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|  | Chemical and Biological Engineering |

###### **Biofilm formation of *Methanosarcina barkeri* on different support materials:**

###### **applications for anaerobic digestion**

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##### September 2016

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A thesis presented to the University of Sheffield in fulfilment of the thesis requirement for the degree of

Doctor of Philosophy in Chemical and Biological Engineering

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I, Vi Nguyen, declare that I am the sole author of this thesis and that the research presented within is the result of my own efforts, unless acknowledged otherwise in the text. I confirm that this work has not been submitted for any other degrees.

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# IV. List of abbreviations

2D-LC Two-dimensional liquid chromatography

AB Lewis acid-base

ABC ATP-binding cassette

AD Anaerobic digestion

ANOVA Analysis of variance

ATP Adenosine triphosphate

ATR Attenuated total reflectance

CLSM Confocal laser scanning microscopy

COD Chemical oxygen demand

ConA-Rho Concanavalin A-rhodamine

CTC Tartarate carbonate

DAPI 4',6-diamidino-2-phenylindole

DLVO Derjaguin, Landau, Verwey, and Overbeek model

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

EL Electrostatic

EPM Electrophoretic mobility

EPS Extracellular polymeric substances

FDR False discovery rate

FITC Fluorescein-5-isothiocyanate

FTIR Fourier transform infrared spectroscopy

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

HCD High collision dissociation

HPLC High-performance liquid chromatography

IEP Isoelectric point

IPP Isopentenyl pyrophosphate

IR Infrared

iTRAQ Isobaric tags for relative and absolute quantification

LC Liquid chromatography

LC-MS/MS Liquid chromatography-tandem mass spectrometry

LW Lifshitz-van der Waals

LW-AB Lifshitz-van der Waals and acid-base model

MMTS Methyl methanethiosulfonate

MS Mass spectrometry

MS/MS Tandem mass spectrometry

MtaA Methylcobamide:coenzyme M methyltransferase

MtaB Methanol-corrinoid co-methyltransferase

MtaC Methanol-corrinoid protein

NAD Nicotinamide adenine dinucleotide

NADH Reduced nicotinamide adenine dinucleotide

PBS Phosphate-buffered saline

PCA Principal component analysis

PE Polyethylene

PETG Polyethylene terephthalate glycol

PP Polypropylene

PTFE Polytetrafluoroethylene

PVC Polyvinylchloride

PVDF Polyvinylidene fluoride

RPLC Reverse-phase liquid chromatography

SDS Sodium dodecyl sulphate

SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM Scanning electron microscopy

SFE Surface free energy

SRT Solids retention time

TAN Total ammonia nitrogen

TCA cycle Tricarboxylic acid cycle

TCEP Tris(2-carboxyethyl)phosphine hydrochloride

TEAB Triethylammonium bicarbonate buffer

TEMED Tetramethylethylenediamine

TFA Trifluoroacetic acid

TPI Triose phosphate isomerase

UHPLC Ultra-high pressure liquid chromatography

UniProt Universal Protein Resource

VFA Volatile fatty acids

xDLVO Extended Derjaguin, Landau, Verwey, and Overbeek model

XPS X-ray photoelectron spectroscopy

# V. Acknowledgements

This PhD has taken me on an emotional rollercoaster from day one. This thesis is the culmination of several years of hard work, and I could not have done it without the help and influences of various people to whom I am extremely grateful to.

To my supervisor, Catherine Biggs, for your endless support, for your positivity when things didn’t work out and your unwavering belief in me. Even with your own personal life challenges, you have always made time for me. I thank you. You are an inspiration.

To my stand-in supervisor, Henriette Jensen, for making time for me, for being ever supportive and for undertaking the daunting task of guiding me through the final stages of my thesis and PhD.

To Esther and Narciso, for voluntarily becoming my mentors in the final stages of my PhD, and for showing me the fun side of research. Your constant positivity, jokes, and guidance pulled me through. I could not have done it without you.

To everyone in the D72 office, and in the B56 dream team (Esther, Liz and Joy). You have been alongside me throughout my PhD and have shown unwavering support and friendship over the years. I will always be grateful for your friendship.

To the technical staff at CBE, especially Dave and James, for always being available to help me with any issues I had with the anaerobic chamber and beyond. Thank you for always being there.

To my dear friend, Kirstine Szifris, for our weekly thesis writing-lunch-coffee days. Thank you for your unwavering support, understanding and for offering an escape from the stresses of my PhD. I look forward to our epic celebration party when it’s all finished.

To all my family and friends, of whom there are too many to mention, for your enduring patience, support and love over the years. I have neglected many of you this past year, but you have always been there for me. I thank you all.

And to Mike Simmonds, my life partner in crime, for being an essential source of support throughout my PhD, for sharing my highs and lows, and for being able to describe microbial physicochemical interactions and adhesion modelling to our friends and family.

# VI. List of publications

**Journal Publications**

# NGUYEN, V., Karunakaran, E., Collins, G. & Biggs, C.A. (2016) Physicochemical analysis of initial adhesion and biofilm formation of *Methanosarcina barkeri* on polymer support material. *Colloids and Surfaces B: Biointerfaces, 143: 518-525.*

# VII. Abstract

Biofilms are sessile microbial communities attached to a surface, and offer a multitude of benefits to various biotechnological applications, such as anaerobic digestion. Therefore, engineering systems to promote biofilm formation is becoming increasingly desirable in the biotechnology sector. This thesis aimed to promote biofilm formation from the robust model methanogen, *Methanosarcina barkeri,* onto polymer support materials as a strategy for optimising the anaerobic digestion of domestic wastewater in peri-urban areas. A first step in this direction was to understand the effect of the support material on the biofilm-forming capabilities of *M. barkeri.*

Various techniques were used throughout this thesis to show that the choice of support material was an important environmental factor in triggering different physiological responses from *M. barkeri* during biofilm formation*.* DLVO modelling, surface characterisation and static adhesion assays revealed the important role of the physicochemical surface properties of *M. barkeri* and the six support materials for initial microbial adhesion. *M. barkeri* was shown to exhibit different abilities to attach to the support materials, with the type of material strongly influencing the extent of initial attachment. Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, zeta potential analysis and fluorescence microscopy suggested that significant modifications to the cell surface occurred in response to attachment to a favourable support material (PVC), with increased levels of cell surface polysaccharides detected in biofilms attached to PVC compared to PETG. Furthermore, microbial attachment to PVC caused a significant higher relative abundance of proteins involved in methanogenesis, metabolism, cell wall biogenesis and EPS production compared to biofilms attached to PETG. The results from this thesis suggest that *M. barkeri* showcases different physiological responses for biofilm formation depending on the support material. Therefore, the choice of support material is an important design parameter for retaining microbial biomass within AD reactors*,* and should be considered in future design frameworks for high rate anaerobic digestion.

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## Chapter 1

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##### Introduction

*“The suspense is terrible. I hope it will last.”*― Oscar Wilde

# 1.1. Domestic wastewater: global challenges

Rapid urbanisation, industrialisation and an ever-growing global human population have led to an increasing generation of domestic wastewater. Domestic wastewater is defined as being comprised of faeces, urine and greywater [1], and is a complex substrate with a high level of particulate suspended matter, and other domestic waste substrates, such as detergents, proteins and fatty compounds [2]. With 50% of the global population currently residing in urban cities and expected to rise to 60% by 2030 [3], it is clear that such drastic changes in the global demography need to be met with upgraded wastewater infrastructure. However, the reality is that many urban cities already lack adequate waste management infrastructure to cope with current levels of urban waste streams [3], let alone a predicted surge in urban population dynamics. The provision of adequate wastewater treatment facilities is both energy-intensive and expensive, and there does not exist a ‘one size fits all’ solution that is applicable to all global urban areas. Therefore, it is a challenge to implement suitable sustainable solutions that fulfil the needs of an urbanised area [4].

Poor global management and treatment of domestic wastewater has serious downstream effects, such as water scarcity, poor energy access and climate change. With untreated domestic wastewater contributing up to 10% of global methane (CH4)emissions each year [5], it is clear that it is a significant source of methane [3]. CH4 is a greenhouse gas with a global warming potential 25 times greater than that of carbon dioxide (CO2) [6]. By 2020, the uncontrolled release of CH4 from untreated wastewater could increase by 50% compared to 1990 levels [3]. This has serious repercussions to the global ecology and to the future stability of the environment and climate.

Domestic wastewater is also a significant source and carrier of water pollutants and pathogenic disease [3,7]. It facilitates the transmission of pathogenic microorganisms via various faecal-oral pathways, such as by contaminating groundwater sources, food, hands and feet, resulting in various sanitation-related diseases, namely diarrhoea and cholera [7]. Diarrhoea is responsible for the deaths of 2.2 million people worldwide, which causes more deaths worldwide than HIV/AIDS, tuberculosis and malaria combined [8]. These frightening statistics highlight the fact that such fatalities can be avoided with the provision of adequate wastewater treatment.

The effects from the poor management of domestic wastewater are especially fatal to the more vulnerable urbanised areas in the Global South. An unprecedented level of migration into urban cities in the Global South has led to the rapid onset of peri-urban settlements around the outskirts of cities [4]. These are temporary settlements that are characterised by poor infrastructure, limited space, dense populations and an uncertain tenure, hindering the provision of wastewater treatment technologies to these areas. Additionally, there is unwillingness by Governments to provide secure financial backing of basic sanitation amenities to these temporary settlements [9]. As a result, the unacceptable reality is that 1 billion people lack access to clean drinking water and 2.6 billion people are without access to basic sanitation [10].

It is clear that the provision of wastewater treatment could contribute towards meeting these basic human necessities. The implementation of locally-appropriate decentralised wastewater treatment solutions within the future urban framework of the Global South could help to provide sanitation, wastewater treatment and the safekeeping of the environment and its population from the effects of untreated domestic wastewater [4]. Most importantly, the provision of such technological solutions for sanitation at the peri-urban interface could also be highly transferable to the Global North, which relies on the use of centralised wastewater treatment technologies with often extremely energy-intensive, expensive and highly technical design requirements that fail to meet environmental sustainability criteria [11]. Technologies based on anaerobic digestion offer a sustainable solution and could play a transformative role in addressing these global challenges.

# 1.2. Anaerobic digestion: opportunities and challenges

Anaerobic digestion (AD) is a naturally occurring process with great promise for resource recovery and energy production. It is a complex biochemical reaction in which organic material is degraded and converted into methane by a specialised consortium of microorganisms in an oxygen-free environment [12]. This process occurs naturally in anaerobic zones, such as in the stomach of ruminants, in landfill sites and in wetlands [6].

The process of AD can be contained and controlled within bioreactors to harness two valuable end-products from waste biomass, bringing economic, environmental and health benefits: biogas, a renewable energy source, and a stabilised solid digestate, rich in nitrogen and phosphorous that can be used as fertiliser [13].

High strength domestic wastewater is typical in peri-urban areas of the Global South, where water consumption and usage is characteristically low, resulting in a wastewater with a concentrated level of organic matter. The definition of high strength wastewater is a wastewater possessing a typical chemical oxygen demand (COD) of approximately 1200 mg/L [2]. With the increasing issues surrounding the disposal and treatment of domestic wastewater in urban areas, the potential of using AD to stabilise, sanitise and recover energy from high strength domestic wastewater in peri-urban areas is extremely promising. AD in this context can also be used to drive innovation in the Global North, where the treatment of high strength wastewater is also a growing concern [14].

However, AD is still largely hindered by poor operational stability, which has prevented its uptake on a larger global scale. This stems from the complex microbial consortium involved in the AD process, which consists of a network of specialised microbial trophic groups possessing different growth and nutritional requirements, metabolic rates and environmental sensitivities [15].

A recurring issue that arises from this complex microbial consortium is the frequent wash-out of the slower growing methanogenic archaea [16,17], which are the microbial trophic group responsible for facilitating the final essential biological step in the AD process [12]. The slow growth rates of the methanogens results in their poor retention within AD reactors, as they are often washed out in the effluent before they have had chance to form established biofilms [16]. In addition, the methanogens have extremely specialised metabolic pathways and environmental conditions for producing methane [18,19]. As a result, the poor retention of methanogens within AD reactors and their high sensitivity towards sudden environmental or operational fluctuations [19] can result in poor AD performance [15].

# 1.3. Opportunities for *Methanosarcina* in anaerobic digestion

Compared to the other methanogenic archaea, the methanogenic genus, *Methanosarcina* is recognised as being the more robust and resilient member, with an ability to tolerate various environmental and operational stressors [19]. Given the robust nature of *Methanosarcina*, it is desirable to selectively retain a high active biomass of this methanogenwithin AD reactors in order to more efficiently treat high strength domestic wastewater. Promoting the biofilm formation of *Methanosarcina* within AD reactors holds promise for heavy duty biomethanation for future biotechnological applications, and this is achievable with the use of appropriate support materials within AD reactors to selectively immobilise *Methanosarcina*. However, the processes underlying biofilm formation in archaea are generally poorly understood [20,21], and this is the same for the genus *Methanosarcina*.

Biofilm formation is a complex process involving a multitude of factors, and is the predominant mode of growth for microorganisms [22–24]. Many researchers have examined single microorganisms in the laboratory to better understand biofilm behaviour in a controlled environment, and often using a wide suite of methods to delve into the underlying physical, chemical and biological factors for biofilm formation in a wide range of microbial species [25]. Various physicochemical, spectroscopic, microscopic and proteomic research methods have provided an unprecedented view of the processes governing biofilm formation in bacterial biofilms, and offer great promise for archaeal biofilm research [20].

The unexplored territory of archaeal biofilms is a promising field of research, and further understanding of biofilm formation in this ubiquitous domain of life would be valuable for various biotechnological applications, such as AD.

# 1.4. Research objectives

The overall objective of this thesis is to promote a microbial-based strategy for optimising the anaerobic digestion of high strength domestic wastewater. This strategy involves the selective retention of the robust methanogenic genus, *Methanosarcina* onto support materials within AD reactors to address the main limitation of the AD process - that is the frequent washout of active methanogenic microbial biomass from AD reactors, which has an adverse effect on biogas yield, quality and start-up times. *Methanosarcina barkeri* will be used as a model species throughout this thesis, owing to its common presence in anaerobic reactors [15].

This thesis will examine the effect of different support materials on the biofilm-forming behaviour of *M. barkeri* in order to ascertain which support materials are the best biofilm carriers for the selective immobilisation of *M. barkeri.* Additionally, the experiments in this thesis will be carried out in high ionic and neutral pH aqueous environments to model the typical characteristics of domestic wastewater in the Global South, and to examine the biofilm-forming behaviour of *M. barkeri* in such environments. Therefore, the main research questions of this thesis are:

1. How does the nature of the support material influence the physicochemical interactions governing the initial adhesion process in *M. barkeri*?
2. Does the nature of the support material influence cell surface modifications in *M. barkeri* during biofilm formation?
3. How does the nature of the support material influence the functional proteome of *M. barkeri* during biofilm formation?

This thesis will utilise a mixed methods approach to elucidate the physicochemical and biological factors governing biofilm formation in *M. barkeri* as a function of support material. The results of this investigation will be presented as follows: chapter 2 will provide an overview of the existing literature on anaerobic digestion, the process of biofilm formation and the different methods currently used in biofilm research that can be applied to *M. barkeri* in this thesis*.*

Chapter 3 combines the extended Derjaguin, Landau, Verwey, and Overbeek model (xDLVO) with surface physicochemical measurements, scanning electron microscopy (SEM) and static biofilm assays to examine the effect of physicochemical interactions on the adhesion of *M. barkeri* to six different polymer support materials at the initial and later stages of biofilm formation.

Chapter 4 combines Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), zeta potential analysis and fluorescence microscopy to analyse changes to the cell surface of *M. barkeri* during biofilm formation on a good and poor biofilm carrier, as determined from chapter 3.

Chapter 5 applies a quantitative proteomic approach to examine the whole suite of proteins expressed by *M. barkeri* during biofilm formation upon attachment to either a good and poor biofilm carrier, along with enzyme assays to validate the results of the quantitative proteomics.

Finally, chapter 6 surmises the main conclusions of the thesis and an outline of future research directions of this work.

## Chapter 2

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##### Literature Review

*“Begin at the beginning," the King said, very gravely,*

*"and go on till you come to the end: then stop.”  
 ―* Lewis Carroll, Alice in Wonderland

# 2.1. Microbiology of anaerobic digestion

AD is a complex multistage biochemical reaction that is carried out by a specialised consortium of syntrophic bacteria and methanogenic archaea in an oxygen-free environment [12,16]. Complex organic macromolecules are broken down into simple one-carbon substrates for the methanogenic archaea to convert into methane. This is one of the most complex natural biosystems known to currently exist [22].

The decomposition of organic biomass occurs in four sequential steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Fig. 2.1). These stages are linked, with the products of one stage serving as the substrate for the next. The first hydrolysis step involves the degradation of complex macromolecules, such as proteins, polysaccharides and lipids into soluble organics, such as amino acids, simple sugars and fatty acids [12]. This is followed by an acidogenesis step, in which the products from hydrolysis are converted into volatile fatty acids (VFA) by acidogenic bacteria, as well as gaseous by-products, such as carbon dioxide (CO2), hydrogen (H2) and hydrogen sulphide. The production of VFAs in this step can often lead to a lowering of the pH due to the accumulation of VFAs [15].



Soluble organics

Organic macromolecules

**Fig. 2.1** Biochemical steps involved in anaerobic digestion (adapted from [12]).

The VFAs produced by acidogenesis are consequently broken down further into mainly acetic acid, as well as CO2 and H2 in the acetogenesis step. This involves the action of H2-producing acetogenic bacteria, whose activity is inhibited by high partial pressures of H2. These bacteria are only able to convert VFAs into acetic acid at very low partial pressures of H2, in the range of 2.6-74 Pa [26]*.* As a result, a symbiotic relationship exists between H2-producing acetogenic bacteria and H2-consuming acetogenic bacteria, which are able to consume H2 in the environment and thus, maintain low partial pressures of H2 for acetogenesis to occur [27].

The final step is methanogenesis, in which a diverse group of methanogenic archaeaconvert a limited number of simple carbon substrates, such as acetic acid, methylamines, CO2 and H2 into CH4 [18,26].

Compared to aerobic wastewater treatment processes, AD waste treatment is much more cost-effective, requiring smaller-sized reactors, less energy and water consumption, and produces little or no waste sludge at the end of the process [12,16]. It is an established low-cost and ecologically sustainable technology that has already shown great success in treating and reusing waste biomass on a global scale [26].

The recent rise of AD in the global arena was largely due to the innovative work of Lettinga and co-workers [16]. Their work led to advancements and important adjustments to the designs of conventional AD reactors, resulting in the establishment of high rate anaerobic reactors, which were initially applied to treat high strength industrial wastewaters in the Global North. These high rate anaerobic reactor designs relied upon the high retention of an adherent well-balanced microbial biomass, or immobilisation, within AD reactors to degrade both low and high strength wastewaters [16], leading to drastic improvements in the efficiency and stability of the AD process [28]. The provision of abiotic support materials for the immobilisation of microorganisms within AD reactors is common [13]. The sessile microbial community attached to these support materials is commonly known as a biofilm [29,30], and these support materials ensure their fixture within AD reactors [13]. Biofilms work extremely well in maintaining a high level of microbial activity within AD reactors, and play an important role in wastewater treatment technologies such as AD [16].

The complex consortium of microorganisms involved in AD grow and perform optimally at mesophilic temperatures (30-35℃) [28] and can degrade a large variety of organic feedstocks into biogas [26]. Biogas is a combustible fuel mixture consisting mainly of CH4 (~50-70%), CO2 (~25-45%) and other trace gases. CH4 has a high calorific value of 21–24 MJ/m3, or 6 kWh/m3, making it a valuable energy resource if captured [5].

As such, AD is an ideal technology for the Global South where the tropical climate provides optimal mesophilic temperatures for the growth of the microorganisms involved in the AD process [2]. This means that no further energy requirements are needed to heat up AD systems to ideal operating temperatures [16]. Additionally, the capacity of implementing AD systems in a decentralised mode without the need for expensive sewerage piping systems [5] makes it a promising technology for treating domestic wastewater in peri-urban areas in the Global South.

Common feedstocks used for AD are typically animal manure, industrial wastewater, food waste and the solid fraction of municipal waste [26]. However, the energy potential of domestic wastewater has often been neglected in favour of the perspective that it is a waste product that needs to be disposed of. This ‘throw away’ attitude undermines the value of domestic waste streams and can hinder progress in combating the issues surrounding wastewater treatment. In fact, past studies have highlighted the energy potential of various domestic waste streams in AD [31], such as faecal sludge [32], urine [33], greywater [34] and sewage sludge [35], with promising results.

# 2.2. Methanogenesis

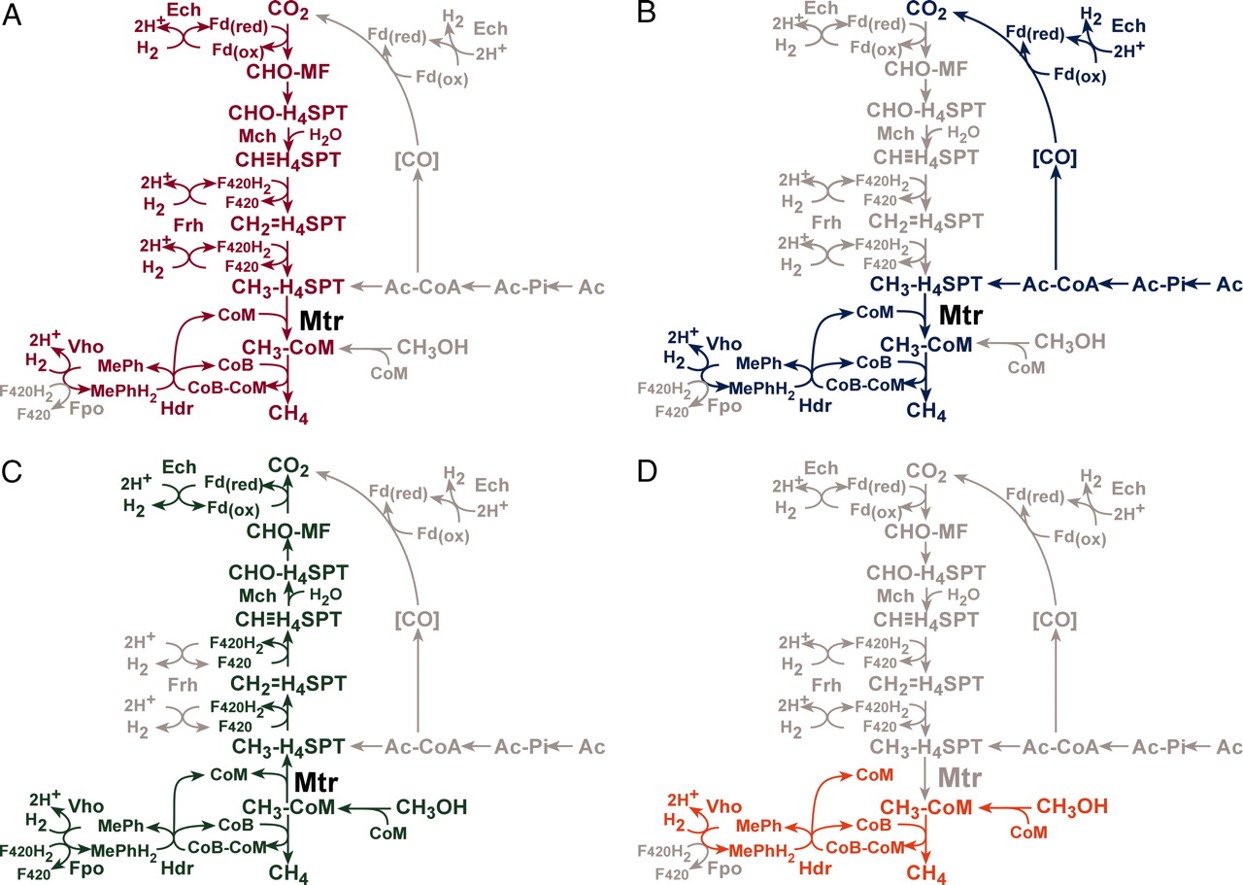
The last biochemical reaction involved in AD, methanogenesis, is responsible for the majority of natural and anthropogenic CH4 gas emissions in the world [36].It is one of the most primitive and ancient known metabolic pathways, pre-dating many forms of respiration, such as photosynthesis, and is an important biochemical reaction that maintains the global flux and balance of CH4 [37]. In fact, methanogens are the only organisms known to biologically produce CH4 [36].

Given the diversity of the methanogenic archaea*,* it is somewhat surprising that they produce CH4 from a limited number of specific substrates using extremely specialised metabolic pathways [38]. These methanogenesis pathways are listed in Table 2.1 and are schematically depicted in Fig. 2.2, along with their associated enzymes.

**Table 2.1** Specialised metabolic pathways of the methanogen archaea [18].

|  |  |  |
| --- | --- | --- |
| **Metabolic pathway** | **Reaction** | **Methanogenic family** |
| CO2 reduction pathway (hydrogenotrophic) | **CO2 + 4H2 → CH4 + 2H2O**  Reduction of CO2 using H2 as reductant | *Methanobacteriales*  *Methanococcales*  *Methanomicrobiales*  *Methanosarcinaceae* |
| Methylotrophic pathway | **4CH3OH → 3CH4 + CO2 + 2H2O**  Conversion of methylated compounds, such as methanol and methylamines into CO2 and CH4 | *Methanosarcinaceae* |
| Methyl reduction pathway | **CH3OH + H2 → CH4 + H2O**  Reduction of methanol using H2 as reductant | *Methanosarcinaceae* |
| Aceticlastic pathway | **CH3-COO- + H+ → CH4 + CO2**  Conversion of acetate into CH4 and CO2 | *Methanosaetaceae*  *Methanosarcinaceae* |

Acetate is the most important substrate for methanogenesis, being the precursor for 70% of the CH4 produced in AD processes [2,39]. Yet despite acetate having such an essential role in the metabolism of the methanogens, only two methanogenic genera are able to convert acetate into methane using the aceticlastic pathway: *Methanosarcina* and *Methanosaeta* [26].



**Fig. 2.2** The four methanogenic pathways found in the methanogenic archaea with their associated enzymes. The hydrogenotrophic pathway is shown in red (A). The aceticlastic pathway is shown in blue (B). The methylotrophic pathway is shown in green (C). The methyl reduction pathway is shown in orange (D). These four methanogenesis pathways overlap. The steps not used in each pathway are shaded in grey. Figure adapted from [40].

*Figure abbreviations of methanogenesis enzymes:* CHO-MF, formyl-methanofuran; CHO-H4SPT, formyl-tetrahydrosarcinapterin; CH≡H4SPT, methenyl-tetrahydrosarcinapterin; CH2=H4SPT, methylene-tetrahydrosarcinapterin; CH3-H4SPT, methyl-tetrahydrosarcinapterin; CH3-CoM, methyl-coenzyme M; CoM, coenzyme M; CoB, coenzyme B; CoM-CoB, mixed disulfide of CoM and CoB; Mph/MphH2, oxidized and reduced methanophenazine; F420/F420H2, oxidized and reduced Factor 420; Fd(ox)/Fd(red), oxidized and reduced ferredoxin; Ac, acetate; Ac-Pi, acetyl-phosphate; Ac-CoA, acetyl-CoA; Ech, ferredoxin-dependent hydrogenase; Frh, F420-dependent hydrogenase; Vho, methanophenazine-dependent hydrogenase; Fpo, F420 dehydrogenase.

*Methanosaeta* is commonly detected in anaerobic sludge and is a strictly aceticlastic methanogen. As such, it is only capable of producing CH4 by metabolising acetate [40]. Alternatively, *Methanosarcina* is not restricted to a single specialised metabolic pathway for producing CH4, and is the only methanogen known to produce CH4 using the four different methanogenesis pathways [18,19]. *Methanosarcina* is therefore able to metabolise a wider range of methanogenic substrates, including acetate, methanol, methylamines, CO2 and H2. This metabolically diverse nature makes *Methanosarcina* a key member of the methanogenic consortium involved in AD [19].

In contrast to the aceticlastic pathway, almost all methanogenic archaea are able to use the hydrogenotrophic pathway (Table 2.1), which is the precursor for 30% of the CH4 produced in AD reactors. The hydrogenotrophic methanogens are important for maintaining low levels of H2 in the environment, which would otherwise inhibit the growth of the obligate H2-producing acetogenic bacteria and the other methanogenic archaeaspecies [2].

Compared to the other members of the AD microbial consortium, the methanogenic archaea are the most sensitive to environmental and operational fluctuations. With the methanogens playing an essential step in the process of AD, their vulnerability to environmental fluctuations is a major cause for system failure [15,19], highlighting the sensitivity and complexity of the methanogenesis process. This issue is exacerbated by imbalances in the dynamics of the AD microbial community, which can be caused by overloading AD reactors with organic feedstock. This consequently promotes the hydrolysis stage and causes the acidogenic and acetogenic bacteria to more rapidly produce VFAs. This can produce a sharp drop in the pH to levels outside of the optimal growing range for methanogens, which is typically between 6.8 and 7.5 [12,19]. The elevated presence of VFAs within AD reactors is inhibitive to methanogen growth, and therefore, the production of CH4 [15].

Other inhibitory substances include high levels of ammonia in AD reactors, to which the methanogens are the most sensitive compared to the rest of the AD community [19]. Methanogenic activity has been reported to be particularly inhibited by free ammonia (NH3) due to its membrane-permeable nature, which can cause changes in intracellular pH and inhibit enzyme activity [15,19]. High levels of total ammonia nitrogen (TAN) exceeding 3000-4000 mg TAN L-1 have been reported to suppress the activity of the methanogens within AD reactors [19]. Additionally, methanogen activity can also be hindered by sudden fluctuations in temperature and salinity levels exceeding 8000 mg of sodium ions within AD reactors [12,15].

The slow growth rates of the methanogens is another issue for the AD process. The methanogens possess typical doubling rates that are 5-15 times slower than that of the other members of their AD consortium [41]. These slow growth rates result in the frequent wash-out of the methanogens in the effluent of AD reactors before they have had chance to establish themselves [16,17]. A combined effect of the accumulation of inhibitory substances within AD reactors, the sensitivity of the methanogens towards various operational and environmental fluctuations and their slow growth rates can cause inefficient biogas production and poor AD performance [42–45]. As such, the vulnerability of the methanogenic consortium is one of the key limitations in the AD process [19].

Efforts to facilitate heavy duty biomethanation for future biotechnological applications should take into account this microbial limitation to AD process stability. Current decentralised and centralised AD reactors are regulated by operational controls, such as measuring the effluent pH, temperature, levels of VFAs and trace metals in the reactor [19,46].

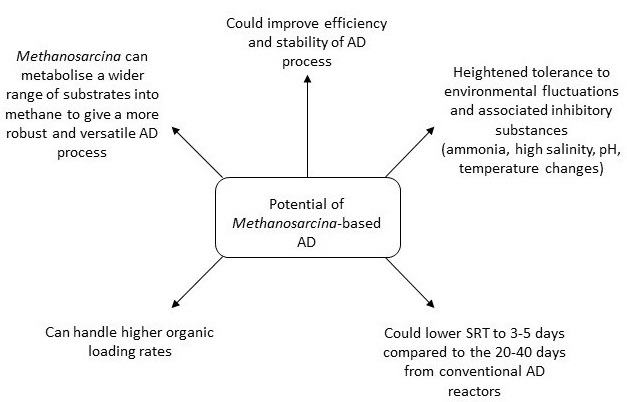
However, it is clear that in order to optimise the AD process to enhance biogas production and process stability, an understanding of the microbial community is also required. Yet, a microbial-based strategy is distinctly lacking in the AD sector, and is greatly needed [46]. Finding resilient and adaptive microorganisms to strengthen the AD microbial community against future reactor changes is one potential microbial-based strategy. The immobilisation of such resilient and adaptive microorganisms within AD reactors is promising for the high rate degradation of domestic wastewater. An extremely promising candidate is the metabolically versatile and robust methanogenic genus, *Methanosarcina* [19].

###### 2.2.1. *Methanosarcina*: a key methanogen in anaerobic digestion

It is widely recognised that *Methanosarcina* is one of the more robust methanogens of the methanogenic consortium in AD [19]. Compared to the other methanogenic archaea, *Methanosarcina* are able to tolerate various environmental and operational stressors. Previous studies have reported a shift in the microbial community to mainly *Methanosarcina* species in AD reactors with high levels of ammonium of up to 7000 mg TAN L-1 [47], high organic loading rates [48], sudden changes in pH of 0.8 to 1 units [49], as well as in AD reactors treating recalcitrant high-solid waste substrates with a total solids content greater than 10% [50] and across a wide temperature range of 15 to 70°C [51,52].

These environmental fluctuations are typical inhibitors to methanogen growth and activity, as described previously [15]. However, the heightened tolerance of *Methanosarcina* to such common inhibitory parameters is desirable for high-rate AD reactors, and their retention within such reactors has the potential to increase process stability and AD performance [39]. An AD reactor based on *Methanosarcina* has been proposed to reduce the typical solids retention time (SRT), or the average amount of time that solids remain in an AD reactor [12] from 20-40 days in conventional AD reactors to 3-5 days, as well as being able to tolerate higher organic loading rates [19,49]. These robust characteristics of *Methanosarcina* are promising for a *Methanosarcina-*based high rate AD system, and are summarised in Fig. 2.3.

This tolerant nature can be attributed to the physical properties of *Methanosarcina. Methanosarcina* typically has a large coccoid cell size with a cell diameter of 0.5-3 μm, with cells growing in aggregated clusters. This physical property can prevent the diffusion of inhibitory substances into the cell [19,52]. *Methanosarcina* is unique in its ability to grow as aggregated cell clusters, which is not observed in the other methanogens. This aggregated mode of growth promotes cellular protection from the external environment, as well as allowing an easier mass transfer of essential nutrients and substrates between individual cells within aggregates [53]. *Methanosarcina*’s physical cell size and aggregated mode of growth is extremely beneficial to its survivalwithin hostile environments, such as in a fluctuating AD reactor.



**Fig. 2**.**3** Schematic of a potential AD system based on *Methanosarcina.*

The robust nature of *Methanosarcina* can also be attributed to itsversatile ability to produce CH4 using all four known methanogenesis metabolic pathways [40]. This makes it less sensitive to inhibitors of any of the four pathways, and gives weight to the concept of enriching AD reactors with *Methanosarcina* to tolerate various environmental scenarios [19].

Moreover, *Methanosarcina* possesses a more efficient aceticlastic pathway than its aceticlastic companion, *Methanosaeta,* possessing a higher maximum growth rate and rate of acetate utilisation [39]. This is an important property of *Methanosarcina,* given that acetate is the main precursor to CH4 production in methanogenesis [2]. In aceticlastic methanogenesis, *Methanosarcina* requires only one ATP molecule per acetate molecule activated in a dual step pathway, compared to the two ATP molecules required in a single step pathway in the *Methanosaeta* [54].

The highly efficient aceticlastic pathway in *Methanosarcina* evolvedalmost 250 million years ago, and was responsible for the largest mass extinction of taxonomic life recorded in history during the end-Permian era, by producing fatally high levels of CH4 in the atmosphere and oceans [54]. Whereas such fatalities have occurred when *Methanosarcina* was left to produce and release CH4 into the atmosphere uncontrollably,

this highly efficient aceticlastic pathway holds promise for the retention of this genus within AD reactors for increased process stability and heavy duty biomethanation [19,39].

The concept of a *Methanosarcina*-based strategy is encouraging for decentralised AD systems treating high strength domestic wastewater in peri-urban areas in the Global South, as well as for centralised systems in the Global North. However, in order to achieve a *Methanosarcina-*based high rate AD system, a framework is required in which to better understand the biofilm-forming capabilities of *Methanosarcina* within AD reactors.

# 2.3. Methanogenic biofilms

The majority of prokaryotic life has a natural tendency to form sessile and immobilised communities or ‘biofilms’ on a wide range of solid surfaces, whilst encased within a sticky polymeric matrix [24,30]. This surface-attached lifestyle is an ancient mode of growth and survival strategy for prokaryotes with first sighting in the fossil record almost 3.25 billion years ago [29], whereas existence as free-floating planktonic cells is uncommon in the natural environment [23]. This sessile and complex gathering of microorganisms is extremely efficient at carrying out highly specialised and complex tasks as a single syntrophic unit [22], and offers various advantages to surviving a constantly changing environment. These advantages include better co-metabolism, interspecies transfer of substrates, cross-feeding and protection from the environment compared to cells living planktonically [22,24]. As a result, biofilms are ubiquitous in a wide range of ecological niches.

Bacteria and archaea co-exist in biofilms in various ecological niches, and this is especially true in AD. The beneficial value of biofilms to AD and various other biotechnological applications is large [55], and is facilitated by the natural tendency of mixed natural populations of microorganisms to adhere to a surface and form biofilms [17]. In AD, biofilms are key during the start-up period to establish the microbial community needed for the degradation of waste biomass [16]. Therefore, promoting methanogenic biofilm formation within AD reactors is advantageous for the performance and productivity of AD. The immobilisation of these biofilms onto support materials within AD reactors is a commonly used and viable option [17].

The methanogenic archaea have attracted much interest over the years [56] for their key role in biogas production and their contribution in maintaining the global flux of CH4 in the Earth’s biota [37]. However, knowledge of the processes underlying biofilm formation in the methanogens is limited, let alone the rest of the AD microbiome. To date, only a small proportion of the methanogenic microbiome has been isolated for study [26]. Thus, current understanding of the methanogens and their biofilm-forming abilities is incomplete, and particularly in the context of understanding the processes governing their biofilm formation to support materials within AD reactors. This gap in knowledge is applicable to the Archaea generally [20,21]. However, the little work that has been conducted on methanogenic archaeal biofilms to abiotic surfaces has shown a general ability to form biofilms, showcasing a variety of different biofilm structures in varying environmental conditions [21].

Bang *et al*. (2014) demonstrated that three methanogenic species, *Methanobrevibacter smithii, Methanosphaera stadtmanae* and *Methanosarcina mazei* strain Gö1 were able to successfully form biofilms on mica and plastic dishes when grown as static pure cultures. The type of abiotic surface and species was shown to influence the morphology of the biofilm. *M. mazei* and *M. smithii* formed multi-layered biofilms with a height of up to 40 μm in the plastic dishes, whereas this was less pronounced in *M. stadtmanae* biofilms with a height of 2 μm [57].

In another study, three methanogen archaeal species, *Methanobacterium formicicum, Methanosaeta concilli* and *M. barkeri* were subjected to polypropylene and glass coupons over 9 days as mixed pure cultures within a modified Robbins device with a flow rate of 9.6 ml/min. All three species were found to form sparse and thin biofilms in these shear conditions, consisting mainly of isolated patches of aggregated cells [58].

The surface structures and cell surface composition of archaea are fundamentally different to those of bacteria. Therefore, the mechanisms by which archaea interact with their external environment and attach to surfaces to initiate biofilm formation can be different to bacteria [59]. Yet some methanogenic archaea show similarities to bacteria in their use of cell surface appendages to facilitate microbial attachment to abiotic surfaces, such as the archaellum and pili [20]. Previous studies have found the archaellum, otherwise known as the archaeal flagellum, to facilitate initial adhesion to a range of abiotic surfaces, as well as mediate a communication network between cells within the biofilm for the methanogens, *Methanocaldococcus villosus* KIN24-T80 and *Methanococcus maripaludis* Mm900 [21]. Desmond *et al.* [60]conducted a phylogenomic analysis of the components of the archaellum and discovered that although *Methanosarcina mazei, Methanosarcina acetivorans* and *Methanosarcina barkeri* are generally regarded as being non-motile, their genomes do contain the genes encoding the components of an archaellum. The role of the archaellum in surface adhesion and motility in *Methanosarcina* is an interesting and relatively new field that warrants further examination [60].

Additionally, the self-secretion of a slime polymeric matrix collectively known as extracellular polymeric substances (EPS) is as prevalent in archaeal biofilms as it is in bacteria [21]. Cells of *Methanosarcina* are typically enveloped by an external heteropolysaccharide matrix consisting of methanochondroitin, whichis responsible for facilitating cell-cell and cell-abiotic surface adhesion [19,53]. The production of this exopolysaccharide layer is regulated by the cell in response to the external environment

and growth conditions [61]. This surface-mediated response to the environment is also observable in other archaeal species. The halophilic archaea, *Haloferax volcanii* and *Haloarcula japonica* T5 have been reported to overproduce the exopolysaccharide mannose when glucose is present in the environment [21]*.*

Biofilms vary not only in their phenotypic appearance and function, but also in the time required for mature development, depending on various environmental factors, such as nutrients, pH, shear stress and the attachment substratum [62]. As such, biofilms are dynamic, and change with their environmental surroundings. The process of biofilm formation seems to have been conserved across a wide range of prokaryotic life [29], consisting of a few distinct steps.

In bacteria, biofilm formation is generally described to occur as follows: when an abiotic surface is in contact with an aqueous environment, the process commences with the formation of a conditioning film on the abiotic surface from the attachment of organic solutes in the surrounding aqueous environment. This is followed by the initial reversible adhesion of microbial cells to the conditioned abiotic surface, then the secondary irreversible adhesion of microbial cells to the surface, and the growth of the attached cells into a mature biofilm (Fig. 2.4) [24,63]. This generalised process of biofilm formation also seems to be the case for archaeal biofilms [21,64].



**Fig. 2.4** Diagram of the steps involved in biofilm formation: establishment of a conditioning film (A), reversible adhesion mediated by physicochemical interactions (B), irreversible adhesion (C) and growth of mature biofilm (D). Figure adapted from [63].

However, biofilm formation is a complex process that is also significantly influenced by multiple external factors, such as the surface composition, hydrodynamic shear, nutrients in the environment and the microbial species, which challenges the complete comprehensive understanding of this process [63,65]. Much research has been conducted in examining the multifaceted factors influencing biofilm formation in bacteria, yet this knowledge is largely lacking for archaea [20,21]. This gap in knowledge highlights a promising area of research for *Methanosarcina,* given its promising potential for high rate AD systems. There is a need to better understand the biofilm-forming capabilities of this methanogen for potential application in heavy duty biomethanation.

###### 2.3.1. Initial adhesion

The initial accumulation of cells at a surface highlights the important role that the interface plays in the first stages of biofilm formation. Initial adhesion originates from the interactions occurring at the microbial and abiotic surface, and the strength of these interfacial interactions are dependent upon the physicochemical surface properties of the microbial and abiotic surface [66]. These interfacial interactions are considered to facilitate the initial reversible adhesion of cells to an abiotic surface, in which cells are loosely attached and can be disrupted easily from the surface [63].

Microbial interfacial interactions are categorised as being either specific or non-specific. Specific interactions comprise of stereo-chemical interactions between localised and specific complementary surface structures. These interactions are spatially confined between specific molecular groups on the surface and take effect over short distances less than 5 nm [63].

On the other hand, non-specific microbial interactions are a result of universal interfacial interaction forces between all molecules of the entire cell and abiotic surface. These interfacial interactions are comprised of the omnipresent Lifshitz-van der Waals forces, electrostatic forces and Lewis acid-base interactions [67,68], and arise in all situations where two surfaces interact. As such, they are non-specific in nature and operate over a longer range than the specific interactions [63]. Both the specific and non-specific interactions are key in the initial adhesion of microbial cells to an abiotic surface, and are both significantly influenced by the surface properties of both interacting surfaces [69].

Planktonic microbial cells are often brought into close contact with an abiotic surface by various means, such as by diffusion, Brownian motion or by the natural mobility of the microorganism [63]. Upon approaching each other, the non-specific interfacial forces are primarily responsible for the initial attachment of microbial cells to an abiotic surface [65]. These longer range non-specific forces, such as the electrostatic and Lifshitz-van der Waals forces, operate at distances over 50 nm and can pull microbial cells towards an abiotic surface [70].

The Lifshitz-van der Waals forces are always present and arise due to induced dipole-induced dipole interactions [63]. They operate over a long distance of greater than 10 nm, as well as extremely short distances of less than 0.2 nm [71]. The van der Waals forces are more influential than the electrostatic forces at short distances, and are not affected by the ionic strength of the aqueous environment. They are predominantly attractive in nature [72,73].

Electrostatic forces occur between charged surfaces, and are typically repulsive if the two interacting surfaces have like charges. The magnitude of these electrostatic forces exponentially decays over large separation distances and in high ionic strength environments [63]. As most microbial and abiotic surfaces exhibit a net negative surface charge under physiological conditions (pH 5-7), they have to overcome these repulsive electrostatic forces in order to come into close contact for adhesion [66].

The Lewis acid-base forces are a more recent addition to the repertoire of accepted interfacial interactions, and constitute all the electron-donating and electron-accepting interactions between polar moieties [71]. As such, a surface is described as being a Lewis acid if it is able to accept electrons, and a Lewis base if it is able to donate electrons [74]. These acid-base forces have been reported to operate at interaction distances of less than 1.5 nm [70], although their force decreases with distance [71]. They can be repulsive or attractive, and take into account the hydrophobicity of surfaces, which has a large influence on initial adhesion [75].

Hydrophobicity is a physical interfacial force that plays a key role in initial microbial adhesion [76]. Hydrophobic interactions in aqueous environments arise due to the incapability of apolar molecules to form hydrogen bonds with the surrounding polar water molecules. This is largely a result of the strong tendency of water molecules to form hydrogen bonds with each other and their strong polar energy of cohesion [71]. The presence of ‘hydrophobic’ surfaces in aqueous environments means that there is insufficient hydrogen bonding between the water molecules and the hydrophobic surface. Therefore, such surfaces are regarded as being able to detract water molecules away from the microbe-surface interface and promote microbial adhesion to the surface [67,71,77]. Hydrophobicity stems from Lewis acid-base interactions and are reported to act over a long-range of up to a distance of 80 nm [71]. They are generally attractive interfacial forces, with a force of up to two decimal orders of magnitude higher than the van der Waals and electrostatic interactions [76,78].

Despite understanding the main interfacial interactions governing adhesion between a microbial and abiotic surface, the process of initial adhesion cannot be fully described in terms of these interfacial interactions. The process of initial adhesion is further complicated by the heterogeneous nature of the microbial surface [63]. Far from being a rigid and homogeneously smooth surface, microbial cells are covered by any number of surface structures, appendages and functional groups, such as polysaccharides, lipids, surface-bound proteins, flagella and pili, to name a few [69,79]. This surface appendage topography is far from smooth, and previous studies have recognised the importance of surface roughness in microbial adhesion [80]. This may happen due to a rough surface having a greater surface area and the presence of surface depressions for favourable attachment [66]. However, the mechanisms by which surface roughness influences microbial adhesion are still not well understood [80].

These cell surface structures and molecular groups can also impart a significant influence on the physicochemical properties of the cell surface, such as the surface charge, surface hydrophobicity and surface free energy [72]. Changes in the pH can induce the dissociation or protonation of carboxyl (COO-), phosphate and amino surface functional groups on the microbial and abiotic surface [72], which can significantly alter the surface charge. High ionic strengths can suppress the effect of microbial and abiotic surface charges [63]. Various studies have demonstrated a difference in the level of initial attachment and preferential microbial attachment to solid surfaces at specific pHs, and at different ionic strengths [81–83].

Therefore, the immediate environment has an influential role on the resultant physicochemical properties of both the microbial and abiotic surface, which in turn, has an effect on the interfacial interactions and initial adhesion [63,69,72]. With the physicochemical surface properties of both surfaces being in a state of constant flux, this can affect initial adhesion and highlights the complexity of this first step in biofilm formation.

###### 2.3.2. Irreversible adhesion

The second phase of microbial attachment occurs over a longer time-scale and relies on the secretion of a polymeric mixture by the cells themselves, or the use of microbial surface appendages, such as pili and fimbriae [63] to facilitate a more irreversible adhesion of cells to an abiotic surface.

Irreversible adhesion is mainly governed by the cellular secretion of EPS outside the cell, consisting of an external network of polysaccharides, DNA, proteins and amphiphilic molecules [23]. EPS is widely recognised to play a role in adhesion, as well as facilitating cell-cell communication, mass transfer of nutrients and substrates within the biofilm, and act as a protective barrier against hostile external environments [84,85].

EPS production is an important facet of irreversible adhesion to a surface, and provides mechanical stability to mature biofilms by way of intermolecular binding, through electrostatic, polar, van der Waals and hydrogen bonding [86]*.* The polysaccharides of the EPS play a key role in cementing the cells and biofilm together by way of electrostatic and hydrogen bonding , and interact with other macromolecular components within a biofilm to create a network of interacting polysaccharides, proteins, lipids, cells and cell products [85].

EPS production is strictly genetically regulated, and it varies as a function of microbial species, mode of growth, environmental conditions, as well as to the type of support material [22,24]. A previous study showed that the choice of support material within a biofilm reactor treating synthetic inorganic wastewater influenced biofilm and EPS formation. Soft combination packing, a fibre material primarily composed of polyvinyl formal, was found to promote higher biofilm and EPS biomass than activated carbon fibre [87].

A study by Veiga and colleagues [88] found that the secreted EPS from anaerobic granules formed within a lab-scale AD reactor treating fatty acid media consisted mainly of rhamnose, mannose, galactose, glucose, and glucosamine from two methanogens, *Methanobacterium formicicum* and *M. mazeii*. The polysaccharide content secreted from both methanogens made up almost a third of the total EPS extracted from the anaerobic granules, highlighting the important role of methanogenic exopolysaccharides in binding these granular biofilms together. In particular, the temperature, type of feedstock and nitrogen and phosphate concentrations had a significant effect on the level of EPS production from *M. formicicum.*

Irreversible microbial adhesion to a surface can also incur significant modifications to the cell surface and differential levels of protein expression to adapt cells to a biofilm lifestyle [62], and are important indicators of biofilm formation. Key cell surface and intracellular changes during biofilm formation have been demonstrated in a wide range of microbial systems [89–92], and highlight some of the importance transitions that cells undergo in order to become more irreversibly attached to a surface.

# 2.4. Selective immobilisation of methanogens onto support material

Given the significant influence of the physicochemical properties of both the microbial and abiotic surface in initial adhesion and consequent biofilm formation in many natural and engineered biosystems [63], it is clear that the choice of abiotic material is an important design parameter for retaining microbial biomass within AD reactors [93]. Different support materials possess variable physicochemical properties that have a differential effect on initial microbial adhesion and biofilm formation [63]. It is becoming widely recognised that the use of appropriately chosen support materials in fixed-film AD reactors can enrich AD reactors with selective methanogenic biofilms, which has potential to increase the efficiency of the AD process, as well as AD performance [16,94]. Fixed-film AD reactors span different technologies such as the upflow anaerobic sludge blanket (UASB), fluidised bed and anaerobic filters [13], which all enhance the performance of AD by providing an increased surface area for adhesion from microbial biomass.

Different support materials for selective microbial immobilisation have been examined in fixed-film AD reactors, ranging from plastic polymers, to zeolites, to carbon fibre [42,94,95]. Support materials applicable to effective microbial immobilisation in AD consist of those made from non-biodegradable materials to ensure high durability in the face of high shear and high rate flows, and those that exhibit a large surface area and high porosity [42] to enhance biofilm formation*.*

However, owing to the variable experimental parameters and feedstocks tested in the literature, it is a challenge to select an obvious set of high performing materials to use as support structures in fixed-film AD reactors. In addition, the enormous diversity of biological and physicochemical mechanisms governing biofilm formation can make it difficult to define the ideal conditions required for optimal initial adhesion and biofilm formation [66]. Yet, studies have reported a correlation between the selective adhesion of methanogenic archaea to particular support materials based on microbial and abiotic physicochemical surface properties [45,96,97]. This information can provide a better insight into the processes underlying selective biofilm formation of methanogens to a surface.

Sanchez *et al.* (1994) demonstrated that sepiolite and dibase attracted a higher attached cell density of methanogens and methane production from domestic sewage after 8 days of incubation, compared to polyvinylchloride (PVC), polyurethane and bentonite. Each of these support materials selected for different species of attached bacteria and methanogens, thus providing a specific localised environment for particular species and types of biofilms to form [98].

In another study, the polymeric materials, PVC and polypropylene (PP) supported the highest attachment of methanogenic archaea in a pilot-scale AD reactor treating anaerobic sludge, resulting in higher COD removal rates. The findings suggested a strong influence of the support material on the archaeal population, whereas this specificity was not significant in the bacterial population [45].

Similarly, a study demonstrated the selective adhesion of *M. concilii* to polytetrafluoroethylene (PTFE), PP and polyethylene (PE), whereas no cell adhesion was observed on polyamide. *M. mazei* did not adhere to any of the tested polymer surfaces, potentially due to the highly hydrophilic nature of its secreted EPS, whereas *M. hungatei* selectively attached to polyacetal and PVC [96].

A comprehensive understanding of howa particular support material affects the physicochemical and biological mechanisms underlying biofilm formation is not well understood. Such an understanding would allow the tailored design and choice of appropriate support materials for the early establishment of selective high-performing biofilms in AD reactors, such as the proposed concept of a *Methanosarcina-*based AD reactorput forward in this thesis.

# 2.5. Current biofilm research methods

The study of biofilms is a complex process, and as such, requires a multidisciplinary approach to better understand the underlying mechanisms governing it [99]. Therefore, various methods have been developed to examine the different steps involved in biofilm formation in bacteria and archaea. These methods span a wide range of disciplines, such as surface and polymer science, microbiology, genetics and chemistry, and highlights how a fully interdisciplinary effort is required for the study of biofilms [25]. These methods will be discussed in further detail in the following sections, and form the basis of the methodology used throughout this thesis.

###### 2.5.1. Examining initial reversible adhesion

Initial adhesion is significantly affected by the physicochemical surface properties of both the microbial and abiotic surface [63], such as surface free energy, surface functional groups, surface chemical composition and surface hydrophobicity. These surface properties can be experimentally measured using standardised laboratory methods, and can further our understanding of key surface-associated factors that influence initial adhesion [99]. In addition, the process of initial adhesion can be quantitatively described by the use of theoretical colloidal models. Some of these methods are listed in Table 2.2. The methods listed in Table 2.2 formed the basis of the methodology used throughout this thesis, because they provided the correct information for the parameters of the colloidal model used in this thesis to describe initial adhesion (please refer to 2.5.2.). However, it has to be stressed that the methods listed in Table 2.2 are not exhaustive and that other methods are available to characterise the microbial cell surface.

For example, previous studies have used atomic force microscopy (AFM) to examine the effect of surface roughness on microbial initial adhesion [75]. A recent study integrated AFM force curves into the Derjaguin, Landau, Verwey, and Overbeek (DLVO) colloidal model [100], and is a promising new methodology and addition to the suite of methods currently in use for examining microbial initial adhesion. Other available surface characterisation methods not included in Table 2.2 include the microbial adhesion to hydrocarbons (MATH) assay for measuring microbial cell surface hydrophobicity [63,78].

The range of different methods available to describe initial microbial adhesion highlights how complex the process is. As such, it would be more accurate to describe initial microbial adhesion by utilising a combination of different approaches and theoretical models to provide different information on the specific physicochemical interactions between microbial cells and abiotic surfaces. From this, a framework can be created to better understand microbial adhesion and biofilm formation. Some of the methods currently used for examining the physicochemical determinants of initial adhesion will be discussed in further detail in the sections below.

**Table 2.2** Methods commonly used in the laboratory to analyse the physicochemical factors governing microbial initial adhesion to abiotic surfaces.

|  |  |
| --- | --- |
| **Physicochemical factor** | **Methodology** |
| Surface hydrophobicity | Contact angles on a flat surface |
| Surface charge | Electrophoretic mobility (zeta potential) |
| Surface tension | Contact angles on a flat surface |
| Surface functional groups | Fourier transform infrared spectroscopy (FTIR) |
| Surface chemical groups | X-ray photoelectron spectroscopy (XPS) |

2.5.1.1. Surface functional groups

In microorganisms, the outer cell surface forges direct contact with the external environment, and as such, it plays a crucial role in the initial adhesion and biofilm-forming capabilities of a microorganism to a solid surface [101]. Understanding the functional group chemistry of the cell surface and how it changes and responds to the surrounding environment can broaden our understanding of how cells adapt to a biofilm mode of growth.

The use of infrared (IR) spectroscopy was first used by microbiologists to rapidly differentiate between different microbial species in the 1950s and 1960s. Fourier transform infrared spectroscopy (FTIR) has become a popular choice for non-destructively analysing the surfaces of microbial cells [102]. It has a high specificity in differentiating between different microbial species or biofilm components without prior preselection [103]. FTIR measures the specific vibrational, rotational and bending modes of particular bonds or functional groups to certain wavelength frequencies of infrared beams [104]. This derives from the fact that the atoms within a molecule are loosely held together [105]. The sample absorbs specific frequencies of the infrared radiation, which gives rise to different forms of vibrations. These patterns of vibration are influenced by the different binding angles and binding forces between the atoms in a molecule. Thus, the unique display of internal vibrations exhibited by a complex molecule in response to infrared radiation produces a unique spectral fingerprint that can be used to identify that specific molecule [106] (Fig. 2.5).

Proteins



Side chain vibrations

Polysaccharides

**Fig. 2.5** Typical ATR-FTIR spectra of a biological sample.

In a complex sample, the functional groups of molecules typically absorb infrared radiation over a wavenumber range of 4000 to 300 cm-1 [105], yet it is common for microbiologists to identify key absorption bands in IR spectra for biological material over a wavenumber range of 800 to 1800 cm-1. This wavenumber range correlates to characteristic absorbance peaks from macromolecules of biological origin, such as proteins, lipids, nucleic acids and carbohydrates [103]. This can provide useful information on the key functional groups and biological components of the surface involved in biofilm formation, and can describe how the cell surface changes during biofilm formation [99].

The use of a diamond attenuated total reflectance (ATR) crystal is often used in FTIR, offering the advantage of being able to directly examine cell suspensions and abiotic surfaces non-invasively directly atop the ATR crystal. The ATR crystal possesses a high refractive index, and an infrared beam penetrates the ATR into the sample at an angle that allows the total internal reflection of the beam [106]. This reflected beam extends 0.5-5 µm beyond the ATR crystal and into the sample, and possesses altered frequencies that are unique to the functional groups of the sample surface and which are measured by ATR-FTIR [103,106].

The ATR-FTIR method provides spectral information on both the cytoplasmic and surface components of a cell, as the sample penetration depth of the reflected beam (0.5-5 µm) is greater than the typical cell wall thickness of bacteria (30-100 nm) [107]. However, a study by Jiang *et al.* (2004) found that the FTIR spectra of intact cells of *Bacillus subtilis* 168, *Bacillus lichenifermis, Pseudomonas stutzeri* 17588 and *Pseudomonas aeruginosa* PAO1 cells correlated with the FTIR spectra from their extracted cell walls [108]. As the IR spectra did not significantly differ between intact cells and isolated cell walls, numerous studies since then have used FTIR to analyse surface-specific changes to the cell surface [87,92,102]. The work of van der Mei *et al.* (2000) also heralded the use of FTIR for analysing modifications to the cell surface [109], with the assumption that 60 - 70% of the total hydrated cell weight is dominated by the cell wall or surface. Therefore, any major changes in surface composition can be attributed to the dominating cell wall or surface.

ATR-FTIR has been used to study biofilm behaviour and formation in archaea, such as in the thermoacidophilic archaeon, *Sulfolobus metallicus.* ATR-FTIR was able to identifythe surface-associated components of the secreted EPS matrix. The conformational protein and polysaccharide changes between intact cells and the extracted EPS was also measured, to monitor how the cell surface changed in response to a biofilm lifestyle [110].

2.5.1.2. Surface elemental chemical composition

The chemical and elemental composition of the surface also plays an important role in initial adhesion by affecting the interfacial forces at the microbe-abiotic interface [111]. X-ray photoelectron spectroscopy (XPS) is a well-established surface-sensitive method that is able to characterise and quantify the elemental composition and chemical environment of any material surface [112]. It has increasingly been used to characterise the surface macromolecular composition of a wide range of microorganisms [99].

XPS is based on a soft X-ray beam of a specific wavelength that irradiates the surface of a sample at a depth of 2-5 nm [102,111]. The X-ray beams are absorbed by the atoms in the sample, leading to the ejection of electrons from their inner electron shells whose binding energies are measured by XPS. Specific elements possess unique binding energies depending on their electron density and chemical states [102]. These elemental binding energies appear as peaks in XPS spectra, and the intensities of the ejected electrons are proportional to the concentration of the element from which they came. As such, they are used as markers to identify the elemental composition of a microbial or abiotic surface [111].

XPS analysis is therefore a high resolution surface analysis technique that analyses the binding energies of emitted electrons from a sample to reveal the composition and bonding environments of the elements in the sample, as well as the surface functional groups and macromolecules in the sample [112].

The quantification of macromolecules in a sample can also be estimated from high resolution XPS spectra using a simple approach based on the work of Rouxhet *et al.* (1994) [111]. This approach calculates the molecular composition of a cell surface based on comparing the measured atomic concentration ratios of oxygen to carbon, and nitrogen to carbon of experimental samples to the known ratios of model compounds for polysaccharides, hydrocarbon-like compounds and proteins. However, careful consideration should be taken into account with the interpretation of these values, as they may differ from the actual cell surface composition due to the oversimplification of the quantification approach used [102]. Despite this, Dufrêne *et al.* (1997) confirmed the validity of using XPS and Rouxhet’s approach to estimate the overall molecular composition of the surface of *Bacillus brevis*, by showing that the cell surface molecular composition as deduced by XPS analysis fitted well with the measured total peptide content from biochemical analysis [113]. In fact, XPS has been increasingly used in numerous microbiological studies to study and quantify surface-associated changes during biofilm formation [75,89,92,101,102].

2.5.1.3. Zeta potential analysis

The surface potential of the microbial and abiotic surface cannot be experimentally determined [72,79]. However, the zeta potential, ζ, is a measurable trait, and is assumed to be the same as the surface potential. Therefore, zeta potential analysis is used to determine the surface charge of microbial and abiotic surfaces [114].

In an aqueous environment, ionised surface functional groups create a surface charge on microbial and abiotic surfaces, which attracts oppositely charged ions in solution to the surface. This has a great effect on the distribution of ions surrounding the charged surface, and creates a double layer of oppositely charged ions closely surrounding the microbial cell surface at a distance of a few nanometres, which is referred to as the Stern layer [72]. A second distribution of electrostatically attracted ions is also observed in an external region consisting of distant diffused ions that are weakly attracted to the charged surface [79]. These two regions of ions create what is known as the double electric layer, and the zeta potential is the electric potential at the boundary (also referred to as the shear plane) between these two regions. This is shown in Fig. 2.6.



**Fig. 2.6** Schematic of the electric double layer and zeta potential (adapted from [115]).

In zeta potential analysis, the application of an electric field causes charged microbial cells to move towards an electrode of opposite charge. The velocity of this movement, known as the electrophoretic mobility (EPM), is related to the zeta potential using Smoluchowski’s equation [116]:

(Equation 1)

where ƞ is the viscosity of the aqueous medium, is the relative permittivity of the liquid, and is the permittivity of a vacuum.

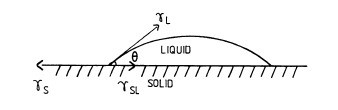
Zeta potential measurements therefore provide valuable information on the surface electrochemical make-up of a microbial or abiotic surface, which is known to vary as a function of microbial activity, growth phase or genetic differences [99]. The zeta potential is often measured as a function of pH, as this has a large effect on the dissociation and protonation of acid-base surface functional groups and therefore, the resultant zeta potential. It is also measured as a function of ionic strength, as high ionic strengths above 0.1 M suppress the thickness of the double layer [72].

2.5.1.4. Surface free energy

The surface free energy (SFE) is a defining property of all solid surfaces and is a function of interfacial forces occurring at the surface [74]. It is an important standard for analysing the adhesive properties of a surface [68] and is an indirect measurement of the hydrophobicity of a surface [117]. A solid surface with a high SFE is generally classed as being hydrophilic and negatively charged, whereas a low SFE surface is classed as being relatively hydrophobic and with a lower surface charge [25].

The quantification of the SFE of a solid surface is mainly based on contact angle measurements [78], owing to their ease of measurement and the reliability of the results [69]. Contact angle (θ) analysis is also the only method available that is able to accurately measure the interfacial forces acting on the actual surface of a solid [68]. The sessile drop technique is often used to measure contact angles, and consists of depositing a single drop of liquid onto the surface of a solid, and measuring the angle at the tangent at which the liquid, air and solid surface meet (Fig. 2.7) [75].

Thomas Young first recognised the association between the SFE of a solid surface (γS), the surface tension of a liquid (γL), the SFE of the interface (γSL) and the contact angle (θ), over 200 years ago (Fig. 2.7) [117]:



**Fig. 2.7** Relationship between the contact angle and the interfacial interactions at the surface of a solid (adapted from [113]).

Young’s equation is still the basis for calculating the SFE of any solid surface, and is based on the assumption that the solid surface in question is flat and smooth [68,117]:

(Equation 2)

where γS is the SFE of a solid surface, γL is the surface tension of a liquid, γSL the SFE of the interface, and θ is the contact angle.

For microbial surfaces, the measurement of their SFE based on contact angles is a little more challenging than for abiotic surfaces, and is achieved by the deposition of a lawn of microbial cells onto membrane filters [118]. The microbial lawn is consequently dried until the measured water contact angle on the surface reaches a ‘plateau phase’ over time, or when the contact angle is stable [78]. This method has allowed many scientists to successfully measure the SFE of a wide range of microorganisms [69].

The method proposed by van Oss, Chaudhury and Good [76] partitions the SFE into three different measurable interfacial components, and also takes into account the contribution of polar acid-base forces to the SFE [74]. This comprehensive partitioning of the surface free energy into its respective measurable components has been used by many scientists in the field of surface science, and particularly by those wanting to understand the process of initial adhesion in biofilm formation [69,81].

The interfacial components that make up the SFE described by van Oss, Chaudhury, and Good [74] consist of the Lifshitz-van der Waals (*γLW*) and acid-base (*γAB*) components, where the polar AB component is partitioned into electron-donating (*γ+*) and electron-accepting (*γ-*) parameters [68].

In order to calculate the three unknown SFE components (*γLW, γ+* and *γ-*), contact angles are measured from three probe liquids, typically diiodomethane, formamide and water, as they yield the best reliable results [78]. Their surface tension components are known [69]. These combine to yield the total SFE of a surface (*γtota****l***) to give an indication of the adhesive properties of a surface.

The apolar *γLW* component is calculated from contact angles using an apolar liquid, typically diiodomethane [68,78]. The polar *γ+* and *γ-* components are calculated from contact angles using two polar liquids, typically water and formamide [78].

###### 2.5.2. Modelling initial adhesion - DLVO model

Microbial cells are considered to be colloids, having a high surface area-to-volume ratio and a size range of 1 nm - 10 μm, which is similar to the properties of colloids [99]. Therefore, microbial aggregation, or biofilm formation, can be modelled on colloidal aggregation. For more than 40 years, microbiologists have examined the underlying physicochemical interactions governing the initial adhesion of microbial cells to a solid surface by using traditional colloidal models and theories [79], such as the commonly used Derjaguin, Landau, Verwey, and Overbeek (DLVO) colloidal model [63,69].

The DLVO model describes the net interaction energy between two interacting colloidal surfaces, and has also been used to describe the interaction between a microbial cell and an abiotic surface [63,69,79]. It takes into account the additive effect of the main interfacial interactions: Lifshitz-van der Waals (LW) and electrostatic forces (EL), and describes their decay with distance [72].

The important contribution of the short-range Lewis acid-base (AB) interactions to initial microbial adhesion (refer to 2.3.2) was further acknowledged and incorporated by Van Oss and co-workers into the classic DLVO model [68]. This gave rise to the extended DLVO model, otherwise known as the xDLVO model. The model is based on the assumption that the microbial cell is rigid and spherical [79].

The xDLVO model is used to determine the total free energy of interaction between a microbial and abiotic surface (GTOT) as a balance of the three main interfacial forces (LW, EL and AB) over a separation distance, *H*. As such, it is partly based on a thermodynamic perspective, which pertains to describe the total Gibbs free energy of adhesion between two interacting surfaces and therefore, whether adhesion is thermodynamically favourable [69,72]. Microbial adhesion is said to be thermodynamically favourable when is negative, and is thermodynamically unfavourable when is positive [63]:

(Equation 3)

When these three physicochemical parameters are plotted as a function of distance, a DLVO energy profile can be ascertained (Fig. 2.8) that typically shows the presence of a shallow energy minimum at large separation distances between the microbial and abiotic surface. This is referred to as the secondary minimum and is when cells are able to reversibly attach to a surface, meaning that they can be easily dispersed by shear forces [79,119]. A deep energy minimum at short separation distances can be observed, and is referred to as the primary minimum at which cells become irreversibly attached to a surface. An energy barrier also exists, which separates cells from an abiotic surface at the minimum distance of separation due to short range repulsive forces [79]. Cells need to overcome this energy barrier if they are to transition from the secondary minimum to a more irreversible attachment to a surface [63].

The size of the energy barrier is dependent on the zeta potential and ionic concentration of the aqueous solution. Increasing the ionic concentration of the aqueous solution decreases the electrostatic interactions due to the shielding effects of the ions in suspension, and this can decrease the energy barrier [72]. Therefore, microbial adhesion can be promoted by changing the ionic concentration of the solution. The interaction energies are generally represented by a kT energy scale, owing to the fact that 1 kT is equal to the Brownian motion energy [63].



Energy

barrier

Electrostatic forces

***Interaction energy (Kt)***

***Distance***

Van der Waals forces

Primary minimum

**DLVO**

Secondary minimum

Energy

barrier

**Fig. 2.8** Typical DLVO interaction energy profiles and the influence of the electrostatic and van der Waals forces.

However, the DLVO model only describes the contribution of a small fraction of the possible interactions occurring at the microbe-abiotic interface that are influential in microbial adhesion [66]. The microbial cell surface is covered by surface polymers and structures, such as pili and capsule material, which can also interact with the abiotic surface and take part in microbial adhesion [79]. These interactions are not taken into account by the DLVO model. Therefore, despite the success that the DLVO model has incurred to describe microbial adhesion to surfaces, it has not always been able to fully explain initial adhesion in some studies [120–122].

However, the DLVO model is still to date the most commonly used method of qualitatively and quantitatively describing the process of initial adhesion at the microbe-abiotic interface. It has been able to successfully further understanding of the initial adhesion of a wide range of microorganisms to a flat surface as a function of the main interfacial interactions at the surface [99].

###### 2.5.3. Examining secondary irreversible adhesion

Secondary irreversible adhesion is typically facilitated by the increased growth and abundance of attached cells and the self-secretion of EPS to bind cells to a surface [63]. As such, numerous standard laboratory methods used to monitor this later stage of biofilm formation focus on the reliable quantification and visualisation of biofilms (extracellular analysis), and on the examination of the biological mechanisms governing biofilm formation (intracellular analysis). There is an exhaustive list of different methods for the quantification and detection of biofilms [25], therefore this thesis will only briefly consider the most commonly used methods.

2.5.3.1. Extracellular analysis - Adenosine triphosphate (ATP) bioluminescence assay

All living cells rely on an internal energy currency in the form of adenosine triphosphate (ATP) that enables them to carry out their metabolic functions. As such, the detection of intracellular ATP in environmental samples has been a common method for indirectly monitoring and quantifying viable biofilms [123].

The ATP bioluminescence assay is a method for quantifying biofilms, and is highly selective and sensitive to low levels of ATP in samples. It is based on a biochemical reaction between intracellular ATP and the luciferin-luciferase enzyme found in the firefly, causing the emission of light (bioluminescence). The reaction involves lysing cells in the sample to release intracellular ATP, adding the luciferin-luciferase enzyme to catalyse the reaction and induce bioluminescence, and measuring the light emission on a luminometer. The bioluminescence is proportional to the amount of biomass in a biofilm, and also gives an indication of the metabolic state of the biofilm [124].

This assay has been used for both pure cultures [124–126] and mixed environmental samples from anaerobic digesters [127] as a rapid cell viability indicator. However, the ATP bioluminescence analysis for pure cultures can be highly sensitive to sources of contamination in samples, which can skew results and overestimate the quantification of biofilms [128].

2.5.3.2. Extracellular analysis - Crystal violet assay

Crystal violet is a surface-associated dye that stains microbial cells attached to a surface, and as such, is a well-established stain for rapidly detecting and quantifying biofilms for a wide range of microbial species [129]. It has been used in numerous static biofilm studies with biofilms grown within microtitre plates, to quantify the growth of biofilms as a function of time [130–133].

Surface-attached cells are stained by the crystal violet dye in the assay, and the adsorbed dye is solubilised by the addition of a solvent such as acetic acid. The solubilised dye can be quantified in terms of optical density, and is proportional to the size of the biofilm [129].

Koerdt *et al.* (2010) used the crystal violet assay to quantify the amount of biofilm formed by the archaeal species, *S. acidocaldarius, S. solfataricus* and *S. tokodaii* when grown within microtitre plates [64]. The authors confirmed that the optical density values of resuspended biofilm cells significantly correlated with the crystal violet values.

However, this indirect method of detecting and quantifying biofilms is limited by the crystal violet dye, in that it stains not only cells, but also any biomaterial attached to the surface, such as EPS [129,132]. It is also unable to differentiate between living and dead cells in the biofilm [134]. Therefore, this assay can overestimate the amount of biofilm attached to a surface.

2.5.3.3. Extracellular analysis - Microscopy

In the last two decades, fluorescence-stained microbial samples combined with epifluorescence or confocal laser scanning microscopy (CLSM) has become a solid part of a microbiologist’s standard suite of biofilm research methods [25]. This method offers a direct insight into the quantification, composition and architecture of surface-attached biofilms in a rapid and reliable way. This is an especially appropriate method for the examination of biofilms formed on non-transparent surfaces, such as plastics and metals [25].

Epifluorescence and CLSM have gained much interest for their ability to non-destructively visualise fluorescently stained microbial cells in situ [135]. CLSM offers the additional advantage of offering higher resolution images than epifluorescence microscopy, and an ability to examine the depths of biofilms to provide further information on the architecture of biofilms. However, CLSM is limited by the fact that it is time-consuming, requires technical expertise and requires expensive equipment and consumables [132].

Epifluorescence microscopy cannot accurately measure the thickness of biofilms, and is more suited for the investigation of thin and sparse biofilms. It also requires less technical expertise and costly equipment than CLSM [65].

Microbial biofilms can be stained with various fluorescent dyes that selectively bind to specific biofilm components to allow a visualisation of the spatial arrangement of biofilms, the composition of the surrounding EPS and a means of quantifying cells or biofilm components [99]. For example, 4',6-diamidino-2-phenylindole (DAPI) binds to nuclear DNA [136], propidium iodide permeates through damaged cell membranes and can detect dead cells [137], concanavalin A (ConA) binds to glucose and mannose residues [20], fluorescein-5-isothiocyanate (FITC) binds to proteins [138] and DDAO (7-hydroxy-9H−1,3-dichloro-9,9-dimethylacridin-2-one) stains extracellular DNA (eDNA) [110].

There is an important place for microscopy in the study of biofilms, as they allow the direct observation and quantification of biofilms [99]. They are also appropriate for analysing large sample sets, and most importantly, make it possible to directly quantify and observe attached cell biomass [25].

Fluorescent staining has been used to quantify and visualise surface-attached cells and EPS for both pure cultures and mixed environmental samples. Chen *et al.* (2006) used four fluorescent stains (FITC, SYTO 63, ConA and calcofluor white) to examine the spatial arrangement of nucleic acids, proteins and polysaccharides in the EPS of biofilms from waste-activated sludge attached to cellulose membranes [139]. A triple fluorescent staining method was utilised by McSwain *et al.* (2005) to visualise the EPS composition of granular sludge, and the authors found that the granules had a protein core with a polysaccharide-rich exterior [140]. Frols *et al.* (2012) used a fluorescent staining method to analyse biofilm formation from 20 different pure cultures of haloarchaea species [141], which highlighted the variable biofilm structures exhibited by each species and the use of surface structures to facilitate adhesion from some haloarchaeal species, such as *Halobacterium salinarum*. A quadruple fluorescent staining method was used to visualise the biofilm architecture of the archaeal species, *S. acidocaldarius, S. solfataricus* and *S. tokodaii* when grown within microtitre plates [64]. Therefore, fluorescent staining coupled with epifluorescence or CLSM imaging shows great promise for studying biofilms.

2.5.3.4. Intracellular analysis - Proteomics

Proteomics is the study of the global protein profile, otherwise referred to as the proteome, of a cell, tissue or organism at a certain time point [142,143]. Understanding the full complement of proteins produced by a cell provides vital information on the intracellular responses of a cell to a changing environment, as well as the proteins responsible for the phenotype and physiological responses of a cell, which cannot be elucidated from studying the genes alone [142,144]. As such, many microbiologists have increasingly taken up proteomics in recent years to elucidate the biological mechanisms underlying the adaptive responses of a cell in a changing environment [99].

Mass spectrometry (MS) has revolutionised the field of proteomics by allowing the accurate and reliable identification of extracted cellular proteins based on their mass (m) and charge (z). MS is highly sensitive, and is able to handle mixed protein samples in high-throughput operations [142]. In recent years, tandem mass spectrometry (MS/MS) has become a widely used technique for the analysis of complex protein mixtures. This consists of the proteolytic digestion of complex protein mixtures into peptides, which are increasingly becoming separated by two independent liquid phase separation systems, otherwise known as two-dimensional liquid chromatography (2D-LC). This separation system is coupled to MS, and provides a comprehensive method of identifying and quantifying proteins in a sample [145,146].

However, a key obstacle in proteomics is the analysis of such complex biological material. A protein sample from a single cell can contain an unfathomable concentration of proteins that is beyond the dynamic range and detection ability of MS. This sample complexity can compromise the ability of MS in detecting low abundance proteins and quantifying proteins over a wide dynamic range [145,147]. In order to address these limitations, sample preparation is key in reducing the complexity of protein mixtures and enriching proteins prior to MS. Therefore, the proteomics workflow consists of a series of different preparation steps that aims to fractionate complex proteins into less complex components for more accurate protein analysis by MS [148].

2.5.3.4.1. Protein extraction and fractionation

A typical proteomics workflow is shown in Fig. 2.9 and proceeds as follows: firstly, cells from a culture are chemically lysed to release the complex intracellular mixture of proteins produced by the cell at a given time point. A typical chemical lysis buffer for protein extraction consists of various buffers, salts and detergents to ensure maximum protein solubility in solution. Protein solubilisation is an integral part of extracting proteins from a cell culture, and involves breaking down the interfacial bonds and interactions involved in protein aggregation, resulting in individually dispersed proteins in solution [149].



**Fig. 2.9** Typical proteomics workflow from a complex mixture of proteins from a cell culture, to protein analysis by MS. Figure adapted from [144].

Detergents are often used to solubilise proteins, and to date, the anionic detergent, sodium dodecyl sulphate (SDS), is the most commonly used detergent for effectively disrupting the hydrophobic and ionic protein-protein interactions in proteins [150]. However, detergents greatly interfere with the downstream proteomic processing steps, and often must be removed from samples [145]. Low levels of SDS (< 0.1%) can complicate peptide separation techniques downstream and are incompatible with MS [148,151]. The removal of SDS and other detergents can be removed by acetone precipitation, and salt removal can be facilitated by the use of centrifugal filtration devices [152].

Thereafter, proteins are commonly enriched and pre-fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular weight [152,153]. This is still one of the most commonly used methods for separating proteins owing to its ability to resolve and separate proteins in a complex protein sample, and is an important method for reducing protein complexity [154]. The success of protein separation is based on the solubilisation of proteins. SDS is again used to solubilise proteins in a sample and to provide a net negative charge to all proteins. Upon application of an electric field in a polyacrylamide gel, these negatively charged and solubilised protein molecules migrate through the gel at a rate based on their net charge and size [145], and are detected by a visible stain. Silver staining is a popular choice of stain, owing to its sensitivity to proteins at the nanogram-level [155].

The separated proteins appear as gel bands at different positions that can be compared to the bands from proteins of known size [153]. Methods for protein quantification, such as the Lowry or Bradford assay, are important steps to ensure that equal amounts of protein from different experimental conditions enter the downstream proteomic workflow [148].

2.5.3.4.2. Protein digestion

Prior to protein digestion, the protein sample undergoes denaturation, alkylation and reduction using various reagents to increase the efficiency of protein digestion, by allowing proteases better access to protein cleavage sites [145]. Denaturation and reduction is required for the disruption of disulphide bonds between cysteine residues in proteins, which are responsible for protein structure [148]. Owing to the incompatibility of SDS to liquid chromatography (LC) separation techniques and MS, surfactants that are compatible to MS, such as the commercially available RapiGest™ (Waters, USA), are often used to enhance the solubilisation and denaturation of proteins [152]. This surfactant has an acid labile moiety that allows it to undergo hydrolysis when exposed to acidic environments, thus allowing its removal from samples prior to LC-MS [148].

Strong reducing agents, such as dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), are commonly used to reduce protein disulphide bonds [145]. Alkylation of cysteines is required to further prevent denatured proteins from reforming disulphide bonds between cysteine residues [148].

Thereafter, the denatured, reduced and alkylated proteins undergo enzymatic digestion and are broken down into smaller and simpler peptides by a sequence-specific protease, such as trypsin. Trypsin specifically cleaves the arginine and lysine residues on proteins on their carboxy-terminal side [144], producing peptides that fall within the mass range detection limits of MS for easier identification. This is a key step, as mass spectrometers are more sensitive to sequencing peptides that are up to 20 residues long compared to proteins [144,147].

2.5.3.4.3. Peptide separation

In order to further reduce the complexity of the peptide sample prior to MS, a 2D-LC approach is often used to fractionate the peptides using two liquid phase separation methods. This approach is indispensable in the analysis of complex protein samples owing to its speed, reproducibility and automation [156]. The most common method for peptide separation is to trap peptides out of solution onto an immobilised phase based on the polarity, hydrophobicity or ionisation of the peptides [156,157].

Reverse-phase liquid chromatography (RPLC) is the most popular separation method, owing to its high efficiency and reproducibility [156]. It is able to elute, or remove peptides in order of their hydrophobicity [144]. The more polar peptides are eluted first (polar mobile phase), and the less polar peptides become adsorbed onto a hydrophobic solid support as they interact with the hydrophobic groups on the support (non-polar immobilised phase) [158]. Using an organic solvent at a gradient of increasing organic content reduces the hydrophobic interactions between the adsorbed peptides and the solid support, thus allowing peptides to become eluted after the polar mobile phase peptides [144].

The ability to use RPLC coupled to MS (online mode) makes RPLC a suitable method for the identification of biological molecules, and for the purification of peptide samples before MS analysis [142]. The versatility of this technique means that RPLC separation is becoming increasingly used in both the first and second dimensions of a 2D-LC system [156].

Prior to injection of peptides into a mass spectrometer in the second dimension of LC separation, liquid peptide samples are vaporised to the gas phase. In 2D-LC, this is carried out by electrospray ionisation [156] which ionises liquid phase peptides with a high electric potential at the end of an LC system, producing a cloud of highly charged peptide ions. These ions become desolvated as the solvent evaporates, yielding smaller peptide ions with a high charge density [142].

2.5.3.4.4. Mass spectrometry

MS is the final step of the proteomics workflow and measures the mass to charge ratio (m/z) of peptides in the gas phase. Within the mass spectrometer, the gas phase peptide ions collide with an inert collision gas, typically nitrogen or argon in a process known as collision induced dissociation. This collision causes the peptides to further fragment into fragment ions, in order to obtain the sequence of peptides and their m/z values [142].

The mass spectrometer organises the ionised peptides based on their m/z for mass analysis. There are three different types of mass spectrometers, which differ in how they measure m/z ratios: quadrupole MS, which uses an electric field produced by four parallel metal rods to transmit ions of a specific m/z ratio for analysis; time of flight MS, which measures m/z based on how much time is needed for an ion to cross the flight tube; and quadruple ion trap MS, which traps ions in a 3D electric field for analysis [142,144].

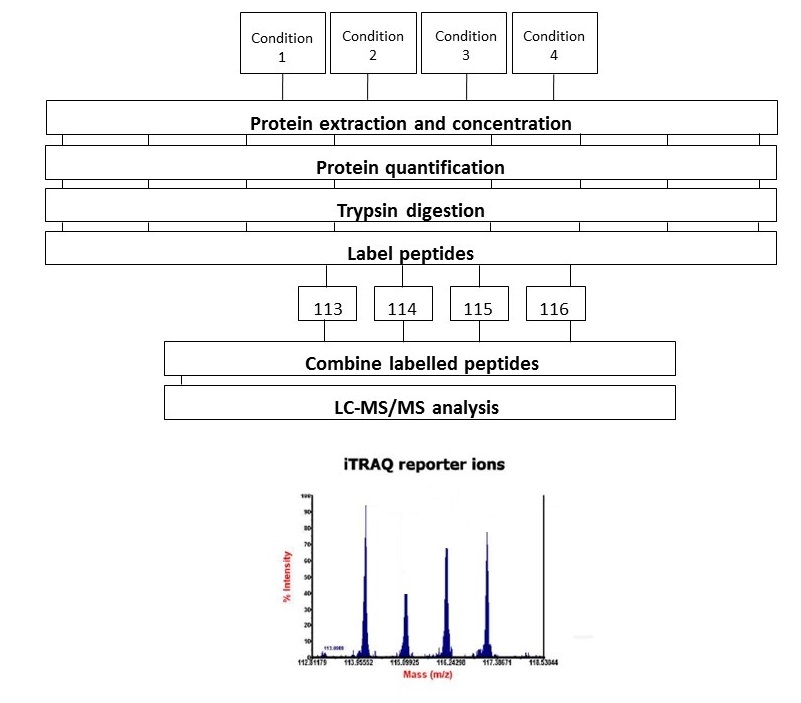
The m/z values of fragment ions in a sample are recorded in a mass spectrum, and the masses of these unknown proteins are matched to the known masses of naturally occurring proteins for a given species within a database [142]. As such, protein identification can only occur if the genome of the organism in question has already been sequenced to enable the comparison of all possible species-specific proteins [144]. Various software programs now exist that specialise in the identification of proteins based on raw MS data, such as Mascot, MaxQuant and SEQUEST. These programs use search algorithms combined with a form of statistical scoring to significantly compare the mass spectra of unknown and known proteins, yielding a closely-matched protein identification [142].

2.5.3.4.5. iTRAQ quantification

In recent years, the emergence of a rapid, reliable and high-throughput protein quantification method based on isobaric tags for relative and absolute quantification (iTRAQ) has been advantageous to proteomic analysis [143]. Other methods for quantitative proteomic analysis exist, such as label-free, stable isotope labelling with amino acids in cell culture (SILAC) and isotope-coded affinity tag (ICAT), but iTRAQ offers the advantage of being an easy to use method with the capability to analyse and quantify the relative abundance of proteins from up to eight different samples simultaneously [159,160]. As such, numerous studies have used iTRAQ to elucidate key differential expression of proteins during biofilm formation for a number of archaeal and bacterial species subjected to various growth conditions in a single experimental run [89,161,162].

iTRAQ labels are chemical tags that react with the N-termini of primary amines of peptides with a neutral mass balance group and a uniquely charged N-methylpiperazine reporter group [147]. The balance group ensures that all labelled peptides have the same mass of 145 Da (isobaric) during tandem MS [163,164]. Following peptide fractionation within a mass spectrometer, the iTRAQ reporter groups break off from their respective peptides and produce ions with distinct m/z values of 114, 115, 116, 118, 119, 121 and 122. These strong reporter ions allow the relative quantification of proteins, where the relative intensity of each reporter ion is a direct indication of the relative abundance of each protein in the sample [163]. Having multiple biological replicates for each experimental condition is recommended in order to increase the statistical robustness of the results [159].

A limitation of the iTRAQ approach stems from the enzymatic digestion of proteins prior to labelling, which can increase sample complexity. Therefore, a 2D-LC approach is required prior to MS of labelled peptides to increase the resolution of proteins in the sample [147]. Another issue is that iTRAQ can be prone to the underestimation of changes in protein expression levels [165] due to sample complexity and isotopic impurities [160]. Therefore, the use of validation experiments is important to verify the conclusions drawn from iTRAQ. A typical iTRAQ workflow is shown in Fig. 2.10.



**115**

**116**

**114**

**113**

**Fig. 2.10** A typical iTRAQ workflow. The different intensities of each reporter ion in the depicted MS/MS spectrum in this diagram provides direct indication of the relative abundance of a protein in the sample.

2.5.3.4.6. Archaeal proteomics

As is clear, the process of proteomics is a complex one, but it has developed rapidly in the face of recent advances in scientific instrumentation and analysis, and now forms an important part of a microbiologist’s repertoire of tools for examining biofilm formation [166]. However, proteomics is still a relatively new research area for studying archaea owing to the challenges involved in cultivating archaeal species for proteomic manipulation [167]. A search on Web of Science (*www.webofknowledge.com*; accessed 9 September 2016) reveals only 136 journal articles on the proteomic analysis of archaea, compared to 2122 journal articles for bacteria. To date, there are only 24 journal articles on the proteomic analysis of *Methanosarcina*, with no known recorded published papers on the proteomic analysis of biofilm formation in *M. barkeri* (Web of Science, *www.webofknowledge.com*, accessed 9 September 2016).

The limited proteomic work that has been carried out in studying the methanogen archaea has revealed various interesting biological functions in response to different growth conditions. Proteomics is an indispensable tool that has revealed the differential expression of proteins involved in methanogenesis for *Methanococcus maripaludis* when grown in limiting hydrogen conditions [168], the overproduction of exopolysaccharide from *M. barkeri* when grown under conditions of desiccation [169], the differential expression of proteins involved in temperature adaptation in *M. barkeri* grown at 15°C compared to 37°C [161], the higher relative abundance of cell surface proteins when *Methanococcoides burtonii* was grown at 4°C compared to 23°C, the dominance of differentially expressed oxidative stress proteins at 23°C [162], and proteins involved in stress response in *Methanosarcina acetivorans* strain C2A when grown in acetate compared to methanol [170,171].

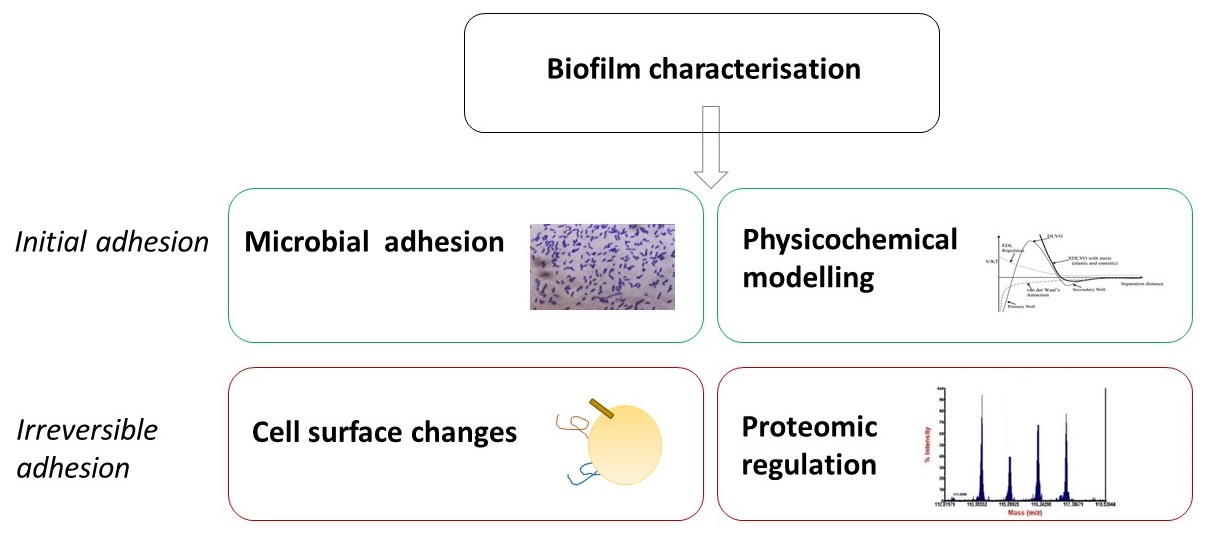
Proteomics has a wide-ranging applicability in microbiological research, and can give insights into the adaptive responses and biological mechanisms underlying various microbial behavioural responses to a changing environment. This holds great promise for analysing the biofilm formation of *M. barkeri* to different support materials, which isan area that has not yet been examined in great detail.

# 2.5. Conclusions

Biofilm formation is a complex developmental process that has garnered much scientific interest in the research community. In order to fully characterise biofilm formation, the physicochemical and biological factors that are key in the initial reversible and later irreversible steps of biofilm formation need to be considered [63]. This knowledge is generally lacking in archaea [20], and little is known of the processes involved during biofilm formation of *M. barkeri* to different support materials.

The overall objective of this thesis is to develop a microbial-based strategy for optimising the AD of high strength domestic wastewater, which is based on the selection of relevant support materials to selectively immobilise *M. barkeri.* Therefore, a mixed methods approach was used to examine some of the key physicochemical and biological factors affecting the initial adhesion and irreversible adhesion steps of biofilm formation in *M. barkeri* to different support materials, chosen for their common usage in AD reactors [94] and evaluated to be of low cost, available globally [13] and durable [42] for applicability in peri-urban areas. Alternative materials that had strong potential for use as a support material in AD reactors included those that were more fibrous or porous, such as straw, woodchip [13] and sisal leaf [172]. However, the biodegradability [173] of such materials ruled these out in the final evaluation.

A summary of the research activities carried out in this thesis is shown in Fig. 2.11.



**Fig. 2.11** A summary of the main research areas examined in this thesis.

## Chapter 3

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##### Physicochemical analysis of initial adhesion and biofilm formation of *Methanosarcina barkeri* on polymer support material

# 3.1. Introduction

The initial accumulation of cells at a surface highlights the important role that the abiotic surface plays in the first stages of biofilm formation [65]. The initial microbial adhesion to an abiotic surface is mediated by short and long-range physicochemical interactions at the microbe-abiotic interface, resulting from the surface components of both interacting bodies. The requirement of a substrate for biofilm formation has been a significant source of scientific interest, with many researchers examining the contribution of the surface properties of both the abiotic and microbial cell surface on initial adhesion and biofilm formation [69,75,120,174]. Therefore, various methods from colloidal cohesion studies have been further developed for biological systems to measure the physicochemical properties of microbial surfaces to provide valuable insight into the physicochemical determinants of initial adhesion and later stage biofilm formation.

Microbial initial adhesion has been described in past studies using the extended Derjaguin-Landau-Verwey-Overbeek theory (xDLVO) [79], where particle adhesion is described in terms of the interfacial Lifshitz-van der Waals, electrostatic double layer and Lewis acid-base interactions as a function of separation distance, and are calculated from experimental surface parameter measurements [63]. To our knowledge, this has not been applied for understanding the adhesion behaviour of key methanogenic species, such as *M barkeri,* in AD reactors.

The objective of the present study was to therefore examine the effect of the surface characteristics of six common polymer support materials on the initial adhesion of the model methanogen, *M. barkeri,* and to assess the potential of these support materials as selective biofilm carriers. Specifically, the focus was on the effect of high ionic strength and neutral pH environments on initial adhesion. These characteristics are typical of domestic wastewater found in arid regions with low household water consumption [2,175], such as in peri-urban areas of the Global South. The quantitative characterisation of initial adhesion obtained from both a theoretical model (xDLVO) and a static adhesion assay are compared within this experiment. Additionally, biofilm formation was examined over a longer time period to examine whether the process of initial adhesion was a critical factor for biofilm formation in *M. barkeri* to the six support materials.

# 3.2. Materials and Methods

###### 3.2.1. Preparation of anaerobic media and stock solutions

Cultures of the archaeal strain *M. barkeri* DSM 800 were obtained from DSMZ (DSMZ, Braunschweig, Germany) and cultivated in a methanol-based anaerobic medium (DSM 120 medium). Frozen glycerol stocks of *M. barkeri* were prepared anaerobically, and stored at -80°C. Anaerobically prepared DSM 120 medium (pH 6.8) [176] was prepared within a 1 litre Duran bottle (Sigma Aldrich, UK) on the bench top, and aliquoted into 50 ml volumes within borosilicate glass serum bottles (Sigma Aldrich, UK). The media consisted of dibasic potassium phosphate (0.35 g), potassium dihydrogen phosphate (0.23 g), ammonium chloride (0.5 g), magnesium sulphide heptahydrate (0.5 g), calcium chloride dihydrate (0.25 g), sodium chloride (2.25 g), iron(II) sulphate heptahydrate (2 mg), trace element solution (1 ml), yeast extract (2 g) and casitone (2 g). All chemical components were purchased from Sigma Aldrich, UK.

Resazurin was used in the media as an oxidation-reduction indicator. In order to exclude oxygen from media, serum bottles were placed on a heat plate at maximum high heat for 10 minutes, whilst simultaneously sparging with 100% nitrogengas (N2). After this duration of heating, the resazurin changed colour from blue to pink to signify oxidation [177], after which the serum bottles were taken off the heat plate to cool, whilst still sparging with N2 for a further 10 minutes.

Thereafter, serum bottles were sealed with butyl rubber seals (Sigma Aldrich, UK) and aluminium caps (Sigma Aldrich, UK) in a swift movement to minimise the entry of oxygen into N2-sparged media. Media bottles were autoclaved at 121˚C for 15 minutes to sterilise media before use.

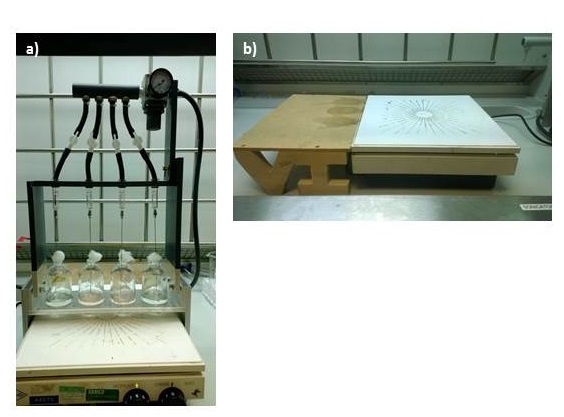
Before inoculation, the strong reducing agents, cysteine-hydrochloride (0.3 g/L) and sodium sulphide nonahydrate (0.3 g/L), were added to sterilised media to produce a redox potential of less than -330 mV, which is a requirement for the cultivation of strict anaerobes [171]. Additional media components were also added at this point, such as the main carbon source, methanol (10 ml/L), a vitamin solution (10 ml/L) and sodium bicarbonate (2 g/L) to maintain a neutral pH for growth [171] using an aseptic syringe method. All inoculations and subculturing were performed in an anaerobic chamber (PLAS-LAB Simplicity 888, PLAS-LABS, U.S.A.).

These media components were prepared anaerobically as stock solutions as described earlier, by heating and sparging with 100% N2 gas before being sealed and autoclaved. Once the media had become reduced at -330 mV, the resazurin became colourless, indicating that media were ready for inoculation.

###### 3.2.2. Development of a sparging station for anaerobic cultivation

The cultivation of anaerobes is dependent upon an oxygen-free pre-reduced media, which requires an efficient gas sparging method to exclude oxygen from bottles of media on the bench-top. At the starting phase of this project, the current setup in the laboratory was deemed inefficient and time-consuming for the preparation of multiple bottles of media for anaerobic cultivation, consisting of a single gassing probe to sparge individual media bottles with N2 gas.

Therefore, a bespoke design of a new gas sparging setup was designed and adapted from Balch & Wolfe (1976) [178], consisting of four sparging probes attached to a N2 gas supply by gas-impermeable butyl rubber tubing (Cole Parmer, UK) and fitted with a gas pressure regulator (Cole Parmer, UK) (Fig. 3.1). The final production of the gas sparging setup was implemented by the University of Sheffield’s Chemical and Biological Engineering workshop.



**Fig. 3.1** Photo of (a) the gas sparging setup as adapted from [172], with details of (b) the personalised cooling platform.

The gas sparging setup was dependent on the use of gas-impermeable materials, such as butyl rubber tubing and borosilicate glass syringes to maintain anaerobic conditions throughout the whole system. Having multiple sparging probes allowed the simultaneous flushing of several media bottles with N2 gas, thus reducing the time needed to prepare anaerobic media and anoxic stock solutions.

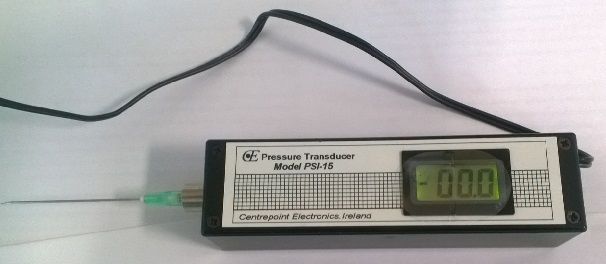
The setup consisted of a tube connector (Cole Parmer, UK) connected to a 100% N2 gas tap to divide the gas flow to four sparging probes, with the flow of gas to each probe controlled by individual gas valves (Cole Parmer, UK). The sparging probes consisted of 2 ml borosilicate glass syringes with a metal Luer lock tip (SAMCO, UK) connected to 21G blunt steel syringe needles (Sigma Aldrich, UK). Butyl rubber tubing was inserted and secured within the opening of the glass syringes in order to deliver N2 gas.

The sparging probes and butyl rubber tubing were supported by a thermoplastic standing structure with a stainless steel base. The stainless steel base was used to withstand the heat from the heat plate, and consisted of a platform with four holes cut into it to allow the insertion of four serum bottles. The holes in the steel platform had the same diameter as the serum bottles used for anaerobic cultivation. The steel platform was fixed to a height that would allow a heat plate to fit underneath it, to optimise the simultaneous heating and sparging of serum bottles that was required for the preparation of anoxic media and stock solutions.

In order to facilitate the cooling of heated media, the transfer of serum bottles away from the heat plate was necessary. In this new sparging setup, after serum bottles had been on the heat plate for 10 minutes, the whole structural support was carefully pushed backwards off the heat plate and onto a custom-made wooden base structure for serum bottles to cool whilst still sparging.

###### 3.2.3. Growth conditions

Growth curves were established for *M. barkeri* upon receipt. *M. barkeri* was subcultured from frozen glycerol stocks or live cultures into 50 ml of freshly reduced media to establish growth and statically incubated at 37˚C. Growth was monitored by optical density readings over 8 days at 600 nm (OD600) on a spectrophotometer (Ultrospec 2100 Pro, Fisher Scientific, UK), and by internal gas pressure readings using a personalised and tailored gas pressure transducer with a syringe needle adapter, courtesy of Dr. Gavin Collins from the National University of Galway. This can be seen in Fig. 3.2. The starting OD600 of growth curves was 0.05. Samples for OD600 measurements were extracted within an anaerobic chamber.



**Fig. 3.2** Modified pressure transducer used to measure the gas pressure in the headspace of cultures.

To maintain reproducibility of results and to avoid phenotypic drift from repetitive culturing, experiments were started from maintained fridge stock cultures and were subcultured no more than 3 times. Fridge stock cultures were maintained monthly at 4˚C, with new stock cultures grown from frozen glycerol stocks every 3 months.

###### 3.2.4. Support material preparation

Six different support materials were used, consisting of common engineering plastics chosen for their low cost, durability and accessibility: polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyvinylidene fluoride (PVDF), polyethylene terephthalate glycol (PETG) and polytetrafluoroethylene (PTFE) (Engineering & Design Plastics Ltd., Cambridge, UK). Each plastic was cut into 1 x 1 cm coupons for the static initial adhesion and biofilm formation experiments, and into 1.5 cm x 6 cm coupons for streaming potential and contact angle measurements.

The plastic coupons were cleaned with 70 % ethanol (Sigma, UK) to remove grease on the surface before being submerged into a 2% v/v PCC-54 detergent solution (Fisher Scientific, U.K.), and subjected to a water sonication bath (Fisherbrand, UK) within a container for 5 minutes. The coupons were then rinsed several times with Milli-Q ultrapure water (Merck Millipore, Germany) until they stopped foaming. They were dried inside a laminar flow cabinet and further UV-sterilised for 3 hours, before being stored in sterile Falcon tubes (Sigma, UK) for future use.

###### 3.2.5. Surface characterisation analysis

3.2.5.1. Contact angle measurement

Contact angles of *M. barkeri* and the support materials were measured using the sessile drop technique using a tensiometer (Attension Theta Lite, Biolin Scientific, Sweden) and 3 probe liquids of different polarity and with known surface energy (please refer to section 2.5.1.4). These liquids were Milli-Q water, diiodomethane (Sigma-Aldrich, UK) and formamide (Sigma-Aldrich, UK). Contact angle measurements were carried out at room temperature (22˚C).

Coupons with dimensions of 1.5 cm x 6 cm were prepared for each of the support materials. The reported contact angle results are based on 3 replicate coupons and a droplet of each probe liquid deposited onto a randomly selected location on the surface of each support material coupon.

*M. barkeri* cells were harvested at mid-exponential phase (96 h growth), as monitored by OD600 measurements, and centrifuged (Heraeus Megafuge 16, Thermo Scientific, USA) at 6000 x g for 5 minutes and rinsed twice with 100 mM potassium chloride solution (KCl) (Sigma-Aldrich, UK) at pH 7. Using a vacuum pump to provide a negative pressure, washed cells were filtered onto a 0.45 µM pore nitrocellulose filter membrane (MilliPore, USA) to obtain an even lawn of microbial cells, which was assessed by visual inspection. A drying time of 20 minutes was used for all contact angle measurements after preliminary experiments using water contact angles were carried out to establish the ‘plateau phase’ over time, or when the contact angle was stable [78]. Results reported are contact angles measured for 3 biological replicates and a droplet of each liquid deposited onto a randomly selected location on the microbial lawn.

3.2.5.2. Calculation of surface free energy components

The van Oss, Chaudhury, and Good [76] approach for measuring SFE was used in this study to take into account not only the surface tension of a liquid and solid, but also the acid-base contributions to the SFE of a solid surface [69]:

(Equation 1)

where θ is the contact angle of the three probe solutions with known surface tensions on the surface (Table 3.1), γ*L* is the surface energy of the probe liquids, and γ*LW*, γ*+*and γ*-*are the Lifshitz-van der Waals, electron acceptor and electron donor parameters of the solid surface (γ*S*).

**Table 3.1** Surface tension components (mJm-2) of three commonly used probe liquids for measuring contact angles for the calculation of SFE [117].

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Probe liquid** | ***γtotal*** | ***γLW*** | ***γAB*** | ***γ+*** | ***γ-*** |
| Water | 72.8 | 21.8 | 51 | 25.5 | 25.5 |
| Formamide | 58 | 39 | 19 | 2.3 | 39.6 |
| Diiodomethane | 50.8 | 50.8 | 0 | 0 | 0 |

Having measured three contact angles for the three probe liquids, equation 1 can be solved simultaneously to calculate the surface tension of γ*LW*, γ*+*and γ*-*for a sample surface [69]. The apolar *γLW* component was calculated from contact angles using diiodomethane [68,78]. The polar *γ+* and *γ-* components were calculated from contact angles using water and formamide [78].

The total contribution of the polar AB component of the SFE was calculated as the geometric mean of the electron-donating (γ-) and electron-accepting (γ+) parameters [69,78,179]:

(Equation 2)

3.2.5.3. Zeta potential analysis

Electrokinetic measurements were made to analyse the streaming potential of each support material, using an EKA Electrokinetic Analyser (Anton Parr GmbH, Austria) at the School of Chemical and Process Engineering, University of Leeds, UK.

Coupons of the support materials with dimensions of 1.5 cm x 6 cm were placed in a rectangular measuring cell between two Ag/AgCl electrodes. A 100 mM KCl solution at pH 7 was circulated around the system and inside the streaming channel of the measuring cell, with a rinse pressure of 300 mbar. The zeta potential was calculated from the streaming potential by the EKA Electrokinetic Analyser software based on the Smoluchowski equation [115].

The electrophoretic mobility (EPM) of *M. barkeri* was measured using phase amplitude light scattering (ZetaPALS, Brookhaven Instruments, UK) in 100 mM KCl solution, adjusted to pH 7. Cells were harvested at mid-exponential phase (96 h growth), washed and resuspended in 100 mM KCl solution at pH 7. An electric field of 2.5 V cm-1 and a frequency of 2.0 Hz were used to measure the EPM, as these settings have successfully been used in previous electrophoretic mobility studies of biosystems [92]. The zeta potentials of *M. barkeri* were calculated from EPM measurements using the Smoluchowski equation on the ZetaPALS software. Results are reported as an average of 20 cycles, 3 biological replicates and 3 independent experiments.

###### 3.2.6. xDLVO energy profiles

The xDLVO model was used to measure the total interaction energy between *M. barkeri* and the support materials in an aqueous environment of 100 mM KCl, pH 7. The model is based on the assumption of a colloidal particle being spherical, which was in accordance with the spherical cell morphology of *M. barkeri*. The sphere-flat plate equations were used in the xDLVO model [69] to model the interaction between the spherical microbial cells and flat plate dimensions of the polymer support materials.

The Lifshitz-van der Waals and acid-base model (LW-AB) developed by Van Oss and co-workers [76] forms the basis of the xDLVO model, and was used to determine the free energy of interaction between microbial and abiotic surfaces (GTOT) over a separation distance, *H*. It takes into account the additive effect of the Lifshitz-van der Waals (LW), electrostatic double layer (EL) and Lewis acid-base (AB) free energy of interaction as a function of separation distance (*H*) [69]:

(Equation 3)

The LW interactions are derived from the Hamaker constant (*A)*, which expresses the magnitude of the omnipresent LW forces in any system in a conventional way [180]. LW interactions were calculated using the following equation:

(Equation 4)

where *a* is the radius of *M. barkeri* cells which was assumed to be 1 µm [53], λ is the correlation length of molecules in liquid, which was taken to be 0.6 nm for hydrophilic bacteria [69] and *A* is the Hamaker constant. The Hamaker constant was calculated from the LW interaction energy (), which was determined from contact angle measurements [63]. The Hamaker constant was calculated as follows:

(Equation 5)

where d0 is the minimum separation distance between two surfaces and is typically 1.57 A ̊ plus the distance from the shear plane, which varies between 5 A ̊ at low and 3 A ̊at high ionic concentration [69]. Therefore, d0 was taken to be 4.57 A ̊ for a high ionic concentration aqueous environment (calculated as the minimum separation distance of 1.57 A ̊ plus 3 A ̊, taking into account the high ionic concentration of the medium used in the adhesion experiments [68].

The zeta potential, , was taken to be the surface potential in the DLVO model. The EL interaction energy was calculated as follows [69]:

(Equation 6)

where is the zeta potential of *M. barkeri* and the support material surfaces and assumed to be the same as the surface potential, is the electrical permittivity of the medium (8.854 x 10-12 C2 J-1 m-1) [81], *H* is the separation distance and is the double layer thickness, which was calculated as [69]:

-1 (Equation 7)

where *I* is the ionic concentration in terms of molarity.

The polar AB interactions originate from the electron-donating and electron-accepting interactions between polar moieties [71]. The AB interactions were calculated as follows [69]:

(Equation 8)

where d0 is the minimum separation distance between two surfaces and was taken to be 4.57 in this study, as described previously. λ is the correlation length of molecules in liquid, and *H* is the separation distance.

###### 3.2.7. Initial adhesion

Static adhesion experiments based on microtiter plates allows the rapid analysis of multiple growth conditions or microbial species within an experiment [132]. Therefore, clean coupons (1 x 1 cm) of each of the support materials were secured into wells of a sterile 24 well plate (Corning Costar, USA) using 10 µl of silicone sealant (Aquarium Sealant, King British, UK), which has been used as a non-toxic adhesive in previous static biofilm studies [129]. Experimental plates were prepared in a laminar flow cabinet and UV-sterilised for 3 h before being placed in an anaerobic chamber for at least 48 h prior to the initial adhesion experiment to remove all residual traces of oxygen from plates and coupons.

Cells of mid-exponential phase (96 h growth) *M. barkeri* cultures were harvested by centrifugation (Heraeus Megafuge 16, Thermo Scientific, U.S.A.) at 6000 x g for 5 minutes in sterile microcentrifuge tubes inside an anaerobic chamber. Cell pellets were rinsed twice and resuspended in 100 mM KCl solution at pH 7.

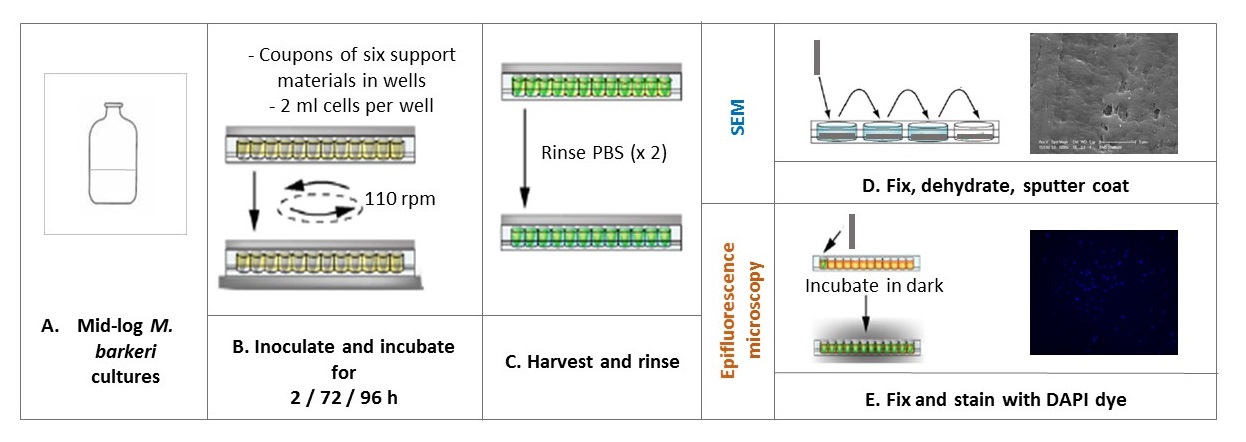
Wells of prepared 24 well plates were filled with 2 ml of cell suspension of *M. barkeri* with an adjusted OD600 of 0.3. Control wells contained clean plastic coupons in sterile 100 mM KCl, pH 7 solution only. Plates were incubated at room temperature (22˚C) and gently shaken at 110 rpm in an incubating mini shaker inside the anaerobic chamber (VWR International, USA) for 2 h. This short duration was chosen to examine initial microbial adhesion and to prevent established biofilm formation from *M. barkeri.* After 2 h, coupons were rinsed twice with anaerobically prepared 100 mM, pH 7 KCl.

###### 3.2.8. Biofilm formation

Cells of mid-exponential phase (96 h growth) *M. barkeri* cultures were washed and harvested as described earlier (section 3.2.7) and finally resuspended in DSM 120 anaerobic medium at pH 7. The biofilm assay was prepared using the same experimental conditions and in the same 24 well plates as used for the initial adhesion assay, except that adhesion occurred over a duration of 72 h and 96 h at 37°C. A higher temperature of 37°C was used to enable cells to grow optimally in order to examine their biofilm-forming capabilities. After these time points, coupons were rinsed twice with anaerobically prepared 100 mM KCl adjusted to pH 7.

###### 3.2.9. Cell biomass quantification methods

Three different methods for detecting and quantifying the abundance of adherent cells of *M. barkeri* to the support materials were tested to ascertain an optimal biofilm quantification method: ATP bioluminescence, crystal violet and fluorescent staining coupled with epifluorescence microscopy. The method that was finally used in this study was fluorescent staining coupled with epifluorescence microscopy method. A finalised general workflow of the initial adhesion and biofilm formation experiments used in this study are shown in Fig. 3.3. Details of each of the 3 tested methods are described in more detail below.



**Fig. 3.3** General workflow of the initial adhesion and biofilm formation experiments used in this study.

3.2.9.1. ATP bioluminescence analysis

A commercial ATP assay kit, BacTiter-Glo Microbial Cell Viability Assay (Promega, WI, USA) was used to lyse cells and react the released intracellular ATP from cells with an enzymatic luciferin-luciferase solution. A standard curve of the luminescence of known ATP concentrations, as well as of known OD600 values of *M. barkeri* cells was performed before each experiment, as described as follows: known concentrations of ATP (Promega, USA) were prepared in culture medium ranging from 106 pM to 10 pM ATP by preparing 10-fold serial dilutions in culture medium.

Each ATP concentration was pipetted in triplicate into a white 96 well plate (Promega, USA) designed for luminescence measurements within a laminar flow hood. An equal volume of the BacTiter-Glo reagent solution containing the luciferin-luciferase system was added to each ATP concentration, and incubated at room temperature in light for 5 minutes. ATP bioluminescence was measured on a GENios Multi-Detection Multiplate Reader (Tecan, UK) and given as relative light units (RLU). A standard curve correlating the concentration of ATP to RLU could then be generated. A similar standard curve was generated for known OD600 values of *M. barkeri* cells (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8) with RLU.

After rinsing within an anaerobic chamber, coupons from both the initial adhesion and biofilm formation assays were left to air dry for 15 minutes. Control samples consisted of DSM 120 anaerobic medium with no cells. 300 µl of phosphate-buffered saline (PBS), consisting of 8 g NaCl, 200 mg KCl, 1.44 g Na2HPO4 and 240 mg KH2PO4, and 300 µl of the BacTiter-Glo reagent solution were added to all samples to lyse cells, and thoroughly mixed by aspiration. The solution was left to incubate at room temperature for 5 minutes within the anaerobic chamber. 200 µl of this reactive solution was pipetted into wells of a white 96 well plate and the ATP bioluminescence was measured on a GENios Multi-Detection Multiplate Reader. RLU was correlated to ATP concentration and OD600 using the standard curves.

3.2.9.2. Crystal violet analysis

Rinsed coupons from both the initial adhesion and biofilm formation assays were stained with 300 μl of 0.001% (w/v) crystal violet for 20 minutes at room temperature. Coupons with adherent cells were then rinsed with sterile Milli-Q water [133]. The crystal violet dye was solubilised by the addition of 300 μl of 95% ethanol, and the OD595 of the suspended biofilm solution was measured using a GENios Multi-Detection Multiplate Reader.

3.2.9.3. Fluorescent staining and epifluorescence microscopy

Rinsed coupons from the initial adhesion and biofilm formation assays were fixed with 4% paraformaldehyde solution (Sigma-Aldrich, UK) for 15 minutes inside an anaerobic chamber. Thereafter, coupons were rinsed twice with PBS. Fixed coupons were stored at 4˚C in PBS for future microscopic imaging.

Paraformaldehyde-fixed coupons from both adhesion assays were labelled with 1 μg/ml DAPI solution (Sigma-Aldrich, UK) for 10 minutes at room temperature in the dark, and rinsed twice with PBS.

Epifluorescence microscopy was used to ascertain the area covered by adhered *M. barkeri* cells. Acquisition was facilitated with a Leica AF6000 inverted microscope (Leica Microsystems GmbH, Germany) attached to a computer with a magnification of 100 in order to obtain a representative surface coverage of coupons. The use of a lower magnification to observe a larger field of view of microbial surface coverage has previously been recommended [132].

20 microscopic fields (1 mm2 per field) were randomly selected for each coupon and measured for surface area coverage. Images were analysed with the ImageJ software in ‘Analyse Particles’ mode to calculate the average percentage of area covered by cells after 2 h, 72 h and 96 h.

###### 3.2.10. Scanning electron microscopy (SEM)

To visualize cell adhesion after 2 h, 72 h and 96 h, a parallel experiment was run alongside the initial adhesion and biofilm formation assays, using the exact same experimental conditions, in which adhering cells on the support materials were viewed under a scanning electron microscope (Electron Microscopy Unit, University of Sheffield, UK).

Coupons of each support material from both adhesion assays were removed from cell suspensions into sterile 24 well plates and rinsed twice with sterile, anaerobically prepared 100 mM KCl inside an anaerobic chamber. Coupons were then fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, UK) at 4˚C for 16 hours. Secondary fixation was carried out in 2% osmium tetroxide solution (Sigma-Aldrich, UK) before fixed samples were dehydrated through a graded series of 75% to 100% ethanol. Fixed samples were sputter-coated with gold before mounted onto stubs and viewed using a Philips XL-20 scanning electron microscope (Philips, Netherlands) at an accelerating voltage of 20 kV.

###### 3.2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., USA). The Brown-Forsythe test was used to determine statistical differences between group variances. The non-parametric Kruskal-Wallis test was used to compare percentage adhesion data after 2 h, 72 h and 96 h between the support materials, followed by *post-hoc* analysis using Dunn’s multiple comparison test. The non-parametric Wilcoxon matched-paired test was also used to test whether incubation time had an effect on the biofilm-forming abilities of *M. barkeri* to the support materials from 72 h to 96 h.

# 3.3. Results

###### 3.3.1. Preliminary experiments: Growth curves of *M. barkeri*

The growth of *M. barkeri* was determined over a duration of 8 days as an increase in the optical density at 600 nm and in gas pressure (Fig. 3.4). The growth curve followed a typical pattern of an exponential phase from 48 h to 130 h (2-5 days), followed by a stationary phase after 130 h. The time points for the biofilm formation experiments were selected based on this growth curve, in order to obtain cells in the mid- to late exponential phase of growth when cells were metabolically active. This is driven by the fact that metabolically active cells readily attach to abiotic surfaces [30].



**Fig. 3.4** Growth curve of *M. barkeri* at 37˚C with methanol as monitored by optical density measurements at 600 nm (⏹) and gas pressure (⏺). Error bars represent the standard deviation of 4 independent growth curve experiments.

###### 3.3.2. Surface characterisation

3.3.2.1. Contact angle measurement

The contact angles of water, formamide and diiodomethane on the surfaces of *M. barkeri* and the support materials are shown in Table 3.2, and were used to calculate surface free energy. Water contact angles were used as an indication of hydrophobicity [78].

The support materials each had similarly low surface free energies. These ranged from 28 mJ.m-2 to 48 mJ.m-2, with PTFE having the lowest total surface free energy and also having the most hydrophobic surface, with a high water contact angle of 116˚. On the other hand, PVC possessed the highest surface free energy and was the most hydrophilic surface with a comparatively lower water contact angle of 72˚. This is a result of PVC’s high polar acid-base (γAB) component, and can be attributable to its high electron-donating (γ-) nature. In addition to PTFE, PP also possessed a hydrophobic surface, whereas the remaining support materials had hydrophilic surfaces.

**Table 3.2** Contact angles, hydrophobicity, surface free energy components and zeta potential measurements of *M. barkeri* and support materials (100 mM, pH 7 potassium chloride). Error values represent standard deviations of 3 independent experiments.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | Contact angle (degrees) | | | Surface free energy (mJ.m-2) | | | | |  |
| Surface | Water | | Diiodomethane | Formamide | γLW | γAB | γ+ | γ- | γtotal | Zeta potential (mV) |
| *M. barkeri* | 8±2 | | 47±5 | 9±1 | 36 | 21.42 | 2.12 | 54 | 57 | -20±3 |
| PE | 77±9 | | 42±4 | 59±1 | 39 | 0.75 | 0.02 | 9 | 39 | -20±5 |
| PP | 91±7 | | 42±3 | 67±3 | 39 | 0.40 | 0.02 | 3 | 39 | -12±3 |
| PVC | 72±6 | | 29±5 | 58±3 | 45 | 3.39 | 0.21 | 14 | 48 | -5±3 |
| PETG | 76±1 | | 36±5 | 60±1 | 42 | 1.80 | 0.08 | 11 | 44 | -11±5 |
| PVDF | 77±6 | | 46±3 | 64±4 | 36 | 1.00 | 0.02 | 11 | 37 | -39±4 |
| PTFE | 116±2 | | 63±8 | 81±7 | 27 | 1.15 | 0.23 | 1 | 28 | -6±5 |

All support materials had a van der Waals component (γLW) that far exceeded the polar acid-base component (γAB). Van Oss et al. (1988) showed that organic materials, such as plastics typically possessed γLW values of 40 mJ.m-2 [76], which is in accordance with these results.

Having a water contact angle of 8o,*M. barkeri* had the most hydrophilic surface compared to the support materials. This is in accordance with the finding that most microorganisms possess hydrophilic surfaces with water contact angles less than 60˚ [78,120]. *M. barkeri* also possessed a more energetic surface than the support materials, with a total surface free energy of 57 mJ.m-2. Having a higher electron-donating component (54 mJ.m-2) compared to its electron-accepting component, this implies that *M. barkeri* has a strong polar surface [120]. Living surfaces are known to typically have strong electron-donating surface properties [78].

3.3.2.2. Zeta potential analysis

The zeta potentials of the support materials determined from streaming potential measurements showed that PVC had the least negative zeta potential at 100 mM KCl and pH 7 (-5 mV), closely followed by PTFE with -6 mV. PVDF had the most negative surface zeta potential with -39 mV (Table 3.1).

All solid surfaces exhibited negative zeta potentials, indicating negative surface charge under the conditions tested. *M. barkeri* also possessed an electronegative surface, with a zeta potential of -20 mV at 100 mM KCl and pH 7.

###### 3.3.3. xDLVO energy profiles

Assumptions for *M. barkeri* with regards to its morphology and surface charge were made when using the xDLVO model. *M. barkeri* has a large spherical shape, which falls within the assumptions of the xDLVO model of spherical particles [79]. Zeta potentials calculated from electrophoretic mobility and streaming potential measurements were assumed to be surface potentials, as this cannot be experimentally determined.

The total interaction energy of adhesion as determined by the xDLVO model between *M. barkeri* and the support materials was calculated as a function of separation distance, in 100 mM KCl at pH 7 at room temperature (22˚C) (Fig. 3.5). For ease of presentation, the Lifshitz-van der Waals, electrostatic double layer and acid-base interaction energies are also illustrated for each support material with *M. barkeri* (Fig. 3.6).



**Fig. 3.5** Total interaction energy for the approach of *M. barkeri* to the support materials (PE, PP, PVC, PETG, PVDF, PTFE) at 100 mM KCl, pH 7 as a function of separation distance (Å).

These predicted DLVO energy profiles demonstrate that repulsion is predicted between *M. barkeri* and all the support materials at small separation distances, except for PTFE and PP, as a result of repulsive short-range GAB forces (Fig. 3.6). These polar interactions create an energy barrier at the surface and prevent the irreversible adhesion of *M. barkeri* to PE, PETG, PVDF and PVC. However, these repulsive forces only operate at small separation distances of 30 Ǻ.



**Fig. 3.6** Calculated interaction energies for the Lifshitz-van der Waals (GLW), electrostatic (GEL), acid-base (GAB) and total interaction energy (GTOT) of the initial adhesion of *M. barkeri* to a) PE, b) PETG, c) PP, d) PTFE, e) PVC and f) PVDF in 100 mM KCl solution, pH 7.

A secondary minimum is present at longer separation distances corresponding to 30 Ǻ from the substratum for PE, PETG and PVC and 45 Ǻ for PVDF (Fig. 3.5). At these separation distances, approaching cells are predicted to reversibly attach to these support materials, but irreversible adhesion is not possible due to the energy barrier. The depths of the secondary minima suggests that irreversible adhesion is more likely to spontaneously occur on PVC with a net attractive interaction energy at secondary minimum of approximately 120 kT, whereas PVDF exhibited the lowest net attractive interaction energy at secondary minimum of approximately 50 kT (Fig. 3.5).

Repulsive electrostatic GEL forces were present for all combinations of *M. barkeri* and support material. As a result of their highly charged surfaces, strong, long-range GEL interactions were predicted between *M. barkeri* and PVDF and PE at 70 Ǻ and 50 Ǻ respectively from the substratum (Fig. 3.6; Table 3.1). PVC and PTFE had less dominant GEL forces due to their low charged surfaces (Table 3.1) and therefore, a reduced electrostatic repulsion. Attractive GLW interactions were experienced by all combinations of *M. barkeri* and support material (Fig. 3.6).

PP and PTFE were the only support materials to have both attractive GLW and GAB interactions (Fig. 3.6). These overcame the repulsive short-range GAB interactions, resulting in a strong net attractive force at the microbial-abiotic interface. Therefore, the xDLVO model predicted irreversible adhesion of *M. barkeri* cells to these surfaces.

###### 3.3.4. Cell biomass quantification

The ATP bioluminescence and crystal violet staining methods for quantifying the amount of cells attached to the six support materials in the initial adhesion and biofilm formation assays were not successful. The results from both these quantification methods proved to be unreliable and inconsistent upon repeat experiments.

The ATP bioluminescence analysis revealed a large increase in ATP concentration from *M. barkeri* to all six support materials after 2 h compared to the control, but no significant changes in ATP concentration were detected after 72 h or 96 h (p > 0.05, Student paired t-test). These results were not consistent with SEM images that showed increased cell attachment to some of the support materials at these time points compared to images taken at 2 h (Figs. 3.7-3.9). The ATP bioluminescence findings from this study can be explained by the findings from Blaut & Gottschalk (1984), who found that in *M. barkeri* grown on methanol, the initial addition of methanol to the medium was linked to a tenfold increase in intracellular ATP concentrations over a short duration of 2 h, as methanogenesis commenced [181]. However, intracellular ATP concentrations decreased rapidly after 2 h to approximate original levels as methanol became depleted. It is feasible that the ATP bioluminescence results in this study reflect the results found by Blaut & Gottschalk (1984). As a result, this method was not used for the time-course quantification of adherent cells of *M. barkeri* in both adhesion assays, as it could not give a reliable quantification of the amount of cells attached to the support materials.

Secondly, the well-used biofilm quantification method of crystal violet staining was also deemed unsuitable for quantifying the amount of adherent cells in the initial adhesion and biofilm formation experiments*,* producing inconsistent and unreproducible results upon repeat experiments. Archaea possess different cell envelope structures to bacteria, which can cause discrepancies in their response to Gram stains, such as crystal violet [182].

*Methanosarcina* possesses a cell wall structure of variable thickness depending on growth conditions [53], which could further affect the results of crystal violet staining. A previous study by Beveridge & Schultze-Lam (1996) showed that the addition of crystal violet to *Methanosarcina mazei* caused cell lysis [182]. It is feasible that the crystal violet results in this study reflect the results found in these previous studies. Therefore, crystal violet staining was not used further for cell biomass quantification in this chapter.

###### 3.3.5. Initial adhesion

The initial adhesion of *M. barkeri* to the support materials after 2 h in 100 mM KCl at pH 7 was quantified in terms of the percentage of the surface area covered by adhering cells per mm2 using DAPI staining coupled with epifluorescence microscopy (Fig. 3.7a). This method proved to be more reliable and reproducible than both the ATP bioluminescence and crystal violet methods, and was used to quantify adhered cell biomass in this study. Epifluorescence microscopy was used instead of CLSM, because preliminary experiments using CLSM revealed that *M. barkeri* biofilms were sparse and thin. As the thickness of biofilms was not being measured in this study, it was more appropriate to use epifluorescence microscopy.

Results showed that *M. barkeri* differentially attached to the support materials after 2 h (Fig. 3.7a), with the type of material strongly influencing the extent of cell adhesion (p < 0.0001, Kruskal-Wallis test).

The percentage of surface coverage varied across all support materials, with PVC performing best in promoting the initial adhesion of *M. barkeri*, with 12% of the surface area/mm2 colonized by cells after 2 h. PTFE and PP also promoted a high percentage surface coverage from cells of *M. barkeri.*

However, PETG, PVDF and PE possessed the poorest surfaces for initial colonization from *M. barker,* exhibiting <5% surface coverage per mm2*.* There was no significant difference in the level of initial adhesion from *M. barkeri to* these three support materials (p > 0.05, Kruskal-Wallis and Dunn’s multiple comparison test). However, there was a distinct difference between these support materials and PVC, PP and PTFE in their ability to promote initial attachment from *M. barkeri* (p < 0.05, Kruskal-Wallis and Dunn’s multiple comparison test).

**Fig. 3.7** Average percentage surface coverage of support materials from *M. barkeri* after an exposure time of a) 2 h in 100 mM pH 7 KCl and b) 72 h and 96 h in basal media after staining with DAPI. Error values represent 95 % confidence intervals from 3 independent experiments.

###### 3.3.6. Biofilm formation

The ability of *M. barkeri* to form biofilms on the support materials was tested after 72 h and 96 h in basal media (DSM 120 medium). The results from the biofilm formation experiments showed different levels of adhesion to the support materials at these time points (Fig. 3.7b), with the type of material strongly influencing the extent of cell adhesion (p < 0.0001, Kruskal-Wallis test).

Similar to the findings from the initial adhesion test*,* PVC, PP and PTFE were shown to promote better biofilm formation from *M. barkeri* after 72 h and 96 h (Fig. 3.7b). There was not a significant difference in the percentage of adherent cells between these three support materials after 72 h and 96 h (p > 0.05, Kruskal-Wallis and Dunn’s multiple comparison test).

PETG, PVDF and PE had the poorest surfaces for biofilm formation. There was a significant difference between these support materials and PVC, PP and PTFE in the level of biofilm formed after 72 h and 96 h (p < 0.05, Kruskal-Wallis and Dunn’s multiple comparison test).

Additionally, a significant increase in the percentage of adherent cells was observed from 72 h to 96 h for all six support materials (p < 0.0001, Wilcoxon matched-paired test), suggesting that biofilm formation increased over time.

###### 3.3.7. Scanning electron microscopy

SEM imaging was used to visualise *M. barkeri* attachment onto the different support materials after 2 h in 100 mM KCl and 72 h and 96 h in basal media. The initial adhesion of *M. barkeri* to the support materials proceeded as a random attachment of cells in isolated patchy areas after 2 h (Fig. 3.8).

After 72 h and 96 h in basal media, SEM images showed different cell morphologies and the potential presence of additional material on the surfaces of PVC and PTFE in particular (Figs. 3.9-3.10) compared to the control coupons (Fig. 3.11). This could be EPS. In comparison, the other support materials were more sparsely covered by cells of *M. barkeri* after 72 h and 96 h*,* particularly PETG and PVDF.



**Fig. 3.8** Scanning electron microscopy images of initial adhesion of *M. barkeri* to support materials after 2 h in 100 mM KCl at pH 7 corresponding to (a-f): PE, PP, PVC, PETG, PVDF and PTFE.



**Fig. 3.9** Scanning electron microscopy images of biofilm formation of *M. barkeri* to support materials after 72 h in basal media corresponding to (a-f): PE, PP, PVC, PETG, PVDF and PTFE.



**Fig. 3.10** Scanning electron microscopy images of biofilm formation of *M. barkeri* to support materials after 96 h in basal media corresponding to (a-f): PE, PP, PVC, PETG, PVDF and PTFE.



**Fig. 3.11** Scanning electron microscopy images of control coupons after 72 h in sterile basal media only, corresponding to (a-f): PE, PP, PVC, PETG, PVDF and PTFE.

# 3.4. Discussion

The absolute requirement for a substrate for biofilm formation has prompted significant scientific interest in the role of the surface properties of the microbial and abiotic substrate in mediating initial adhesion and consequent biofilm formation. Various surface characterisation methods have been developed to examine the properties of microbial and abiotic surfaces [63], and their effect on the interfacial physicochemical forces during initial adhesion. These methods have been applied in this chapter to examine the role of the support material in the initial adhesion process of *M. barkeri* to six different polymer support materials.

The results from this study showed that *M. barkeri* is capable of forming biofilms over a duration of 96 h, and that PVC, PTFE and PP possessed the best surfaces for initial adhesion and biofilm formation from *M. barkeri* (Fig. 3.6a) after 2 h in 100 mM KCl at pH 7, and after 72 h and 96 h in basal media (Fig. 3.6b)*.* The interaction energies between the microbial and abiotic substrata, and therefore, the extent of microbial adhesion were predicted by the xDLVO model by taking into account the additive effect of attractive van der Waals, repulsive electrostatic and acid-base forces [63].

There was a close correlation in the results obtained from the xDLVO model and those from the initial adhesion and biofilm formation experiments, with both showing that PTFE, PP and PVC provided the best surfaces for adhesion from *M. barkeri* (Fig. 3.6). The xDLVO model showed that PTFE and PP were the only support materials to exhibit both attractive GLW and GAB interactions (Fig. 3.3). These attractive interfacial interactions superseded the repulsive GEL interactions, resulting in a strong net attractive force between microbial and abiotic substrata.

Hydrophobic and low surface free energy surfaces, such as PTFE and PP in this study, are reported to have a better tendency to remove water from the area between two contacting surfaces, therefore leading to a stronger level of microbial adhesion [67]. This is a role of hydrophobicity, and these interfacial interactions arise from the inability of apolar molecules from the hydrophobic surface to form hydrogen bonds with the surrounding polar water molecules in aqueous environments [71]. The presence of ‘hydrophobic’ surfaces in aqueous environments means that there is insufficient hydrogen bonding between the water molecules and the hydrophobic surface [71]. Such surfaces are therefore regarded as being able to detract water molecules away from the microbe-surface interface and promote microbial adhesion to the surface [67,77].

Additionally, the xDLVO model prediction of *M. barkeri* irreversibly attaching to PTFE can be described in terms of the polar parameters of both the microbial and abiotic surface. The xDLVO model showed a strong attraction between the strong electron-donating surface (γ-) of *M. barkeri* to the strongest electron-accepting (γ+) surface of PTFE (Table 3.2).

The xDLVO model was also able to account for the low percentage of surface coverage of cells to PE, PETG and PVDF after 2 h in 100 mM KCl, with experimental initial adhesion results correlating with the depths of the predicted secondary minima in the xDLVO energy profile (Fig. 3.3). *M. barkeri,* PVDF and PE possess highly negatively charged surfaces (Table 3.2), and were predicted by the xDLVO model to produce strong repulsive GEL interactions between *M. barkeri* and PVDF and PE at 70 Ǻ and 50 Ǻ respectively from the surface (Fig. 3.4). Negative zeta potentials of surfaces in neutral pH environments [71,183] typically arise from COO− surface functional groups, and high levels of COO− surface functional groups could be applicable to PVDF and PE.

PVC has proven to be a good support material for promoting high levels of archaeal attachment within a 2 h timeframe in previous studies using mixed methanogenic consortia [45,94].This has also been demonstrated in this study where PVC fared best from all the polymer support materials tested in promoting the selective attachment of *M. barkeri* after 2 h in 100 mM KCl at pH 7.

This observation can be explained by further analysing the surface properties of PVC. PVC possessed the most hydrophilic surface with a water contact angle of 72˚, and the highest surface free energy (Table 3.2). This is due to the high polar acid-base (γAB) component of PVC’s surface, which can be accredited to its high electron-donating (γ-) nature (Table 3.2). *M. barkeri* exhibited an extremelylow water contact angle of 8˚ (Table 3.2), which is suggestive of a strong hydrophilic nature that could be a feature of its external methanochondroitin layer. This consists of glucuronic acid and acetylgalactosamine [53,184], both of which are hydrophilic in nature. *M. barkeri* also has a strong polar surface (Table 3.2), having a high electron-donating surface component (54 mJ.m-2)compared to a lower electron-accepting surface component (2 mJ.m-2). This strong electron-donating surface property is typical of living surfaces [78].

The strong initial adhesion of *M. barkeri* to PVC as determined from the initial adhesion experiment could be a result of PVC’s polar surface and high *γAB*. This surface property could facilitate an increased number of interfacial acid-base interactions between the cells of *M. barkeri* and the surface of PVC [185].

However, the xDLVO model predictions for the initial adhesion of *M. barkeri* to PVC deviated slightly from the results of the initial adhesion experiment (Fig. 3.6a). The xDLVO model showed the presence of a secondary minimum between *M. barkeri* and PVC at a separation distance of 30 Ǻ from the substratum, suggesting that cells were only reversibly attached to PVC.

This can be explained by the fact that the DLVO model only takes into account the main physicochemical interactions occurring at the interface (Lifshitz-van der Waals, electrostatic and acid-base) [186]. In reality, initial adhesion is a complex process that can also be significantly influenced by various non-DLVO parameters. For example, hydrodynamic shear forces arising from the flow of fluid over a surface can have a significant effect on the transport of microorganisms to a surface and therefore, affect the rates of initial microbial adhesion [30]. Hydrodynamic shear forces can also significantly affect the shape and structure of established biofilms [22,65,66]. Past studies have tested the effect of defined hydrodynamic shear conditions on initial microbial adhesion with the use of parallel plate flow chambers and rotating discs [66].

In addition, the microbial cell surface is covered by surface polymers, which can have a high affinity to different abiotic surfaces and facilitate attractive surface polymer interactions in initial adhesion [72,79]. These polymer interactions are not taken into account by the DLVO model, and could explain the observed selective adhesion of *M. barkeri* to PVC in this study [63].

*Methanosarcina* species are known to typically possess an external surface layer consisting of a thick polymeric network of methanochondroitin fibrils, which can extend 20-200 nm from the inner microbial surface layer and are responsible for cell-cell adhesion [53]. Microbial surface structures are able to cross the repulsive energy barrier at separation distances of 20-100 nm from the substratum [79], to allow cells in the secondary minimum to come into close contact with each other [69]. Therefore, microbial surface structures play a role in the initial adhesion process by facilitating the transition of cells into the primary minimum and a more irreversible attachment [186].

In this study, it is feasible that the highly polar surface of PVC combined with specific polymer interactions between the surface structures of *M. barkeri* and the surface of PVC could have promoted a more irreversible attachment in the primary minimum. Previous studies have reported deviations from the predictions of the DLVO model, which have been explained by the presence of microbial surface polymers or structures [63,185].

This study showed that the support materials promoting the highest affinity of adhering *M. barkeri* cells after 2 h in 100 mM KCl were also the best support materials for longer term attachment after 72 h and 96 h in basal media (Fig. 3.6b); a media that is also highly ionic and neutral in pH [176]. This finding agrees with those from previous studies on mixed methanogenic samples [45,94], and suggests that initial adhesion is a critical factor for biofilm formation in *M. barkeri*.

EPS could have played a role in the selective attachment of *M. barkeri* cells to PVC and PTFE after 72 h and 96 h, as suggested by SEM imaging (Figs. 3.8-3.9). There are certainly distinct differences between the topography and roughness of the surfaces of the coupons at these time pointscompared to the control coupons (Figs. 3.8-3.10). The inclusion of support materials within AD reactors have been reported to undergo rapid attachment from mixed methanogenic consortia within a few hours [187,188], and can promote the early production of EPS [67].

Surface roughness is another non-DLVO parameter that is recognised as having a significant effect on initial adhesion and can be measured using AFM [75]. A recent study proposed a simple deposition model that incorporated surface roughness measurements garnered from AFM into the DLVO model to better describe initial adhesion [189]. It is clear that the microbiology toolkit for examining initial microbial adhesion and biofilm formation on surfaces is changing and becoming more expansive. For future work, such a model could be used to provide further insight into the effect of the surface roughness of *M. barkeri* and the support materials on initial adhesion, which could alter the interpretations garnered from the DLVO model in this study.

It is known that phenotypic and physiological changes in the cell are initiated by the initial attachment of microbial cells to an abiotic surface, which can promote adhesion by the release of EPS or other surface-associated polymers [23,29]. Hydrophobic and low surface free energy materials have been reported to promote an earlier production of surface-associated polymers compared to hydrophilic high surface free energy materials, and is a demonstrable role of hydrophobicity [67]. This finding could explain the observed polymeric material on the surfaces of PVC and PTFE in the SEM images (Fig. 3.6). However, further investigation would be needed to identify the role of the abiotic surface in promoting distinct changes to the cell surface during biofilm formation, and the role of these cell surface changes for irreversible microbial attachment. This will be examined in further detail in the next chapter.

# 3.5. Conclusions

This study highlights the important role that the surface characteristics of support materials have in influencing microbial adhesion. *M. barkeri* was shown to exhibit different abilities to attach to the six support materials, with the type of material strongly influencing the extent of attachment (p < 0.05).

The results from the xDLVO model and the initial adhesion experiments were in close agreement with each other during the 2 h timeframe tested in this study for initial adhesion. This highlights the potential of using the xDLVO model to rapidly select the most suitable support materials for the selective immobilization of *M*. *barkeri,* which could be applicable for a *Methanosarcina-*based AD reactor design.

The findings from this study will not only provide a framework in which to better describe and understand the initial adhesion and biofilm formation of *M. barkeri*, but could also aid in the selection of support materials for the targeted immobilization of key *Methanosarcina* species to enhance the start-up of AD reactors.

## Chapter 4

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##### Influence of the support material on the cell surface properties of *Methanosarcina barkeri*

# 4.1. Introduction

The last chapter revealed that the physicochemical surface properties of the support materials elicited different initial adhesion behaviour from *M. barkeri*. Previous studies have also shown the nature of the abiotic surface to cause different patterns of adhesion from methanogens, as well as influencing the composition of adherent microorganisms in mixed biofilms from environmental samples [42,94,96,126]. This suggests that the abiotic surface is an important environmental factor in triggering different microbial responses to facilitate biofilm formation on abiotic surfaces [91].

The abundance of bacterial biofilm studies in the literature has provided valuable insight into some of the influential physiological changes that occur in the cellular transition to a biofilm lifestyle, such as substantial changes to the cell surface [85,92,190]. It is well known that the microbial outer cell surface forges direct contact with the external environment, and that it plays a crucial role in sensing and responding to environmental factors to mediate irreversible adhesion and mature biofilm formation [101]. Previous studies have established that changes to the cell surface are important during biofilm formation for various microbial systems [89,91,92,133]. The cell surface of *Methanosarcina* is also known to be involved in adhesion [19,53], yet the question of how the cell surface responds to an abiotic surface to adapt cells for a more irreversible attachment is not well understood.

It is already established that initial adhesion is mediated by various physicochemical surface interactions, influenced by surface hydrophobicity, surface charge and surface free energy [63]. The progression towards a more irreversibly attached biofilm is mediated by more biological factors, such as the production of EPS [96] or significant compositional changes to the cell surface to mediate biofilm formation [89]. Whereas the DLVO model can provide a means of describing initial adhesion in terms of the underlying physicochemical interactions at the microbe-abiotic interface, it does not take into account the effect of cell surface macromolecules on adhesion [79]. As microbial cell surfaces are naturally heterogeneous in this respect, this is a large limitation of the DLVO model [63]. Providing a more comprehensive description of biofilm formation also requires understanding of the non-DLVO factors involved in adhesion. Therefore, understanding the role of the microbial cell surface in helping cells attach better to abiotic surfaces is important.

Important modifications to the cell surface during biofilm formation have been characterised by combining X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared spectroscopy (FTIR), zeta potential analysis, and fluorescently staining cell surface components [89,92,102]. Mukherjee *et al.* (2012) used FTIR, zeta potential measurements, XPS, and proteomics to reveal that an increase in surface polysaccharide production and a decreased surface hydrophobicity and surface charge was important for biofilm formation in *Bacillus cereus*. The increased production of surface-exposed proteins and in surface hydrophobicity was also identified for *Escherichia coli* during biofilm formation [89]. Koerdt *et al.* (2011) also used FTIR and XPS to reveal that the overexpression of surface carbohydrate functional groups and surface polysaccharide moieties were important for *S. acidocaldarius* and *S. solfataricus* during biofilm formation [89].

It is understood that cells of *Methanosarcina* are typically enveloped by an external heteropolysaccharide matrix consisting of methanochondroitin, whichis responsible for facilitating cell-cell and cell-abiotic surface adhesion [19,53]. The production of this exopolysaccharide layer is regulated by the cell in response to the external environment and growth conditions [61]. Therefore, this chapter aims to examine whether important cell surface changes are involved during biofilm formation in *M. barkeri* upon adhesion to different support materials with a special interest in the production of the exopolysaccharide layer in response to attachment to a good biofilm carrier (PVC) compared to a poor biofilm carrier (PETG). A combination of spectroscopic and microscopic approaches were used to explain the role of cell surface modifications in the irreversible adhesion of *M. barkeri* to PVC and PETG. Fully characterising the cell surface of *M. barkeri* during biofilm formation provides an insight into whether different mechanisms for irreversible adhesion are observed in *M. barkeri* as a function of support material, and to provide better understanding of the mechanisms involved in biofilm formation.

# 4.2. Materials and Methods

###### 4.2.1. Support material preparation

Two different polymer support materials were tested in this study based on their previous performance in chapter 3 for selectively immobilising *M. barkeri:* PVC as a model representative of a good biofilm support carrier, and PETG as a model representative of a poor biofilm support carrier. Each polymer material was cut into 1 x 4 cm length coupons for each experimental approach.

The coupons were cleaned as described previously (section 3.2.4), with 70 % ethanol and 2% (v/v) PCC-54 detergent solution. Rinsed coupons were dried inside a laminar flow cabinet and further UV-sterilised for 3 h, and stored in sterile tubes within an anaerobic chamber for at least 48 h before use.

###### 4.2.2. Batch culture setup and biofilm sampling

*M. barkeri* was grown in DSM 120 medium from a fridge stock culture and incubated at 37˚C, with growth continuously monitored by OD600. This was regarded as the parent culture.

For biofilm sampling, twenty 125 ml serum bottles containing 50 ml of freshly reduced media were inoculated with mid-exponential phase cells (96 h growth) from the parent culture with an adjusted starting OD600 of ~0.05. Each serum bottle contained four coupons of the same support material. The coupons from these twenty cultures were pooled together and regarded as a biological replicate in order to accrue enough biofilm biomass for further analysis. The decision to pool together twenty cultures was determined from a preliminary experiment that aimed to estimate the amount of cellular biomass from five, ten and twenty pooled cultures. It was found that the amount of cellular biomass obtained from twenty pooled cultures was sufficient for further analysis, and this number of cultures was used for the rest of this experiment.

Sterile coupons from each biofilm experimental condition (PVC or PETG) were added to media bottles on the day of experimentation within an anaerobic chamber. Four biological replicates were established for each experimental approach.

Due to the high numbers of cultures involved for each biofilm experimental condition, it was not feasible to perform this whole experiment in one run. Therefore, this experimental setup was staggered over a duration of 8 days. Duplicate cultures of *M. barkeri* for each experimental condition (PVC versus PETG) were grown from the same parent culture on days 1, 2, 7 and 8 to allow a biological replicate of each experimental condition to be set up over staggered days for easier handling, as shown in Figure 4.1.



**Fig. 4.1** Schematic of the batch setup for growth of biofilms on support material.

These staggered *M. barkeri* parent cultures were all started from the same stock culture. Their growth curves were replicated and are shown in Fig. 4.2. They were not significantly different (p > 0.05, one-way ANOVA), justifying the decision to regard these staggered cultures as the same parent culture across all biological replicates and experimental days.

Once inoculated, the experimental cultures were incubated at 37°C and gently shaken at 110 rpm for 96 h to allow biofilm formation on the coupons. This time point was chosen to coincide with the later time point used in the biofilm assay in the previous chapter. This biofilm setup was carried out individually for each experimental approach and repeated twice.



**Fig. 4.2** Growth curves of the four parent cultures over 96 h in the biofilm setup, with each parent culture staggered over 8 days from the same stock culture.

###### 4.2.3. Fourier-transform infrared spectroscopy (FTIR) analysis

After 96 h of growth, a biological replicate from each biofilm experimental condition (corresponding to twenty pooled cultures and eighty coupons per biological replicate) were opened within an anaerobic chamber. Coupons with attached biofilm were carefully and aseptically removed from all culture bottles using sterile tweezers. The planktonic cell suspension was discarded and coupons from each biological replicate were twice washed with sterile anaerobically-prepared 100 mM KCl solution within the anaerobic chamber, and then placed into a sterile container containing 80 ml of 100 mM KCl. Cells were removed from coupons by vigorous manual shaking for 2 minutes.

The detached biofilm cell suspension was transferred into sterile glass centrifuge tubes. Biofilm cells were washed with sterile 100 mM KCl and harvested by centrifugation at 6000 x g for 10 minutes at 4˚C outside the anaerobic chamber.

Thereafter, 5 μl of the cell pellets from the both the PETG and PVC biofilm conditions were pipetted onto the diamond crystal of the attenuated total reflectance (ATR) attachment of the Fourier-transform infrared spectrophotometer (IR-Prestige 21, Shimadzu, UK), and left to air dry for 45 minutes. At least 64 scans were made on each sample using the Happ-Genzel apodisation function with a resolution of 4 cm-1. The FTIR spectrum was analysed over a wavenumber range of 600 to 4000, but manual analysis of the spectra was limited to a wavenumber range of 800 to 1800, as this range correlates to characteristic absorbance peaks from macromolecules of biological origin [103].

Spectral processing was carried out using IR Solution software (Shimadzu, UK) where FTIR spectra was atmosphere and baseline-corrected to remove any interfering background noise from the spectra, as well as normalised to the intensity of a peak at 2930 cm-1 to ensure that any differences in the amount of cells loaded onto the ATR were accounted for. This peak absorbance is attributed to the asymmetric stretching of C-H groups [103] and is present in all microorganisms [109].

Principal component analysis (PCA) was carried out using XLSTAT 2014.4 software using the Pearson correlation.

###### 4.2.4. X-ray photoelectron spectroscopy (XPS)

As described previously, biofilm cells were removed from coupons after 96 h of growth by vigorous manual shaking for 2 minutes. The detached biofilm cell suspension was transferred into sterile glass centrifuge tubes and washed with sterile Milli-Q water and harvested by centrifugation at 6000 x g for 10 mins at 4˚C, before transferring washed cells into sterile 2 ml microcentrifuge tubes (Eppendorf, Germany).

Using the flame of a Bunsen burner to create a sterile field to prevent contamination, parafilm tape (Sigma Aldrich, UK) was used to cover the opening of microcentrifuge tubes containing washed cell pellets, and a sterile syringe needle was used to pierce a hole within the parafilm tape to allow dehydration of samples. Samples were dehydrated by freeze drying for 24 h before XPS analysis.

XPS analysis was carried out using an AXIS Ultra DLD Photoelectron spectrometer (Kratos Analytical, UK). Survey scans were collected between 1200 to 0 eV binding energy (160 eV pass energy; 0.5 eV intervals). High-resolution scans for C 1*s*, N 1*s*, and O 1*s* were collected over an appropriate energy range (20 eV pass energy; 0.05 eV intervals), with spectra decomposed into their respective elemental components to identify the functional groups present on the cell surface.

The carbon peak (C 1s) was analysed at high resolution and was best fit with four components: a component due to carbon bound only to carbon and hydrogen, C-(C,H), at 285 eV; a component due to carbon singly bound to oxygen or nitrogen, such as from ethers, alcohols, amines, and/or amides, C-(O,N), at 286.5 eV; a component at 288.2 eV corresponding to carbon doubly bonded to oxygen or singly bonded to two oxygen atoms from amides, carbonyls, carboxylates, esters, acetals, and/or hemiacetals, C=O, O-C-O; and a weak component due to carboxyl activity, COOR, at 289.8 eV.

The oxygen peak (O 1s) at high resolution was also best fit with two components: a component at 531.6 eV due to carbon sharing a double bond with oxygen in carboxylic acid, carboxylates, esters, carbonyls or amide (C=O); and a component due to attributable to hydroxide, acetal, or hemiacetal (C-OH, C-O-C) at 533 eV. Nitrogen was apparent at a binding energy of 398 eV, and arises mainly as a result of the amine or amide groups in proteins [102].

Three analyses were recorded per sample with an analysis area of 700 µm x 300 µm. The data was calibrated for binding energy by using the C 1*s* main carbon peak at 285 eV, and correcting all data for each sample analysis accordingly. XPS spectra peaks were fitted using the Casa XPS 2.3.1250 software.

###### 4.2.5. Zeta potential analysis

The EPM of *M. barkeri* was measured using phase amplitude light scattering in 100 mM KCl solution at a pH of 2, 4, 6, 7, 8 and 10, with the pH adjusted with potassium hydroxide (KOH) and hydrochloric acid (HCl). The EPM of cells at each different pH was measured, using the same methods described previously in section 3.2.5.3.

###### 4.2.6. Multiple fluorescent staining of cell surface components

After 96 h of growth, biofilm cells on PVC and PETG were collected and harvested as described previously, by rinsing with anaerobically-prepared PBS and harvested by centrifugation at 6000 x g for 5 minutes. The supernatant was removed from harvested cells. Cells were resuspended in 1 ml of PBS and aliquoted into sterile microcentrifuge tubes, before adding 4% (w/v) paraformaldehyde solution to fix cells for 15 minutes inside an anaerobic chamber. Thereafter, fixed cells were harvested by centrifugation at 6000 x g for 5 minutes and the fixing agent supernatant was removed. 1 ml of PBS was added in order to rinse cells of fixing agent, before further centrifugation to harvest cells.

Fluorescent staining of harvested biofilm cells was carried out within foil-wrapped microcentrifuge tubes [140] in order to determine the amount of polysaccharides and proteins on the cell surface. Following the staining methodology used by Fish *et al.* (2015) [138], a triple fluorophore combination of DAPI, fluorescein-5-isothiocyanate (FITC) and concanavalin A lectin conjugated with rhodamine (ConA-Rho) was used to detect cellular DNA, amines and amino-sugars, and extracellular α-mannopyranosyl and α -glucopyranosyl sugar residues [57], with staining carried out in this order.

A volume of 1 ml of 1 µg/ml of DAPI was used to stain samples for 10 minutes at room temperature in the dark, followed by staining with 1 ml of 100 µg/ml of FITC for 1 h at room temperature in the dark, and 1 ml of 100 µg/ml of ConA-Rho for 30 minutes at room temperature in the dark. An intermediate washing step with Milli-Q water followed each staining stage. Standards on glass coverslips (Sigma-Aldrich, UK) were established prior to experimental analysis to confirm the binding specificity of the chosen fluorophores, by immersing coverslips into sterile 50 ml Falcon tubes containing 1 µg/ml of bovine serum albumin (to model proteins) and glucose solution (to model polysaccharides) for 1 h (Sigma-Aldrich, UK). Thereafter, glass coverslips were removed from their standard solutions and left to air dry within a laminar flow hood, before staining with DAPI, ConA-Rho and FITC fluorophores as described previously.

After staining, cells were centrifuged at 6000 x g for 5 minutes, the supernatant removed and cells resuspended in PBS. Immediately before imaging on epifluorescence microscopy, 10 µl of cell suspension from the biofilm and planktonic samples were pipetted onto clean glass microscope slides and covered with a glass coverslip (Sigma-Aldrich, UK) for microscopic viewing.

Acquisition was facilitated with a Leica AF6000 inverted microscope attached to a computer with a magnification of 100 in order to obtain a representative surface coverage. The fluorophores were visualised on three channels corresponding to excitation and emission wavelengths of 358/461 nm (DAPI), 488/520 nm (FITC) and 543/580 nm (ConA-Rho). Five microscopic fields (1 mm2 per field) were randomly selected for each sample and measured as percentage of surface area coverage. Images were analysed with the ImageJ software in ‘Analyse Particles’ mode to calculate the average percentage of area covered by cells after 96 h.

###### 4.2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism. The Brown-Forsythe test was used to determine statistical differences between group variances. A one-way ANOVA with Tukey’s *post-hoc* test was used to compare the growth curves between the staggered parent cultures, and an unpaired student t-test to compare the surface chemical information obtained from XPS and fluorescent staining results for both biofilm samples.

# 4.3. Results

###### 4.3.1. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectra was used to characterise the dominant functional groups found on the cell surface of *M. barkeri* between 800 and 1800 cm-1 when grown as a biofilm attached to either PVC or PETG.

The normalised FTIR spectra for PVC and PETG-biofilm cells showed identical dominant surface functional groups (Fig. 4.3a). In particular, there were major peak intensities of absorption bands in the spectral regions correlating to both polysaccharides and the phosphate groups of nucleic acids (1200-900cm-1), amide III components of proteins (1310-1240 cm-1), and amide I (C=O) and amide II components (N-H) (1700-1500 cm-1) [103].

A comparison of the FTIR spectra of both biofilm samples (Fig. 4.3a) reveals an increase in the intensity of absorption bands corresponding to polysaccharide functional groups (1200-900 cm-1) and protein functional groups (1700-1500 cm-1) in PVC-biofilm cells compared to PETG-biofilm cells.

Previous studies have combined FTIR with PCA analysis to differentiate between cells in different stages of growth and lifestyle, showing the potential and relevance of this method in biofilm studies [89,92]. Cluster analysis using PCA analysis was used to identify any variations between the two biofilm samples. PCA analysis identified distinct clustering of both experimental conditions from each other (Fig. 4.3b). These results suggest that there are distinct cell surface differences between the two biofilm populations.

**a)**



**b)**



**Fig. 4.3** Comparison between a) the FTIR spectra of PETG-biofilm (blue line) and PVC-biofilm cells (red line) of *M. barkeri,* and b) the principal component analysis of FTIR spectra of biofilms formed on PETG and PVC.

###### 4.3.2. X-ray photoelectron spectroscopy (XPS) analysis

XPS analysis was used to analyse the cell surface chemical composition of *M. barkeri* when grown as a biofilm attached to either PVC or PETG, and a summary of the results are presented in Table 4.1.

**Table 4.1** Quantification of the elemental surface composition of *M. barkeri* grown as a biofilm attached to either PVC or PETG. Errors represent standard deviation of two independent experiments.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **Biofilm** | |
| **Element** | **Assignment** | **PVC**  **(atomic conc. %)** | **PETG**  **(atomic conc. %)** |
| Total C |  | 64.1 ± 1.4 | 64.8 ± 2.4 |
| Total O |  | 25.7 ± 1.2 | 21.6 ± 3.1 |
| Total N |  | 6.9 ± 0.3 | 7.5 ± 1.8 |
| C1s | C-(C,H) | 38.9 ± 3.6 | 44.8 ± 6.6 |
| C1s | C-(O,N) | 37.9 ± 2.2 | 34.3 ± 4.4 |
| C1s | C=O, O-C-O | 18.9 ± 1.0 | 20.9 ± 2.5 |
| C1s | COOR | 4.2 ± 0.5 | 7.6 ± 1.8 |
| O1s | C=O | 40.4 ± 5.9 | 51.05 ± 10.28 |
| O1s | C-OH, C-O-C | 66.3 ± 11.2 | 48.95 ± 10.28 |
| O/C |  | 40.0 | 33.3 |
| N/C |  | 10.8 | 11.5 |

A wide survey scan revealed the dominance of carbon (C), nitrogen (N) and oxygen (O) on the cell surface of each experimental condition at a 2-5 nm penetration depth. The amount of C, N and O on the cell surface of each biofilm condition was quantified as a

function of N/C and O/C atomic concentration ratios, to identify the proportion of carbon linked to nitrogen or oxygen atoms, respectively (Table 4.1). Each biofilm condition had an abundance of O/C linkages on the cell surface, suggesting the presence of polysaccharide moieties [89,109]. However, PETG-biofilm cells had significantly lower concentrations of oxygen atoms on their cell surface compared to the PVC-biofilm cells (p < 0.05, unpaired t-test), suggesting lower levels of cell surface polysaccharide moieties.

A comparison of the atomic concentration ratios of oxygen doubly bonded to carbon (O=C/C), and carbon doubly bonded to oxygen (C=O/C) as a function of N/C of both biofilm samples showed an abundance of C=O and O=C functional groups with respect to amides (N/C). These suggest an excess of carbonyl groups (C=O) on the cell surface that may arise from polysaccharide moieties, as a 1:1 ratio is not observed between these two parameters to signify a proteinaceous surface [111].

The N/C values provide an estimate of the level of protein moieties on the cell surface [101,109]. In this study, there were no significant differences in the N/C values between the two biofilm communities (p > 0.05, unpaired t-test), suggesting that the abundance of protein moieties on the cell surface of biofilms attached to both PVC and PETG were similar.

The molecular composition of the cell surface could also be calculated by further analysing the XPS spectra. Rouxhet *et al.* (1994) developed a simple approach to calculate the molecular composition of a cell surface based on the assumption that biological cell surfaces consisted of three model components that only changed in a narrow range: polysaccharides, proteins and hydrocarbon-like compounds [111]. These basic cell surface macromolecules can be quantitatively determined from the measured atomic concentrations of carbon, nitrogen and oxygen [102].

This approach is based on comparing the measured atomic concentration ratios of O/C and N/C of samples to the known ratios of model compounds for polysaccharides, hydrocarbon-like compounds and proteins. These model compounds consist of glucan to model polysaccharides (O/C = 0.833, N/C = 0.000, carbon atomic concentration = 37.0 mmol/g), (CH2)n for hydrocarbon-like compounds (O/C = 0.000, N/C = 0.000, C-(CH)/C = 1.00, carbon atomic concentration = 71.4 mmol/g), and the amino acid composition of the major outer membrane of *Pseudomonas fluorescens* for proteins (O/C = 0.325, N/C = 0.279, carbon atomic concentration = 43.5 mmol/g)[111].

Therefore, three equations can be solved to give the proportions of carbon linked to each molecular cell surface component [102,111]:

O/C = 0.325 (CPEP/C) + 0.833 (CPS/C) (Equation 1)

N/C = 0.279 (CPEP/C) (Equation 2)

1 = (CPEP/C) + (CPS/C) + (CHC/C) (Equation 3)

where O/C and N/C are the measured atomic concentration ratios of oxygen and nitrogen, with respect to carbon in the experimental sample; CPEP, CPS, and CHC are the atomic concentrations of carbon present in proteins, polysaccharides, and hydrocarbon-like products.

There were no significant differences in the surface-associated concentrations of proteins or hydrocarbon-like compounds between PVC and PETG-biofilm samples (p > 0.05, unpaired t-test) (Fig. 4.4). However, the PVC-biofilm cells possessed a significantly higher amount of polysaccharides on the cell surface compared to PETG-biofilm cells (p < 0.05, unpaired t-test), as evidenced by a larger weight fraction of polysaccharides in the biofilm (0.33±0.03) compared to PETG-biofilms (0.25±0.04).



**Fig. 4.4** Quantification of proteins, polysaccharides and hydrocarbon-like compounds on the cell surface of *M. barkeri* grown as a biofilm on PVC or PETG, as calculated from XPS spectral data. Errors represent standard deviation of two independent experiments.

###### 4.3.3. Zeta potential analysis

The zeta potential is a useful indicator of the cell chemistry of a microbial cell, and arises due to the presence of anionic and cationic functional groups on the surface [72]. The pH of the external environment controls the ionisation of surface functional groups, and an important surface parameter is the isoelectric point (IEP), or the pH at which the zeta potential is zero [183]. This is a balance between the charging of anionic and cationic functional groups on the surface. The IEP can provide information on the chemical moieties and composition of the cell surface [183]. Therefore, the EPM of biofilm cells was measured over a pH range of 2-10 and converted to zeta potentials using the Smoluchowski equation to determine the IEP (Fig. 4.5).



**Fig. 4.5** The zeta potentials (mV) of *M. barkeri* grown as a biofilm on either PVC (blue line) or PETG (red line) as a function of pH, at pH 2, 4, 6, 7, 8 and 10. Error bars represent the standard deviation of two independent experiments.

The transition from a basic pH to an acidic pH range incurred a decline (less negative) in the electronegativity of the cell surface, which is a typical characteristic of microbial cells [183]. Upon biofilm formation at pH 7, the zeta potential of biofilms attached to PVC and PETG were -22.9 mV and -22.1 mV, respectively, which point to very subtle differences in the cell surface chemistry of both biofilms.

It was not possible to determine the IEP of biofilms attached to PVC and PETG in the tested pH range, showing that biofilm cells possessed a strong net negative charge. The EPM was not measured for cells below the lower limit of pH 2 for risk of losing the integrity of the cell surface.

It can be presumed from Fig. 4.5 that the IEP of both PVC and PETG biofilms would have been less than pH 2. According to Rijnaarts *et al.* (1995), an IEP of less than 2.8 indicates a significant amount of anionic polysaccharides containing negatively charged carboxyl and phosphate groups at the cell surface, which have pKa values of less than 2.8 [177]. The deprotonation of carboxyl and phosphate groups associated with cell surface polysaccharides may contribute to the highly negative zeta potentials of biofilms attached to PVC and PETG at pH 7.

###### 4.3.4. Epifluorescence microscopy of cell surface components

Biofilms attached to either PVC or PETGwere triple-stained with DAPI, FITC and ConA-Rho in order to determine the amount of surface polysaccharides and proteins after 96 h. Cells from both experimental conditions were washed and centrifuged to ensure the removal of loosely-bound EPS for the analysis of the cell surface [92,191], and to ensure that results were comparable with those from the cell surface-sensitive methods of XPS and FTIR.

The amine-reactive stain, FITC was not successful in the identification of surface-associated proteins, and instead stained proteins throughout the whole cell. This has been observed in previous studies [140]. As a result, this stain was not quantified. DAPI was able to stain cellular DNA throughout the cell, whereas ConA-Rho was successfully able to distinguish surface-associated polysaccharides from cells.

The levels of *M. barkeri* cells and exopolysaccharides were quantified as the percentage of cells and glucose and mannose sugar residues covering the surface of a 1 mm2 microscopic field, as described previously in section 3.2.9.3. The results show that there were significant differences in the surface coverage of DAPI-stained cells between both biofilm samples (p < 0.05, two-tailed t-test), with PVC-biofilm cells having the highest cellular biomass after 96 h (10.4% surface coverage) (Fig. 4.6a), as also found in chapter 3.

The detection of higher levels of extracellular α-mannopyranosyl and α -glucopyranosyl sugar residues (1.9% surface coverage) was also observed for PVC-biofilm cells compared to lower levels in PETG-biofilm cells (0.2% surface coverage) after 96 h (p < 0.05, two-tailed t-test) (Fig. 4.6b).

**Fig. 4.6.** Quantification of cellular DNA and surface-associated α-mannopyranosyl and α -glucopyranosyl sugar residues by fluorescently staining 96 h cultures of *M. barkeri* with a) DAPI and b) ConA-Rho respectively, when grown as a biofilm on PVC and PETG. Error bars represent standard deviation of two independent experiments.

# 4.4. Discussion

It is becoming clear that abiotic surfaces are important environmental factors that can promote differential patterns of adhesion by acting as indicators of favourable conditions for biofilm growth and development [22]. Therefore, abiotic surfaces can signal for cellular changes to mediate the spatial reorganisation of the cell surface to allow microbial cells to irreversibly attach to an abiotic surface [192,193]. In spite of this, little work has been done to elucidate whether cell surface modifications are involved in the irreversible adhesion of *M. barkeri* to abiotic surfaces. This study aimed to shed light on this question to provide a framework in which to better understand biofilm formation in this methanogen.

A multifaceted approach using FTIR, XPS, and fluorescent microscopy analysis revealed significantly higher levels of polysaccharides on the cell surface of biofilm cells attached to PVC compared to biofilms attached to PETG (Figs. 4.3, 4.4, 4.6). FTIR and XPS analysis suggested that there were distinct changes in the chemical composition of the cell surface of biofilm cells as a response to the support material. FTIR spectral data showed that surface polysaccharides may have played a key role in the establishment of biofilms of *M. barkeri* on PVC after 96 h, producing a surface profile that was significantly different to that of PETG-biofilm cells (Fig. 4.3). This spectral region (1200-900cm-1) also overlaps with the spectral region of nucleic acids [103] and it is possible that eDNA plays a role in the development of biofilm formation in *M. barkeri.* Past studies on bacterial biofilm formation have shown that eDNA is a significant factor for microbial attachment and biofilm formation [193]. It has also been detected in the EPS from *Sulfolobus* species and haloarchaeal strains during the initial stages of biofilm formation [20]. However, the mechanisms underlying the role of eDNA in archaeal biofilms is still not well understood [20].

As such, the surface-sensitive methods used throughout this chapter were especially selected to focus on the production of exopolysaccharides from *M. barkeri* during biofilm formation owing to the key role of this surface component in cell-surface adhesion [19]. Therefore, the role of polysaccharides was only considered in the examination of the cell surface during biofilm formation on PVC and PETG in this chapter.

These findings were further supported by XPS spectral data, which similarly suggested that biofilms attached to PVC had increased levels of surface polysaccharides during biofilm formation (p < 0.05), as evidenced by a larger weight fraction of polysaccharides in the biofilm (0.33±0.03) compared to PETG (0.25±0.04) (Table 4.1; Fig. 4.4). These results also corresponded with those from the fluorescent staining analysis, which again detected significantly higher levels (p < 0.05) of the major EPS components, glucose and mannose sugar residues, in biofilms attached to PVC (1.9% surface coverage) compared to PETG (0.2% surface coverage) (Fig. 4.6).

This suggests that the surface of PVC provided a more favourable surface for microbial attachment as shown by the higher levels of DAPI stained cells in this study (Fig. 4.6a). It can be hypothesised that the higher levels of cell attachment were associated with an increased production of polysaccharides to perhaps aid in a more irreversible attachment of cells to PVC.

Zeta potential analysis was able to identify the nature of these polysaccharides in thebiofilms. As the IEP of both biofilms from PVC and PETG were presumed to be less than 2, this implies that biofilms possessed a cell surface dominated by negatively charged polysaccharides associated with carboxyl and phosphate groups [183]. This conclusion is also supported by the ConA-Rho staining, which detects only negatively charged mannose and glucose sugar residues. These sugar residues are known to be major polysaccharide components of EPS in archaea [57], and their increased levels in PVC-biofilms suggests a biofilm-specific adaptation.

These observed changes in cell surface properties in response to environmental factors have been reported in previous studies, and often in comparison to planktonic cells. For example, the cell surface of biofilm cells of *B. cereus* was found to be more hydrophilic and had increased levels of polysaccharides compared to their planktonic counterparts [92]. Similarly, biofilms of *S. acidocaldarius*, *S. solfataricus* and *S. tokodaii* produced significantly different surface profiles compared to their planktonic counterparts, having increased levels of polysaccharide moieties on the cell surface [64]. Phenotypic and physiological differences are commonly observed between biofilm and planktonic cells owing to the adhesive requirements of the biofilm [22]. This study has also shown that different phenotypic and physiological differences can exist between biofilm populations attached to different abiotic surfaces, owing to the different adhesive requirements from an abiotic surface. This is testament to the ability of microorganisms to readily adapt to changing environments, such as growth on PVC or PETG, and highlights the importance of the abiotic surfaceas a key environmental factor for biofilm formation.

It is feasible that biofilm formation in *M. barkeri* may proceed in a similar fashion to bacteria, in which the initial attachment of cells to a surface induces key cell surface changes, such as the production of exopolysaccharides to facilitate irreversible attachment [22,85]. Indeed, a mechanism of biological adaptation to abiotic surfaces is the production of EPS in response to attachment to a surface, and this is well known [23]. Exopolysaccharides are known to interact with themselves, multivalent cations, cell surface proteins and glycoproteins to produce a biological layer that interacts with the external environment [85], and forms an important physiological response to a surface-associated lifestyle [22,194]. Fluorescent staining of the major EPS components, mannose and glucose residues, with ConA, have shown the production of these sugar residues in *S. acidocaldarius*, *S. solfataricus* and *S. tokodaii* [64], and in the methanogens, *M. stadtmanae* and *M. smithii* [57] after initial attachment to a surface.

Therefore, it is feasible that the preferential attachment of *M. barkeri* to PVC in this study triggers an early production of surface polysaccharides for irreversible cell attachment, which could be regarded as an adaptive response for biofilm development on PVC.

However, the poor microbial attachment to PETG could be associated with a lag phase in polysaccharide production, as observed in this study (Figs 4.3, 4.4, 4.6). As PETG elicits slower biofilm development from *M. barkeri*, which was determined from chapter 3 as being a result of its electrostatically repulsive surface properties, a less developed biofilm is expected compared to PVC. As such, cell attachment to PETG is not expected to trigger the same programme of cell surface changes as observed in cells attached to PVC [22,62].

This study has compared the two different biofilms of *M. barkeri* after 96 h on two different support materials. A valuable validation of whether the production of exopolysaccharides from *M. barkeri* on PVC during this timeframe was a result of preferential adhesion from *M. barkeri*, future work could focus on comparing these biofilms with a planktonic culture as control.

# 4.5. Conclusions

A variety of surface characterisation methods were used to assess changes in the cell surface of *M. barkeri* during biofilm formation on PVC and PETG. This study showed that during biofilm formation, *M. barkeri* actively changed the chemical properties of its surface in response to PVC and PETG. Biofilms were shown to increase the levels of polysaccharides on their cell surface in response to attachment to PVC, as evidenced from FTIR, XPS, zeta potential and fluorescence microscopy analysis. The increased levels of surface polysaccharides are likely to be associated with the higher levels of cell adhesion to PVC, and suggest a key role of surface polysaccharides in the irreversible attachment of *M. barkeri* cells during biofilm development.

However, PETG elicited lower levels of cell attachment and surface polysaccharides from *M. barkeri.* This cell surface phenotype could imply a less developed biofilm on PETG compared to PVC. Taken together, the results from this study show that *M. barkeri* is able to sense and readily respond to an abiotic surface. It is clear that the choice of support material not only influences the physicochemical interactions for initial adhesion, but also the cell surface composition of adherent cells.

The biological mechanisms underlying these observable cell surface modifications in *M. barkeri* during biofilm formation are not well understood, and further work is needed to address this question. This information would provide a much more comprehensive framework in which to better understand the effect of the support material on the biofilm-forming capabilities of *M. barkeri,* and could guide the selection of appropriate support materials for the immobilisation of *Methanosarcina* within AD reactors. Therefore, the next chapter examines these biological mechanisms for biofilm development in more detail by investigating the proteomic profiles of *M. barkeri* biofilms upon attachment to PVC and PETG.

## Chapter 5

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##### Influence of a biofilm lifestyle on the functional proteome of *Methanosarcina barkeri* as a function of support material

# 5.1. Introduction

Distinct adaptive differences in the cell adhesion and cell surface characteristics between biofilms of *M. barkeri* to PVC and PETG have been established in the previous chapters. The different physicochemical properties of PVC and PETG have been influential in the process of initial adhesion of *M. barkeri* to these surfaces, and have elicited distinctly different phenotypical characteristics and biofilm behaviour from *M. barkeri.* It is apparent that the nature of the support material is an important environmental factor that can promote unique metabolic and physiological changes during biofilm formation, and that *M. barkeri* is able to carry out abiotic surface sensing*.* Therefore, an examination of the biological mechanisms that are occurring whilst *M. barkeri* is adapting to a biofilm lifestyle on PVC or PETG is desirable in order to provide a comprehensive understanding of the biological factors underlying biofilm formation in this methanogen.

Biofilm research reached a pinnacle with the advent of proteomic analysis, and it is now possible to determine all the expressed proteins of a cell, or the functional proteome, in order to understand cellular behaviour in different environmental conditions [142]. Quantitative proteomics has proven to be a powerful and potent tool that has provided insight into the inner workings of a wide range of biological matter [144]. Since its inception in 2004 [195], quantitative proteomics based on isobaric tags for relative and absolute quantification (iTRAQ) has become extremely popular with researchers owing to its ease of use and ability to quantify multiple protein samples simultaneously [159].

iTRAQ has been used in previous studies to quantitatively uncover the complete protein expression and therefore, the adaptive responses of *M. barkeri* and *Methanococcoides burtonii* to different growth temperatures [161,162], as well as the adaptation of planktonic cells of *S. acidocaldarius, S. solfataricus,* and *S. tokodaii* to a biofilm lifestyle [89]. However, to date, there has not been a proteomic study of the biofilm-forming behaviour of *M. barkeri* on different support materials. This information would be extremely useful for fixed biofilm-based biotechnological applications, such as AD, and especially with regards to a transition towards a more *Methanosarcina*-­basedAD system. Such information would greatly enhance understanding of biofilm formation from this key methanogen on different support materials.

The aim of this study was to examine the whole suite of proteins expressed by *M. barkeri* after 96 h of growth on either PVC or PETG to assess whether the nature of the support material had a significant effect on the functional proteome. As with the previous chapter, these two support materials were chosen based on their previous performance as good and poor biofilm carriers for *M. barkeri,* respectively. Since these two support materials had elicited distinct physiological and adhesive responses from *M. barkeri* within this time frame in the previous chapters, this study sought to obtain a more global and integrated biological view of biofilm formation from *M. barkeri* as a function of support material.

# 5.2. Materials and Methods

###### 5.2.1. Support material preparation

As in chapter 3, two different polymer support materials were used based on their previous performance for selectively immobilising *M. barkeri:* PVC to represent a good biocompatible surface for adhesion, and PETG to represent a poorly biocompatible surface. Each polymer material was cut into 1 x 4 cm length coupons for each biofilm formation experiment. The plastic coupons were cleaned and sterilised as described previously in section 3.2.4.

###### 5.2.2. Biofilm accumulation experiment

A static biofilm experiment based on 24 well plates was carried out, as described previously in section 3.2.8. Biofilms were established on coupons of PVC and PETG in an anaerobic chamber in basal media over a duration of 6 days, with sampling times at 2, 4 and 6 days. Four replicates were established for each plastic at each sampling time point, and at time of sampling, coupons were rinsed and fixed in 4% paraformaldehyde solution as described before.

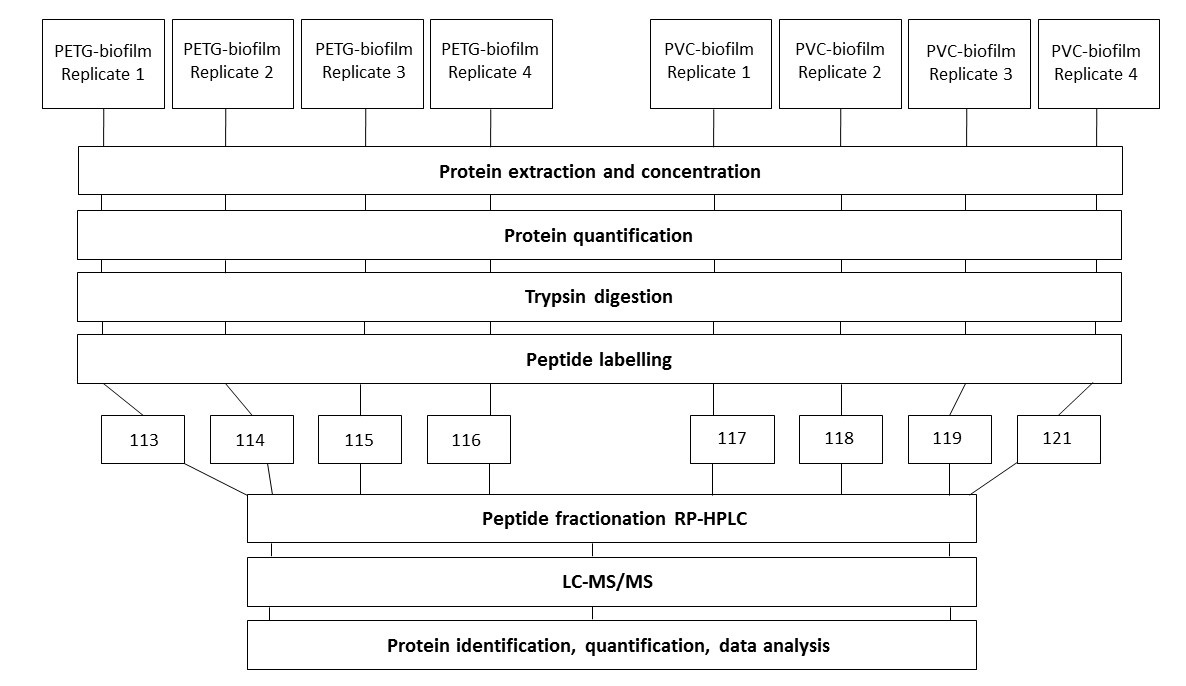
Fixed coupons were fluorescently stained with DAPI (please refer to 3.2.9.3) and ConA-Rho (please refer to 4.2.6) before biofilms were visualised with epifluorescence microscopy as described in 4.2.6. Five randomly assigned microscopic fields were chosen (1 mm2 per field) and biofilm coverage was measured as the percentage of coupon area covered by DAPI and ConA-Rho-stained cells and mannose and glucose sugar residues, respectively. Images were analysed with ImageJ software as described in 4.2.6. Results are based on three independent experiments.

###### 5.2.3. Batch culture setup and biofilm sampling for proteomics

*M. barkeri* was grown in DSM 120 medium from a stock culture and incubated at 37˚C, with growth continuously monitored by optical density measurements at 600 nm (OD600). Biofilms of *M. barkeri* on PVC and PETG were established using the exact same setup as described in section 4.2.2. Four biological replicates were obtained for PVC and PETG.

###### 5.2.4. Protein extraction and concentration

After 96 h of growth, two biological replicates (corresponding to twenty pooled cultures and eighty coupons per biological replicate) were handled at any one time, and processed within an anaerobic chamber. The workflow for proteomics analysis can be seen in Fig. 5.1. Cells of *M. barkeri* attached to coupons were carefully and aseptically removed from all culture bottles using sterile tweezers. The planktonic cell suspension was discarded and coupons from each biological replicate were thrice washed with sterile anaerobically-prepared PBS within the anaerobic chamber, and then placed into a sterile container (Nalgene, USA) containing 80 ml of freshly prepared lysis buffer on ice.



**Fig. 5.1** Workflow for the iTRAQ-based quantitative proteomics used in this study.

The lysis buffer contained 150 mM sodium chloride (Sigma Aldrich, UK), 15 mM dithiothreitol (DTT) (Sigma Aldrich, UK), 10 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, UK), 1% (w/v) sodium dodecyl sulphate (SDS) (Sigma Aldrich, UK), 0.5% (v/v) NP-40 detergent (Sigma Aldrich, UK) and 1600 μl of a protease inhibitor cocktail (Sigma Aldrich, UK), with all solutions prepared in 200 mM of triethylammonium bicarbonate buffer (TEAB) (Sigma Aldrich, UK). All lysis buffer components were prepared in advance as stock solutions, aliquoted into smaller volumes and stored at -20˚C for future use.

Coupons were sonicated with the lysis buffer in an ice-filled sonication bath (Fisherbrand, UK) within the anaerobic chamber to ensure that the first stages of protein extraction would occur in an anaerobic environment where the cells would not be subjected to any environmental stress that could affect protein expression. Sonication was applied in five rounds of 1 minute pulses to prevent thermal damage to cellular proteins. Thereafter, coupons were transferred to an orbital shaker (Yellow Line, IKA®- Werke GmbH & Co. KG, Germany) and shaken overnight in the lysis buffer for 16 h at 4˚C.

Coupons were removed from the lysate after 16 h. The dilute nature of the lysate from each of the biological replicates called for a method to concentrate the proteins. Therefore, the dilute lysate was concentrated using Vivaspin® 20 ml centrifugal concentrators with a molecular weight cut-off of 3000 Da (Sigma Aldrich, UK) that had previously been centrifuged with 50 mM TEAB buffer to remove any traces of sodium azide and glycerin from the filter membrane. The lysate in the concentrators was centrifuged at 4500 x g for 2 h at 20˚C to ensure optimal concentration of proteins from samples. These centrifugal conditions were performed for all biological replicates unless otherwise stated.

Thereafter, the flow-through was discarded and the centrifugal concentrators were topped up with additional lysate and further centrifuged, until the lysate from each of the four biological replicates was maximally concentrated. The protein concentrate was then desalted in three cycles of centrifugation to remove excess salt and detergents with 50 mM TEAB buffer for optimal concentration.

The protein concentrate was pipetted out from the centrifugal concentrators for each biological replicate, collected in sterile 2 ml Protein LoBind tubes (Eppendorf, Germany) and stored at -20˚C for future use.

###### 5.2.5. Protein quantification

To remove any traces of salt from the detergents in the protein samples, 400 μl of protein concentrate from each biological replicate was incubated with ice-cold acetone (Sigma Aldrich, UK) in a ratio of 4:1 (v/v) at -20˚C for 4 h to allow protein precipitation. Thereafter, samples were centrifuged at 21,000 x g for 20 minutes at 4˚C to collect the precipitated proteins. The supernatants were discarded and the pellets resuspended in 35 μl of 100 mM TEAB buffer.

Protein quantification is necessary for the downstream processes to ensure that equal amounts of protein are present in each sample for gel electrophoresis and to calculate how much trypsin is required for optimal protein digestion and quantitative mass spectrometry. Therefore, protein quantification was carried out using the Lowry method and qualitative verification was determined using SDS-PAGE.

Acetone-precipitated protein samples were first diluted 1:100 in Milli-Q water to ensure that the protein concentrations for all biological replicates would fall within the linear range of the modified Lowry method [196]. A range of concentrations of bovine serum albumin (BSA) (Sigma Aldrich, UK) from 0-200 μg/ml were prepared from a 1 mg/ml stock solution of BSA, to act as a protein standard.

According to the modified Lowry method, 300 μl of each concentration of the BSA protein standards were added to 700 μl of Milli-Q water, before the addition of 100 μl of 0.15% (w/v) sodium deoxycholate (Sigma Aldrich, UK). Samples were vortexed and incubated at room temperature for 10 minutes. 100 μl of 72% (v/v) trichloroacetic acid (Sigma Aldrich, UK) was added to all samples. Samples were thoroughly vortexed and centrifuged at 3000 x g for 15 minutes at room temperature.

The supernatants from all samples were discarded and the pellets left to air dry. Pellets were resuspended in 0.5 ml of Milli-Q water. Thereafter, 0.5 ml of a freshly prepared reagent containing an equal 1:1:1:1 ratio of 0.8 M sodium hydroxide (Sigma Aldrich, UK), 10 % (w/v) SDS, Milli-Q water and copper tartarate carbonate (CTC) solution (0.2% (w/v) potassium sodium tartarate tetrahydrate, 0.1% (w/v) copper sulphate, 10% (w/v) sodium carbonate) (Sigma Aldrich, UK), was added to all samples and incubated at room temperature for 10 minutes. 0.25 ml of 1:6 diluted Folin’s phenol reagent (Sigma Aldrich, UK) was added to all samples, vortexed thoroughly and left to incubate at room temperature for 30 minutes for the colour to develop. Thereafter, the absorbance of all samples were measured at a wavelength of 750 nm, using the 0 μg/ml BSA standard as a blank.

Aliquots from each biological replicate for both the PVC and PETG conditions containing 4 μg of protein were also resolved on polyacrylamide gels, consisting of two components: a 12% polyacrylamide resolving gel containing 12 ml of 30% (v/v) acryl-bisacrylamide (Sigma Aldrich, UK), 7.5 ml of 1.5 M Tris(hydroxymethyl)aminomethane (Tris) (pH 8.8), 0.3 ml of 10% (v/v) SDS, 0.3 ml of 10% (v/v) ammonium sulphate (Sigma Aldrich, UK), 12 μl of tetramethylethylenediamine (TEMED) (Sigma Aldrich, UK) and 9.9 ml of Milli-Q water; and a 4% polyacrylamide stacking gel containing 0.83 ml of 30% (v/v) acryl-bisacrylamide, 0.63 ml of 1.5 M Tris (pH 6.8), 50 μl  of 10% (v/v) SDS, 50 μl of 10% (w/v) ammonium sulphate, 5 μl of TEMED and 3.4 ml of Milli-Q water. The stacking gel was added on top of the resolving gel after it had polymerised.

A running buffer was prepared as a 10 x stock solution containing 14.4% (w/v) glycine (Sigma Aldrich, UK), 3% (w/v) Tris (Sigma Aldrich, UK) and 1% (w/v) SDS in Milli-Q water. This was diluted 1:10 before use in gel electrophoresis.

The protein samples were thoroughly vortex-mixed before appropriate volumes from each sample corresponding to 4 μg of protein were pipetted into newly labelled 2 ml Eppendorf tubes (Eppendorf, Germany). Milli-Q water was added to each of the samples in a 1:1 ratio and the volume brought up to a final volume of 15 μl with loading dye containing bromophenol blue (x 2 concentration) (Sigma Aldrich, UK). After vortex mixing, all samples were incubated at 95˚C for 5 minutes in a heat block.

Thereafter, the polymerised polyacrylamide gel was immersed in a holding tank containing the running buffer. 5 μl of each sample was loaded into each lane of the gel, as well as 5 μl of a protein marker (Sigma Aldrich, UK) in the first lane. Electrophoresis was run initially at 80 V before increasing the voltage to 120 V until the bromophenol blue dye had reached the end of the gel.

###### 5.2.6. Silver staining of proteins from SDS-PAGE

The gel was then rinsed with Milli-Q water and Silver stained in order to detect proteins from SDS-PAGE with a sensitivity at the nanogram level [155]. The gel was soaked in a fix solution containing 50% (v/v) methanol (Sigma Aldrich, UK) and 12% (v/v) acetic acid (Sigma Aldrich, UK) for 40 minutes.

The fix solution was replaced with fresh fix solution and incubated for a further 30 minutes at room temperature. Afterwards, the fix solution was discarded and the gel was soaked in 50% (v/v) ethanol solution in three cycles of 20 minutes.

Thereafter, the gel was soaked in a 0.02% (w/v) sodium thiosulphate solution (Sigma Aldrich, UK) for one minute, before being washed twice with Milli-Q water and incubated in a 0.1% (w/v) silver nitrate (Sigma Aldrich, UK) solution for 20 minutes in the dark. After incubation, the gel was rinsed in Milli-Q water and the silver stain was developed in a solution containing 0.04% (v/v) formalin (Sigma Aldrich, UK) and 2% (w/v) sodium thiosulphate (Sigma Aldrich, UK), until gel bands became visible. At this point, the gel was rinsed in distilled water to prevent the silver stain from developing further. The rinsed gel was finally immersed in a fix solution for 30 minutes.

###### 5.2.7. Trypsin digestion

To ensure that a sufficient concentration of protein was available for iTRAQ labelling, the precipitated proteins from duplicate acetone-precipitated samples for each biological replicate for PVC and PETG were combined and resuspended in 70 μl of 100 mM TEAB buffer. This doubled the protein concentration for each biological replicate, as verified by the Lowry method.

For each sample, volumes corresponding to 80 μg of protein were pipetted from each sample into sterile 2 ml Protein LoBind tubes (Fisher Scientific, UK). This amount of protein was chosen to override the risk of having unlabelled peptides entering the mass spectrometer and increasing the chance of protein estimation errors [163].

Sample volumes were standardised to a final volume of 50 μl by adding 100 mM TEAB buffer. In order to enhance the solubilisation of proteins for enzymatic digestion, RapiGest™ surfactant (Waters Corporation, USA) was added at a final concentration of 0.1% (w/v). The reduction of the disulphide bonds between cysteines residues in the proteins was carried out using Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma Aldrich, UK). TCEP was added to all samples to a final concentration of 8 mM. Samples were consequently vortex mixed and incubated at 55˚C for 30 minutes.

Thereafter, the reduced cysteines were alkylated with a blocking reagent, methyl methanethiosulfonate (MMTS) (Sigma Aldrich, UK), which was added to a final concentration of 16 mM and incubated in the dark at room temperature for 30 minutes. Sequencing grade trypsin (Promega, UK) was reconstituted in 100 μl of 100 mM TEAB buffer to a final concentration of 0.2 ug/ul. Trypsin was added to all samples following an enzyme to protein ratio of 1:30 to ensure sufficient enzymatic digestion, as determined by preliminary experiments. Samples were incubated at 37˚C for 16 h whilst shaking at 350 rpm.

To ensure that the trypsin had successfully digested all proteins, aliquots from each digested sample corresponding to 2 μg of protein were resolved on 12% polyacrylamide gels at 120 V and subsequently Silver stained, as described previously.

###### 5.2.8. iTRAQ labelling

8-Plex iTRAQ reagents (Applied Biosystems, USA) were brought to room temperature before adding 50 μl of isopropanol (Sigma Aldrich, UK) to each label. After being thoroughly vortex mixed, 50 μl of each iTRAQ label was added to a specific sample digest as follows: labels 113-116 for the four biological replicates of the PETG conditions, and labels 117-121 for the four biological replicates of the PVC conditions.

Isopropanol was added to each sample at a final concentration of 60% v/v and samples were thoroughly vortex mixed. The pH of the peptide solution was checked before iTRAQ labelling. Samples were incubated at room temperature (22˚C) and shaken at 350 rpm for 2 h. Hydroxylamine hydrochloride (Sigma Aldrich, UK) was added to a final concentration of 10 mM to each of the samples in order to quench the excess of any unreacted iTRAQ labels. Samples were vortex mixed and incubated at 37˚C for 10 minutes.

Thereafter, RapiGest™ was removed from labelled samples. RapiGest™ undergoes hydrolysis in acidic conditions, which is useful for removing it easily from samples [151] to prevent it from interfering with iTRAQ reagents. Thus, a formic acid solution diluted with HPLC-grade water (Sigma Aldrich, UK) was added to each sample to a final concentration of 0.5% and vortex mixed in order to hydrolyse RapiGest™ from samples. Samples were incubated at 37˚C for 15 minutes, and centrifuged at 16,000 x g for 10 minutes to remove the hydrolysed RapiGest™.

The supernatants containing the labelled peptides from all samples were pooled together into a single sterile 2 ml Protein LoBind tube. The pooled sample was dried in a centrifugal vacuum concentrator (Speed Vac, Thermo Scientific, UK) and stored at -20˚C for further analysis.

###### 5.2.9. RP-HPLC separation of digested proteins

In order to reduce the complexity of the proteome and to better improve the identification and quantification of iTRAQ labelled peptides, reverse phase high-performance liquid chromatography (RP-HPLC) was used to pre-fractionate the labelled sample before mass spectrometry in the first dimension of LC separation.

200 μl of buffer A consisting of 97% (v/v) HPLC-grade water (Sigma Aldrich, UK), 3% (v/v) acetonitrile (Sigma Aldrich, UK) and 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma Aldrich, UK), was added to the dried iTRAQ-labelled sample. The sample was vortex mixed for 5 minutes, water bath sonicated for 1 minute, and vortex mixed and centrifuged at maximum speed (13,000 x g) for 1 minute in order to thoroughly mix the sample with buffer A and remove particulate matter, if present. The sample was then pipetted into an autosampler vial (Agilent Technologies, USA). Another autosampler vial containing 200 μl of buffer A served as a blank. Prior to peptide fractionation in an ultra-high pressure liquid chromatography (UHPLC) system (Dionex UltiMate™ 3000 UHPLC, Thermo Fisher Scientific, UK), the blank was used to equilibrate the UHPLC column until a stable baseline was achieved.

The peptides were fractionated in offline mode using the UHPLC system on a Hypercarb porous graphite carbon column (Thermo Scientific, USA) (50 x 2.1 mm, 3 μm particles with 250 Å pores) set at 30˚C with a flow rate of 0.2 ml/minute. Samples were eluted using a linear gradient of an organic buffer (buffer B) containing 97% (v/v) acetonitrile, 3% (v/v) HPLC-grade water and 0.1% (v/v) TFA ranging from 0-90% over 1 h. Peptide elution was monitored using the Chromeleon software (Thermo-Dionex, USA) at 214 nm. Peptide fractions were collected every 3 minutes between 10 and 60 minutes in sterile 1.5 ml Protein LoBind tubes using a fraction collector (Foxy Junior, Isco, USA), of which 16 peptide fractions were collected. These fractions were dried in a centrifugal vacuum concentrator before being stored at -20˚C prior to mass spectrometry analysis.

A peptide fraction was analysed by LC-MS/MS prior to identification and quantification by tandem mass spectrometry. This was to check the success of the iTRAQ labelling. Peptide separation was performed offline in the first dimension using a UHPLC system as described above, and separated in the second dimension using an online LC-MS/MS system.

###### 5.2.10. RP-LC-MS analysis

The 16 dried peptide fractions were resuspended in 10 μl of loading buffer (97% (v/v) HPLC grade water, 3% (v/v) acetonitrile and 0.1% TFA). The 16 fractions were then pooled depending on peak intensities in the chromatogram from the first dimension LC separation, yielding a total of five fractions. These five pooled peptide fractions were pipetted into autosampler vials and analysed by an LC-MS/MS system in online mode, which forms the second dimension of LC separation.

The iTRAQ labelled peptides were separated by RPLC on a Dionex UltiMate™ 3000 UHPLC system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, UK) for mass analysis. The UHPLC system separated peptides using a 75 µm x 50 cm, 2 µm particle size, EASY-Spray™ analytical column. Peptides were loaded on the column in mobile phase A (0.1% (v/v) formic acid in water) and separated using a linear gradient of 3-35% mobile phase B (0.1% (v/v) formic acid in acetonitrile) with a flow rate of 300 nL/min over a 90 min time period.

The instruments were operated in a data dependent mode and controlled by Xcalibur software (Thermo Fisher Scientific, UK). The following specifications were used to acquire data: survey scan was acquired over the range m/z350-1500 at a mass resolution of 60,000 and 30,000 for MS and MS/MS survey scans respectively. The top 15 most intense precursor ions were subjected to high collision dissociation (HCD) with a normalised collision energy of 34 with the precursor ion isolation window set to 1.2 m/zunits.

###### 5.2.11. Protein identification and quantification

Raw mass spectrometry data was loaded onto MaxQuant 1.5.5.1 (Max Planck Institute, Germany) and peptide identification was carried out using a *Methanosarcina barkeri* proteome database downloaded from UniProt (accessed 12 August 2016).For peptide identification using MaxQuant, oxidation of methionine and deamidation of asparagine and glutamine were set as variable modifications. Methylthio of cysteine was set as a fixed modification. Both the peptide to spectrum match false discovery rate (FDR) and protein FDR were set to below 1% on the search parameters of MaxQuant. The default values for isotopic corrections were applied for each identified peptide by MaxQuant. The set of identified proteins was further refined by removing contaminants. Only proteins with two or more unique peptides were used for protein quantification.

To extract information about the relative quantification of proteins, the intensity of the isotopic corrected values of the reporter ions (114 – 121) for each protein was normalised to the isotopic corrected intensity of reporter ion 113. The median of the normalised values for the reporters corresponding to each biological condition was calculated. Principal component analysis (PCA) was applied to the normalised dataset using XLSTAT 2014.4 to provide quality assurance of the biological replicates.

Fold changes in protein abundance between biological conditions were calculated as the ratio of the median normalised reporter intensities (PVC proteins/PETG proteins). Perseus 1.5.5.3 (Max Planck Institute, Germany) was used to statistically analyse whether the fold changes were significant. A one-way ANOVA was carried out on the normalised iTRAQ dataset with multiple test correction based on the Benjamini-Hochberg test. This test is based on the FDR, or the expected proportion of errors in the null hypothesis, and ranks protein ratios at a specified FDR to establish a reasonable cut-off [197]. By comparing the error rate within a set of tests, the Benjamini-Hochberg test provides a balance between the discovery of statistically significant proteins and false positives, which are less strict than the Bonferroni correction [198]. Fold changes were considered to be significant if the *p*-value was less than or equal to 0.05 after multiple test correction.

###### 5.2.12. Validation of iTRAQ results

Biofilm cells were grown and established on PVC and PETG using the same protocol as described previously (please refer to section 4.2.2.) for the proteomics analysis. Washed biofilm cell pellets were stored at -20˚C for future analysis.

Two enzyme assay kits (Biovision, USA) were used to validate fumarase and triose phosphate isomerase from biofilm cells grown on PVC or PETG, based on their significant difference in protein abundance as deduced from the results of the iTRAQ experiment and the availability of these enzyme kits.

Fumarase activity was measured spectrophotometrically at 485 nm by its ability to convert fumarate into malate. Fumarase activity in samples was measured as μmol of NADH generated by fumarase during a specified reaction time. Firstly, 150 μl of assay buffer from the fumarase activity colorimetric assay kit (BioVision, USA) was added to cell pellets from each biofilm sample, and homogenised using 200 μm zirconium beads (VWR, UK) in 10 cycles of high-speed vortexing on a Genie Disruptor (Scientific Industries, USA) for 30 seconds followed by 1 minute on ice. Thereafter, biofilm samples were centrifuged at 10000 x g for 10 minutes at 4˚C, and the supernatant collected.

Protein quantification was carried out on both biofilm samples using the Bradford assay in order to standardise the amount of protein for the fumarase and triose phosphate isomerase activity assays. A standard curve was established using prepared BSA concentrations of 0-10 μg/ml, with three replicates for each standard concentration. 3 μl of biofilm sample from each experimental condition was diluted 1:500 in HPLC water (Sigma Aldrich, UK) to give two technical replicates for each sample. 50 μl of BSA standard or diluted biofilm sample was added to disposable cuvettes (VWR, UK) along with 50 μl of Bradford reagent (Sigma Aldrich, UK) and incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm with Milli-Q water used as a blank. The protein concentration of the biofilm samples was calculated from the BSA standard curve. 4 μg of total proteins from each biofilm sample was used in the fumarase activity assay.

A standard curve of reduced nicotinamide adenine dinucleotide (NADH) (BioVision, USA) ranging from 0-12.5 nmol of NADH per well was established in a clear 96-well plate (Sigma Aldrich, UK), with three replicates for each concentration. The volume was adjusted to 50 μl with fumarase assay buffer from the assay kit. Volumes corresponding to 4 μg of protein from each biofilm sample was added to wells and adjusted to 50 μl with fumarase assay buffer, with two technical replicates per sample. Thereafter, 50 μl of reaction mix from the assay kit containing the fumarase enzyme mix and fumarase substrate were added to wells containing the NADH standard concentrations and biofilm samples, and the absorbance at 485 nm (OD485) was measured immediately in kinetic mode for 1 h at 37˚C.

NADH standard readings from 0 nmol/well were subtracted from all other readings. The fumarase activity in samples was measured as μmol of NADH generated by fumarase during a specified reaction time. It was calculated from the difference in OD485 between two time points taken from the linear range of the biofilm samples, and comparison with the NADH standard curve.

Triose phosphate isomerase activity (TPI) was measured using the same protocol as described for the detection of fumarase, but using the triose phosphate isomerase activity colorimetric assay kit (BioVision, UK). TPI activity was measured spectrophotometrically at 485 nm by its ability to convert dihydroxyacetone phosphate into glyceraldehyde-3-phosphate. TPI activity in samples was measured as μmol of NADH generated by TPI during a specified reaction time, as described above.

# 5.3. Results

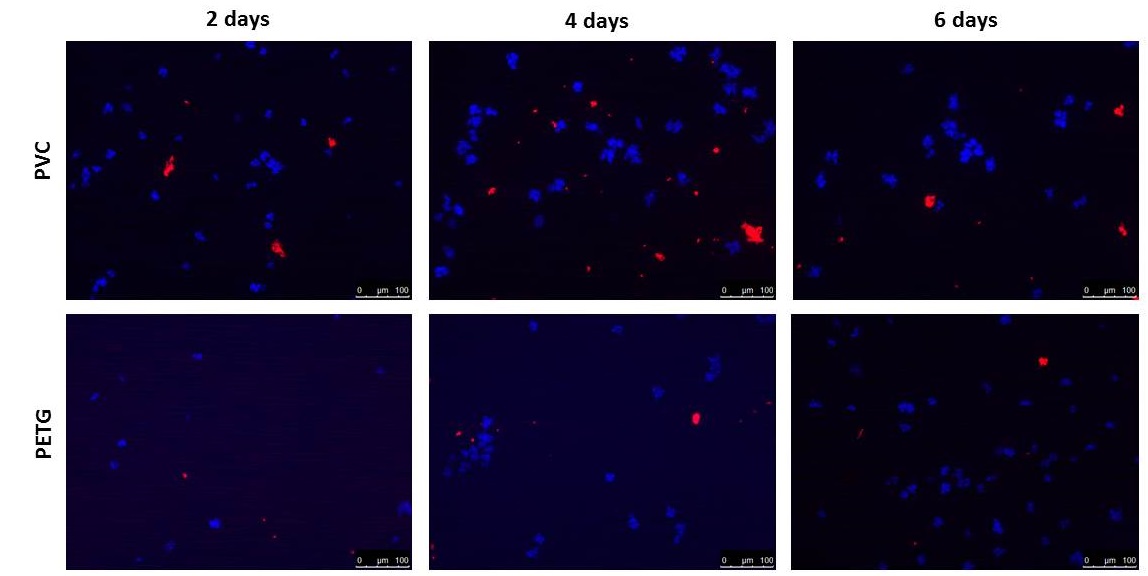
###### 5.3.1. Biofilm accumulation experiment

The results from the biofilm accumulation experiment are presented in Fig. 5.2, and show that significantly higher levels of cell and mannose and glucose residues were produced over time by biofilms attached to PVC compared to PETG (two-way ANOVA with Sidak’s multiple comparison test, p < 0.05).



**Fig. 5.2** Percentage surface coverage of PVC and PETG by cells (stained by DAPI) and mannose and glucose residues (stained by ConA-Rho) of *M. barkeri* over 6 days as an indication of biofilm accumulation over time. Error bars represent the standard deviation of 3 independent experiments.

In biofilms attached to PVC, the percentage of adherent DAPI-stained cells was significantly different between all tested time points (two-way ANOVA with Tukey’s multiple comparison test, p < 0.05), whereas cell number did not significantly differ over the time course for biofilms attached to PETG (two-way ANOVA with Tukey’s multiple comparison test, p > 0.05), suggesting poor accumulation of cells at the surface of PETG. The images from epifluorescence microscopy are shown in Fig. 5.3.



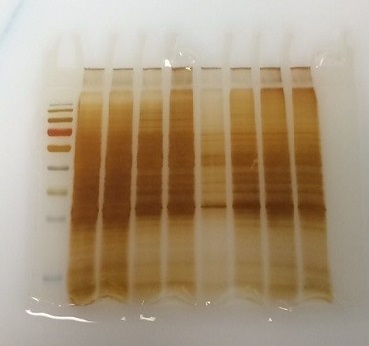
**Fig. 5.3** Epifluorescence images taken of DAPI stained cells (blue) and ConA-Rho stained mannose and glucose residues (red) for biofilms of *M. barkeri* attached to PVC and PETG after 2, 4 and 6 days at 37°C.

In biofilms attached to PVC, the percentage of mannose and glucose residues covering coupons was significantly higher at the later stages of the time course (days 4 and 6) compared to the start of the time course (day 2) (two-way ANOVA with Tukey’s multiple comparison test, p < 0.05). However, in biofilms attached to PETG, the percentage of mannose and glucose residues covering coupons did not significantly differ over the time course of the experiment (two-way ANOVA with Tukey’s multiple comparison test, p > 0.05), suggesting poor accumulation of mannose and glucose residues from biofilms on the surface of PETG (Fig. 5.3).

###### 5.3.2. Protein quantification

Quantification of the protein concentrations from the four replicates of both the PETG and PVC-biofilm experimental conditions was calculated by the modified Lowry method, showing that biofilm cells of *M. barkeri* attached to PVC had an average of 1.46±0.24 mg/ml of protein, whereas cells attached to PETG had 0.86±0.25 mg/ml of proteins.

SDS-PAGE was used to qualitatively verify the results determined from the modified Lowry method. 4 µg of extracted proteins from samples were analysed by SDS-PAGE and silver staining (Fig. 5.4). Visual inspection of the intensity of the bands of the resolved proteins between the two biological conditions from the SDS-PAGE analysis supported the results of the Lowry method, suggesting a difference in protein expression between the PVC and PETG-biofilm conditions

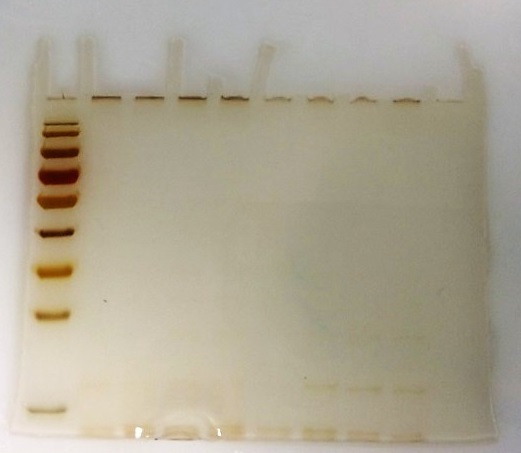


**b)**

**a)**

**Fig. 5.4** SDS-PAGE analysis of 4 µg of extracted proteins from four replicates of a) PVC biofilm experimental condition and b) PETG-biofilm experimental condition.

For enzymatic digestion of proteins with trypsin, SDS-PAGE was again used to check that the trypsin had successfully digested all proteins. Aliquots from each digested sample corresponding to 2 μg of protein were resolved on 12% polyacrylamide gels at 120 V and subsequently Silver stained. No gel bands were observed after staining, indicating successful protein digestion by the trypsin treatment (Fig. 5.5).



**b)**

**a)**

**Fig. 5.5** SDS-PAGE analysis of 2 µg of extracted proteins from four replicates of a) PVC biofilm experimental condition and b) PETG-biofilm experimental condition.

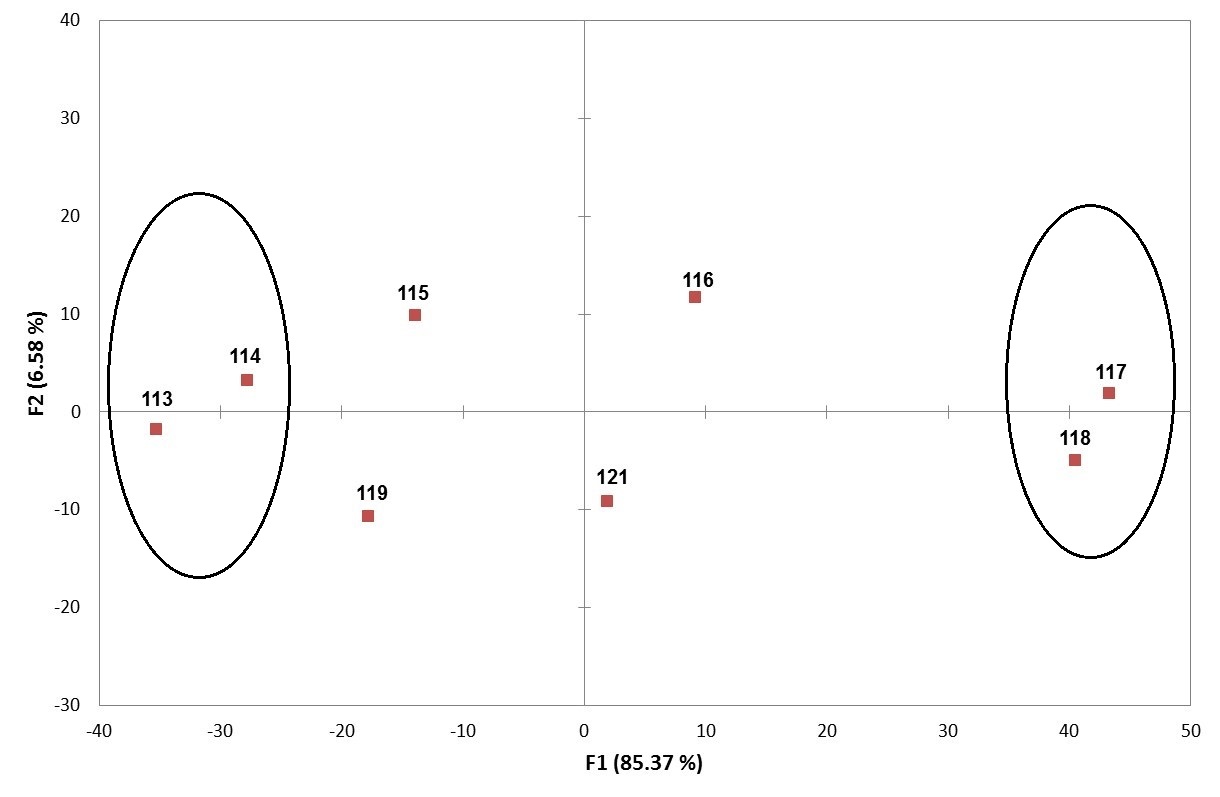
###### 5.3.3. PCA analysis of mass spectrometry data

Four biological replicates for each biofilm condition were established to improve the robustness of the experiment, and also because the pooling together of a set number of coupons from each biological replicate from different bottles would incur some level of variation between replicates.

Cluster analysis using principal component analysis (PCA) was performed on the normalised relative reporter intensities for identified peptides from tandem mass spectrometry. The relative reporter ion intensities were associated with the relative abundance of each peptide, and PCA analysis can test for variances in protein expression between the biological replicates and as a means of quality control.

The first principal component of a PCA analysis describes the maximum variance in the original dataset [199], and the PCA analysis in Fig. 5.6 showed a large level of variation between the four biological replicates of each biofilm condition. Such variation is to be expected from such a large number of batch cultures where differences in inoculum levels, media or starter cultures can all be contributing factors. Consequently, the ‘noise’ from such variables can mask any important statistical trends in protein expression between the biofilm conditions [199].

However, the PCA analysis revealed distinct clustering of two biological replicates in each biofilm condition (iTRAQ labels 113 and 114 corresponding to two PETG-biofilm replicates, and labels 117 and 118 corresponding to two PVC-biofilm replicates). These closely clustered biological replicates suggest a similar level of protein expression and an underlying biological relationship. Therefore, it was decided to focus only on the most closely clustered labels (113, 114, 117 and 118) for further data analysis in order to conceal some of the noise and complexity of the dataset from the labels with the most variation (115,116, 119, 121).



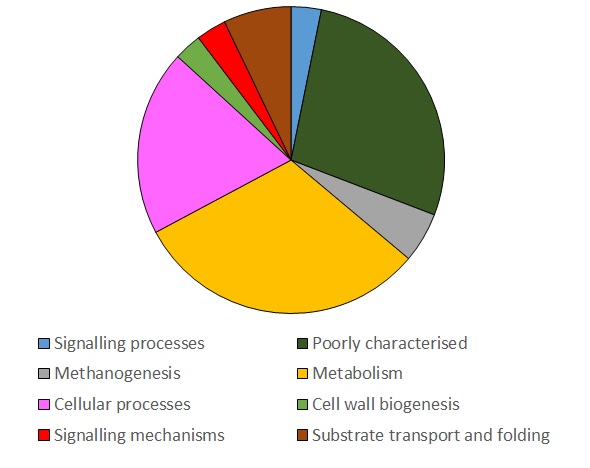
**Fig. 5.6** PCA analysis of the relative reporter intensities of each iTRAQ label shows distinct clustering of labels 113 and 114 (corresponding to two PETG-biofilm replicates) from labels 117 and 118 (corresponding to two PVC-biofilm replicates).

###### 5.3.4. iTRAQ protein identification and quantification

The spectroscopic and microscopic results from chapter 4 revealed significant adaptive features from *M. barkeri* depending on the support material. Therefore, quantitative proteomics based on iTRAQ was used to elucidate whether the patterns of protein expression differed between *M. barkeri* biofilms grown on two different support materials.

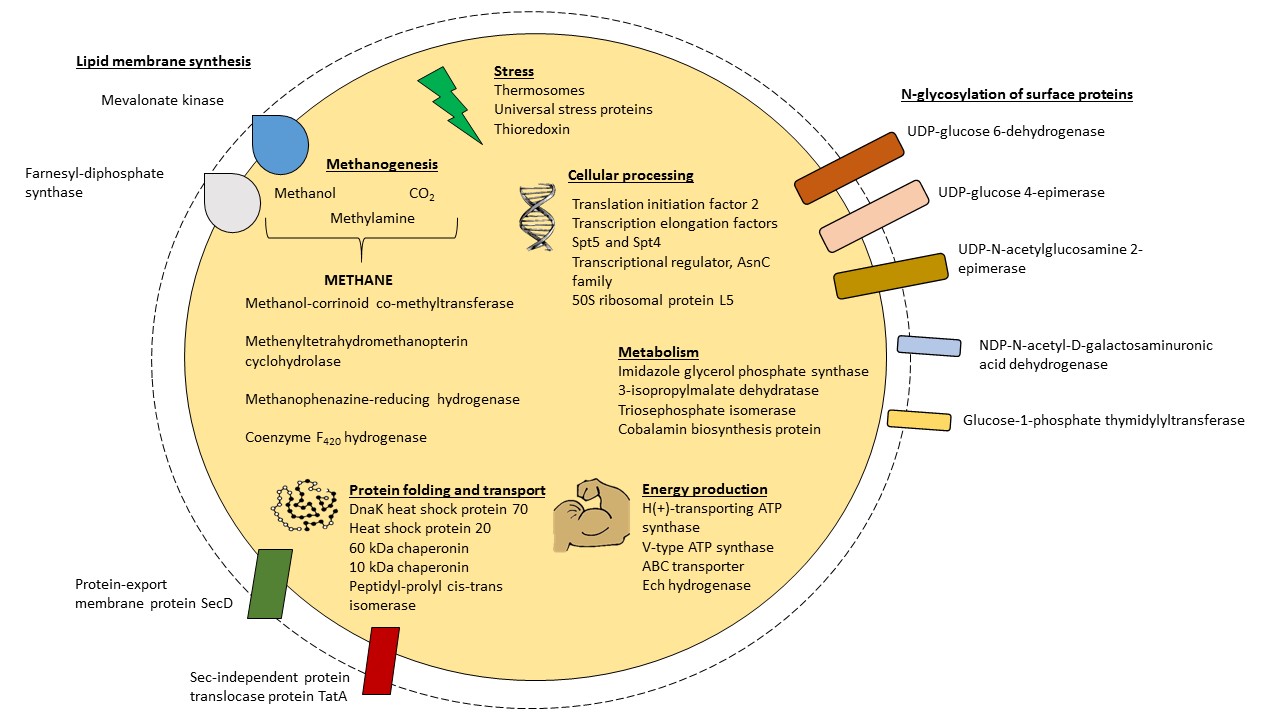
Further refinement of the iTRAQ dataset was performed by validating only those proteins that had two or more unique peptides, the removal of contaminants, and a false discovery rate (FDR) of 1%, which yielded 900 proteins in total. The FDR was automatically calculated on MaxQuant software by matching the identified MS/MS spectra to a reverse concatenated database based on the original *M. barkeri* FASTA file containing identified amino acid sequences. A fold change greater than 1 indicated a higher relative abundance of protein from cells attached to PVC compared to PETG. A fold change of less than 1 indicated a lower relative abundance of protein from cells attached to PETG compared to PVC.

However, after the application of a *p*-value cut-off of 0.05 after multiple testing correction, 732 total proteins were finally identified as having a significant fold change (20.2% of the total proteome of *M. barkeri*). Of these, 730 proteins were found in higher relative abundance in biofilms of *M. barkeri* grown with PVC as the support material, and 2 proteins were found in higher relative abundance in biofilms of *M. barkeri* grown with PETG as the support material. Of the total 732 identified proteins, 28.6% were poorly characterised or hypothetical proteins (Fig. 5.6). According to UniProt, 46% of the total proteome of *M. barkeri* consists of uncharacterised proteins, and it is therefore unsurprising that 28.6% of the identified proteins in this study were poorly characterised.



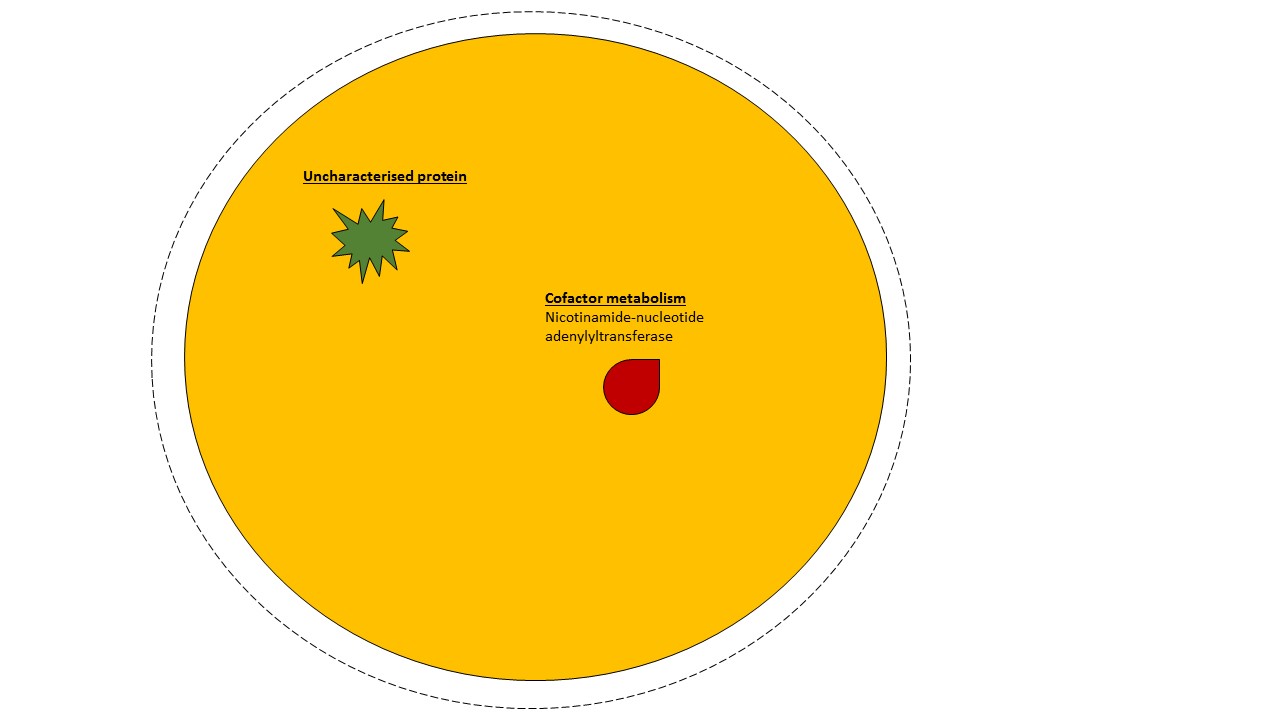
**Fig. 5.7** The main functions assigned to the identified proteins from iTRAQ analysis of *M. barkeri* cells attached to PVC and PETG.

The majority of identified proteins were involved in metabolism (32.1%), followed by cellular processes (20.2%), substrate transport and folding (7.4%), methanogenesis (5.5%), signalling mechanisms (3.3%) and cell wall biogenesis (3.0%) (Fig. 5.7). These main functions were further decomposed into subfunctions to provide a better understanding of the functional proteome of *M. barkeri* in response to the support material after 96 h of growth (Tables 5.1-5.7). Some of the proteins found in higher relative abundance from *M. barkeri* whilst attached to PVC and PETG after 96 h are schematically shown in Fig. 5.8.



**A**

**B**



**Fig. 5.8** Schematic of some of the proteins found in higher relative abundance in *M. barkeri* when attached to PVC (A) and the 2 proteins found in higher relative abundance in biofilms of *M. barkeri* attached to PETG (B) after 96 h.

###### 5.3.5. Investigation of cellular adaptation strategies during biofilm formation

5.3.5.1. Methanogenesis

*Methanosarcina* are the most metabolically versatile within the methanogenic archaea, with the ability to metabolise not only acetate, H2 and CO2, but also methanol and methylamines using all four methanogenesis pathways [18,200]. As such, various proteins involved in methanogenesis from these different pathways were found in higher relative abundance in *M. barkeri* attached to PVCin this study compared to cells attached to PETG (Table 5.1). These proteins are schematically shown in Fig. 5.9.



**Fig. 5.9** A schematic of the proteins found in higher relative abundance involved in the different methanogenesis pathways from biofilms of *M. barkeri* attached to PVC compared to PETG. **Red** boxes correspond to proteins involved in methylamine methanogenesis, **green** boxes correspond to proteins involved in CO2 methanogenesis, **blue** boxes correspond to proteins involved in methanol methanogenesis, and **pink** boxes correspond to proteins common to all methanogenesis pathways. Map constructed from KEGG Mapper (http://www.genome.jp).

*Enzyme commission numbers:*

**Pink boxes**: MttC (dimethylamine corrinoid protein), 2.8.4.1. (methylcoenzyme M reductase system, component A2), 1.8.98.1 (CoB--CoM heterodisulfide reductase subunit B), 1.5.98.2 (methylenetetrahydromethanopterin reductase)

**Green boxes**: 2.1.1.86 (tetrahydromethanopterin S-methyltransferase, subunit A), 23.1.101 (formylmethanofuran-tetrahydromethanopterin formyltransferase), 3.5.4.27 (methenyltetrahydromethanopterin cyclohydrolase), 1.5.98.1 (methylenetetrahydromethanopterin dehydrogenase), 1.12.98.1 (coenzyme F420-reducing hydrogenase, beta subunit), 1.2.99.5 (formylmethanofuran dehydrogenase, subunit E)

**Red boxes**: MtbA (methylamine-specific methylcobalamin:coenzyme M methyltransferase, MtbC (dimethylamine corrinoid protein)

**Blue boxes**: MtaA (methanol-specific methylcobalamin: coenzyme M methyltransferase), MtaB (methanol:corrinoid methyltransferase), MtaC (methanol corrinoid protein)

Three homologs of MtaB encoding the enzyme methanol-corrinoid co-methyltransferase (Mbar\_A0741, Mbar\_A1064 and Mbar\_A3638), and the downstream gene MtaC encoding methanol-corrinoid protein (Mbar\_A1063) were 2 fold higher in relative abundance in *M. barkeri* cells attached to PVC compared to cells attached to PETG. These two subunits, MtaB and MtaC, comprise methyltransferase I, which is an essential protein complex responsible for transferring a methyl group from methanol to coenzyme M for methanogenesis [201], and is catalysed by MtaA encoding the enzyme methylcobamide:Coenzyme M methyltransferase. Two homologs of MtaA (Mbar\_A1054, Mbar\_A3639) were also in higher relative abundance by up to two fold by *M. barkeri* attached to PVC compared to PETG. This methyltransferase unit plays a key role in the terminal stages of methanol methanogenesis, and is coupled to ATP production [181].

**Table 5.1** Proteins involved in methanogenesis and found in higher relative abundance in biofilms on PVC with significant fold changes (p ≤ 0.05).

|  |  |  |  |
| --- | --- | --- | --- |
| **Loci** | **Protein** | **Fold change** | |
| *Methylamine methanogenesis* | | |
| Mbar\_A0843 | Monomethylamine methyltransferase MtmB1 | 2.16 | |
| Mbar\_A3604 | Dimethylamine corrinoid protein | 2.04 | |
| Mbar\_A1506 | Dimethylamine methyltransferase MtbB1 | 1.93 | |
| Mbar\_A1502 | Trimethylamine methyltransferase MtbB | 1.85 | |
| Mbar\_A0841 | Methylamine-specific methylcobalamin:coenzyme M methyltransferase | 1.75 | |
| Mbar\_A1501 | Dimethylamine corrinoid protein | 1.53 | |
|  |  |  | |
| *Methanol methanogenesis* | | |
| Mbar\_A1054 | Methylcobamide:CoM methyltransferase MtaA | 2.23 | |
| Mbar\_A0741 | Methanol--corrinoid protein co-methyltransferase MtaB | 2.00 | |
| Mbar\_A3638 | Methanol:corrinoid methyltransferase MtaB | 1.98 | |
| Mbar\_A3639 | Methanol-specific methylcobalamin: coenzyme M methyltransferase MtaA | 1.90 | |
| Mbar\_A1063 | Methanol corrinoid protein MtaC | 1.72 | |
| Mbar\_A1064 | Methanol:corrinoid methyltransferase MtaB | 1.65 | |
|  |  |  | |
| *CO2 methanogenesis* | | |
| Mbar\_A1256 | Tetrahydromethanopterin S-methyltransferase subunit G | 2.24 | |
| Mbar\_A2923 | Formylmethanofuran dehydrogenase, subunit D | 2.20 | |
| Mbar\_A1845 | Methanophenazine-reducing hydrogenase, cytochrome B subunit | 2.16 | |
| Mbar\_A2233 | Methenyltetrahydromethanopterin cyclohydrolase | 2.05 | |
| Mbar\_A1292 | Formylmethanofuran dehydrogenase, subunit B | 2.01 | |
| Mbar\_A0980 | Formylmethanofuran--tetrahydromethanopterin formyltransferase | 1.93 | |
| Mbar\_A1288 | Formylmethanofuran dehydrogenase, subunit F | 1.92 | |
| Mbar\_A1095 | F420-dependent methylenetetrahydromethanopterin dehydrogenase | 1.89 | |
| Mbar\_A1290 | Molybdenum-containing formylmethanofuran dehydrogenase 1 subunit C | 1.79 | |
| Mbar\_A1289 | Formylmethanofuran dehydrogenase, subunit A | 1.72 | |
| Mbar\_A1287 | Formylmethanofuran dehydrogenase, subunit E | 1.69 | |
| Mbar\_A2922 | Formylmethanofuran dehydrogenase, subunit B | 1.67 | |
| Mbar\_A2589 | CoB--CoM heterodisulfide reductase subunit A | 1.65 | |
| Mbar\_A0662 | Coenzyme F420 hydrogenase beta subunit | 1.60 | |
| Mbar\_A0452 | Coenzyme F420 hydrogenase subunit alpha | 1.39 | |
|  |  |  | |
| *Common to all methanogenesis pathways* | | |
| Mbar\_A0202 | Acetyl-CoA decarbonylase/synthase complex subunit beta | 1.60 | |
| Mbar\_A0430 | Acetyl-CoA decarbonylase/synthase complex subunit delta | 1.54 | |
| Mbar\_A0148 | Ech hydrogenase subunit E | 1.70 | |
| Mbar\_A0639 | CoB--CoM heterodisulfide reductase subunit B | 2.11 | |
| Mbar\_A0254 | 5,10-methylenetetrahydromethanopterin reductase | 2.10 | |
| Mbar\_A0894 | Methyl-coenzyme M reductase subunit gamma | 2.08 | |
| Mbar\_A0676 | Methylcoenzyme M reductase system, component A2 | 2.05 | |
| Mbar\_A1610 | Archaeal flavoprotein | 2.00 | |
| Mbar\_A0895 | Methyl-coenzyme M reductase operon protein C | 2.00 | |
| Mbar\_A0897 | Methyl-coenzyme M reductase subunit beta | 1.96 | |
| Mbar\_A1503 | Dimethylamine corrinoid protein | 1.95 | |
| Mbar\_A0896 | Methyl-coenzyme M reductase operon protein D | 1.81 | |
| Mbar\_A0893 | Methyl-coenzyme M reductase subunit alpha McrA | 1.78 | |
| Mbar\_A2588 | Formylmethanofuran dehydrogenase, subunit F | 1.70 | |
| Mbar\_A3541 | Cysteate synthase | 2.04 | |

Coenzyme M is an important substrate for all four methanogenic metabolic pathways, acting as the terminal methyl carrier in methanogenesis [202]. The higher relative abundance of various enzymes involved in the production or handling of coenzyme M across all methanogenesis pathways from cells attached to PVC (Mbar\_A0841, Mbar\_A1054, Mbar\_A3639, Mbar\_A0639, Mbar\_A0894, Mbar\_A0676, Mbar\_A0895, Mbar\_A0897, Mbar\_A0896, Mbar\_A0893) suggests that biofilms attached to PVC are producing more methane from methanol compared to biofilms on PETG.

Cysteate synthase is involved in the synthesis of coenzyme M [202] and was found in higher relative abundance in biofilms of *M. barkeri* attached to PVC compared to PETG. This enzyme (Mbar\_A3541) correlates with the detection of methylcobamide:CoM methyltransferase, MtaA, and methanophenazine-reducing hydrogenase, which both require coenzyme M for methanogenesis. Therefore, the higher relative abundance of cysteate synthase suggests that a high level of coenzyme M is needed to support high rates of methanogenesis when *M. barkeri* preferentially attaches and forms biofilms on PVC.

Several proteins linked to the reduction of CO2 by H2 to methane were in higher relative abundance by *M. barkeri* when attached as a biofilm to PVC compared to PETG. These include methenyltetrahydromethanopterin cyclohydrolase (Mbar\_A2233; EC 3.5.4.27) and methanophenazine-reducing hydrogenase (Mbar\_A1845). The first enzyme, methenyltetrahydromethanopterin cyclohydrolase (Mbar\_A2233; 3.5.4.27), is a cytoplasmic enzyme that has previously been purified from *M. barkeri* [203], and is involved in the reversible formation of N5,N10-methenyltetrahydromethanopterin (methenyl-H4MPT+) from N5-formyltetrahydromethanopterin (formyl-H4MPT) [204,205]. This compound is subsequently converted to methyl-coenzyme M, an important substrate in the final stages of methanogenesis [202].

The second enzyme, methanophenazine-reducing hydrogenase (Mbar\_A1845), is associated with energy conservation in *M. barkeri.* *Methanosarcina* is the only order of methanogenic archaea in possession of cytochromes and methanophenazine [196], which are membrane-bound protein complexes responsible for electron transport [206]. In the reduction of CO2 with H2 to methane in *Methanosarcina* sp., methanophenazine-reducing hydrogenase transfers electrons from H2 to methanophenazine in an extremely exergonic reaction, causing the reduction of coenzyme M and coenzyme B. This exergonic reaction is coupled to the accumulation of a transmembrane electrochemical proton gradient used for ATP synthesis [207,208].

Another interesting observation was the higher relative abundance of various V-type ATP synthases (Mbar\_A0384, Mbar\_A0385, Mbar\_A0386, Mbar\_A0387, Mbar\_A0388, Mbar\_A0389, Mbar\_A0391), and two H(+)-transporting ATP synthases (Mbar\_A3099, Mbar\_A0392) (see Table 5.6) from biofilms of *M. barkeri* attached to PVC compared to PETG. These ATP synthases are responsible for energy conservation and production. Such energy conservation and production functions complement the detection of methylcoenzyme M reductase (Mbar\_A0676), which requires an energy input in order to facilitate the conversion of carbon sources into methane [170].

Coenzyme F420 is an important cofactor involved in methanogenesis and is the main electron carrier in the cytoplasm of methanogens [207,208]. Coenzyme F420 is reduced by coenzyme F420 hydrogenase, and is the sole electron donor for the reduction of methylenetetrahydromethanopterin (methylene-H4MPT) in the initial stages of methanogenesis from CO2 [207]. The higher relative abundance of the two subunits of coenzyme F420 hydrogenase (Mbar\_A0452, Mbar\_A0662) in cells of *M. barkeri* grown on PVC compared to PETG suggests that high levels are required to start methanogenesis from CO2. Indeed, the higher relative abundance of a larger number of proteins linked to methanogenesis in cells attached to PVC compared to PETG is a reflection of the role of PVC in promoting biofilms of *M. barkeri* to produce more methane compared to those on PETG.

5.3.5.2. Cell wall biogenesis

A more developed biofilm is associated with an increasing accumulation of cells to a substrate, and therefore, an increased growth of cells. This increasing level of attached cell growth is accompanied by modifications to the morphology of the cell surface and the synthesis of exopolymers to facilitate irreversible adhesion and cell growth [63,209]. Various proteins involved in modifying the cell surface of *M. barkeri* were found to be in higher relative abundance in biofilm cells of *M. barkeri* attached to PVC compared to PETG*,* including proteins linked to the biogenesis or N-glycosylation of the lipid membrane, S-layer and externalmethanochondroitin layer of *M. barkeri* (Table 5.2) Their role in *M. barkeri* are discussed in more detail below.

**Table 5.2** Proteins involved in cell wall and membrane biogenesis and cell growth and found in higher relative abundance in biofilms on PVC with significant fold changes (p ≤ 0.05).

|  |  |  |
| --- | --- | --- |
| **Loci** | **Protein** | **Fold change** |
| *Cell wall/membrane biogenesis* | | |
| Mbar\_A2140 | UDP-N-acetylglucosamine 2-epimerase | 2.14 |
| Mbar\_A1421 | Mevalonate kinase | 2.1 |
| Mbar\_A0291 | Glycerol-1-phosphate dehydrogenase | 2.07 |
| Mbar\_A1417 | Farnesyl-diphosphate synthase/geranylgeranyl-diphosphate synthase | 2.04 |
| Mbar\_A3422 | Digeranylgeranylglycerophospholipid reductase | 1.86 |
| Mbar\_A0394 | Polyprenyl synthetase | 1.82 |
| Mbar\_A0675 | Geranylgeranylglyceryl phosphate synthase | 1.81 |
| Mbar\_A0231 | dTDP-glucose 4,6-dehydratase | 1.78 |
| Mbar\_A2020 | Glucose-1-phosphate thymidylyltransferase | 1.75 |
| Mbar\_A2021 | Probable phosphoglucosamine mutase | 1.72 |
| Mbar\_A1420 | Isopentenyl phosphate kinase | 1.69 |
| Mbar\_A1937 | Mannose-1-phosphate guanylyltransferase | 1.66 |
| Mbar\_A0021 | UDP-glucose 4-epimerase | 1.58 |
| Mbar\_A2853 | Surface layer protein B | 1.58 |
| Mbar\_A1119 | UDP-glucose 6-dehydrogenase | 1.56 |
| Mbar\_A1887 | dTDP-4-dehydrorhamnose 3,5-epimerase | 1.55 |
| Mbar\_A1139 | NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase | 1.46 |
|  |  |  |
| *Cell cycle control, mitosis and meiosis* | | |
| Mbar\_A1885 | CODH nickel-insertion accessory protein | 1.77 |
| Mbar\_A0618 | Cell division protein FtsZ | 1.77 |
| Mbar\_A0191 | Cell division protein FtsZ | 1.72 |
| Mbar\_A0908 | Cell division control protein 48 | 1.68 |
| Mbar\_A0529 | Cell division cycle protein 48-like protein | 1.66 |

Mevalonate kinase is involved in the mevalonate pathway and is responsible for the synthesis of isopentenyl pyrophosphate (IPP) [210]. IPP is the intermediate building block for the synthesis of isoprenoids, which constitute the major constituents of the ether-linked archaeal lipid membrane layer [211,212]. Farnesyl-diphosphate synthase/geranylgeranyl-diphosphate synthase is involved in the subsequent condensation of IPP, producing archaeal membrane lipids with the commonly found chain lengths of C20 (geranylgeranyl diphosphate) and C25 (farnesylgeranyl diphosphate) [210]. In this study, both mevalonate kinase (Mbar\_A1421) and farnesyl-diphosphate synthase/geranylgeranyl-diphosphate synthase (Mbar\_A1417) were found at relative abundance levels two-fold higher in biofilm cells of *M. barkeri* attached to PVC compared to PETG (Table 5.2), suggesting an increased use of the mevalonate pathway for the biosynthesis of membrane lipids in *M. barkeri* in response to biofilm attachment to PVC.

In addition, four uncharacterised proteins (Mbar\_2011, Mbar\_A1557, Mbar\_A2016 and Mbar\_A1034) were found in higher relative abundance in biofilm cells attached to PVC compared to PETG. A previous study has suggested that there is a strong similarity between these four uncharacterised proteins to a major S-layer surface protein in *M. barkeri* (Mbar\_1758) [213]. In turn, this major S-layer protein (Mbar\_1758) was found to be analogous to the major S-layer proteins, MM1976 and MA0829, from *M. mazei* and *M. acetivorans* respectively [214]*.* Therefore, this could suggest that the four uncharacterised proteins found in higher relative abundance in this study (Mbar\_2011, Mbar\_A1557, Mbar\_A2016 and Mbar\_A1034) are linked to S-layer surface proteins and that a higher level of S-layer protein modification is required for *M. barkeri* to attach to PVC compared to PETG.

Various proteins specific to the N-glycosylation of surface proteins were also in higher relative abundance from *M. barkeri* biofilms attached to PVC compared to PETG. The N-glycosylation of surface proteins plays a key role in helping archaeal cells adapt and respond to physical stresses and in maintaining cell viability, cell shape and protection. The process involves the covalent addition of polysaccharides to alter the lipid and protein components of the cell membrane in response to the external environment [215,216]. These higher abundance proteins included UDP-glucose 6-dehydrogenase (Mbar\_A1119), NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase (Mbar\_A1139), UDP-glucose 4-epimerase (Mbar\_A0021) and UDP-N-acetylglucosamine 2-epimerase (Mbar\_A2140).

In addition, proteins involved in the synthesis of components of the external methanochondroitin cell layer of *M. barkeri* were found to be in higher relative abundance in *M. barkeri* cells attached to PVC compared to PETG. These proteins included NDP-N-acetyl-D-galactosaminuronic acid dehydrogenase (Mbar\_A1139) and glucose-1-phosphate thymidylyltransferase (Mbar\_A2020), which are both responsible for the synthesis of the glucoronic acid, galactosamine and rhamnose components of the methanochondroitin cell layer [88,212]. The higher relative abundance of these proteins has been described in bacterial systems for specifically synthesising high-molecular-weight sugar-rich EPS molecules [89], and it is feasible that their higher relative abundance in biofilms of *M. barkeri* attached to PVC in this study is indicative of the requirement for the synthesis of EPS and cell surface components in response to biofilm formation.

Biofilm formation results in the binary division of adherent cells to form cell clusters during biofilm growth [62]. Various proteins involved in the binary division of cells during cellular growth in biofilm formation were found in higher relative abundance in *M. barkeri* cells attached to PVC compared to PETG, including four cell division proteins (Mbar\_A0618, Mbar\_A0191, Mbar\_A0908, Mbar\_A0529). This suggests that biofilm cells attached to PVC were in a higher active growth phase compared to those on PETG.

The ability to transport proteins across membranes is vital for cell viability, and is an indicator of the physiological state of a cell. Protein secretion is a key player in allowing cells to interact with their external environment [209], and has been linked to the production of EPS, extracellular structures and biofilm in archaea [217]. In this study, numerous ABC transporters with two-fold increased relative abundance that were involved in substrate uptake and export were observed in biofilms attached to PVC compared to PETG (Table 5.6), along with their associated ATPases (Mbar\_A1078, Mbar\_A1889), which hydrolyse ATP in order to translocate substrates across the membrane. These proteins have a high affinity for specific substrates and allow *M. barkeri* to efficiently thrive in environments with low levels of these substrates [218].

Other proteins involved in energy production and conservation were also in significantly higher relative abundance in biofilm cells attached to PVC, compared to PETG (Table 5.6), including the H(+)-transporting ATP synthases (Mbar\_A3099, Mbar\_A0392) and V-type ATP synthases (Mbar\_A0388, Mbar\_A0384, Mbar\_A0385, Mbar\_A0386, Mbar\_A0391, Mbar\_A0389, Mbar\_A0387). The higher relative abundance of these proteins suggest that biofilm cells attached to PVC require an elevated energetic input to support growth and the transport of substrates from the cell compared to those attached to PETG.

Other important transmembrane enzymes specific for protein export across the cytoplasmic membrane were also detected in biofilms attached to PVC compared to PETG. Two homologs of Sec-independent protein translocase protein TatA (Mbar\_A2548, Mbar\_A2549) involved in the transport of folded proteins [217], and protein-export membrane protein SecD (Mbar\_A0851) involved in the transport of unfolded proteins, were both found in higher relative abundance (Table 5.5). The higher relative abundance of substrate proteins and cell surface proteins in biofilms attached to PVC highlights the importance of the production and secretion of EPS and glycosylated proteins into the environment for biofilm formation [209].

5.3.5.3. Signalling mechanisms

Signalling mechanisms are important in microorganisms to allow them to respond to important extracellular signals in their environment, which can promote survival, defence and growth [219]. Various signal proteins involved in nitrogen regulation and defence responses were found in higher relative abundance in *M. barkeri* biofilms attached to PVC compared to PETG (Table 5.3), and are described in more detail below.

**Table 5.3** Proteins involved in signalling mechanisms and found in higher relative abundance in biofilms on PVC with significant fold change (p ≤ 0.05).

|  |  |  |
| --- | --- | --- |
| **Loci** | **Protein** | **Fold change** |
| *Signal transduction mechanisms* | | |
| Mbar\_A3247 | Sensory transduction histidine kinase | 3.77 |
| Mbar\_A2180 | L-glutamine synthetase | 2.25 |
| Mbar\_A3420 | MoaA/NifB/PqqE family protein | 2.14 |
| Mbar\_A2347 | Nitrogen regulatory protein P-II | 2.07 |
| Mbar\_A3129 | GTP-binding protein | 2.05 |
| Mbar\_A0729 | NifS protein | 1.98 |
| Mbar\_A0122 | Ferric uptake regulation protein | 1.80 |
| Mbar\_A2425 | NifU protein | 1.79 |
| Mbar\_A0159 | Nitrogen regulatory protein P-II | 1.74 |
| Mbar\_A2703 | Nitrogen fixation protein NifH/NifE | 1.70 |
| Mbar\_A0665 | Glutamate synthase (NADPH) GltB1 subunit | 1.69 |
| Mbar\_A0347 | Mo-nitrogenase iron protein subunit NifH | 1.52 |
| Mbar\_A0167 | Mo-nitrogenase MoFe protein subunit NifK | 1.40 |
|  |  |  |
| *Defense mechanisms* | | |
| Mbar\_A1182 | Nucleoid protein MC1 | 2.29 |
| Mbar\_A3527 | Universal stress protein | 2.24 |
| Mbar\_A0479 | Peroxiredoxin | 1.98 |
| Mbar\_A3529 | Universal stress protein | 1.98 |
| Mbar\_A1084 | Thermosome subunit | 1.95 |
| Mbar\_A0825 | Universal stress protein | 1.85 |
| Mbar\_A2310 | Thioredoxin | 1.80 |
| Mbar\_A0309 | CRISPR-associated helicase, Cas3 family | 1.82 |
| Mbar\_A1201 | Thermosome subunit | 1.78 |
| Mbar\_A2898 | Thioredoxin reductase | 1.64 |
| Mbar\_A2586 | Universal stress protein | 1.34 |

Nitrogen fixation is an important biological process for living organisms. This process produces ammonium from atmospheric nitrogen for incorporation into cell material to facilitate growth and biogenesis, and is a highly regulated process owing to its ATP-intensive requirements [220]. Nitrogenase catalyses this process, and is an enzyme consisting of two subunits: a molybdenum-iron and an iron protein, which are encoded by *nif* genes [221]. It is only in the *Methanosarcina* that such a wide range of *nif* genes and nitrogen fixation proteins are conserved, highlighting the metabolic diversity of this genus not only in methanogenesis, but also in nitrogen fixation [220]. In this study, several alternative nitrogenases involved in ammonium assimilation were found in higher relative abundance in biofilms of *M. barkeri* attached to PVC compared to PETG (Table 5.3). These proteins included two nitrogen regulatory proteins P-II (Mbar\_A2347, Mbar\_A0159), the two subunits of nitrogenase (Mbar\_A0347, Mbar\_A0167), and nitrogen signal transduction proteins (Mbar\_A3420, Mbar\_A0729, Mbar\_A2425, Mbar\_A2703).

Additionally, glutamine synthetase and glutamate synthase are the major enzymes involved in ammonium assimilation and amino acid synthesis, which were both found in higher relative abundance in *M. barkeri* biofilms attached to PVC compared to PETG (Mbar\_A2180, Mbar\_A0665). This suggests that biofilms of *M. barkeri* attached to PVC may require increased levels of nitrogen fixation to support higher levels of cellular growth during biofilm formation on PVC.

Mature biofilm formation is also associated with environmental stress, with the different metabolic activities of a biofilm creating varying gradients in nutrients, signalling compounds, oxygen and microbial waste within the biofilm [222]. Therefore, various stress-related proteins were found in higher relative abundance in *M. barkeri* biofilms attached to PVC, including two thermosome proteins (Mbar\_A1084, Mbar\_A1201), which are group II chaperonins essential for protein folding and for promoting cell survival in the face of environmental stress [206].

Six universal stress proteins were also two-fold higher in abundance in cells attached to PVC compared to PETG (Mbar\_A3527, Mbar\_A3529, Mbar\_A0825, Mbar\_A0487, Mbar\_A3147, Mbar\_A2586). These proteins are indicative of the establishment of a more developed biofilm on the surface of PVC, which is usually accompanied by higher levels of different metabolic activity and therefore, localised stress within the biofilm. This can result in the observed higher relative abundance of stress-related proteins in *M. barkeri* biofilms attached to PVC.

The higher relative abundance of other proteins related to stress from biofilms attached to PVC compared to PETG were observed (Table 5.5), including the group I chaperonins, DnaK heat shock protein 70 and heat shock protein 20 (Mbar\_A3433, Mbar\_A0859). These proteins have been reported to be differentially regulated in *M. barkeri* as a mechanism of cell survival under conditions of environmental stress [206] such as heat shock [161,223]. Additionally, the group I chaperonins, 60 kDa chaperonin (GroEL protein) (Mbar\_A1543) and 10 kDa chaperonin (GroES protein) (Mbar\_A1542) were identified in cells attached to PVC. These higher relative abundance proteins suggest that they may play an important role in the stress response of *M. barkeri* during biofilm formation on PVC.

5.3.5.4. Cellular processes

Multiple proteins involved in cellular processes, such as translation, transcription, replication and repair were found in higher relative abundance in biofilm cells of *M. barkeri* when attached to PVC compared to PETG (Table 5.4).

**Table 5.4** Proteins involved in cellular processes and found in higher relative abundance in biofilms on PVC with significant fold change (p ≤ 0.05).

|  |  |  |
| --- | --- | --- |
| **Loci** | **Protein** | **Fold change** |
| *Translation* | | |
| Mbar\_A0097 | 50S ribosomal protein L5 | 2.50 |
| Mbar\_A0095 | 30S ribosomal protein S8 | 2.33 |
| Mbar\_A0089 | 50S ribosomal protein L30P | 2.29 |
| Mbar\_A3224 | tRNA (guanine(26)-N(2))-dimethyltransferase | 2.25 |
| Mbar\_A3687 | 30S ribosomal protein S7 | 2.24 |
| Mbar\_A0094 | 50S ribosomal protein L6 | 2.21 |
| Mbar\_A0077 | 30S ribosomal protein S4 | 2.20 |
| Mbar\_A1008 | Arginine--tRNA ligase | 2.18 |
| Mbar\_A0101 | 30S ribosomal protein S17P | 2.13 |
| Mbar\_A0901 | Threonylcarbamoyl-AMP synthase | 2.12 |
| Mbar\_A3383 | 30S ribosomal protein S6e | 2.12 |
| Mbar\_A1873 | 30S ribosomal protein S15 | 2.10 |
| Mbar\_A1428 | 50S ribosomal protein L18e | 2.09 |
| Mbar\_A0078 | 30S ribosomal protein S13 | 2.09 |
| Mbar\_A0534 | Protein translation factor SUI1 homolog | 2.09 |
| Mbar\_A3231 | 50S ribosomal protein L21e | 2.08 |
| Mbar\_A0884 | Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B | 2.08 |
| Mbar\_A0614 | 50S ribosomal protein L1 | 2.08 |
| Mbar\_A3509 | RNase L inhibitor | 2.08 |
| Mbar\_A0092 | 50S ribosomal protein L19e | 2.06 |
| Mbar\_A0110 | 50S ribosomal protein L3 | 2.05 |
| Mbar\_A0282 | 30S ribosomal protein S24e | 2.03 |
| Mbar\_A0098 | 30S ribosomal protein S4e | 2.03 |
| Mbar\_A0454 | 30S ribosomal protein S19e | 2.02 |
| Mbar\_A2127 | 30S ribosomal protein S3Ae | 2.01 |
| Mbar\_A1332 | tRNA pseudouridine synthase A | 2.00 |
| Mbar\_A0839 | Pyrrolysine--tRNA ligase | 2.00 |
| Mbar\_A1149 | 30S ribosomal protein S17e | 1.99 |
| Mbar\_A0458 | 50S ribosomal protein L31e | 1.98 |
| Mbar\_A1427 | 50S ribosomal protein L13 | 1.98 |
| Mbar\_A3686 | Elongation factor 2 | 1.97 |
| Mbar\_A1569 | 50S ribosomal protein L44e | 1.97 |
| Mbar\_A3173 | Aspartate--tRNA(Asp/Asn) ligase | 1.95 |
| Mbar\_A3688 | 30S ribosomal protein S12 | 1.94 |
| Mbar\_A0615 | 50S ribosomal protein L11 | 1.92 |
| Mbar\_A0105 | 50S ribosomal protein L22 | 1.92 |
| Mbar\_A0108 | 50S ribosomal protein L23P | 1.91 |
| Mbar\_A3684 | 30S ribosomal protein S10 | 1.89 |
| Mbar\_A0279 | Probable bifunctional tRNA threonylcarbamoyladenosine biosynthesis protein | 1.88 |
| Mbar\_A2508 | 50S ribosomal protein L37Ae | 1.88 |
| Mbar\_A1470 | Glutamate--tRNA ligase | 1.87 |
| Mbar\_A0100 | 50S ribosomal protein L14 | 1.87 |
| Mbar\_A0109 | 50S ribosomal protein L4 | 1.86 |
| Mbar\_A0613 | 50S ribosomal protein L10 | 1.86 |
| Mbar\_A0971 | Translation elongation factor | 1.85 |
| Mbar\_A2092 | Alanyl-tRNA editing protein AlaX-M | 1.83 |
| Mbar\_A2048 | Leucine--tRNA ligase | 1.83 |
| Mbar\_A3388 | 50S ribosomal protein L7Ae | 1.82 |
| Mbar\_A1507 | Lysine--tRNA ligase | 1.82 |
| Mbar\_A0460 | 50S ribosomal protein L18Ae | 1.81 |
| Mbar\_A1976 | Valine--tRNA ligase | 1.80 |
| Mbar\_A0107 | 50S ribosomal protein L2 | 1.80 |
| Mbar\_A3465 | 30S ribosomal protein S8e | 1.79 |
| Mbar\_A1745 | Tyrosine--tRNA ligase | 1.79 |
| Mbar\_A3230 | tRNA pseudouridine synthase Pus10 | 1.78 |
| Mbar\_A3260 | Ribosomal RNA large subunit methyltransferase E | 1.77 |
| Mbar\_A3387 | 30S ribosomal protein S28e | 1.77 |
| Mbar\_A3384 | Probable translation initiation factor IF-2 | 1.77 |
| Mbar\_A0090 | 30S ribosomal protein S5 | 1.76 |
| Mbar\_A3229 | tRNA (pseudouridine(54)-N(1))-methyltransferase | 1.75 |
| Mbar\_A1007 | Peptide chain release factor subunit 1 | 1.75 |
| Mbar\_A2752 | Phenylalanine--tRNA ligase beta subunit | 1.74 |
| Mbar\_A1372 | Translation initiation factor 2 subunit beta | 1.73 |
| Mbar\_A1371 | 50S ribosomal protein L10e | 1.72 |
| Mbar\_A0104 | 30S ribosomal protein S3 | 1.72 |
| Mbar\_A0076 | 30S ribosomal protein S11 | 1.71 |
| Mbar\_A1204 | O-phosphoserine--tRNA(Cys) ligase | 1.70 |
| Mbar\_A0106 | 30S ribosomal protein S19 | 1.70 |
| Mbar\_A1375 | Phenylalanine--tRNA ligase alpha subunit | 1.70 |
| Mbar\_A0541 | Serine--tRNA ligase | 1.67 |
| Mbar\_A3685 | Elongation factor 1-alpha | 1.67 |
| Mbar\_A1567 | Translation initiation factor 2 subunit alpha | 1.63 |
| Mbar\_A1361 | Alanine--tRNA ligase | 1.63 |
| Mbar\_A1426 | 30S ribosomal protein S9 | 1.62 |
| Mbar\_A3440 | Elongation factor 1-beta | 1.58 |
| Mbar\_A0956 | tRNA-archaeosine synthase | 1.56 |
| Mbar\_A1789 | Ribonuclease Z | 1.55 |
| Mbar\_A0287 | Translation initiation factor 2 subunit gamma | 1.53 |
| Mbar\_A1088 | tRNA-guanine(15) transglycosylase | 1.51 |
| Mbar\_A1423 | 30S ribosomal protein S2 | 1.50 |
| Mbar\_A3187 | tRNA (cytidine(56)-2'-O)-methyltransferase | 1.47 |
| Mbar\_A2945 | Threonine--tRNA ligase | 1.47 |
| Mbar\_A1667 | Lysine--tRNA ligase | 1.44 |
| Mbar\_A0099 | 50S ribosomal protein L24P | 1.34 |
| Mbar\_A0543 | Methionine--tRNA ligase | 1.85 |
| Mbar\_A3613 | Isoleucine--tRNA ligase | 1.95 |
|  |  |  |
| *Transcription* | | |
| Mbar\_A3597 | Small nuclear ribonucleoprotein, LSM family | 2.48 |
| Mbar\_A2807 | Type 2 DNA topoisomerase 6 subunit B | 2.47 |
| Mbar\_A1333 | Transcriptional regulator, ArsR family | 2.38 |
| Mbar\_A3695 | DNA-directed RNA polymerase subunit H | 2.22 |
| Mbar\_A1459 | Transcriptional regulator, AsnC family | 2.14 |
| Mbar\_A2808 | Transcriptional regulator, AsnC family | 2.05 |
| Mbar\_A0196 | Transcription factor E | 2.00 |
| Mbar\_A1637 | DNA-directed RNA polymerase subunit L | 1.98 |
| Mbar\_A2509 | DNA-directed RNA polymerase subunit P | 1.97 |
| Mbar\_A0616 | Transcription elongation factor Spt5 | 1.97 |
| Mbar\_A0284 | Transcription elongation factor Spt4 | 1.96 |
| Mbar\_A1424 | DNA-directed RNA polymerase subunit K | 1.96 |
| Mbar\_A2535 | DNA-directed RNA polymerases I, II, and III | 1.92 |
| Mbar\_A2865 | Transcriptional regulator, ArsR family | 1.90 |
| Mbar\_A1033 | Transcriptional regulator, XRE family | 1.88 |
| Mbar\_A0285 | DNA-directed RNA polymerase, subunit E | 1.87 |
| Mbar\_A3689 | Probable transcription termination protein NusA | 1.85 |
| Mbar\_A0621 | Transcriptional regulator, XRE family | 1.83 |
| Mbar\_A2079 | Transcriptional regulator, XRE family | 1.82 |
| Mbar\_A3448 | Putative transcriptional regulator | 1.81 |
| Mbar\_A2410 | DNA topoisomerase 1 | 1.79 |
| Mbar\_A1729 | Transcriptional regulator, ArsR family | 1.79 |
| Mbar\_A0507 | Putative nickel-responsive regulator | 1.78 |
| Mbar\_A1270 | Transcriptional regulator | 1.76 |
| Mbar\_A3694 | DNA-directed RNA polymerase, subunit B | 1.76 |
| Mbar\_A3232 | DNA-directed RNA polymerase, subunit F | 1.75 |
| Mbar\_A2965 | Transcriptional regulator, ArsR family | 1.75 |
| Mbar\_A3693 | DNA-directed RNA polymerase subunit beta | 1.73 |
| Mbar\_A0787 | Transcriptional regulator, TetR family | 1.73 |
| Mbar\_A3691 | DNA-directed RNA polymerase subunit A | 1.72 |
| Mbar\_A3692 | DNA-directed RNA polymerase subunit | 1.70 |
| Mbar\_A1394 | Transcription initiation factor IIB | 1.65 |
| Mbar\_A3464 | Transcriptional regulator, AsnC family | 1.65 |
| Mbar\_A0193 | Cleavage and polyadenylation specificity factor | 1.63 |
| Mbar\_A3663 | Transcriptional regulator, MarR family | 1.57 |
| Mbar\_A0595 | TATA-box-binding protein | 1.85 |
| *Replication, recombination and repair* | | |
| Mbar\_A3500 | Putative snRNP Sm-like protein | 2.16 |
| Mbar\_A0879 | DNA polymerase sliding clamp | 2.09 |
| Mbar\_A0963 | Replication factor-A protein | 2.03 |
| Mbar\_A1205 | Translin family protein | 2.01 |
| Mbar\_A1899 | DNA ligase 2 | 1.98 |
| Mbar\_A2503 | Proteasome subunit alpha | 1.91 |
| Mbar\_A3508 | ATP-dependent DNA helicase Hel308 | 1.89 |
| Mbar\_A1418 | Ribonuclease J | 1.86 |
| Mbar\_A2507 | Exosome complex component Rrp42 | 1.86 |
| Mbar\_A2181 | D-aminoacyl-tRNA deacylase | 1.85 |
| Mbar\_A0959 | DNA primase DnaG | 1.82 |
| Mbar\_A0569 | Endonuclease III | 1.79 |
| Mbar\_A2014 | CCA-adding enzyme | 1.78 |
| Mbar\_A1582 | Replication factor C small subunit | 1.78 |
| Mbar\_A0145 | ATP-dependent RNA helicase | 1.75 |
| Mbar\_A0047 | Transposase, IS4 family | 1.73 |
| Mbar\_A2805 | DNA gyrase subunit B | 1.72 |
| Mbar\_A1037 | Anaerobic ribonucleoside-triphosphate reductase | 1.71 |
| Mbar\_A0455 | DNA-binding protein | 1.69 |
| Mbar\_A1593 | Replicative DNA helicase Mcm | 1.68 |
| Mbar\_A2708 | UvrABC system protein B | 1.67 |
| Mbar\_A1495 | DNA mismatch repair protein MutS | 1.63 |
| Mbar\_A1212 | Flap endonuclease 1 | 1.59 |
| Mbar\_A1777 | DNA polymerase | 1.50 |
| Mbar\_A2338 | DNA repair and recombination protein RadA | 1.49 |
| Mbar\_A2710 | UvrABC system protein A | 1.45 |
| Mbar\_A2804 | DNA gyrase subunit A | 1.26 |

Increased levels of protein synthesis are a recognised requirement for biofilm formation to support elevated levels of cell growth in the biofilm [224]. In this study, various ribosomal proteins, translation initiation factors (Mbar\_A0287, Mbar\_A1567, Mbar\_1372), and translation elongation factors (Mbar\_A0971, Mbar\_A3440) were found in higher relative abundance for biofilm cells attached to PVC compared to PETG, suggesting the establishment of a metabolically active and growing biofilm on PVC compared to PETG.

In addition, significant transcriptional changes were observed in biofilms of *M. barkeri* attached to PVC compared to PETG. The approximate two-fold increase in relative abundance of transcription initiation factor IIB (Mbar\_A1394) was observed, which is essential for initiating transcription in archaea, and is a homologue of the eukaryotic transcription initiation factor IIB [56]. Various transcriptional regulators were also found in higher relative abundance from different families, including those from the AsnC family (Mbar\_A1459, Mbar-A2808), the ArsR family (Mbar\_A2865, Mbar\_A1729, Mbar\_A2965), and the XRE family (Mbar\_A1033, Mbar\_A0621, Mbar\_A2079), as well as transcription elongation factors Spt5 and Spt4 (Mbar\_A0616, Mbar\_A0284). This suggests a key role of these proteins for transcription during biofilm formation of *M. barkeri* to a favourable support material, such as PVC.

Archaeal proteins involved in DNA replication are homologous to those from eukarya [225], and such proteins were found in higher relative abundance in cells of *M. barkeri* attached to PVC compared to PETG, such as replication factor-A protein (Mbar\_A0963) and replication factor C (Mbar\_A1582). The high abundance (20% of the identified proteome) of proteins involved in cellular processing in biofilms attached to PVC suggests an important role of these housekeeping proteins during biofilm formation in *M. barkeri*.

5.3.5.5. Substrate transport and folding

Biofilms of *M. barkeri* attached to PVC showed a higher relative abundance of various proteins involved in post-translational modification, chaperones and substrate transport compared to a lower protein abundance in biofilms attached to PETG (Table 5.5).

**Table 5.5** Proteins involved in substrate transport and folding and found in higher relative abundance in biofilms on PVC with significant fold change (p ≤ 0.05).

|  |  |  |
| --- | --- | --- |
| **Loci** | **Protein** | **Fold change** |
| *Post-translational modification, chaperones* | | |
| Mbar\_A1543 | 60 kDa chaperonin (GroEL protein) | 2.03 |
| Mbar\_A1542 | 10 kDa chaperonin (GroES protein) | 2.33 |
| Mbar\_A3434 | Protein GrpE (HSP-70 cofactor) | 2.22 |
| Mbar\_A1128 | Glycosyltransferase | 2.20 |
| Mbar\_A2249 | Peptidyl-prolyl cis-trans isomerase | 2.14 |
| Mbar\_A1209 | Glycyl-tRNA synthetase | 2.09 |
| Mbar\_A3433 | Chaperone protein DnaK (HSP70) | 2.07 |
| Mbar\_A2893 | O-linked N-acetylglucosamine transferase | 2.06 |
| Mbar\_A0883 | Glutamyl-tRNA(Gln) amidotransferase subunit A | 2.05 |
| Mbar\_A1032 | Putative aliphatic sulfonate binding protein | 2.03 |
| Mbar\_A3203 | SSU ribosomal protein S6P modification protein | 2.02 |
| Mbar\_A0859 | Heat shock protein Hsp20 | 1.90 |
| Mbar\_A3530 | PmbA/TldD family protein | 1.88 |
| Mbar\_A0061 | Probable deoxyhypusine synthase | 1.87 |
| Mbar\_A3532 | Lon-B peptidase, Serine peptidase, MEROPS family S16 | 1.87 |
| Mbar\_A0461 | Prefoldin subunit alpha | 1.86 |
| Mbar\_A3039 | Aminoacyl-histidine dipeptidase | 1.85 |
| Mbar\_A0194 | Proteasome subunit beta | 1.84 |
| Mbar\_A2319 | tRNA(Ile2) 2-agmatinylcytidine synthetase TiaS | 1.83 |
| Mbar\_A1004 | Signal sequence peptidase | 1.60 |
| Mbar\_A1806 | Heat shock protein Hsp20 | 1.58 |
| Mbar\_A3017 | UDP-galactopyranose mutase | 1.56 |
| Mbar\_A1615 | Oligopeptide ABC transporter, solute-binding protein | 1.90 |
| Mbar\_A3490 | ATPase, PilT family | 1.90 |
| Mbar\_A0400 | Proteasome-activating nucleotidase | 1.79 |
| Mbar\_A2900 | Diphthine synthase | 1.77 |
| Mbar\_A1307 | Molybdate-binding protein | 1.71 |
| Mbar\_A2175 | Ketoisovalerate ferredoxin oxidoreductase, gamma subunit | 1.70 |
| Mbar\_A0620 | Proteasome-activating nucleotidase | 1.67 |
| Mbar\_A1237 | Putative regulatory protein | 1.66 |
| Mbar\_A1238 | 4-methyl-5-(Beta-hydroxyethyl)thiazole monophosphate synthesis protein ThiF | 1.65 |
| Mbar\_A1316 | Methionine aminopeptidase | 1.64 |
| Mbar\_A3650 | Nascent polypeptide-associated complex protein | 2.25 |
|  |  |  |
| *Transport* | | |
| Mbar\_A1078 | Iron-regulated ABC transporter ATPase subunit SufC | 2.38 |
| Mbar\_A3459 | ABC transporter, ATP-binding protein | 2.21 |
| Mbar\_A1030 | ABC transporter, ATP-binding protein | 2.04 |
| Mbar\_A0995 | Zinc ABC transporter, ATP-binding protein | 1.86 |
| Mbar\_A1286 | ABC transporter, solute-binding protein | 1.84 |
| Mbar\_A1558 | Molybdenum ABC transporter, solute-binding | 1.63 |
| Mbar\_A1872 | Iron(III) dicitrate transport ATP-binding protein | 1.63 |
| Mbar\_A1265 | Putative molybdenum transport protein ModA | 1.62 |
| Mbar\_A0062 | ABC transporter, ATP-binding protein | 1.51 |
| Mbar\_A2562 | ABC transporter, ATP-binding protein | 2.01 |
| Mbar\_A3677 | Oligopeptide ABC transporter, oligopeptide-binding protein | 1.89 |
| Mbar\_A1284 | Oligopeptide ABC transporter, solute-binding protein | 1.83 |
| Mbar\_A3678 | Oligopeptide ABC transporter, oligopeptide-binding protein | 1.75 |
| Mbar\_A1269 | Transporter, RND superfamily | 1.74 |
| Mbar\_A0473 | Nickel ABC transporter, solute-binding protein | 1.71 |
| Mbar\_A2811 | Potassium channel protein | 1.71 |
| Mbar\_A0707 | ABC transporter, ATP-binding protein | 1.67 |
| Mbar\_A0851 | Protein-export membrane protein SecD | 2.25 |
| Mbar\_A2548 | Sec-independent protein translocase protein TatA | 2.17 |
| Mbar\_A1889 | P-type copper-transporting ATPase | 1.84 |
| Mbar\_A2549 | Sec-independent protein translocase protein TatA | 1.98 |

Archaea have a similar chaperonin system to bacteria for the folding of newly synthesised polypeptides and proteins [202]. The heat shock protein 70 (DnaK) system ensures that newly synthesised nascent polypeptides do not aggregate before or after translation, and are finally folded by the GroEL/GroS system [223,226]. In biofilms of *M. barkeri* attached to PVC, there was an observed higher relative abundance of heat shock protein 70 (Mbar\_A3433), GroEL protein (Mbar\_A1543) and GroES protein (Mbar\_A1542) chaperonins, as well as other chaperonins for protein folding, such as heat shock protein 20 (Mbar\_A3433, Mbar\_A0859).

Furthermore, peptidyl-prolyl cis-trans isomerase (Mbar\_A2249) and a nascent polypeptide-associated complex protein (Mbar\_A3650) were also found in higher relative abundance in biofilms of *M. barkeri* attached to PVC compared to PETG. These proteins are also involved in the folding of newly synthesised proteins and polypeptides [216]. Their expression in response to attachment to PVC could suggest a necessity for elevated levels of protein synthesis to keep up with increased growth rates as cells grow and irreversibly attach to PVC as a biofilm.

In association with the higher relative abundance of various proteins involved in the folding of newly synthesised proteins and polypeptides, was the higher relative abundance of numerous transmembrane enzymes specific for protein export across the cytoplasmic membrane (Table 5.5) in cells attached to PVC. The ability to transport proteins across membranes is vital for cell viability and is regulated by the external environment [217]. Various ABC transporters and Sec proteins for translocation were found in two-fold higher levels of abundance. Therefore, the higher relative abundance of numerous protein and polypeptide folding and transport proteins in biofilms of *M. barkeri* attached to PVC compared to PETG suggests an important role of these proteins in the production and secretion of EPS and biofilm components during biofilm formation on PVC.

5.3.5.6. Metabolism

A large abundance of proteins (comprising 32% of the identified proteome in this study) involved in central metabolic processes for amino acid, lipid, nucleotide, energy and cofactor transport and synthesis were found in higher relative abundance in cells attached to PVC compared to PETG. These proteins can be seen in Table 5.6.

**Table 5.6** Proteins involved in metabolism and found in higher relative abundance in biofilms on PVC with significant fold change (p ≤ 0.05).

|  |  |  |
| --- | --- | --- |
| **Loci** | **Protein** | **Fold change** |
| *Amino acid transport and metabolism* | | |
| Mbar\_A1100 | Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase | 2.47 |
| Mbar\_A2320 | NH(3)-dependent NAD(+) synthetase | 2.40 |
| Mbar\_A1514 | Imidazole glycerol phosphate synthase subunit HisF subunit HisF) | 2.25 |
| Mbar\_A0753 | 2-amino-3,7-dideoxy-D-threo-hept-6-ulosonate synthase | 2.23 |
| Mbar\_A0921 | 3-dehydroquinate synthase | 2.18 |
| Mbar\_A0482 | Gamma-glutamyl phosphate reductase | 2.15 |
| Mbar\_A0920 | 2-amino-3,7-dideoxy-D-threo-hept-6-ulosonate synthase | 2.13 |
| Mbar\_A1023 | 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase | 2.12 |
| Mbar\_A1041 | Putative methylthioribose-1-phosphate isomerase | 2.12 |
| Mbar\_A3512 | Ornithine carbamoyltransferase | 2.11 |
| Mbar\_A1565 | 3-isopropylmalate dehydratase | 2.11 |
| Mbar\_A1294 | Phosphoserine aminotransferase | 2.10 |
| Mbar\_A1641 | Diaminopimelate decarboxylase | 2.09 |
| Mbar\_A3654 | Phosphoribosyl-AMP cyclohydrolase | 2.08 |
| Mbar\_A2049 | Threonine synthase | 2.07 |
| Mbar\_A1339 | 3-isopropylmalate dehydrogenase | 2.04 |
| Mbar\_A2427 | O-acetylhomoserine sulfhydrolase | 2.03 |
| Mbar\_A3624 | Anthranilate phosphoribosyltransferase | 2.00 |
| Mbar\_A0220 | Ketol-acid reductoisomerase | 2.00 |
| Mbar\_A1638 | O-phospho-L-seryl-tRNA:Cys-tRNA synthase | 1.99 |
| Mbar\_A3476 | Imidazole glycerol phosphate synthase subunit HisH | 1.99 |
| Mbar\_A2257 | Asparagine synthetase | 1.99 |
| Mbar\_A1067 | Hydantoinase | 1.96 |
| Mbar\_A0004 | Argininosuccinate lyase | 1.96 |
| Mbar\_A3625 | N-(5'-phosphoribosyl)anthranilate isomerase | 1.96 |
| Mbar\_A3626 | Anthranilate synthase, component I | 1.95 |
| Mbar\_A0942 | 2-isopropylmalate synthase | 1.94 |
| Mbar\_A3252 | 5'-deoxyadenosine deaminase | 1.93 |
| Mbar\_A3503 | Tryptophan synthase beta chain | 1.93 |
| Mbar\_A2069 | Dihydroxy-acid dehydratase | 1.92 |
| Mbar\_A2422 | Cysteine synthase | 1.92 |
| Mbar\_A2756 | Phosphoribosylformylglycinamidine synthase PurS | 1.91 |
| Mbar\_A0058 | Indolepyruvate oxidoreductase subunit IorA | 1.91 |
| Mbar\_A1590 | Phosphoserine phosphatase | 1.90 |
| Mbar\_A3251 | Adenosylhomocysteinase | 1.90 |
| Mbar\_A3507 | Histidinol dehydrogenase | 1.88 |
| Mbar\_A0666 | Glutamine synthetase | 1.87 |
| Mbar\_A2428 | Homoserine O-acetyltransferase | 1.86 |
| Mbar\_A0624 | 2-isopropylmalate synthase | 1.85 |
| Mbar\_A3140 | Mannose-6-phosphate isomerase, type 2 | 1.85 |
| Mbar\_A2192 | 2-isopropylmalate synthase | 1.83 |
| Mbar\_A0760 | Aspartate-semialdehyde dehydrogenase | 1.82 |
| Mbar\_A0210 | ACT domain protein | 1.82 |
| Mbar\_A3627 | Anthranilate synthase, component II | 1.81 |
| Mbar\_A1312 | ATP phosphoribosyltransferase | 1.79 |
| Mbar\_A0872 | Histidinol-phosphate aminotransferase | 1.76 |
| Mbar\_A2375 | Carbamoyl-phosphate synthase small chain | 1.76 |
| Mbar\_A2421 | Serine O-acetyltransferase | 1.76 |
| Mbar\_A2177 | Ornithine decarboxylase | 1.75 |
| Mbar\_A2933 | Hydantoinase/oxoprolinase | 1.75 |
| Mbar\_A2083 | Aminotransferase | 1.74 |
| Mbar\_A0861 | Aspartokinase | 1.70 |
| Mbar\_A3622 | Tryptophan synthase beta chain | 1.70 |
| Mbar\_A2080 | Phosphoserine aminotransferase apoenzyme/L-aspartate aminotransferase apoenzyme | 1.67 |
| Mbar\_A0481 | Glutamate 5-kinase | 1.65 |
| Mbar\_A0924 | Prephenate dehydrogenase | 1.64 |
| Mbar\_A2373 | Argininosuccinate synthase | 1.64 |
| Mbar\_A3438 | 1-pyrroline-5-carboxylate synthetase | 1.64 |
| Mbar\_A0217 | Putative (R)-citramalate synthase CimA | 1.64 |
| Mbar\_A1068 | Hydantoinase | 1.63 |
| Mbar\_A1431 | D-3-phosphoglycerate dehydrogenase | 1.59 |
| Mbar\_A1338 | 3-isopropylmalate dehydratase small subunit | 1.56 |
| Mbar\_A1313 | S-adenosylmethionine synthase | 1.55 |
| Mbar\_A1961 | 3-isopropylmalate dehydratase large subunit | 1.55 |
| Mbar\_A1137 | Aspartate aminotransferase | 1.54 |
| Mbar\_A2167 | 3-isopropylmalate dehydratase large subunit | 1.48 |
| Mbar\_A0576 | Branched chain amino acid aminotransferase apoenzyme | 1.43 |
| Mbar\_A2491 | Hydantoinase | 1.30 |
|  |  |  |
| *Carbohydrate transport and metabolism* | | |
| Mbar\_A0934 | Triosephosphate isomerase | 2.76 |
| Mbar\_A0902 | Ribulose bisphosphate carboxylase | 2.28 |
| Mbar\_A2222 | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase 2 | 2.17 |
| Mbar\_A2100 | Putative ribose 1,5-bisphosphate isomerase | 2.09 |
| Mbar\_A2189 | Glyceraldehyde-3-phosphate dehydrogenase 1 | 2.01 |
| Mbar\_A1170 | 3-hexulose-6-phosphate synthase | 1.94 |
| Mbar\_A2022 | Glutamine--fructose-6-phosphate aminotransferase | 1.92 |
| Mbar\_A1750 | Glucose-6-phosphate isomerase | 1.92 |
| Mbar\_A2997 | Glycogen phosphorylase | 1.91 |
| Mbar\_A2315 | Bifunctional protein FolD | 1.83 |
| Mbar\_A0214 | Cellulase | 1.82 |
| Mbar\_A2850 | Enolase | 1.81 |
| Mbar\_A1808 | Phosphoglycerate kinase | 1.79 |
| Mbar\_A3188 | AMP phosphorylase | 1.75 |
| Mbar\_A0860 | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | 1.74 |
| Mbar\_A0935 | Bifunctional enzyme Fae/Hps phosphate synthase | 1.73 |
| Mbar\_A2004 | ADP-dependent phosphofructokinase | 1.71 |
| Mbar\_A0623 | Isocitrate dehydrogenase | 1.71 |
| Mbar\_A1120 | UDP-glucose 6-dehydrogenase | 1.68 |
| Mbar\_A2223 | Phosphoenolpyruvate synthase | 1.56 |
| Mbar\_A3132 | 3-hexulose-6-phosphate isomerase | 1.52 |
| Mbar\_A1836 | Fructose-1,6-bisphosphatase | 1.50 |
| Mbar\_A0538 | Alpha-amylase | 1.46 |
|  |  |  |
| *Cofactor transport and metabolism* | | |
| Mbar\_A2316 | Serine hydroxymethyltransferase | 2.26 |
| Mbar\_A1101 | (5-formylfuran-3-yl)methyl phosphate synthase | 2.25 |
| Mbar\_A0160 | UbiE/COQ5 methyltransferase | 2.21 |
| Mbar\_A3191 | Probable cobyric acid synthase | 2.18 |
| Mbar\_A0131 | Thiamin-monophosphate kinase | 2.16 |
| Mbar\_A2565 | Cobalamin biosynthesis protein | 2.12 |
| Mbar\_A0575 | Molybdopterin molybdochelatase | 1.98 |
| Mbar\_A1178 | GMP synthase | 1.98 |
| Mbar\_A1463 | Delta-aminolevulinic acid dehydratase | 1.97 |
| Mbar\_A0862 | Phosphoribosylformylglycinamidine cyclo-ligase | 1.96 |
| Mbar\_A1367 | Molybdenum cofactor biosynthesis protein B | 1.95 |
| Mbar\_A2129 | Phosphomethylpyrimidine synthase 2 | 1.93 |
| Mbar\_A1599 | CoB--CoM heterodisulfide reductase iron-sulfur subunit D | 1.90 |
| Mbar\_A0977 | Probable L-tyrosine/L-aspartate decarboxylase | 1.90 |
| Mbar\_A3731 | Cobaltochelatase CobN subunit | 1.87 |
| Mbar\_A1493 | Cobalt-precorrin-5B C(1)-methyltransferase | 1.87 |
| Mbar\_A2082 | 6,7-dimethyl-8-ribityllumazine synthase | 1.85 |
| Mbar\_A0597 | Phosphomethylpyrimidine synthase 1 | 1.85 |
| Mbar\_A3418 | 2-phospho-L-lactate guanylyltransferase | 1.84 |
| Mbar\_A1866 | Probable L-aspartate dehydrogenase | 1.83 |
| Mbar\_A0630 | Precorrin-8X methylmutase | 1.79 |
| Mbar\_A0627 | Cobalt-factor II C20-methyltransferase | 1.78 |
| Mbar\_A1183 | GTP cyclohydrolase MptA | 1.77 |
| Mbar\_A2872 | Phenylacetate-CoA ligase | 1.76 |
| Mbar\_A0489 | 2, 5-diamino-6-(5-phosphoribosylamino)pyrimidin-4(3H)-one reductase | 1.75 |
| Mbar\_A1865 | Quinolinate synthase A | 1.75 |
| Mbar\_A3580 | Pyridoxal 5'-phosphate synthase subunit PdxT | 1.71 |
| Mbar\_A3453 | L-threonine O-3-phosphate decarboxylase | 1.71 |
| Mbar\_A1524 | 3,4-dihydroxy-2-butanone 4-phosphate synthase | 1.70 |
| Mbar\_A3417 | FO synthase subunit 2 | 1.70 |
| Mbar\_A1187 | Cobyrinate a,c-diamide synthase | 1.68 |
| Mbar\_A1631 | 2-phospho-L-lactate transferase | 1.67 |
| Mbar\_A0714 | Aconitase | 1.65 |
| Mbar\_A2081 | Riboflavin synthase | 1.63 |
| Mbar\_A1279 | UbiE/COQ5 methyltransferase | 1.63 |
| Mbar\_A0348 | Cobyrinate a,c-diamide synthase | 1.62 |
| Mbar\_A1523 | Riboflavin kinase | 1.62 |
| Mbar\_A1776 | Protoporphyrin IX magnesium-chelatase | 1.62 |
| Mbar\_A1458 | Heme biosynthesis protein | 1.61 |
| Mbar\_A1588 | Biotin--(Acetyl-COA-carboxylase) synthetase | 1.60 |
| Mbar\_A1867 | Nicotinate-nucleotide pyrophosphorylase | 1.60 |
| Mbar\_A0625 | Probable cobalt-precorrin-6B C(15)-methyltransferase | 1.57 |
| Mbar\_A1222 | Beta-ribofuranosylaminobenzene 5'-phosphate synthase | 1.54 |
| Mbar\_A0806 | Beta-ribofuranosylaminobenzene 5'-phosphate synthase | 1.51 |
| Mbar\_A3254 | Pantothenate synthetase | 1.50 |
| Mbar\_A2986 | Phenylacetate-CoA ligase | 1.49 |
| Mbar\_A0951 | (S)-2-hydroxy-acid dehydrogenase | 1.49 |
| Mbar\_A1190 | Probable cyclic pyranopterin monophosphate synthase | 1.46 |
| Mbar\_A0244 | Methanogen homoaconitase small subunit | 1.45 |
| Mbar\_A0344 | Sirohydrochlorin cobaltochelatase | 1.44 |
| Mbar\_A0629 | Precorrin-3B C17-methyltransferase | 1.43 |
| Mbar\_A3416 | FO synthase subunit 2 | 1.37 |
| Mbar\_A0256 | Nicotinamide-nucleotide adenylyltransferase | 0.68 |
|  |  |  |
| *Energy production and conversion* | | |
| Mbar\_A3099 | H(+)-transporting ATP synthase, subunit alpha | 3.27 |
| Mbar\_A1597 | Sulfite reductase, assimilatory-type | 2.39 |
| Mbar\_A0388 | V-type ATP synthase subunit C | 2.21 |
| Mbar\_A0086 | Adenylate kinase | 2.15 |
| Mbar\_A2618 | Sulfite reductase, beta subunit | 2.11 |
| Mbar\_A1258 | Tetrahydromethanopterin S-methyltransferase subunit A | 2.04 |
| Mbar\_A1847 | Methanophenazine-reducing hydrogenase, small subunit | 2.01 |
| Mbar\_A0392 | H(+)-transporting ATP synthase, subunit H | 2.00 |
| Mbar\_A0384 | V-type ATP synthase subunit D | 1.95 |
| Mbar\_A2455 | Ech hydrogenase subunit C | 1.95 |
| Mbar\_A3458 | ABC transporter, ATP-binding protein | 1.91 |
| Mbar\_A0255 | F420H2 dehydrogenase subunit F | 1.90 |
| Mbar\_A0385 | V-type ATP synthase beta chain | 1.90 |
| Mbar\_A0386 | V-type ATP synthase alpha chain | 1.89 |
| Mbar\_A1255 | Tetrahydromethanopterin S-methyltransferase subunit H | 1.88 |
| Mbar\_A0391 | V-type ATP synthase subunit I | 1.83 |
| Mbar\_A0389 | V-type ATP synthase subunit E | 1.81 |
| Mbar\_A0387 | V-type ATP synthase subunit F | 1.75 |
| Mbar\_A1002 | Pyruvate synthase subunit PorC | 1.71 |
| Mbar\_A1000 | Pyruvate synthase subunit PorA | 1.62 |
|  |  |  |
| *Inorganic ion transport and metabolism* | | |
| Mbar\_A3651 | HesB protein | 2.02 |
| Mbar\_A0228 | Sulfide dehydrogenase | 1.99 |
| Mbar\_A2290 | Coenzyme F420-reducing hydrogenase, beta subunit | 1.98 |
| Mbar\_A0503 | Aldehyde dehydrogenase | 1.98 |
| Mbar\_A1793 | Metallo cofactor biosynthesis protein | 1.93 |
| Mbar\_A0187 | K(+)-insensitive pyrophosphate-energized proton pump | 1.90 |
| Mbar\_A1195 | Polyphosphate kinase | 1.89 |
| Mbar\_A1851 | Hydrogenase expression/formation protein | 1.86 |
| Mbar\_A1868 | Iron(III) dicitrate-binding protein | 1.84 |
| Mbar\_A1848 | Hydrogenase expression/formation protein | 1.76 |
| Mbar\_A1846 | Methanophenazine-reducing hydrogenase, large subunit (EC 1.12.98.3) | 1.73 |
| Mbar\_A3322 | NADH-dependent flavine oxidoreductase | 1.73 |
| Mbar\_A1781 | Phosphate import ATP-binding protein PstB | 1.71 |
| Mbar\_A0227 | Sulfide dehydrogenase | 1.69 |
| Mbar\_A1852 | Hydrogenase expression/formation protein | 1.56 |
| Mbar\_A1849 | Hydrogenase expression/formation protein | 1.54 |
| Mbar\_A3112 | Potassium channel protein | 1.53 |
| Mbar\_A0696 | Aldehyde ferredoxin oxidoreductase | 1.49 |
| Mbar\_A0771 | Zinc-binding alcohol dehydrogenase | 1.47 |
| Mbar\_A0634 | Iron-sulfur cluster carrier protein | 1.43 |
|  |  |  |
| *Lipid transport and metabolism* | | |
| Mbar\_A1040 | Myo-inositol-1-phosphate synthase | 2.00 |
|  |  |  |
| *Nucleotide transport and metabolism* | | |
| Mbar\_A1467 | Probable dihydroorotate dehydrogenase B | 2.15 |
| Mbar\_A2389 | Glutamyl-tRNA(Gln) amidotransferase subunit E | 2.02 |
| Mbar\_A1192 | 5'-nucleotidase SurE | 1.99 |
| Mbar\_A0403 | Adenylosuccinate synthetase | 1.90 |
| Mbar\_A1168 | Aspartate carbamoyltransferase | 1.88 |
| Mbar\_A2317 | Formyltetrahydrofolate-dependent phosphoribosylglycinamide formyltransferase | 1.88 |
| Mbar\_A0060 | Orotidine 5'-phosphate decarboxylase | 1.84 |
| Mbar\_A1167 | Aspartate carbamoyltransferase regulatory chain | 1.77 |
| Mbar\_A0571 | IMP cyclohydrolase | 1.60 |
| Mbar\_A0258 | Putative adenylate kinase | 1.48 |
|  |  |  |
| *Secondary metabolites transport and metabolism* | | |
| Mbar\_A1438 | Chorismate synthase | 2.52 |
| Mbar\_A0626 | Cobalt-factor II C20-methyltransferase | 2.19 |
| Mbar\_A2424 | Cysteine desulfurase IscS | 2.03 |
| Mbar\_A1972 | 3-hydroxy-3-methylglutaryl coenzyme A reductase | 2.00 |
| Mbar\_A3126 | Chorismate mutase | 1.98 |
| Mbar\_A3127 | Shikimate kinase | 1.90 |
| Mbar\_A2214 | 2-oxoacid ferredoxin oxidoreductase, subunit alpha | 1.88 |
| Mbar\_A0721 | Alpha-phosphoglucomutase | 1.88 |
| Mbar\_A0363 | Ribose-phosphate pyrophosphokinase | 1.86 |
| Mbar\_A0137 | 7-cyano-7-deazaguanine synthase | 1.86 |
| Mbar\_A3192 | Alanine dehydrogenase | 1.85 |
| Mbar\_A1462 | Glutamyl-tRNA reductase | 1.85 |
| Mbar\_A0550 | Acetyl-CoA acyltransferase | 1.84 |
| Mbar\_A0199 | Acetyl-CoA decarbonylase/synthase gamma subunit | 1.82 |
| Mbar\_A3513 | Phosphoribosylamine--glycine ligase | 1.80 |
| Mbar\_A3057 | dCMP deaminase | 1.80 |
| Mbar\_A1396 | Pyruvate phosphate dikinase | 1.80 |
| Mbar\_A1792 | Uroporphyrinogen-III synthase | 1.79 |
| Mbar\_A3471 | PyrE-like protein 2 | 1.77 |
| Mbar\_A1465 | Probable porphobilinogen deaminase | 1.77 |
| Mbar\_A0211 | Phenylacetate-CoA ligase | 1.76 |
| Mbar\_A0673 | Adenylosuccinate lyase | 1.76 |
| Mbar\_A1821 | Phosphotransacetylase | 1.72 |
| Mbar\_A0057 | Indolepyruvate ferredoxin oxidoreductase, subunit iorB | 1.70 |
| Mbar\_A0531 | Phosphoribosylaminoimidazole-succinocarboxamide synthase | 1.70 |
| Mbar\_A1791 | S-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase | 1.69 |
| Mbar\_A0762 | AIR carboxylase | 1.68 |
| Mbar\_A1586 | Pyruvate carboxylase subunit B | 1.67 |
| Mbar\_A1587 | Pyruvate carboxylase subunit A | 1.66 |
| Mbar\_A0536 | Phosphoribosylformylglycinamidine synthase subunit PurL | 1.64 |
| Mbar\_A1464 | Glutamate-1-semialdehyde 2,1-aminomutase | 1.58 |
| Mbar\_A0891 | 3-phosphoshikimate 1-carboxyvinyltransferase | 1.52 |
| Mbar\_A3498 | Amidophosphoribosyltransferase | 1.51 |
| Mbar\_A2417 | Fumarase beta subunit | 1.40 |
| Mbar\_A2755 | Phosphoribosylformylglycinamidine synthase subunit PurQ | 1.40 |

Nicotinamide-nucleotide adenylyltransferase is involved in the synthesis of nicotinamide adenine dinucleotide (NAD+). NAD+ is a main substrate in various intracellular metabolic and regulatory processes, involved in the breakdown of nutrients, and the biosynthesis of new macromolecules in archaea [227]. This protein (Mbar\_A0256) was found in higher relative abundance in biofilms attached to PETG, and may suggest that biofilms on PETG are metabolically active, but have not yet triggered biofilm formation to the same extent as biofilms on PVC. This finding corresponds to the results from the biofilm accumulation experiment (Figs. 5.2-5.3).

Evidence for changes in carbon and energy metabolism were given by the higher relative abundance of proteins involved in gluconeogenesis and the tricarboxylic acid (TCA) cycle. Archaea use a modified Embden-Meyerhof pathway for gluconeogenesis, which is the synthesis of glucose 6-phosphate from pyruvate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is exclusively involved in gluconeogenesis in archaea, and catalyses the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate [228]. This protein was two-fold higher in abundance in biofilms of *M. barkeri* attached to PVC compared to PETG, as well as other proteins involved in gluconeogenesis, such as fructose-1,6-bisphosphatase (Mbar\_A1836), phosphoenolpyruvate synthase (Mbar\_A2223), phosphoglycerate kinase (Mbar\_A1808) and enolase (Mbar\_A2850) [229]. The higher relative abundance of these proteins in biofilms attached to PVC may correspond to the similarly high relative abundance of glycosyltransferases, suggesting that cells may be using precursors from gluconeogenesis for N-glycosylation.

In terms of energy production from carbohydrate metabolism, methanogens are known to utilise an incomplete TCA cycle for the generation of energy from acetyl-CoA [230]. Key biosynthetic intermediates arising from this incomplete TCA cycle were found in higher relative abundance in *M. barkeri* cells attached to PVC compared to PETG, such as aconitase (Mbar\_A0714) and isocitrate dehydrogenase (Mbar\_A0623). Therefore, this could suggest a larger requirement for energy from more established biofilms attached to PVC compared to PETG in order to drive the large number of amino acid, carbohydrate, nucleotide, cofactor and lipid metabolism pathways observed in *M. barkeri* when attached to PVC (Table 5.6), as well as cell growth and metabolism during biofilm formation. Indeed, the increased abundance of proteins involved in energy production and carbohydrate metabolism has been reported in more developed biofilms of bacterial systems [90].

###### 5.3.6. Validation of iTRAQ results

Two proteins (fumarase and triose phosphate isomerase) were chosen to validate the protein quantification results obtained from the iTRAQ analysis, based on the significance of their fold changes and on the commercial availability of these enzyme assay kits.

The results from the triose phosphate isomerase activity (TPI) assay revealed that *M. barkeri* cells attached to PVC had a TPI activity of 0.012 nmol/min/µl compared to cells attached to PETG which exhibited a TPI activity of 0.006 nmol/min/µl. This is a 2-fold increase of TPI activity from PVC-biofilm cells, which corresponds with the expected fold change of 2.76 for TPI in PVC-biofilm cells as determined by iTRAQ analysis (Table 5.6).

However, the fumarase activity assay revealed that *M. barkeri* cells attached to PVC had a fumarase activity of 0.017 nmol/min/µl compared to cells attached to PETG which exhibited a fumarase activity of 0.0012 nmol/min/µl. This is a 14-fold increase of fumarase activity from PVC-biofilm cells, whereas a much lower fold change of 1.40 from PVC-biofilm cells was determined by iTRAQ analysis (Table 5.6).

# 5.4. Discussion

Despite the increasing interest in biofilms, little is known of the effect of the attachment substratum on the cell physiology of adherent cells. This study aimed to describe the changes in protein expression from biofilms of *M. barkeri* attached to two different support materials, PVC and PETG, after 96 h, and revealed that biofilms were physiologically distinct depending on the support material they were attached to. A biofilm mode of growth on PVC caused the increased relative abundance of 730 proteins involved in methanogenesis, cellular growth, central metabolism, energy conservation, and cellular processes compared to biofilms attached to PETG. A biofilm mode of growth on PETG caused the increased relative abundance of 2 proteins that were found in lower relative abundance in biofilms attached to PVC.

Past studies have shown that environmental conditions, such as temperature, nutrients and pH, signal the modified regulation of protein expression in cells to facilitate adhesion and biofilm formation [89,231,232]. The support materials tested in this study, PVC and PETG, can also be regarded as an environmental factor that can signal an altered proteome in *M. barkeri* for biofilm formation.

The results suggest that PVC provides an ideal attachment substratum and microenvironment that promotes a higher level of adhesion and cell accumulation compared to PETG. Preferential adhesion to PVC was predicted to be a result of a net attractive interaction energy and possible polymer bridging between the surfaces of *M. barkeri* and PVC in chapter 3. In this chapter, the more rapid biofilm formation of *M. barkeri* cells to PVC after 96 h was associated with the higher relative abundance of numerous proteins involved in a more developed stage of biofilm formation (Tables 5.1-5.6).

The previous two chapters have shown that PETG has a surface that is relatively incompatible for biofilm formation from *M. barkeri* compared to PVC, eliciting low levels of attachment. In this chapter, results showed that the lower level of adhesion and slower accumulation of cells to the surface of PETG produced a proteomic profile of a less developed biofilm compared to PVC. Cells of *M. barkeri* could be experiencing an extensive initial lag phase in growth in biofilms when grown with PETG, in which cells were slowly adapting to attachment and growth on PETG. Such a lag phase in biofilm development has been reported for *Bacillus cereus* [231], where an extensive lag time in growth was experienced by cells after initial attachment to glass wool and glass slides, respectively.

This observation is supported by the results from the biofilm accumulation experiment in this study, where PETG experienced a low accumulation of cells, mannose and glucose residues throughout the time course compared to biofilms on PVC (Fig. 5.2). In biofilms attached to PVC, distinct phenotypic differences were observed at each time point, which could correlate to three distinct developmental stages of biofilm formation: the lower levels of cell and mannose and glucose residues detected at day 2 could describe the initial adhesion of cells to the abiotic substratum. The irreversible adhesion of cells may be indicated by the higher accumulation of cells and sugar residues at the PVC surface on day 4, followed by the establishment of a more developed biofilm presumably experiencing nutrient depletion and potential biofilm dispersal, as indicated by the slightly lower levels of cell and sugar residue accumulation at day 6 (Fig. 5.2). It is interesting to note that these distinct biofilm phenotypes were lacking in cells attached to PETG, suggesting a much slower biofilm developmental process on this surface compared to PVC.

It can be observed from Fig. 5.3 that the ConA signals detected for *M. barkeri* attached on PVC and PETG did not overlap or closely correspond with the DAPI-stained cells. Previous studies have reported the overlapping of ConA and DAPI signals from the EPS of *Sulfolobus* biofilms[64] and in microbial granules from granular sludge [140]. It is possible that some disruption to the structure of *M. barkeri* biofilms may have occurred during the multiple washing steps involved in fluorescent staining the biofilms. However, Strathmann *et al.* [135]observed an uneven distribution of ConA-stained components from biofilms of *Pseudomonas aeruginosa,* and that some cells were not surrounded by ConA-stained components. This observation is typical of microbial aggregates and can be attributed to the heterogeneous production of EPS from biofilm-forming communities [135].The different phenotypic characteristics of biofilm development in *M. barkeri* observed in the biofilm accumulation experiment have also been observed in various microbial systems, and have been linked to the differential regulation of proteins [231]. Sauer *et al.* (2002) detected five distinct phases of biofilm development from the constant microscopic analysis of biofilms of *Pseudomonas aeruginosa* over a time course of 12 days, from initial adhesion through to biofilm dispersion. Each biofilm developmental phase was also associated with a distinct expression of proteins [87]. In this study, PVC and PETG was shown to elicit different developmental stages of biofilm formation from *M. barkeri* over 96 h as evidenced by results from the biofilm accumulation and proteomic experiments.

It is well known that the microbial cell surface is the interface with the environment and in archaea, significant changes to the lipid membrane [210,233] and surface-exposed proteins, including those in the S-layer and methanochondroitin layer of *Methanosarcina,* have been reported [59,234]. In this study, proteins involved in lipid membrane biogenesis, such as mevalonate kinase and farnesyl-diphosphate synthase/geranylgeranyl-diphosphate synthase, and proteins involved in EPS production and in the N-glycosylation of surface-exposed proteins in the methanochondroitin and S-layer were all found in higher relative abundance in biofilms of *M. barkeri* attached to PVC compared to PETG (Table 5.2). For example, glucose-1-phosphate thymidylyltransferase (Mbar\_A2020) was detected in cells attached to PVC, and has previously been identified in *Haloferax volcanii* [235] and *M. voltae* [184] as being a key enzyme involved in the *N-*glycosylation of S-layer glycoproteins.

All archaeal surface-exposed proteins are modified by N-glycosylation [59], which is the attachment of an oligosaccharide specifically to the asparagine residue of a target protein [215]. This process is naturally conserved across all three domains of life [184], and in archaea, N-glycosylation of surface proteins provides essential S-layer stability and maintenance, and plays a key role in allowing cells to adapt and respond to various environmental triggers [59,215,234].

Previous studies have shown that *S. acidocaldarius* differentially regulates proteins involved in N-glycosylationin response to high temperatures, as has *Haloferax volcanii* in response to high salt levels[234]. In this study, PVC was the environmental trigger for the induction of a more developed biofilm from *M. barkeri*, causing the higher relative abundance of key cell surface-associated proteins to facilitate irreversible attachment. Similar findings have also been observed in mature biofilms of *Pseudomonas aeruginosa,* which had a higher relative abundance of proteins involved in outer membrane biogenesis compared to biofilms at the initial stages of biofilm formation [90], and in *Escherichia coli*, which upregulated outer membrane proteinsduring biofilm formation [91]. These results also further emphasise the important role of extracellular polymers and proteins in biofilm development, as previously reported [30,62].

Additionally, the higher relative abundance of four uncharacterised proteins that could be associated with major S-layer proteins [213] (Mbar\_2011, Mbar\_A1557, Mbar\_A2016 and Mbar\_A1034) from biofilms of *M. barkeri* attached to PVC, is further suggestive of a role of the microbial surface in mediating irreversible adhesion and a more developed biofilm. This study has demonstrated the important changes to the cell surface that occur in *M. barkeri* during the transitional episodes of biofilm development, from early stage biofilms on PETG to more developed biofilms on PVC.

The irreversible attachment of cells to an abiotic surface is characterised not only by the production of EPS, but also with a rapid increase in cell population [236]. Therefore, the formation of a more developed biofilm requires a higher input of energy, cell material and metabolism to keep up with the demands of a biofilm lifestyle [63,224]. In this study, numerous proteins involved in amino acid metabolism, the TCA cycle, methanogenesis, gluconeogenesis, protein synthesis and energy production were found in higher relative abundance in the more developed biofilms attached to PVC compared to PETG. Similar findings have been found in mature biofilms of *P. aeruginosa* compared to early stage biofilms [90] and in biofilms of *S. acidocaldarius*, *S. solfataricus*, and *S. tokodaii* compared to planktonic cells [89].

However, biofilms attached to PVC showed a lower relative abundance of nicotinamide-nucleotide adenylyltransferase, which was found in higher relative abundance in biofilms on PETG (Table 5.6). This enzyme is involved in the synthesis of NAD+, which is an important cofactor for redox reactions and for controlling cell metabolism [237,238]. This may suggest that biofilms on PETG are metabolically active, but have not yet triggered biofilm formation to the same extent as biofilms on PVC. This finding corresponds to the results from the biofilm accumulation experiment.

In eukarya, levels of intracellular NAD+ can increase two-fold in response to environmental stress, such as in situations of nutrient depletion [238]. Many archaeal cellular processes share more similarities to the eukarya than to bacteria [239,240], and it is possible that the growth of *M. barkeri* to PETG, which has a demonstrable poor surface for attachment and biofilm formation, may constitute stressful conditions for *M. barkeri,* and promote an increased metabolic requirement for early-stage biofilm formation. Therefore, nicotinamide-nucleotide adenylyltransferase may play a role in kick-starting biofilm formation on PETG.

One of the mechanisms of biofilm formation is having a stress response, owing to the fact that cells within a biofilm are under increased environmental stress. This is due to living in close proximity of each other, and the increasing levels of cell numbers, cell layers, and nutrient depletion within the biofilm [222]. This study revealed the higher relative abundance of multiple proteins associated with a stress and defence response from *M. barkeri* when attached to PVC compared to PETG, such as universal stress proteins, thermosomes and thioredoxin. As described previously, the relatively low levels of adhesion and proteins involved in biofilm formation from *M. barkeri* to PETG suggests a possible extensive lag phase in the initial attachment of cells to PETG (Fig. 5.2). Therefore, with the higher levels of adhesion and more developed biofilm formation of *M. barkeri* to the surface of PVC, the higher relative abundance of proteins involved in stress and defence can be expected compared to PETG biofilms.

Using an iTRAQ approach using the two most clustered biological replicates from each biofilm condition gave rise to the conclusion that PVC induced a higher level of microbial adhesion and a faster accumulation of cells to the surface compared to PETG, producing a proteomic profile of a more developed biofilm. This conclusion was also applicable to the normalisation technique used, which was based on normalising the intensity of the isotopic corrected values of the reporter ions (114 – 121) for each protein against the isotopic corrected intensity of reporter ion 113. The median of these normalised reporter values was consequently calculated for the fold changes in protein abundance. However, using median correction as a method of normalisation is more often used to compensate for high variability in complex data sets [160, 163]. Such a method would have been valuable in this experiment and should have been done. Whether the same conclusions hold in a more complex protein dataset from the analysis of all four biological replicates for each biofilm condition and corrected for using median normalisation is a matter for future work.

iTRAQ is a popular tool in determining the function of proteins for a wide range of eukaryal, bacterial and archaeal species, and has gained popularity over the years for its ease of use and ability to quantify multiple samples simultaneously [159]. In order to validate these conclusions from the iTRAQ approach, there was a need to verify the reliability of the results from iTRAQ, which in this case, was carried out by measuring the activity of fumarase and triose phosphate isomerase in PVC and PETG biofilm cells.

The results from the triose phosphate isomerase activity enzyme assay were comparable with those from the iTRAQ approach, with both approaches showing an approximate two-fold higher relative abundance of triose phosphate isomerase in cells attached to PVC. For the validation of fumarase, the iTRAQ analysis gave a fumarase fold change of 1.4 for cells attached to PVC, whereas the enzyme assay showed a 14-fold increase in fumarase activity from cells attached to PVC.

This discrepancy in fumarase quantification could be explained by the fact that malate was the intermediate that was spectrophotometrically measured as an indicator of fumarase activity. Malate is a key intermediate in the incomplete TCA cycle for carbohydrate and energy metabolism in *M. barkeri* [230], and is produced from fumarate by the action of fumarase [221]. However, malate is also produced from oxaloacetate by malate dehydrogenase in the incomplete TCA cycle [241,242]. Additionally, the genome of *M. barkeri* includes homologs of malic enzymes, which are ubiquitous in nature and have been proposed to be involved in the interconversion of pyruvate to malate in the carbohydrate metabolism of the archaeon, *Thermococcus kodakaraensis* KOD1 [243]. It is feasible that the proposed different pathways for malate synthesis in *M. barkeri* may have increased the levels of malate in biofilm cells of *M. barkeri* above that which were solely a result of the activity of fumarase. This may have led to the observed high levels of fumarase activity determined by the enzyme assay for biofilms attached to PVC.

Despite this, both the iTRAQ and enzyme assay approach gave the same conclusion that there was an increase in triose phosphate isomerase and fumarase produced in *M. barkeri* biofilms attached to PVC compared to PETG, suggesting that validation of the iTRAQ results was possible. These two different approaches complement each other and the biofilm accumulation experiment, and suggests a key role of PVC in promoting the distinct expression of proteins and phenotypes involved in biofilm formation in *M. barkeri.*

# 5.5. Conclusions

This study has shown that the nature of the support material is a key factor in biofilm-dependent protein expression. A combination of the iTRAQ approach and static biofilm and enzyme assay experiments successfully highlighted that PVC provided the most suitable surface for microbial attachment and biofilm accumulation over time, resulting in a phenotypic and proteomic profile of a more developed biofilm. However, PETG provided a poor surface for microbial attachment and biofilm formation, yielding a phenotypic and proteomic profile of a less developed biofilm. The results reinforce the idea that the nature of the support material is an important environmental factor for biofilm formation, which can be sensed by *M. barkeri* to signal changes in the cell surface, cellular growth, methanogenesis, energy production and central metabolism to facilitate irreversible adhesion onto a favourable support material, such as PVC.

## Chapter 6

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##### Conclusions and Future Work

*“The truth is rarely pure and never simple.”   
―*[Oscar Wilde](http://www.goodreads.com/author/show/3565.Oscar_Wilde)

# 6.1. Introduction

This thesis aimed to develop a microbial-based strategy for optimising the AD of domestic wastewater in peri-urban areas, by promoting biofilm formation from key resilient methanogens, such as *Methanosarcina,* within AD reactors. Previous studies have found methanogens to preferentially attach to specific support materials in both pure and mixed cultures [94,96], yet little is known of the underlying processes for this selective attachment. This information could provide guidance to the AD sector on which support materials could be prioritised for use in AD reactors to retain a higher active methanogenic biomass to improve the stability and performance of the AD process [16].

Additionally, current understanding of archaeal biofilm formation is limited [20,21], and this thesis would provide a framework in which to better understand this natural, yet complex process in the archaeon, *M. barkeri.* Therefore, as a first step in proposing a *Methanosarcina-*based AD system, the overall question being addressed in this thesis was *how* different support materials affected the biofilm-forming capabilities of *M. barkeri*. The experiments in this thesis were carried out in high ionic and neutral pH aqueous environments to model the typical characteristics of domestic wastewater in peri-urban areas of the Global South, and the tested support materials were chosen based on their common usage in AD reactors [94] and evaluated to be of low cost, available globally [13] and durable [42] for applicability in peri-urban areas.

A wide range of chemical, physical, spectroscopic, microscopic and proteomic methods were utilised throughout this thesis in order to understand the physicochemical and biological factors influencing biofilm formation in *M. barkeri* as a function of support material. These methods have allowed for an unprecedented view of biofilm formation for a diverse range of bacteria in previous studies [25], and show great promise in understanding the regulatory processes for archaeal biofilm formation [20]. Some of these methods have been used in previous studies to reveal details on the mechanisms underlying archaeal biofilm formation in *M. smithii*, *M. stadtmanae* and *M. mazei* [57], and *S. acidocaldarius, S. solfataricus,* and *S. tokodaii* [89]. However, this suite of methods has not been extensively used to examine the biofilm-forming capabilities of *M. barkeri* on different support materials.

# 6.2. Understanding biofilm formation in *M. barkeri*

This thesis showed that the surface characteristics of the microbial and abiotic surface were influential factors in the level of adhesion from *M. barkeri.* The support materials,PVC, PTFE and PP were proven to have the most attractive surfaces for initial attachment and subsequent biofilm formation from *M. barkeri,* as evidenced by the significantly higher percentage of cell coverage to these surfaces after 2 h (initial adhesion) and 96 h (irreversible adhesion) in chapter 3. The xDLVO model suggested that the preferential attachment of cells to PTFE and PP was a result of attractive Lifshitz-van der Waals and acid-base interaction forces at the microbial-abiotic interface, and suggests a key role of hydrophobicity in the adhesion of *M. barkeri* to PTFE and PP. Hydrophobicity is an important surface parameter in the initial adhesion process, and has been reported in various microbial systems [78].

However, out of all the tested support materials, PVC fared best at promoting the selective adhesion of *M. barkeri.* It is feasible that the highly polar surface of PVC could have facilitated an increased number of interfacial acid-base interactions between the cells of *M. barkeri* and the surface of PVC [185]. PVC’s highly polar surface combined with non-DLVO interactions, such as polymer interactions between the surface structures of *M. barkeri* and the surface of PVC, could have promoted a more irreversible attachment. The other tested support materials proved to be poor biofilm carriers for *M. barkeri,* as a result of highly negatively charged surfaces, which were predicted by the xDLVO model to produce strong repulsive electrostatic interactions between the microbial and abiotic surface.

Chapters 4 and 5 sought to understand the biological factors underlying the patterns of differential adhesion of *M. barkeri* to a good biofilm carrier (PVC) and to a poor biofilm carrier (PETG) during biofilm formation. Biofilm formation on PVC seemed to be associated with increased levels of polysaccharides on the cell surface. This finding was concomitant across different surface characterisation techniques, such as FTIR, XPS, zeta potential and fluorescence microscopy. These methods highlighted different cell surface chemical profiles between biofilms attached to PVC and PETG, suggesting that biofilm formation on PVC incurred significant modifications to the polysaccharides on the cell surface to perhaps facilitate a more irreversible attachment. Indeed, the production of surface polysaccharides during irreversible microbial adhesion is well documented in various microbial systems, and is as an adaptive response to a surface and the transition to a biofilm lifestyle [22,23,65].

Upon examining the protein expression profiles of biofilms attached to PVC, it was interesting to find the higher relative abundance of 730 proteins involved in methanogenesis, metabolism, energy production and cell growth compared to biofilms on PETG. The findings of increased cell surface polysaccharides in PVC biofilms as determined from the surface characterisation techniques used in chapter 4, were confirmed by the identification of numerous proteins in higher relative abundance involved in cell surface biogenesis and EPS production in PVC biofilms compared to PETG biofilms.

Biofilm formation in *M. barkeri* could proceed in a similar fashion to bacteria, in which the initial attachment and increasing accumulation of cells to a surface triggers key cell surface changes, such as the production of surface-associated polymers and EPS to facilitate irreversible attachment [22,190,193]. The increased levels of cells, cell surface polysaccharides and higher relative abundance proteins involved in central metabolism, EPS production and energy production in biofilms attached to PVC, imply that biofilms were developing faster on this support material compared to PETG. Therefore, PVC could be said to possess a surface that was physicochemically and biologically compatible to *M. barkeri* biofilm development, incurring significant cell surface and proteomic changes to facilitate the transition to a biofilm lifestyle.

PETG elicited significantly lower levels of cells, cell surface polysaccharides and proteins involved in central metabolism, which are indicative of a much slower developmental stage of biofilm formation. This is unsurprising given that the repulsive surface characteristics of PETG may be delaying cell accumulation at the surface and therefore, consequent biofilm formation.

This thesis has shown that abiotic surfaces are important environmental factors for triggering different patterns of adhesion and biofilm formation, and can act as indicators of favourable conditions for biofilm growth and development [22]. The selection of a good biofilm carrier, such as PVC, could promote the growth and biofilm development of *Methanosarcina* within AD reactors, whereas a poor choice of support material, such as PETG, could decrease the chances of retaining a high active biomass of methanogenic biofilms. The results from this thesis suggest that appropriate support materials can be rationally chosen for the immobilisation of *M. barkeri* based on an initial assessment of their surface characteristics, as suggested by previous studies [45,94], which could be used to guide the design of high rate AD reactors in both the Global North and South. The experimental workflow used in this thesis could provide a means for assessing the compatibility of support materials for the attachment of key methanogens within AD reactors.

# 6.3. Future work

The work from this thesis provided a framework in which to better understand biofilm formation in *M. barkeri* on different polymer support materials in high ionic and neutral pH aqueous environments. However, this has opened up further lines of investigation and opportunities for future work in understanding biofilm formation in *M. barkeri.*

Future work should focus on the role of EPS in biofilm formation in *M. barkeri*, as the secretion of EPS is an important facet of facilitating irreversible adhesion to an abiotic surface [23]. This thesis has suggested a key role of cell surface polysaccharides and EPS during biofilm formation in *M. barkeri,* but it would be interesting to further examine the composition of EPS and how it changes in response to attachment to different support materials. Previous studies have highlighted distinct changes in EPS composition in biofilms as an adaptive response to different environmental conditions [29]. There is no universal method for extracting EPS from microbial systems for analysis [23], but a combination of different EPS extraction methods and lectin-staining of EPS components could be used to provide further insight in the role of EPS during biofilm formation on different support materials in *M. barkeri*.

Another future research direction is to examine the cell surface composition and functional proteome of planktonic cells of *M. barkeri* in comparison to the studied biofilms in this thesis. It is widely understood that the transition from a planktonic to a biofilm lifestyle incurs significant changes in the metabolic capacity, cell surface composition and genetic programming of cells, and highlights the important intra- and extracellular changes that planktonic cells must undergo in order to adapt to a more sessile, biofilm lifestyle [22]. Knowledge of the underlying regulatory processes governing biofilm formation from the planktonic state in archaea are still not well known [20]. Such information would be complementary to the work carried out in this thesis on biofilms of *M. barkeri*, and could shed light on some of the key processes underlying the transition of cells to a biofilm mode of growth on different support materials.

In chapter 5, numerous uncharacterised proteins were identified in the proteome of biofilms attached to PVC. This is unsurprising given the large percentage of uncharacterised proteins in the total proteome of *M. barkeri,* yet investigating the correlation between these uncharacterised proteins to biofilm development would be an interesting field of research.

This thesis has largely undertaken a biological investigation of biofilm formation in *M. barkeri,* yet in order to apply this knowledge to AD reactors in wastewater management, future research should examine whether PVC remains to be a good selective biofilm carrier for *M. barkeri* when placedwithin a pilot-scale AD reactor treating actual high-strength domestic wastewater. Previous studies have found PVC to possess a good capacity for retaining methanogenic archaea from a mixed species inoculum and improving biogas production within lab-scale AD reactors [45]. Yet whether PVC brings about the same selective capacity for *M. barkeri* biofilms as observed in this thesis within AD reactors is an interesting area of research. In a complex substrate such as domestic wastewater, there exists a plethora of different microorganisms involved in the AD process, which could have a differential effect on the dynamics and development of *M. barkeri* biofilms on PVC. Therefore, monitoring changes in the composition of methanogenic biofilms on PVC within AD reactors in response to competition from other microorganisms of the AD microbiome would provide a more comprehensive understanding of biofilm formation in *M. barkeri* to this support material.

The process of biofilm formation is complex, and there are further complexities in understanding biofilm formation within mixed environmental samples where multiple interactive relationships between different microorganisms can influence biofilm development [22]. This highlights the need for a multidisciplinary approach in the study of biofilms. This thesis has scratched the surface of understanding biofilm formation in *M. barkeri* to different support materials by using a multifaceted approach spanning different disciplines. Such an approach is needed for studying complex biofilms in environmental samples, such as in AD reactors, and requires an interdisciplinary way of thinking to uncover the mechanisms of methanogenic biofilm formation to facilitate heavy duty biomethanation from AD.

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**Appendix**

**DSMZ 120 media recipe for *Methanosarcina barkeri***

|  |  |  |
| --- | --- | --- |
| K2HPO4 | 0.35 | g |
| KH2PO4 | 0.23 | g |
| NH4Cl | 0.50 | g |
| MgSO4 x 7 H2O | 0.50 | g |
| CaCl2 x 2 H2O | 0.25 | g |
| NaCl | 2.25 | g |
| FeSO4 x 7 H2O solution (0.1% w/v in 0.1 H2SO4) | 2.00 | ml |
| Trace element solution SL-10 (see below) | 1.00 | ml |
| Yeast extract (OXOID) | 2.00 | g |
| Casitone (BD BBL) | 2.00 | g |
| Na-resazurin solution (0.1% w/v) | 0.50 | ml |
| NaHCO3 | 0.85 | g |
| Vitamin solution (see below) | 10.00 | ml |
| Methanol | 10.00 | ml |
| L-Cysteine-HCl x H2O | 0.30 | g |

|  |  |  |
| --- | --- | --- |
| *Vitamin solution:* |  |  |
| Biotin | 2.00 | mg |
| Folic acid | 2.00 | mg |
| Pyridoxine-HCl | 10.00 | mg |
| Thiamine-HCl x 2 H2O | 5.00 | mg |
| Riboflavin | 5.00 | mg |
| Nicotinic acid | 5.00 | mg |
| D-Ca-pantothenate | 5.00 | mg |
| Vitamin B12 | 0.10 | mg |
| p-Aminobenzoic acid | 5.00 | mg |
| Lipoic acid | 5.00 | mg |
| Distilled water | 1000.00 | ml |

|  |  |  |  |
| --- | --- | --- | --- |
| *Trace element solution SL-10:* | |  |  |
| HCl (25%; 7.7 M) | | 10.00 | ml |
| FeCl2 x 4 H2O | | 1.50 | g |
| ZnCl2 |  | 70.00 | mg |
| MnCl2 x 4 H2O | | 100.00 | mg |
| H3BO3 |  | 6.00 | mg |
| CoCl2 | x 6 H2O | 190.00 | mg |
| CuCl2 | x 2 H2O | 2.00 | mg |
| NiCl2 x 6 H2O | | 24.00 | mg |
| Na2MoO4 x 2 H2O | | 36.00 | mg |
| Distilled water | | 990.00 | ml |

First dissolve FeCl2 in the HCl, then dilute in water, add and dissolve the other salts.

Finally make up to 1000 ml.