sizes of pores in the used PES membranes obtained from our sampling dairy plants were not available. Thus, the results discussed in this study did not consider the pore size of the membrane.

With the increased whey protein concentration in the medium, the biofilm became denser. Mixed *Klebsiella* strains generated more biofilm on membranes than single strains. Used PES membrane enhanced biofilm formation compared with new membranes, while new PES showed less biofilm density than new PVDF membranes. Therefore, monitoring membrane age and selecting material for use in industry will help to reduce biofilm development.

The highest biofilm density was produced on used PES membranes, so the two *Klebsiella* strains (B006 and TR002) grown on used PES membranes were used for the investigation of biofilm removal from membranes. The standard CIP procedure used in dairy membrane plants was tested by measuring the culturable counts of *K.* B006 and

TR002 remaining on the membrane surfaces after cleaning. The results showed that the standard CIP failed to eliminate cells and left a density of 1.90 – 2.09 $\log_{10}$ CFU cm^{-2}. Others have also reported that the limitation of CIP effectiveness is the residual micro-organism concentration on the equipment surfaces, resulting in rapid resumption of biofilm formation (Bremer et al., 2006; Kumar & Anand, 1998; Sharma & Anand, 2002).

Cleaning is the first step and is important for the successful sanitation of the processing equipment (Forsythe & Hayes, 1998). As disinfectants do not penetrate the biofilm matrix left on a surface after an ineffective cleaning, disinfectants used under those conditions do not kill all the biofilm living cells (Simoes et al., 2006). It is also important to remove food debris and other residues that may contain microorganisms or promote microbial growth (Simoes et al., 2010). Enzymatic detergents are easily neutralized, biodegradable and known as "green chemicals" (Farone & Cahn, 1970), causing fewer pollution problems compared with acid or caustic cleaning regimes (D'Souza & Mawson, 2005). They effect significant hydrolysis of whey proteins (Arguello et al., 2002; Simoes et al., 2010) and can lengthen membrane lifespan being less aggressive to the membranes as they are highly substrate and reaction specific (D'Souza & Mawson, 2005). However, the use and study of enzymes in biofilm control is limited, owing to the competitive price of the chemicals used today and the patent-protection of most of the enzymatic detergents (Simoes et al., 2010).

Three enzymatic cleaners (Reflux® E1000, Reflux® E2001 and QuatroZyme®), containing pure protease, protease and lipase or mixed enzymes respectively, were used in our study. Their performances in removing cells in biofilms were compared with hypochlorite (200 ppm FAC, pH 10.8 -11.0) used in standard CIP, which is regarded as a cleaning and sanitising step by the dairy industry. However, using these enzymatic cleaners did not make a significant difference from the standard CIP, although QuatroZyme® - a mixture of enzymes - worked slightly better than others. Simoes et al. (2010) also stated that a mixture of enzymes might be required for sufficient biofilm degradation because of the EPS heterogeneity.

Since cleaners were not sufficient in biofilm removal, we considered that use of a sanitiser is an essential step that should be added to the standard CIP procedure. The

sanitising step is responsible for reducing the membrane surface population of viable cells left after cleaning and preventing microbial growth on surfaces before commencement of processing in a membrane plant. Sanitation using the anolyte of EW following a cleaning cycle with enzymatic detergent was found most effective in removing/killing cells from *K. oxytoca* biofilms on used PES membrane. However, the limitation of this study is that the laboratory work was conducted using a CBR 90 biofilm reactor that was not able to mimic all the parameters used in dairy membrane plants i.e. membrane flux, pressure and spiral-wound structure. Therefore pilot plant trials must be carried out to test the efficiency of the improved CIP on an industrial scale. As we discussed in previous chapters, membrane materials are sensitive to chemicals, so monitoring of membrane life while performing membrane cleaning is essential. The alteration of membrane properties can be investigated by several techniques, including AFM, Attenuated Total Reflection – Fourier Transform Infrared spectroscopy (ATR-FTIR) and SEM (Lee et al., 2010b).

In conclusion:

(1) *K. oxytoca* was representative of microorganisms responsible for developing biofilm on dairy UF and RO membranes.

(2) Surface hydrophobicity and charge were not the predominant factors affecting adhesion of *K. oxytoca* to microtitre plates.

(3) The growth of *K. oxytoca* as a biofilm was significantly affected by strain type, medium concentration (i.e. whey protein concentration) and membrane type (i.e. membrane material and age).

(4) Using enzymatic detergent combined with an extra step of sanitation, using the anolyte of EW, in a CIP procedure would improve membrane cleaning in terms of removing biofilms from membrane surfaces.

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Appendix I: A full factorial experimental design for testing the responses of three factors (whey protein concentration, membrane type and strain) to growth of *Klebsiella* biofilm in a CDC biofilm reactor

Run No.	Strain	Membrane Type	Whey Protein Concentration (%)
1	*K*.B006	New PES	1
2	*K*.B006	New PES	5
3	*K*.B006	New PES	20
4	*K*.B006	New PVDF	1
5	*K*.B006	New PVDF	5
6	*K*.B006	New PVDF	20
7	*K*.B006	Used PES	1
8	*K*.B006	Used PES	5
9	*K*.B006	Used PES	20
10	*K*.TR002	New PES	1
11	*K*.TR002	New PES	5
12	*K*.TR002	New PES	20
13	*K*.TR002	New PVDF	1
14	*K*.TR002	New PVDF	5
15	*K*.TR002	New PVDF	20
16	*K*.TR002	Used PES	1
17	*K*.TR002	Used PES	5
18	*K*.TR002	Used PES	20
19	*K*.B006 & *K*.TR002	New PES	1
20	*K*.B006 & *K*.TR002	New PES	5
21	*K*.B006 & *K*.TR002	New PES	20
22	*K*.B006 & *K*.TR002	New PVDF	1
23	*K*.B006 & *K*.TR002	New PVDF	5
24	*K*.B006 & *K*.TR002	New PVDF	20
25	*K*.B006 & *K*.TR002	Used PES	1
26	*K*.B006 & *K*.TR002	Used PES	5
27	*K*.B006 & *K*.TR002	Used PES	20

Appendix II: Information on ingredients of some chemicals.

Chemicals	**Main Components**
Reflux® 7C	Ethylenediaminetetraacetic acid (EDTA) tetrasodium salt 20 - 35%
Reflux® B615	Potassium hydroxide 10 - < 30% Sequesterant 10 - < 30% Amphoteric surfactant < 1% Anionic surfactant < 1%
Reflux® S800	Sodium hypochlorite 10 - < 30% Sodium hydroxide < 1%
Reflux® R400	Nitric acid 30 – 60% Phosphoric acid 10 - < 30%
Perform®	Hydrogen peroxide 10 - < 30% Acetic acid < 10% Peracetic acid < 10%
D/E Neutralizing Solution	Approximate formular per liter: Pancreatic digest of casein 5.0 g Yeast extract 2.5g Dextrose 10.0 g Sodium thioglycollate 1.0 g Sodium thiosulfate 6.0 g Sodium bisulfite 2.5 g Polysorbate 80 5.0 g Lecithin 7.0 g Bromcresol purple 0.02 g