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NANYANG
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**ENHANCING EXTRACELLULAR BIOCATALYSIS THROUGH
BIOFILM ENGINEERING AND MUTUALISTIC INTERACTIONS**

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SINGAPORE CENTRE FOR ENVIRONMENTAL LIFE

SCIENCES ENGINEERING (SCELSE)

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2016

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LIST OF ABBREVIATIONS

× g	g-force in centrifuge
μg	Microgram
μL	Microlitre
μM	Micromolar concentration
μm	Micrometre
μmoles	Micromoles
3-CA	3-chloroaniline
A2O process	Anaerobic-anoxic-oxic process
AHL	Acyl homoserilne lactone
ALP	Alkaline phosphatase
AQDS	Anthraquinone-2,6-disulfonate
ATCC	American type culture collection
ATP	Adenosine triphosphate
Bap	Biofilm associated protein
BCA	Bicinchoninic acid
bEPS	Bound extracellular polymeric substances
BIND	Biofilm Integrated Nanofiber Display
BOD	Biochemical oxygen demand
bp	Base-pair
c-di-GMP	Cyclic di-guanylate monophosphate
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
CSSPR	Continuously stirred suspended planktonic reactors
CSTR	Continuously stirred tank reactor
CV	Crystal violet
CYP	Cytochrome P450
d	Time in days
DAPI	4', 6-diamidino-2-phenylindole
DCP	2,4-dichlorophenol
DHA	Dihydroxyacetone
DMRB	Dissimilatory metal reducing bacteria
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DSSN+	(4,4'-bis(4'-N,N-bis(6''-(N,N,N-trimethyl ammonium) hexyl) amino)-styryl) stilbene tetraiodide)
DTT	Dithiothreitol
EAB	Electroactive biofilm
eDNA	Extracellular deoxyribonucleic acid

EPS	Extracellular polymeric substances
FAD	Flavin adenine dinucleotide
FBBR	Fluidized bed biofilm reactor
Fdh	Formate dehydrogenase
FI	Fluorescence intensity
FMN	Flavin mononucleotide
g	Gram
G6PDH	Glucose-6-Phosphate Dehydrogenase
Gdh	Glucose dehydrogenase
GFP	Green fluorescent protein
GW	Groundwater
h	Time in hour
HfMBR	Hollow fiber Membrane bioreactor
HRT	Hydraulic retention time
INP	Ice nucleation protein
kDa	Kilo Dalton
L	Litre
IaEPS	Loosely associated extracellular polymeric substances
LB	Luria Bertani medium
M	Molar concentration
M9	M9 minimal media
MBBR	Moving bed biofilm reactors
MFC	Microbial fuel cell
mg	Milligram
min	Time in minute
mL	Millilitre
mM	Millimolar concentration
mm	Millimetre
MM1	Modified M1 minimal media
mmoles	Millimoles
Mtr	Metal reduction
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
nm	Nanometre
NTA	Nitrilo triactetic acid
O.D	Outer diameter
OA-SGW	Synthetic groundwater amended with organics
°C	Degree Celcius
OD	Optical density
OMCs	Outer membrane cytochromes
PAHs	Poly aromatic hydrocarbons
PBS	Phosphate buffer saline
PCE	Perchloroethylene

PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
pm	Picometre
q-PCR	Quantitative polymerase chain reaction
RBC	Rotating biological contactor
RFU	Relative fluorescence units
RNA	Ribonucleic Acids
roGFP	Redox sensitive green fluorescent protein
RPKM	Reads Per Kilobase of transcript per Million mapped reads
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acids
SDS	Sodium dodecyl sulfate
sec	Time in Seconds
SGW	Synthetic groundwater
SR-SIM	Super resolution structured illumination microscopy
TCE	Tetrachloroethylene
TEAB	Triethylammonium bicarbonate buffer
TOC	Total organic carbon
UASBR	Upward anaerobic sludge blanket reactor
v/v	Volume by volume
w/v	Weight by volume
YFP	Yellow fluorescent protein

LIST OF PUBLICATIONS

Journal publication

Sivakumar K, Wang VB, Chen X, Bazan GC, Kjelleberg S, Loo SCJ, Cao B* (2014). Membrane permeabilization underlies the enhancement of extracellular bioactivity in *Shewanella oneidensis* by a membrane-spanning conjugated oligoelectrolyte. Appl Microbiol Biotechnol. **98(21)**: 9021-9031.

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Conferences

Sivakumar K, Mukherjee M and Cao B* (2014). Display of roGFP on Cell Surface Enables in Situ Quantification. 14th AIChE Annual Meeting, Atlanta, GA, USA (**Poster presentation**)

Sivakumar K, Liu W and Cao B* (2014). Understanding the Role and Harnessing the Power of a Matrix-Associated Protein BpfA. IWA Conference: The Perfect Slime, Essen, Germany (**Oral presentation**).

Sivakumar K and Cao B* (2015). Engineering Catalytic biofilms for extracellular hydrolysis of Mannan polysaccharides. 15th AIChE Annual Meeting, Salt Lake City, UT, USA (**Oral presentation**).

ABSTRACT

Biocatalysis is an environmentally benign process that is applied extensively in chemical industry. Whole-cell biocatalysis has been studied extensively; however, it is limited to batch and fed-batch processes employing planktonic cells. In addition, the chemical toxicity of the substrate and/or product has often hampered the biocatalytic performance of whole-cell biocatalysts.

Biofilms are surface-associated, robust microbial communities embedded in an extracellular matrix. In contrast to free-living cells, biofilms are highly tolerant to environmental stresses, rendering such surface consortia attractive catalytic systems for biocatalysis. Although biofilms have many advantages, the application of biofilms as biocatalysts in chemical and biotechnological industries is still in its infancy, because of the challenges associated with the intrinsic biofilm properties such as mass transfer limitations in biofilms.

To alleviate mass transfer issues in biofilm-based biocatalysis, this project has focused on the development of robust and efficient biofilm-based extracellular biocatalytic systems through matrix engineering to make the biofilm matrix a “micro-factory” for biocatalysis. The major challenge for biofilm matrix-enabled biocatalysis is to functionalize the biofilm matrix through the display of catalytic centres.

Using *Shewanella oneidensis* as a model organism, we developed a novel approach to engineer the biofilm matrix to exhibit extracellular biocatalytic activity. This approach was demonstrated through the display of redox sensitive green fluorescent protein (roGFP) in the matrix of *S. oneidensis* biofilms by genetically

fusing it to the C-terminus of BpfA, a previously reported matrix-associated protein. BpfA-roGFP cells exhibited characteristic fluorescence signal, when exposed to oxidation inducing chemicals such as H_2O_2 , Ag^+ and SeO_3^{2-} . We then used BpfA-roGFP to spatially resolve the extracellular redox status in the biofilm matrix. This study provided a non-invasive approach for monitoring extracellular redox status within biofilm microenvironments, which could be used to understand redox responses of biofilms to environmental perturbations.

The potential of biofilm matrix-enabled biotransformation in biotechnology was explored through the construction of fusion strain BpfA-Man5C, in which β -1,3-glucomannanase Man5C was genetically fused to the C-terminus of BpfA. BpfA-Man5C cells demonstrated highly recyclable Man5C activity in hydrolysing amorphous mannan substrate glucomannan. We further used this engineered strain to develop biofilms in a fluidized bed biofilm reactor and elucidated the factors influencing the extracellular Man5C activity exhibited by the catalytic biofilms. Our results showed that the BpfA-Man5C biofilms displayed a significantly higher hydrolysis activity than the planktonic cultures. We also examined the effect of biofilm thickness on the overall extracellular Man5C activity in the fluidized bed bioreactor

To enhance extracellular bioactivity, we explored two approaches: (1) Use of conjugated oligoelectrolyte DSSN⁺ and (2) Mutualistic interactions between different organisms. In previous studies, DSSN⁺ has been reported to be able to enhance bioelectricity generation in bioelectrochemical devices, although the mechanism remained elusive. In our study, we showed that DSSN⁺ enhanced extracellular ferrihydrite reduction by *S. oneidensis* in a growth-dependent manner. The incorporation of DSSN⁺ into *S. oneidensis* cell membrane increased the

extracellular concentration of flavins and extracellular enzyme activities without significantly decreasing cell viability. Our results suggested that membrane permeabilization is the dominant mechanism for the enhancement of extracellular bioactivity in *S. oneidensis* by DSSN+. We further demonstrated that the interaction between DSSN+ and *S. oneidensis* cells enhanced biofilm formation and stability without compromising the overall biofilm activity. The deployment of membrane intercalating conjugated oligoelectrolytes, of which, DSSN+ is one of many possible molecular structures, may be applied to enhance extracellular bioactivity in bacteria towards more efficient biofilm-based biocatalysis.

Further, we also showed how mutualistic interactions could improve extracellular bioactivity. In this study, we reported a mutualistic system consisting of fermentative bacterium *Escherichia coli* and metal-reducing bacterium *S. oneidensis* in ferrihydrite reduction. Our results showed that the extracellular bioactivity of *S. oneidensis* is significantly enhanced with the presence of *E. coli* and formate exchange was identified to be the key mechanism in this mutualistic system.

CHAPTER 1: INTRODUCTION

1.1. Background

The last few decades have witnessed the development of several sustainable green techniques in the field of chemical and biochemical processes. Most of these processes have biotransformations as the fundamental mechanism, underpinning the pivotal role of biological systems (Alcalde et al. 2007). Biocatalysis is an environmentally benign process that has proved to be an alternative to chemical synthesis of value added products. Biocatalysis initiates industrial catalytic conversions with the aid of renewable, low energy consuming and biodegradable catalysts (Alcalde et al. 2007; Coward-Kelly and Chen 2007; Leresche and Meyer 2006). Hence, biocatalysis tends to reduce the environmental perturbations resulting from chemical catalytic reactions.

Majority of the biocatalytic reactions involve either the simple conversion of a substrate to value added products or biodegradation of a recalcitrant substrate. It also encompasses redox reactions such as the extracellular reduction of heavy metals and generation of clean energy. Hence, biocatalysis provides an effective platform for harnessing industrial applications (Alcalde et al. 2006; Gross et al. 2007; Hartmann and Jung 2010; Illanes et al. 2012). It exploits the potential of microbial cells or isolated enzymes to mediate chemical transformations (Hartmann and Jung 2010; Leresche and Meyer 2006; Santacoloma et al. 2010).

Isolated enzymes are often sensitive to operational conditions such as pH and temperature, which restricts the application of enzyme-based biocatalysis (Alcalde et al. 2006; Bornscheuer et al. 2012; Illanes et al. 2012; Popov and

Lamzin 1994; Rosche et al. 2009; Schüürmann et al. 2014; Zhao et al. 2002). Hence, most industrial biocatalytic conversions utilize the catalytic power of whole-cells (Leon et al. 1998; Nikolova and Ward 1993). However, such cell-mediated reactions are operated in batch and fed-batch modes, which often results in low catalytic stability, short retention time and relatively lower rates of biocatalyst regeneration (Halan et al. 2012a; Nikolova and Ward 1993; Rosche et al. 2009).

Biofilms are surface-associated microbial communities embedded within a self-produced extracellular matrix composed of extracellular polymeric substances (EPS). In contrast to free-living cells, biofilms are highly tolerant to environmental stresses and exhibit a long-term stability (Flemming and Wingender 2010; Halan et al. 2012b). Employing biofilms for biocatalytic reactions might be an alternative for sensitive enzymes and whole-cells (Tsoligkas et al. 2011a). Self-immobilization of whole-cells within the biofilm matrix further enhances the biocatalytic activity (Betancor and Luckarift 2008; Cirino and Sun 2008; Dalby 2007).

Biofilms also facilitate coupling of metabolic activities with biocatalytic reactions through the mutualistic intercellular interactions in biofilms (Gross et al. 2007; Rosche et al. 2009). However, high heterogeneity and environmental dynamics within the biofilms coupled with mass transfer limitations render the control of biofilm-based bioprocesses very challenging (Flemming and Wingender 2010; Illanes et al. 2012; Parsek and Fuqua 2004; Zhang et al. 2014). Hence, to date, the biofilm-based biocatalysis is still in its infancy.

1.2. Research gaps and challenges

Biofilms are capable of integrating the functional requirements of whole-cell catalysts and serve as a platform that can enhance the stability of catalytic enzymes. However, biofilm-based biocatalysis has rarely been implemented in industrial applications on a larger scale due to certain bottlenecks.

In biofilm-based biocatalysis, diffusion of substrates and intermediates in biofilms has a profound influence on biocatalytic efficiency, because it drives mass transport within biofilm microstructures (Halan et al. 2012a; Stewart 2003; Tsoligkas et al. 2011b). Poor diffusion within biofilm microenvironments results in concentration gradient of substrates, nutrients, oxygen, or other electron acceptors as well as accumulation of waste products (Stewart 2003). Hence, diffusion limitations significantly hamper the efficiency of biofilm-based biocatalysis (Bishop et al. 1995; Pamp et al. 2007; Stewart 2003; Sutherland 2001b). Further, intracellular retention of the products might alter the physiology of the biofilm, compromising its catalytic activity. The techniques used for product isolation are disruptive in nature and will perturb the biofilm architecture.

Harnessing the power of biofilm matrix for extracellular biocatalysis may alleviate the mass transfer issues in biofilm-based biocatalysis. Since the catalytic centres are localized in the biofilm matrix, neither substrate nor product has to cross the membrane barrier (Schüürmann et al. 2014). Further, the biofilm matrix enables the retention and immobilization of electron carriers and enzymes for extracellular biocatalysis (Branda et al. 2005; Flemming et al. 2007). The extracellular products can be isolated and extracted by simple techniques from the biofilm matrix without disturbing the biofilm architecture (Bornscheuer et al. 2012; Halan et al. 2012b; Hibbert et al. 2005; Leon et al. 1998; Polizzi et al. 2007).

The facts outlined here encourage the broader application of biofilm matrix-enabled extracellular biocatalysis in industrial biotechnology and biochemical engineering. The deployment of catalytic centres in biofilm matrix and continuous maintenance of the catalytic activity are the key challenges associated with biofilm matrix-enabled extracellular biocatalysis.

1.3. Objectives

To address the key challenges outlined in the previous section, this study aims at enhancing the extracellular biocatalysis through biofilm engineering, mutualistic interactions and by incorporating membrane intercalating synthetic molecules. This study has focussed on engineering catalytic biofilms through immobilization of enzymes in biofilm matrix by using matrix-associated proteins. In addition, this study has explored the use of conjugated oligoelectrolytes and mutualistic intercellular interactions to enhance the extracellular bioactivity.

In this study, we used *Shewanella oneidensis* MR-1 as the model organism. Its genome sequence is well annotated and its biofilms has been reported to have wide range of environmental and biotechnological implications (Arai et al. 2010; Lies et al. 2005; Ng et al. 2013; Zhang et al. 2014). *S. oneidensis* is capable of coupling the anaerobic oxidation of organic matter with the reduction of metal ions, metal oxides and solid electrodes (Coursolle et al. 2010; Coursolle and Gralnick 2012; Fredrickson and Gorby 1996).

To address the matrix-enabled extracellular biocatalytic approach, we engineered the biofilm matrix of *S. oneidensis* to display enzymes empowered with specific catalytic activity. *S. oneidensis* fusion strains were constructed through the genetic fusion of specific enzymes onto biofilm promoting factor BpfA

(285 kDa), a significantly large cell surface and matrix-associated protein (Cao et al. 2011a; Theunissen et al. 2010a).

1.4. Scope

This thesis contains seven chapters including an introduction, a literature review, four research chapters and conclusions with future recommendations.

In Chapter 1, a brief background on biocatalysis and biofilm-based biocatalysis is outlined. This chapter lays emphasis on the significance of matrix-enabled extracellular biocatalysis as compared to conventional biocatalytic approaches. The objective and scope of the work is described in detail.

Chapter 2 covers the literature review for the whole thesis. This chapter briefly describes the relevance of biocatalysis, conventional biocatalysts such as whole-cells and isolated enzymes with their benefits and drawbacks and aspects of biofilm-based catalysts. It also outlines the aspects of surface-display of enzymes and intercellular interactions within a biofilm.

Chapter 3 presents the effects of membrane intercalating conjugated oligoelectrolyte DSSN⁺ on extracellular bioactivity and biofilm stability.

Chapter 4 reports the role of intercellular interactions in extracellular biocatalysis by using extracellular ferrihydrite reduction as experimental platform.

Chapter 5 presents the display of redox sensitive green fluorescent protein roGFP on the biofilm matrix of *S. oneidensis* MR-1 to resolve the extracellular redox state in biofilm matrix.

Chapter 6 illustrates the biotechnological application of the biofilm matrix-enabled biocatalysis. This chapter reports on the extracellular hydrolysis of mannan polysaccharides by 1,3-glucomannanase Man5C displayed on the biofilm matrix of *S. oneidensis* MR-1.

Chapter 7 provides a summary of the thesis work and proposes some future recommendations.

CHAPTER 2: LITERATURE REVIEW

2.1. Background on biocatalysis

Biocatalysis refers to the catalytic conversion of a substrate into the desired product by a biological catalyst. It can either be in the form of simple single step bioconversion or complex biotransformations ranging from biodegradation or bioremediation (Coward-Kelly and Chen 2007; Hudlicky and Reed 2009). It serves as an environmentally friendly technique for industrial catalysis (Alcalde et al. 2007). Biocatalysis reduces the amount of waste generated, as relative to chemical catalysis (Erable et al. 2010; Gross et al. 2013). It provides considerable benefits such as biodegradability, low power requirements and is easily replenished (Leresche and Meyer 2006; Nestl et al. 2011; Wohlgemuth 2010).

Biocatalysis in commercial scale encompass synthesis of value added chemicals, manufacture of antibiotics, pharmaceutical products and intermediates, recycling and bleaching in paper manufacture as well as catalytic conversions of fibres and biomass. These successful cases have been reported in literature (Cirino and Sun 2008; Hudlicky and Reed 2009; Panke and Wubbolts 2005; Pollard and Woodley 2007; Santacoloma et al. 2010; Schmid et al. 2001; Yamazawa et al. 2013; Yim et al. 2011). Either whole-cells or isolated enzymes mediate these biotransformations (Alcalde et al. 2007; Gross et al. 2007; Halan et al. 2012b).

Whole-cells are generally preferred over enzymes as industrial biocatalysts for large-scale applications, because this method does not require extensive purification steps (Leon et al. 1998; Winn 2012). The function of whole-

cells as catalysts is augmented by the metabolic activity of the cells hence, expanding their influence over a range of reactions (Smith et al. 2015). The whole-cell-based approach can be applied at all process conditions through the manipulation of cellular metabolic circuits (Gross et al. 2007). Whole-cells provide the flexibility of being reused and recycled in repetitive reactions. Whole-cell approach is the favoured option, whenever the reaction occurs in sequential steps, where each step is catalysed by a specific enzyme. Whole-cells also provide feasible conditions, if the reaction requires continuous recirculation of cofactors (Santacoloma et al. 2010; Zhao et al. 2002).

The cellular platform has made it possible to genetically engineer an enzyme into a recombinant protein with enhanced catalytic activity by integrating protein engineering with directed evolution (Blank et al. 2010; Hibbert et al. 2005). When this combination is applied in a process environment, it might generate genetically engineered organisms with enhanced biocatalytic capability (Hibbert et al. 2005). Literatures have reported cases of such phenotypic variants with much improved catalytic activity, when compared to wild type (Hibbert et al. 2005; Leon et al. 1998; Nikolova and Ward 1993; Wohlgemuth 2010).

However, most of the previous works in whole-cell catalysis has been associated with batch systems mediated by planktonic cells. These systems have severe drawbacks such as absence of long-term catalytic stability, lower rates of catalytic retention and regeneration and relatively low tolerance to toxic substrates and products (Gross et al. 2007; Halan et al. 2012b). Exposure to harsh environmental conditions will further disrupt the process efficiency.

Whole-cell biocatalysis requires the substrate to diffuse into the cell across the cell membrane for the intracellular enzymes to process the reaction. Further,

the product formed within the cell has to be retrieved using energy-intensive approaches, which severely hinders the catalytic activity of whole-cells (Kim et al. 2007; Polizzi et al. 2007; Schüürmann et al. 2014). The toxicity issues related to the product, if retained within the cell, might affect the stability of the whole-cell biocatalyst (Schüürmann et al. 2014).

The cellular environment is composed of diverse intracellular and extracellular enzymes that might inhibit a specific enzyme activity. The hindrance from other enzymes is another major restricting factor for whole-cell-mediated biocatalysis (Smith et al. 2015). The batch systems also create an environment characterized with low rate of cell entrapment due to dispersed aggregation of cells.

Enzymes are biological catalysts, which mediate most of biotransformations (Walsh 2001). Most enzymes are proteins having a specific three-dimensional structure, and may require organic and inorganic cofactors like biotin or magnesium to mediate catalytic conversions (Walsh 2001). Enzymes are highly biodegradable and take part in a reaction only as a catalyst.

Certain enzymes are purely selective and specific towards a particular substrate and reaction, whereas certain others have the ability to catalyse diverse reactions (Illanes et al. 2012; Kim et al. 2006; Kim et al. 2007). The respective enzymes regulate the metabolic pathways taking place in such conversions. The equilibrium of the reaction remains unaltered due to the non-consumption of enzymes in the reaction (Hibbert et al. 2005). The enzymes isolated from microbial cells are used for most of the biotransformations. Enzyme-mediated reactions include oxidation-reduction reactions, amination reactions, hydrolysis and reverse hydrolysis reactions and glycosylation reactions (Dalby 2007; Walsh

2001; Zhao et al. 2002). Table 2.1 describes the functional classification of enzymes.

Table 2.1. The International Union of Biotechnology has broadly categorized enzymes under six different classes based on their specific application (Hudlicky and Reed 2009; Wohlgemuth 2010).

Enzymes	Function
Oxidoreductases	Catalyze the oxidation and reduction of organic molecules like alcohols and ketones. Highly cofactor dependent
Transferases	Mediate the translocation of functional groups like amino group and phosphoryl group in a reaction
Hydrolases	Mediate the hydrolysis of organic molecules like esters and peptides
Lyases	Responsible for the addition of multiple bonds
Isomerases	Monitor the isomerization reaction
Ligases	Initiate the formation of carbon bonds like C-O, C-N, C-S.

Among the different categories, the most widely used enzymes are the hydrolases (Hudlicky and Reed 2009). The most extensively used hydrolases include proteases, carbohydrases and lipases. Each of these enzymes are characterized with core substrate specificity (Hudlicky and Reed 2009; Nestl et al. 2011). In addition to organic synthesis, enzymatic biocatalysis also find wide application in the generation of biofuels such as bioethanol production from the enzymatic hydrolysis of lignocellulose biomass. The benefits and drawbacks of whole-cell and enzymatic approaches are summarized in Table 2.2.

Table 2.2. General features of whole-cell and enzymatic biocatalysis (Hudlicky and Reed 2009; Illanes et al. 2012; Leresche and Meyer 2006; Wohlgemuth 2010)

Type of Biocatalysis	Features
Whole-cell biocatalysis	Benefits
	<p>No requirement of expensive enzyme purification techniques</p> <p>Whole-cell environment protects the intracellular enzymes and provide more stability</p> <p>High surface to volume ratio increases its scope of application</p> <p>Facilitate the multi-step biotransformations mediated by series of enzymes</p> <p>Facilitates recycling of catalysts in multiple cycles as compared to isolated enzymes</p> <p>Cofactor regeneration is much easier within the cellular environment</p>
	Drawbacks
Enzymatic biocatalysis	<p>The substrate and product has to negotiate the cell membrane barrier</p> <p>Highly limited to batch and fed batch systems</p> <p>Product recovery is very difficult</p> <p>Intracellular enzymes within the cellular environment might interfere with the specific enzyme activity</p> <p>Overall reaction rate is considerable lower</p>
	Benefits
	<p>Extreme degree of tolerance towards wide range of chemical transformations</p> <p>High degree of substrate specificity and reactions occur under mild conditions</p> <p>Ease in availability as most of the enzymes are extracted from microbial cells</p> <p>Low waste generated to product ratio</p> <p>Well compatible with the concept of “green technology”</p> <p>Waste effluent from enzymatic reactions are suitable for discharge with low degrees of treatment</p>
	Drawbacks
	<p>Highly dependent on operational conditions</p> <p>Requires expensive techniques for enzyme extraction and purification</p> <p>Enzyme extraction techniques are highly time consuming</p> <p>Enzyme activity is highly dependent on folded structure. Too much of measures to improve the activity will restrict the activity</p> <p>Interference from other enzymes will hamper the yield of</p>

enzyme isolation

Enzyme-based biocatalysts are hardly recyclable and prone to rapid degradation

Very low heat tolerance and not active in non-aqueous environment

However, several bottlenecks have hampered the implementation of enzymatic biocatalysis for industrial applications on a larger scale. The major obstacles include expensive techniques like liquid chromatography and liquid-liquid extraction involved with enzyme isolation and purification (Schüürmann et al. 2014; Smith et al. 2015). The process is extremely costly and occupies a significant operational time (Kim et al. 2006; Sheldon and van Pelt 2013). Furthermore, it is challenging to recycle and reuse enzymes for multiple rounds of catalytic activity. Another drawback is the preservation of enzyme stability under different process configurations and physico-chemical environments (Halan et al. 2012b; Rosche et al. 2009). The stability of the enzymes often gets perturbed with operational factors like pH, temperature, chemical gradients, and other process related factors (Coward-Kelly and Chen 2007; Illanes et al. 2012; Kim et al. 2006). The specificity of enzymes to respective substrates in a catalytic reaction draws a narrow borderline towards its application in industrial processes (Hudlicky and Reed 2009).

Isolated enzymes have been reported to be functionally inactive in non-aqueous environments (Smith et al. 2015). In cases of chemical transformations mediated by some cofactor-dependent enzymes, the continuous recirculation of the cofactors poses a serious issue. Such reactions require the coupling of catalytic reaction with redox reactions to regenerate the cofactors, rendering such systems highly expensive and difficult to control (Moore et al. 2014; Polizzi et al. 2007; Popov and Lamzin 1994).

Another major drawback for enzymatic biocatalysis is the sufficient lack of structural integrity for the natural enzymes evolved from the microbial cells. Enzyme activity is highly dependent on its precise folded structure. The fluid nature of the enzymes causes structural changes during the catalytic activity (Winn 2012). To overcome these limitations, an approach integrating directed evolution with protein engineering is required to be implemented (Nestl et al. 2011; Polizzi et al. 2007; Ran et al. 2008; Santacoloma et al. 2010; Zhao et al. 2002).

Although, biocatalysis as an alternative to chemical reactions is promising in industrial applications, its implementation on a commercial scale has been limited. It is highly speculated that a biological system capable of conferring functional stability to both whole-cells and catalytic enzymes is required (Alcalde et al. 2006; Botyanszki et al. 2015; Dalby 2007; Gross et al. 2007; Halan et al. 2012b).

2.2. Biofilms and biofilm-based biocatalysis

2.2.1. The highly dynamic and heterogeneous microenvironments in biofilms

Biofilms are the most ubiquitous and resilient form of microbial life on this planet. Biofilms consist of either single specie or diverse group of microorganisms adhered to a specific surface (Ding et al. 2014a; Halan et al. 2012b; Singh et al. 2006). Biofilms are made up of clusters of microbial aggregates immobilized within a network of self-produced extracellular polymeric substances (EPS) referred to as the biofilm matrix, as shown in Figure 2.1 (A).

The biofilm environment is often characterized with spatial and temporal nutrient gradients, diverse metabolic activities and varying mass transfer rates (Parsek and Fuqua 2004; Singh et al. 2006; Sutherland 2001b). The development and performance of a biofilm is influenced by its intrinsic heterogeneity and physiological dynamics. As such, the biofilms consist of microbial communities with distinct phenotypes characterized by varying levels of gene expression and physiological state (Rosche et al. 2009).

In the course of biofilm formation, the cells make transition from the free-swimming planktonic lifestyle to the sessile surface-attached mode. Figure 2.1 (B) illustrates the different phases of biofilm development. Biofilm formation commences with reversible attachment of planktonic cells to the substratum (Halan et al. 2012b; Noguera et al. 1999). The attached cells secrete EPS that firmly hold and bind the cells to the surface as a single layer. Bacterial surface appendages such as pili, flagellum and EPS components such as polysaccharides and structural proteins provide anchorage and promote surface attachment of cells (Flemming et al. 2007; Flemming and Wingender 2010). Cell-surface hydrophobic interactions, electrostatic forces and van der Waals forces also contribute to this second phase of biofilm development, referred to as irreversible attachment.

The EPS imparts structural integrity and stability to the biofilm as well as provide resistance against toxic and harsh environments by serving as an external barrier. The EPS also contributes to the physico-chemical parameters influencing the maintenance of the biofilms such as hydrophobicity, nutrient gradient, roughness, porosity and overall biofilm heterogeneity (Flemming 2011; Flemming et al. 2007; Flemming and Wingender 2010; Pamp et al. 2007).

The biofilm attachment phase is followed by vertical development through the formation of microcolonies from the surface-attached cells. The microcolonies develop into mushroom-shaped towering structures laced with porous channels for the mass transfer of nutrients and water within the biofilm microenvironments (Bishop et al. 1995; O'Toole and Kolter 1998; Singh et al. 2006; Stephens 2002). This is described as the biofilm maturation phase, which culminates with the formation of three-dimensional scaffolds (Lee et al. 2014).

The final stage of biofilm development is biofilm dispersal. The depletion of nutrient supply and the accumulation of waste products trigger the biofilm dispersal (Davies and Geesey 1995; Kaplan 2010). Biofilm dispersal is normally initiated from the centre of biofilms, which peaks with the displacement of biofilm cells into freely suspended planktonic form (Winn 2012).

The biofilm dispersal facilitated by shear forces or external stresses, such as prophage driven predator attack, is commonly referred to as passive dispersal. The hydrolysis of the EPS by glycosidases and proteases resulting in cell detachment, is also categorized under passive biofilm dispersal. Active biofilm dispersal involves the gradual transition of cells from biofilm mode to planktonic mode (Branda et al. 2005; Coombs et al. 2010; Flemming and Wingender 2010; Halan et al. 2012b; Kaplan 2010; O'Toole and Kolter 1998; Pamp et al. 2007; Stephens 2002; Winn 2012). The complete biofilm development cycle is illustrated in Figure 2.1 (B) (Stoodley et al. 2002).

A

B

Figure 2.1. (A) Schematic illustration of biofilm and biofilm matrix with EPS components, reproduced from (Flemming 2011) with permission from “Nature Publishing Group” (a) Biofilm development cycle; (b) The principal EPS components that make up the biofilm matrix; (c) The physicochemical interaction between the EPS components that build the stable scaffold which encases the biofilm; (d) A simulation molecular model showing the interaction between an EPS component alginate (right) and an extracellular enzyme lipase (left) of *Pseudomonas aeruginosa* biofilm matrix. (Flemming et al. 2007; Flemming and Wingender 2010); (B) Biofilm development cycle, reproduced from (Stoodley et al. 2002) with permission from “Annual Reviews”.

What makes biofilms the most distinct form of microbial lifestyle is the immobilization of cells in a matrix composed of self-produced polymeric substances such as polysaccharides, proteins, nucleic acids and lipids and extracellular DNA (eDNA) (Pamp et al. 2007; Singh et al. 2006; Sutherland 2001a; Sutherland 2001b). The matrix occupies approximately 90% of the overall biofilm biovolume (Rosche et al. 2009). The matrix builds the scaffold and encase the cells, providing the hydrodynamic environment (Branda et al. 2005; Lee et al. 2014; Parsek and Fuqua 2004; Rosche et al. 2009).

The overall biofilm structure is conserved through the mass transfer of substrates and nutrients. The channels and pores within the biofilm matrix facilitate the inward diffusion of water and nutrients as well as the outflow of waste products generated (Singh et al. 2006; Stewart 2003). This convective mode of transfer is essential to drive the biofilm-mediated bioprocesses (Gross et al. 2007; Renslow et al. 2010; Singh et al. 2006; Stewart 2003; Sutherland 2001b). The biofilm matrix also imparts mechanical stability and structural integrity to the slimy biofilms and provides resistance against various physicochemical stresses (Lee et al. 2014; O'Toole and Kolter 1998; Parsek and Fuqua 2004; Stewart 2003). Functions that the EPS in biofilms offer are summarized in Table 2.3.

Table 2.3. The EPS imparts the following functions to biofilms (Allison 2003; Flemming et al. 2007; Flemming and Wingender 2010)

Functional aspect	Functions
Structural unit of biofilm	Impart the mechanical stability and structural integrity as well as builds the three dimensional scaffold
Biofilm formation	Initiate the formation of hydrated microenvironments characterized with spatial and temporal heterogeneity
Protective barrier	Act as an external barrier and protects the biofilm against physicochemical stresses and toxic compounds, predator grazing and anti-microbial agents Imparts the viscoelastic properties required to keep the biofilm intact when subjected to environmental perturbations
Interactions in biofilms	Promotes horizontal gene transfer and enhanced enzyme activity in biofilm matrix Facilitate redox activity in biofilm matrix Enable the sorption of cations Enzymatic degradation of complex molecules to simple products Facilitate the accumulation of signaling molecules and other mediators of intercellular interactions

Exopolysaccharides form a major EPS component of biofilm matrix.

Polysaccharides are thin strands with a complex structure that remain on the cell surface (Flemming et al. 2007; Flemming and Wingender 2010). They are ubiquitous in natural biofilms and engineered settings such as soil and waterbodies and wastewater treatment systems. They also form the vital component of biofilms causing clinical infections (Pamp et al. 2007; Parsek and Fuqua 2004). The polysaccharides play a pivotal role in each stage of biofilm development. For example *P. aeruginosa* produces three distinct type of polysaccharides: alginate, Pel and Psl, each of them performing a significant function towards biofilm development (Branda et al. 2005; Flemming et al. 2007; Flemming and Wingender 2010; Parsek and Fuqua 2004; Sutherland 2001a).

The biofilm matrix is also composed of extracellular proteins mainly in the form of enzymes and structural proteins (Flemming and Wingender 2010). After

the maturation phase certain enzymes such as proteases and cellulases are released into the matrix, which hydrolyze the EPS components contributing to biofilm dispersal (Flemming and Wingender 2010; Tsoligkas et al. 2011a; Winn 2012).

Structural proteins are those, which are either bound to the outer membrane or expressed on the cell surface (Flemming and Wingender 2010). Their key role is to impart stability to the three dimensional network and forge a bridge between the cells and the extracellular surface. Structural proteins impart stability to biofilm matrix through key interactions with cations, such as between Ca^{2+} and polysaccharides (Cao et al. 2011a; Cao et al. 2011b). Structural proteins include special class of high molecular weight proteins known as biofilm-associated surface proteins (Bap) that is specifically involved with biofilm formation (Bornscheuer et al. 2012; Cao et al. 2011b; Lasa 2006; Lasa and Penadés 2006; Theunissen et al. 2010a).

Cell to cell communication within the biofilm is a major factor contributing to intercellular interactions within biofilms (Lee et al. 2014; O'Toole and Kolter 1998; Winn 2012). Quorum sensing is one of the well-characterized intercellular signalling systems (Decho et al. 2010a; Kjelleberg and Molin 2002; Platt and Fuqua 2010). Quorum sensing-based signaling is enabled by a specific family of compounds known as N-acyl-L-homoserine lactones (AHLs), which are specific to respective bacterial species. The AHL levels secreted by the cells are regulated by Autoinducer synthase LuxI and Autoinducer receptor LuxR. The AHLs synthesized by LuxI are secreted to the extracellular space, where it interacts with LuxR, which initiates the activation of signaling. The specific binding capability of LuxI and LuxR in each cell ensures accurate and precise response

to a certain signal (Decho et al. 2010a; Decho et al. 2010b; Kjelleberg and Molin 2002; Platt and Fuqua 2010). The critical role played by the AHL-mediated quorum sensing in biofilm matrix development and maintenance has been studied in detail for *P. aeruginosa* biofilms (Decho et al. 2010b; Hentzer et al. 2002; Hentzer et al. 2003; Lee et al. 2014; Winn 2012).

Further, intracellular signaling molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) also plays a prominent role in biofilm formation and detachment. The rise in c-di-GMP levels generally enhances biofilm formation with increase in EPS synthesis and decreased levels of cell motility (Chua et al. 2015; Güvener and Harwood 2007; Hickman and Harwood 2008; Steiner, Lori et al. 2013; Wu et al. 2014).

To harness a complete understanding of biofilm biology, several biofilm model systems and biofilm reactors have been configured. These systems enhance our knowledge about biofilm development and provide a comprehensive understanding of the fundamentals of biofilm biology. They serve as a relevant tool to mimic the applications of biofilms in natural and engineered settings as well as study the detrimental effects of clinical biofilms (Coenye and Nelis 2010; Heydorn et al. 2000; Qureshi et al. 2005). Table 2.4 describes the model systems used to study biofilm biology and biofilm reactors used for biofilm applications in different settings.

Table 2.4. Biofilm model systems and reactors used for elucidating biofilm biology and biofilm-based bioprocesses

Type	Features	Application	Reference
Biofilm model systems for elucidating biofilm biology			
Microtiter plate-based model system	A static batch reactor system Biofilms grown on bottom or side wells Media has to be replaced at regular time intervals Screening of several factors related to biofilm biology	Facilitates the study on nutrient gradients Effects of waste accumulation Biofilm formation capability Inhibitory effects of antibiotics and disinfectants Effects of shear stress in biofilms	(Cerca et al. 2005; Coenye and Nelis 2010; De Beer et al. 1994; O'Toole and Kolter 1998; Pitt et al. 1993; Walker and Sedlacek 2007)
Modified Robbins Device	A rectangular channel with a longitudinal array of ports Each port can support biofilm growth Made of plastic or stainless steel Plug flow reactor	Evaluate the effect of anti-microbial materials on biofilm formation Biofilm formation under continuous hydrodynamic conditions	(Cerca et al. 2005; Honraet and Nelis 2006; Krom et al. 2007)
Flow cell	Plug flow reactor with single or multiple channels (3 x) for biofilm growth Made of acrylic glass Can be operated over different flow rates Possible to operate in aerobic and facultative mode Flow only in single direction	Facilitate the real time quantification of biofilm development and processes non-destructively using microscopy Continuous monitoring of spatial heterogeneity, biofilm attachment and dispersal, cell to cell interactions within the biofilm Study biofilm-based bioprocesses like bioremediation of xenobiotics Analysis of biofilm matrix components by using specific dyes and microscopy	(Lee et al. 2014; Mohanty et al. 2014; Mohanty et al. 2015; Wu et al. 2015a; Wu et al. 2015b)
Constant	A plug flow reactor designed to study oral biofilms	Mimics the oral biofilm setting, study	(Hope and Wilson

depth film fermenter	Biofilm formation is always limited to a pre-determined depth	the attachment and detachment of oral biofilms and effects of shear stress on oral biofilms Also used to elucidate the effects of antimicrobial agents Quantify the surface characteristics on biofilm formation	2004; Morgan and Wilson 2001)
Tubing biofilms	Plug flow reactor with biofilms formed on the inner surface of a cylindrical plastic tube of known length and diameter	Employed to study biofilm-based bioprocesses such as biocatalysis and bioremediation Quantify the nutrient gradients, surface characteristics and other factors affecting biofilm biology	(Alonso 2015; Ding et al. 2014a; Williams et al. 2013)
Annular biofilm reactor	A rotating cylinder fitted with coupons that support biofilm growth	Used in biofilm control studies for drinking water disinfection systems	(Cerca et al. 2005)
Biofilms entrapped in polymeric scaffolds	Cells are immobilized in synthetic polymeric matrix like hydrogel Mimics real biofilms in nature Potential to replace real biofilms for mediating biotransformations Possess long-term stability and efficiency than real biofilms Easy to control, predict and could be recycled Still at early stages	A potential tool to improve the longevity of biofilm-based bioprocesses A tool to scale up the laboratory models for industrial applications	(Zhang et al. 2014)
Microfluidic devices	A pattern consisting of several microchannels supported on a photosensitive polymeric substratum Microchannels (50-500 μm wide and 30-250 μm deep)	Microscopic analysis of biofilms with highest degree of resolution and precision Facilitate manipulation of nutrient gradients and spatial heterogeneity	(Benoit et al. 2010; De La Fuente et al. 2007; Ding et al. 2010; Kim et al.

	Whole assembly is integrated inside a square chamber (20-50 mm length) Facilitate flow along both sides of the square chamber Operated in low flow rates with low Reynold's number ensuring highly laminar flows	High throughput estimation of biofilm biology	2010a; Liu et al. 2015; Richter et al. 2007b)
Biofilm reactors for harnessing engineering applications			
Continuously stirred tank reactor with fixed media (CSTR)	Continuous system provided with longitudinal mixing Biofilm growth on fixed bed support Small specific biofilm surface area Low substrate and oxygen mass transfer rate Suitable for high biomass retention and low substrate concentration Uniform feed and effluent flow rate.	Wastewater treatment (larger volume with less organic loading) Bioreactor for biofuel production and industrial fermentation processes with lower substrate concentration Bioremediation of xenobiotics from ground water	(Huang et al. 2004; Qureshi et al. 2005; Tay and Yang 2002)
Moving bed biofilm reactors (MBBR)	Biofilm is formed on suspended biofilm carriers Larger biofilm surface area High substrate and oxygen mass transfer rate Higher rate of substrate conversion Substantial reduction in reactor footprint	Wastewater treatment with high organic loading Suitable for bioprocesses like bioremediation, biodegradation, fermentation with high substrate loading	(Nicolella et al. 2000; Qureshi et al. 2005; Rusten et al. 2006)
Packed bed biofilm reactor	A plug flow reactor Reactor is packed with the synthetic media for biofilm growth Feed is provided from the bottom at a low flow rate, which keeps the biofilm carriers intact Biofilm is submerged within the reactor Lack of mechanical shear reduces cell dispersal rate from the media High cell retention due to large surface area of the biofilm carrier	Biofuel generation Biodegradation of xenobiotics Biosorption and reduction of heavy metals Industrial fermentation Removal of inorganic waste gases such as H ₂ S and NH ₃	(Ding et al. 2014a; Nicolella et al. 2000; Ottengraf 1987; Qureshi and Maddox 1988; Qureshi et al. 2000)

	Excessive cell growth can lead to poor diffusion and blockage Large gradients in concentration of nutrients and oxygen and cell density across the bed Turbulent flow regime within the reactor		
Fluidized bed biofilm reactor	Biofilm are formed on low-density particles that expands due to fluidization conditions Fluidization is maintained either with high upward flow rate or through aeration or mechanical stirring Large surface area for cells to attach but fluidization conditions checks excessive cell growth More contact between cells and the substrate Biofilms are in the form thin layers of attached cells Diffusion is not a limiting factor Since the particle size is small, no likely chances of clogging or blockage Attrition and shear induced by fluidization is much lower than mechanical agitation in stirred reactors Can be modified into granular biofilm reactors	Biocatalysis with immobilized enzymes and whole-cells Industrial fermentation Wastewater treatment (lower volume with high organic loading) Well suited for anaerobic bioprocesses in upward anaerobic sludge blanket configuration (UASB) such as anaerobic wastewater treatment, dechlorination, anaerobic treatment of municipal leachate, anaerobic biodegradation of phenolic compounds	(Iza et al. 1992; Lettinga et al. 1980; Qureshi and Maddox 1988; Seghezzo et al. 1998; Tsuno et al. 1996; Veeresh et al. 2005; Wobus et al. 1995)
Hollow fiber Membrane bioreactor (HfMBR)	Biofilms are grown on the hollow fibers, which is encased together in silicon tubing Large surface area to volume ratio for biofilm formation High rate of cell entrapment which can be controlled Stable biofilms and easily reproducible With a radial flow, no diffusion limitations Biofilms can be easily harvested by reversing the flow direction Facilitates control of bioprocesses	Wastewater treatment Biosorption and heavy metal immobilization Synthesis of value added products such as pharmaceutical products and intermediates Biocatalytic reactions which involves cofactor recirculation Characterization of biofilm matrix and recovery of value added metabolites from the biofilm	(Brindle and Stephenson 1996; Cao et al. 2011a; Cao et al. 2012; Cao et al. 2011b; Casey et al. 1999; Ding et al. 2014a)

2.2.2. Biofilm-mediated biocatalysis

As described in the previous section, biofilms provide an excellent platform to explore different routes of biocatalytic reactions and biotransformations (Betancor and Luckarift 2008; Bornscheuer et al. 2012; Dalby 2007; Flemming and Wingender 2010; Gross et al. 2013; Gross et al. 2007; Hartmann and Jung 2010). Biofilms provide advantages such as long-term catalytic retention and stability, high rate of catalyst regeneration, wider range of substrate specificity and potential to operate as a continuous system (Gross et al. 2007; Halan et al. 2012b).

Biofilm-mediated synthesis of value added products originated with the production of ethanol from the fermentation of sugars Biofilm facilitates biocatalysis with productivity and stability several folds higher than planktonic cells (Kunduru and Pometto III 1996; Rosche et al. 2009; Todhanakasem et al. 2014; Zhang et al. 2004). Microbial electrosynthesis and clean energy generation using electroactive biofilms have gained a lot of attention in the biocatalytic arena (Bond et al. 2012; Erable et al. 2010; Rabaey et al. 2007; Gross et al. 2007). These processes lead to the formation of by-products such as hydrogen, hydrogen peroxide, methane, ethanol and other resourceful organic compounds.

Biofilm-mediated biofuel production from waste biomass is also an area, which has been well studied (Franks et al. 2010; Halan et al. 2012b; Rosche et al. 2009). Majority of the reports have highlighted the longevity and stability as key features of biofilm-mediated biocatalysis. Cell entrapment within the biofilm matrix enables the continuous processing of biocatalytic reactions with minimum available biomass. The incorporation of suitable biofilm carriers further enhance

cell entrapment and ,, ensures better substrate accessibility for biofilm catalyst (Gross et al. 2007; Halan et al. 2012b; Rosche et al. 2009; Winn et al. 2012).

Biofilms are widely used for bioremediation of polycyclic aromatic hydrocarbons (PAHs) such as chlorinated or nitro-aromatic compounds (Singh et al. 2006; Lu et al. 2011). Polycyclic aromatic hydrocarbons are discharged into soils and water bodies from industrial effluents. They are highly carcinogenic and persist in natural environment for a long period (Lu et al. 2011; Wick et al. 2011). PAHs are highly non-biodegradable and their biotransformation may result in the generation of more toxic products. Hence, bioremediation of PAHs cannot be processed in a single step microbial conversion (Lu et al. 2011; Wick et al. 2011). The self-immobilized and well-protected biofilm framework provides the best setting for the degradation of such xenobiotics (Singh et al. 2006; Wick et al. 2011). Several cases of biofilm-mediated bioremediation have been reported, as listed in Table 2.5.

Biofilms also facilitate the sequestration and immobilization of heavy metals like Cr and As. Heavy metal bioremediation by biofilms is mediated through several types of microbe-mineral interactions (Cao et al. 2010; Coombs et al. 2010; Gadd 2004; Singh et al. 2006). EPS secreted by biofilms entrap and accumulate the heavy metals hence, playing a major role in such interactions. Biofilms of dissimilatory metal reducing bacteria (DMRB) such as *Shewanella* can couple bioremediation of organic contaminants with heavy metal reduction or power generation (Bretschger et al. 2007; Chaudhuri and Lovley 2003; Lies et al. 2005). Several studies have shown the vast potential of electroactive biofilms to support the microbial respiration with higher power yields and degradation of the substrate (Bond et al. 2012; Erable et al. 2010; Franks et al. 2010; Gadd 2004).

The electrostatic interactions cause the adsorption of metallic ions onto EPS components or bacterial cell wall, resulting in metal precipitation.

The secretion of metal chelating molecules like siderophores and electron shuttles like flavins enhance biofilm-mediated heavy metal remediation (Watanabe et al. 2009; Patil et al. 2012; Neilands 1995). Biofilms with cells that express catabolic genes encoded specifically for degradation of toxic compounds are best suited for biofilm-mediated bioremediation (Blank et al. 2010). This can be further enhanced through engineering environmental strains by integrating molecular approaches.

These may include genetically manipulating specific metabolic pathways or by engineering enzymes proficient at degradation or by multiplying the copy number of catabolic genes (Bailey 1991; Cirino and Sun 2008; Yim et al. 2011; Yang et al. 2015). Biofilms of recombinant strains expressing specific catabolic genes have enhanced bioremediation capability (Singh et al. 2006; Yim et al. 2011). Indigenous microorganisms at the contaminated sites develop their own phenotypes, which exhibit greater tolerance towards environmental contaminants. In natural settings, indigenous microbes typically mediate bioremediation and hence, they form a crucial link towards biofilm-mediated biotransformations (Singh et al. 2006).

Another alternative strategy involves the development of mixed communities, which mediate the biotransformation through mutualistic interactions by coupling diverse metabolic activities (Gross et al. 2007; Lower et al. 2001; Marsili et al. 2008; Richardson et al. 2012; Rosche et al. 2009; Sheldon and van Pelt 2013; Singh et al. 2006). The biofilm microenvironments facilitate interspecies interactions that drive certain biofilm-mediated bioprocesses (Morris

et al. 2013b; Parsek and Fuqua 2004). A typical example of such interspecies interactions within a self-immobilized framework could be observed in microbial mats (Stal and Caumette 2013; Wilmotte et al. 2015). Interspecies interactions such as syntrophy mediate the complete removal of carbonaceous organic matter in anaerobic wastewater treatment (Roest et al. 2005; Terada et al. 2011).

2.2.3. Role of mutualistic cell to cell interactions in biofilm-based bioprocesses

Microorganisms contribute extensively to the geochemical cycling of organic carbon and minerals (Fredrickson and Gorby 1996; Lies et al. 2005; Schuetz et al. 2009; Walsh 2001). Most of these reactions are catalysed by biofilms in natural environment (Wang et al. 2015b). Biofilms can colonize even the most hostile environments with its potential capacity to metabolize a variety of substrates (Handley et al. 2012). The cell to cell interactions within the biofilm contribute significantly to this unique biofilm activity (Wang et al. 2015b).

Several different types of microbial interactions take place in a natural biofilm. Neutralism refers to the interactions when the function of one bacterial species is completely independent of other species (Morris et al. 2013a). Competition is the type of interaction when different bacterial species within the biofilm compete for the limiting resources (Morris et al. 2013a). Interactions involving certain benefits for a certain community without causing any impacts (positive or negative) on any other community are referred to as commensalism. When two species gain benefits by the presence of each other, the interaction is referred to as mutualism (Curl and Truelove 1986; James et al. 1995; Morris et al. 2013a).

Mutualistic interactions may occur when the partners with different metabolic functions combine together for deriving mutual benefits. In addition, if the partners have to associate each other in a certain environment, where both cannot survive on its own, mutualistic interactions happen (Morris et al. 2013a). The benefits of mutual interactions exceed the functional output from each partner. Different forms of mutualistic interactions include synergy, syntrophy, symbiosis and obligatory mutualistic metabolism (Curl and Truelove 1986; James et al. 1995; Morris et al. 2013a). Hence, in natural environments, mutualism represents a long-term robust partnership between diverse bacterial species.

During a typical anaerobic mutualistic interaction, a complex organic molecule is hydrolysed or fermented to simple metabolites such as formate, acetate, ethanol, and butyrate. These fermentation products serve as electron donor for reduction of sulfate, iron and nitrate as well as methanogenesis. These interactions mediate the anaerobic wastewater treatment, biodegradation of aromatic compounds, oils and complex sugars (Clouthier and Pelletier 2012; Morris et al. 2013a; Rinaldi et al. 2008; Yamazawa et al. 2013; Yang et al. 2015b). Table 2.5 enlists several biofilm-mediated biotransformations.

Table 2.5. Biofilm-mediated biotransformations

Biofilm-mediated biotransformation	Reactor	Organism	Key findings	Reference
Ethanol production from lignocellulosic acid hydroxylate	Flow cell	<i>Zymomonas mobilis</i> (4 strains)	Biofilm formation was found to be significantly higher on hydrophobic plastic surfaces than glass surface. Biofilm-mediated fermentation (~72% theoretical yield) was found to be significantly higher than the planktonic fraction (~4% theoretical yield). Presence of toxic fermentation inhibitors did not affect the metabolic activity in biofilms owing to the presence of a stable EPS.	(Todhanakas et al.2014)
Synthesis of octanol and styrene oxide	Membrane biofilm reactor (Tubular silicon membrane)	<i>Pseudomonas sp.</i> VLB120ΔC	The study is based on biofilm-mediated synthesis of octanol and styrene oxide using multiple enzymatic systems. Intrinsic styrene monooxygenase in the model strain catalyze the conversion of styrene to styrene oxide, whereas octanol synthesis is processed through the expression of alkane hydroxylase. Very high volumetric productivity and long-term biocatalytic activity were observed for biofilm-mediated synthesis. An optimization study to magnify the process to industrial scale (production capacity of 1000 t/year) suggested that the biofilm-mediated synthesis of styrene oxide is highly feasible in case of product yield (5-folds) and biomass wastage (400-folds less) as compared to stirred suspended reactors with planktonic cells.	(Gross et al. 2013)
Synthesis of Packed trickle		<i>Gluconobacter</i>	DHA is an important component in	(Hekmat et

dihydroxyacetone (DHA) from glycerol	bed reactor		<i>oxydens</i>	cosmetic industry and a significant intermediate in the synthesis of several chemicals. Repeated fed batch biofilm-mediated fermentation process eliminates the pre-treatment steps and significantly enhances the longevity and volumetric productivity of the process. The tolerance capacity of the cells to DHA was increased in biofilm mode.	al. 2003)
Ethanol synthesis from glucose	Packed reactor	bed	<i>Z. mobilis</i> and <i>Saccharomyces cerevisiae</i>	The study reports on significantly higher production of ethanol (15 to 100-folds high) by biofilms cultivated on different plastic supports as relative to suspended fraction. Immobilization of cells in the biofilm prevented the wash out of biocatalysts as observed in suspended reactors. With increased productivity, reactor plugging was raised as an issue.	(Kunduru and Pometto III 1996)
Continuous production of lactic acid	Packed reactor	biofilm	<i>Lactobacillus casei</i> subsp. <i>ramnosus</i>	Lactic acid is an important component in food, cosmetic, pharmaceutical and plastic industries. High productivity and low cell washout with biofilms. Higher mass transfer rates and no destruction of support material as compared to cells immobilized in hydrogels. Reactor also facilitates the adjustment of biofilm thickness.	(Cotton et al. 2001)
Production of hydrogen from sucrose	Trickle reactor	bed	<i>Caldicellulosiruptor saccharolyticus</i>	Biofilms are grown on polyurethane foam support at high temperature (~70°C with continuous gas flow under low hydrogen partial pressure on a large surface area. Hydrogen formed is immediately removed. Volumetric production capacity of hydrogen	(Van Groenestijn et al. 2009)

				by the thermophilic bacteria was found to be same as that of mesophilic with a higher biomass yield. Biofilm environment provided advantages such as higher biomass yield and better cell entrapment rate.	
Production of lactic acid from glucose and cornstarch	Rotating fibrous bed reactor	<i>Rhizopus oryzae</i>		Fungal biofilm-mediated fermentation of glucose to produce lactic acid. Significant effects of pH and dissolved oxygen were recorded on fermentation. Biofilm environment in reactor facilitated better control over the operation but hampered the diffusion of oxygen creating a spatial gradient.	(Tay and Yang 2002)
Simultaneous production and recovery of fumaric acid	Rotary biofilm contactor with an adsorption column	<i>Rhizopus oryzae</i>		Cells are immobilized on the rotating plastic discs, alternatively exposed to aerobic and anaerobic conditions during the course of rotation. On a weight yield basis, biofilms (91% of theoretical yield) produced higher amounts of fumaric acid than planktonic freely suspended cells (63%). Operated continuously for two weeks without compromising the activity.	(Cao et al. 1996)
Ethanol production from glucose	Expanded bed biofilm reactor	<i>Z. mobilis</i>		Cell immobilization resulted in high rate of cell entrapment with minimum wash out at high dilution rate. Large biofilm surface area for cell attachment, mass transfer and biocatalytic reaction. The expansion of the bed facilitates efficient contact between the biofilm and substrate.	(Bland et al. 1982)
Production of acetone, butanol	Packed bed reactor	<i>Clostridium beijerinckii</i> BA101		Cells are adsorbed onto the support media. Biofilm-mediated fermentation (5-folds	(Qureshi et al. 2004)

and ethanol				higher than planktonic fraction) scaled up by maintaining the same productivity rate. Excessive cell growth on support media due to spore formation caused blockage and affected the diffusion of nutrients leading to decrease in productivity.	
Ethanol production from hydrolyzed waste starch	Fluidized bed reactor	bed	<i>Z. mobilis</i>	Sterilization of the waste starch (to remove lactic acid bacteria that inhibit <i>Z. mobilis</i> activity) is eliminated by adopting short residence time (HRT) using biofilm-mediated fermentation. High conversion efficiency with low HRT. ~3-folds increase in ethanol production by biofilms as compared to suspended cells.	(Weuster-Botz et al. 1993)
Polyhydroxybutyrate (PHB) synthesis from pulp fibre sludge	Packed reactor	bed	<i>Alcaligenes eutrophus</i>	The study reports on the synthesis of PHB from glucose released into the media by the cellulose degradation of pulp fibre sludge. Biofilms are grown on positively charged microcarrier. <i>A. eutrophus</i> has weak biofilm formation capability. Electrostatic interactions between the cells and the microcarrier hold the bacterial carriers on the biofilm support. Hence, ionic strength of the biofilm carrier has a huge influence on biocatalysis mediated by biofilm defective organisms. Most of the activity was observed in the presence of excess carbon source and less nitrogen source. The volumetric yield was comparable with that of the planktonic fraction.	(Zhang et al. 2004)
Biodegradation of	Rotating		<i>Pseudomonas</i>	Diffusion limitations in biofilms with high	(Kargi and

2,4-dichlorophenol (DCP)	perforated tube biofilm reactor	<i>putida</i> DSM6978	biomass concentration mitigate the toxic effects of DCP and its intermediates. Activated sludge supplemented with <i>P.putida</i> was used as the inoculum. High rates of degradation were observed at low organic loading and high surface area to discharge ratio. Initial concentration of DCP also had a significant effect on biofilm-mediated biodegradation.	Eker 2005)
Bioremediation of chlorophenol contaminated ground water	Fluidized bed biofilm reactor	Selectively enriched microorganisms from the site	Study reported strategies for enriching chlorophenol-degrading consortia from the site, reaction kinetics of chlorophenol remediation, troubleshooting techniques and toxicity removal. Chlorophenols are utilized as sole source of carbon and energy. The laboratory results were also scaled up into an onsite pilot plant.	(Puhakka et al. 1995)
Reductive dechlorination	Membrane bioreactor	Enriched anaerobic sludge	In the presence of hydrogen as electron donor, the enriched anaerobic sludge from swine wastewater treatment plant reduced dichlorophenol to phenol. Anaerobic degradation replaces chlorine with hydrogen hence, producing less toxic intermediates. Nitrate has an inhibitory effect as an alternate electron acceptor. Sulfate inhibited the biodegradation without being utilized as electron acceptor. The silicon tubing for membrane bioreactor alleviated the hydrogen diffusion limitations in the biofilm.	(Chang et al. 2003)
Biodegradation of 4-chlorophenol	Granular activated	Enriched consortia	Adsorption combined with biofilm-mediated biodegradation. Biofilms cultivated on GAC	(Carvalho et al. 2001)

	carbon biofilm reactor		are more tolerant to shock loading than planktonic cultures. The biofilm is able to survive long periods of contaminant starvation and also displays a long-term activity. Removal efficiency between 60-100% is attained.	
Biodegradation of a mixture of C ₁₄ , C ₁₅ and C ₁₆ alkanes	Rotating biological contactor (RBC)	<i>Prototheca zopfii</i>	Algal biofilms developed on the rotating plastic disks partially exposed to aerobic and anaerobic conditions were operated in single stage as well as three stages in series. Single stage RBC achieved a degradation rate of 65%. The three stage RBC was modelled on the basis of results from the single stage RBC.	(Yamaguchi et al. 1999)
Biodegradation of carbon tetrachloride	Fixed biofilm reactor	<i>Pseudomonas cepacia</i> and <i>Providenci astuartii</i>	Continuous flow fixed biofilm reactor inoculated with two strains with high degradative capability. Almost 100% degradation. A good correlation was observed between the experimental results and the predicted models.	(Jin and Englande 1998)
Biodegradation of toluene	Hollow fibre membrane bioreactor	Secondary sludge from wastewater treatment consisting of aerobic and anaerobic bacteria	Hollow fibre membranes for biofilm formation provide advantages such as high cell density, high surface to volume ratio for cell attachment, no clogging due to excessive cell growth and better control over the bioprocess. The reactor is used to degrade toluene in contaminated air. Air flow rate and toluene initial concentration has a major influence on the degradation efficiency.	(Parvatiyar et al. 1996)
Decolourization of textile dye	Laboratory scale activated	<i>Coriolus versicolor</i> with activated	White rot fungi degrade the dyes through extracellular ligninolytic enzyme system.	(Kapdan and Kargi 2002)

	sludge unit with inclined plates for biofilm formation	sludge	Diffusion limitations of substrate are eliminated with the extracellular localization. Biofilm environment provides more stability to the extracellular enzyme activity. Fungal biofilms are combined with activated sludge to couple biosorption with enzymatic degradation. Combined treatment methodology provided a biodegradation efficiency of 82%	
Biodegradation of phenoxy herbicides	Granular activated carbon biofilm reactor	Mixed culture of herbicide degrading bacteria	The consortia used for the study were isolated from the manufacturing site of the phenoxy herbicides used in the study. Degradation is a combined effect of metabolic activity and biosorption	(Oh and Tuovinen 1994)
Bioremediation of phenol	Membrane bioreactor	<i>Neurospora crassa</i>	Fungal biofilms expressing oxidative enzyme activity for phenol degradation. Biofilms displayed a significantly higher activity (~1.4-folds) than suspended cells. The activity was also exhibited for a long-term, confirming the phenolic degradative capability of fungal biofilms	(Luke and Burton 2001)
Biotransformation of benzaldehyde to benzylalcohol	Packed bed reactor	<i>Z. mobilis</i>	Biofilms increase the substrate tolerance by 6-folds with respect to substrate concentration and exposure time as compared to planktonic cells. Biofilms also displayed steady productivity throughout the reaction. The study proposes biofilms as “factories” for the synthesis of fine chemicals.	(Li et al. 2006)
Continuous synthesis of epoxypropane	Fluidized bed biofilm reactor	Mixed culture methanotrophs	Methane monooxygenase induced oxidation of propene to epoxypropane by methanotroph biofilms. This reaction does	(Xin et al .2003)

			not contribute to biomass growth or energy and also results in the accumulation of toxic products. Biofilm-mediated synthesis of epoxypropane enhances the stability and decreases the toxicity levels. Also facilitates long-term operation and potential for scaling up towards commercial scale.	
Biodegradation of 3-chloroaniline (3-CA)	Submerged biofilm reactor	<i>Comamonas testosteroni</i> WDL7	Several studies have shown that the intracellular signaling molecule c-di-GMP controls the biofilm formation and dispersal. In this study the role of c-di-GMP in biofilm-mediated bioprocess is elucidated with the biofilm-mediated degradation of 3-CA as the model biotransformation. Constitutive expression of c-di-GMP synthase in <i>C.testosteroni</i> elevated the c-di-GMP levels, EPS production and biofilm formation capability (~10-folds higher than wild type). Further the 3-CA degradation efficiency of <i>C. testosteroni</i> biofilms expressing YeDQ was much higher than the WT with better cell entrapment rate and significantly lower rate of cell dispersal. Study presents a novel strategy for biofilm-mediated biodegradation through c-di-GMP over expression.	(Wu et al. 2015a)
Biosorption of heavy metals	Anaerobic-anoxic-oxic (A2O) process with rotating biological contactor	Wasted biomass from A2O process	EPS of floc forming activated sludge microorganisms are negatively charged which can adsorb positive metallic ions. RBC is slotted next to A2O process and is enriched with the floc forming activated sludge biofilms on its rotating discs.	(Chang et al. 2006)

					Biosorption of Cd, Ni and Zn was evaluated using this network. A rapid uptake of the metals was observed within 20 min. Initial metal concentration, pH, temperature and sludge types influenced the metal uptake	
Biosorption and bioprecipitation of heavy metals	of	Moving sand bed filter biofilm reactor	<i>Ralstonia eutropha</i> CH3 (bioprecipitating), <i>Pseudomonas mendocina</i> AS302 and <i>Arthrobacter</i> sp. BP7/26 (biosorption)		A moving sand bed reactor is enriched with biofilms of heavy metal bioprecipitating and biosorbing cells. About 80-90% removal of Cu, Zn, Ni and Co was observed for 18 months along with removal of organic molecules. The metals immobilized in the sludge could be recycled and reused in industries. Steep pH gradients induced by the biofilm metabolic activity results in the precipitation and electrostatic interactions cause the biosorption. This method exhibits potential for the treatment of leachate	Diels et al. 2003)
Cr(VI) immobilization by biofilms	by	Submerged biofilm reactors	<i>S. oneidensis</i> MR-1		Through the disruption of putrescine biosynthesis, the biofilm cohesiveness is enhanced, which leads to higher rate of cell entrapment and hence, thicker biofilms. Higher biofilm cohesiveness improves the immobilization rate of heavy metals; Theoretically 56% of Cr(VI) added was immobilized by the hyper adherent mutant biofilm whereas WT biofilms could accumulate only 27%. The study also highlights the role of putrescine in the deconstruction of biofilm matrix	(Ding et al. 2014a)
Bioimmobilization of	Constant		<i>S. oneidensis</i> MR-1		The metabolic activities within the	(Cao et al.

Cr(VI) and U(VI)	depth fermenter	film		microenvironments of biofilm upon exposure to U(VI) and Cr(VI) were elucidated and resolved spatially and temporally. Exposure to environmental contaminants induces changes in the structure and hydrodynamic properties of the biofilm, rendering the biofilms to adapt and mediate the bioremediation with a custom-developed approach. The results suggested the reduction of Cr(VI) to Cr(III) by the biofilm, which had an inhibitory effect on lactate metabolism by the cells. Similarly U(VI) immobilization also affected the metabolic activity within the biofilm. The study also underpins how the interactions EPS with the toxic cations alter the mass transfer and nutrient uptake within the biofilm microenvironments	2012)
Bioimmobilization of U(VI)	Packed reactor	bed	Indigenous subsurface sediments from the site	The study is an attempt to elucidate the mechanism involved in the removal of U(VI) through interactions between the subsurface sediments, oxic ground water and river water which involved reduction as well as adsorption and desorption. To decipher the mechanism, packed bed sediment biofilm reactors were fed with synthetic groundwater (SGW), synthetic groundwater amended with organics (OA-SGW) and deionized water, each containing U(VI). Further the reactors were fed with river water to study the remobilization of U(VI). The results suggested reductive immobilization of	(Ahmed et al. 2012)

			<p>U(VI) with OA-SGW, which were also highly resistant to desorption by river water (93%). In case of GW sediments, U(VI) immobilization was achieved only through adsorption. The study confirmed that supplementing the natural sediments with organics leads to better removal of U(VI) from the contaminated site through reductive immobilization. Also the remobilization of U through river water intrusion is minimal and occurs through desorption</p>	
Bioimmobilization of U(VI)	Hollow fibre membrane biofilm reactor	<i>Shewanella</i> sp.HRCR -1	<p>The study was conducted to elucidate the effect of EPS in biofilm-mediated U(VI) immobilization using biofilms grown in hollow fibre membrane bioreactors. Bound EPS (bEPS) is a functional extension of the cells in the biofilm and is enriched with redox active outer membrane cytochromes, which facilitate the reduction of U(VI). Further bEPS also accumulates U(VI) through sorption by the polysaccharides Sorption dominated the U(VI) immobilization by loosely associated EPS (laEPS).</p>	(Cao et al. 2011a)
Biosynthesis of nisin	Packed bed biofilm reactor	<i>Lactococcus lactis</i>	<p>Biofilm-mediated continuous production of nisin and effect of whey permeate in improving the productivity was studied in packed bed biofilm reactors. The effect of pH, temperature, cell density, and substrate concentration and dilution rate was also investigated. The reactor was operated for 6 months without any</p>	(Liu et al. 2005)

			mechanical failure. The study illustrates the biofilm-mediated synthesis of nisin continuously with great stability and also shows potential for scaling up.	
Bioelectrochemical production of ethanol	Bioelectrochemical system	Acetate reducing inoculum	Ethanol production from acetate reduction with hydrogen as electron donor results in very low yield. In this study the electrode is used as the donor and the role of methyl violet as a mediator in acetate reduction is also investigated. Ethanol productivity increased by 6-folds in the presence of mediator. The electron flow occurred across the biofilm. After 5 days, the cell density had a cascading effect on the electron flow that affected the productivity	(Steinbusch et al. 2009)
Bioelectrochemical production of organic compounds from CO ₂	Bioelectrochemical system	Acetogenic <i>Sprormusa ovata</i>	Biofilm-mediated reduction of CO ₂ to acetate using electrode as the electron donor	(Nevin et al. 2010)
Combined nitrification and denitrification	Membrane aerated biofilm reactor	Sludge consisting of nitrifying bacteria and heterotrophs	Biofilm-mediated combined nitrification and denitrification as well as removal of organic loading. Heterotrophic bacteria and denitrifiers occupied the biofilm-bulk liquid interface while ammonia-oxidizing bacteria were localized on the outer surface of the membrane. DO was completely consumed in the biofilm. Denitrification occurred primarily through nitrite. Wastewater exhibited a low TOC/N ratio as it entered the biofilm.	(Terada et al. 2003)

One key functional element of biofilm-mediated biocatalysis is the biofilm matrix. An ideal biofilm matrix should have the following characteristics: (1) Porous enough to facilitate the diffusion of water, energy source and nutrients; (2) Have sufficient channels to permit outflow of waste products generated within the biofilm; (3) Anchor the biofilms with better cell retention capacity; (4) Impart resistance against the external stresses that tend to impede the biofilm robustness (Branda et al. 2005; Erable et al. 2010; Flemming and Wingender 2010; Polizzi et al. 2007; Singh et al. 2006; Sutherland 2001b). However, there are certain factors that reduce the effectiveness of biofilm-mediated bioprocesses, which needs to be addressed.

2.2.4. Limitations associated with biofilm-mediated biocatalysis

Diffusion has a profound influence on the efficiency of biofilm-based biocatalysis, since it drives the mass transfer of substrate and intermediate products within biofilm microenvironments (Stewart 2003). The diffusion limitations cause detrimental effects to biofilm robustness by drastically hindering the mass transfer and fluid flow. Two major factors contributing to the diffusion restrictions are excessive production of EPS and accumulation of biofilm biomass (Singh et al. 2006; Stewart 2003). High biomass density and significant amount of EPS reduce the convective mass transfer (Gross et al. 2013). This creates a high nutrient gradient throughout the whole biofilm, which affects biofilm cohesiveness.

Biofilm cohesiveness is a major factor contributing to biofilm-based bioprocesses. The disruption of biofilm cohesiveness leads to perturbations in biofilm functional activities (Ding et al. 2014a; Zhang et al. 2014). In biofilms, the diffusion distance should be considered with respect to multicellular aggregates, whereas in planktonic cells the diffusion distance can be normalized to a single

cell (Stewart 2003). The diffusion restrictions also lead to the accumulation of waste within the biofilm (Gross et al. 2007). Non-uniform distribution of nutrients coupled with waste accumulation disrupts the metabolic activities of cells in the biofilm. The overall productiveness of the biofilm-based bioprocesses depends extensively on the physiological state and metabolic activities of the biofilm cells. Therefore, the diffusion profile contributes to the intrinsic heterogeneity within the biofilm microenvironments.

The mass transfer of nutrients and water through the biofilm is often represented by the diffusion coefficient (Lewandowski and Beyenal 2007; Renslow et al. 2010). The experimental findings from the previous works have shown that the diffusion coefficient is highly dependent on space dimensions (biofilm thickness) (Stewart 2003; Renslow et al. 2010). Normally the diffusion coefficient exhibits a marked decrease with biofilm thickness (Beuling et al. 1998; Lewandowski and Beyenal 2007; Renslow et al. 2010).

The diffusion coefficient (relative) is expressed as the ratio of diffusion coefficient of water in biofilm to the diffusion coefficient of water at a distance of 1000 μm from the aqueous surface (Beyenal et al. 1998; Renslow et al. 2010). During initial biofilm development phase, the diffusion is maximum at top layers and minimum at bottom layers. In this phase, the relative diffusion coefficient decreases from 100% at the top layers (biofilm-liquid interface) to about 55-65% at the bottom layers, as shown in Figure 2.2 (A) (4-day old biofilms) (Renslow et al. 2010). This is because the cell adhesion and cell aggregation is initiated from the bottom depths, accounting for more cells there during attachment phase (Bishop et al. 1995; Renslow et al. 2010).

During the maturation phase, cell aggregation extends to the middle, resulting in an increasing cell density (Figure 2.2 (B), 8-day biofilm), which results in decreased diffusion rates at the middle layers. The lower layers display increasing or decreasing diffusion rates, as cells start to disperse or shift to the middle layers (Bishop et al. 1995; Renslow et al. 2010).

For biofilms of age more than 8 or 10 days (Figure 2.2 (C), 10-day biofilm), cells at bottom layers tend to detach and shift to planktonic mode. Hence, the relative diffusion coefficient is minimum near middle layers, where more biomass is concentrated. An increasing trend is observed in upper and lower layers, which is almost devoid of cells (Beuling et al. 1998; Renslow et al. 2010).

From Figure 2.2, it is also evident that the diffusion in biofilm varies with the biofilm age (Bishop et al. 1995; Renslow et al. 2010). The profiles observed for 4-day (Figure 2.2 (A)), 8-day (Figure 2.2 (B)) and 10-day (Figure 2.2 (C)) biofilms demonstrate the temporal shift in biofilm diffusion coefficients with each biofilm development stage. Such an inconsistent profile is due to the spatio-temporally varying cell density within the biofilm microenvironments (Renslow et al. 2010).

For most of the biofilms, the aerobic zone exists only in first few layers ranging up to 100 μm from the biofilm-liquid interface (Beyenal et al. 1998). Further, the relative diffusivity of organic solutes like lactate, acetate and glucose is limited to 25% (Stewart 2003). These organic molecules form the driving force for all metabolic activities within the biofilm. Hence, the constraints imposed on mass transfer through the biofilm tend to bring down the effectiveness of the biofilm-based biocatalysis (Halan et al. 2012b; Polizzi et al. 2007; Singh et al. 2006; Stewart 2003).

Figure 2.2. Spatial and temporal profile of biofilm diffusivity, reproduced from (Renslow et al. 2010) with permission from “John Wiley and Sons”. (A) 4-day old biofilm; (B) 8-day old biofilm; (C) 10-day old biofilm.

Other factors also disrupt the productivity of biofilm applications. Even though biofilms stand out as attractive biocatalytic systems as compared to conventional biocatalysts, the intracellular localization of the catalytic centre poses several bottlenecks.

Intracellular biocatalysis occurs within the confines of microbial cell. The intracellular enzymes mediate the catalytic reaction and the product is generated within the cell (Halan et al. 2012b; Hibbert et al. 2005). Intracellular biocatalysis involves wide range of complexities. The products formed intracellularly might become toxic at high concentrations. The biofilm is a constantly evolving heterogeneous system, which will eventually lose its catalytic activity due to toxic effects of the product.

Intracellular biocatalysis requires the application of several extensive techniques to extract the product from the cells. The techniques used in the isolation and extraction of the formed products might hamper the yield and quality of the respective product. In most of the cases, the cells have to be lysed to retrieve products, which will destroy the structural integrity of biofilms. Such product isolation strategy challenges the application of biofilms for continuous processing of biocatalysis (Bornscheuer et al. 2012; Halan et al. 2012b; Leon et al. 1998; Polizzi et al. 2007).

The biofilm environment includes extensive variety of enzymes that might inhibit the activity of a specific enzyme through interference. The membrane barrier, which both substrate and the product have to encounter, is another well-cited drawback associated with intracellular biofilm-mediated biocatalysis (Alcalde et al. 2006; Schüürmann et al. 2014; Smith et al. 2015). Hence, in order to make the biofilm-mediated biocatalysis more productive, an alternative solution is to shift the catalytic activity to biofilm matrix.

2.2.5. *Biofilm-based extracellular biocatalysis*

The robustness imparted by biofilm matrix is the most significant feature for biofilm-based bioprocesses. The shift in catalytic activity to biofilm matrix might alleviate some of the constraints addressed in the previous section. Extracellular biofilm-based biocatalysis have the capacity to mitigate diffusion limitations, since there is no requirement for substrate or product to diffuse across the membrane barrier. One obvious advantage for extracellular catalysis is that it avoids intracellular accumulation of end-products that might be detrimental to metabolic activity.

Biofilm matrix-enabled biocatalysis facilitates easy recovery of the product without perturbing the overall structural integrity of the biofilm. This also provides the advantage of using biofilms for multiple rounds of catalysis (Alcalde et al. 2006; Branda et al. 2005; Flemming et al. 2007; Hoffman and Decho 1999; Rosche et al. 2009; Schüürmann et al. 2014; Smith et al. 2015; Tsoligkas et al. 2011a).

The retention of enzymes within the matrix improves the overall catalytic activity of the matrix (Branda et al. 2005; Flemming et al. 2007). The extracellular enzymes hydrolyze complex macromolecules into low molecular weight monomers, which can diffuse through the microchannels within a biofilm. Polymeric substances within the matrix comprise the substrates for extracellular enzyme activity. Polysaccharides either serve as substrates or anchor the enzymes at the periphery of the cells. These molecules alternatively serve as additional sources of energy and carbon for cells under nutrient limiting conditions (Hoffman and Decho 1999).

A study on *Pseudomonas* sp. biofilm has reported on an increase in enzyme activity with increased polysaccharide production under nutrient and energy limiting conditions (Albertson et al. 1990; Chróst 1991). Biofilm-based extracellular enzymatic hydrolysis of complex insoluble organic matter is highly effective as compared to cell free and planktonic whole-cell-based hydrolysis (Zhang et al. 2007). The accumulation of low molecular weight products enhances the rate of mass transfer and mobility within the biofilm matrix.

The abundant supply of polysaccharides facilitates the EPS to serve as an ion exchange resin within the biofilm matrix. Polysaccharides bearing a negative charge form electrostatic interactions with cations. This aspect of EPS has been

used extensively for extracellular remediation of heavy metals (Beech and Sunner 2004; Fredrickson and Gorby 1996; Gadd 2004; Lies et al. 2005; Singh et al. 2006).

Most common mode of EPS-mediated heavy metal remediation is through biosorption. Planktonic cell EPS belonging to *Rhizobium*, *Paenibacillus* and *Cyanobacteria* adsorb heavy metals such as Pb, Cd, Cu, Zn, Ni, Co and Mn (De Philippis et al. 2001; Chen et al. 1995; Morillo et al. 2006). Marine whole-cell isolates of sulfate reducing bacteria produce EPS, which chelate with Ni, Cr and Mo (Beech et al. 1995). Although most metals interact with the polysaccharides, proteins also bind with heavy metals, as reported in the case of *Rhodopseudomonas acidophila* (Sheng et al. 2005a).

Biofilm-based EPS facilitate better heavy metal sorption than that of planktonic cells. The *P. aeruginosa* biofilm EPS have shown enhanced ability to bind with Cu, Pb, Cr, Ni, Fe and Au, as relative to its planktonic cells (Teitzel et al. 2003; Kang et al. 2006). High rate of Pb immobilization (~90%) were displayed by the EPS fractions of *Burkholderia cepacia* and *Desulfovibrio desulfuricans* biofilms (Templeton et al. 2003; Kang et al. 2006). Studies have also reported on biosorption of Zn, Cd and Cr by EPS fractions of photosynthetic biofilms (Morris et al. 2006). The electrostatic interactions between the biofilm matrix-based anionic groups and metal ions were confirmed through the Cr uptake by *Arthrobacter viscosus* biofilm matrix (Lameiras et al. 2007).

The EPS extracted from activated sludge has high metal complexation potential, enabling heavy metal removal with wastewater treatment. Previous studies have reported that granular sludge matrix serves as an effective

biosorbent for the removal of heavy metals from industrial wastewater (Guibaud et al. 2005; Liu et al. 2001; Liu et al. 2003).

Another alternative mechanism for matrix-enabled heavy metal bioremediation is based on the ability of biofilms to mediate reduction of heavy metals. Biofilm matrix of DMRB like *Shewanella* and *Geobacter* is enriched with redox active outer membrane cytochromes (OMCs), which facilitates the extracellular reductive immobilization of heavy metals (Bond et al. 2012; Cao et al. 2011a; Coursolle et al. 2010; Coursolle and Gralnick 2012; Yong et al. 2013). *S. oneidensis* mediate the synthesis of catalytic nanoparticles such as Pd nanoparticles from the reduction of tetrachloropalladate (Ng et al. 2013). Such biofilms enriched with catalytic nano-particles in its matrix can be further exploited as a recyclable nano-biocatalyst with certain modifications, offering great potential in nano-biotechnology (Ng et al. 2013).

Significant amount of OMCs, especially MtrC and OmcA in the matrix contributed to extracellular U(VI) reduction by *S. oneidensis* biofilms (Cao et al. 2010; Cao et al. 2011a). MtrC and OmcA are also involved with the *S. oneidensis*-mediated extracellular synthesis of Ag and Ag₂S nanoparticles. Further, the Ag and Ag₂S nanoparticles retained in the supernatant displayed significantly higher catalytic activity, highlighting the importance of extracellular retention of the product (Ng et al. 2012). Sulfate reducing *Desulfovibrio desulfuricans* G20 biofilm matrix has also shown the ability for reductive immobilization of U(VI) (Beyenal et al. 2004).

EPS interaction with metals also leads to biomineralization and biocorrosion. Extracellular biopolymers within biofilm matrix interact with cations to produce their respective minerals and drive either their precipitation, or

dissolution. Functional anionic groups within biofilm matrix serve as the binding sites for cations and influence their precipitation or dissolution. Biofilm-induced biomineralization occurs primarily in natural environments such as the formation of stromatolites, thrombolites and soil precipitates (Decho 2010).

Biocorrosion is the biofilm-induced deterioration of the metal surface. Extracellular enzyme activity is considered one of the important factors leading to biocorrosion. Oxygen respiration by the biofilms accelerates the rate of biocorrosion. Treatment of biofilms with sodium azide, which often inhibits biofilm-based enzyme and respiratory activities, were found to suppress biocorrosion (Beech and Sunner 2004).

Typically extracellular biocatalysis mediated by biofilms are employed in the field of power generation, heavy metal reduction and reductive dechlorination using bioelectrochemical systems such as microbial fuel cells (Bretschger et al. 2007; Chaudhuri and Lovley 2003; Coursolle and Gralnick 2012; Lower et al. 2001). Microbial fuel cells combine electrochemistry and biocatalysis by using renewable and cheap feedstocks such as wastewater and carbohydrates with biofilms growing on electrode as the catalyst.

The electroactive biofilms (EAB) in MFCs often couple the oxidation of substrate with the reduction of electrode (Erable et al. 2010; Franks et al. 2010; Gadd 2004). Alternatively, electrodes may serve as electron donor for certain biofilm-mediated bioprocesses (Strycharz et al. 2008). The catabolic reactions associated with the substrate oxidation either involve anaerobic respiration or fermentation or combination of both.

In the absence of other terminal electron acceptors, some microorganisms transfer electrons extracellularly to solid-phase electron acceptors (Clarke et al. 2011; Coursolle et al. 2010; Coursolle and Gralnick 2012; Reguera et al. 2005). The strategies by which electroactive biofilms discharge electrons to a solid insoluble electron sink include: (1) direct mode of electron transfer through outer membrane cytochromes; (2) indirect mode of electron transfer through the secretion of redox active electron shuttles such as flavins (Patil et al. 2012; Richardson et al. 2012; Shi et al. 2009); (3) electrically conductive nanowires especially in cases of severe electron acceptor limitation (Gorby et al. 2006a; Shi et al. 2009).

Under fermentative metabolism, electrode biofilms utilize complex substrates such as starch, cellulose, and xylose. The metabolic cycle involved here produces a series of simple metabolites such as formate, lactate, ethanol and pyruvate, which can also be used as a substrate for catabolic process (Torres et al. 2007; Goud and Mohan 2011). The flexibility with respect to metabolic activity and catabolic substrates make MFCs and other bioelectrochemical systems sustainable for several extracellular biocatalytic reactions.

Wastewater treatment in MFCs is mediated by anode-based electroactive biofilms using the electron flow generated from the oxidation of organic matter in wastewater. EABs stay close to anode and hence, proliferate into the deeper layers of biofilms (Clauwaert et al. 2008; Harnisch et al. 2011; Hoffman and Decho 1999; Rozendal et al. 2008). The wastewater treatment mediated by anodic biofilms can be coupled with the synthesis of value added products such as hydrogen and ethanol in cathode. By using hydrogen or cathode as the donor,

the biofilms on cathode can also mediate extracellular reductive catalysis such as reduction of glycerol to 1,3-propanediol or reduction of acetate to ethanol (Hoffman and Decho 1999; Rozendal et al. 2008).

The extracellular reductive ability of MFC biofilms has been used for bioremediation of soil and groundwater from toxic environmental contaminants such as chlorinated hydrocarbons and Uranium. Reductive dechlorination is the most effective technique for remediation of chlorinated hydrocarbons such as perchloroethylene (PCE) and tetrachloroethylene (TCE). Reductive dechlorination occurs in sequential steps through the formation of several intermediates with ethylene being the end-product (Hoffman and Decho 1999). By using electrode as the sole electron donor, *G. lovleyi* biofilms could reduce PCE to dichloroethylene and *Dehalococcoides* sp. biofilms could mediate the complete degradation of TCE to ethylene (Aulenta et al. 2009; Strycharz et al. 2008). A single stage granular biofilm consisting of aerobic methanotrophic bacteria in its outer layers and anaerobic population capable of reducing PCE in inner layers, has been reported, which could mediate methanogenesis with reductive dechlorination using hydrogen as the electron donor (Guiot et al. 2008).

Dissimilatory nitrate and perchlorate reduction is another extracellular reduction mediated by MFC biofilms. *G. metallireducens* biofilms reduce nitrate to nitrite with electrode as the sole donor (Gregory et al. 2004). The ability of EABs to facilitate extracellular electron transfer has been used in engineering biosensors for monitoring the biodegradative activity in contaminated sites. For the construction of such MFC-based biosensors, xenobiotics was used as the catabolic substrate. The extent of degradation was quantified based on the

current generated by the biofilm-mediated extracellular electron transfer to anode (Hoffman and Decho 1999; Su et al. 2011; Tront et al. 2008).

The extracellular electron transfer to the solid electron sink controls the extracellular reactions mediated by electrode biofilms. EABs, which catalyse these reactions, are widespread in diverse environments including natural ecosystems such as soil, water bodies and sediment as well as wastewater treatment plants. Hence, biofilm-based extracellular bioprocesses have great implications in the environmental cycling of organic matter (carbon and nitrogen), heavy metals and recalcitrant xenobiotics.

To enhance the extracellular reactions, better strategies to modify the cell-matrix interactions such as extracellular electron transfer pathways have to be harnessed. Alternative methods to enhance extracellular reductive processes include the addition of polymeric redox mediators like methylene blue or redox electron shuttles such as anthraquinone-2,6-disulfonate (AQDS) (Rabaey et al. 2007; Patil et al. 2012). The exogenous addition is both expensive as well as requires continuous recharging, which may not be feasible for long-term applications.

Further, biofilm-based extracellular biocatalysis require the continuous exchange of bioactive components such as flavins, nicotinamide adenine dinucleotide (NADH) between the cells and the matrix (Blank et al. 2010; Hernandez and Newman 2001; Kotloski and Gralnick 2013b; Lies et al. 2005; Marsili et al. 2008; Popov and Lamzin 1994; Tsofigkas et al. 2011b; Watanabe et al. 2009). A potential route to harness better exchange between the cells and matrix is by adopting a synthetic biology approach (Yang et al. 2015a; Yang et al.

2015b). Such an approach can engineer the biofilm matrix with enhanced biocatalytic capability (Bazan et al. 2013; Patil et al. 2012).

2.2.6. Conventional immobilization techniques for industrial biocatalysts

Biofilm matrix-enabled extracellular biocatalysis require the enzymes to be displayed continuously in the biofilm matrix. However, the employment of biofilms in continuous reactions presents the risk of enzyme loss due to washout. To ensure the continuous processing of biofilm-based bioprocesses using extracellular enzymes, it has to be immobilized in the matrix. Enzyme immobilization facilitates easy handling and ensures its long-term stay at the site of application.

Enzymes are immobilized either by chemically binding it to a support material or by entrapping it in a polymeric matrix or through crystallization. The enzyme is attached to a hydrophobic organic physical carrier such as resins or inorganic carriers like zeolite under the action of weak van der Waals force, rendering it brittle for industrial applications.

The entrapment technique includes anchoring the enzymes in an organic or inorganic polymer network or a hollow fibre membrane. The bond here is made much stronger by introducing covalent interactions between the binding matrix and enzyme.

Another promising approach involves the use of carrier free immobilized enzymes such as cross-linked enzyme crystals or cross-linked enzyme aggregates that provide advantages such as high degree of stability and catalytic activity at low production cost. This approach initiates the physical aggregation of enzymes followed by chemical crosslinking (Winn 2012).

The disadvantages of these methods are that, the presence of a carrier effectively decreases the active region of the catalyst, as the enzyme occupies only a fraction of the overall carrier. Secondly, the carriers can be subjected to mechanical failures in a continuous system. It is also difficult to optimize the most feasible physico-chemical immobilization technique. Addressing these drawbacks, an approach integrating cellular biology and protein engineering could be adopted to derive utmost benefits from matrix-enabled extracellular biocatalysis (Smith et al. 2015).

2.3. Immobilization of enzymes in biofilm matrix

Extracellular enzymes form a major part of the EPS and play a part in the biofilm metabolic cycle. These enzymes also impart specific catalytic functions to the biofilm matrix. The stability of an extracellular enzyme depends primarily on their three dimensional structures and the extracellular microenvironments within the biofilm matrix. The fluctuations within the highly heterogeneous and dynamic extracellular microenvironments could denature enzymes. Extracellular enzymes also lack the protective cellular environment to counter these fluctuations (Smith et al. 2015).

However, like cells are preserved within the biofilm matrix, the matrix serves as protective scaffold for extracellular enzymes. The interactions between the cells and the matrix stabilize the fluctuating local environment. Hence, the matrix anchorage enhances the stability of the enzyme and longevity of the enzyme activity. Since the enzyme is anchored to the biofilm matrix, there is less chance of degradation and interference from other enzymes (Smith et al. 2015).

However, shifting biocatalysis to biofilm matrix has its own drawbacks. Reactions such as biocatalytic oxidoreductions used for the synthesis of pharmaceutical compounds and intermediates often require the continuous supply of expensive cofactors like NADH and NADPH (Zhang et al. 2009). The disruption in the free flow of cofactors stifles such reactions. In order to regenerate the cofactors within the biofilm, the cells have to harvest a significant amount of energy. The external addition of the cofactors is not a cost effective option in biotransformations.

Hence, in order to reduce the metabolic and energy costs of maintaining the cofactor recirculation, extracellular enzymes should be anchored close to the cells. Further, the localization of extracellular enzymes at the periphery of the cells provides better accessibility to the substrate (Schüürmann et al. 2014; Smith et al. 2015). To immobilize the enzymes in the matrix, an approach, which engineers the extracellular matrix into a functionalized and scalable catalytic framework, is required. Additionally, the immobilized enzymes should not be compromised on its activity, stability and modularity.

The matrix immobilization of enzymes is typically carried out in the following ways, irrespective of the host organism (Schüürmann et al. 2014; Smith et al. 2015): (1) The target enzyme is tagged with a signal peptide, which mediates the extracellular secretion of the enzyme (Figure 2.3 (A)); (2) The enzyme of interest shall be genetically fused to a native matrix-associated protein to enable a stable matrix anchorage (Figure 2.3 (B)) (Smith et al. 2015).

Figure 2.3. Schematic illustration of matrix-displayed enzymes with the industrial applications. Inner circle in dotted lines show the cytoplasmic membrane and outer circle in thick bold line denotes the cell membrane. (A) The enzyme tagged with a signal peptide is secreted to the matrix. Signal peptide directs the translocation of enzyme into the protein secretion pathway from the cytoplasmic membrane. After the translocation across the cytoplasmic membrane, signal peptides are often removed; (B) The carrier protein with the fused enzyme is localized on the cell surface, enabling the matrix display (Hawkes 2008; Schüürmann et al. 2014; Smith et al. 2015).

The selection of the immobilization strategy depends on complexity of the target enzyme, its application, and the mode of operation (batch or continuous system). The enzyme expression may be regulated through an inducible promoter, which requires a specific substrate to initiate the transcription of the target protein (Semenza, Jiang et al. 1996, Kleinert, Pautz et al. 2004). In such cases, expression of the target enzyme might require a very high concentration of the inducible substrate that may be toxic to cells. The enzyme expression under inducible promoter is most suited for batch reactions (Smith et al. 2015).

The enzymes may be expressed continuously under the control of constitutive promoter of a native matrix-associated protein (Ryu and Karim 2011;

Schmid et al. 2001; Smith et al. 2015; van Bloois et al. 2011). Whenever the native protein is expressed, the target enzyme will also be displayed simultaneously on the matrix. The native carrier protein dictates the transportation and localization of the enzyme. The secretory pathway of the carrier protein will regulate the enzyme display (Schüürmann et al. 2014). As a result, such a strategy is more suitable for continuous reactions as it also eludes the possibility of enzyme loss due to washout.

Recent studies have shown how curli fibres can be engineered to function as the primary extracellular catalytic component for *E. coli* biofilms. Curli fibres are responsible for cell adhesion, aggregation and biofilm stability. Curli belongs to the family of amyloid nanofibers with a diameter of 4-8 nm, localized on the cell surface. Its transcription is operated by 7 proteins: CsgA (structure), CsgB (nucleation), CsgE and CsgF (processing), CsgC and CsgG (secretion) and CsgD (direct transcriptional regulation) (Nguyen et al. 2014).

The extracellular self-assembly of a single protein CsgA constitutes the curli fibres. Hence, the curli system serves as the most optimized platform towards engineering the biofilm matrix as compared to other EPS components.

A study has reported that the peptide constructs fused to CsgA is capable of being displayed as amyloid nanofibers on the matrix, without compromising its function and activity (Nguyen et al. 2014). Amyloids are highly resistant towards stress induced by detergents and organic solvents and show high stiffness and mechanical strength (Hammar et al. 1995; Knowles et al. 2010; Smith et al. 2006). Amyloid fibres also contribute to 10-35% of the total biofilm biovolume and can engineer matrix towards attributes such as nanoparticle biotemplating, covalent immobilization of enzymes (Nguyen et al. 2014).

Recently a novel strategy using curli fibres of *E. coli* to immobilize catalytic enzymes in biofilm matrix has been developed. This approach defined as Biofilm Integrated Nanofiber Display (BIND), has laid the foundation for the matrix display of enzymes (Botyanszki et al. 2015; Nguyen et al. 2014).

In this system, a recombinant α -amylase is immobilized onto the curli nanofiber network through a covalent bond formed between a peptide component and a protein component (Figure 2.4 (A)). The peptide component known as SpyTag (13-amino acid) is fused to curli protein CsgA and the protein component SpyCatcher (15 kDa) is fused to α -amylase. The peptide-protein coupling enables the covalent bond formation, which mediates the immobilization of α -amylase onto the curli fibres (Figure 2.4 (B)) (Botyanszki et al. 2015).

Further tests suggested that the α -amylase activity (Figure 2.4 (C)) is highly stable over a range of pH (4-10) and organic solvents, as compared to the purified enzymes. The biofilm matrix-enabled α -amylase activity is also exhibited over a long-term (28 days) with high stability (Botyanszki et al. 2015). This study deduced the following confirmations (Botyanszki et al. 2015; Nguyen et al. 2014): (1) The matrix immobilization of enzymes can be performed without the addition of any chemicals and facilitates a site specific display of enzymes; (2) The curli fibres provide a high surface area for immobilization, an attribute associated with most of the matrix-associated proteins; (3) Since the enzyme is immobilized to the curli fibres, the amount of curli produced by the cells is equivalent to the enzymes displayed on the matrix; (4) Biofilm matrix-enabled biocatalysis described in the study is produced by integrating the concepts of protein engineering and synthetic biology.

The studies on curli fibres have confirmed that engineering curli fibres could be an effective solution for site-specific matrix immobilization of catalytic enzymes. Such engineered catalytic biofilms could be used in several biocatalytic applications such as bioremediation of ground water and natural sediments, biofuel and bioenergy production, enzymatic hydrolysis of complex organic molecules and synthesis of several value added products (Botyanszki et al. 2015; Nguyen et al. 2014; Smith et al. 2015).

Figure 2.4. Biofilm matrix-enabled immobilization of enzymes on curli fibres. (A) *E. coli* expresses CsgA fused to SpyTag (CsgA-ST), which self-assembles into curli fibres on the cell surface; (B) This polymer matrix is covalently modified with an enzyme fused to SpyCatcher. The peptide-protein coupling ensures the enzyme immobilization through covalent bond formation; (C) Biofilm matrix mediated extracellular biocatalysis occurs on the high surface-area catalytic fibers. pNPMP is 4-nitrophenyl- α -D-maltopentaoside and R is the hydrolyzed α -D-maltopentaoside. The Figure is reproduced from (Botyanszki et al. 2015) with permission from “John Wiley and Sons”.

Another study has presented matrix-enabled biocatalysis in a microbial fuel cell through the surface display of redox active glucose oxidase (Fishilevich et al. 2009). The anodic biofilm, which mediates the biocatalysis is made of *Saccharomyces cerevisiae* with surface-displayed glucose oxidase. The power density generated by the engineered biofilm was 2-folds higher than the purified

enzyme and unmodified biofilm (with no surface-displayed glucose oxidase) (Fishilevich et al. 2009). This study has explored the potential of integrating the application of cell surface-displayed enzymes in matrix-enabled biocatalysis with positive implications. .

Cell surface display of enzymes refers to immobilization or display of enzymes on the exterior of cells (Schüürmann et al. 2014; Smith et al. 2015). The cell surface display alleviates several restrictions such as the enzyme isolation, physical barrier in the form of cytoplasmic and outer membranes, which makes enzyme accessibility to the substrate becomes much easier. It also eludes any interference from the intracellular enzyme system.

It has also been reported that enzyme immobilisation on the cell surface increase its resilience towards temperature and organic solvents (Jung et al. 2006; Schmid et al. 2001; Schüürmann et al. 2014; Shiraga et al. 2005; Smith et al. 2015). The cell surface display systems serve as a platform to functionalize enzymes with upregulated activity. The cell surface display of enzymes originated through the integration of directed evolution strategy with protein engineering (Schüürmann et al. 2014; van Bloois et al. 2011; Wang et al. 2012).

Several studies have reported on generation of novel whole-cell catalysts with surface-displayed enzymes in laboratory scale. Recent studies have focussed on autotransporter proteins and ice nucleation proteins as two specific carrier proteins for displaying the target enzymes. Autotransporters have a modulated structure and are associated with type V secretion systems. The most commonly used autotransporters are the adhesion AIDA-I from enteropathogenic *E. coli* and the esterase EstA from *P. aeruginosa* (Jose et al. 2012; Nicolay et al. 2012; Nicolay et al. 2013; Schüürmann et al. 2014).

Ice nucleation proteins (INPs) are outer membrane-associated proteins composed of a hydrophilic C-terminal region, an N-terminal, which shows high affinity to outer membrane and a repetitive internal domain. INPs have been used as surface anchor proteins in *E. coli*, *Pseudomonas putida* and *Xanthomonas campestris* (Jose et al. 2012; Jung et al. 1998; Schüürmann et al. 2014; van Bloois et al. 2011).

Further, the outer membrane proteins, such as OprF of *P. aeruginosa* and OmpW of *E. coli*, have been applied as anchoring proteins (Smith et al. 2015). The PgsA anchor protein from *Bacillus subtilis* has also been used for biocatalytic applications. Endospores from *Bacillus* species also offer a potential alternative as surface anchorage systems (Jemli et al. 2014; Jose et al. 2012; Kobayashi et al. 2000; Schüürmann et al. 2014; Tsuchiya et al. 1999).

A study on cytochrome P450 (CYP) CYP11A1 in combination with surface-displayed bovine adrenodoxin demonstrated the applicability of surface-displayed CYPs (Jemli et al. 2014; O'Reilly et al. 2011; Schmid et al. 2001; Schumacher and Jose 2012; Smith et al. 2015). The esterification mediated by lipase from *Pseudomonas fluorescens* SIK W1 fused with OprF anchor motif was found to be active in hexane for more than 1 week (Jemli et al. 2014; Jung et al. 2006; Schmid et al. 2001; Shiraga et al. 2005; Tsuchiya et al. 1999). The surface display of nitrilase through autotransporters demonstrated how the mobile and fluidic nature of anchor protein facilitated the display of multimeric enzymes with high long-term stability and high rate of reusability (Detzel et al. 2011; Detzel et al. 2013). These studies have clearly demonstrated that cell surface-display systems overcome the rate limiting steps associated with whole-cell and enzymatic biocatalysis.

Exploiting the amyloid fibres to immobilize enzymes has demonstrated the successful implementation of site-specific matrix display of enzymes. The cell surface-displayed systems have significantly improved the productivity of whole-cells-based biocatalysis. If the cell surface-displayed systems are integrated into the biofilms, it could result in a versatile strategy characterized with elevated mass transfer, enhanced enzyme stability, improved tolerance to physicochemical stresses, and better accessibility to substrate.

CHAPTER 3: EFFECT OF CONJUGATED OLIGOELECTROLYTE DSSN+ ON EXTRACELLULAR BIOACTIVITY OF *S. oneidensis*

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3.1. Introduction

Biocatalysis, which has been suggested as an alternative to chemical transformations, deploy microbial cells or isolated enzymes as catalysts (Hartmann and Jung 2010; Leresche and Meyer 2006; Santacoloma et al. 2010). High sensitivity to operational conditions often limits the applications of enzyme-based biocatalysis (Tsoligkas et al. 2011b). The expensive methods required to isolate and purify the enzymes is another impeding factor. Hence, most of the industrial biocatalytic conversions use planktonic whole-cells (Leon et al. 1998; Nikolova and Ward 1993). Whole-cell-based systems are characterized with low catalytic stability, short retention time and relatively lower regeneration of the biocatalysts (Halan et al. 2012a; Nikolova and Ward 1993; Rosche et al. 2009).

In contrast to free-living cells, surface-adhered biofilms are highly tolerant to environmental stresses and exhibit long-term stability (Flemming and Wingender 2010; Halan et al. 2012b). The biofilm lifestyle renders such surface consortia attractive catalytic systems for continuous and robust biotransformations (Rosche et al. 2009). In biofilms, diffusion drives the mass

transport of nutrients, substrate and intermediate products within the layers of biofilm microstructure (Stewart 2003). Diffusion limitations may cause detrimental effects to the biofilm robustness by drastically impeding mass transfer and fluid flow (Branda et al. 2005; Stewart 2003). Previous studies have shown that the diffusion coefficient in biofilms is spatially heterogeneous and exhibit a reduction with depth, as schematically illustrated in Figure 2.2 (Beuling et al. 1998; Lewandowski and Beyenal 2007; Renslow et al. 2010). Hence, diffusion limitations in biofilms could cause a significant decrease in the efficacy of biofilm-based biocatalysis (Halan et al. 2012b; Polizzi et al. 2007; Singh et al. 2006; Stewart 2003).

Extracellular biocatalysis in biofilms offer a solution to ameliorate diffusion limitation in biofilm-based biocatalysis. Biofilm-based extracellular biocatalysis mediate the biotransformations in biofilm matrix and hence, eliminates the membrane as a barrier that cripples the diffusion of nutrients and substrates. Enhancing extracellular bioactivity becomes a critical step towards more efficient biofilm-based biocatalysis, since it also facilitates the easy recovery of the products without damaging the structural integrity of the biofilms. To this end, various biological and chemical approaches to boost extracellular bioactivity have been reported (Chen et al. 2012; Liu et al. 2013a; Yang et al. 2015a).

Recently, a conjugated oligoelectrolyte (4,4'-bis(4'-N,N-bis(6''-(N,N,N-trimethyl ammonium) hexyl) amino)-styryl) stilbene tetraiodide) (DSSN+) has been reported to be able to enhance power generation in bioelectrochemical devices, typically microbial fuel cells (MFC). Conjugated oligoelectrolytes are synthetic molecules that possess a very strong inclination to align within the lipid bi-layer membranes. These molecules are characterized with π - conjugated

aromatic backbone that facilitates charge delocalization and pendant ionic groups on either side, which render them soluble (Bazan et al. 2013; Garner et al. 2012; Hou et al. 2013; Wang et al. 2013; Yan et al. 2015).

The insertion of DSSN⁺ has been reported to have improved the performance of yeast MFC by 7--fold (Garner et al. 2012). Another study has described an improvement (~3.5-folds higher current generation than the control) in the performance of a MFC driven by a typically non-electrogenic *E. coli* K-12 (Wang 2014; Wang et al. 2013). Since power generation in such devices is based on the extracellular reduction of the electrode by biofilms, we hypothesize that DSSN⁺ may also enhance extracellular bioactivity in biotransformations, which can be further harnessed to improve environmental microbial processes in bioreactors (Bazan et al. 2013; Hinks et al. 2014; Wang 2014; Wang et al. 2014).

The objective of the study was to elucidate the impacts of membrane intercalating conjugated oligoelectrolyte like DSSN⁺ on extracellular bioactivity in biotransformations, and study how the cell-DSSN⁺ interactions affect the biofilm stability, metabolic activity and the exchange between cells and the matrix. To test this hypothesis, we used reduction of ferrihydrite, an insoluble iron (III) mineral, by the metal-reducing bacterium *S. oneidensis* MR-1 as a model extracellular biotransformation. Further, the influence of cell-DSSN⁺ interaction on its biofilm stability and metabolic activity were evaluated in a hydrodynamic flow cell system.

3.2. Material and Methods

3.2.1. Microorganisms and growth conditions

The *S. oneidensis* strains used in this study are listed in Table 3.1. The wild-type strain MR-1 was obtained from ATCC (no: 700550) and all the mutant strains were kindly provided by Dr. Liang Shi in the Pacific Northwest National Laboratory, USA. Seed cultures were prepared aerobically by transferring a loop of the frozen *S. oneidensis* stock to 10 mL Luria Bertani (LB) medium and then incubated for 16 h at 30°C in a rotary shaker (200 rpm).

Table 3.1. List of bacterial strains used in this study

Strains	Description	Reference
MR-1	Manganese reducing strain (WT)	Myers and Nealson 1988)
$\Delta cymA$	<i>cymA</i> (SO4591) deletion in MR-1	(Marshall et al. 2008a)
$\Delta mtrA$	<i>mtrA</i> (SO1777) deletion in MR-1	(Marshall et al. 2008a)
$\Delta mtrC$ - <i>omcA</i>	<i>mtrC</i> (SO1778) and <i>omcA</i> (SO1779) deletion in MR-1	(Marshall et al. 2008a)

3.2.2. Preparation of ferrihydrite

A weakly crystalline to amorphous ferrihydrite suspension was prepared by neutralizing a 0.2 M solution of FeCl₃ to a pH of 7 with NaOH (Bretschger et al. 2007; Lies et al. 2005). The resulting suspension was then harvested and washed several times using MilliQ water and was resuspended in MilliQ water to a concentration of 445 mM.

3.2.3. Synthesis of DSSN+

DSSN+ was synthesized and characterized according to previously reported procedures (Garner et al. 2010).

3.2.4. Reduction of ferrihydrite by *S. oneidensis* under growth conditions

The reduction of ferrihydrite was performed in 25 mL serum bottles under anaerobic conditions. About 0.4 mL (2% v/v) of the LB seed culture was mixed with 20 mL of the modified minimal M1 (MM1) media that contained 20 mM lactate (electron donor), and 30 mM HEPES, 7.5 mM NaOH, 28.04 mM NH₄Cl, 1.34 mM KCl, 4.35 mM NaH₂PO₄ and 0.68 mM CaCl₂ that is further supplemented with traces of vitamins, minerals and amino acids at pH 7.0 (Bretschger et al. 2007; Wang et al. 2008; Zhang et al. 2014). Insoluble amorphous ferrihydrite suspension was injected into the respective anaerobic bottles to a final concentration of 10 mM. When needed, 5 µM DSSN+ was added. The cultures were bubbled with nitrogen for several min and sealed with a butyl rubber stopper and then incubated at 30° C under dark conditions. Fe²⁺ was quantified using ferrozine assay described elsewhere (Coursolle and Gralnick 2012; Lies et al. 2005; Wang et al. 2008). Briefly, 100 µL of the extracted cell-free sample was acidified with equal volume of 1 M HCl (100 µL) and then incubated for 30 min at room temperature. 20 µL of the treated sample was mixed with 200 µL of ferrozine reagent (0.05% w/w of ferrozine in 50 mM HEPES buffer at pH 7.0). The absorbance at a wavelength of 562 nm was taken using a UV-VIS spectrophotometer. The experiments were carried out in triplicates.

3.2.5. Reduction of ferrihydrite using resting cells

The *S. oneidensis* cells from the LB seed culture were harvested by centrifugation (5000 x g, 15 min, 4° C) and washed three times using 30 mM HEPES buffer (pH 7.0). The cells were then re-suspended in the same buffer to a final cell density of 1.2 × 10⁹ cells/mL. Ferrihydrite (FeOOH) (10 mM) and lactate (20 mM) was added as the electron acceptor and electron donor respectively.

When needed, 5 μ M DSSN+ was supplemented. The suspensions were bubbled with nitrogen gas and maintained under anaerobic conditions. Fe²⁺ was quantified using ferrozine assay as briefly mentioned in the previous section.

3.2.6. Effect of DSSN+ on cell viability

Cells from overnight LB cultures were pelleted, washed with 30 mM HEPES buffer (pH 7.0) and resuspended in the same buffer containing 20 mM lactate in the presence and absence of 5 μ M DSSN+. Cell viability was quantified by plating the suspension onto LB agar and enumerating the colony forming units (CFU).

3.2.7. Proteomic analysis

S. oneidensis MR-1 preculture was grown in 100 mL LB for 16 hours. About 2% by volume of the LB culture was added to anaerobic Wheaton serum bottles (25 mL) containing MM1 medium with 20 mM lactate and 10 mM ferrihydrite with or without DSSN+. The cells were pelleted by centrifugation at 5000 x g for 20 min, which was washed twice with PBS buffer. The harvested cells were lysed at 4° C in lysis buffer (0.1% SDS, 0.5 M TEAB, 50 mM protease inhibitor tablet) with intermittent vortexing and sonication. The lysate was centrifuged at 20,000 x g for 1 h at 4° C and supernatant stored at -80° C. Protein quantification was performed using BCA assay. The iTRAQ based proteomic analysis was conducted as mentioned elsewhere (Mohanty et al. 2013; Zhang et al. 2014).

3.2.8. Quantitative Polymerase Chain Reaction (qPCR)

S. oneidensis MR-1 was cultivated in MM1 medium with 20 mM lactate as carbon source and 10 mM ferrihydrite as electron acceptor in the presence and absence of 5 μ M DSSN+ in anaerobic serum bottles (25 mL). The RNA extraction was carried out after 12 h (late-exponential growth phase). The RNA concentration was determined using Nanodrop spectrophotometer (Thermoscientific, DE, USA). The qPCR reactions and the data analysis were conducted as described elsewhere (Mohanty et al. 2013). The primers used in qPCR are listed in table 3.2.

Table 3.2. List of primers used for qPCR

Primers	Sequence
SO3468-f	GTGCGTTTAGGCGACAGTATC
SO3468-r	CCTCCGCCTTGGGTTTCTTTG
<i>bfE</i> -f (SO0702)	TTCCGCCTTCCCCTTTGATGTGTG
<i>bfE</i> -r (SO0702)	CCTGCGGGGCGTTACCTGAA
<i>frdA</i> -f(SO0398)	GATCCCCGTGCGTCCAACC
<i>frdA</i> -r(SO0398)	CGCAGCGCCTCCACCAACTCT
<i>ushA</i> -f	TGATGAAGCGGGTAACAAGAAAAAG
<i>ushA</i> -r	TGCGCATAACCACCAGAACCAA
<i>fccA</i> -f	GCGCGAGCGTTAACCGTAGTC
<i>fccA</i> -r	CACCCGCTGGAGAGTAAGTTGG

3.2.9. Flavin quantification

Flavins were quantified using fluorescence measurements (Clark et al. 2012; Wang et al. 2015b). Approximately 0.5 - 1 mL of the samples were taken at periodic time intervals and centrifuged at 12,000 $\times$ g for 10 min at 4°C. About 200 μ L of the cell free supernatants were transferred to a clear 96 well plate and the fluorescence intensity was read at 440 nm excitation and 525 nm emission in a Tecan multiwall plate reader (TECAN M200, Männedorf, Switzerland) (Covington

et al. 2010; Wang et al. 2015b). MM1 media with 5 μ M DSSN+ was used as an abiotic control to eliminate the background fluorescence. Flavins secreted by *S. oneidensis* are mainly flavin mononucleotide (FMN) and riboflavin (RF). Both FMN and RF can serve as electron shuttles facilitating extracellular electron transfer (EET) (Brutinel and Gralnick 2012; Covington et al. 2010; Von Canstein et al. 2008). In this study, the goal of flavin measurement was to elucidate whether the electron shuttle-mediated EET was influenced by DSSN+ or not. Hence, total flavins instead of individual flavin species were quantified. The standard curve for the measurement of total flavins was prepared using riboflavin as described in previous reports (Covington et al. 2010; Je et al. 2007; Pan 2015; Von Canstein et al. 2008)

.3.2.10. Glucose-6-phosphate dehydrogenase (G6PDH) activity assay

The G6PDH assay was performed using Glucose-6-Phosphate Dehydrogenase Assay Kit (Catalog Number MAK015, Sigma-Aldrich) following manufacturer's instructions. About 50 μ L of the master reaction mix prepared was mixed with 50 μ L of the sample in duplicates (samples were tested in several dilutions to bring the readings within the standard curve range) and mixed well in a horizontal shaker. The absorbance at 450 nm was noted using the Tecan multiwall plate reader at 37°C.

3.2.11. Quantification of extracellular ATP

The BacTiter-Gio Microbial Cell Viability Assay Kit (Catalog Number G8230, Promega) was used to quantify extracellular ATP in cell free supernatants instantaneously after drawing out samples. Equal volumes of BacTiter-Gio reagent and the test samples were mixed in duplicates in a 96 well

plate and the luminescence was recorded as an indicator of the ATP concentration.

3.2.12. Impacts of DSSN+ on biofilm growth under hydrodynamic conditions

The biofilms were grown at room temperature in a three-channel flow cell of dimensions 400 × 40 × 4 mm (Ding et al. 2014a; Thormann et al. 2004). The optical density at 600 nm (OD₆₀₀) of the overnight LB culture of *S. oneidensis* MR-1 tagged with yellow fluorescent protein (YFP) was diluted to 0.1 using MM1 buffer before inoculating into the flow cell (Zhang et al. 2014). Upon inoculation, the flow cell was kept in an inverted position for 2 h to ensure the initial attachment of bacteria on the substratum. The MM1 medium with 20 mM lactate with or without 5 µM DSSN+ was introduced at a constant flow rate of 4 mL/h using a peristaltic pump (Ding et al. 2014a; Wu et al. 2014).

3.2.13. Image acquisition and processing

A Carl Zeiss Confocal Laser Scanning Microscopy (CLSM) model LSM 780 with 34 channels and transmitted light-PMT equipped with an Axio observer inverted microscope and different objective lenses (20× /0.4 N.A, 40× /0.6 N.A, 40× /1.3 N.A Oil, 63× /1.4 N.A Oil and 100× /1.4 N.A Oil) was used to image the biofilms. The biofilms in the flow cell were imaged at excitation wavelengths of 488 nm and 514 nm to image DSSN+ and cells (YFP), respectively (Wang et al. 2013; Yan et al. 2015). The images were taken in triplicates in each channel localized along the inlet, middle, and outlet of the channel. The CLSM images were processed using ZEN 2011 software. The biofilm biovolume and surface

coverage were estimated using IMARIS software package (Bitplane AG, Zurich, Switzerland) (Ding et al. 2014a; Zhang et al. 2014).

3.2.14. Quantitative analysis of cell detachment and extracellular flavins

The cell detachment rate and extracellular flavins were quantified during biofilm growth. Cell detachment rate (CFU/mm²/h) was quantified using a drop-plate method (Chen et al. 2003a; Ding et al. 2014a; Zhang et al. 2014). The extracellular flavins in the effluent samples were estimated as mentioned in section 3.2.9.

3.2.15. Extracellular alkaline phosphatase (ALP) activity in biofilms

Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules like proteins located in the periplasmic space (Monte and Chiang 1975). The ALP activity in the effluent samples was quantified using ALP assay following the manufacturer's instructions (Genetex, CA, USA).

3.2.16. High performance liquid chromatography (HPLC) analysis

The concentration of lactate and acetate were quantified using HPLC equipped with a HPX-87 H (Bio-Rad, CA, USA) ion exchange column (300 x 7.8 mm) and a UV detector. The 0.004 M H₂SO₄ was used as the mobile phase at a flow rate of 0.6 mL/min (Lies et al. 2005; Simonides et al. 1988; Wang et al. 2015b).

3.3. Results and Discussion

3.3.1. DSSN+ enhances extracellular reduction of ferrihydrite by *S. oneidensis*

S. oneidensis is capable of reductively releasing Fe^{2+} from insoluble ferrihydrite under anaerobic condition with appropriate electron donors (Bretschger et al. 2007; Coursolle and Gralnick 2012; Wang et al. 2015b). Figure 3.1 (A) shows Fe^{2+} concentration in the cultures of *S. oneidensis* MR-1 respiring on ferrihydrite in the absence or presence of 5 μM of DSSN+.

Figure 3.1. (A) DSSN+ enhances extracellular reduction of ferrihydrite by *S. oneidensis* MR-1 growing in MM1 medium with 20 mM lactate. Results are presented as mean $\pm$ standard error (n=3); (B) Schematic illustration of the bacterial cell membrane incorporated by DSSN+; (C) Epifluorescence image of *S. oneidensis* MR-1 cells grown in MM1 medium in the presence of 5 μM DSSN+.

Negligible amount of Fe^{2+} (<0.6 mM in 24 h) was detected in the cell-free abiotic controls with or without DSSN⁺, suggesting that the release of Fe^{2+} mainly arises from cellular activity. The addition of DSSN⁺ (5 μM) into the MR-1 cultures respiring on ferrihydrite increased the initial Fe^{2+} release rate from 0.26 ± 0.01 mM/h to 0.31 ± 0.01 mM/h. The concentration of Fe^{2+} in 24 h cultures with or without DSSN⁺ was 4.32 ± 0.04 mM and 3.78 ± 0.11 mM, respectively. The addition of DSSN⁺ (5 μM) into the MR-1 cultures respiring on ferrihydrite did not promote bacterial growth; Hence, the enhancement in the extracellular reduction could be attributed to cell-DSSN⁺ interactions. Figure 3.1 (B) shows the schematic illustration of membrane intercalation of DSSN⁺. After examining the cells in the culture containing DSSN⁺, it was observed that DSSN⁺ was intercalated into the membrane of *S. oneidensis* (Figure 3.1 (C)).

In extracellular respiration of *S. oneidensis*, the electrons from the oxidation of electron donors such as lactate are transported to the electron acceptors at the cell exterior through the direct electron transport chains consisting of membrane-bound electron carrier proteins. For example, the metal-reducing (Mtr) pathway, a cytochrome-based electron transport chain featuring cytochromes such as CymA, MtrA, OmcA and OmcA, has been well characterized in *S. oneidensis* (Coursolle et al. 2010; Richardson et al. 2012; Shi et al. 2009). The incorporation of DSSN⁺ into the cell membrane raised a question of whether DSSN⁺ interacts directly with the cytochromes in the electron transport chain. To address this question, we further investigated the impact of DSSN⁺ on *S. oneidensis* mutants lacking cytochromes in the Mtr pathway.

As expected, the mutants lacking cytochrome CymA, MtrA or MtrC/OmcA exhibited a significantly lower capability of extracellular reduction compared to the

WT (Figure 3.2). The addition of DSSN+ to these cytochrome mutants did not improve the extracellular reduction of ferrihydrite (Figure 3.2), suggesting no direct interactions between DSSN+ and the Mtr pathway. In addition, there was no significant difference in ferrihydrite reduction by resting cells of *S. oneidensis* MR-1 WT with or without DSSN+ (Figure 3.2), implying that the enhancement effect of DSSN+ on extracellular reduction is highly growth-dependent.

Figure 3.2. The concentration of Fe^{2+} in cell suspension of the WT and mutant strains containing lactate as an electron donor and ferrihydrite as an electron acceptor, with or without DSSN+. Results are presented as mean $\pm$ standard error ($n=3$).

3.3.2. DSSN+ increases the concentration of extracellular flavins

The growth-dependent effect of DSSN+ on extracellular reduction of ferrihydrite by *S. oneidensis* suggested an involvement of certain growth-associated metabolic intermediates that can facilitate extracellular reduction. Among the metabolic intermediates in *S. oneidensis*, flavins, dominated by flavin mononucleotide and riboflavin, are a group of redox molecules capable of

shuttling electrons between the outer membrane cytochromes and the insoluble electron acceptors (Kiely et al. 2011; Kotloski and Gralnick 2013a; Kotloski and Gralnick 2013b; Marsili et al. 2008; Yang et al. 2015a). Literature precedent reports that *S. oneidensis* can reduce insoluble electron acceptors without direct contact through electron shuttling by flavins (Kotloski and Gralnick 2013b; Marsili et al. 2008). To elucidate whether flavins are involved in the DSSN+-enhanced extracellular reduction, the concentration of extracellular flavins in the MR-1 cultures were quantified in the presence or absence of DSSN+ and the results are shown in Figure 3.3.

Figure 3.3. The concentration of extracellular flavins in MR-1 culture at different growth stages (3 h-early exp, 6-9 h-mid exp, 11 h-late exp, 24 h-stationary) in the presence or absence of DSSN+. Results are presented as mean $\pm$ standard error (n=3).

During the growth of *S. oneidensis*, the concentration of extracellular flavins in the cultures with or without DSSN+ increased with time. The presence of DSSN+ significantly increased the concentration of extracellular flavins at early exponential (3 h), middle exponential (6-9 h) and stationary growth phases (24 h). Highest concentrations were attained at the stationary phase in the batch cultures,

reaching up to $\sim 0.23 \mu\text{M}$ and $\sim 0.73 \mu\text{M}$ for the cultures in the absence or presence of DSSN+, respectively (Figure 3.3). Increase of extracellular flavins has been reported to be able to enhance the extracellular electron transfer to ferrihydrite and solid electrode by *S. oneidensis* (Kiely et al. 2011; Kotloski and Gralnick 2013b; Shi et al. 2009). The DSSN+-enhanced extracellular reduction of ferrihydrite by *S. oneidensis* could be attributed to the increased concentration of extracellular flavins in the presence of DSSN+.

3.3.3. No significant effects of DSSN+ on flavin biosynthesis

To further elucidate whether the increase of extracellular flavins was enhanced by DSSN+-induced biosynthesis, the expression of several key genes involved in flavin biosynthesis were quantified. Flavin production in MR-1 is initiated through a protein (encoded by gene SO3468) synthesizing flavin adenine dinucleotide (FAD) in the cytoplasm. FAD is translocated across the inner membrane into the periplasm by a bacterial FAD exporter encoded by gene *bfe* (SO0702) (Kotloski and Gralnick 2013b). FAD is processed to flavin mononucleotide (FMN) in the periplasm by the 5'-nucleotidase encoded by *ushA* (SO2001) (Covington et al. 2010). Excess FAD is incorporated into the periplasmic fumerate reductase encoded by *fccA* and quinol:fumarate reductase FAD binding subunit encoded by *frdA* (SO0398) (Kotloski and Gralnick 2013a; Kotloski and Gralnick 2013b; Yang et al. 2015a).

The qPCR results revealed that there was no difference in the expression level of these key genes with or without DSSN+ ($p > 0.05$) (Figure 3.4). This was further validated at the protein level by proteomics analysis. Among the proteins identified were quinol:fumarate reductase FAD binding subunit FrdA, bifunctional UDP-sugar hydrolase/5'-nucleotidase UshA, and riboflavin synthase α subunit

RibC-like protein (Table 3.3). The proteomics results showed that the abundance of these proteins remained unchanged with or without DSSN+ (Table 3.3), confirming that DSSN+ had no significant impact on flavin biosynthesis.

Figure 3.4. Influence of 5 μ M DSSN+ on the expression levels of key genes involved in flavin production. Values were normalized to that of the housekeeping gene *envZ*. Results are presented as mean $\pm$ standard error (n=6).

Table 3.3. Proteomic results showing no effect on proteins involved in flavin synthesis and Mtr pathway under the influence of 5 μ M DSSN+. Ratio* indicates the relative abundance of proteins in DSSN+ treated cells relative to the control and $p < 0.05$ were considered to be statistically significant.

Locus tag	Protein name	Ratio *	P-value
SO4591	CymA	1.38	0.53
SO1777	MtrA	1.29	0.62
SO1778	MtrC	1.35	0.49
SO1779	OmcA	1.22	0.83
SO2001	Bifunctional UDP-sugar hydroase/5'-nucleotidase UshA	1.37	0.78
SO3468	Riboflavin synthase alpha subunit RibC- like protein	1.24	0.82
SO0398	Quinol: fumarate reductase FAD binding subunit FrdA	1.02	0.96

3.3.4. DSSN+ increases extracellular ATP content and G6PDH activity without significantly decreasing cell viability

The increased concentration of extracellular flavins and unaffected flavin biosynthesis in the presence of DSSN+ led to a hypothesis that membrane permeabilization could be the dominating mechanism. To test it, we further quantified extracellular ATP content and extracellular G6PDH activity. The results are shown in Figure 3.5 (A) and (B).

Figure 3.5. (A) The concentration of extracellular ATP; (B) Extracellular enzyme activity of G6PDH in the presence or absence of DSSN+. Results are presented as mean $\pm$ standard error (n=3).

In the presence of DSSN+, the concentration of ATP and the enzyme activity of G6PDH in the cell-free supernatant at the late-exponential (12 h) and stationary (24 h) growth stages were significantly higher (Figure 3.5 (A) and (B)), suggesting that DSSN+ could have caused membrane permeabilization, which led to an increased release of flavins. Intriguingly, membrane permeabilization caused by DSSN+ exhibited only marginal impacts on cell viability as evidenced by viability assay after 24 h exposure to 5 μ M; DSSN+: $2.57 \pm 0.03 \times 10^8$ CFU/mL (treated) vs. $2.84 \pm 0.005 \times 10^8$ CFU/mL (control).

Although the integration of the DSSN+ into the cell membrane does not significantly affect short term cell viability and activity, whether cell-DSSN+ interaction imposes a long-term negative effect on cell growth and cellular energetic still remains elusive and requires further investigation.

3.3.5. Cell-DSSN+ interaction does not compromise biofilm formation and activity

To further understand the impacts of DSSN+ on biofilm formation and activity, *S. oneidensis* MR-1 biofilms were grown using flow cells under hydrodynamic conditions in the presence or absence of DSSN+. Figure 3.6 shows the results obtained from quantitative analyses of the biofilms developed in flow cells with or without DSSN+.

Figure 3.6. (A) Biofilm biovolume; (B) Surface coverage calculated based on confocal images; (C) Cell detachment rate from the biofilms. Results are presented as mean ± standard error (n=3).

The biofilm biovolume and surface coverage (Figure 3.6 (A) and (B)) were higher in the presence of DSSN+. After 72 h growth, a biovolume of $8.12 \pm 0.11 \times 10^7 \mu\text{m}^3$ and a surface coverage of $1.22 \pm 0.01 \times 10^7 \mu\text{m}^2$ were observed in case of biofilms with DSSN+, in comparison to control biofilms (biovolume of $2.88 \pm 0.02 \times 10^7 \mu\text{m}^3$ and surface coverage of $7.13 \pm 0.04 \times 10^6 \mu\text{m}^2$). Further, biofilms with DSSN+ showed substantially lower detachment rates (Figure 3.6 (C)) during the first 3 days: $79 \pm 5.90 \text{ CFU/mm}^2/\text{h}$ (48 h) and $588 \pm 32.08 \text{ CFU/mm}^2/\text{h}$ (72 h) in comparison to control biofilms ($465 \pm 43.60 \text{ CFU/mm}^2/\text{h}$ (48 h) and $837 \pm 73 \text{ CFU/mm}^2/\text{h}$ (72 h)). After 3 days, a comparable cell detachment rate was obtained for both the biofilms with or without DSSN+: $1150 \pm 72.05 \text{ CFU/mm}^2/\text{h}$ (DSSN+) vs. $1230 \pm 72.33 \text{ CFU/mm}^2/\text{h}$ (control) after 96 h and $1630 \pm 59.76 \text{ CFU/mm}^2/\text{h}$ (DSSN+) vs. $1880 \pm 102.27 \text{ CFU/mm}^2/\text{h}$ (control) after 120 h. The

data indicates that the biofilm detachment rates in the presence of DSSN+ were approximately 6-folds lower than the control after 48 h. The quantitative biofilm data indicated that DSSN+ favours the formation of a stable biofilm.

As implied by the results discussed above, effluents from the biofilms with DSSN+ had a higher concentration of extracellular flavins and a higher enzyme activity of the periplasmic alkaline phosphatase (ALP) (Figure 3.7 (A) and (B)). To further examine whether DSSN+ affects overall biofilm metabolic activity, the lactate consumption rate as well as the acetate production rate was determined. A comparable or slightly higher rate of oxidative conversion of lactate to acetate was observed for biofilms with DSSN+ (Figure 3.7 (C)), suggesting that the presence of DSSN+ in biofilms does not compromise biofilm activity.

Figure 3.7. (A) Extracellular flavin concentration; (B) Extracellular enzyme activity of ALP; (C) Concentration of lactate and acetate in the effluent during biofilm growth. Results are presented as mean $\pm$ standard error (n=3).

3.3.6. A working model on impacts of DSSN+ on extracellular bioactivity

Based on our results, a working model illustrating the mechanism of how DSSN+ enhances extracellular ferrihydrite reduction by *S. oneidensis* (Figure 3.8) was proposed. DSSN+ does not interact with the Mtr pathway nor induce flavin biosynthesis. However, it permeabilizes the cell membrane, which underlies an increase in extracellular concentration of flavin electron shuttles. The increased amounts of flavins enhance the extracellular reduction of ferrihydrite.

Figure 3.8. Working model on the mechanism of DSSN⁺. DSSN⁺ does not interact with protein electron carriers such as cytochromes CymA, MtrA, and MtrC/OmcA in the Mtr-pathway. There is no significant effect of DSSN⁺ on the expression of genes involved in flavin biosynthesis such as SO3468, *bfe*, *frdA* (SO0398), and *ushA* (SO2001). However, it permeabilizes cell membrane and results in an increase in extracellular electron shuttles such as flavins that enhances extracellular reduction of ferrihydrite. We do not have sufficient evidence to show that the cytoplasmic membrane is affected.

Previous reports on the enhancement of power generation by DSSN⁺ in bioelectrochemical systems using *E. coli* suggested that DSSN⁺ might function as an artificial electron conduit and promotes direct cross membrane electron transfer to the electrode (Wang et al. 2013; Yan et al. 2015). Here, we show that DSSN⁺ causes membrane permeabilization and facilitates the release of flavins that can be used as electron shuttles to enhance shuttle-mediated electron transfer. Recent studies have also shown that the membrane intercalation of DSSN⁺ can cause membrane perturbation and increase the ion conductance, which corroborates with our findings (Du et al. 2013; Hinks et al. 2015; Hinks et al. 2014). A recent study has reported on the release of electroactive cytosolic

compounds with DSSN+ insertion, which underpins potential effects on cytoplasmic membrane as well (Wang et al. 2014). However, we do not have evidence to suggest potential effects on cytoplasmic membrane. Hence, these results provide novel implications for cell-DSSN+ interactions: in addition to potentially promote direct extracellular electron transport, DSSN+ also mediates the shuttle-enabled extracellular electron transfer. In fact, in *S. oneidensis*, we found that the latter is the key mechanism.

The drastic increase in electron transfer capability of *E. coli* observed in the previous reports could also be at least partially attributed to the release of flavins caused by DSSN+ treatment. *E. coli* is not considered an electrogenic bacterium because it does not have an efficient cross-membrane electron transfer pathway and also does not secrete extracellular electron shuttles such as flavins (Wang 2014; Wang et al. 2013). Overexpression of porins in *E. coli* membranes resulted in a greatly enhanced extracellular electron transfer capability through the facilitation of the release of flavins to the cell exterior (Yong et al. 2013). The exogenous addition of flavins to this mutant caused further improvement (Yong et al. 2013).

In contrast to the effect of DSSN+ in *E. coli* systems, the performance boosting effect in *S. oneidensis* was relatively marginal. *S. oneidensis* is one of the most effective and best studied electrogenics and it produces outer membrane cytochromes and extracellular flavins for transferring electrons across the cell membrane through a direct electron transfer pathway and a flavin mediated route (Marsili et al. 2008; Patil et al. 2012; Shi et al. 2009; Watanabe et al. 2009). Our results showed that DSSN+ could not rescue the *S. oneidensis* mutants with disrupted direct electron transfer pathway, suggesting no or very

limited role of DSSN⁺ in direct electron transfer in *S. oneidensis*. The increase of extracellular flavins caused by DSSN⁺ might be limited and could not lead to a marked improvement in extracellular electron transfer capability of *S. oneidensis*, which was evidenced by a slight increase in ferrihydrite reduction in this study and a moderate enhancement in extracellular electron transfer to electrode (Wang 2014; Wang et al. 2013).

This study demonstrates that the incorporation of a membrane spanning conjugated oligoelectrolyte DSSN⁺ into biofilm cells of *S. oneidensis* MR-1 i) increases the extracellular concentration of redox shuttles, *i.e.*, flavins, and extracellular enzyme activities without significantly compromising cell viability, and ii) enhances biofilm formation and stability without negating the overall biofilm activity. Hence, membrane spanning conjugated oligoelectrolytes such as DSSN⁺ can induce the exchange of bioactive molecules between cells and the biofilm matrix and thereby achieve enhanced extracellular bioactivity and more efficient biofilm matrix-enabled biocatalysis. Taken together, our results suggest that membrane spanning conjugated oligoelectrolytes, of which DSSN⁺ is one of many possible molecular structures, may be applied to enhance extracellular bioactivity in bacteria towards more efficient biofilm-based biocatalysis.

CHAPTER 4: EFFECT OF MUTUALISTIC CELL TO CELL INTERACTIONS ON EXTRACELLULAR BIOACTIVITY

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4.1. Introduction

Most of the organic carbon in nature occurs in the form of sugars released either through photosynthetic activity or as waste biomass from different sources (Pandey et al. 2000; Sheen et al. 1999). Hence, in deeper sediment layers, where there is abundant supply of sugars, most of the microorganisms support their growth by fermentation of sugars (Chaudhuri and Lovley 2003; Lovley and Coates 2000; Pandey et al. 2000). The release of secondary metabolites by fermentative microorganisms supports the growth of microbial communities such as *S. oneidensis*, which do not have sugar utilization pathways (Yang et al. 2015). Hence, the degradation of sugars in nature is facilitated by the intercellular interactions between microbial communities harbouring diverse metabolic functions.

Among the different intercellular interactions, mutualism occurs, when the microbial partners associate by either exchanging metabolites or molecular signals and cues. Mutualism represents a cooperative scenario, which integrates diverse metabolic functions to achieve a desired output (Brenner et al. 2008).

Further, by compartmentalizing the task among the microbial partners, the steps required to achieve the desired outcome is reduced.

Mutualism occurs by metabolite exchange, when the members in a community survive by sharing the nutrients involved in growth and metabolism (Morris et al. 2013b; Schink 2002; Sieber et al. 2012). Under such a scenario, the presence of a cooperative partner drives the survival of even the functionally redundant organism in the mixed community (Schink 2002; Sieber et al. 2012). Literatures have coined this scenario as “obligatory mutualistic metabolism” to highlight the mutualistic interactions, which provides beneficial aspects for both the partners (Morris et al. 2013b). Mutualistic interaction between microbial communities encompass closely associated cell to cell interactions and plays important roles in biogeochemical cycling of carbon and solid minerals (McInerney et al. 2011). Mutualistic interactions also form the driving force in several biofilm-based bioprocesses such as anaerobic wastewater treatment, biodegradation of recalcitrant molecules like lignocellulose and sludge digestion (Brenner et al. 2008).

The collective output through mutualistic interactions is typically greater than that of each of the monoculture systems. For example, a defined binary culture of fermentative *Clostridium cellulolyticum*, and EAB *G. sulfurreducens* respire on an extracellular electrode to convert a specific cellulosic biomass to useful energy (Ren et al. 2007). Hence, there is a need to elucidate mutualistic interactions in engineered systems.

The objective of the study was to explore the function driven intercellular mutualistic interactions in extracellular biocatalytic reactions. To this end, a mutualistic system, consisting of a fermentative bacterium and a dissimilatory

metal-reducing bacterium in extracellular ferrihydrite reduction is explored.

Escherichia coli K-12 was chosen as representative fermentative microorganism, as it is easy to culture with simple nutritional requirements and its genome sequence is relatively well annotated (Blattner et al. 1997), while *S. oneidensis* was used as model anaerobically respiring bacteria. We further deciphered that the structural assembly of both communities in our system is driven by their specific metabolic functions. In a parallel study, mutualistic interactions between *E. coli* and *S. oneidensis* were also established in a microbial fuel cell, as reported in (Wang. et al. 2015).

4.2. Materials and Methods

4.2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 4.1. *E. coli* K-12 (ATCC #10798) was purchased from the American Type Culture Collection (Manassas, VA). Stock cultures were stored in LB medium with 25% glycerol at -80°C. Monocultures of *E. coli* or *S. oneidensis* were prepared aerobically in LB medium at 37°C and 30°C, respectively.

Table 4.1. List of bacterial strains used in the study

Strains	Description	Reference
<i>S. oneidensis</i> MR-1	Manganese reducing strain (WT)	(Myers and Nealson 1988)
$\Delta hydA$	<i>hydA</i> (SO3920) deletion in MR-1	(Marshall et al.2008b)
$\Delta hyaB$	<i>hyaB</i> (SO2098) deletion in MR-1	(Marshall et al.2008b)
$\Delta hydA-hyaB$	<i>hydA-hyaB</i> deletion in MR-1	(Marshall et al.2008b)
<i>E. coli</i> K-12	Fermentative strain	(Clark 1989)

4.2.2. Selective minimal media composition

The minimal media used in this study was designed specifically to stimulate growth conditions for both *E. coli* and *S. oneidensis* strains. The media was prepared as a mixture of MM1 and M9 buffer, mixed in equal proportions (1:1 v/v) with 20 mM glucose as the carbon source. The M9 minimal buffer is composed of 261.14 mM of NaHPO₄·7H₂O, 111.88 mM of KPO₄, 42.77 mM of NaCl, 93.46 mM of NH₄Cl and supplemented with traces of MgSO₄ (about 2 mM) and 0.68 mM CaCl₂. The MM1 minimal buffer consisted of 30 mM Hepes, 7.5 mM NaOH, 1.34 mM KCl, 4.35 mM NaH₂PO₄ and further supplemented with traces of mineral, vitamin and amino acids. *E. coli* K-12 and *S. oneidensis* MR-1 pre-cultures were prepared in LB medium.

4.2.3. Reduction of ferrihydrite under growth conditions

The ferrihydrite stock suspension was prepared as outlined in section 3.2.2 (Sivakumar et al. 2014). The anaerobic reduction of ferrihydrite was performed in several 25 mL anaerobic Wheaton serum bottles. Ferrihydrite suspension was injected into the respective anaerobic bottles at an initial concentration of 10 mM. The inoculum volume was maintained at 2% for monoculture systems and 1% each for all the co-culture systems. Three replicates were maintained for each condition. The tubes were maintained under anaerobic conditions at room temperature by initially bubbling with nitrogen gas for several min and then by sealing it with a butyl rubber stopper and aluminum crimp. Tubes without cells were used as abiotic control. Ferrozine assay was used to quantify the Fe³⁺ reduction (Sivakumar et al. 2014).

4.2.4. Flavin quantification using fluorescence

Approximately 0.5 - 1 mL of the samples were taken at periodic time intervals and centrifuged at 12,000 × g for 10 min at 4° C. For fluorescence measurements 200 µL of the cell free supernatants were transferred to a clear 96 well plate and read at 440 nm excitation and 525 nm emission. The background fluorescence was corrected by using the selective minimal media as the blank. Total flavins instead of individual flavin species were quantified to elucidate whether flavin-mediated extracellular electron transfer was influenced by mutualistic interactions. The standard curve for the measurement of total flavins was prepared using riboflavin as described in previous reports (Covington et al. 2010; Je et al. 2007; Pan 2015; Von Canstein et al. 2008).

4.2.5. Community dynamics in ferrihydrite reduction

The community dynamics in the mutualistic system were quantified using drop-plate method (Chen et al. 2003a; Ding et al. 2014a; Zhang et al. 2014). The plates were prepared in duplicates. The drops were allowed to dry on the plates before turning it over for incubation at 30° C for 16-20 h. The *S. oneidensis* cells tagged with green fluorescent protein were counted in dark using a fluorescent torch (MeCan Imaging Inc., Japan) and the non-fluorescent *E. coli* cells were estimated by deducting the fluorescent colonies from the total number of colonies. The viable bacterial cells were expressed as CFU/mL.

4.2.6. Metabolite analysis

The methods for metabolite analysis are as described in section 3.2.16 (Andersson and Hedlund 1983; Bover-Cid and Holzapfel 1999).

4.2.7. RNA extraction

The seed cultures of *E. coli* K-12 and *S. oneidensis* MR-1 WT were incubated in LB medium for 16 h. For ferrihydrite reduction systems, 2% (v/v) of the seed cultures (*E. coli* and *S. oneidensis*) were added into anaerobic Wheaton serum bottles (25 mL) filled with M1-M9 media (1:1, v/v) with 20 mM glucose and 10 mM ferrihydrite. The anaerobic conditions were maintained as mentioned above and the experiment was performed using three replicates. Tubes inoculated with *E. coli* K-12 alone were used as the control to validate the effects of communal metabolism. The cells were pelleted for RNA extraction after achieving approximately 30% of Fe³⁺ reduction in co-culture system. The bacterial cell pellets were thawed in ice and treated with lysozyme in TE buffer. Total RNA was extracted with RNeasy Mini Purification kit (Qiagen). On-column DNase digestion with the RNase-free DNase Set (Qiagen) was carried out to facilitate the removal of DNA. The concentration of RNA and presence of DNA contamination was assessed using a Qubit® 2.0 Fluorometer (Invitrogen). The integrity of RNA was assessed with the Agilent 2200 TapeStation System (Agilent Technologies).

4.2.8. RNA sequencing

The quality of the RNA samples was confirmed with the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) and Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) on a Bioanalyzer RNA 6000 Nano Chip (Agilent). Subsequently, next-generation sequencing library was prepared by adopting the TruSeq RNA Sample Preparation v2 protocol (Illumina) with modifications. mRNA purification was excluded and 200 ng of total RNA was added to the elute-fragment-prime step instead. PCR was amplified stepwise, which enriches

selectively for library fragments with adapters ligated on both ends. These steps were executed according to the manufacturer's protocol but the amplification cycles were minimized to 12 steps. Each library was specifically tagged with barcodes from Illumina's TruSeq LT RNA to facilitate library pooling for sequencing. Library quantization was produced using Invitrogen's Picogreen assay. The average library size was confirmed by referencing the libraries on a Bioanalyzer DNA 1000 chip (Agilent). The library concentration was adjusted to 2 nM and the concentration was determined by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), employing qPCR primers in Illumina's qPCR protocol and Illumina's PhiX control library as a standard. All the libraries were then combined equally and sequenced in two lanes of an Illumina HiSeq2500 rapid run at a final concentration of 7.2 pM and a read-length of 101 bp paired-end (Mohanty et al. 2013).

4.2.9. Computational Analysis

The combined sequence reads were analyzed in RNA-Seq and expression analysis application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The *E. coli* K-12 (<http://www.ncbi.nlm.nih.gov/nucore/556503834>) and *S. oneidensis* MR-1 (<http://www.ncbi.nlm.nih.gov/nucore/414561716>) genomes were used as the reference genome for the computational analysis of sequencing data. These conditions were used to filter the unique sequence reads: minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of 2 mismatches. Data were normalized by calculating the reads per kilobase per million mapped reads (RPKM) for each gene (Mortazavi et al. 2008). Statistical analyses ANOVA and t-test were performed. The fold changes were considered statistically significant for all the genes whose p-value ≤ 0.05 . The fold

changes ($\log_2 R$) in the gene expression for *E. coli* and *S. oneidensis* were quantified by taking the ratio (R) of RPKM in treated to control (Wu et al. 2014).

4.3. Results and Discussion

4.3.1. Mutualistic interaction is established in ferrihydrite reduction

S. oneidensis MR-1 is capable of reducing Fe^{3+} to Fe^{2+} under anaerobic conditions (Coursolle and Gralnick 2012; Fredrickson and Gorby 1996). To establish the mutualistic interactions between *E. coli* and *S. oneidensis*, we used ferrihydrite reduction as the model extracellular biocatalytic reaction. Figure 4.1 shows the Fe^{2+} concentrations in co-culture and monoculture systems respectively. The results suggested that the co-culture system displayed substantially higher rate of Fe^{3+} reduction. The concentration of Fe^{2+} in co-culture system after 60 h was 4.65 ± 0.11 mM, whereas no Fe^{2+} was detected in monoculture systems. (Figure 4.1). These rates of reduction are comparable to that of *S. oneidensis* in presence of lactate as electron donor (Lies et al. 2005). These results suggest the potential role of mutualistic interaction between *E. coli* and *S. oneidensis* in extracellular ferrihydrite reduction. The same mutualistic system deployed in glucose fed MFC generated 2-fold higher current density, as compared to monoculture systems (Wang et al. 2015).

Figure 4.1. Ferrihydrite reduction kinetics for mono and co-cultures of *E. coli* and *S. oneidensis*. Results are presented as mean $\pm$ standard error (n=3).

4.3.2. Hydrogen is not an electron carrier in mutualistic interaction between *E. coli* and *S. oneidensis*

Hydrogen and formate are common metabolites of exchange in mutualistic systems and are produced from glucose fermentation by *E. coli* (Walker et al. 2009). It has been shown that hydrogen transfer can mediate electron flow in mutualistic systems efficiently through hydrogen utilization catalyzed by hydrogenases (McInerney et al. 2011). Hydrogenase is a class of highly characterized enzymes in *Shewanella* that plays a vital role involving hydrogen (Boyd et al. 2010). The annotated genome sequence of *Shewanella* encodes an Fe only hydrogenase [FeFe], *hydA* (SO3920) and a NiFe hydrogenase [NiFe], *hyaB*(SO3921). HyaB is capable of performing the dual role of producing and consuming hydrogen (Marshall et al. 2008b). HydA is expressed only for hydrogen formation with a capacity 10-100 times higher than HyaB (Boyd et al. 2010; Marshall et al. 2008b).

To elucidate the presence of hydrogen in the mutualistic system, we conducted ferrihydrite reduction using co-cultures of *E. coli* with *S. oneidensis* hydrogenase mutants. There was no significant difference in ferrihydrite reduction between *S. oneidensis* WT and hydrogenase mutant co-culture systems. After 24 h, the Fe^{2+} concentrations in co-culture systems (Figure 4.2) were 3.35 ± 0.005 mM (ΔhydA), 3.55 ± 0.02 mM (ΔhyaB) and 3.31 ± 0.03 mM ($\Delta\text{hydA-hyaB}$) respectively. These findings rule out the possibility of hydrogen as a potential metabolite of exchange in interactions between *E. coli* and *S. oneidensis*. Hydrogen was also not involved in the interactions between *E. coli* and *S. oneidensis* in our study with MFC (Wang et al. 2015).

Figure 4.2. Ferrihydrite reduction kinetics for mono- and co-cultures of *E. coli* and *S. oneidensis* WT and hydrogenase mutants. Results are presented as mean $\pm$ standard error (n=3).

4.3.3. Increase in extracellular flavins enhances ferrihydrite reduction in mutualistic system

Electron shuttles or redox mediators are soluble redox active molecules, which transfer the electrons to the extracellular insoluble electron sink (Marsili et

al. 2008; Watanabe et al. 2009). *S. oneidensis* produce riboflavin and flavin mononucleotide (FMN) that serve as electron shuttles facilitating the transfer of electrons to solid substrates such as ferrihydrite and electrode (Kotloski and Gralnick 2013b; Marsili et al. 2008). The role of flavins as an indirect electron transfer mediator is already well reported (Kotloski and Gralnick 2013b; Marsili et al. 2008; Richardson et al. 2012). The flavin secretion pathway is described in Section 3.3.3 (Chapter 3) and is illustrated in Figure 3.8.

Temporal profiles of extracellular flavins were observed for monoculture and co-culture systems (Figure 4.3). As expected, the concentration of extracellular flavins increased with time. The amount of extracellular flavins in co-culture system was ~2-folds higher than the monoculture systems. After 60 h (~50% Fe^{3+} reduction by co-culture), extracellular flavins in co-culture system accumulated upto 30 ± 0.01 nM, whereas *S. oneidensis* and *E. coli* monocultures accounted for 17 ± 0.22 nM and 11 ± 0.27 nM flavins respectively. The flavin profiles and Fe^{3+} reduction kinetics have implicated a direct correlation, especially at the peak phase of Fe^{3+} reduction (20-60 h).

Figure 4.3. Flavin electron shuttles enhance the extracellular reduction of ferrihydrite in co-culture system. Results are presented as mean $\pm$ standard error (n=3).

Certain degree of cell lysis might lead to the release of intracellular flavins in *E. coli* monocultures. The enhanced current generation observed in the mutualistic MFC system were also attributed to increased extracellular flavins secreted by *S. oneidensis*, which is consistent with previous reports (Kiely et al. 2011; Kotloski and Gralnick 2013a; Shi et al. 2009; Wang et al. 2015). A previous study also reports that the addition of flavins boost the current production in *E. coli* monoculture systems (Yong et al. 2013). These findings confirm the potential role of flavin electron shuttles in enhanced ferrihydrite reduction by the co-culture.

4.3.4. Formate is the principal metabolite of exchange in the mutualistic co-culture system

To determine the metabolite of exchange, HPLC was used to quantify the key metabolites in mutualistic system. Formate was identified as the principal electron carrier along with lactate and pyruvate at lower concentrations, as shown in Figure 4.4.. The formate concentration reached a maximum of ~20 mM after 40 h and thereafter decreased to ~5 mM after 60 h, indicating a high rate of consumption during the peak phase of ferrihydrite reduction. Formate was reported as the main metabolite of exchange in the MFC mediated by this mutualistic system (Wang et al. 2015).

Figure 4.4. Metabolite analysis using HPLC identified formate as the principle electron carrier in the mutualistic interactions between *E. coli* and *S. oneidensis*. Results are presented as mean $\pm$ standard error (n=3).

4.3.5. Mutualistic interactions determine the community structure in ferrihydrite reduction

Community dynamics are fundamental in determining how mutualistic systems function as an entity in various ecological settings (Parameswaran et al. 2009; Ren et al. 2007; Stolyar et al. 2007. To understand the functional ecology in ferrihydrite reduction), we observed the community profile for the co-culture mediated ferrihydrite reduction (Haruta et al. 2009; Morris et al. 2013b). A 2-fold (log scale) increase in *E. coli* cell density was observed after 20 h (from $2 \times 10^6 \pm 0.16 \times 10^4$ CFU/mL to $2.7 \times 10^8 \pm 0.24 \times 10^6$ (CFU/mL) (Figure 4.5 (A)). *S. oneidensis* exhibited a 1-fold (log scale) increase only after 40 h ($1.4 \times 10^6 \pm 0.41 \times 10^6$ CFU/mL to $1.55 \times 10^7 \pm 0.87 \times 10^4$ CFU/mL), which was followed by a period of steady growth ($8.33 \times 10^7 \pm 0.12 \times 10^6$ CFU/mL (60 h)) (Figure 4.5 (A)).

The % community distribution plot (Figure 4.5 (B)) show that the relative proportion of *S. oneidensis* increased from ~10% (at 20 h) to ~35% at 60 h. The increase in *S. oneidensis* between 40th and 60th hour indicates the presence of potential substrates for *S. oneidensis* released from the fermentation of glucose (Figure 4.4). The community dynamics data implies that the fermentation of glucose by *E. coli* enabled its proliferation in the initial stages (upto 40 h). The release of fermentation products facilitated growth of *S. oneidensis*, enhancing the rate of ferrihydrite reduction.

Figure 4.5. Community dynamics in the mutualistic system. (A) CFU counts of *E. coli* and *S. oneidensis* strains in ferrihydrite reduction; (B) Relative proportion (%) of *E. coli* and *S. oneidensis* strains in ferrihydrite reduction. Results are presented as mean $\pm$ standard error (n=3).

The resolved structure-function relationship of the mutualistic MFC system, showed that *E. coli* accounted for majority of the planktonic cells (~98%), whereas *S. oneidensis* dominated the biofilms on the electrode (biovolume fraction ~60%) (Wang et al. 2015). It has been reported that fermentation and anaerobic respiration together is more thermodynamically favourable than a sole fermentative process, based on the energy yield per electron transferred in the

respective reactions (Lovley 2006; McInerney and Beaty 1988). Further, the preferential localization of *S. oneidensis* on the anode was attributed to its capability to respire on anode by utilizing formate released from glucose fermentation by *E. coli* (El-Naggar et al. 2010; Gorby et al. 2006b; Von Canstein et al. 2008). This observation implied that the distribution of each species in this mutualistic community is driven by its metabolic functions towards an optimum communal metabolism (Wang et al. 2015).

In addition, a mass balance performed on the glucose fermentation by *E. coli* planktonic cells revealed that ~60% of glucose is consumed by *E. coli* planktonic cells, leading to the release of fermentation products and *S. oneidensis* accounts for only ~3-4% of the total electrons derived from the co-culture system (Wang et al. 2015). *E. coli* contributed to majority of the electrons (~96-97%) derived. This confirmed flavin-mediated electron transfer as the main extracellular electron transfer mechanism in this co-culture system.

These results provided a novel perspective for mutualistic interactions. The presence of *S. oneidensis* improves the electrogenic properties of *E. coli* through the release of flavins. *E. coli* in turn releases simple metabolites for *S. oneidensis* to undergo anaerobic respiration. Hence, the mutualistic community enriched with species of diverse metabolic functions is able to drive itself towards an optimum communal metabolism.

4.3.6. Preferential localization of *S. oneidensis* cells near to ferrihydrite favours extracellular reduction through mutualistic interaction

RNA-sequencing-based transcriptomic study on *S. oneidensis* biofilms formed on the electrode revealed that the expression profiles of extracellular

electron transfer related genes were upregulated. In particular, expression levels of genes involved in the MTR pathway (*cymA*, *mtrA*, *mtrB*, *mtrC*, *omcA*) and flavin biosynthesis (*fccA*, *frdA*, SO 3468 and *ribE*) were significantly increased. These observations along with the CFU and biovolume data supported the stronger *S. oneidensis* biofilm presence in the anode (Wang et al. 2015).

Similarly, a transcriptomic study was conducted to understand the effects of communal metabolism on *S. oneidensis* in ferrihydrite reduction. Our results indicated that all the genes involved with the Mtr pathway (*cymA*, *mtrA*, *mtrB*, *mtrC* and *omcA*) were upregulated significantly (Table 4.2). The expression level of genes involved with the secretion of flavins (*ribE*, SO 3468, *ushA*, *frdA*) to the extracellular space also increased remarkably (Table 4.2), which underpins role of flavin electron shuttles (Figure 4.3) in enhanced extracellular ferrihydrite reduction. It has been reported that *S. oneidensis* localize near the solid electron acceptor in order to undergo anaerobic respiration. From Figure 4.5, it is clear that *S. oneidensis* comprises only a fraction of the total biomass, which might have accumulated within the periphery of ferrihydrite crystals. The findings from MFC mediated by the same mutualistic system corroborate this observation (Wang et al. 2015).

In addition to the results mentioned above, we also observed a substantial increase in the expression levels of key formate dehydrogenase genes involved with the oxidation of formate (*fdhC*, *fdhA*, *fdhX*, *fdhT*), as shown in Table 4.2. These findings further reinforce formate as the principal metabolite of exchange (Figure 4.4), which is also consistent with the MFC reported data (Wang et al. 2015).

Table 4.2. Selected gene expression changes in *S. oneidensis* imparted by the presence of *E. coli* in ferrihydrite reduction

Gene	Annotation	Fold change
Genes involved in Mtr pathway		
<i>cymA</i>	Membrane anchored tetraheme cytochrome CymA	3.89
<i>mtrA</i>	Extracellular iron oxide respiratory periplasmic decaheme cytochrome component MtrA	4.83
<i>mtrB</i>	Extracellular iron oxide respiratory outer membrane component MtrB	4.73
<i>mtrC</i>	Extracellular iron oxide respiratory surface decaheme cytochrome component MtrC	4.79
<i>omcA</i>	Extracellular iron oxide respiratory surface decaheme cytochrome component OmcA	5.52
Genes involved in flavin secretion		
<i>ribE</i>	Riboflavin synthase beta subunit RibE	3.68
<i>SO3468</i>	Riboflavin synthase alpha subunit RibC-like protein	3.19
<i>ushA</i>	Bifunctional UDP-sugar hydrolase/5'-nucleotidase UshA	3.13
<i>fccA</i>	Periplasmic fumarate reductase FccA	3.68
<i>frdA</i>	Quinol:fumarate reductase FAD-binding subunit FrdA	3.42
Formate dehydrogenase genes		
<i>fdhC</i>	Formate dehydrogenase cytochrome b subunit FdhC	3.80
<i>fdhA</i>	Formate dehydrogenase molybdopterin-binding subunit FdhA	3.75
<i>fdhX</i>	Formate dehydrogenase accessory protein FdhX	3.71
<i>fdhT</i>	Formate dehydrogenase chaperone FdhT	3.57

Transcriptomic data from MFC mutualistic system also indicated that genes involved in carbohydrate metabolism in *E. coli* were upregulated (Wang et al. 2015). This suggested a mutualistic relationship, where *S. oneidensis* takes up formate for respiration, which releases electrons, whereas *E. coli* ferments glucose to produce formate. Specifically in MFC-based mutualistic system, *ydiQ*,

which is a flavoprotein in *E. coli*, is significantly up-regulated, which suggests that secreted flavins from *S. oneidensis* can potentially induce increased *E. coli* metabolism (Wang et al. 2015). For ferrihydrite reduction-based co-culture system, the presence of *S. oneidensis* induced upregulation of several *E. coli* genes (*csrA*, *poxB*, *uxuA*, *waaJ*) involved in carbon metabolism. Hence, our transcriptomic findings strongly establish the mutualistic interactions between *E. coli* and *S. oneidensis* in both MFC and ferrihydrite reduction.

Table 4.3. Selected gene expression changes in *E. coli* imparted by the presence of *S. oneidensis* in ferrihydrite reduction

Gene	Annotation	Fold change
<i>csrA</i>	Pleiotropic regulatory protein for carbon source metabolism	1.48
<i>poxB</i>	Pyruvate dehydrogenase (pyruvate oxidase), thiamine triphosphate-binding, FAD-binding	2.14
<i>pblB</i>	Formate C-acetyltransferase 1, anaerobic; pyruvate formate-lyase 1	1.98
<i>uxuA</i>	Carbohydrate metabolism; mannonate hydrolase	2.15
<i>manY</i>	Mannose-specific enzyme IIC component of PTS	2.27
<i>rffG</i>	dTDP-glucose 4,6-dehydratase (EC:4.2.1.46)	2.02
<i>waaJ</i>	UDP-D-glucose:(galactosyl)lipopolysaccharide glucosyltransferase (EC:2.4.1.58)	1.79
<i>rfbA</i>	Glucose-1-phosphate thymidyltransferase	1.84
<i>rffH</i>	Glucose-1-phosphate thymidyltransferase	1.31

4.3.7. Proposed mutualistic interaction model between *E. coli* and *S. oneidensis*

Based on the results obtained from this study, we propose a mutualistic interaction model between fermentative *E. coli* and dissimilatory metal reducing *S. oneidensis* (Figure 4.6). *E. coli* initiates the mutual interaction with the fermentation of glucose releasing simple organic molecules like formate, lactate and acetate. *S. oneidensis* utilizes these fermentation products and respire on

solid ferrihydrite directly through Mtr pathway or indirectly with the secretion of flavin electron shuttles (Marsili et al. 2008; Shi et al. 2009). In addition, these electron mediators can also enhance the metabolic activity of *E. coli* in the mutualistic community. A direct correlation is observed between all the findings that underpins the mutualistic interactions in our model.

Figure 4.6. Model showing the mutualistic interaction between *E. coli* and *S. oneidensis* fed with glucose as the sole carbon source and ferrihydrite as the electron acceptor. The proposed model is based on interspecies metabolite transfer. *E. coli* ferments glucose to release metabolites such as lactate and formate for *S. oneidensis* to serve as electron donor. *S. oneidensis* secrete flavins to facilitate the extracellular electron transfer to ferrihydrite. Flavins also stimulate the metabolic activity and extracellular electron transfer capability of *E. coli*, as indicated by the double-headed curved dotted arrow. No inward electron transfer is involved. The extracellular electron transfer to ferrihydrite is shown in thin straight dotted lines.

In this study, we have demonstrated that extracellular biocatalytic processes can be mediated through intercellular interactions between bacterial communities characterized with diverse metabolic functions. It was also shown,

how the mutualistic interactions enable in harnessing mutual benefits for both the communities. Mutualistic interactions with *E. coli* enabled *S. oneidensis* to balance the functional redundancy associated with glucose as the main substrate. In turn, the flavins secreted by *S. oneidensis* stimulated the electron transfer capability and glucose metabolism in *E. coli*. Further studies on similar intercellular mutualistic interactions are required to gain a complete understanding of communal metabolism in natural environments and engineered settings.

CHAPTER 5: BIOFILM MATRIX DISPLAY OF roGFP FOR QUANTIFYING EXTRACELLULAR REDOX STATUS OF BIOFILM MICROENVIRONMENTS

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5.1. Introduction

In natural and engineered habitats, microorganisms often exist in the form of surface-adhered biofilms (Ding et al. 2014a; Flemming and Wingender 2010). The interplay between biofilm metabolism and physicochemical processes in biofilm matrix make the biofilm microenvironments highly dynamic and heterogeneous with spatiotemporally varying concentration of nutrients, chemical cues, and signaling molecules (Parsek and Fuqua 2004; Singh et al. 2006; Sutherland 2001b). Previous studies have suggested an important role of physicochemical processes in biofilm development (Flemming and Wingender 2010; Mark and Rebecca 2012; McLean et al. 2008; Stewart and Franklin 2008; Sutherland 2001b; Wimpenny et al. 2000).

Among these diverse processes, redox changes influence a range of cellular respiration processes (Cannon and Remington 2009; Yu and Bishop 1998). Redox reactions are often associated with the biological processes involved with the release or retention of energy (Bjornberg et al. 2006; Cannon and Remington 2009; Jiang et al. 2006). It has been reported that the redox status is highly critical for the microorganisms to cope with environmental stresses such as oxidative stress induced by environmental contaminants like heavy metals and metalloids (Cannon and Remington 2009; Morgan et al. 2011).

The intracellular redox state has been demonstrated to have a great impact in driving the morphological development of biofilms (Dietrich et al. 2013). To understand the role of redox state in biofilm development and responses of biofilms to environmental perturbations, it is essential to quantify the redox state of microenvironments in biofilms.

The redox monitoring methods in biofilm encompass variety of techniques ranging from microelectrode-based investigations to the application of redox dyes and genetically tagged fluorescent proteins. Microsensors have been widely used to quantify physicochemical parameters such as pH, dissolved oxygen, redox potential, and metabolites in biofilms with high spatial resolution and precision (Cao et al. 2012; Halan et al. 2012b; Li and Bishop 2004; Rasmussen and Lewandowski 1998). Most of the microsensor-based studies in biofilms have focused on understanding the biofilm physiology based on the mass transfer (Cao et al. 2012; Halan et al. 2012b).

In addition, certain redox sensitive dyes can be introduced into biofilms to map redox state of microenvironments (Nielsen et al. 2000; Staal et al. 2012; Teal et al. 2006; Werner et al. 2004; Wolfaardt et al. 1994). However, these

methods might perturb the biofilm structure, as they are locally invasive (Halan et al. 2012b). Another intriguing fact is that all these methods can be deployed only to elucidate the intracellular redox profile in a biofilm.

Genetically encoded redox biosensors offer a non-invasive alternative approach to quantify the redox state in real time within biofilms and biofilm matrix (Bjornberg et al. 2006; Teal et al. 2006). Redox sensitive green fluorescent protein (roGFP) is one such redox biosensor that monitors the redox status through the ratiometric quantification of relative fluorescence intensity at two excitation maxima (400 nm and 480 nm) (Cannon and Remington 2009; Morgan et al. 2011). The roGFP probe is constructed by the formation of disulfide bonds between the cysteine residues on the surface of GFP β -barrel. The disulfide formation during the oxidation of roGFP increases the excitation peak at 400 nm relative to 480 nm excitation peak. Similarly when the roGFP is in its reduced state, a higher excitation peak is exhibited at 480 nm (Arias-Barreiro et al. 2010; Cannon and Remington 2009; Morgan et al. 2011). Therefore, measuring the ratio between the fluorescence intensity at 400 nm and 480 nm excitation maxima can quantify the redox state. The intracellularly expressed roGFP has been reported to be effective to monitor cellular redox state in the presence of membrane permeable oxidants like hydrogen peroxide (H_2O_2) or reductant like dithiothreitol (DTT) (Cannon and Remington 2009). However, the roGFP redox probe has never been exploited to quantify redox state in extracellular microenvironments of biofilms.

The objective of the study was to functionalize the biofilm matrix with the matrix display of the roGFP through the genetic fusion of roGFP to BpfA and to quantify and resolve the extracellular redox state in the biofilm matrix

microenvironments. The study was also conducted to validate and decipher whether the matrix-displayed enzyme approach sustain the catalytic activity and stability in biofilm mode and in continuous systems

To address these questions, we developed an approach for quantifying redox state in extracellular microenvironments of biofilms by displaying roGFP at the cell exterior. Specifically, we used *S. oneidensis*, as the model organism and genetically fused roGFP (~ 27 kDa) onto the C-terminus of biofilm promoting factor, BpfA, a large protein (~ 285 kDa) mainly expressed on cell surface and in biofilm matrix. BpfA belongs to repeats in toxin (RTX) family of proteins and have high affinity towards Ca^{2+} . BpfA has been reported to play a crucial role in stabilizing the biofilm matrix through the electrostatic interactions with Ca^{2+} (Cao et al. 2011a; Cao et al. 2011b; Theunissen et al. 2010b). Then we grew biofilms using this reporter strain and demonstrated the quantification of depth-resolved redox state in the extracellular microenvironments of the biofilms.

5.2. Materials and methods

5.2.1. Microorganisms and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 5.1. Stock cultures were maintained in LB medium with 20% glycerol at -80°C. Unless otherwise stated, cultures were grown aerobically at 30°C in LB medium or modified M1 defined minimal medium (MM1) (Zachara et al. 1988). For biofilm growth, MM1 with 1/10th LB (by volume) was used (Zhang et al. 2014).

Table 5.1. List of bacterial strains, plasmids, and primers used in the study

Strains	Description	Reference
<i>S. oneidensis</i>		
MR-1 WT	Manganese reducing strain	(Nealson and Myers 1992)
BpfA-roGFP	Reporter strain with roGFP fused to BpfA	This study
<i>E. coli</i>		
DH5α	Host for roGFP expression vector	(Tolia and Joshua-Tor 2006)
Plasmids	Description	Reference
pMC1-EPS-rtGFP	Redox sensitive rtGFP expression vector	(Madhaiyan et al. 2013)
pmC1-EPS-roGFP	roGFP expression vector derived from pmC1-EPS-rtGFP	This study
pUC57-roGFP-T5-APH-loxP-6-aggC	Vector used for the in-frame fusion of roGFP to BpfA	This study
Primers	Sequence	Reference
<i>bpfA</i> -F1- <i>EcoRI</i>	AAAGAATTCCCAGTAATCAGGATA CAGCGCAAGGC	This study
<i>bpfA</i> -RI- <i>SmaI</i>	AAACCCGGGTACAGGATCGACTG TTTTTAAGGGATC	This study
roGFP-F1	AAAGATATCATGGTCTCGAAGGGC GAGGAG	This study
roGFP-R1	AAAAGTAGTCTTGTAGAGCTCGTC CATGCCGA	This study
<i>bpfA</i> -F2	ACGGTAGGCAAAGATGGCACTG	This study
<i>roGFP</i> -R2	TGTTGCCGTCCTCCTTGAAGTC	This study
<i>roGFP</i> -F2	GACTTCAAGGAGGACGGCAACA	This study
<i>aggC</i> -R	ACTGCACGGTCAGATTGAGGTC AA	This study

5.2.2. Construction of the vector expressing roGFP

The plasmid pUC57-roGFP-T5-APH-loxP-H6-aggC contains a DNA cassette composed of a coding sequence for the redox sensitive roGFP2, kanamycin resistant aminoglycoside phosphotransferase (*aph*) of *E. coli*, and a 305 bp 5' fragment of *aggC* gene fragment derived from *S. oneidensis* MR-1

chromosome DNA (Bagdasarian et al. 1983; Madhaiyan et al. 2013; Heidelberg et al. 2002). The DNA cassette was designed, synthesized and cloned in pUC57 (Dargeviciute et al. 2002). *S. oneidensis* do not have very high codon usage bias pattern. Hence, the genetic fusion of *roGFP* with *bpfA* was performed without much codon optimization (Fox and Erill 2010; Sybirna et al. 2008). The *aph* encoding sequences were designed according to the codon preference of *S. oneidensis* MR-1. The *roGFP*-encoding gene was derived from the vector pMC1-EPS-*roGFP2*, a derivative of pMC1-EPS-rtGFP (Figure 5.1 (A)) (Madhaiyan et al. 2013). The *rtGFP* sequence in pMC1-EPS-rtGFP is modified to contain the S147C/Q204C/S65T triple mutations, creating the redox-sensitive *roGFP2*-encoding gene (Figure 5.1 (B)) (Dooley et al. 2004; Liu et al. 2013b). To create the *roGFP* knock-in construct, the *roGFP2* sequence was amplified by PCR using *roGFP*-F1 and *roGFP*-R1 as primers (sequence shown in Table 5.1) and pMC1-EPS-*roGFP2* as the template. The PCR products were digested with *EcoRV* and *SpeI*, purified using the QIAquick Gel Extraction Kit (Qiagen, USA) and inserted into the pUC57 vector at the same restriction sites to create pUC57-*roGFP*-T5-APH-loxP-H6-aggC. A gene knock-in homology arm corresponding to the last 986 bp coding sequence of *bpfA* was amplified by PCR with *bpfA*-F1-*EcoRI* and *bpfA*-R1-*SmaI* as the primers (sequence shown in Table 5.1) and *S. oneidensis* MR-1 genomic DNA as the template using Expand Long Template PCR system according to the manufacturer's instruction (Roche, Germany). The PCR product was cut with *SmaI* and *EcoRI*, gel-purified and inserted to pUC57-*roGFP*-T5-APH-loxP-H6-aggC at the corresponding sites to create the knock-in (KI) *roGFP* expression vector pUC57-KIroGFP. In short, the *roGFP* expression vector is described as pUC57-*bpfA*-*roGFP*-aggC (Figure 5.1 (C)), whose plasmid map is shown in Figure A-1. To make in-frame fusion of *roGFP* to the C-terminus of BpfA

(Figure 5.1 (D)), the pUC57-bpfA-roGFP-aggC was digested with *EcoRI* and *HindIII* and the vector-free fragment was purified and transformed to *S. oneidensis* MR-1 by electroporation as described below.

5.2.3. Construction of *S. oneidensis* BpfA-roGFP

The expression vector pUC57-bpfA-roGFP-aggC was transformed to *E. coli* DH5α competent cells through electroporation. The plasmid was extracted from *E. coli* DH5α cells using the plasmid extraction kit (Invitrogen). The expression vector was digested into two fragments prior to electroporation, since the linearized expression vector has high site specific recombination frequency. The digestion of the plasmid DNA (~ 20 µg) was carried out with the restriction enzymes *HindIII* (20 U) and *EcoRI* (10 U) in a volume of 200 µL. The digestion of the gel into two fragments was checked using gel electrophoresis. The digested DNA fragments were purified using a PCR cleaning kit (Invitrogen). By adding 1 /10th volume of 3 M sodium acetate (pH 4.8) and equal volumes of ethanol the purified DNA was concentrated. The mixture was incubated at -20°C for 30 min and then centrifuged at 20,000 × g for 20 min. The supernatant was discarded and the pellet was washed twice with 70% ethanol. The washed pellet was dried for 15 min under vacuum, which was then re-dissolved in 5 µl of Milli-Q water to a concentration of ~1000 ng/µl. Approximately 2 µl was used to electroporate (1800 V, 2 mm cuvette, Bio-Rad) *S. oneidensis* MR-1 electrocompetent cells. Kanamycin (20 µg/L) was used in LB agar to select colonies of MR-1 BpfA-roGFP. The fusion of *roGFP* with *bpfA* was confirmed by colony PCR, for which the 5' primer was selected from upstream of the homology region of *bpfA* (*bpfA*-F2) and 3' primer from the *roGFP* (*roGFP*-R2) sequence. It was further reconfirmed by using a similar strategy with the 5' primer designed from the homology region of

roGFP (roGFP-F2) and the 3' primer designed from *aggC* (*aggC*-R), the gene downstream to *bpfA* in *S. oneidensis* MR-1 genome. The primers used for this step is shown in Table 5.1 with sequence. Further, the sequence of the PCR product was identified through Capillary Sequencing facility (AITbiotech Pte Ltd). Using the National Centre for Biotechnology Information Blast online facility (NCBI Blast, www.ncbi.nlm.nih.gov/BLAST), the PCR product sequence was checked for nucleotide sequence similarity with that of *bpfA* and *roGFP*. The details of the vector construction are illustrated in Figure 5.1.

Figure 5.1. The steps involved in the inframe fusion of roGFP to BpfA are illustrated here. (A) The vector pMC1-EPS-RtGFP from which the *roGFP* gene used in this study, *roGFP2* is derived; (B) The roGFP expression vector pMC1-EPS-roGFP2; (C) The roGFP knock in vector used for inframe fusion of roGFP to BpfA consist of a *bpfA* fragment (993 bp), *roGFP2* gene (720 bp), kanamycin resistant aminoglycoside phosphotransferase (*aph*) of *Escherichia coli* (792 bp) and a 305 bp 5' portion of *aggC* gene of *S. oneidensis* MR-1. The vector was constructed in two steps; (D) The in-frame fusion of *roGFP* to *bpfA* through homologous recombination.

5.2.4. Planktonic growth and biofilm formation assay

The planktonic growth kinetics for the WT and BpfA-roGFP was conducted using MM1 medium with 20 mM lactate. About 40 μ L of the overnight LB cultures ($OD_{600} \sim 1.0$) was mixed with 200 μ L of MM1 medium in each well of a polystyrene black flat bottom 96-well plate (Costar, Corning Inc) and incubated at 30°C. The OD_{600} was recorded continuously for 24 h in a microplate reader (TECAN M200, M200, Männedorf, Switzerland). Biofilm formation assay was conducted as reported elsewhere (Ding et al. 2014a; Merritt et al. 2005). About 40 μ L of the overnight LB cultures ($OD_{600} \sim 1.0$) suspended in MM1 (200 μ L) was incubated at 30°C for 24 or 48 h. The planktonic cells were then discarded and the cells attached to the wells were washed with 0.9% NaCl. The cells were then stained by adding 100 μ L of crystal violet (CV, 1% aqueous solution). After removing excess CV from each well, attached cells were air dried and resuspended in 100 μ L of 96% ethanol. The biofilm biomass was quantified by measuring OD_{590} (Ding et al. 2014a; Merritt et al. 2005).

5.2.5. Fluorescence spectrum of *S. oneidensis* BpfA-roGFP

The *S. oneidensis* BpfA-roGFP cells grown aerobically in LB broth at 30°C were harvested and washed twice with PBS buffer (1 $\times$, pH 7.4) and resuspended in the same buffer to a final OD_{600} of ~ 0.6 . Cell suspensions (900 μ L each) were treated with 10 mM H_2O_2 or dithiothreitol (DTT) and the fluorescence excitation spectra (emission at 530 nm) were obtained by scanning excitation wavelength from 350 to 500 nm with an interval of 5 nm. Each measurement was conducted in triplicates.

5.2.6. Response of BpfA-roGFP to chemically induced oxidation

Cell suspensions prepared as mentioned in section 5.2.5 were treated with H₂O₂ (10mM – 10 µM), AgNO₃ and K₂SeO₃ at different concentrations (50 ppm, 25 ppm, 10 ppm, 5 ppm and 1 ppm) in the wells of black flat bottom 96-well plates (Costar, Corning Inc). The fluorescence (emission at 530 nm) was recorded in relative fluorescence units (RFU) with an excitation wavelength of 390 nm and 460 nm for 2 h with an interval of 5 min. The fluorescence intensity ratio (390/460 nm) was determined. Cells with no chemical treatment were used as the control.

5.2.7. Proteinase treatment

BpfA-roGFP cells from overnight LB cultures were washed twice and then resuspended in PBS buffer to an OD₆₀₀ of 0.6. Aliquots of the cell suspension were centrifuged at 12,000 × g for 10 min. Each cell pellet resuspended in 200 µL proteinase K (1 mg/mL in 0.05 M Tris, pH 7.5) and incubated at 30°C for 2 h (Cheung and Fischetti 1988). After treatment, cells were pelleted and then resuspended in PBS buffer with 10 mM H₂O₂ and transferred to the wells of black flat bottom 96-well plates (Costar, Corning Inc). The fluorescence intensity (emission at 535 nm) with excitation at 390 nm and 460 nm were recorded in a microplate reader (TECAN M200, Männedorf, Switzerland) for 2 h. The experiments were carried out in triplicates.

5.2.8. Super resolution imaging

Preparation of cells for super resolution microscopy was performed by inoculating 200 µL overnight BpfA-roGFP culture into each well of an 8-well µ-slide (µ-Slide 8 well, ibiTreat, Ibidi, Martinsried, Germany). After 2 h attachment,

planktonic cells were removed and replaced by either 200 μ L 10 mM H₂O₂ or PBS buffer (control) and incubated for 1 h. Imaging was carried out using a super resolution microscope (ZEISS Super Resolution ELYRA PS.1, Oberkochen, Germany) with an excitation/emission at 405/510 nm.

5.2.9. Biofilm growth

Biofilms of the MR-1 WT and BpfA-roGFP were grown at room temperature in three-channel flow cells (BioCentrum-DTU, Denmark). Diluted overnight LB cultures (OD₆₀₀ of ~0.2) were inoculated into the flow cells. Upon inoculation, medium flow was stopped and the flow cells were inverted for 2 h to facilitate cell attachment on the glass slides (Sivakumar et al. 2014). The media was supplied at a constant flow rate of 6 mL/h using a peristaltic pump. The biofilms were grown for 3 days and then treated with the same media containing 5 mM H₂O₂ for 1 h. Biofilms without H₂O₂ treatment were used as control.

5.2.10. CLSM image acquisition and processing

A Carl Zeiss Confocal Laser Scanning Microscopy (CLSM) model LSM 780 equipped with an Axio observer inverted microscope was used to image the flow cell biofilms. The CLSM images of the biofilms were obtained with an emission at 530 nm and excitation at 405 nm and 488 nm. The images were processed using ZEN, (ZEISS 2012, Oberkochen, Germany) software (Ding et al. 2014a; Sivakumar et al. 2014). ImageJ (Version 1.46r, National Institute of Health, USA) was used to quantify horizontal plane-averaged fluorescence intensity.

5.2.11. Quantitative analysis of BpfA-roGFP expressed in biofilm matrix

The mature biofilms of the WT and BpfA-roGFP were initially treated with 5 mM H₂O₂ for 1 h and then stained with nucleic acid specific red fluorescent dye Syto 59 (Invitrogen, Singapore). The CLSM images were obtained with excitations at 405 nm and 488 nm for roGFP and at 561 nm for Syto 59 and emission at 530 nm. The images were analyzed using IMARIS software (version 7.6.4, Bitplane, Zurich, Switzerland) (Ding et al. 2014a; Lee et al. 2013). The redox status at different depth in the biofilms exposed to H₂O₂ was indicated by the fluorescence excitation ratio (405/488 nm) quantified through imaging analyses.

5.3. Results and Discussion

5.3.1. Genetic fusion of roGFP onto BpfA does not affect biofilm formation

Through a homologous recombination approach, we constructed a fusion strain BpfA-roGFP with genetic fusion of roGFP onto BpfA at the C-terminus (Figure 5.1). Although the reporter strain BpfA-roGFP exhibited a longer lag phase than the WT, both BpfA-roGFP and the WT have comparable specific growth rate ($\sim 0.14 \pm 0.01$ /h) (Figure 5.2 (A)). Hence, the genetic fusion has only minimal effects on cell growth in planktonic cultures. Previous studies have implicated an important role of BpfA in biofilm formation (Cao et al. 2011b; Theunissen et al. 2010b). BpfA is a large protein (~ 285 kDa) and roGFP (~ 27 kDa) is only about 1/10th of BpfA. Hence, the fusion of roGFP into BpfA may not cause a significant change in the folded structure and function of BpfA. To confirm it, we quantified biofilm formation capability of BpfA-roGFP cells and the results suggested that it exhibited a comparable biofilm formation capability with

the WT (Figure 5.2 (B)). After an incubation of 24 h, the biofilm biomass in terms of OD₅₉₀ exhibited by WT and BpfA-roGFP were: 0.63 ± 0.03 (WT) vs. 0.58 ± 0.03 (BpfA-roGFP) and after 48 h, 0.75 ± 0.06 (WT) vs. 0.82 ± 0.03 (BpfA-roGFP), respectively (Figure 5.2 (B)). These results confirmed that the fusion of roGFP with BpfA has marginal effect on cell growth and no negative impact on biofilm formation, which underlies potential biotechnological applications of this genetic fusion approach.

Figure 5.2. (A) Growth kinetics of *S. oneidensis* MR-1 WT and the roGFP reporter strain BpfA-roGFP in MM1 medium with 20 mM lactate; (B) Quantitative analysis of the biofilm formation by *S. oneidensis* MR-1 WT and BpfA-roGFP based on static biofilm assay (96-well plate method) in MM1 medium with 20 mM lactate (* $p \leq 0.05$). Results are presented as mean $\pm$ standard error (n=6).

5.3.2. Oxidation and reduction induce fluorescence of BpfA-roGFP

Previous studies have reported that the addition of H₂O₂ or DTT results in the oxidation or reduction of roGFP, emitting fluorescence signals (Arias-Barreiro et al. 2010; Cannon and Remington 2006). Figure 5.3 (A) shows the fluorescence spectral response of the BpfA-roGFP planktonic cells to H₂O₂ and DTT.

Treatment of BpfA-roGFP cells with 10 mM H₂O₂ or DTT resulted in two distinct

peaks corresponding to the oxidized (excitation at 390 nm) and reduced state (excitation at 460 nm), respectively (Figure 5.3 (A)). These results demonstrated a characteristic spectral response of roGFP in the BpfA-roGFP to oxidative and reductive agents. The fluorescence intensity ratio at 390 nm to 460 nm is associated with the redox equilibrium of the local environments, where the roGFP is expressed. Intriguingly, both peaks at 390 and 460 nm were observed at a comparable intensity for cells not treated with H₂O₂ or DTT (Figure 5.3 (A)), which could have resulted from a redox equilibrium controlled by surface redox potential of the respiring cells.

With the addition of H₂O₂ or DTT, the fluorescence signal of the BpfA-roGFP cells could be observed within several mins and the signal intensity increased over time and eventually reached a stable fluorescence signal. To determine an optimal exposure time, the cells exposed to 10 mM H₂O₂ or DTT were excited at 390 and 460 nm and the ratio of fluorescence intensity at two excitation wavelengths (390/460 nm) were monitored for 2 h (Figure 5.3 (B)). The fluorescence intensity ratio (390/460 nm) gradually increased from 1.150 ± 0.014 and reached a constant of 1.44 ± 0.02 after 1 h of H₂O₂ treatment. Similarly, with DTT treatment, the fluorescence ratio decreased from 0.930 ± 0.002 and stabilized at 0.77 ± 0.01 after 1 h (Figure 5.3 (B)). The ratio of fluorescence intensity from excitation at 390 and 460 nm indicates the extent of roGFP oxidation/reduction and hence, can be used to report the redox status.

Figure 5.3. (A) Spectral response of *S. oneidensis* BpfA-roGFP cells in the presence of H₂O₂ and DTT. Results are presented as mean $\pm$ standard error (n=3); (B) The ratiometric kinetic analysis of BpfA-roGFP cells on exposure to 10 mM H₂O₂ and DTT respectively. Results are presented as mean $\pm$ standard error (n=3).

To further confirm whether the BpfA-roGFP cells could report the redox changes imparted by environmental contaminants, the characteristic fluorescence signal of the BpfA-roGFP cells were monitored upon exposure to oxidation inducing chemicals such as heavy metal(oids) Ag⁺ and SeO₃²⁻. The

spectral pattern similar to H₂O₂ exposure was observed within 10 mins upon exposure to different concentrations of Ag⁺ and SeO₃²⁻ and respective fluorescence intensity ratio is illustrated in Figure 5.4. Compared with the control, the treated BpfA-roGFP cells exhibited a higher fluorescence intensity ratio (390/460 nm) (Figure 5.4), clearly confirming that the BpfA-roGFP could be used to monitor the extracellular redox status triggered by environmental contaminants.

Figure 5.4. Response of extracellular roGFP fluorescence in BpfA-roGFP planktonic cells to oxidation induced by the presence of H₂O₂, AgNO₃ and K₂SeO₃ at different concentrations. Results are presented as mean ± standard error (n=3).

5.3.3. BpfA-roGFP is expressed on cell surface

To further elucidate whether the recombinant protein BpfA-roGFP is expressed on cell surface, the BpfA-roGFP cells were treated with proteinase K, an enzyme that digests the surface proteins (Cheung and Fischetti 1988). The proteinase-treated cells were exposed to 10 mM H₂O₂ for 1 h to examine the oxidation-induced fluorescence of roGFP. Compared with the control, the cells treated with proteinase K exhibited much less fluorescence when excited at 390 nm and 460 nm (Figure 5.5 (B)). After 1 h of incubation, the fluorescence

intensity of the treated cells was only about 55% of that of the control. The treated cells also showed a decreased ratio of fluorescence intensity (390/460 nm) (Figure 5.5 (C))). Upon 1 h exposure to H₂O₂, the fluorescence intensity ratio (390/460 nm) of the control and treated cells was 1.650 ± 0.001 and 1.420 ± 0.007 , respectively. The results imply that the roGFP expressed in the BpfA-roGFP cells is displayed at the exterior of the cells and are accessible to proteinase K.

We further used super-resolution structured illumination microscopy (SR-SIM) to directly examine the localization of BpfA-roGFP. The BpfA-roGFP cells were treated with 10 mM H₂O₂ and imaged under SR-SIM (Figure 5.5 (A)). The cells display a characteristic fluorescence at 405 nm excitation, induced by the oxidation of roGFP by H₂O₂. The SR-SIM image clearly shows that the roGFP is localized on the cell surface, which is consistent with the proteinase treatment results.

Figure 5.5. Surface display of roGFP in BpfA-roGFP. Cells with no proteinase treatment are described as the control here. (A) Localization of BpfA-roGFP by SR-SIM in the reporter strain MR-1 BpfA-roGFP. The fluorescence (green) observed at 405 nm excitation is induced by the oxidation of roGFP fused to BpfA secreted to the cell surface upon exposure to 10 mM H_2O_2 for 1 h; (B) The fluorescence excitation intensity at 390 and 460 nm normalized with the control for the BpfA-roGFP cells treated with proteinase K; (C) Fluorescence excitation intensity ratio (390/460 nm) of BpfA-roGFP cells treated with proteinase K and control. Results are presented as mean $\pm$ standard error ($n=3$).

5.3.4. Monitoring redox status in biofilm matrix of BpfA-roGFP biofilms

Using the established correlation between H_2O_2 concentration and the fluorescence intensity of roGFP in BpfA-roGFP, we attempted to use this reporter strain to quantify the extracellular redox state in biofilm matrix. Biofilms of WT and BpfA-roGFP were grown in multi-channel flow cells under hydrodynamic

conditions. After 3 days, the biofilms were exposed to 5 mM H₂O₂ for 1 h. Figure 5.6 (A) illustrates the oxidation-induced fluorescence of roGFP. The exposure to H₂O₂ induced the oxidation of roGFP in the BpfA-roGFP biofilms, resulting in the generation of its characteristic fluorescence (Ex 405 nm). The negligible fluorescence intensity in BpfA-roGFP biofilms without H₂O₂, as shown in Figure 5.6 (A) and (B), confirmed that the fluorescence of the biofilms was caused by the oxidation of roGFP by H₂O₂.

Figure 5.6. (A) CLSM images of *S. oneidensis* biofilms illustrating the redox events; Top panel show the WT biofilms, control (left hand side) and exposed to 5 mM H₂O₂ for 1 hour (right hand side); Bottom panel show the reporter strain BpfA-roGFP biofilms, control (left hand side) and treated with 5 mM H₂O₂ for 1 hour (right hand side); (B) Display of roGFP in *S. oneidensis* MR-1 BpfA-roGFP biofilm matrix. The top panel illustrates the MR-1 BpfA-roGFP biofilms with no H₂O₂ treatment, whereas the bottom panel shows the BpfA-oxidized roGFP channels in BpfA-roGFP biofilms exposed to 5 mM H₂O₂ for 1 h.

To further evaluate the distribution of BpfA-roGFP in biofilm matrix, the WT and the BpfA-roGFP biofilms were stained with nucleic acid specific red fluorescent dye Syto 59, after exposing the biofilms to 5 mM H₂O₂ for 1 h (Figure 5.7 (A)). For the WT biofilms, only cells (red) could be observed. In contrast, the cells (red) and the oxidized BpfA-roGFP sites (green) were distinctively visualized in BpfA-roGFP biofilms (Figure 5.7 (A)). After 72 h of growth, the total biofilm biovolume of BpfA-roGFP biofilms were estimated as $2.13 \pm 0.45 \times 10^6 \mu\text{m}^3$, while the biovolume for the oxidized roGFP sites were $6.61 \pm 0.11 \times 10^5 \mu\text{m}^3$. The quantitative analysis of the CLSM images indicated that the oxidized roGFP sites contributed to about $31.92 \pm 2.16\%$ of the overall biofilm biovolume (Figure 5.7 (B)).

Figure 5.7. (A) Localization of BpfA-roGFP on the biofilm matrix. The green channels in the bottom panels correspond to the matrix localized oxidized roGFP sites in BpfA-roGFP biofilms, upon exposure to 5 mM H₂O₂ for 1 hour. The cells in the biofilm, stained with 5 μ M red fluorescent nucleic acid stain SYTO 59, can be seen in red colour. As a control, the MR-1 WT biofilms lacking roGFP, is shown in the top panel, in which only the red colour indicating the cells in biofilm can be seen; (B) BpfA-roGFP biofilm biovolume showing the volumetric distribution of oxidized roGFP sites.

The green fluorescence of the extracellular roGFP in the BpfA-roGFP biofilms allows the quantification of the extracellular redox status in biofilm matrix with spatial resolution. The fluorescence intensity ratio (405/488 nm) obtained from each CLSM image obtained at different depth of the biofilms indicates the extracellular redox status at a specific depth in the biofilm matrix. The fluorescence intensity ratio (405/488 nm) varied at different biofilm depth (Figure 5.8). The depth-resolved spatial redox profile of the biofilm matrix suggests an increasing extracellular oxidation state from the biofilm-liquid interface till $\sim 15 \mu\text{m}$ below the interface and followed by a steady decline towards the bottom. A similar depth resolved profile was also observed for Cr(III) in the top layers of *S. oneidensis* MR-1 biofilm, where the local concentration of Cr(III) reached as high

as 2.5 mM, when the biofilm was only exposed to 0.2 mM chromate (Cao et al. 2012). The depth resolved extracellular redox status within the biofilm matrix profiled by using BpfA-roGFP provides important experimental data facilitating our understanding of physiological impacts of oxidative contaminants on cells in biofilms.

Figure 5.8. Depth resolved extracellular redox status in the *S. oneidensis* BpfA-roGFP biofilm is illustrated in terms of the ratiometric fluorescence change (405/488 nm RFU/RFU) with the biofilm depth.

In summary, by using *S. oneidensis* as a model organism, we demonstrated a novel approach to quantify extracellular redox status in biofilm microenvironments. We displayed a redox sensitive fluorescent protein roGFP onto the cell surface of *S. oneidensis* by fusing it to the C-terminal of BpfA, a large surface protein. The fusion has marginal impact on cell growth and no negative effect on biofilm formation capability. *S. oneidensis* BpfA-roGFP cells exhibit a characteristic fluorescence of roGFP with an increased fluorescence

intensity ratio (390/460 nm) upon exposure to oxidation inducing chemicals such as H_2O_2 , Ag^+ and SeO_3^{2-} . Using H_2O_2 as a model oxidizing agent, we further demonstrated the use of BpfA-roGFP for monitoring extracellular redox status in biofilm matrix with a precise spatial resolution. This study provides a novel approach to non-invasively quantify extracellular redox status in microenvironments within biofilms, which can be used to understand redox responses of biofilms to environmental perturbations.

CHAPTER 6: BIOFILM MATRIX DISPLAY OF Man5C FOR BIOFILM-BASED EXTRACELLULAR HYDROLYSIS OF MANNAN SUBSTRATES

This chapter has been presented in oral format as Sivakumar K and Cao B. (2015).

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6.1. Introduction

Hemicellulose is a major constituent of plant cell wall polysaccharides, which accounts for approximately one third of the cellulosic dry biomass, by weight (Chauhan et al. 2012; Lüthi et al. 1991). Hemicellulose is composed of hexose and pentose sugars linked together in a branched structure. The branched structure imparts an amorphous form that is highly receptive to enzymatic hydrolysis (Lüthi et al. 1991).

Hemicellulases cause the hydrolysis of hemicelluloses by cleaving the glycosidic bonds of the sugar backbone chain, releasing monomeric sugars (Chauhan et al. 2012; Dhawan and Kaur 2007). Hemicellulases have emerged as a key biotechnologically important enzyme for biofuel synthesis (Chauhan et al. 2012; Dhawan and Kaur 2007; Ghose and Bisaria 1987; Hogg et al. 2003).

β -1,3-glucomannanases are hemicellulases that hydrolyze the internal glycosidic bond, which links the linear mannose chain backbone of hemicelluloses such as mannan, glucomannan and galactomannan (Hogg et al. 2003). β -1,3-glucomannanases are grouped under glycoside hydrolase families (GHs) 5 and 26 on the basis of their amino acid sequences (Chauhan et al. 2012; Hogg et al. 2003).

Isolated mannanes might be sensitive to operational conditions, which might inhibit pure enzyme-based hydrolysis (Zhao et al. 2002). Further whole-cell-based hydrolysis are restricted only to batch and fed batch systems (Rosche et al. 2009). The diffusion limitations in biofilms hinder substrate accessibility for the immobilized mannanase and hence, restrict the continuous hydrolysis of mannan substrates (Branda et al. 2005; Halan et al. 2012b; Sutherland 2001b).

The objective of the study was to functionalize the biofilm matrix with the display of Man5C, through the genetic fusion of Man5C to the C-terminus of BpfA for the extracellular hydrolysis of mannan substrates. Bacterial biofilm matrix generally has an EPS composed of cellulose backbone (Vu et al. 2009). Hence, the β -1,4 Man5C which is specific to hemicellulose-based polysaccharides will not cause the hydrolysis of inherent biofilm matrix polysaccharides (Arai et al. 2010; Gibbs et al. 1992; Hogg et al. 2003; Sunna 2010; Tunnicliffe et al. 2005).

Specifically we used *S. oneidensis* as a model organism and glucomannan as a model mannan substrate. To test the hypothesis, we genetically fused Man5C (~36 kDa) onto the C-terminus of BpfA (~285 kDa) (Cao et al. 2011a; Cao et al. 2011b; Theunissen et al. 2010a). We further used the fusion strain to develop biofilms in fluidized bed biofilm reactors and illustrated the depth resolved Man5C activity in the extracellular microenvironments of the biofilms. The fluidized bed biofilm reactors (FBBR) are considered superior in processes involving high mass transfer rate of substrate and the catalyst (Begum and Radha 2013; Nikolov and Karamanev 1987; van Ommen et al. 2012). The fluidized suspended solid media offer a larger contact surface for biofilm formation hence, effectively increasing the adhesion of cells and overall biofilm development (Nikolov and Karamanev 1987).

6.2. Material and Methods

6.2.1. Microorganisms and growth conditions

The bacterial strains, plasmids and primers used in the current study are shown in Table 6.1. The growth media and culture incubation conditions for bacterial growth in both planktonic and biofilm mode were described in detail previously (Ding et al. 2014a; Sivakumar et al. 2015). Unless otherwise stated, the bacterial cultures in planktonic growth mode were incubated under aerobic conditions in a rotary shaker (200 rpm) at 30°C in LB medium or highly defined modified M1 minimal medium (MM1) with 20 mM lactate as the carbon source. The composition of the MM1 medium was described in detail in Section 3.2.1 (Chapter 3) (Ding et al. 2014a; Ng et al. 2013; Sivakumar et al. 2015). For biofilm growth, diluted LB medium (10 × dilution) was used (Zhang et al. 2014).

Table 6.1. List of bacteria strains, plasmids and primers used in the study

Strains	Description	Reference
<i>S. oneidensis</i> MR-1		
WT	Manganese reducing strain	(Nealson and Myers 1992)
BpfA-Man5C	Fusion strain with Man5C fused to BpfA	This study
<i>E. coli</i>		
DH5α	Host for Man5C expression vector	(Tolia and Joshua-Tor 2006)
Plasmids	Description	Reference
pUC57-bpfA-Man5C-T5-APH-loxP-H6-aggC	Vector used for the in-frame fusion of Man5C to BpfA	This study
Primers	Sequence	Reference
<i>bpfA</i> -F1- <i>Eco</i> RI	AAAGAATTCCCAGTAATCAGGATACAGC GCAAGGC	(Sivakumar et al. 2015)
<i>bpfA</i> -RI- <i>Sma</i> I	AAACCCGGGTACAGGATCGACTGTTTTT AAGGGATC	(Sivakumar et al. 2015)
<i>man5C</i> -F1	ATATCGCTAATTCTTTTTCTGCCTCTAAC TTGTACTAC	This study
<i>man5C</i> -R1	ACTAGCAACAACCACTTAGAAAAATCAAA AGCAGCTTGAGCC	This study
<i>bpfA</i> -F2	ACGGTAGGCCAAAGATGGCACTG	(Sivakumar et al. 2015)
<i>man5C</i> -F2	TACTTTAAGCAAAGAATTGCTCATG	This study
<i>man5C</i> -R2	CACCACCAGTAGAAACCAAAATA	This study
<i>aggC</i> -R	ACTGCACGGTCAGATTCGAGGTCAA	(Sivakumar et al. 2015)

6.2.2. Construction of the vector expressing Man5C

The Man5C expressing strain was created essentially as described previously (Sivakumar et al. 2015). The Man5C expression vector pUC57-Man5C-T5-APH-loxP-H6-aggC contains a DNA cassette that encodes the β -1,3-glucomannanases, Man5C (*man5C*); codon optimized kanamycin resistant aminoglycoside phosphotransferase Aph (*aph*) of *Escherichia coli*, and the 305 bp *aggC* gene fragment derived from *S. oneidensis* MR-1 chromosome DNA

(Bagdasarian et al. 1983; Heidelberg et al. 2002). The Man5C-coding sequence (Figure 6.1 (A)) was synthesized according to the codon preference of *Pichia pastoris* based on the protein sequence of *Penicillium lilacinum* ATCC 36010 (Yang et al. 2011). The synthetic Man5C coding sequence in pUC57 was amplified by polymerase chain reaction (PCR) by using *man5C*-F1 and *man5C*-R1 as primers (sequence listed in Table 6.1) and digested with *EcoRV* and *SpeI* and sub-cloned in pUC57-roGFP-APH-loxP-H6-aggc (Figure A-1) at the same site to construct pUC57-Man5C-APH-loxP-H6-aggc, which was inserted with a 986 bp *bpfA* fragment to create pUC57-bpfA-Man5C-T5-APH-loxP-H6-aggc (Figure 6.1 (B)) (Sivakumar et al. 2015). The plasmid map of the Man5C expression vector is shown in Figure A-2.

6.2.3. Construction of the Man5C fusion strain *S. oneidensis* BpfA-Man5C

S. oneidensis strain expressing BpfA-Man5C was made as described (Sivakumar et al. 2015). pUC57-bpfA-Man5C-T5-APH-loxP-H6-aggc (Figure 6.1 (B)) was digested with *EcoRI* and *HindIII* and the fragment containing the Man5C sequence was gel-purified, concentrated and transformed to *S. oneidensis* MR-1 by electroporation (Figure 6.1 (C)). The positive transformants (BpfA-Man5C colonies) were selected from LB agar plates containing 20 µg/L of Kanamycin. Positive colonies were confirmed through colony polymerase chain reaction and through the sequencing of PCR products using the Capillary Sequencing facility (AITbiotech Pte Ltd). For colony PCR, the forward primer (*bpfA*-F2) was designed from the *bpfA* region and the reverse primer (*man5C*-2) was designed from the *man5C* region. The nucleotide sequence of the PCR product was checked for similarity using the National Centre for Biotechnology Information Blast online

program (NCBI Blast, www.ncbi.nlm.nih.gov/Blast) (Sivakumar et al. 2015). The steps involved in the construction of BpfA-Man5C are illustrated in Figure 6.1.

Figure 6.1. The steps involved in the construction of the Man5C fusion strain *S. oneidenis* MR-1 BpfA-Man5C are illustrated here. (A) The *man5C* gene fragment derived from the *Penicillium lilacinum* ATCC 36010; (B) The Man5C expression vector pUC57-bpfA-Man5C-T5-APH-loxP-H6-aggC; (C) The genetic fusion of Man5C to the C-terminal of BpfA through homologous recombination.

6.2.4. Planktonic growth kinetics and biofilm formation assay

MM1 media with 20 mM lactate was used to conduct the growth kinetics and biofilm formation capability assay for WT and BpfA-Man5C strains. Both the assays were conducted as mentioned elsewhere (Ding et al. 2014). To 200 μ L of MM1 media, 40 μ L of the overnight seed culture (in LB broth) was added. The absorbance at 600 nm (OD_{600}) was monitored every 15 min until stationary phase using a microplate reader (Tecan). For the static biofilm assay, about 40 μ L of the overnight seed culture mixed with 200 μ L of the modified MM1 media was incubated at 30°C in polystyrene flat bottom 96-well plate (Thermoscientific). The

biofilm biomass in each well was quantified after 24 and 48 h in terms of OD₅₉₀ after discarding the planktonic cells. The attached cells were washed with 100 µL of 0.9% NaCl and then stained with 100 µL of 1% crystal violet (CV, 1% aqueous solution). After staining, the cells were further washed with 100 µL of 96% ethanol, which was followed by OD₅₉₀ measurement (Ding et al. 2014; Wu et al. 2014).

6.2.5. Substrate for Man5C activity

Konjac Mannan (glucomannan) was used as the Mannan substrate to evaluate the Man5C activity in both planktonic and biofilm mode of bacterial growth (Dhawan and Kaur 2007; Chauhan et al. 2012). A 2% (w/v) stock suspension of glucomannan was prepared in 100 mM Sodium Acetate buffer (pH 5.5) with intermittent heating for about 1 h until boiling followed with cooling at room temperature (Ghose 1987; Lüthi et al. 1991)

6.2.6. Estimation of Man5C activity

The total mannose released from glucomannan was estimated by Dubois assay (Taylor 1995; Masuko et al. 2005). About 1 mL of the cell free supernatants (100 × diluted) was mixed with 1 mL of phenol (4% w/v) and 4 mL of H₂SO₄ (98%) and incubated at room temperature for 10-15 min. The OD₄₉₀ of the reaction mixture was measured (Lüthi et al. 1991; Taylor 1995). From the mannose (mg/mL) concentration, the total mmoles of mannose accumulated was calculated. One Unit (U) of Man5C activity is defined as the amount of Man5C that is required to release 1 µmole of mannose per minute. Further, the specific Man5C activity was defined in terms of total µmoles of mannose released per unit time per cell dry biomass (U/ mg Dry Biomass). The Man5C activity was performed in triplicates.

6.2.7. *Man5C activity of resting cells*

Overnight LB culture of WT and BpfA-Man5C were incubated at 30°C for about 20 h. The cells were harvested by centrifugation (7000 × g for 15 min) and then washed (2 ×) with 100 mM sodium acetate buffer (pH 5.5) and re-suspended in the same buffer to an OD₆₀₀ of approximately 1.0 (Ng et al. 2013; Sivakumar et al. 2014). For the Man5C activity tests, the cell pellets were re-suspended in 100 mL of 100 mM sodium acetate buffer (pH 5.5) containing 0.5% (w/v) of glucomannan. Samples were withdrawn at regular intervals and the total mmoles of mannose released was quantified (Lüthi et al. 1991; Taylor 1995; Masuko et al. 2005). The specific Man5C activity was calculated as mentioned above.

6.2.8. *Man5C activity of recycled whole-cell catalysts*

The resting cell-based enzymatic hydrolysis of glucomannan was conducted in 7 cycles, each cycle lasting for 20 h. For the first cycle, the BpfA-Man5C cell pellets harvested (at OD₆₀₀~1) were subjected to Man5C activity in 100 mM sodium acetate buffer (pH 5.5) containing 0.5% (w/v) of glucomannan in a working volume of 100 mL. After 20 h, the second cycle was initiated by harvesting the BpfA-Man5C cells from the first cycle (7000 × g for 15 min). The cells were recycled by further re-suspending it in 100 mL of 100 mM sodium acetate buffer (pH 5.5), containing 0.5% of glucomannan to an OD₆₀₀ of ~1.0. Similar steps were performed for the subsequent cycles. For every cycle, the total mmoles of mannose released and specific Man5C activity were quantified.

6.2.9. Proteinase treatment

The overnight BpfA-Man5C culture was washed twice with and then resuspended in 0.9%NaCl at an OD₆₀₀ of 1.0. The proteinase treatment assay was conducted, as described in our previous work (Sivakumar et al. 2015). The cell suspension was centrifuged at 12,000 × g for 10 min and the pellets were resuspended in 200 µL proteinase K in several aliquots (1 mg/mL in 0.05 M Tris, pH 7.5) (Cheung and Fischetti 1988). The proteinase treatment was carried out for 1 h at 30°C. The effect of the proteinase treatment was monitored in intervals of 15 min. Every 15 min, a set of aliquots (triplicates) were centrifuged and the proteinase treated BpfA-Man5C cells were tested for Man5C activity, as mentioned above.

6.2.10. Reactor set up for continuous Man5C activity by BpfA-Man5C biofilms and whole-cells

The continuous Man5C activity by BpfA-Man5C planktonic cells were conducted in continuously stirred suspended planktonic reactors (CSSPR) (Figure 6.2 (A)). The biofilm-based Man5C activity was tested using fluidized bed biofilm reactors (FBBR) (Figure 6.2 (B)) filled with low-density polystyrene chips (100 µm × 100 µm × 20 µm) (4 mL inside the reactor) for biofilms to attach and grow. The fluidization conditions were achieved by using a multipoint magnetic stirrer UM-12T (USUN, China) at room temperature (Yong et al. 2014). The magnetic stirring of the polystyrene chips hinders the formation of bubbles within the reactors and impart a uniform mixing of the fluidized media. With the continuous stirring of cylindrical magnetic bars at 500 rpm (optimized after several trials), the polystyrene chips fluidized uniformly and the bed expanded in a homogenous manner hence, providing a non-porous surface for biofilm growth

(van Ommen et al. 2012). A set of reactors with and without chips was also set up to function as abiotic controls. The details of the design parameters used in the reactor are shown in Table 6.2. The reactors were inoculated with diluted overnight LB cultures (OD~0.1) followed by a 2 h phase without any flow to facilitate the attachment of cells to the chips (Wu et al. 2014). MM1 medium with 1/10th LB was then continuously supplied at a flow rate of 5 mL/h to support the biofilm growth (Zhang et al. 2014). After 60 h of biofilm growth, 0.1% glucomannan (w/v) suspended in MM1 medium with 1/10th LB (v/v), was introduced into the reactors. The effluent flow rate from the reactors was also maintained at 5 mL/h hence, harnessing a steady state continuous system. The effluent samples from FBBR & CSSPR were collected to estimate the planktonic biomass concentration (mg/mL dry planktonic biomass). To quantify the biofilm biomass in FBBR, a portion of the polystyrene chips was removed from the reactors. The chips-attached cells were re-suspended in 5 mL 0.9%NaCl that was vigorously shaken for 10-20 min to facilitate the cell detachment from the chips. The CFU/mL of the cell suspension was determined using drop plate method, which was converted to the dry biofilm biomass concentration (mg/mL) in the reactor (Sivakumar et al. 2014; Wu et al. 2014).

Table 6.2. The process design parameters used in CSSPR and FBBR

Reactor parameters		Design values
Working volume		100 mL
Influent flow rate		5 mL/h
Effluent flow rate		5 mL/h
% Flow through volume in reactors		96%
Surface area of the polystyrene chips in FBBR		100 μm $\times$ 100 μm
Hydraulic retention time		20 h

Figure 6.2. (A) Schematic illustration of the BpfA-Man5C planktonic reactor (CSSPR) used to evaluate the Man5C activity; (B) Schematic illustration of the BpfA-Man5C biofilm reactor (FBBR) used to evaluate the Man5C activity. The cross sectional view shows the fluidized bed biofilm attached to the chips.

6.2.11. Imaging of fluidized bed biofilms

A portion of the chips (taken out from the BpfA-Man5C FBBR for biomass estimation) was transferred to each well of an eight-well μ -slide chamber (μ -slide 8 well, ibiTreat, Ibidi, Martinsried, Germany). The chips were then stained with nucleic acid specific Syto 9 (Invitrogen, Carlsbad, CA) (Zhang et al. 2014). The chips were imaged using a Carl Zeiss Confocal Laser Scanning Microscopy (CLSM) model LSM 780 equipped with an Axio observer inverted microscope (Ding et al. 2014a; Ng et al. 2013). The CLSM images of the biofilms were obtained with an emission at 530 nm and excitation at 488 nm. The images were processed using ZEN (ZEISS, 2012, Oberkochen, Germany) software (Zhang et al. 2014). ImageJ (Version 1.46r, National Institute of Health, Bethesda, MD) was used to quantify the horizontal plane-averaged biofilm thickness (Sivakumar et al. 2015). The images were analyzed using IMARIS software to estimate the biofilm biovolume and surface coverage (version 7.6.4, Bitplane, Zurich, Switzerland) (Zhang et al. 2014).

6.2.12. Man5C activity in CSSPR and FBBR

BpfA-Man5C biofilms and planktonic cells were allowed to grow in FBBR and CSSPR respectively for 60 h before 0.1% glucomannan (w/v) was introduced into the reactors. After the addition of glucomannan, the effluent samples were collected at pre-determined time points. The CFU/mL and dry biomass (planktonic and biofilms) were determined. The overall Man5C activity in the reactors was expressed in the following ways: (i) Total mmoles of Mannose released from the Man5C hydrolysis of glucomannan, (ii) Specific Man5C activity (U/mg Dry Biomass). Further, the Man5C activity imparted by planktonic cells is considered to be same in both FBBR and CSSPR. The biofilm-imparted Man5C

activity in FBBR is determined by deducting the planktonic cells facilitated Man5C activity from the total Man5C activity observed in FBBR.

6.3. Results and Discussion

6.3.1. Genetic fusion of Man5C onto BpfA does not affect the biofilm formation capability

The fusion strain BpfA-Man5C was constructed with the genetic fusion of Man5C onto the C-terminus of BpfA through a homologous recombination approach (Figure 6.1 (C)) (Hogg et al. 2003; Sivakumar et al. 2015). The details of the fusion strain construction are illustrated in the Figure 6.1. As compared to the WT, the lag phase for the fusion strain BpfA-Man5C was significantly longer. However no significant difference was observed between the specific growth rate of BpfA-Man5C ($\sim 0.14 \pm 0.01 \text{ h}^{-1}$) and WT ($\sim 0.15 \pm 0.005 \text{ h}^{-1}$) (Figure 6.3 (A)). These results showed that the genetic fusion has no potential impacts on the cell growth. The Man5C fragment ($\sim 36 \text{ kDa}$) used for the genetic fusion is approximately $1/8^{\text{th}}$ of the BpfA (285 kDa). Hence, it is suggested that the genetic fusion of Man5C onto BpfA will have no significant impacts on the function and structure of BpfA.

To validate the hypothesis, the quantitative analysis of the biofilm formation capability of WT and BpfA-Man5C were examined. After 24 h and 48 h, the biofilm biomass in terms of OD_{590} exhibited by WT and BpfA-Man5C were: 0.68 ± 0.04 (WT) vs. 0.71 ± 0.04 (BpfA-Man5C) (24 h) and 0.90 ± 0.03 (WT) vs. 0.97 ± 0.03 (BpfA-Man5C) (48 h), respectively (Figure 6.3 (B)). The results suggest that the biofilm formation capability of BpfA-Man5C is comparable with that of the WT and is not compromised with the genetic fusion of Man5C. Further

from these results, it can be deduced that the genetic fusion has no negative impacts on the folded structure and function of BpfA, which is related to biofilm formation

Figure 6.3. (A) Growth kinetics of *S. oneidensis* MR-1 WT and the Man5C fusion strain BpfA-Man5C in MM1 medium with 20 mM lactate; (B) Quantitative analysis of the biofilm formation by *S. oneidensis* MR-1 WT and BpfA-Man5C based on static biofilm assay (96-well plate method) in MM1 medium with 20 mM lactate (* $p \leq 0.05$). Results are presented as mean $\pm$ standard error (n=6).

6.3.2. BpfA-Man5C planktonic cells hydrolyze glucomannan

Previous studies have reported that Man5C belonging to the family of β -1,3-glucomannanas hydrolyzes glucomannan releasing mannose (Lüthi, et al. 1991; Gibbs, et al. 1992; Hogg et al. 2003; Dhawan and Kaur 2007; Yang et al. 2011). Most of the industrial bio-transformations exploit the power of whole-cell-based planktonic cultures (Leon et al. 1998, Pfruender et al. 2004; Schewe et al. 2009; Sivakumar et al. 2014).

To characterize the fusion strain BpfA-Man5C, we tested the enzymatic hydrolysis of glucomannan under planktonic non-growth based batch culture conditions. The WT was used as a negative control to evaluate the rate of Man5C activity. Figure 6.4 (A) shows the total mmoles of mannose released over time from the hydrolysis of glucomannan. As expected, the BpfA-Man5C cells

hydrolyzed glucomannan significantly generating 36.36 ± 0.35 mmoles of mannose, ~5-folds higher than the WT (7.78 ± 0.58 mmoles of mannose) after 20 h.

Figure 6.4 (B) illustrates the specific Man5C activity (U/mg dry biomass) after 20 h. The Man5C activity exhibited by the BpfA-Man5C and WT whole-cells were: 0.272 ± 0.002 U/mg dry biomass (BpfA-Man5C) and 0.012 ± 0.0002 U/mg dry biomass (WT), significantly higher than the WT. Compared with the WT, the BpfA-Man5C cells exhibited a higher capability to hydrolyze glucomannan. The enhanced rate of hydrolysis underpins better accession of insoluble glucomannan by Man5C, implying the localization of Man5C on the cell exterior. These results clearly suggest that BpfA-Man5C cells could be used for the extracellular hydrolysis of mannan substrates.

Figure 6.4. The hydrolysis of glucomannan by WT and BpfA-Man5C planktonic cells. (A) Total mmoles of mannose released from the Man5C hydrolysis of glucomannan after 20 h; (B) Specific Man5C activity (U/mg dry biomass) displayed by WT and BpfA-Man5C cells after 20 h. Results are presented as mean $\pm$ standard error (n=3).

6.3.3. BpfA-Man5C is expressed on the cell surface

To elucidate the expression of Man5C on the cell surface, the BpfA-Man5C cells were treated with proteinase K, an enzyme that digests the surface proteins (Cheung and Fischetti 1988; Sivakumar et al. 2015). After every 15 min, the proteinase treated BpfA-Man5C cells were tested for glucomannan hydrolysis. With proteinase treatment, there was a notable decrease in the amount of mannose released. With 60 min proteinase treatment, the total amount of

mannose released (20.76 ± 0.78 mmoles of mannose after 20 h) decreased by 2.1-folds, as relative to the control (43.81 ± 1.55 mmoles of mannose after 20 h) (Figure 6.5 (A)). From Figure 6.5 (A), it is evident that the proteinase treatment for 15 and 30 min also lowered the mannose release rate.

The BpfA-Man5C cells with 60, 30 and 15 min proteinase treatment displayed a specific Man5C activity of 0.16 ± 0.006 (U/mg dry biomass) 0.171 ± 0.005 (U/mg dry biomass) and 0.200 ± 0.002 (U/mg dry biomass) respectively, as compared to the control (0.331 ± 0.006 U/mg dry biomass) (Figure 6.5 (B)). These observations suggest that the proteinase treatment of BpfA-Man5C cells had a deleterious effect on its Man5C activity (~2-folds reduction with 1 h treatment). The continuous shredding of Man5C with proteinase treatment leads to a reduction in Man5C activity, which points towards the cell surface localization of Man5C, as it makes BpfA-Man5C more accessible to proteinase degradation. Hence, from these findings it can be deduced that the BpfA-Man5C is displayed on the cell surface, which is in line with our previous study (Sivakumar et al. 2015).

Figure 6.5. Surface display of Man5C in BpfA-Man5C. (A) The total mannose (mmoles) generated by the Man5C hydrolysis of 0.5% glucomannan by proteinase treated BpfA-Man5C cells. Cells with no proteinase treatment are used as the control; (B) Specific Man5C activity (U/mg dry biomass) by the proteinase treated BpfA-Man5C cells. Results are presented as mean $\pm$ standard error (n=3).

6.3.4. Long-term Man5C activity is displayed by the recycled BpfA-Man5C cells

Previous studies have reported that BpfA, the biofilm promoting factor in *S. oneidensis* is predominantly expressed on the cell surface and biofilm matrix (Theunissen et al. 2010; Cao et al. 2011). A significantly higher amount of BpfA has been reported to be associated with the loosely associated EPS of *Shewanella* biofilms (Cao et al. 2011). The decreased Man5C activity with proteinase treatment confirmed the expression of Man5C on the cell surface of BpfA-Man5C cells. However, the surface localization of Man5C in BpfA-Man5C

cells presents the risk of high rate of enzyme loss, when operated in continuous mode.

To elucidate the long-term stability of surface-anchored Man5C, we observed the recyclability of BpfA-Man5C cells in repetitive batch mode glucomannan hydrolysis in multiple cycles. Each cycle lasted for 20-24 h. After each cycle, the cells were harvested and the supernatant was discarded. The subsequent cycle was initiated by re-suspending the harvested cells from the previous cycle in fresh medium with 0.5% glucomannan. Figure 6.6 (A) shows the total mmoles of mannose released from glucomannan in different cycles and Figure 6.6 (B) illustrates the specific Man5C activity. Figure 6.6 (A) and (B) show that the BpfA-Man5C whole-cells could be recycled (~5 times) without substantial loss in Man5C activity. From Figure 6.6 (A), it is clear that there was only a marginal decrease in the mannose released during the first 4 cycles. During the first cycle the BpfA-Man5C cells hydrolyzed glucomannan releasing 17.74 ± 0.62 mmoles of mannose (after 20 h) which decreased to 15.62 ± 0.12 mmoles of mannose during the fifth cycle (~80% decrease) and 8.52 ± 0.46 mmoles in the seventh cycle. Hence, the continuous recycling of BpfA-Man5C cells resulted in ~50% suppressed activity after 7 cycles. As expected, the BpfA-Man5C cells displayed a significantly higher mannose release rate (Figure 6.6 (B)) during the first three cycles (First cycle: 0.121 ± 0.004 U/mg dry biomass; Second cycle: 0.119 ± 0.008 U/mg dry biomass; Third cycle: 0.124 ± 0.004 U/mg dry biomass) followed by gradual decrease for the next cycles (Fourth cycle: 0.101 ± 0.005 U/mg dry biomass; Fifth cycle: 0.106 ± 0.0008 U/mg dry biomass). A steep decline was observed in the final two cycles (Sixth cycle: 0.086 ± 0.001 U/mg dry biomass; Seventh cycle: 0.058 ± 0.003 U/mg dry biomass). The findings suggest that BpfA-Man5C cells could be recycled multiple times without a major loss in

the activity. From these findings, it also becomes apparent that the surface-displayed BpfA-Man5C whole-cell catalysts are highly stable for long-term biotransformations in buffered medium.

Figure 6.6. High recyclability of BpfA-Man5C cells. (A) The total mmoles of mannose released from the hydrolysis of 0.5% glucomannan by BpfA-Man5C cells in seven repetitive cycles; (B) Specific Man5C activity displayed by BpfA-Man5C whole-cells in multiple rounds of glucomannan hydrolysis. Results are presented as mean $\pm$ standard error (n=3).

6.3.5. BpfA-Man5C biofilms exhibit higher Man5C activity relative to planktonic cells

To further elucidate how the Man5C activity is displayed in the self-immobilized biofilm framework, we used BpfA-Man5C biofilms to quantify the extracellular Man5C activity in biofilm matrix. Biofilms of BpfA-Man5C were developed on fluidized bed biofilm reactors (FBBR). The biomass in FBBR is a mix of both planktonic and biofilm fractions. The whole-cell-based Man5C activity in continuous mode was also monitored using continuously stirred suspended planktonic reactors (CSSPR).

After 60 h, the biofilms attached on polystyrene chips in FBBR and suspended cells in CSSPR were continuously exposed to 0.1% glucomannan. The Man5C activity was observed between the planktonic and biofilm fractions respectively after addition of glucomannan. The dry biomass (mg dry biomass) was used as the biomass quantitative indicator for within the FBBR and CSSPR. Figure 6.7 shows the mg dry planktonic biomass in CSSPR and FBBR (estimated from the reactor effluent) and the biofilm biomass in FBBR (after the addition of glucomannan). The results showed that the BpfA-Man5C planktonic biomass in FBBR were significantly lower (~6-7-folds) than their counterparts in CSSPR (Figure 6.7) hence, suggesting a high cell entrapment capacity for the fluidized polystyrene chips. However, there was no significant difference between biofilm and planktonic fractions in FBBR. Further, a marginal decrease in BpfA-Man5C biofilm biomass was observed towards the end, pointing towards potential biofilm dispersal (Figure 6.7).

Figure 6.7. The planktonic and biofilm biomass expressed in terms of mg dry biomass. The biofilm is developed in MM1 media with $1/10^{\text{th}}$ LB and 0.1% glucomannan is introduced after 60 h of biofilm development. Time from 0-60 h indicate biofilm development and after 60 h corresponds to biofilm matrix-enabled Man5C activity. Low planktonic biomass in FBBR indicates high rate of cell entrapment within the FBBR biofilms. Results are presented with mean $\pm$ standard error (n=3).

To compare the Man5C activity between the planktonic and biofilm phase, we estimated the total mmoles of mannose released over time and the results are shown in Figure 6.8 (A). Our results showed that the overall mannose released in FBBR was ~2-folds higher than in CSSPR. After 144 h of Man5C activity, BpfA-Man5C biofilms and planktonic cells in FBBR hydrolyzed glucomannan to release about 89.28 ± 2.33 mmoles of mannose, as compared to the BpfA-Man5C planktonic cells in CSSPR (46.82 ± 1.66 mmoles of mannose). Further ~39% of the total mannose delivered to the reactor was recovered through Man5C hydrolysis in FBBR, while ~20% could be recovered in CSSPR.

The BpfA-Man5C FBBR-based biofilms also displayed a much higher specific Man5C activity as shown in Figure 6.8 (B). At 36 h, the BpfA-Man5C biofilms displayed ~5-folds higher specific Man5C activity of 0.019 ± 0.001 U/mg dry biomass (Figure 6.8 (B)) than its planktonic counterparts (0.0033 ± 0.0002 U/mg dry biomass). The specific Man5C activity by BpfA-Man5C FBBR biofilms increased by 7-folds after 72 h and further by 8-folds after 108 and 144 h, as shown in Figure 6.8 (B). The substantial amount of planktonic biomass in CSSPR (Figure 6.7) could be an impeding factor for its significantly decreased specific Man5C activity. Biomass accumulation within CSSPR significantly limits the diffusion of glucomannan, which could be a major factor accounting for the suppressed activity.

Further, we also confirmed that the biofilms contributed to approximately 54% Man5C activity in FBBR till 72 h, which decreased to about 40% during 108 and 144 h (Figure 6.8 (C)). The high initial Man5C activity by the BpfA-Man5C FBBR biofilms can be attributed to the high rate of cell retention within the FBBR. The cell dispersal from the FBBR biofilms contributes to the decrease in Man5C activity. Due to the fluidized conditions prevailing in FBBR, there might be a continuous exchange of cells between the planktonic and biofilm phase. The fluidized conditions also make the FBBR attached cells liable for easy disassembly. Hence, the Man5C activity in FBBR is mediated together by both BpfA-Man5C biofilms and planktonic cells.

Figure 6.8. BpfA-Man5C FBBR biofilms exhibit very high Man5C activity. (A) Total mmoles of mannose released by BpfA-Man5C biofilms (FBBR) and suspended planktonic cells (CSSPR); (B) Specific Man5C activity; (C) %Man5C activity exhibited by BpfA-Man5C biofilms and planktonic fraction in FBBR. Results are presented as mean $\pm$ standard error (n=3).

6.3.6. Resolving the optimum biofilm thickness for biofilm matrix-enabled *Man5C* activity

The BpfA-*Man5C* biofilms developed on the polystyrene chips were imaged using CSLM every 36 h, after the introduction of glucomannan. The biofilms were relatively unstructured and distributed in the form of cell aggregates around the chips, as shown in Figure 6.9 (A). The confocal images showed the proliferation of cells around the periphery of the chips, a trend maintained throughout the cycle (Figure 6.9 (A)), suggesting a very thin layer of biofilms.

Figure 6.9 (B) shows the results of the quantitative analysis (biofilm biovolume) for the FBBR biofilms. The biofilm biovolume increased after the addition of glucomannan (0h: $4.63 \times 10^6 \pm 1.76 \times 10^6 \mu\text{m}^3$; 36 h: $1.54 \times 10^7 \pm 6.24 \times 10^5 \mu\text{m}^3$ and 72 h: $1.35 \times 10^7 \pm 1.75 \times 10^6 \mu\text{m}^3$). Further a slight decrease in biovolume was observed during 108 h ($1.19 \times 10^7 \pm 7.68 \times 10^5 \mu\text{m}^3$) and 144 h ($1.10 \times 10^7 \pm 2.10 \times 10^5 \mu\text{m}^3$) respectively. The decrease in biofilm biovolume towards the end indicates chances of cell dispersal from the FBBR biofilms.

Figure 6.9. (A) CLSM images of *S. oneidenis* BpfA-Man5C FBBR biofilms analyzed during 0, 36, 72, 108 and 144 h; (B) Quantitative analysis (Biofilm biovolume) of the *S. oneidenis* BpfA-Man5C FBBR biofilms. Results are presented as mean $\pm$ standard error (n=3).

Biofilm thickness is a critical factor for biofilm-based bioprocesses (Ding et al. 2014a). Increase in biofilm thickness induces mass transfer related issues and

significant decrease in biofilm thickness affects the overall biofilm stability and architecture. To further elucidate how the biofilm thickness regulates the extracellular enzymatic hydrolysis of glucomannan, the biofilm-mediated specific Man5C activity is resolved against biofilm thickness, as illustrated in Figure 6.10.

Prior to glucomannan addition, we observed a thickness of 38.14 ± 9.29 μm for BpfA-Man5C biofilms (Figure 6.10). After 36 h, a 1.5-fold increase in biofilm thickness (47.56 ± 5.41 μm) was observed followed with a remarkable decrease (~ 2.4 -folds) in biofilm thickness at 72 h (24.30 ± 4.33 μm). Marginal change in biofilm thickness was observed during 108 h (23.14 ± 2.25 μm) and 144 h (19.41 ± 2.20 μm) (Figure 6.10). Intriguingly, the decrease in biofilm thickness between 36 h and 72 h induced a much higher activity for the BpfA-Man5C biofilms, as the biofilm-based Man5C activity increased from 0.01 ± 0.0008 U/mg dry biomass (36 h) to 0.038 ± 0.002 U/mg dry biomass (72 h) and 0.045 ± 0.005 U/mg dry biomass (108 h). The drop in biofilm thickness alleviated the substrate diffusion within the biofilm matrix, which effectively increased the matrix-enabled Man5C activity.

Figure 6.10. The biofilm-based extracellular Man5C activity is resolved against the biofilm thickness. Bar-chart indicates the BpfA-Man5C biofilm matrix-enabled extracellular Man5C activity and the scatter smooth line-marker plot shows the biofilm thickness variation over time after the introduction of glucomannan. Results are presented as mean $\pm$ standard error (n=3).

The biofilm thickness is often associated with the intrinsic heterogeneous and dynamic properties of biofilm (Ding et al. 2014). In the case of FBBR biofilms, the biofilm thickness is highly dependent on mixing, fluidization and the bed expansion characteristics (Nikolov and Karamanev 1987; van Ommen et al. 2012). Further, with respect to the biofilm biomass data ((Figure 6.9 (B) and Figure 6.7), the cell dispersal from the chips is minimal.

The maximum amount of mannose released was detected between 36 h & 72 h at a biofilm thickness of about 25 μ m (Figure 6.10). The fluidization conditions and aberration between the chips might induce the delocalization of cells between biofilm and planktonic phase, which could be a major factor

responsible for the drop in biofilm thickness. The observations indicate that the drop in biofilm thickness did not hinder the extracellular Man5C activity. The drop in biofilm thickness alleviates the mass transfer limitations associated with diffusion of glucomannan. The matrix localization of BpfA-Man5C further increases the substrate accessibility. These observations imply that a significantly enhanced Man5C activity could be harnessed from the BpfA-Man5C biofilm matrix, irrespective of the biofilm thickness. The specific Man5C activity plot (Figure 6.8 (B) and Figure 6.10) indicates that the Man5C activity maintains an increasing trend between 36 and 72 h and attains a plateau between 72 and 108 h, suggesting that the extracellular enzyme activity might be optimum between 36 and 108 h. From Figure 6.10, it is also clear that the drop in biofilm thickness is not an impeding factor for sustained enzyme activity.

In summary, using a homologous recombination approach, we constructed a fusion strain *S. oneidensis* BpfA-Man5C, in which the β -1,3-glucomannanase Man5C is genetically fused to the C-terminal of the biofilm-promoting factor BpfA. We demonstrated that the BpfA-Man5C is expressed on the cell surface and we also showed that the BpfA-Man5C whole-cells displayed the characteristic Man5C activity by hydrolyzing the mannan substrate glucomannan releasing mannose in non-growth mode. The enzymatic activity is stable over long-term, as evidenced by the application of recycled BpfA-Man5C whole-cells in repetitive enzymatic cycles as well as the prolonged activity in biofilms. The matrix-enabled Man5C activity resolved against the biofilm thickness of BpfA-Man5C biofilms advance the fundamental understanding on regulating the factors required to optimize the enzyme activity. This study provides novel implications to monitor the extracellular enzymatic hydrolysis within the microenvironments of biofilms.

7.1. Summary and Conclusions

The study focussed on engineering biofilm matrix to display catalytic enzymes on the matrix for mediating extracellular biocatalytic processes. To improve extracellular bioactivity, membrane intercalating conjugated oligoelectrolyte DSSN+ and intercellular-based mutualistic interactions were deployed.

Membrane spanning conjugated oligoelectrolyte DSSN+ permeabilize the cell membrane and release flavins and intracellular enzymes into the biofilm matrix without compromising cell viability and biofilm activity. Our findings (Chapter 3) clearly implicated that DSSN+ enhances extracellular biotransformation and may be used to harness the exchange of bioactive molecules between cells and the matrix (Sivakumar et al. 2014). The findings from Chapter 4 have demonstrated how a mutualistic community drive their diverse metabolic functions towards a communal metabolism. This study has underpinned how intercellular mutualistic interactions enhance extracellular bioactivity and modulate better biofilm-based extracellular biocatalysis (Wang et al. 2015b).

The matrix display of enzymes was achieved through the genetic fusion of specific enzymes to the C-terminus of biofilm promoting factor BpfA, a major matrix-associated protein in *S. oneidensis* (Cao et al. 2011a; Cao et al. 2011b; Theunissen et al. 2010b). We used BpfA as a delivery vector for the secretion and immobilization of enzymes in the biofilm matrix. Specifically the redox sensitive green fluorescent protein roGFP and mannan hydrolysing β -1,3-

glucomannanase Man5C were genetically fused to BpfA to be displayed on the matrix of *S. oneidensis* biofilms. The findings in Chapter 5 confirmed the ability of BpfA-roGFP cells to report the extracellular redox changes induced by environmental contaminants. Further, BpfA-roGFP facilitated the non-invasive spatial resolution of extracellular redox state within the biofilm matrix (Sivakumar et al. 2015). The findings from Chapter 6 clearly implicated that the BpfA-Man5C biofilms can be used for the extracellular hydrolysis of mannan substrates. The localization of Man5C on the biofilm matrix alleviated the diffusion barrier for glucomannan hydrolysis and facilitated the product (mannose) recovery without perturbing the overall structural integrity of the biofilm. It was also shown that the biofilm-based extracellular Man5C activity is highly robust and its longevity is well retained in continuous systems.

This study coherently affirms how the matrix-displayed enzyme approach alleviates the diffusion limitation and sustains the catalytic activity and stability in continuous systems. Further, this study has also articulated the potential of biofilm matrix-enabled extracellular biocatalysis in biotechnological applications. Taken together, the approaches explored in this study engineer the biofilms with enhanced extracellular bioactivity, which could be deployed for more coherent and productive biofilm-based biocatalysis.

7.2. Future Recommendations

The findings of this thesis work have demonstrated that the catalytic enzymes immobilized within the biofilm matrix is well retained in continuous systems and is capable of exhibiting stable catalytic activity over a longer duration. Future recommendations involve the exploration of approaches to sustain extracellular biocatalysis, which requires regeneration of enzyme

cofactors and the potential biotechnological applications of biofilm-mediated hydrolysis of mannan substrates.

7.2.1. Sustainable redox biotransformations in biofilm matrix through the genetic construction of a cofactor regeneration system

The mass transfer limitations in biofilm often occlude the free flow of cofactors and hence, stifle redox biotransformations, which require cofactor recirculation. The external addition of the cofactors is not a cost effective option in biotransformations. In order to process such reactions, either a cofactor regeneration system or a system capable of continuously supplying cofactors is required to be integrated into the catalytic biofilms.

Formate dehydrogenase (Fdh) and Glucose dehydrogenase (Gdh) are most commonly used for regenerating cofactors like NADH and NADPH (Wang et al. 2015a; Zhang et al. 2009). Formate dehydrogenase mediates the oxidation of formate coupled with the reduction of NAD⁺ to NADH (Kratzer et al. 2008; Popov and Lamzin 1994). The genetic fusion of Fdh or Gdh to BpfA might facilitate cofactor recycling in biofilms with no requirement for cell permeabilization.

Cells harbouring BpfA-Fdh on its surface may be coupled with cells expressing an NADH-dependent oxidoreductase in biofilms. As the NADH-dependent enzyme mediates the desired biotransformation with NADH oxidation, BpfA-Fdh will process the reduction of NAD to NADH in the presence of formate. NADH-dependent enzymes can be displayed either on the cell surface or secreted to cell exterior. Several cell surface-associated anchor protein systems such as autotransporter proteins or ice nucleation proteins may be used for the fusion of NADH-dependent oxidoreductase.

Mutualistic interactions between *E. coli* and *S. oneidensis* (Chapter 4)

take place with formate as the principal metabolite of exchange (Wang et al. 2015b). Table 7.1 lists potential scenarios of enzyme coupling for continuous cofactor recycling. The matrix display (BpfA-Fdh) will negotiate the mass transfer limitations associated with the free flow of cofactors.

Table 7.1. Potential scenarios of NAD(P)H/NAD(P) recycling coupled enzyme approach in biofilm matrix-enabled oxidoreductions

NAD/NADP dependent enzyme fused to BpfA	NADH/NADPH dependent	Enzyme activity	Reference
BpfA-Fdh	Butanol dehydrogenase (Bdh)	Bdh mediates the conversion of butanal to butanol with NADH oxidation	(Conway et al. 1987; Walter et al. 1992)
BpfA-Fdh	Cytochrome P450 monooxygenase Cyp101	NADH-dependent. Cyp101 mediates the hydroxylation of PAHs like naphthalene, indole and anthracene. Also catalyses the rapid conversion of propane to propanol and ethane to ethanol	(Bernhardt 2006; Hannemann et al. 2007; Urlacher and Eiben 2006)
BpfA-Gdh	Cytochrome P450 fatty acid decarboxylase OleT _{JE}	NADPH-dependent. Catalyzes the decarboxylation of long chain fatty acids to α -Alkenes	(Belcher et al. 2014)
BpfA-Fdh	Urease and Glutamate dehydrogenase	NADH-dependent Glutamate dehydrogenase use NH_3 , ketoglutarate as substrates to produce glutamic acid which gets inhibited with the presence of heavy metals. Can be used to develop a qualitative assay for heavy metal screening	(Rodriguez et al. 2004)
BpfA-Gdh	Aldoreductase	Reduction of aldehydes or monosaccharides like glucose	(Zhang et al. 2006; Zhang et al. 2009)

7.2.2. Exploring the potential of the *Man5C* fusion strain *BpfA-Man5C* for extraction of carotenoids and lipids

The ability of BpfA-Man5C to extracellularly hydrolyze mannan substrates could be harnessed for other biotechnological applications. Red yeast cell wall is composed of a framework rich in hemicellulose (Utsugi et al. 2002; Vidal et al. 2003). Red yeast such as *Rhodospiridium toruloides*, *Rhodotorula glutinis* and *Sporobolomyces roseus* produce significant amount of photosynthetic pigments called carotenoids, which serve as antioxidants as well as a precursor for vitamin A. Carotenoids are hydrophobic molecules localized in the lipid bilayer of the membrane. *R. toruloides* also produces abundance of lipids, also localized within the lipid bilayers (Lee et al. 2015). Carotenoids and lipids are normally extracted by permeabilizing the membrane through physicochemical methods (Lee et al. 2015). Mechanical cell wall disruption is highly energy intensive and affects the purity and yield of the extracted carotenoids and lipids. Alternate extraction methods include treatment of red yeast with chemicals, which affects the yield and purity of the products. Chemical methods of extraction increases the overall production cost and poses environmental issues related to the disposal of used chemicals.

Treatment of red yeast with BpfA-Man5C cells might cause the hydrolysis of red yeast cell wall, facilitating the extraction of membrane-associated carotenoids and lipids from red yeast. Preliminary results have shown that the presence of BpfA-Man5C inhibits red yeast growth in planktonic cultures (data not shown). Hydrolyzing the red yeast cell wall with BpfA-Man5C cells is an environmentally benign and low energy consuming process as compared to the physico-chemical extraction techniques.

However, it is difficult to separate the carotenoid fraction from the lipids. Recently, a study reported the use of a membrane transporter in *R. toruloides*, which facilitated the continuous secretion of carotenoids to the extracellular space (Lee et al. 2015). A co-culture catalytic system consisting of red yeast harbouring the carotenoid exporter (*R. toruloides* Pdr10) and *S. oneidensis* BpfA-Man5C allow the extraction of carotenoids and lipids in two phases. The treatment with BpfA-Man5C will hydrolyze the red yeast cell wall to release only lipid fraction. The carotenoids shall be secreted directly to extracellular space. Other factors also need to be considered in BpfA-Man5C biofilm-mediated extraction of carotenoids and lipids. The bioreactor has to be configured only after optimizing the minimum thickness required for BpfA-Man5C biofilms to extracellularly hydrolyze red yeast cell walls. Further, the cell density ratio (between red yeast and BpfA-Man5C) for the most effective extraction must be ascertained. The operating conditions for the highest Man5C activity also need to be optimized.

APPENDIX

Figure A-1: The plasmid map of the roGFP expression vector pUC57-bpfA-roGFP-aggC. The vector is cleaved into two fragments through digestion with *EcoRI* and *HindIII*. The fragment encoding *bpfA*, *roGFP* and *aggC* is transferred to *S. oneidensis* MR-1 competent cells to generate the roGFP reporter strain *S. oneidensis* BpfA-roGFP.

Figure A-2: The plasmid map of the Man5C expression vector pUC57-bpfA-man5C-aggC. The vector is cleaved into two fragments through digestion with *EcoRI* and *HindIII*. The fragment encoding *bpfA*, *man5C* and *aggC* is transferred to *S. oneidensis* MR-1 competent cells to generate the Man5C fusion strain *S. oneidensis* BpfA-Man5C.

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