Analysis of microbial components of two-liquid phase bioreactors for improved volatile organic compound biofiltration

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**ANALYSIS OF MICROBIAL COMPONENTS OF
TWO-LIQUID PHASE BIOREACTORS FOR
IMPROVED VOLATILE ORGANIC
COMPOUND BIOFILTRATION**

NG CHOW GOON

SCHOOL OF BIOLOGICAL SCIENCES

2014

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TWO-LIQUID PHASE BIOREACTORS FOR
IMPROVED VOLATILE ORGANIC
COMPOUND BIOFILTRATION**

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Journal papers

Chow Goon NG, Huang MIAO, Benoit GUIEYSSE and Chun Chau SZE

Alternative microbial combinations influence non-aqueous-liquid/aqueous phase interfacial and emulsion dynamics in two phase partitioning bioreactors

Manuscript in preparation for submission to Journal of Hazardous Materials

Conference proceeding

Chow Goon NG, Huang MIAO and Chun Chau SZE (2011) Microbial dynamics in two-phase-partitioing bioreactors for hexane removal show partitioning of distinct groups into non-aquoues phase. Presented as poster at 15th International Biodeterioration & Biodegradation Symposium, Vienna, Austria. Abstract P201.

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LIST OF ABBREVIATIONS

Δt	Rate of change
μg	Microgram
μl	Microliter
μM	micromolar
16S rDNA	16S ribosomal RNA gene
18S rDNA	18S ribosomal RNA gene
PG	Pattern ARDRA Group
AH	Alkane hydroxylase
ARDRA	Amplified ribosomal DNA restriction analysis
ATP	Adenosine triphosphate
BD	Band Distribution
BSA	Bovine serum albumin
BTEX	Benzene, Toluene, Ethylbenzene, and o-Xylene
CFU	Colony Forming Units
CLSM	Confocal laser scanning microscopy
CO_2	Carbon dioxide
Cq	Quantification cycle
CSH	Cell surface hydrophobicity
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
Dy	Dynamics
FID	Flame Ionization Detector
Fo	Functional organization
GC	Gas chromatography
gDNA	Genomic DNA
IF	Interfacial fraction
LA	Luria-Bertani Agar
LC	Ladder (reference ladder) constructed from selected clones of the 16S rDNA library
LCI	Ladder (reference ladder) based on combination of LC and LI
LI	Ladder (reference ladder) constructed from selected isolates sampled from bioreactors

M	Molar
MATH	Microbial adhesion to hydrocarbons
MDS	Multi-dimensional scaling
mg	milligram
ml	milliliter
mM	Milimolar
MPB	Monophasic bioreactor
MSM	Minimal salt medium
MSM-glucose	MSM medium with 0.1% glucose
NAL	Non-aqueous liquid
nM	Nanomolar
NTU	Nephelometric Turbidity Unit
O ₂	Oxygen
°C	Degree Celsius
OD	Optical density
PB	Prominent Band
PCA	Principal component analysis
PCR	Polymerase-chain reaction
P-L	Pareto-Lorenz
PG	Pattern group based on ARDRA
qPCR	Quantitative (real-time) polymerase chain reaction
RDP	Ribosome Database Project
rpm	revolutions per minute
Rr	Range-weighted richness
RT	room temperature
SD	standard deviation
TBE	Tris-borate-EDTA
TCA	Tricarmonic acid cycle
TCD	Thermal Conductivity Detector
TE	Tris-EDTA
TPPB	Two-liquid-phase partitioning bioreactor
TPPB-IF	TPPB interfacial fraction
TX	Triton X-100

UV	Ultraviolet
v/v	volume per volume
VOC	Volatile organic compound
w/v	weight per volume

ABSTRACT

The dynamics of the microbial communities in a two-liquid phase partitioning bioreactors (TPPBs) for treatment of volatile organic compounds (VOCs) was investigated. The TPPBs were set up in 0.5 L stirred reactors using silicone oil (at 10% v/v) as the non-aqueous liquid (NAL) phase and hexane as the model VOC, which also served as the sole carbon source in the environment of mineral salt medium (MSM). These TPPBs showed better microbial growth, higher biosurfactant production and greater efficiency in hexane removal compared to the conventional monophasic bioreactors (MPBs) over the 52 weeks of study. Properties such as rate of community change, microbial diversity richness and the dominance of certain bacterial groups showed some degree of similarity when the two types of bioreactors were compared, but the actual microbial compositions between TPPBs and MPBs revealed differences across time as analyzed by a strategy combining 16S rDNA sequencing with PCR-amplified ribosomal DNA restriction analysis (ARDRA), phylogenetic analysis and DGGE. The approach also allowed us to distinguish the culturable isolates from the bioreactors and the clones from the 0th week community's full-length 16S rDNA library, down to the genus level. This was mapped to the DGGE profiles to derive the community structures based on key genera over time. Dominant subpopulations with a biased presence in one type of bioreactor or other were identified. It was revealed that TPPBs supported a higher abundance of *Mycobacterium* while the MPBs supported more *Rhodococcus* across time. Monoculture study of the isolates were able to verify their intrinsic hexane catabolic capacity and growth on hexane. The association between each strain's level of growth on hexane, cell surface hydrophobicity and biosurfactant production were examined with respect to the influence of the NAL phase, and five functional Groups each defined by a profile of these properties were derived. Interestingly, a highly similar distribution of these five Groups across both types of bioreactors and all time surfaced, implying distinct advantages associated with this functional group distribution. A hexane uptake scenario involving bacterial interactions within a microbial community carrying the five functional Groups of bacteria in the monophasic and biphasic contexts will be discussed.

Chapter 1: General Introduction

1.1 Volatile organic compounds (VOCs)

One of the major environmental concerns, well recognized and continually being addressed worldwide, is air pollution. Air pollution has been associated with the increase in agricultural, transportation, and industrial activities, and over the past decade, these have led to an unprecedented high rate of emission of pollutants into the air (Xing et al, 2011). Although air pollutants, which typically include sulphur oxides, nitrogen oxides, particulate matter and volatile organic compounds (VOCs), may be derived from natural occurrences such as volcanic eruptions, processes driven by human activity are still the main contributors, be it natural (from agricultural wastes) or anthropogenic (from vehicles and industries) in origins (Crocker & Schnelle, 1998). The contribution by the latter can be substantial, particularly in industrialized or developed countries. Efforts had been made to control the emission of airborne contaminants through implementing regulations and the development of newer technologies both to reduce the production and to enhance the removal of the air pollutants. Despite these efforts, one group of air pollutants, the VOCs, still pose significant problems as there are challenges in effecting their removal.

VOCs are a group of organic compounds, which have – as part of their physical properties – high vapour pressures, and therefore will vaporize readily into the atmosphere (Kennes & Veiga, 2001). These VOCs include aliphatic hydrocarbons such as hexane and octane, and aromatic hydrocarbons such as benzene, pyrene and toluene. They are most often generated as evaporative emissions from industries and during burning of fuels from vehicles (Economopoulos, 1993). Anthropogenic sources of VOCs are not limited to emission by vehicles and industries into the open air and could also be released indoors by items such as paints, fabrics and glue that formed part of the household and office furnishings (Health, 2012). Frequent exposure to such environments and hence constant inhalation of VOCs has detrimental effect on human health. The acute effects included symptoms such as fatigue, sleepiness,

headaches, and nausea (EPA, 2012) while symptoms such as respiratory problems (Wichmann et al., 2009) and nervous system degeneration and lung adenocarcinoma (Barnes, 1998) may arise upon chronic exposure. Such adverse health effect is due to the varying levels of toxicity and carcinogenicity associated with specific VOCs. Other than affecting health, some of the VOCs are also directly involved in the degradation of the ozone layer, or implicated in the greenhouse effect.

As a result of the awareness to these negative impacts, there is increasing urgency in controlling the emissions of toxic VOCs. This is tackled firstly at the policy-making level to bring about tighter global control, e.g. through regulations such as the U.S. EPA National Emission Standards for Hazardous Air Pollutants (EPA, 2012). Concurrently, to meet such regulatory standards, much efforts are in place to develop technologies, which can effectively reduce their release from industrial sources (Hunter & Oyama, 2000; Iranpour et al, 2005).

1.1.1 Current treatment methods for control of VOC release

To date, a number of strategies ranging from physico-chemical methods to biological treatment have been applied to control VOC release. The choices and designs of such strategies depend on the operating conditions (flow rate, VOC concentration, temperature, humidity, etc) and the nature of the pollutants (Crocker & Schnelle, 1998).

Physico-chemical methods for removal of waste gas containing VOCs include condensation (Buonicore, 1992), adsorption (Hines et al, 1993), absorption (Hines et al, 1993), incineration (de Nevers, 1995), membrane filtration systems (Wijmans et al, 1997) and ultraviolet treatment (Gschwandtner & Fairchild, 1992). These methods are less efficient and effective in dealing with large volumes of air containing low concentration of VOCs (Crocker & Schnelle, 1998; Shareefdeen & Singh, 2005). In addition, high investment and operational costs of physico-chemical methods are driving the industries to develop and adopt alternative cost-effective waste gas treatment technologies such as biological treatment (Delhoménie & Heitz, 2005).

Biological treatment methods primarily involve the use of microorganisms to degrade pollutants present in the air stream into carbon dioxide, water, inorganic compounds, and biomass (Hernández et al, 2010), although phytoremediation i.e. through the use of plants, have sometimes been considered. Microorganisms such as *Aspergillus niger* (Spigno et al, 2003), *Pseudomonas aeruginosa* (Hernández & Muñoz Torre, 2011; Muñoz et al, 2006) and *Rhodococcus sp.*(Lee et al, 2010) have been applied in various biological removal processes of VOCs. These treatment methods show good operational stability under ambient operational conditions (room temperature and atmospheric pressure) and are environmentally friendly (Spigno et al, 2003).. Unlike physico-chemical methods which are effective in removing high VOC concentration, the biological methods such as bioscrubbers, biofilter and biotrickling filters (Table 1.1) are more effective in eliminating low to moderate VOC concentrations (1 to 1000 ppm), which is relevant for most industrial gaseous effluents (Kim, 2004).

Biofiltration and biotrickling filtration are the most commonly used biological methods, whereby waste gas is streamed through or over a culture (in the forms of liquid cultures or biofilms) carrying microorganisms capable of degrading VOCs. However, biofiltration technology has been facing various operational difficulties such as bed plugging, packing acidification, nutrient shortages, temperature increase and moisture content loss depending on the configuration of the treatment processes (Delhoménie & Heitz, 2005; Deshusses & Johnson, 1999; McNevin & Barford, 2000). In addition to these operational issues, biological factors such as bioavailability and toxicity of VOCs to the biodegradative microorganisms pose great challenges too.

Table 1.1 Characteristics of the various biological methods to remove VOCs.

Adapted from Delhoménie & Heitz (2005) and Economopoulos (1993)

Bioprocess	Bioscrubber	Biofilter	Biotrickling filter
Mode of Microorganisms	<ul style="list-style-type: none"> • Suspended in the aqueous growth medium 	<ul style="list-style-type: none"> • Immobilized on the filter 	<ul style="list-style-type: none"> • Immobilized on the filter
Liquid phase	<ul style="list-style-type: none"> • Mobile • Continuously dispersed • Recycled 	<ul style="list-style-type: none"> • Occasional bed irrigation with nutrient solutions 	<ul style="list-style-type: none"> • Mobile • Continuous trickling over the filter bed • Possible recycling
Advantages	<ul style="list-style-type: none"> • No clogging problem as no solid media was used • pH control allows for treatment of compounds with acidifying properties 	<ul style="list-style-type: none"> • Low cost and ease of operation • More efficient for treating poorly water-soluble VOCs 	<ul style="list-style-type: none"> • pH control allows for treatment of compounds with acidifying properties • Continuous liquid recirculation minimise filter clogging and pressure build-up
Disadvantages	<ul style="list-style-type: none"> • Inefficient to treat low solubility VOC • Low specific surface areas for gas/liquid transfer • Production of sedimented sludge and waste water to be treated/disposed 	<ul style="list-style-type: none"> • Lack of pH control • Biomass buildup at the filter bed • Requires frequent change of filter material • Lack of long-term stability unless carefully controlled 	<ul style="list-style-type: none"> • Additional operation and maintenance cost for liquid recirculation system and chemical requirement • Potential development of pH gradient in the axial direction of the filter bed.

1.2 Bioavailability and toxicity of VOCs to microorganisms

The underlying criteria of biological treatment is that firstly the target VOCs must be metabolizable substrates to the microorganisms present within the system, and secondly, these must be in a form or distributed in a milieu that can be readily taken up by the microorganisms. The substrates taken up are then enzymatically transformed to simpler compounds, which is interpreted as the “removal” of the target compounds from the (biological treatment) system.

In the case of VOCs, typically, these hydrophobic compounds have low rates of dissolution into the aqueous liquid cultures, the introduction of which will result in formation of separate phases and only the minute amounts dissolved in the aqueous phase are in fact available for the biodegradative microorganisms residing in it (Deshusses & Johnson, 2000). VOCs such as short-chain alkanes (e.g. pentane, hexane) (Arriaga & Revah, 2005a), benzene, toluene and their derivatives belong to this category of VOCs. Hence they are not effectively removed via the biofiltration or biotrickling filtration configuration, despite the presence of microorganisms capable of metabolizing them.

From another perspective, even if higher concentration of VOCs could be made available to the microorganisms, the often toxic nature of the VOCs may inhibit or kill the microorganisms, possibly including the active biodegraders, leading to poor removal rates (Delhoménie & Heitz, 2005; Deshusses, 1997; McNevin & Barford, 2000). This may occur, for instance, during bouts of sudden high loads of VOCs in industrial off-streams which are very much variable and uncontrolled, unlike in a laboratory setting.

The two-liquid phase partitioning bioreactors (TPPBs), also known as biphasic bioreactors, have therefore been conceptualized by several authors to address these limitations, and studies on TPPBs have been conducted over the past two decades in order to assess its applicability to treat VOC-contaminated air (Muñoz et al, 2012; Quijano et al, 2009).

1.3 Two-liquid-phase partitioning bioreactor (TPPB) system

A TPPB system consists of an aqueous phase that carries pollutant-degrading microorganisms and nutrients (to support their growth), and a water-immiscible non-aqueous liquid (NAL) phase which exhibits high solubilisation capacity for the hydrophobic target pollutants. The NAL phase serves to dissolve, store and transfer the apolar organic pollutants to the aqueous phase where biodegradation can occur (Deziel et al, 1999), or can itself serve as substrates for the microorganisms. For the former purpose, the NAL phase chosen should be biocompatible (non-toxic to the microbial community), non-biodegradable by the microbial community, have high pollutant partitioning capacity and low volatility, and preferably, of low cost (Bruce & Daugulis, 1991; Morrish et al, 2008; Muñoz et al, 2006).

The hydrophobic VOCs, once dissolved in large quantity in the NAL phase, are delivered to the microorganisms in the aqueous phase based on the equilibrium partitioning coefficients of the VOCs (Figure 1.1). As the VOCs are consumed by the microorganisms in the aqueous phase, the partitioning equilibrium will drive more VOCs into the aqueous phase, creating a steady stream of feed at non-toxic levels (Daugulis & Boudreau, 2003; Deziel et al, 1999). In addition to this mechanism, some microorganisms have been observed to be in direct contact with the NAL phase (Ascón-Cabrera & Lebeault, 1995b; MacLeod & Daugulis, 2005) at the aqueous/NAL interface, suggesting a direct uptake of the organic compounds from the NAL, further improving the efficiency of VOC removal. The increased bioavailability of the VOCs in this biphasic scheme can therefore result in an enhanced level of biodegradation (Muñoz et al, 2006). Furthermore, the NAL can act as a reservoir to buffer the toxic effects of suddenly high levels of VOCs in the feed stream. In this way, the TPPB system allows for the retention of higher concentration of hydrophobic organic compounds in the overall system, yet continues to maintain in the aqueous phase a low enough concentration of the compound that is not inhibitory to the microorganisms (Vermue et al, 1993).

To date, various studies have shown that TPPBs can indeed enhance the biodegradation of compounds such as benzene (Yeom & Daugulis, 2001), phenol

(Amsden et al, 2003), toluene (Daugulis & Boudreau, 2003) and hexane (Muñoz et al, 2006). For example, Daugulis and Boudreau (2003) reported a toluene elimination capacity (EC) of 727 g per m³ of reactor volume per hour in a TPPB that contained hexadecane. Likewise, Arriaga et al. (2006) achieved hexane EC of 160 g m_{reac}⁻³h⁻¹ in a fungal biofilter supplied with silicone oil. This elimination capacity was significantly higher than the highest EC of 100 g m_{reac}⁻³h⁻¹ and 60 g m_{reac}⁻³h⁻¹ reported in classical fungal and bacterial biofilters, respectively (Arriaga & Revah, 2005b; Kibazohi et al, 2004). Similarly, Cesário et al. (1997) increased oxygen transfer by 120% using 10% of a perfluorocarbon FC40 in an oxygen limited toluene biodegradation process. Besides improving on the VOC retention and bioavailability, the presence of NAL has also been observed to increase the ability of the bioreactors to handle quantitative variations in the VOC loads, as expected. For instance, Boudreau and Daugulis (2006) demonstrated that when the VOC loading were cyclically increased by nearly 10-fold, the TPPB system maintained a constant removal efficiency of 97 %, unlike the monophasic system which showed a drop in the efficiency from 95 % to 69 %. This signifies the benefits of applying TPPBs to industrial off-gases where the VOC load might vary considerably across time.

As a result of such promising outcomes, there are increasing interests to develop TPPB systems that can enhance the biodegradation of toxic organic compounds to be applied in actual off-gas treatment settings. However, prior to industrial application of the TPPBs, further studies are needed to understand more about the TPPBs, which are operating on long term. To date, most studies conducted on TPPBs have been on short-term bases (Table 1.2) and examined from the engineering point of view. The longest study on TPPBs was for only up to six months as reported by Van Groenestijn and Lake (1999), with a primary focus on the removal rates and kinetics. To make applications of TPPBs a reality, satisfactory functional output (i.e. VOC removal) by the microbial community of the system over the long term is desirable. However, at this point in time, there is considerable lack of knowledge of the microorganisms and its microbial dynamics within a TPPB and the biology associated with such microbial communities (refer Section 1.3), and certainly not over long-term operation (one

year or more). Greater understanding on the microbial dynamics that may affect the performance of TPPBs will be useful for improving the long-term stability of TPPBs under full-scale industrial continuous operation. This was the primary motivation in initiating the study described in this thesis.

Figure 1.1 Predicted flow of hydrophobic VOC (e.g. hexane) in (A) a conventional monophasic system and (B) a biphasic system. In (A), hexane removal by microorganisms occurs at a low rate as microbial activity is limited by the slow substrate transfer (block arrows) to the aqueous phase. In (B), hexane removal rate is enhanced overall as the substrate is first transferred to the non-aqueous liquid (NAL), e.g. silicone oil, which has a high affinity for hexane. The hexane concentration in the aqueous phase remains low but as it gets consumed by microorganisms, a continuous release from the NAL occurs, driven by the water-NAL partitioning equilibrium. This pathway increases the overall substrate transfer to the aqueous phase. Cells at the aqueous/NAL interface (shaded black) may access the substrate directly and further enhance the overall removal rate. Figure is adapted and modified from Muñoz et al (2007a).

Table 1.2 Duration of studies on TPPBs for treatment of VOCs.

Type of VOC	Duration of Study	Reference
Benzene	50 hours	Yeom and Daugulis (2001)
Benzene	140 hours	Davidson and Daugulis (2003)
Benzene	30 days	Nielsen et al (2006)
Hexane	6 months	Van Groenestijn and Lake (1999)
Hexane	30 days	Spigno et al (2003)
Hexane	8 days	Muñoz et al (2006)
Hexane	50 days	Hernández et al (2010)
Hexane	80 days	Muñoz et al (2013)
Isopropylbenzene	38 days	Aldric and Thonart (2008)
Pyrene, perylene, chrysene, benzo[a]pyrene	26 days	Marcoux et al (2000)
Pyrene	15 days	Mahanty et al (2008)
Styrene	29 days	Osswald et al (1996)
Toluene	262 hours	Daugulis and Boudreau (2003)

1.3.1 Use of single strain versus microbial consortium in TPPB

Various single species of microorganisms have been used as inocula in TPPBs and demonstrated to be effective in enhancing the degradation of VOCs (Table 1.3). Although these were useful as experimental models, from the application perspective they face the challenges of compromised performance when subjected to fluctuations in operating conditions. Most TPPBs were instead preferentially operated with mixed cultures enriched from activated sludge or from soil (Table 1.3), because of the purported higher resilience and robustness conferred by the diversity present in microbial communities (Muñoz et al, 2012). These mixed-culture TPPBs have exhibited enhanced VOC degradation (Ambujom, 2001; Guieysse et al, 2001; Guieysse et al, 2008; Hernández et al, 2012; Muñoz et al, 2013; Olguin-Lora et al, 2003; Prpich & Daugulis, 2004) and also the capability to degrade a variety of toxic substrates without the accumulation of toxic intermediates (Acuna-Arguelles et al, 2003; Prpich & Daugulis, 2004).

In a mixed culture, various groups of microorganisms may have different degradation potentials for the target VOC due to the diversity of biochemical pathways present and, as a result, the community as a whole can utilize varied compounds (intermediates and by-products) as substrates. The synergistic effect of members within the microbial consortium is likely to have contributed to increased metabolic capabilities and greater stability of the bioreactors (Ambujom, 2001; Prpich & Daugulis, 2005). However, even before the question of interaction and metabolic cooperation can be tackled, there remains a lack of basic knowledge regarding microbial consortia found in TPPB systems, such as their community structures and key microbial players. The mixed-culture TPPBs demonstrated to be effective in VOC (Ambujom, 2001; Guieysse et al, 2001; Guieysse et al, 2008; Hernández et al, 2012; Muñoz et al, 2013; Olguin-Lora et al, 2003; Prpich & Daugulis, 2004) have been operating as “black boxes” and remained uncharacterized, which makes it difficult to control or make prediction about performance. Hence, there are growing interests among environmental engineers to develop strategies or capabilities to customize reliable microbial consortia as TPPB inocula (Muñoz et al, 2012).

An understanding of the temporal development of microbial communities in a TPPB context would be able to provide insights to work towards this end. In order to achieve this, an experimental TPPB model would be used in this study that targeted the removal of the VOC, hexane.

Table 1.3 Single species pure cultures and consortia/mixed cultures used as inocula in TPPB studies.

Type of microbial inocula	Target VOC	Reference
Single species pure cultures		
<i>Alcaligenes xylosoxidans</i>	Benzene	Yeom and Daugulis (2001)
<i>Alcaligenes xylosoxidans</i>	Toluene	Daugulis and Boudreau (2003)
<i>Aspergillus niger</i>	Hexane	Spigno et al (2003)
<i>Pseudomonas aeruginosa</i>	Hexane	Muñoz et al (2006)
<i>Pseudomonas aeruginosa</i>	Hexane	Hernández et al (2010)
<i>Rhodococcus erythropolis</i>	Isopropylbenzene	Aldric and Thonart (2008)

<i>Mycobacterium frederiksbergense</i>	Pyrene	Mahanty et al (2008)
Consortia/mixed cultures		
Mixture of <i>Pseudomonas</i> strains	2,4,6-	Ascón-Cabrera and Lebeault
SP1 and SP2, <i>Arthrobacter</i> sp.	trichlorophenol	(1995a)
AK2 and <i>Alcaligenes</i> sp. AL2		
Activated sludge	Hexane	Van Groenestijn and Lake (1999)
Activated sludge	Hexane	Hernández et al (2012)
Activated sludge	Hexane	Hashemi et al (2012)
Activated sludge	Methane	Rocha-Rios et al (2009)
Creosote-contaminated soil	Pyrene, perylene, chrysene, benzo[<i>a</i>]pyrene	Marcoux et al (2000)
Petroleum contaminated soil	BTEX	Littlejohns and Daugulis (2009)
Marine microbial consortium	Styrene	Osswald et al (1996)

1.4 Hexane

Several classes of VOCs are listed under the United States Toxic Substances and Disease Registry (ATSDR, 2012) and United States Environment Protection Agency (EPA, 2012). One of them is the family of short- to medium-chain alkane hydrocarbons, to which n-hexane, also simply referred to as hexane, with the chemical formula of C₆H₁₄, belongs. Hexane has been chosen as the model VOC in this study due to its hydrophobicity (aqueous solubility of 9.5mg/L) (Chiou et al, 1988) and environmental relevance. It has also posed a notorious challenge for most of the current biofiltration systems (Muñoz et al, 2006; Spigno et al, 2003).

Hexane is a significant constituent of crude oil, gasoline and natural gas, and is emitted by petroleum and related industries and vehicles. In industries involving shoe-making, leather joining, and roofing, hexane is used as a component of glues (Bedino, 2005). In addition, it can be used for degreasing purposes (Nijem et al, 2001) and in textile manufacturing (Bedino, 2005).

Besides being a common industrial pollutant, hexane is also found in some common household products such as cleaning agents, contact cement and spray adhesives (NRDC, 2010). As such, almost everyone in most city dwellings can be expected to be exposed to varying levels of hexane in the air, making the removal of this VOC of great practical relevance.

1.4.1 Impact on environment and health

Once hexane is released into the environment, it will immediately vaporize due to its strongly volatile nature. According to Australia's National Pollutant Inventory of, in 2010/2011, the total industrial emission of hexane in Australia amounted to 8.9×10^4 kg (Department of Sustainability, 2012). Also, in the reports by Toxics Release Inventory (TRI) of the United States, in 2011, a total of 14,564,200 kg hexane was released to the environment by the industries within the United States, approximately 92% of which were produced by the food, beverages, tobacco, petroleum and chemical manufacturing industries (EPA, 2012). In Singapore, similar inventory reports are not publicly available but with the heavy petroleum-related industry sector and the high density of vehicles on road, the level of hexane emission is expected to be substantial. In view of the substantial hexane produced by the industries in the form of waste emissions with a clear outlet of release, such portion could be channeled to constructed bioreactors. Hence this portion of hexane in the environment could be excellent target for removal via TPPB.

As hexane vaporizes easily, inhalation of hexane-contaminated air is the most common form of exposure. The short-term exposures of hexane can give rise to symptoms ranging from headaches and dizziness to other effects similar to (alcoholic) intoxication. However, prolonged and high concentration exposure can result in lasting and possibly permanent damage to the central nervous system. A medical condition called peripheral neuropathy has been associated with high level of hexane exposure, and patients would suffer symptoms such as numbness and tingling in the feet, legs and hands, and even paralysis in some severe cases (Services, 1999).

1.4.2 Biodegradation/mineralization of hexane

In general, alkanes which are not volatile are more readily degraded in the environment due to physical accessibility to biodegradative microorganisms, compared to the volatile short chain alkanes (C2-C9) including hexane. Studies have also demonstrated that hexane is more cytotoxic to many bacterial species compared to other solvents such as n-dodecane and dimethyl sulfoxide (de Carvalho & da Fonseca, 2004; Lee et al, 2010) as it could partition well into and lead to accumulation of itself at high concentration in cell membranes, causing cell death (Sardesai & Bhosle, 2004).

Despite this, microorganisms which are metabolically capable of degrading hexane have been found. The isolation and identification of these (bacteria) have led to the elucidation of short/medium chain n-alkane catabolic pathways under both aerobic and anaerobic conditions (Lee et al, 2010; Wilkes et al, 2002). Aerobic degradation of hexane in bacteria (Figure 1.2) typically begins with the terminal or sub-terminal insertion of a molecular oxygen atom into the n-alkane by the enzyme, alkane hydroxylase (AH). These AHs are responsible for the activation of n-alkanes to 1-alkanols, which are then further metabolized by alcohol and aldehyde dehydrogenases to fatty acids, to be fed into the β -oxidation and/ or tricarboxylic acid cycle (TCA), leading to complete mineralization (Amouric et al, 2010; Berthe-Corthi & Fetzner, 2002; Rojo, 2010; van Beilen et al, 2003). In the case of anaerobic biodegradation, hexane is first converted to (1-methylpentyl) succinate in the presence of the co-substrate fumarate by the enzyme (1-methylpentyl) succinate synthase, and further degraded via the TCA cycle (Cravo-Laureau et al, 2005; Wilkes et al, 2002).

As the experimental model set up intended for this study was a TPPB scheme for hexane removal in an aerobic setting, we anticipated that a large proportion of the microorganisms surfaced through this work would be capable of hexane mineralization, and likely via the AH metabolic pathway(s) described above.

Figure 1.2 Aerobic degradation of Hexane via the alkane hydroxylase (AH) pathway. The intermediates of the aerobic hexane degradation pathway have been elucidated and reported by Lee et al (2010) using GC/MS and SPME. Adapted from Berthe-Corthi and Fetzner (2002) and Rojo (2010).

1.5 Silicone oil as NAL phase in TPPB for hexane removal

Because of the presence of the NAL, TPPB systems are able to circumvent the limitations faced by monophasic (aqueous phase) biofiltration with respect to the dissolubility of VOCs (Muñoz et al, 2006). Hence one of the crucial steps in designing a TPPB system is the selection of a suitable NAL.

Various studies have shown that silicone oil exhibits a high affinity for hydrophobic organic compounds (Ascón-Cabrera & Lebeault, 1995a; Guieysse et al, 2001; Muñoz et al, 2006; Muñoz et al, 2003). The partitioning coefficient of hexane in silicone oil ($K_{\text{air/silicone oil}}=0.003$) has been determined experimentally (Arriaga et al, 2006), which is 4 orders of magnitude lower than that of hexane in water ($K_{\text{air/water}}=30.9$) (Arriaga et al, 2006). This means that an inverse magnitude of bias for dissolved hexane will exist in favor of silicone oil relative to water. Hence, given a fixed volume of hexane, in a TPPB system, most of the hexane would be present in the silicone oil, but as hexane is microbially consumed in the

aqueous phase, more of it will be released into the aqueous phase from the silicone oil (Figure 1.1). This is unlike in a monophasic system where most hexane remains in the gas phase. As the silicone oil contains a higher amount of hexane and 30 times more oxygen (Arriaga et al, 2006) compared to the aqueous phase, certain groups of microorganisms have been observed to migrate from the aqueous phase towards the silicone oil and/or grow at the interface (Deziel et al, 1999; Muñoz et al, 2006).

In addition to having a high affinity for hexane, silicone oil has been demonstrated to be biocompatible to various microbial cultures (Ascón-Cabrera & Lebeault, 1995a; Guieysse et al, 2001; Muñoz et al, 2006; Muñoz et al, 2003). Its greater stability and stronger resistance to microbial degradation compared to others such as hexadecane, tetradecane, 1-decanol, diethyl sebacate, and 2-undecanone have also been shown through a series of systematic experiments (Arriaga et al, 2006; Muñoz et al, 2006).

Therefore, on the basis of these properties, silicone oil was selected to serve as the NAL phase in our experimental model of TPPB for hexane removal.

1.6 Stirred tank TPPB configuration

TPPBs devoted to hexane biodegradation have been operated in several reactor configurations such as stirred tank reactors, airlift, biotrickling filters, biofilters and bubble columns (Muñoz et al, 2012). The stirred tank reactor configuration is probably the most commonly adopted in TPPB studies, although it is also the most energy-demanding due to the need for mechanical stirring (Littlejohns & Daugulis, 2009). This is necessary both for aeration as well as to disperse the NAL phase into the aqueous phase (Muñoz et al, 2007b). The former is important if the operation desires to exploit the microbes' more efficient hexane biodegradation routes (Berthe-Corthi & Fetzner, 2002) occurring in the aerobic mode. The latter increases the interfacial area of the NAL in water, thereby increasing the rate of substrate transfer between the two phases.

Stirred tank TPPBs may be operated in a continuous (i.e. where influent and effluent flow rates are equal) (Ascón-Cabrera & Lebeault, 1995a; Hernández et al, 2012; Muñoz et al, 2013) or a batch mode (Arriaga et al, 2006; Ascón-Cabrera & Lebeault, 1995b; Guieysse et al, 2005). In industrial off-gas treatment contexts, the continuous mode is considered less practical due to the inefficiency inherent in a situation where microbial wash-outs constantly occur and the fluid volumes required to be handled are large. A batch mode, on the other hand, is not conducive for long-term operation as nutrient depletion and metabolic waste accumulation will eventually bring a halt to microbial activity. Replacement of old media with fresh ones will have to be incorporated as part of the process design, and some authors have therefore proposed the semi-batch mode of cultivation, whereby half the volume of the media are replaced by fresh ones periodically while the microbial biomass is completely retained within the system (Quijano et al, 2009).

For the purpose of our study, a stirred tank configuration operating on the semi-batch mode would be set up. The intention was to keep the hexane load as a constant parameter, but derive some dynamics in terms of the microbial communities' environment by exploiting the nature of the semi-batch mode of cultivation.

1.7 Objectives of this study

As discussed in earlier sections, in order to bring about greater success in TPPB operations in treatment settings, the microbial dynamics of a TPPB system has to be analyzed to obtain insights about productive microbial interactions that may give us clues regarding effective inoculum selection approaches.

The TPPBs which we intended to establish for this study would make use of a hexane-acclimated soil microbial consortium, which would be maintained in mineral salt medium (MSM) (Muñoz et al, 2006) with 10% of the volume comprising of silicone oil (the NAL phase). Hexane, our target VOC, would be supplied daily to the bioreactors in pure liquid form and serve as the sole carbon source. This would allow us to analyze the characteristics of a complex microbial

community of environmental origin that has been adapted to utilize a target VOC, and has further developed over a long period of operation in a TPPB context. Concurrently, a monophasic system would be initiated with the same inoculum to serve as a reference for comparison in the study.

The objective of this project is to establish such a long-running TPPB system in order to gain an understanding of the resident microbial consortia and their dynamics through temporal analyses (culture-based and molecular) and comparison with the monophasic counterparts. We further aimed to characterize the various bacterial strains isolated from the microbial consortia with respect to properties related to the hexane biodegradative functions, e.g. presence of catabolic genes, biosurfactant production and cell surface hydrophobicity. It was hoped that an evaluation of possible mechanisms in the TPPBs (particularly involving microbial interplays or interactions) that have led to enhanced hexane biodegradation in the TPPB context might be made from these findings.

1.8 Thesis organization

This thesis has been structured into five chapters, in such a way to provide the reader with the ease of reading and hence not always presented according to the chronology of my work. Furthermore, background information relevant to this study is only partially provided in the current chapter (i.e. Chapter 1). The remaining portions are distributed among the Introduction sections of the later chapters to allow for better association of those information with my results. Likewise, the relevant Materials and Methods are parceled out into the respective chapters for easier reference. The scopes of the chapters are described below.

Chapter 1 discusses the importance of VOCs as anthropogenic pollutants and provides an overview of the motivation for and limitations in VOC biodegradation research. It also explains the rationale behind the use of TPPBs for VOC removal and the choice of the TPPB system to be set up as experimental model for our study. Finally, the objectives of this work are presented.

Chapter 2 includes work related to the establishment of the TPPBs and monophasic bioreactors (MPBs) and the verification of enhanced performance of TPPBs during the one year (52 weeks) of operation, through analysis of the biomass and the degradation efficiency of hexane. The necessity to examine both the aqueous phase and interfacial fraction (IF) for microbial contribution would also be surfaced through this chapter.

Chapter 3 presents the microbiological analysis of the temporally sampled bioreactors, including culture-based and molecular approaches to determine community structures and dynamics of the aqueous phase and IFs, and the identification of key bacteria with biased presence in the microbial consortia of TPPBs compared to MPBs.

Chapter 4 focuses on the biological properties of the collection of TPPB-biased bacterial strains revealed in Chapter 3, such as growth on alternative substrates in the presence and absence of NAL, biosurfactant production, and cell surface hydrophobicity. The findings are discussed with respect to the possible mechanisms and microbial interactions taking place in the TPPBs that may have conferred advantage during VOC biodegradation compared to the MBPs.

Chapter 5 summarizes the essential contributions of this study and also proposes several potential areas to look into to bring this knowledge further.

Chapter 2: Development of Microbial Biomass in Aqueous and Interfacial Fractions of TPPB

2.1 Introduction

Although many TPPBs have shown good pollutant removal rates and kinetics (Daugulis & Boudreau, 2003; Hernández et al, 2012; Muñoz et al, 2006), there were also instances whereby no stable biodegradation performance was achieved during the whole experimental time (Aldric & Thonart, 2008; Muñoz et al, 2006) of a study. Hence, if TPPBs were set up in this project, they could not be automatically assumed to be enhanced in hexane biodegradation compared to their MPB controls. In this chapter, the establishment of the TPPBs and MPBs will first be described, followed by a series of experiments to verify that the TPPBs were showing enhanced biological activities compared to the MPBs. It was also at this stage that the nature of microbial distribution to the NAL phase and/or the interfacial region would be examined. These studies served to provide the contexts upon which the community analysis presented in the next chapter (Chapter 3) would be anchored.

2.1.1 Use of acclimated microbial consortia as bioreactor inocula

It is vital to use the appropriate type of microbial consortia as seed cultures for initiating any kind of bioreactors because it would affect the bioprocessing efficiency of the target substrates (van der Gast et al, 2002). Studies have shown that the use of acclimated microbial populations, isolated from contaminated sites or targeted waste materials, resulted in more rapid responses and greater resistance to parameter fluctuations within bioreactors (Fewson, 1988; Hamer, 1997; van der Gast et al, 2002). These enriched microbial communities clearly exhibited better performance when used as inocula compared to their unenriched counterparts. For example, an enriched consortium of aerobic denitrifying organisms obtained from a sludge sample has been shown to effect a high level of ammonia removal when applied to wastewater treatment

processes as opposed to its pre-enriched state which exhibited no signs of removal activities (Yao et al, 2013). Similar observations have been made by Amouric et al (2006) who reported that a hexane-enriched microbial consortium showed progressively enhanced hexane degradation when applied to a biofilter across time. During acclimatization, microbial consortia have been observed to reduce incompleteness with respect to their composition, enriching a group of more specialized microorganisms which are presumably capable of degrading the substrates of interest, while others which failed to adapt in the acclimatization conditions declined in quantity (Chanika et al, 2011; Hilyard et al, 2008; Luo et al, 2009; Yao et al, 2013).

Most of the enriched microbial consortia reported to be used for seeding TPPBs originated from activated sludge or from soils (Muñoz et al, 2012). In this study, soil samples were collected to be assessed and the more appropriate sample was then put through acclimatization to be used as the initial inocula for the TPPBs. During the operation of the bioreactors over the 52 weeks, some aspects discussed in the next two subsections (Section 2.1.2 and 2.1.3) were examined.

2.1.2 Biological manifestation of hexane removal/utilization

Studies on TPPBs designed for hexane removal have evaluated the systems' performances using varied parameters and methodologies. Those, which were looking at process or system efficiency, generally evaluate the influent and effluent gaseous hexane contents (Arriaga et al, 2006; Hernández et al, 2012). Others, which were attempting to dissect mechanisms such as oxygen and substrate mass transfer, would instead measure the concentrations of the relevant compounds in the aqueous and NAL phases (Muñoz et al, 2013; Quijano et al, 2010). These parameters have been particularly informative when the overall efficiency of the engineered system and physico-chemical mechanisms were being scrutinized (Muñoz et al, 2012).

When the performance of TPPB systems has to be investigated from the biological point of view, microbial growth would usually be the first output to be

evaluated. This is based on the fact that when microorganisms completely mineralize a carbon source (e.g. the target substrate in a TPPB), the metabolic processes are linked to energy generation and biosynthesis (Alexander, 1981), and these will lead to “growth”. For instance, microbial utilization of hexane as the sole carbon source have been reported to lead to an accumulation of biomass (Li et al, 2010; Lobos et al, 1992; Yanzekontchou & Gschwind, 1994). Direct measurements of cell densities and/or total dry mass to assess the efficiency of TPPBs were therefore common practices (Muñoz et al, 2012). In addition, growth is associated with an increase in protein biosynthesis, hence assaying for the total cellular content would be a valid means of monitoring the level of biodegradative activities of microbial communities which were relying on the target compound as the only carbon source (Hattori et al, 2003; Stanley, 1986; Venkateswaran et al, 2003). The link of metabolic processes to energy generation provides another indicator for evaluating biological performance – the concentration of ATP may be used to reflect the collective activity level of a microbial consortium (Hammes et al, 2010; Karl, 1980). If the biochemical pathway(s) of the relevant target have already been deciphered, measuring the concentrations of pathway intermediates may also serve the purpose (Muñoz et al, 2013) of performance assessment, although when applied in a microbial consortium context, interpretation may not always be straight forward due to the metabolic heterogeneity present in the community.

2.1.3 Microbial association at the aqueous-NAL interface

Conventionally, metabolic activities of microorganisms in TPPBs are considered to be mainly occurring in the aqueous phase (Daugulis, 2001), and the higher biodegradation performance of TPPBs over monophasic systems was attributed to the establishment of a new substrate transfer pathway via the gas/NAL/water route as a result of the presence of the NAL (Muñoz et al, 2012). However, increasingly more studies have highlighted the need to examine the tendency of certain microorganisms to associate with the NAL phase. Ascón-Cabrera and Lebeault (1993), and Ascón-Cabrera and Lebeault (1995a) first noted that a considerable proportion of the total microbial biomass appeared to be

adhered to the aqueous/NAL interface during the biodegradation of chlorobenzenes and ethyl butyrate in a TPPB. Other reports, such as the exclusive occurrence of *Mycobacterium* PYR-1 at the aqueous/NAL interface during the biodegradation of PAHs, and the varied distribution of different species of *Pseudomonas* between the aqueous phase and the interface (Hernández et al, 2012), highlighted the fact that association profiles of microorganisms to the NAL phase could have consequence on the overall substrate removal efficiency of TPPBs.

As a result of these observations, the presence of microorganisms accumulated around the aqueous/NAL interface has been, in the last one decade, viewed as a factor that contributed to the enhanced biodegradation performance of TPPBs. This school of thoughts has been further encouraged by a very recent demonstration that the hexane degradation efficiency of a TPPB could be enhanced by 8 times when a consortium with extremely high affinity for the NAL phase (and therefore closely associated with the interface) was used as the inoculum. Such enhancement was maintained stably for up to 80 days, and the efficiency was much higher compared to the one operating under similar conditions but inoculated with a consortium of only aqueous phase-associated microorganisms (Hernández et al, 2012; Muñoz et al, 2013).

Since the association of microorganisms to the NAL phase can differ greatly among various TPPB systems (presumably according to the microbial composition of the inoculum), this characteristic of the microbial consortia would also be examined during the 52-week operation of our TPPBs.

2.2 Materials and methods

2.2.1 Culture media

The mineral salt medium (MSM) used in this experiment was prepared according to Muñoz et al (2006). The composition is as shown in Table 2.1. The final pH of the medium is 7. MSM agar plates were made by adding 15 g of Bacto Agar (BD Difco) per litre of MSM medium.

Table 2.1 Composition of mineral salt medium (MSM).

Chemicals	Amount (per litre)
KH ₂ PO ₄ (Sigma Aldrich)	1 g
K ₂ HPO ₄ (Sigma Aldrich)	2 g
NH ₄ Cl (Sigma Aldrich)	0.75 g
MgSO ₄ .7H ₂ O (Sigma Aldrich)	0.5 g
CaCl ₂ (Merck)	0.018 g
Trace element solution	1ml
Contains the following (per litre):	
concentrated HCl (Merck)	6.75 ml
FeCl ₂ .4H ₂ O (Sigma Aldrich)	1.5 g
H ₃ BO ₃ (Sigma Aldrich)	0.06 g
MnCl ₂ .2H ₂ O (Sigma Aldrich)	0.1g
CoCl ₂ .6H ₂ O (Sigma Aldrich)	0.12 g
ZnCl ₂ (Sigma Aldrich)	0.07g
NiCl ₂ .6H ₂ O (Sigma Aldrich)	0.025
CuCl ₂ .2H ₂ O (Sigma Aldrich)	0.015
NaMoO ₄ .2H ₂ O (Sigma Aldrich)	0.025 g
EDTA (Sigma Aldrich)	5.2 g

2.2.2 Culturable cell count of microbial consortia from soil samples before and after enrichment by hexane

Soil samples were collected from two locations in Nanyang Technological University (NTU): (i) from a grassy patch along the road and (ii) from a petroleum-contaminated patch in the car park. Approximately 10 g of the soil samples were suspended in 10 ml of MSM and shaken for 5 minutes at 200 rpm. The suspension was subsequently diluted 100- fold with MSM and 20 ml aliquots of this diluted suspension were transferred to closed 120 ml serum bottles. Subsequently, using a 100 μ l syringe, 0.1g of hexane (Merck) was injected into each bottle and incubated for 17 days at 25°C on a shaker set at 100 rpm.

Microbial cultures were analyzed by spread plating on MSM plates, then incubating at room temperature in a dessicator infused with hexane for 7 days. The increase in biomass due to hexane utilization was indicated by the fold increase in the growth after 17 days of growth. The increase was calculated by dividing the viable cell count (CFU/ml) at day 17 over that of the original cultures at day 0.

2.2.3 Collection and acclimatization of microbial consortium from petroleum-contaminated soil

The analysis above had showed that the microbial community from the petroleum-contaminated soil sample was more enhanced in growth after enrichment by hexane (Figure 2.3 under Section 2.3.1). Hence, such soil sample was put through a hexane acclimatization process to be used as an inoculum for the TPPBs and MPBs. A schematic of the process is shown in Figure 2.1 below. The soil was processed according to the procedure stated in Section 2.2.2 except that an additional step of sieving through metal grids (to remove large soil particles) was included after its suspension in MSM, before the 100-fold dilution.

A total of nine serum bottles (labeled A to I), each containing 20 ml of the MSM-diluted microbial cultures, were prepared and sealed with butyl rubber and aluminum caps. The bottles A to D was injected with 0.1 g of hexane (Merck) using a 100 μ l syringe on days 1 and 15. Bottles E and F, serving as positive controls, were injected with glucose to a final concentration of 0.00125g/ml in addition to 0.1 g of hexane. Bottles G to I served as negative controls with no addition of exogenous source of carbon. All bottles were incubated at 25°C for 24 days with shaking at 100 rpm. Bottles A to F were then pooled together on day 24 and subcultured into three new 120 ml serum bottles, numbered 1, 2 and 3. Bottles G, H and I were also pooled together and subcultured into two new 120 ml serum bottles, numbered 4 and 5. Subculturing was carried out by diluting 0.2 ml of the pooled microbial cultures 10-fold in MSM and transferring 20 ml aliquots to each of the fresh serum bottles. Hexane (0.1 g) was injected into the sealed bottles 1 to 3 on days 24 and 28. Bottles 1 to 5 were each further subcultured into new serum bottles on day 40 with injection of 0.1 g of hexane into bottles 1 to 3. All cultures in the bottle continued to be incubated at 25 °C with shaking at 100 rpm, until day 50. The hexane-acclimated cultures in bottle 1 to 3 were pooled and this final sample was designated as the initial bioreactor inoculum.

Microbial cultures in the serum bottles were monitored for growth via measurement of O₂ and CO₂ concentrations in the headspace of the bottles at 3-5 days intervals as indicated in Figure 2.4 (Section 2.3.2) during the first 28 days, using thermal conductivity detector gas chromatography (TCD-GC, Agilent 7890 equipped with columns: HP-Plot/Q, 30 m, 0.53 mm, 40 micron and HP-Molesieve, 30 m, 0.53 mm, 50 micron).

Figure 2.1 Schematics of acclimatization process of soil microbial community to generate a starting inoculum for TPPBs and MPBs. Bottles A to I contained the microbial cultures from the petroleum-contaminated soil collected from the car park, suspended in 20 ml of MSM as the base medium. One set of bottles (A-D) was put through enrichment by hexane according to the steps shown, to generate the starting inoculum of the bioreactors. The other two sets served as positive (E and F, fed with hexane and glucose) and negative (G-I, no carbon source fed) controls. All bottles were incubated at 25 °C with constant shaking at 100 rpm, until day 50.

2.2.4 Bioreactor operating conditions

Double sidearm Celstir bioreactors of 500 ml capacity (Wheaton, Scientific, Millville, NJ) were incubated at room temperature (23 to 25 °C) and mechanically stirred at 150 rpm with constant exposure to lamp light. Such lamplight exposure is due to the setting of the laboratory where the light is on for 24 hours. TPPBs were prepared by inoculating 5 ml of acclimated culture (refer Section 2.2.3) into each of the duplicate bioreactors containing 450 ml MSM and 50 ml silicone oil (poly(dimethylsiloxane)) 200® fluid viscosity 20 cSt (Sigma Aldrich). The control MPBs were prepared similarly but with 500 ml MSM instead, i.e. without the addition of silicone oil. The initial cell turbidity at 600 nm (OD_{600}) was 0.053 in all bioreactors.

Hexane was added at a volumetric load of $100 \text{ g m}^{-3} \text{ day}^{-1}$ by injecting 38 μl of hexane twice a day to each reactor. Aliquots of 20 ml and 5 ml respectively of the aqueous phase and the interfacial fraction (IF) in the bioreactors were removed weekly and either immediately analyzed or stored at -20 °C for further analysis. Equivalent volume of fresh MSM and silicone oil were added back into the bioreactors after weekly sampling.

The pH of the cultures in the bioreactors was monitored weekly (Figure 2.2) and when it fell below 5, it was taken as an indicator that the system required replenishing by new media. This was done by removing 200 ml of the aqueous phase of each bioreactor, followed by centrifugation at 3,000 g for 20 minutes. The pellet was then resuspended in 200 ml of fresh MSM and re-introduced into the bioreactors. This ensured that the cells were not removed from the bioreactor while a portion of the MSM was replenished.

Figure 2.2 pH of the aqueous media in the monophasic bioreactors (MPBs) and the biphasic bioreactors (TPPBs) over 52 weeks. The media of any duplicate set of bioreactors were changed when the pH of either one of the bioreactors fell to 5 or below. It was observed that TPPB1 and B2 showed variation in pH between duplicates after 30th week whereas MPB1 and 2 demonstrated greater consistency whereby their pH remained mostly above 5.

2.2.5 Monitoring to biomass and biological activities

2.2.5.1 Cell turbidity at OD₆₀₀

The cell turbidity of the aqueous phase of the bioreactors' microbial cultures was monitored weekly for 52 weeks by measuring the optical density at 600 nm (OD₆₀₀) with a spectrophotometer (Shimadzu Biospec) immediately after sample collection. Appropriate dilutions were made to ensure that measurements were made within the linear range of the spectrophotometer.

For IF samples, 200 µl aliquots were spun down at 16,000 g and the cell pellets were washed with 0.1 % Triton X-100 (Bio-Rad) in MSM for 3 times. The pellets were then resuspended in 200 µl of MSM and the appropriately diluted suspensions' OD₆₀₀ were measured.

2.2.5.2 Quantification of total cellular protein

The cell pellets from 0.5 ml of aqueous phase samples from TPPBs and 1 ml of samples from MPBs were obtained by centrifuging the cultures at 16,000 *g* for 10 minutes. The cells were lysed by boiling the pellets suspended in 0.5 ml 0.5 M NaOH for 5 minutes. The concentration of each of these total solubilized protein samples was determined by Bio-Rad Bradford protein assay (Bradford, 1976). In brief, 10 µl of the samples was aliquoted in triplicates to wells in a 96-well black-wall clear-bottom microplate (Costar). The dye reagent concentrate (Bio-Rad) was then prepared according to manufacturer's instructions and 200 µl of it was added to each well with thorough mixing. The mixtures were then incubated at room temperature for 10 minutes before measuring the absorbance at 595 nm using a microplate reader (Tecan Genios). Protein concentrations were determined from calibration curves using bovine serum albumin (BSA) (Sigma Aldrich) as protein standards over the concentration range of 0.1 to 0.5 mg/ml.

2.2.5.3 Quantification of adenosine triphosphate (ATP)

BacTiter-GloTM Microbial cells viability (Promega) assay was used to measure the concentration of ATP in the bioreactor samples. The preparations of the reagents were according to the manufacturer's instructions.

The aqueous phase samples (100 µl) from TPPBs were diluted ten times with fresh MSM, while samples from the MPBs were used directly. Triplicate aliquots of 100 µl were dispensed into wells in a 96-well black-wall clear-bottom microplate (Costar) and 100 µl of BacTiter-GloTM was then added to each well. The luminescent signal was measured using a microplate reader (Tecan Genios) at 400 nm. ATP concentrations were determined from calibration curves of ATP standards over the concentration range of 1 to 100 nM.

2.2.5.4 Culturable cell count

For both aqueous and IF samples, 10-fold serial dilutions of the microbial cultures were performed in sterile water. An additional set for the IF samples was also serially diluted using 0.1 % Triton X-100. The concept of using Triton X-100 as a diluent for the organic phase samples was adapted from (Efroymson & Alexander, 1991). The diluted samples were spread onto MSM agar plates and incubated at room temperature in a desiccator infused with hexane vapour for 7 days. The infusion was achieved by dispensing 1 ml of hexane onto 5 g of activated carbon in a ceramic bowl in the bottom compartment of a desiccator before each incubation. The numbers of colonies on the plates were separately enumerated according to the colony morphotypes (Table 3.2 under Section 3.2.4) and the sum total was calculated to obtain the total viable cell count. To store isolates for further analysis, colonies on these MSM agars were randomly selected, streaked onto fresh MSM plates and incubated similarly in a desiccator infused with hexane vapour. These colonies were harvested and suspended in MSM containing 20 % glycerol, then stored at -80 °C.

2.2.6 Microscopy

2.2.6.1 Brightfield and fluorescence microscopy

To view the microorganisms, a small drop (20 µl) of culture was wet-mounted on microscopic slides (CellPath), covered with glass coverslip (22 x 22mm, CellPath) and viewed under 10x objective or 100x oil-immersion objectives on an Eclipse 80i upright brightfield microscope (Nikon). This microscope were equipped with differential inference contrast (DIC) as well as Intensilight C-HGHI mercury lamp and filers (blue light: Ex 450-490/ DM 505/ BA 520, green light: Ex 510-560/ DM 575/ BA 590) for viewing green and red fluorescence respectively. Images were captured with DS-U2 Digital Sight camera (Nikon) and presented using ImagePro Plus 6.2 software (Media Cybernetics Inc.).

2.2.6.2 Confocal Laser Scanning Microscope (CLSM)

The interfacial fractions were evaluated as a hanging drop on a slide with a centrally dented region, under an Eclipse 90i CLSM (Nikon) equipped with 488 nm and 543 nm lasers. For the observation of green fluorescence, the channel was set at 488 nm excitation and 515/30 nm emission; for red fluorescence, 543 nm excitation and 605/25 nm emission. Confocal images of the green and red fluorescence were taken sequentially to minimize crosstalk between the channels. For observation, 60x (with oil) objectives were used. Microscope controls were manipulated by iControl software (Nikon). Images were processed by and EZ-C1 3.2 FreeViewer software (Nikon) respectively

2.2.7 Hexane degradation kinetic study

Hexane degradation efficiencies of the microbial consortia in the bioreactors were determined by taking duplicates samples of aqueous cultures (2.5 ml) from all 4 bioreactors and the IF cultures (0.17 ml) from the 2 TPPBs. These were centrifuged for 10 minutes at 1,000 g. The cell pellets were washed twice with MSM, resuspended in 2.5 ml of sterile MSM and added into the serum bottle before sealing the lid with butyl rubber and an aluminum cap. At the start of the experiment, 20 μ l of hexane was injected into the serum bottle using a syringe. Serum bottles containing only 2.5 ml of sterile MSM served as negative controls. Bottles were incubated at 25 °C with agitation at 100 rpm. Hexane gas was measured from the headspace by GC-flame ionization detector using HP-Plot/Q column with an oven temperature of 170 °C. The gas samples were collected using syringes, at 2 hour-interval in the first 12 hours, and 12 hour interval for the later time points. The concentrations of the measured hexane gas were obtained from calibration curves prepared prior to the sample analysis. Specific hexane degradation rate was calculated from the gradient of the consumption kinetics divided by the average amount of protein of the cultures which was calculated as $[(\text{Protein}_{\text{before}} + \text{Protein}_{\text{after}})/2]$. The carbon dioxide production and oxygen utilization were measured using thermal conductivity detector gas chromatography as described in Section 2.2.3.

2.3 Results

2.3.1 Assessment of soil microbial consortia for suitability as bioreactor inocula

Before the bioreactors were set up, two types of soils with differing context of exposure to hexane were collected and the microbial consortia obtained from them were evaluated for their suitability as bioreactor seed cultures. These samples were chosen because they could potentially carry hydrocarbon degrading microorganisms– the samples collected from the grassy patch along the road have been exposed to gaseous VOCs from the exhaust fumes of vehicles on the road while the soils from the car park have been exposed to oil discharges from parked vehicles.

Microbial communities from these soil samples were first put through a culturable count analysis on solid media using hexane as the sole exogenous carbon source, in order to assess their inherent hexane biodegradative capacity. Figure 2.3A shows that at the initial state when the soils were collected (purple bars), the counts of culturable hexane degraders from the grass patch consortia were higher than those from the car park by almost one order of magnitude. However, after enrichment by hexane over a period of 17 days (pink bars), the car park soil consortia showed higher CFU counts than the grass patch consortia. Comparing to the pre-enrichment state, the number of culturable hexane degraders in the car park soil microbial communities have increased over the 17-day enrichment by more than 600-fold, which is significantly higher than the less than 100-fold increase by the grass patch communities (Figure 2.3B).

This implies that between the two types of soil communities exposed to VOCs, the ones from the car park soil have higher potentials to adapt to and degrade hexane efficiently, resulting in better growth. This microbial community was therefore put through a hexane acclimatization process to be used as inocula for the bioreactors.

Figure 2.3 Effects of 17-day hexane enrichment on culturable cell counts of two types of soils. Soils were collected from a grassy patch along the road and from an oily patch in the open car park. Microbial communities obtained were and spread plated on MSM agar infused with hexane vapour, to obtain (A) absolute CFU counts before/after 17-day hexane enrichment and (B) fold increase in CFU count after 17 day hexane enrichment. The fold increases between the soils have been compared using 1-sided Student's *t*-Test, and found to be significantly different ($p < 0.05$). Values and errors bars represent the means and the standard deviation of duplicate experiments.

2.3.2 Acclimatization of the car park soil microbial consortia

A fresh batch of petroleum-contaminated soil from the car park was subsequently collected and the microbial consortium obtained was divided into 3 sets to be put through different treatments (Figure 2.1 under Section 2.2.3). The first set (Bottles A to D) was cultivated in MSM with hexane as the sole carbon source, while the second set (Bottles E and F) was cultivated in MSM with glucose and hexane. The latter set served as the positive control since glucose is the most commonly metabolizable carbon source and would be expected to support microbial growth regardless of the presence of hexane degraders. The third set (Bottles G, H and I) was cultivated in MSM in the absence of any carbon sources and was established as negative controls. These negative controls were included in this experiment to demonstrate that growth of microorganisms is dependent on the presence of carbon source, and that there are no presence of

autotrophism which may result in false positive conclusion drawn on the biomass measured in the samples carrying hexane.

As microorganisms proliferated in the serum bottles where they were placed, oxygen (O_2) in the gas space (also referred to as the headspace) would be utilized during respiration, and carbon dioxide (CO_2) would be released when the carbon sources were mineralized. The amount of CO_2 and O_2 in the headspace was therefore monitored using TCD-GC (refer Section 2.2.3) and the data are shown in Figure 2.4 below. A high percentage of CO_2 [$>9.5\%$ (v/v)] and low percentage of O_2 [$<7.9\%$ (v/v)] were measured from the headspace of the positive control set (cultivated in glucose and hexane, pink symbols) as early as after Day 3, indicating that the microbial cultures in the bottles were indeed viable. Since the positive control was able to establish the viability of the seed community fairly early, beyond day 12, we deemed unnecessary to continue monitoring the activities of the consortia. Conversely, the negative controls (no carbon source added, yellow symbols) exhibited no significant generation of CO_2 [$<0.07\%$ (v/v)] and maintained levels of O_2 [$>18.1\%$] which were comparable to that present in the atmospheric air. Hence, this result revealed that no growth in the absence of organic carbon source, indicating that there were no autotrophic microorganisms. As this occurred in the presence of lamplight exposure, photoautotrophic microorganisms were clearly absent in the soil samples.

The four cultures of the set which was solely acclimated with hexane (blue symbols) started to show CO_2 production and O_2 utilization varying between Days 3 and 11, and these activities also quantitatively differed across samples. Clearly, there were differences in growth rates by microorganisms in each sample, despite the fact that they were inoculated with identical volumes of aliquots from the same pool of soil consortium. This observation was not surprising as minute differences in the starting composition of microbial cultures due to uneven distribution often result in a divergence in the overall community growth, which could be what was happening in the different serum bottles. This chaotic nature of microbial consortium has been observed in many consortia-related studies (Roelke et al, 2003).

Monitoring of the positive control set was stopped after Day 11 because the viability of the inoculated community was already clearly established. The hexane-acclimated set was pooled together on day 24 and subcultured into serum bottles 1 to 3 while the negative control were likewise pooled and subcultured into bottles 4 and 5 (Figure 2.1 under Section 2.2.3). For the hexane-acclimated set, the CO₂ generation and O₂ continued after the subculturing, and it was again observed that there were quantitative differences among the different bottles measured, but the variations were minor compared to the earlier period before pooling and subculturing. The microbial cultures from bottles 1- 3 were subsequently pooled on Day 50 to be used as initial microbial consortia for the bioreactors.

Figure 2.4 (A) Carbon dioxide (CO₂) production and (B) oxygen (O₂) utilization by the microbial consortium from car park soil, as analyzed by TCD-GC. Bottles A to D were fed with 0.1 g of hexane as the sole carbon source. The positive controls, Bottle E and F were fed with 0.1 g of hexane and 0.025 g of glucose and showed carbon dioxide production and oxygen utilization, indicating viability of the starting microbial cultures. As such, the monitoring for these bottles was stopped after 11 days. As for the negative controls, Bottle G, H and I, no organic carbon source was provided and they showed little microbial growth. On day 24, Bottles A to D were pooled together and distributed into Bottles 1 to 3 (fed with hexane) while Bottles G to I were pooled and distributed into Bottles 4 and 5 (no carbon source).

2.3.3 Development of biomass and biological activities in the bioreactors

Studies conducted on TPPB systems previously have demonstrated good pollutant removal rates and kinetics (Daugulis & Boudreau, 2003; Hernández et al, 2012; Muñoz et al, 2006), but knowledge of microbial dynamics within the TPPBs remains lacking, especially with long-term operation. Hence, we set up a TPPB system of MSM medium with 10% silicone oil as the NAL phase as described in Section 2.2.4, using the 50-days acclimated microbial consortium as the seed culture, to study the temporal development of this complex microbial community fed with hexane for over 52 weeks. A control monophasic system was also initiated with the same acclimated microbial inoculum to serve as reference for comparison, in order to understand the influence of the NAL to the microbial dynamics in the TPPBs.

2.3.3.1 Microbial biomass in aqueous phase of TPPBs versus MPBs

As the aqueous phase of TPPB systems contains nutrients required to support microbial growth, most of the microorganisms are expected to reside in this phase (Daugulis, 2001). Hence, sampling from the aqueous phase was done weekly to monitor biomass and metabolic activity indicators such as cell turbidity (OD_{600}), total cellular protein level, ATP concentration and culturable cell count, in order to assess the level of collective activities that had arisen from the microorganisms utilizing hexane directly and indirectly as the only source of carbon.

2.3.3.1.1 Cell density(OD_{600}) of aqueous phase cultures

Since an increase in cell density would be an indicator of microbial growth as a consequence of hexane metabolism within the bioreactors, the accumulation of biomass in the cultures were tracked through turbidimetric measurement at 600 nm (OD_{600}). Figure 2.5 showed that the cell densities of the aqueous phase of TPPBs (blue symbols) were consistently greater, by one to two orders of magnitude, than those in the MPBs (pink symbols) across time. The

TPPBs experienced an initial surge in cell density over Weeks 0 to 12 followed by stabilization in the cell density from then onwards. However, the MPBs continued to show an increase in the cell density across 52 weeks of operation, albeit much more slowly after week 12. Within the control TPPB and MPB which were not fed with hexane or any other organic carbon sources (star-shaped symbols), no detectable increase in cell density was observed even after three months of operation. This showed that the biomass increment across time in the TPPBs and the MPBs were indeed due to the utilization of hexane by the microbial consortia in both types of bioreactors.

The determination of cell turbidity makes use of light scattering by cells, i.e. when the number of microbial cells increases, turbidity increases as light passing through is scattered to a greater extent by microbial cells. Although turbidity indirectly reflects the accumulation of biomass in the cultures, it is unable to distinguish the live cells from the dead ones. To complement this set of data, further measurements such as total protein level and ATP concentration were made.

Figure 2.5 Cell density (as analyzed by turbidity) of aqueous phase cultures of the bioreactors. Growth of microbial cultures in the TPPBs and the MPBs, which fed with hexane as sole carbon source, was monitored by optical density at 600 nm (OD₆₀₀) over a period of 52 weeks. TPPB and MPB controls with no carbon (C) source were also set up and showed little/no

increase in their cell densities over 12 weeks. Values and errors bars represent the means and the standard deviation of measurements in duplicates.

2.3.3.1.2 Total cellular protein content of aqueous phase culture

Monitoring the total cellular protein contents serves as an alternative means to estimate biomass accumulation by microbial communities. For this, Bradford protein assay (Bradford, 1976) was carried out weekly over the 52-week operation of the TPPBs and MPBs. Figure 2.6 clearly showed that the total protein contents were higher by more than 10-fold in the aqueous phase microbial communities of TPPBs than those of the MPBs across time even though they were supplied with the same level of hexane daily.

Interestingly, the total cellular protein level of the TPPBs, after the initial rapid increase, appeared to stabilize over Weeks 16 to 32, but thereafter entered another period of steady increase until Week 52. The latter trend was not observed in the turbidity profiles (Figure 2.5), shown in the previous section implying that even though the OD₆₀₀ (which is dependent on the number of “particles”, i.e. cells that scatter light) have remained relatively unchanged, and protein biosynthesis within the cells have continued to increase even after a long period of operation.

Figure 2.6 Total cellular protein content of aqueous phase cultures of the bioreactors. Total protein contents of the cell pellets collected from the microbial cultures in the TPPBs and the MPBs over a period of 52 weeks were determined by Bradford protein assay. Values and errors bars represent the means and the standard deviations of measurements in duplicates.

2.3.3.1.3 ATP quantification of aqueous phase cultures

As ATP is only produced by viable cells undergoing metabolism, it serves as an indicator for the presence of metabolically active cells in the microbial communities (Hammes et al, 2010; Karl, 1980). By quantifying the concentration of ATP of the microbial cultures sampled from TPPBs and the MPBs using BacTiter-Glo™ Microbial Cell Viability Assay, we were able to compare the level of metabolic activities occurring in these bioreactors. As shown in Figure 2.7, the ATP levels in both TPPBs were varying within the range of 400-800nM but that in the MPBs remained within a much lower range of 0-100nM. These data reinforced the fact that there were greater degrees of biological activities occurring in the TPPBs compared to the MPBs, even though same amounts of hexane were fed to the two types of bioreactors.

Similarly to the temporal profiles of cell densities and protein contents in the TPPBs, it was possible to discern a period of stable ATP concentration level after Weeks 12-16. In addition, as in the case of protein level, a further increase occurred from Weeks 32 to 44, but it was noted that the degree of increase was more substantial in TPPB1 than in TPPB2. Complexity leading to divergence is inherent to any consortium-based inocula especially after long-term culturing, and in this instance, between our duplicate set of TPPBs, a divergence in the ATP level has apparently manifested.

Figure 2.7 ATP concentrations of aqueous phase cultures of the bioreactors. ATP levels in the aqueous phases of the TPPBs and the MPBs were determined using BacTiter-GloTM Microbial Cell Viability Assay (Promega) over a period of 52 weeks. Values and errors bars represent the means and the standard deviation of measurements in triplicates.

2.3.3.1.4 Culturable cell count of aqueous phase cultures

In addition to assessing changes in the overall cell densities and metabolic activities of the bioreactor cultures, temporal development of their culturable subpopulation was determined. This was executed by spread plating the cultures on MSM agar infused with hexane vapour, and the results are presented as Colony Forming Units (CFU) per unit volume (Figure 2.8). After the rapid initial increment from 10^5 CFU/ml at Week 0 to 10^9 CFU/ml at Week 2, the CFU counts from the TPPBs remained fairly stable (varying within one order of magnitude) over the rest of the 52 weeks. In comparison, the CFU counts of the MPB communities showed an initial increase from Week 0 to Week 2 but only to 10^8 CFU/ml, and thereafter, the counts sometimes dropped to the 10^6 CFU/ml range. Clearly, the TPPBs were better able to support the growth of the hexane-utilizing culturable subpopulation than the MPBs, and this was observed to be sustained over the long term.

The turbidity assay (Figure 2.5) showed increasing cell density up to Week 12 but this increase was not correspondingly reflected in the culturable cell count profiles shown here. This implies that a proportion of the cells in the liquid cultures were non- culturable on solid media when hexane was supplemented as the sole carbon source. Because a colony is in principle formed from a single cell, if the microorganism does not have the capacity to mineralize hexane completely on its own, a colony may not be generated. In the liquid culture context, however, after the initial steps of biotransformation by, for instance, a hexane degrader, the intermediates (which more readily diffuse in a liquid medium) may be fed into the metabolic pathways of non-hexane-degraders, allowing them to proliferate as well. Therefore, in this study, the culturable subpopulation would be loosely interpreted as the subpopulation of hexane degraders. The data shown in Figure 2.8 therefore suggest that the TPPBs were able to sustain a larger population of hexane degraders than the MPBs.

Figure 2.8 Culturable cell count of aqueous phase cultures of the bioreactors as analyzed by spread plating on MSM agar infused with hexane. Culturable cell counts of the aqueous phase of the TPPBs and the MPBs were determined by spread plate colony-counting method and expressed as colony forming units (CFU)/ml, over a period of 52 weeks. Values and errors bars represent the means and the standard deviation of CFU counts from duplicate experiments.

2.3.3.1.5 Hexane degradation efficiency of aqueous phase cultures

Collectively, the data presented in Section 2.3.3.1.1 - 2.3.3.1.4 allowed us to validate the ability of our TPPB system to sustain more active and abundant microbial growth than MPBs over this 52-week long operation. Although an enhanced level of substrate transfer due to the presence of the NAL (refer Section 1.5) would have led to this, an inherent difference in the hexane utilization efficiency of the microbial consortia in the TPPBs versus the MPBs may have developed over time and contributed to this phenomenon further. To explore this possibility, the hexane removal kinetics of the cultures within the bioreactors was determined, at Weeks 28 and 44. The time points were chosen because Week 28 was within the period when all of the biological activity indicators (Figures 2.5-2.8) in the TPPBs appeared to have attained some level of stability after Week 16,

while Week 44 was within the period whereby the microbial consortia have progressed from this stable state to a somewhat higher activity state (refer Figures 2.6 and 2.7 after Week 32).

In this experiment, a known dose of hexane was injected into sealed serum bottles containing microbial cultures sampled from the MPBs and the aqueous phases of the TPPBs. The quantity of hexane remaining in the headspace of the serum bottles were measured by GC-FID and plotted against time to derive the rates of hexane degradation (mg/h). In order to calculate the specific hexane degradation rates for comparison across the samples, the hexane degradation rates were normalized against the total cellular protein content in each consortium.

Figure 2.9A shows that at Week 28, microbial consortia from the aqueous phase of TPPBs had a lower specific hexane degradation rate [0.12 and 0.11 mg hexane/hour/mg protein)] compared to that of the MPBs [0.23 and 0.40 mg hexane/hour/mg protein)]. However, the overall hexane removal efficiencies of the TPPBs were higher than that of the MPBs due to the higher total protein contents (i.e. greater biomass present) in the TPPBs (Figure 2.9B). At the later Week 44, the TPPBs showed at least 16-fold increase in the specific hexane degradation rates compared to those at Week 28. The MPBs, on the other hand, seemed to have maintained almost similar specific rates of degradation between Weeks 28 and 44. This set of data suggests that the microbial consortia of TPPBs have developed in such a way as to become better adapted (per unit biomass) for hexane removal across time, unlike the MPBs. With the improved specific hexane degradation rates, the overall TPPB system has become even more efficient in removing hexane at Week 44 than the MPBs which continued to show no significant increase in activity over time (Figure 2.9B). The higher biological activities reflected by total cellular protein level and ATP concentration of the TPPBs after Week 32 (Figures 2.6 and 2.7) corroborated well with the notion that communities were better able to develop into higher efficiency states in the biphasic context than in a monophasic environment.

Figure 2.9 Specific (A) and total (B) hexane degradation rates of the microbial consortia in the aqueous fractions of TPPBs and MPBs at 28th and 44th weeks. (A) Specific hexane degradation rate of the aqueous phase cultures in the bioreactors were analyzed by GC-FID to obtain the rates of hexane degraded per hour (in mg/hour), which was then normalized to the average of total cellular protein content between the start and end of the assay, present in each sample (mg protein). (B) Total hexane degradation rates were calculated by multiplying the specific rates for the respective samples to their total cellular protein (mg protein) to obtain the overall hexane degradation rates exhibited by the aqueous phase of the TPPBs and the MPBs. Data pairs of 28th and 44th weeks were compared using 1-sided Student's *t*-Test and * indicates statistically significant difference ($p < 0.05$). Values and errors bars in both panels represent the means and the standard deviations of measurements in duplicates.

2.3.3.2 Microbial biomass associated with TPPB interfacial fractions

It was clear that compared to the MPBs, there were significantly enhanced hexane removal activity by the microbial consortia residing in the aqueous phase of the TPPBs. However, 10% of the TPPB's volume was composed of the NAL, i.e. silicone oil, and so there was a possibility of microbial growth occurring within the NAL phase (Hernández et al, 2012; Muñoz et al, 2013) and/or at the aqueous/NAL interface (Ascón-Cabrera & Lebeault, 1993; Ascón-Cabrera & Lebeault, 1995a), which should be looked into.

As early as in Week 2, it was observed that the initially clear layer of NAL phase in the TPPBs gradually decreased in volume to be replaced by stable emulsion layers of about 3 cm thick. This was in contrast to the NAL layer in the control TPPB operating with no organic carbon (Figure 2.5 under Section 2.3.3.1.1) which maintained its clarity throughout the three months of operation. We inferred that the emulsion layers in the hexane-fed TPPBs were likely due to the influence of the microorganisms, since emulsion was not generated in the absence of microbial growth in the control TPPB. The localization and growth of microorganisms in association with the aqueous/NAL interfacial fraction (IF) was therefore examined.

Figure 2.10 Photographic image of the emulsion layer (interfacial fraction) and the aqueous phase observed in TPPB1 (at Week 45 of operation). In place of the clear NAL was a dense emulsion layer, approximately 3 cm thick, which was formed as early as after Week 2 and remained present throughout the 52 weeks of operation. TPPB2 displayed a similar phenomenon as TPPB1 (data not shown).

2.3.3.2.1 Microscopic examination of microorganisms within IF

In order to determine how microorganisms interacted with the IFs which made up the emulsions in the TPPBs, aliquots of IFs were mounted on glass slides in a hanging-drop manner and observed under the bright-field microscope for the presence of microorganisms. A considerable number of microbial cells was found to be associated with the globules at the aqueous/NAL interface (Figure 2.11). The microbes (M) appeared to be localized on the outside of the globules (G), and not within.

The samples were further analyzed by confocal laser scanning microscopy (CLSM). Firstly, to determine whether the globules shown in Figure 2.11 were oil-in-water or water-in-oil or a combination of both (Dorobantu et al, 2004; Janiyani et al, 1994), an oil-binding fluorescent dye, Nile red, was applied. The specificity of Nile red in staining the NAL silicone oil is demonstrated in Figure 2.12 – when Nile red was applied to a mixture of MSM medium and silicone oil, no fluorescence was observed from the aqueous phase (Figure 2.12A) but the silicone oil fraction registered a bright red fluorescence (Figure 2.12B). Together with SYTO- 9 which could stain microbial cells fluorescent green, Nile red was applied to the IF samples of the TPPBs, and viewed under the CLSM. The images (Figure 2.13) clearly showed the oil-in-water nature of the globules, i.e. the silicone oil was the dispersed phase, and MSM was the dispersion medium, in the IFs of our TPPBs. It was clear from the three dimensional images that the microbial cells were actually present in the aqueous phase and adhered to the surface of the oil globules, but not residing within the oil phase per se. As silicone oil has a high affinity for hexane (Muñoz et al, 2006; Muñoz et al, 2007a), there will be greater availability of hexane (food source) in the silicone oil globules. Some microorganisms which are able to utilize hexane may therefore prefer to cluster/ associate with the oil globules, resulting in a direct substrate uptake from the aqueous/NAL interface (Ascón-Cabrera & Lebeault, 1993; Ascón-Cabrera & Lebeault, 1995a) .

Figure 2.11 Bright-field microscopy at 600x magnification showing microorganisms (refer to M) associated with globules (refer to G) in the aqueous/NAL emulsion layer from TPPB1. Similar observation was made from TPPB2 (data not shown). Size bar: 25 μm .

Figure 2.12 Fluorescence Microscopy at 100x magnification showing (A) MSM medium stained with Nile red and (B) mixture of silicone oil (right) and MSM medium (left) stained with Nile red. Nile red stains the silicone oil fluorescent red but not the aqueous phase. Size bar: 100 μm .

Figure 2.13 CLSM images at 600x magnification, showing the interaction of microorganisms with oil-in-water globules under (A) composite x-y/x-z/y-z 2-dimensional plane views and (B) 3-dimensionally rendered-space view. The microorganisms were stained with SYTO-9 (fluorescent green) while the oil globules were stained with Nile red. Some microorganisms with possibly hydrophobic surface and/or NAL attached on its surface were stained by Nile Red as well, and appeared orange/yellow (indicated by white arrows in A). Size bar: 30 μm .

2.3.3.2.2 Cell density(OD_{600}) of TPPB IFs

Since microorganisms were found within the IFs, cell densities of the weekly samples of IFs collected from the TPPBs were measured (OD_{600}) over the 52 weeks of operation. Figure 2.14 shows that cell densities within the IFs from Week 2 onwards were comparable to that from the aqueous phase of the TPPBs (Figure 2.5 under Section 2.3.3.1.1). The temporal cell density profiles of the IFs showed a similar growth trend as the aqueous phase cultures of the TPPBs, i.e. there was an initial surge in cell density over the first 12 weeks followed by stabilization.

It should be remembered that the IF was in fact formed through the mixing of two phases, i.e. the aqueous MSM and the silicone oil. The aqueous portion accounted for approximately half of the total volume of the IF. Given that the microorganisms were presiding exclusively in the aqueous portion of the IF as shown by the earlier CLSM images (Figure 2.13), the actual cell density within the aqueous phase volume of the IF may be estimated to be double that of the values shown in Figure 2.14. The IF could therefore be viewed as a region in the TPPB where the microbial cells are locally concentrated.

Figure 2.14 Cell density (as analyzed by turbidity) of cultures in the IFs of TPPBs. Growth of microbial cultures in the IFs of TPPBs (TPPB1-IF and TPPB2-IF), fed with hexane as sole carbon source, was monitored by optical density at 600 nm (OD_{600}) over 52 weeks. Values and errors bars represent the means and the standard deviations of measurements in duplicates.

2.3.3.2.3 Culturable cell count of TPPB IFs

To gain a fuller picture of the microbial subpopulation associated with the IFs of the TPPBs, culturable cell count of the IFs were conducted. An additional factor taken into consideration when executing this analysis compared to that of the aqueous phase fraction presented earlier (Section 2.3.3.1.4) was that prior to spreading on the agar plates, i.e. during serial dilution, there was a need for microbial cells to be dissociated from the oil globules and from each other. As discussed (Figure 2.11), we have observed that cells were adhered to the surfaces of the oil globules in the IFs of the TPPBs. The clumping of these microorganisms would affect the final CFU counts obtained, resulting in an underestimation (Heritage et al, 2000).

To dissociate the microorganisms from the oil globules, we treated the IF samples with Triton X-100, a chemical surfactant that has previously been shown not to affect the viability of bacteria such as *Arthrobacter* species (Efroymson & Alexander, 1991). As it was unknown if such treatment would affect other species of microorganisms in our consortia, the Triton X-100 surfactant treatment was first performed on the aqueous phase samples from TPPBs to validate that such treatment would not affect the viability of the microbial cells. The treated sets indeed showed no significant difference in CFU counts compared to the non-treated sets (Figure 2.15A, black symbols). When the Triton X-100 treatment was applied to the IFs, a higher count by one order of magnitude could be obtained (Figure 2.15A, pink symbols) and microbial cells associated with the oil globules were verified via microscopy to be released and dispersed (Figure 2.15B and C).

The culturable subpopulations in the IFs of the TPPBs were thus enumerated (Figure 2.16) and comparison was made to that of the respective aqueous phase of the TPPBs (Figure 2.8). The CFU counts from the IFs were found to be slightly higher than the aqueous phase cultures. If the culturable subpopulation may be interpreted to be the hexane-degrading subpopulation as discussed in Section 2.3.3.1.4, our data would suggest that there was an enrichment of hexane degraders within the IFs from the main bulk aqueous phase cultures of the TPPBs. This is likely since the hexane degraders are the ones that

can benefit most from the proximity to the high concentration of hexane dissolved in the NAL.

Figure 2.15 Treatment of microbial cultures with Triton X-100. (A) Samples from the aqueous phase and IFs of TPPBs were treated with and without 0.1 % Triton X-100 before culturable cell count (CFU/ml) was performed. Values and errors bars represent the means and the standard deviations of measurements in duplicates. The culturable cells obtained from the treated set were compared with the non-treated sets using 2-sided Student's *t*-Test respectively and * indicates significant difference between the two sets of data ($p < 0.05$). Brightfield microscope images at 1000x magnification showed the IF of TPPB1 before (B) and after (C) Triton X-100 treatment. The microorganisms were aggregated and associating with the NAL globules at first (B) but became dispersed in the cultures after Triton X-100 treatment, and no clusters of microorganisms were observed to be associated with the oil globules (C). Size bar: 10 μ m. Similar observation could be made from the IF of TPPB2 (data not shown).

Figure 2.16 Culturable cell count of the IF of TPPBs as analyzed by spread plating on MSM agar infused with hexane. Culturable cell counts of the IFs of the TPPBs were determined by spread plate colony-counting method and expressed as CFU/ml, over a period of 52 weeks. The IF samples were treated with 0.1% Triton X-100 to facilitate aggregate/globule dispersal. Values and errors bars represent the means and the standard deviations of CFU counts from duplicate experiments.

2.3.3.2.4 Hexane degradation efficiency of TPPB IFs

If indeed the IFs of the TPPBs carried an enrichment of hexane degraders as speculated above, the microbial consortia may exhibit higher hexane degradation efficiency than those in the aqueous phases of the TPPBs. We therefore proceeded to assay for the hexane degradation rates of the microbial populations present in the IFs of the TPPBs at Week 44. Figure 2.17 shows that the specific hexane degradation rates of the consortia in the IFs were significantly higher than that of the corresponding aqueous phase consortia. This suggests that the IF microbial subpopulation were more efficient at degrading hexane compared to those within the bulk aqueous phase of the TPPBs, and that there could be some differences in the microbial makeup between the two fractions. Nevertheless, since microbial communities present in both the IF and aqueous phase of TPPBs could degrade hexane more efficiently than those in the MPBs (see horizontal

line drawn in Figure 2.17), the TPPBs as a whole would be able to remove more hexane per unit time in comparison.

Figure 2.17 Specific hexane degradation rates of the microbial consortia in the interfacial fraction (IF) and aqueous phase of TPPBs at 44th week. Specific hexane degradation rate of the cultures in the IFs of TPPBs were analyzed by GC-FID to obtain the rates of hexane degraded per hour (in mg/hour), which was then normalised to the average of total cellular protein content between the start and end of the assay, present in each sample (mg protein). The results of aqueous phase samples of TPPBs presented here have been shown previously in Figure 2.9 (Section 2.3.3.1.5) under a different context. The horizontal line representing the average specific hexane degradation rate of the MPBs (derived from Figure 2.9) has been included as a reference for comparison. Values and errors bars represent the means and the standard deviations of measurements in duplicates. The specific hexane degradation rates between pairs of data were compared using 1-sided Student's *t*-Test and * indicates statistically significant difference ($p < 0.05$).

2.4 Discussion and Conclusions

2.4.1 Enhanced microbial growth and metabolic activities in TPPBs

In past studies, productive mineralization of sole carbon source by microorganisms has been noted through outcomes such as microbial biomass accumulation (Li et al, 2010; Lobos et al, 1992; Yanzekontchou & Gschwind, 1994). Similarly, in our study, feeding the bioreactors with hexane as the only exogenous carbon source had led to the consequence of microbial growth in both the TPPBs and MPBs (Figure 2.5). Although both types of bioreactors were supplied daily with the same amount of hexane over the 52 weeks of operation, the TPPBs were able to support a greater level of microbial growth (Figure 2.5 and 2.8), protein synthesis (Figure 2.6) and activities (Figure 2.7) than the MPBs.

These results were comparable to other studies (Aldric & Thonart, 2008; Hernández et al, 2012; Muñoz et al, 2013) which also demonstrated enhanced microbial growth in TPPBs compared to MPBs. For example, Muñoz et al. (2013) reported that an enhanced microbial growth in the aqueous phase of the TPPBs, reaching a maximum OD₆₅₀ value of 2.6 by day 41 of the bioreactor operation. Microbial growth was also observed to accumulate at the aqueous/NAL interface in this system. Such growth was significantly higher compared to the control deprived of NAL. These experimental findings were similar to the significant differences in microbial growth observed between the TPPBs and MPBs in our study.

Our data have given us the assurance that the TPPB set-up established in this study was able to manifest the advantage of a biphasic system over its monophasic control, not only briefly, but over as long as 52 weeks. This, to the best of our knowledge, is the longest reported stable operation of TPPB to date (Table 1.1 under Section 1.3).

2.4.2 Localization of microbial subpopulations at aqueous/NAL interface

Optical density (Figure 2.14) and culturable cell count (Figure 2.16) analyses have revealed that microorganisms were present at a fairly high cell density in the IFs of the TPPBs. Microscopic observation was able to localize these cells to the aqueous/NAL interface and the aqueous milieu of the emulsion (Figures 2.11 and 2.13). This high cell density distribution to IF was similar to what was reported by Ascón-Cabrera and Lebeault (1993), and Ascón-Cabrera and Lebeault (1995a). The fact that no microorganisms were found embedded or presiding in the NAL phase itself suggests that the nature of our microbial consortium was only moderately hydrophobic. This contrasts with the consortia used by Hernandez et al (2012) and Muñoz et al (2013) which were extremely hydrophobic, to the extent that the microorganisms were found completely immersed within the NAL phase.

Enhanced biodegradation of hydrophobic pollutants in TPPBs have been attributed to the attachment of microbial cells to the NAL/aqueous interface previously (Ascón-Cabrera & Lebeault, 1995b; Hernández & Muñoz Torre, 2011; MacLeod & Daugulis, 2005). For microorganisms that are able to metabolize the substrate dissolved in the NAL, associating with the interface may well be a consequence of their chemotactic response (Hernández et al, 2012; MacLeod & Daugulis, 2005). In addition, the higher cell surface hydrophobicity of a subpopulation of microorganisms may have driven them to interfacial adherence (Ascón-Cabrera & Lebeault, 1995a). Compounded with the fact that an emulsion (by nature of its stable NAL-in-water structure) increases the aqueous/NAL interfacial surface area, microorganisms associating with the IF would clearly benefit from a greater bioavailability of the substrate than within the bulk aqueous phase, as the latter relies on a balance of partitioning equilibrium, diffusion and fluid mixing to deliver the hydrophobic substrates to the resident microorganisms. The significant presence of microorganisms in the IFs of our TPPBs implies that the original inoculum (acclimated from petroleum-contaminated soil) carried a subpopulation with properties that could leverage on these benefits, and this subpopulation was further enriched over time through localization to the IF.

2.4.3 Development of higher hexane degradation efficiency by microbial consortia in TPPB over time

The greater microbial growth in our TPPBs sustained over a long-term operation has affirmed that our biphasic system was able to provide a more viable environment for the microbial consortia to utilize hexane effectively than the monophasic system (discussed in Section 2.4.1). An enrichment of microbial subpopulation within the IFs of our TPPBs via interfacial association was also demonstrated (discussed in Section 2.4.2), and this factor very likely compounded the increased bioavailability of hexane already afforded by the presence of the NAL through establishment of the gas/NAL/water substrate transfer pathway. In addition to these findings, comparison of the specific hexane degradation rates of the bioreactors' microbial consortia (Figures 2.9 and 2.17) was able to provide further insights to the enhanced performance of the TPPBs over the MPBs.

Hexane degradation rate analysis conducted on Week 44 samples of the bioreactors revealed that, compared to the microbial communities in the MPBs, those in the aqueous phase and the IFs of TPPBs possessed higher hexane removal efficiency per unit mass of total cellular protein (Fig 2.17, compare the bars against the horizontal line). In accordance to our expectation that the IF-associated microbial subpopulations may be more enriched in hexane degraders (refer Section 2.3.3.2 and 2.4.2), the specific hexane degradation rates of the IF samples were indeed found to be significantly higher than those of the aqueous phase samples (Figure 2.17). Overall, these data suggest to us that the differences between the MPBs and the TPPBs with respect to their microbial consortia's capacity to metabolize hexane must have some basis in their community composition.

However, the significant difference in specific hexane degradation rates apparently only manifested after considerable temporal development in the respective microbial communities. At Week 28, although the overall hexane degradation rates of the TPPBs were also higher than those of the MPBs (Figure 2.9B), the specific rates, i.e. per unit biomass, exhibited by the MPB communities were in fact slightly (although not significantly) higher than those of the TPPBs

(Figure 2.9A). Hence in the earlier period of TPPB operation (at least up to Week 28), it was by virtue of the higher microbial biomass generated within the TPPBs that resulted in an enhanced hexane degradation over the MPBs. Over the period between Weeks 28 and 44, the microbial communities within TPPBs have apparently developed into greater efficiency per unit biomass by at least 16-fold, but this was not observed in the MPBs (Figure 2.9A). As far as we are aware, this is the first time that a TPPB environment's ability to enhance microbial communities' inherent degradation capacity across time was illustrated. The long term exposure to non-toxic levels of hexane through the biphasic system might have potentially helped the microbial community to arrive at a composition highly efficient in degrading hexane. The community structure may be one whereby the members are working together in a synergistic manner to generate a collectively more effective output in terms of hexane biodegradation.

In conclusion, we would not have been able to gain this insight if the study was not performed over a sufficiently long period, because even at 28 weeks of operation—which was already longer than any of the previously reported duration of studies (refer Table 1.1) – the changes in the efficiency of microbial communities were not yet detectable. Hence, the microbial samples collected over this long-term operation would be a valuable material for study, the analysis of which may bring about an understanding of how microbial community develop into more effective biodegradation, perhaps through better coordination and interaction. In particular, we were motivated to further examine the distribution of microorganisms between the aqueous phase and the IFs and these two fractions of the TPPBs would continue to be characterized separately in order to dissect the underlying interactions and mechanisms. These studies shall be presented in the next chapter.

Chapter 3: Bioreactor Microbial Community Analysis

3.1 Introduction

In this chapter, the focus will be placed on the analysis of the bioreactors' microbial community structure across time, not only in terms of their dynamics but also in relation to the identification of component microbial groups. Several approaches have been engaged to provide, as much as possible, a holistic and relevant picture. Ultimately, the microorganisms identified in the TPPBs and the MPBs may provide valuable clues to the collective scheme of hexane utilization within the communities, and some of their properties and interactions could be further examined (Chapter 4).

3.1.1 Microbial community analysis

Microbial communities occur in varied contexts, ranging from those with only several taxa existing under extreme conditions, to those with greater diversity and exhibiting a high level of complexity due to the myriad of interactions within (Desai et al, 2010). The analyses of microbial communities in natural habitats such as soil, river sediments and marine environments (Deziel et al, 1999; Hammes et al, 2010; Hilyard et al, 2008; van Elsas & Boersma, 2011; Webster et al, 2010) as well as artificial systems such as wastewater treatment facilities, industrial effluents and bioremediation sites (Sanz & Köchling, 2007; van der Gast et al, 2002; Van Hamme et al, 2003; Wang et al, 2007) have been undertaken with differing purposes. All these studies have contributed to our understanding of microbial ecology and interactions to different extent but serious challenges remain. This is partly due to the fact that microbial communities are often exceedingly diverse. For example, soils have been estimated to carry between 4,000 and 10,000 different microbial genomes per gram (Desai et al, 2010).

Unfortunately, the tools for discriminating (identification of taxa) and measuring (quantifying) microbial populations within a complex mixture (the community) are far from ideal. Approaches for analysing microbial diversity and dynamics can be broadly classified into culture-dependent or culture-independent methods. Up to several decades ago, culture-based methods were the mainstay for the analysis and identification of microorganisms present in sampled microbial communities, through the use of a repertoire of physiological and biochemical tests (Lynch et al, 2004). Early pioneering culture-independent “molecular” approaches then attempted to directly analyze the whole microbial community’s collective DNA with respect to their re-annealing and/or hybridization behaviour (van Elsas & Boersma, 2011). This was meant to provide an “overall view” of community diversity and make-up but is understandably low in resolution. From the late 1980’s, other microbial community analysis techniques progressively evolved in the wake of the incredible advances in molecular biological methods. The molecularly-based approaches were able to not only produce “snapshots” of the whole complex communities, but also to provide identification of specific microorganisms and genes therein (Desai et al, 2010; van Elsas & Boersma, 2011). As the field moves into the current era, we see a surge in the high and ultra-high throughput technologies making it possible to employ approaches such as metagenomics and metaproteomics.

Ultimately, the choice of methods and approaches leading to the respective levels of resolution of microbial community structures has to be made based on a balance between the purposes motivating our scientific inquiries and the availability of technical resources.

3.1.2 Culture-based approaches for analysis of microbial community

Traditional culture-based methods, as the name implies, typically require the cultivation of microorganisms. These microorganisms are then characterized and distinguished based on differential morphology, metabolic traits, and physiological responses (Kämpfer et al, 1991; Moore & Holdeman, 1972). Quantification is also possible through the enumeration of colonies on solid media (Reasoner & Geldreich, 1985; Wrenn & Venosa, 1996), as proliferation of

each viable cell will in principle give rise to a single colony (Akkermans et al, 1994; Bruns et al, 2003). With the advent of molecular techniques, identification of the cultivated microorganisms by the phenotype-based approaches was eventually replaced by ribosomal RNA gene sequence-based methods (Section 3.1.3.1) due to their higher level of reliability in taxonomic classification (Dahllof, 2002). Therefore, it should be emphasized that although the methods in these two consecutive sections here are organized as “culture-based” and “culture-independent”, they are not mutually exclusive – the former has to be brought to completion by some of the latter approaches, and vice versa.

Culture-based methods are saddled by considerable limitations, because more often than not, only a small fraction of the microbiota (particularly in natural environments) can be accessed on the basis of cultivation (van Elsas & Boersma, 2011). The ideal growth conditions and growth requirements for most microorganisms are in fact not well-known, hence only a small proportion of microorganisms in complex communities will appear to be “culturable” under the conventional cultivation conditions used (Amann & Fuchs, 2008; Huijsdens et al, 2003; Langendijk et al, 1995; Zoetendal et al, 1998). Another limiting factor arises from the situation that growth of some species in the microbial communities may be selected against, even when deliberate care has been taken to use non-selective media in the analysis. These species may be inhibited in growth due to the competition imposed by the more abundant species growing in that culture medium. Alternatively, inhibition may have occurred as a result of high proportions of nutrients in the medium overriding the metabolism of the bacteria, even leading to its death – a phenomenon referred to as “substrate-accelerated death” (Joint et al, 2010).

Overall, it is reported that only about 0.001 % to 40 % of the microorganisms in microbial communities are culturable by common microbiological techniques (Amann & Fuchs, 2008; Hill et al, 2005; Kirk et al, 2004). Hence relying only on culture-based methods for community analysis can be expected to produce a significant degree of bias in the interpretation of both the diversity and the dynamics of the microbial consortia under study.

3.1.3 Culture- independent approaches based on DNA sequences

Researchers soon realized that the more over-arching way to understand the complexity of a microbial community was by developing direct molecular assessments without relying on the need for cultivation. A number of culture-independent methods have therefore been developed over the years to address the gap left by the culture-dependent methods. These analytical techniques allow the characterization of microbial communities' DNA, RNA, protein, structural components or metabolites, or combinations thereof (Cole et al, 2010; Kirk et al, 2004; Torsvik et al, 1998; Zhao et al, 2011).

In particular, the application of PCR in combination with the extraction of DNA and RNA from sampled microbiota has been the driving force that propelled the development of culture-independent approaches forward (Smith & Osborn, 2009). A wide collection of PCR-based methodologies have emerged to study specific groups of microorganisms and specific genes, and to evaluate overall community profiles (Dahllof, 2002). While these techniques have eliminated the reliance on cultivation and circumvented the associated biases, we need to be aware that PCR itself has also inherent limitations (Smith & Osborn, 2009). For instance, biases in the template-to-product ratios of target sequences amplified during PCR of mixed cultures have been reported (Polz & Cavanaugh, 1998), and the bias is observed to be more severe when the number of PCR cycles is increased. These limitations caution against over-reliance on one type of methodology when probing the microbial community structures and dynamics.

3.1.3.1 Choice of ribosomal RNA gene as candidate for phylogenetic classification

Most culture-independent methods applied for detection, identification and quantification of microbial communities are exploiting the sequence diversity of genes encoding for rRNA (Kirk et al, 2004; Lynch et al, 2004; Rajendhran & Gunasekaran, 2011; Ross et al, 2000; Weisburg et al, 1991). This class of genes is prime candidate because they are universally present, constituting of highly conserved domains yet also possessing stretches of very variable regions.

Analyses of rRNA sequences of microorganisms of the same species have revealed high conservation (≥ 99 % sequence similarity), while varying degrees of inter-species differences have been used to infer phylogenetic relationships. For example, taxonomic identification to the genus levels is often set at ≥ 95 % similarity (Bosshard et al, 2003; Giongo et al, 2010). However, there are also exceptional cases, such as within the genera of *Bacillus*, *Vibrio* and *Mycobacterium*, whereby the nature of sequence diversity of their rRNA may not be adequate to distinguish closely related species (Cilia et al, 1996; Roth et al, 1998). For these genera, additional identification methods complementary to rRNA sequencing will have to be used to distinguish between species.

An additional complication presents itself in the form of genes of 16S rRNA occurring in multiple copies per genome in some species of bacteria, and furthermore, exhibiting sequence heterogeneity among them. To solve this problem, alternative single-copy markers such as *rpoS*, *gyrB* and *recA*, have been considered, and some are showing promises. However, the limited collection of sequence information of these genes stands in contrast to the enormous number of 16S rDNA sequences that are present in the databases, making the latter still the better choice among these few candidates currently (van Elsas & Boersma, 2011).

3.1.3.2 Real time (quantitative) PCR

While limitations of PCR (end-point PCR) presents a challenge in the absolute quantification of taxa abundance within microbial communities, an adaptation of the PCR method, known as real time or quantitative PCR (qPCR), is able to reduce the bias generated by multiple cycling in PCR. In this technique, detection of PCR products (via the use of fluorescent probes) in real time after each cycle facilitates quantitative determination of the initial template copy numbers. Quantification is carried out during exponential amplification when there is a proportional relationship between the amount of amplicons and the initial numbers of target sequences, circumventing the bias posed by end-point PCR (Smith & Osborn, 2009; van Elsas & Boersma, 2011).

However, the application of the qPCR technique is restricted to situations where there is a prior knowledge of the specific target gene sequence. For example, it can be an effective means to quantify the relative abundance of bacteria, archaea and fungi that make up a microbial community, through the use of their respective universal primers for amplifying ribosomal RNA sequences. In the case when a totally uncharacterized microbial consortium has to be studied, qPCR can only be used to detect and quantify groups from specific taxa potentially likely to occur, but will not be able to discern beyond what has been “speculated to be present” by the researchers. Furthermore, the technique is at least one order of magnitude more expensive to carry out than end-point PCR, both in terms instrumentation and reagents. This limits the scale of sampling, and consequently, the scope of scientific queries that may be addressed (Smith & Osborn, 2009).

3.1.3.3 Sequencing of full-length rRNA genes

In the above-mentioned situation whereby the “unknowns” of an uncharacterized microbial community need to be identified, sequencing of the microorganisms’ rRNA genes would be one of the most direct methods (Janda & Abbott, 2007). The full-length rRNA sequences can be aligned against those of the known microorganisms available in public databases such as the Ribosomal Database Project (RDP) (Cole et al, 2009) and the levels of similarity compared to determine the most probable identification match. As of 7 December 2012, the RDP database has garnered a total of 2,639,157 sequences of 16S rRNA genes for such comparison of bacteria and archaea.

If the unknown microorganism is culturable and available as an isolate (as happens when culture-based methods are used), PCR amplification of its rRNA gene using the genomic DNA (or whole cells) of the microorganism as template can yield sufficient material for DNA sequencing. This methodology works in principle if there is only a single copy of rRNA gene in the microorganism’s genome, or if the multiple copies of rRNA genes present are completely identical to each other in sequence. However, problems arise when the numerous copies of the rRNA genes exhibit sequence heterogeneity, i.e. polymorphism exists among

the pool of rRNA originating from a single microorganism. Sequencing this pool of heterogeneous rRNA genes directly will result in “noise” in the sequence data, and interfere with the determination of phylogenetic affiliation of the unknown isolate (Crosby & Criddle, 2003). This problem can be resolved by obtaining a pool of clones, each of which carries a recombinant plasmid with a single sequence of rRNA amplicon insert, a concept that has been expanded to a larger scale in the construction of rRNA libraries – one of the commonly used approaches in microbial community analysis (Section 3.1.3.4).

Since a single rRNA sequence may well represent a single microorganism if its genome carries only a single rRNA “sequence-type”, or be part of the rRNA make-up of a microorganism carrying a heterogeneous set of rRNA sequences, the identity derived after sequence alignment is usually assigned, not as “strains” or “species” but as “molecular operational taxonomic unit” (MOTU). This practice came about because very often, the conventional concepts of the extent and boundaries of ‘species’ fail when molecular diversity is examined. To circumvent the “deadlock” of unresolved status due to the deficit of described species in comparison to the estimated extant number of taxa, defining an unknown organism to the level of MOTU has been introduced as an alternative to ease analytical operations (Blaxter, 2004).

3.1.3.4 Use of 16S rRNA (rDNA) library in community analysis

In microbial communities, there are numerous unknown microorganisms with unknown numbers of rRNA types. In the case of a single unknown isolate (as described in the previous section), the problem created by rRNA gene (rDNA) sequence heterogeneity may be easily resolved but the influence of this phenomenon on community analysis techniques is much more serious (Crosby & Criddle, 2003). The construction of rDNA library from a microbial community will be able to reduce the level of confusion to data output brought about by rRNA sequence heterogeneity, while ensuring that the non-culturable subpopulation of the community can be captured (Spiegelman et al, 2005). In fact, this approach has been viewed as the “gold” standard approach for identifying the

diversity of bacteria in the field of microbial ecology during the last decade (Rastogi & Sani, 2011).

In rDNA clone library analyses, PCR amplicons of rDNAs generated from whole microbial communities are ligated into suitable vector plasmids. The resulting pool of constructs are introduced into *Escherichia coli*, and single colonies (clones) that received vectors with inserts (the amplicons) can be isolated by plasmid extraction, sequenced and the sequences analyzed by comparison to databases (Dahllof, 2002; Van Hamme et al, 2003). Although in theory (based on rarefaction analysis), to achieve satisfactory coverage of bacterial diversity in a particular ecosystem, over 1,500 to 2,000 sequences of clones are often required, this is unrealistically high considering the constraints of conventional sequencing (i.e. Sanger dideoxynucleotide method) technology. Practical considerations and the conscientious focusing on specific and relevant questions that can be addressed “even if coverage is low” have led the scientific community to also accept data obtained with smaller-sized libraries in the lower hundreds (van Elsas & Boersma, 2011). However, when the development of community changes across time needs to be surveyed, the scale of analysis required based on the rDNA library approach makes its application to the study of dynamics not practicable.

3.1.3.4.1 Amplified ribosomal DNA restriction analysis (ARDRA)

Over the last decade, a range of PCR-based molecular fingerprinting techniques, which allow a direct comparative overview of the composition and diversity of microbiota, have emerged. These are more suitable than 16S rDNA library analysis in examining microbial dynamics, since multiple samples, taken at intervals over time, can be “profiled” for comparison against each other. The techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), single-strand conformational polymorphism (SSCP), ribosomal internal spacer analysis (RISA), length heterogeneity-PCR (LH-PCR), and amplified ribosomal DNA restriction analysis (ARDRA) (van Elsas & Boersma, 2011). However, we shall consider the use of DGGE in this context (i.e.

community dynamics) only in the next section (Section 3.1.3.5), and discuss instead an alternative application of ARDRA here, as was used in our study.

ARDRA is a method whereby restriction endonuclease digestion of amplified 16S rDNA (Gich et al, 2000) is employed to generate “molecular fingerprints”. The “fingerprinting” aspect has been exploited in various environmental bacterial community analyses for the determination of the diversity of microorganisms (Lagace et al, 2004; Ross et al, 2000; Zeng et al, 2007). In addition to molecular fingerprinting, there has been attempts to use ARDRA to identify the species or genera of unknown isolates through matching of the molecular fingerprints of known (i.e. taxonomically assigned) bacterial strains to those of the unknown. The method has been verified extensively through comparison of ARDRA profiles across different strains and the matching of their 16S rDNA sequences through the RDP database. Through such studies, it was possible to conclude that ARDRA is a suitable tool for differentiation of unknown bacteria up to the genus level (Sklarz et al, 2009).

Therefore, in analyzing 16S rDNA library clones, instead of having to put through to sequence a large number of clones, ARDRA patterns may be used to preliminarily group isolates together instead. The genus identity of members within these groups may then be inferred by sequencing the rDNA inserts of the representative clones from each ARDRA pattern group (Rastogi & Sani, 2011).

3.1.3.5 Denaturing gradient gel electrophoresis (DGGE)

Among the methods which are able to directly fingerprint microbial communities, DGGE of the PCR amplicons of rRNAs has been most widely accepted (Green et al, 2009; Heuer & Smalla, 1997; Muyzer et al, 1993). The technique exploits the denaturing gradient established (via the use of urea) across a polyacrylamide gel to bring about the separation of mixtures of rDNA amplicons generated from microbial consortia of interest, in accordance with their G-C content (Ercolini, 2004; Luo et al, 2009; Smalla et al, 2007; Tzeneva et al, 2008). Because the G-C content of rDNA influences melting behavior, it will

result in differential mobility of the rDNAs on denaturing gels (Lerman et al, 1984).

However, DNA fragments that can be analysed by DGGE is restricted to below 500 bp in size. Initially, researchers have relied on the sequencing of DNA within excised gel bands of DGGE to determine the identities of microorganisms present in the microbial consortium, but this DNA size limit implies that the length of rDNA that can be used for phylogenetic identification by sequence comparison is too short (Muyzer et al, 1993). Besides this, co-migration of multiple sequences (possibly with similar G-C contents) to the same location in the same gel (Ercolini, 2004; van Elsas & Boersma, 2011) and the formation of multiple DGGE bands by a single microorganism due to heterogeneity of multiple copies of rDNA (Green et al, 2009; Kang et al, 2010; Sekiguchi et al, 2001) further pose challenges. Furthermore, in light of the PCR biases discussed in previous sections, the method is clearly limited to the dominant members (generally referred to as the “top-1,000”) of the target microbial community (van Elsas & Boersma, 2011), and caution is required in the interpretation of DGGE data.

Despite the limitations faced by DGGE, this technique is able to provide a fairly quick and semi-quantitative assessment of the diversity and dynamics of the dominant microbial groups present in complex communities (Yeung et al, 2011). In recent years, Marzorati et al (2008) and Wittebolle et al (2008) have also proposed a systematic processing of the molecular DGGE fingerprints to derive three levels of analysis. These are (i) range-weighted richness (Rr) to describe the diversity of the system, (ii) dynamics (Dy) to reflect the stability of the community and (iii) functional organization (Fo) to define the relation between the structure of the microbial communities and its functionality. The parameters serve as handles to describe the development of a microbial community across time more objectively and to make comparison between numerous communities.

3.1.3.6 Metagenomic and high throughput approaches

Development in DNA microarray technologies in recent years has attracted the attention of environmental microbiologists as they offer the possibility for more rapid throughput to track thousands of genes at one time (Van Hamme et al, 2006). Even more recently, metagenomics approaches using high- and ultra-high-throughput sequencing technology such as the 454-pyrosequencing platform (Dinsdale et al, 2008; Edwards et al, 2006), Illumina Genome Analyzer II and the Applied Biosystems SOLiD system have gained popularity. These systems make it possible for sequences of multiple microorganisms to be analysed simultaneously, without the need to clone the DNA. This takes away firstly the PCR-associated biases and secondly the sampling limit often encountered in the context of sequencing via conventional methods the collection of clones from a 16S rDNA library. The most recent launch of the third generation single molecular real time technology (SMRT) in 2012 by Pacific Biosciences and Oxford Nanopore Technologies offer to expedite the sequencing process even further and with longer reads.

The major disadvantages of these technologies are the problem in deciphering the large datasets generated and the extremely high costs (Desai et al, 2010). The former will require more sophisticated softwares to manage in order to mine from the overwhelming quantity of data sufficiently meaningful biological insights.

3.1.4 Culture-independent approaches based on other cellular components

Analysis of other cellular materials besides DNA, such as protein, lipids and various groups of metabolites, forms the basis of other culture-independent approaches in microbial community analysis. For instance, in fatty acid methyl ester (FAME) analysis, community extracts are profiled by gas chromatography. Fatty acid peaks detected can then be named by the Microbial Identification System software and isolates within the microbiota identified by matching these to FAME database in the software. Since the sensitivity of instruments employed in any of these non-DNA based methods dictates the quantity of microbial

community samples required, their application is only possible if the scale of biomaterials derived sufficiently exceeds the instruments' sensitivity limit.

Current advances in instrumentation have brought down the threshold of biomaterials required considerably, making these methods more viable for microbial community analysis. For instance, metaproteomics is an uprising approach which serves as an alternative to the DNA-based approaches. The analysis allows identification of the full collection of proteins expressed by whole microbial communities at the moment of sampling, making it possible to elucidate the community's network of metabolic pathways (Hettich et al, 2012).

3.1.5 Strategies applied in this thesis to identify the microorganisms and its dynamics changes in the population

There is no single technique available today that can capture all aspects that define a microbial community. Each method has its own level of resolution, limitations and biases (Dahllof, 2002; van Elsas & Boersma, 2011), but they are complementary to one another. While molecular approaches are powerful and have enabled reliable identification of members of the microbial community (Torsvik et al, 1998), such "genetic" information is insufficient to extrapolate community function (Van Hamme et al, 2003). In this respect, the availability of culturable isolates allows for valuable biological characterization and functional studies. Hence, only through an iterative process where a combination of culture-based and culture independent techniques is used, can we satisfactorily further our understanding of the target microbial community. It is important to be very clear about the nature of the scientific queries in order to select the more effective combination of techniques to use. In this study, we aim to study the changes of the bioreactors' microbial communities across time and to identify key microorganisms in the bioreactors that could possibly be responsible for the enhanced hexane removal capacity in the TPPBs over the MPBs. Figure 3.1 shows an overview of the various strategies used.

Firstly, in order to select the relevant taxa of microorganisms to focus on, qPCR technique was applied to determine the relative abundance of bacterial,

fungal and archaeal populations in the microbial consortia of TPPBs and MPBs across time (Section 3.3.1). After the major focus group (bacteria) for study has been decided, a survey of the bacterial communities' dynamics was conducted (Section 3.3.2), preliminarily through colony morphotype distribution of the culturable subpopulations, and subsequently at a higher resolution through DGGE of 16S rDNA. As our bioreactor systems were operated under aseptic conditions, the initial 0th week rDNA would be the origin of both culturable and unculturable subpopulations. A 16S rRNA clone library was therefore constructed (Section 3.3.3.1). Together with a sampling of culturable isolates obtained from the bioreactors, phylogenetic assignment of component groups in the bioreactors' microbial communities was carried out (Section 3.3.3.3). This was achieved through a combination of ARDRA (grouping of isolates and clones based on molecular fingerprints of 16S rDNA) and DNA sequencing (of 16S rDNA from representative isolates and clones of each group). The identified MOTUs were profiled by DGGE in order to provide a level of association between DGGE band positions and possible taxa (Section 3.3.4.1). The dynamics revealed by DGGE were then re-examined to include this level of analysis (Section 3.3.5.2).

Figure 3.1 Schematic diagram outlining the combination of strategies used to analyze microbial communities within the various bioreactors.

3.2 Materials and Methods

3.2.1 Extraction of genomic DNA from bioreactor samples

Appropriate volumes of microbial cultures were collected from the bioreactor fractions in accordance to their respective cell density ranges (Figure 2.5 and 2.14): (i) TPPB aqueous phase (0.2 ml), (ii) TPPB IF (0.2 ml) and (iii) MPB aqueous phase (1.0 ml) in order to extract DNA for the later qPCR and DGGE (Section 3.2.2.2 and 3.2.7).

Cell lysis

Cell pellets were first collected by centrifuging the cultures at 12,000 *g* for 8 minutes and the supernatant carefully and completely aspirated. For the IF pellets, they were further washed three times with 200 μ l of 0.1 % Triton X-100 in 1X Tris-EDTA (TE) buffer (pH 8.0) and centrifuged at 12,000 *g* for 8 minutes each time to completely remove the presence of the NAL, silicone oil. The cells in all the pellets were then lysed by bead beating using Mini-BeadBeater-1 (BioSpec) in the presence of 10 mM Tris 2 mM EDTA with 1% Triton X-100 for 1 minute. To each of these, 3.6 mg of lysozyme (Sigma-Aldrich) was added and the mixture was incubated at 37 °C for one hour to hydrolyse the glycosidic bonds of cell wall peptidoglycans. Subsequently, 0.056 mg of Proteinase K (Macherey Nagel) was added, followed by incubation at 56 °C in Thermomixer (Eppendorf) for at least 12 hours, until microbial cells were completely lysed.

DNA extraction

Extraction of DNA from the cell lysate was carried out using Macherey-Nagel Nucleospin® kit (Macherey Nagel) according to the procedures as described by the manufacturer, with modifications at the final DNA elution step. Instead of adding 50 μ l of elution buffer to elute the DNA bound to the silica membrane in the Nucleospin® Tissue Spin Column, the elution was performed in two rounds using 25 μ l of elution buffer each time, to increase the yield of DNA. The DNA content was then measured using the Nanodrop Spectrophotometer (BioFrontier Technology) at 260 nm.

3.2.2 Polymerase-chain reaction (PCR)

3.2.2.1 Primers

Primer sets, which were used to amplify the target regions of 16S rDNA for bacteria and archaea, and 18S rDNA for fungi, for experiments such as real time PCR (qPCR), DNA sequencing, PCR-ARDRA, 16S rDNA library construction and DGGE, are presented in Table 3.1.

Table 3.1 Sequences and annealing temperatures (Ta) of primers

Experiment	Target group	Primer	Sequence (5'→3')	T _a (°C)	Size of product (bp)	Reference
qPCR	Bacteria 16S rDNA	Tbf	GTG ITG CAI GGI IGT CGT CA	60	323	Maeda et al (2003)
		Tbr	ACG TCI TCC ICI CCT TCC TC			
	Fungi 18S rDNA	Tff	AIC CAT TCA ATC GGT AIT	50	390	Chemidlin Prévost-Bouré et al (2011)
		Tfr	CGA TAA CGA ACG AGA CCT			
DNA sequencing and PCR-ARDRA	Archaea 16S rDNA	Taf	ATT AGA TAC CCS BGT AGT CC	60	273	Stiverson et al (2011)
		Tar	GCC ATG CAC CWC CTC T			
	Bacterial 16S rDNA (full- length)	Fbf	AGA GTT TGA TCC TGG CTC AG	51	1500	Dojka et al (1998)
		Fbr	GGT TAC CTT GTT ACG ACT T			
Amplification of 16S rDNA clone library inserts	Clone inserts	M13f	GTA AAA CGA CGG CCA GT	55	1636	manual from TOPO® TA Cloning® Kit for Sequencing with One Shot® MAX Efficiency® DH5α-T1R <i>E. coli</i>
		M13r	CAG GAA ACA GCT ATG AC			
DGGE	Bacterial 16S rDNA (V3)	V3f- GC	<u>CGC CCG CCG CGC GCG GCG GGC GGG GCG</u> <u>GGG GCA CGG GGG GCC TAC GGG AGG CAG</u> CAG	55- 65	233	Muyzer et al (1993)
		V3r	ATT ACC GCG GCR GCT GG			
	Fungi 18S rDNA	Vff	GGC TGC TGG CAC CAG ACT TGC	55- 65	230	van Elsas et al (2000)
		Vfr- GC	<u>CGC CCG CCG CGC GCG GCG GGC GGG GCG</u> <u>GGG GCA CGG GGG GGT AAA AGT CCT GGT</u> TCC C			
Amplification of DGGE excised band	Bacterial 16S rDNA (V3)	V3f	C TAC GGG AGG CAG CAG	55- 65	233	Muyzer et al (1993)
		V3r	ATT ACC GCG GCR GCT GG			

Underlined sequence refers to bases included to serve as GC clamp, and not part of the *16S/18S rRNA* sequence.

3.2.2.2 Real time PCR (qPCR)

Optimisation for primers concentration

The concentration of primers used during PCR reaction will influence qPCR outcome, hence different quantities (5, 10 and 25 pmoles μl^{-1}) of primers (Table 3.1 under Experiment “qPCR”) were added to the 20 μl reaction mixture (refer next paragraph) to determine the optimal concentration for amplification. The template DNAs (2 μl) used were genomic DNA extracted from the strains *Escherichia coli* K-12 (ATCC 10798), *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (ATCC 9763) and *Methanocaldococcus jannaschii* JAL-1 (ATCC 43067). These microorganisms also served as reference microorganisms for bacteria, fungi and archaea respectively. The optimal primer concentrations were found to be 10 pmoles μl^{-1} for each of the forward and reverse primers respectively.

Conditions for qPCR run

The qPCR was carried out by an Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems) using SYBR-green as the detection fluorochrome. To 18 μl of the reaction mixture Power SYBR® Green PCR Master Mix, which included AmpliTaq Gold® DNA Polymerase, optimized buffer components, dNTP mix, SYBR® Green I Dye and passive reference (ROX), 2 μl of 0.5 ng/ μl genomic DNA (extracted from the reference strains or various samples collected from TPPBs and MPBs) was added as the template. The qPCR programme consisted of an initial step of 10 minutes at 95 °C for enzyme activation, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at T_a (refer Table 3.1) for 1 minute and elongation at 72 °C for 1 minute. The qPCR assay for each target group was performed in duplicates on 8-stripped MicroAmp® microtubes (Applied Biosystems).

To obtain qPCR standard curves (for quantification of target groups/genes), serial dilutions of genomic DNA of the three reference strains were performed to obtain an equivalent of 10^1 , 10^2 , 10^3 , 10^4 , 10^5 and 10^6 gene copies per reaction as adapted from Mufty (2008). qPCR were then carried out as described above, targeting the 16S rRNA genes of *Escherichia coli* K-12 and *Methanocaldococcus jannaschii* JAL-1, and the 18S rRNA gene of

Saccharomyces cerevisiae Meyen ex E.C. Hansen. Data were used to plot the standard curve (refer next paragraph).

qPCR data analysis

Standard curves were constructed by plotting the observed quantification cycle (C_q) against the quantity of template DNA [log(number of gene copies)] used. The C_q of an unknown sample could then be compared against the standard curves to determine the concentrations of target genes present.

The efficiencies of PCR were determined using the formula:

$$\text{PCR efficiency} = [(10^{-1/\text{slope}}) - 1] \times 100\%$$

whereby the slope (gradient) was derived from the standard curve plotted.

Standard curves generated from qPCR of the reference microorganisms *E.coli*, *M. jannaschii* and *S.cerevisiae* were subsequently used for quantification of the total bacteria, archaea and fungi in the microbial communities, respectively.

3.2.2.3 End-point PCR

The PCR reaction mixture was prepared according to the manufacturer's recommendations. In brief, a 50 µl PCR mixture contained 20 µM of each primer (forward and reverse), 200 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 1 U Taq DNA polymerase (Fermentas), 1x Taq buffer with KCl (Fermentas), 1.5 mM MgCl₂ (Fermentas) and sterile distilled water. For DNA sequencing and PCR-amplified rDNA restriction analysis (PCR-ARDRA) of selected isolates or clones, one colony of each strain was directly added into the reaction mixture using a sterile pipette tip to serve as a source of DNA template. For 16S rDNA clone library construction and DGGE of the 16S rDNA V3 regions, total genomic DNA extracted from the microbial communities were used as DNA templates instead. PCR amplification was performed using a Thermocycler (Eppendorf).

The PCR condition for full-length 16S rDNA for DNA sequencing and PCR-ARDRA was modified from Dojka et al (1998). The program consisted of an initial cycle of denaturation of template DNA at 95 °C for 10 minutes. This

was then followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 51 °C for 1 minute and elongation at 72°C for 2 minute. The final extension step was set at 72°C for 5 minutes. Similar program was used for the amplification of the clone library full-length 16S rDNA inserts with a higher annealing temperature of 55 °C (Table 3.1).

The PCR condition for 16S rDNA V3 region of the bacterial communities for DGGE was modified from Muyzer et al (1993). The program consisted of an initial cycle of denaturation of the genomic DNA at 94 °C for 3 minutes. This is then followed by 20 touchdown cycles (denaturation at 94 °C for 1 minute, annealing at 0.5 °C per cycle from 65-55 °C and elongation step at 72 °C for 1 minute) and the next 10 cycles (denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and elongation at 72 °C for 1 minute). The final step involved elongation at 72 °C for 7 minutes.

3.2.3 Agarose gel electrophoresis

All PCR amplified products were electrophoresed in a 1 % agarose gel for 45 minutes at 90 V in 1X Tris-borate-EDTA (TBE) buffer to check for the presence of amplicons. For PCR-ARDRA (Section 3.2.6.1), to obtain the molecular fingerprints, 2.5 % agarose gels were used instead.

A 6 X loading buffer (Fermentas) was mixed with the PCR product before loading into the wells in the agarose gel. The molecular weight marker, GeneRuler™ 1 kb and 100 bp DNA Ladder Plus (Fermentas) were used for easy identification of the band sizes in the gel. The ethidium bromide stained gels were photographed using filtered ultraviolet (UV) illumination by Chemi-genuis Bio imaging system (Syngene).

3.2.4 Distribution of colony morphotypes in microbial communities

Colonies on MSM agar plates infused with hexane for seven days (Section 2.3.3.1.4 and 2.3.3.2.3) were grouped based on their morphologies as shown in Table 3.2. The colony morphotype distributions of the bioreactor

microbial communities at each time point were plotted based on the percentage of the number of colonies of each morphotype to the total number of colonies in Figure 3.6.

Table 3.2 Colony morphotypes observed on MSM agar plates infused with hexane

Colony morphotype	Size (mm)	Shape	Colour	Remarks
Halo	Pinpoint to 3.0	Round	Cream/ White	Lighter cream-coloured transparent ring around the colony, like a halo
Flower-shaped	Pinpoint to 3.0	Irregular	White/ Orange/ Yellow	-
Orange	Pinpoint to 5.0	Round	orange/ pink/ red/ yellow	Referring to colonies with colours ranging from red to yellow hue
Small	Pinpoint to 1.0	Round	Cream/ White	-
Medium	> 1.0	Round	Cream/ White	-

3.2.5 Construction of 16S rDNA clone library

A 16S rDNA clone library was constructed using the total genomic DNA of the week 0 microbial culture (i.e. the inoculum of the bioreactors) as DNA template to PCR-amplify the collection of full-length 16S rDNA as described in Section 3.2.2.3.

The library of PCR products were ligated to pGEM-T easy vector (Promega) and transformed into InvitrogenTM MAX Efficiency DH5 α *E. coli* cells, according to the respective manufacturers' instructions. From the 200 colonies obtained, 168 white clones carrying full-length 16S rDNA inserts were selected based on X-Gal-IPTG blue-white colony screening, followed by PCR screening using M13f/M13r primers (Table 3.1) to confirm the presence of inserts (Section 3.2.2.3).

3.2.6 Molecular analysis of bacterial isolates and clones

3.2.6.1 PCR-ARDRA

The PCR-ARDRA was performed with the assistance of Miss Tay Yu Ling and Dr Miao Huang of our research team.

Bacterial full-length 16S rDNA were amplified (Section 3.2.2.3) from culturable isolates (Section 3.2.4) using the bacterial 16S rDNA universal primers, Fbf/Fbr, as shown in Table 3.1. Genomic DNA extractions of isolates were performed (Section 3.2.1) and 50 ng/μl of gDNA was used as template for PCR amplification according to the procedures described earlier (Section 3.2.2.3).

In order to amplify the inserts of the 16S rDNA library clones, using Fbf/Fbr primers directly would have amplified the 16S rDNA of the *E. coli* host as well and causing undue contamination. Hence, they were first amplified using the vector primers, M13f/M13r (Table 3.1). The PCR products were subsequently purified using NucleoSpin® Gel and PCR Clean-up (Macherey Nagel) according to the procedures as described by the manufacturer and 50 ng/μl of these were used as templates in a second round of PCR with Fbf/Fbr (Table 3.1).

The PCR products were digested with the restriction enzymes *RsaI* (Promega) or *HaeIII* (Fermentas) (Table 3.3). Each reaction was carried out in a total volume of 15 μl which contained the manufacturer-recommended buffer at 1x concentration, 1.25 U of enzyme, 100 μg/ml bovine serum albumin (BSA) (New England Biolabs) and approximately 375 ng DNA. The reactions were then incubated at 37 °C overnight for complete enzymatic digestion. The molecular fingerprints were obtained by performing electrophoresis on a 2.5 % agarose gel (Section 3.2.3).

Table 3.3 Restriction enzymes used in ARDRA

Restriction enzymes	Manufacturer	Buffer	Restriction Sites
<i>RsaI</i>	Promega	C	5'...GG^CC...3' 3'...CC^GG...5'
<i>HaeIII</i>	Fementas	R	5' ...GT^AC...3' 3' ...CA^TG...5'

3.2.6.2 16S rDNA sequencing and analysis

Full-length bacterial 16S rDNA amplicons obtained as described in Section 3.2.2.3 where 100 ng of this template DNA was sent to 1st BASE Group (Singapore) for sequencing using the dideoxy chain termination method in the sense (primer Fbf) and anti-sense (primer Fbr) direction. The sequence of these primers were shown in Table 3.1. The resulting data were viewed using the Chromas Version 1.62 software.

Low quality (noisy) sequence data from the ends of the sense and the anti-sense strands were trimmed manually. The remaining good quality sequences were then reconstructed based on the sense and anti-sense reads to derive credible contigs using the Bioedit software package (Hall, 1999). These were then submitted to the Ribosome Database Project (RDP) database (<http://rdp.cme.msu.edu/>) for similarity comparison with the 16s rDNA sequences of the type strains available in the database. The similarity score, in percentage, were recorded.

3.2.6.3 Sequence analysis and dendrogram construction

The 16S rDNA sequences were aligned with those of the type strains obtained from Genbank and compared via the MEGA5.10 software (Tamura et al, 2011). The evolutionary distances of a 5,000-replicate bootstrapped Neighbor-Joining phylogenetic tree (Saitou & Nei, 1987) were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The numbers listed next to the nodes of the phylogenetic tree referred to confidence values where such node had been observed in 5,000 bootstrap replicates of the dataset.

3.2.7 Denaturing Gradient Gel Electrophoresis (DGGE)

3.2.7.1 Gel Electrophoresis and visualization

DGGE was carried out according to the protocol described by Muzyer et al (1993). The 16S rDNA V3 region PCR amplicons was obtained by PCR-amplifying the fragment from 50 µg of genomic DNA samples extracted from the microbial communities using the 16S rDNA V3 primer set (Table 3.1), according to the PCR procedure described in Section 3.2.2.3. The PCR amplicons of size 233 bp were checked by electrophoresis (Section 3.2.3) and semi-quantified by comparing against the intensity of the GeneRuler™ 100 bp DNA ladder (Fermentas). This allowed the amount of amplicons loaded into each well in the DGGE gel to be adjusted to approximately 1.8 µg per well.

The PCR amplicons were separated in a 8 % polyacrylamide gel with 40-70 % urea/formamide gradient, using D-code electrophoresis system (Bio-Rad). A 6X loading buffer (Fermentas) was mixed with 15 µl of PCR product before loading into the wells. The DGGE was run in 1X Tris-acetate-EDTA (TAE) buffer at 85 V for 16 h at 60 °C. The gels were stained in 1X SYBR-gold (Molecular Probes) for 20 minutes and visualized using Chemi-Genius Bio Imaging System (Syngene).

3.2.7.2 DGGE data analysis

The DGGE profiles obtained were subsequently analysed using Gelcompar® II software (Version 6.5; Applied Maths, Saint-Martens-Latem, Belgium). During gel processing, the lanes were defined, the background was subtracted and differences in the lane intensities were compensated, to obtain densitometric curves based on the band patterns (Jing et al, 2012; Zhang et al, 2009). A matrix of percentage similarities between pairs of densitometric curves were generated, calculated using the Pearson correlation coefficients. Dendrograms were created using unweighed-pair group method using average linkages (UPGMA) (Scheirlinck et al, 2008; Wittebolle et al, 2005).

The reproducibility of the DGGE profiles was checked by running a duplicate set of DGGE for the same samples that were PCR-amplified on a separate occasion.

Richness

The range-weighted richness (Rr) was calculated as $Rr = N^2 \times Dg$ (Economopoulos, 1993; Marzorati et al, 2008), where N is the total number of bands for each sample and Dg is the denaturing gradient between the first and the last band of the pattern in the DGGE gel.

Dynamics

Moving window analysis (Marzorati et al, 2008; Wittebolle et al, 2008) was performed on the data set of percentage similarities (obtained through Pearson correlation coefficients). Similarities between DGGE profiles of consecutive time points were plotted against time to evaluate the level of community stability. The similarity values were also used to calculate the percentage of change, via this equation:

$$\text{Change\%} = 100 - \text{similarity \%}$$

The rates of change (Δt) were calculated as the average percentage of changes within the period of interest (Marzorati et al, 2008).

Functional organization

Pareto-Lorenz (P-L) distribution curves distribution curves was plotted as described by Wittebolle et al (1993). In brief, the bands in each DGGE lane were ranked from high to low based on their intensities. The cumulative proportion of the bands was presented along the x-axis and the respective cumulative normalized band intensities are presented along the y-axis. The resulting curves represented the degree of evenness in the molecular composition of the PCR products and serves as an estimator of the degree of evenness in the microbial community. To assess the functional organization (Fo), the PL curves were evaluated by a horizontal y-axis projection of their intercepts with the vertical 20% x-axis (Wittebolle et al, 2008).

Cluster analysis by multidimensional scaling (MDS) and principal component analysis (PCA)

The band data were subjected to MDS and PCA using the software package SPSS for Windows (version 20.0; SPSS, Inc., Chicago, IL, USA). By using MDS and PCA analysis, a data set generated from each complex band profile (i.e. each sample) could be reduced to one point in a three-dimensional space. MDS analyses the band profiles based on the ranks of similarities using Pearson correlation (Boon et al, 2002). For further analysis of the bacterial community, PCA analysis was performed by generating a band-matching table first where all bands were divided into classes of common bands and for each pattern, a particular band class can have two states: present or absent (binary matrix) (Boon et al, 2002). The standardised data for MDS and PCA were plotted in three dimensions, and examined for clustering behaviour.

The MDS and PCA analysis were also applied to study the distribution of key bacterial genera across time where the DGGE band profiles were substituted with the relative abundance of the key bacterial genera in the microbial community.

3.2.7.3 Sequencing of excised bands

The DGGE bands of interest were excised from the gel, eluted in 30 µl of sterile ddH₂O at 37 °C for 30 min with gentle shaking and centrifuged at 500 g for 2 minutes to pellet down the gel debris. The supernatant containing the eluted DNA was used as template (2 µl) for PCR amplification using primer set V3f/V3r (Table 3.1), according to the procedure in Section 3.2.2.3. The amplicons were subsequently purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel, France) before being put through sequencing as described in Section 3.2.6.2 using primers V3f/V3r (Table 3.1).

3.3 Results

3.3.1 Determination of bacterial/fungal/archeal abundance by qPCR

Microbial communities present within soils have been known to carry members from the Domains Bacteria and Archaea and the Kingdom Fungi (van Elsas & Boersma, 2011). As our bioreactors' inocula originated from soils, it was necessary to survey the relative abundance of these taxonomic groups in order to determine which one or more we should focus our studies on. It will have implications on the various methodologies, e.g. in the case of the eukaryote fungi, primers targeting 18S rRNA instead of the prokaryotic 16S rRNA will need to be used. If one group is particularly dominant, it may not be necessary to devote limited resources on the analysis of very minor or non-existent populations. Hence, the relative abundance of total bacterial, fungal and archaeal populations in the bioreactors' microbial consortia was determined across time. This was carried out using qPCR, as the technique is acknowledged to be fast and specific for the enumeration of target microorganisms and widely used in microbial ecology to determine gene and/or transcript numbers present within microbial community samples.

3.3.1.1 Specificity of universal primers for bacterial, fungal and archaeal rRNA genes

Prior to running qPCR analysis of the bioreactors' samples, the primers intended to be used for the quantification of total bacteria, fungi and archaea in the microbial communities (refer Table 3.1) were tested for specificity via PCR. Universal primers targeting the conserved sequences of bacterial and archaeal 16S rRNA and fungal 18S rRNA were put through PCR with the genomic DNA of the reference strains *E. coli* K-12 (bacteria), *M. jannaschii* JAL-1 (archaea) and *S. cerevisiae* Meyen ex E.C. Hansen (fungi) as templates. As shown in Figure 3.2, amplicons were detected only when the respective pairs of primers were added to the intended target templates.

Optimization of primer concentration was then performed in the subsequent runs of qPCR (Section 3.2.2.2) and found to be 10 pmoles/ μ l. During the qPCR run of a series of standards and samples from the bioreactors, the specificity of primer binding was further verified through melting curves analysis. The melt curves were generated by increasing the temperature at 0.5 °C every 30 seconds after the end of a qPCR run. A representative series of melting curves is shown in Figure 3.3. All the primer sets have generated a single product peak, indicating their specificities in binding.

Figure 3.2 Agarose gel electrophoresis of PCR products amplified using conserved bacterial, fungal and archaeal primers. Genomic DNA of *E. coli* (Ec), *S. cerevisiae* (Sc) and *M. jannaschii* (Mj) were put through PCR using each of the three sets of primers as respectively shown. L: 100 bp DNA marker.

Figure 3.3 Representative melting curves of qPCR of the standards and samples using (A) bacterial (Tbf/Tbr) and (B) fungal (Tff/Tfr) primers. The results showed only PCR products that peaked at about 83°C, when various standards (of intended target gDNA) and samples (bioreactor microbial community gDNA obtained across 52 weeks at 4-week interval) were used, indicating specificity of the primers for the target PCR. Only selected melting curves had been shown to prevent too many lines from cladding the specific temperature peaks observed. Similar observation was made when the archaeal primers were likewise used (data not shown).

3.3.1.2 Standard curves and detection limits

In qPCR, test samples can be quantified (i.e. the target gene or transcript numbers determined) by recording the amplification status during each cycle in the form of fluorescent signal level which is associated with PCR product formation, and then correlating the C_q values (threshold cycle) values derived (Maeda et al, 2003) to the standard curves of reference template DNA.

The standard curves for qPCR in this study were generated for each primer set (bacterial, fungal and archaeal) by plotting the C_q values as a function of the logarithm of the number of DNA copies (over several orders of magnitude) of the targeted gene. The gradients (slope values) derived from the standard curves for bacteria, fungi, and archaea, as shown in Table 3.4, were very close to the theoretical optimum of -3.322 (Higuchi et al, 1993) and the R² (square correlation coefficient) values were close to 1, indicating that quantification

based on Cq values of these standard curves could be performed with good confidence. The amplification efficiencies were 99.7, 98.2 and 98.6 for bacteria, fungi, and archaea respectively. The quantification (or detection) limit of the respective qPCR conditions, defined as the lowest quantity of template at which linearity is maintained (Ibarburu et al, 2010), was found to vary among the targeted populations – for bacteria and archaea, they were down to 10^2 gene copies per μl while for fungi it was down to 10^1 gene copies per μl , implying higher sensitivity.

Table 3.4 Evaluation of suitability of primer sets for SYBR Green-based qPCR to quantify bacteria, archaea and fungi.

Sample	Slope of std curve ^d	R ² ^e	PCR amplification efficiency ^f (%)	Detection range (copy number)
Bacteria ^a	-3.328	0.963	99.7	10^2 - 10^6
Fungi ^b	-3.366	0.983	98.2	10^1 - 10^6
Archaea ^c	-3.324	0.991	98.6	10^2 - 10^6

^a Universal bacterial 16S rRNA gene primers Tbf/Tbr with *E.coli* gDNA.

^b Universal fungal 18S rRNA gene primers Tff/Tfr with *S. cerevisiae* gDNA.

^c Universal archaeal 16S rRNA gene primers Taf/Tar with *M. jannaschii* gDNA.

^d The slope was calculated from the standard (std) curve plotted with Cq on the y-axis and $\log_{10}$ (DNA copy number) on the x-axis.

^e R² represents square correlation coefficient obtained from plotting the standard curve of different concentration of target gDNA sample.

^f PCR amplification efficiency = $[(10^{-1/\text{slope}}) - 1] \times 100\%$

3.3.1.3 Proportion of bacterial, fungal and archaeal population in the microbial consortium

Having established these standard curves, qPCR was carried out on the samples collected from the bioreactors and the number of copies of bacterial, archaeal and fungal target genes present in the microbial consortia were estimated by comparing the Cq values of the samples with the standard curve. The relative abundance (in percentage) of each group in the MPBs and the aqueous and interfacial fractions of TPPBs across time is shown in Figure 3.4. The bacterial population constituted the highest proportion throughout all bioreactors and across time, while the fungal population remained low in abundance, not

exceeding 5 % even at its highest abundance at week 28 in the TPPBs. Archaea were not detectable in both the initial microbial consortium and across the 52 weeks. In addition, the presence of bacteriophages were also not detectable in the bioreactors across 52 weeks as determined by the absence of plaque formation on the bacterial lawn formed by the samples collected from the bioreactors across time (data not shown). Clearly, the bioreactors were conducive in maintaining the high abundance of bacteria found in the initial microbial inoculum and did not favour shifting into fungal dominance despite allowing its occasional surge in abundance. The bacterial communities in the bioreactors would therefore be expected to play a critical and major role in hexane biodegradation/utilization. The specific hexane degradation rates at week 28, as presented in previous chapter (Figure 2.9 under Section 2.3.3.1.5), did not differ considerably between the MPBs and TPPBs, but here the data showed that there was apparent difference in the fungal population's abundance at Week 28 (compare Figures 3.4A against B and C). However, in the later period of Week 44, when specific hexane degradation rates were much higher in the TPPBs (Figure 2.17 under Section 2.3.3.2.4), the fungal population appeared to have decreased. This lack of correlation between fungal presence and hexane biodegradative efficiency of the microbial consortia implies that fungi do not contribute majorly to the hexane degradation. In addition, a quick check on the fungal population's diversity using DGGE showed only very few bands (data not shown). The bacterial population that remained dominant across time, and therefore its dynamics and diversity, would likely have contributed much more significantly to hexane utilization.

Indeed, a review of the literature impresses upon us that overwhelmingly more groups of bacteria have been reported to play dominant roles in various bioremediation situations than fungi. Despite the presence of significant fungi co-residing within the polycyclic aromatic hydrocarbons polluted sites, the contribution of bacteria were found to be major and predominant (Wang et al, 2012). Bacterial species such as *Pseudomonas fluorescens*, *Rhodococcus erythropolis* and *Mycobacterium frederiksbergense*, and sometimes mixed bacterial cultures, have been extensively validated to be responsible for the biodegradation of VOCs (Aldric & Thonart, 2008; Guieysse et al, 2005; Mahanty et al, 2008; Muñoz et al, 2013; Muñoz et al, 2007b; Osswald et al, 1996). To date,

only very few fungi have been reported to be capable of degrading VOC compounds. These include *Fusarium solani* (Arriaga et al, 2006) and *Aspergillus niger* (Spigno et al, 2003).

Figure 3.4 Relative abundance of bacterial, fungal and archaeal populations in the microbial consortia. The microbial consortia from various bioreactors were analyzed via

qPCR on Weeks 0, 4, 8, 12, 28, 44 and 52. The relative abundance of bacterial (in green) and archaeal (in cyan) 16S rRNA genes and fungal (in purple) 18S rRNA genes are obtained by expressing the number of copies of the respective rRNA genes as percentages of the summation of all 3 classes of rRNA gene copies. As the distributions of the populations were similar between duplicate bioreactors, only representative graphs from (A) MPB1, (B) TPPB1 aqueous phase and (C) IF are shown.

The greater abundance of bacteria in our bioreactors' microbial consortium across time, together with the findings from literature, has driven us to focus on the dynamics of the bacterial communities in order to elucidate the key bacterial activities and interactions responsible for the enhanced hexane degradation in the TPPBs.

The remaining portion of this chapter shall be organized into three parts. The first part will focus on a brief survey of the community dynamics (Section 3.3.2). In order to derive more meaningful interpretation from these, the second part was undertaken – which focuses on identifying the various taxonomic groups of bacteria present in the bioreactors (Section 3.3.3). This was then followed by the integration of these two types of information in the last part to derive new insights to the microbial community development in long-term operating TPPBs (Section 3.3.3.4 and 3.3.5).

3.3.2 Dynamics of bacterial communities in bioreactors

Since temporal changes in hexane degradation rate suggests that the microbial consortia in TPPBs have developed over time (discussed in Section 2.4.3), the dynamics for both culturable and non-culturable bacterial populations in the microbial consortia were preliminarily surveyed. This was firstly done by a crude observation of colony morphotype (hence only the culturable subpopulation) distribution across time, followed by a more in-depth analysis of whole communities using DGGE of PCR-amplified 16S rRNA pools of the bacterial communities.

3.3.2.1 Community dynamics based on colony morphotype distribution

We took advantage of the readily available data of the culturable cell count performed over the 52 weeks (Section 2.3.3.1.4 and 2.3.3.2.3) to obtain a crude gauge of bacterial dynamics. The colonies have been enumerated weekly according to five types of morphology and then summed up to generate the total culturable cell count data shown in Figure 2.8 and 2.16. This categorization was based on to the most distinguishing features of the colonies (Figure 3.5) – those which bore Halo (H) around themselves, were Flower-shaped (F), or Orange-colored (O). Colonies with none of these distinguishing features were more generically categorized based on sizes, such as Small-sized (S) or Medium-sized (M). For a more detailed description or criteria of classification, please refer to Table 3.2 in the Materials and Methods (Section 3.2.4). The proportion of each colony morphotype within the culturable subpopulation of the bioreactors' microbial consortia were plotted (Figure 3.6) at weekly intervals during the first 12 weeks (corresponding to the exponential phase of biomass accumulation, Figure 2.5 and 2.14 -for OD), followed by monthly intervals for the more stable phases that come after, to get an impression of changes occurring across time with respect to microbial compositions.

Figure 3.5 Appearances of the 5 colony morphotypes. Colonies from the MSM plates which were used in the weekly counting of culturable cells were categorized based on their

morphologies which are described as: Halo (A), Flower-shaped (B), Orange (C), Small (D) and Medium (E).

The proportions of these colony morphotypes differed depending on the origin of the microbial consortia (i.e. whether from the TPPB aqueous phase or IF or the MPPBs) and the period that the samples were taken. All of them showed dominance by the S colony morphotypes, but this being an extremely generic and non-discriminatory morphotype, not much may be inferred from it. At some periods in the time course, there were high proportions of H and O morphotypes in the MPBs, H and M morphotypes in the aqueous phase of the TPPBs, and O and F morphotypes in their IFs. Crude as it may be, this analysis affirmed that community structures in these three types of environment (i) have prominent differences from each other and (ii) show considerably dynamism across time.

It was also observed that although the duplicate pairs of bioreactors (i.e. comparing panels A vs B, C vs D and E vs F in Figure 3.6) showed similar colony morphotype distributions in general, there was sufficient variation between the pairs in the earlier weeks (up to approximately Week 20) and greater variation in the later weeks (after Week 20) to be interpreted as divergence in the duplicate's community structures. As discussed before, this is inevitable in a complex system such as a microbial community. The chaotic nature of complex systems tends to accumulate variance as time progresses and this manifests as greater divergence (Roelke et al, 2003). This could be the basis of the significant difference in the specific hexane degradation rates of TPPB1 and TPPB2 observed in Week 44 previously (Figure 2.17 under Section 2.3.3.2.4).

Figure 3.6 Distribution of colony morphotypes present in MPB1 (A), MBP2 (B), TPPB1(C), TPPB2 (D), TPPB1-IF(E) and TPPB2-IF (F). The relative abundance of each colony morphotype was plotted as a percentage based on duplicate CFU counts. H: Halo, O: Orange, S: Small, M: Medium and F: Flower-shaped.

3.3.2.2 Community dynamics based on DGGE of 16S rRNA V3 region

Subsequent to the colony morphotype distribution analysis described above, DGGE of the PCR-amplified 16S rDNA preparations of the bioreactors' microbial consortia was carried out. Essentially, amplicons of the variable V3 regions of 16S rDNAs were separated by DGGE (Muyzer & Smalla, 1998) and this allowed us to obtain higher resolution “snap shots” of the bacterial communities across the 52 weeks of operation in order to better understand their dynamics. Again, this analysis was carried out at weekly intervals over the first 12 weeks and 4-weekly intervals from then onwards. A reference ladder (LCI), the profile (Figure 3.21) and construction of which shall be described later (Section 3.3.4.1) was used as an internal control for lab-to-lab (e.g. running conditions and hardwares) and gel-to-gel (voltage and gel gradient) standardization (Neufeld & Mohn, 2005). For an objective analysis of the numerous DGGE profiles (Figure 3.7 and 3.8) allowing temporal cross-bioreactor and/or cross-fraction comparisons, the densitometric rendering of each profile was processed and used to calculate Pearson correlation coefficients to obtain the percentage of similarity between pairs of DGGE profiles (Section 3.2.7.2). Also, to address the issue of the DGGE profiles' overall coverage of analysis later (Section 3.3.5), all band positions that have surfaced in any of the samples' DGGE were mapped relative to each other to form a reference Band Distribution profile (refer “BD” in Figure 3.7 and 3.8).

Figure 3.7 DGGE profiles of the V3 regions of 16SrDNA of bacterial communities from the aqueous phase (A) and interfacial fractions (IF) of TPPB1 and TPPB2 at 4-weekly intervals. The positions of all the bands that have appeared in the complete set of DGGE profiles (including from MPBs, refer Figure 3.8) were consolidated to map out a single Band Distribution (BD) profile. The bands which corresponded to the DGGE reference ladder LCI described and discussed in Section 3.3.4.1 are indicated in orange and numbered (similarly in Figure 3.21 and Table 3.7) for easier reference, while bands which did not are shown in blue. The bands numbered in red correspond to the dominant bands (DB) discussed in Section 3.3.5.1 and are given Prominent Band (PB) number designation. Dominant bands (DB) not corresponding to positions in LCI have been gel-excised (GE) and sequenced. GE1 was taken from 8th week-TPPB2-IF, GE2 from 36th week-TPPB2-A, GE3 from 8th week-TPPB1-A.

Figure 3.8 DGGE profiles of the V3 regions of 16S rDNA of bacterial communities from MPB1 and MPB2 at 4-weekly intervals. The positions of all the bands that have appeared in the complete set of DGGE profiles (including from MPBs, refer Figure 3.28) were consolidated to map out a single Band Distribution (BD) profile. The bands which corresponded to the DGGE reference ladder LCI described and discussed in Section 3.3.4.1 are indicated in yellow and numbered (similarly in Figure 3.21 and Table 3.7) for easier reference, while bands which did not are shown in blue. The bands numbered in red correspond to the dominant bands (DB) discussed in Section 3.3.5.1 and are given Prominent Band (PB) number designation. Dominant bands (DB) not corresponding to positions in LCI have been gel-excised (GE) and sequenced. GE4 was taken from 36th week-MP1.

3.3.2.2.1 Community dynamics based on moving window analysis

In order to quantify the parameter of Dynamics (Dy) of the bacterial communities according to Marzorati et al (2008), the percentage of change between consecutive DGGE profiles of a bioreactor across time, e.g. Week 12 and 16, 16 and 20, and so on for the 4-weekly-interval period and Week 2 and 3, 3 and 4, 4 and 5, and so on for the weekly-interval period of TPPB1, was calculated (Section 3.2.7.2) and plotted over time (Figure 3.9). This is known as a “moving window analysis” where the “window” may be 4 weeks (Figure 3.9A) or 1 week (Figure 3.9B), depending on the context. The percentage of changes across time reflects the degree of dynamism of the community being studied.

From Figure 3.9, it is clear that higher instability in bacterial community structures occurred during the first 12 weeks of operation for both types of bioreactors and their respective fractions. This coincided with the exponential biomass growth phase of the microorganisms in each bioreactor, suggesting that various bacterial subpopulations in the bioreactors were adjusting themselves against each other to adapt to the operating conditions as they proliferated. The microbial consortia then attained stability after 12 weeks, although continuing to maintain levels of changes at 10-20 %. Using the respective data points from the moving window the average weekly and 4-weekly rates of change from 0 to 12 weeks and 12 to 52 weeks were respectively (refer insets in Figure 3.9), which confirmed that the bacterial communities experienced greater changes during the initial 12 weeks of operation. The reduction in the degree of changes thereafter, coinciding with the stationary phase of biomass growth (Figure 2.5 and 2.14) implies that these communities have started to reach a certain level of biological equilibrium, although it remained dynamic.

Overall, the dynamics of the microbial communities between the TPPBs and MPBs were comparable across the whole operation period. During the first 12 weeks, percentage of changes of the TPPB IFs was slightly lower than the aqueous phase samples, but this may not be significant. Therefore, it appears that the presence of NAL in the TPPBs may not have much influence on the bacterial consortia in terms of rate of changes in community structures.

Figure 3.9 Moving window analyses and the rates of change of DGGE profiles

across time. These analyses were performed using pairwise similarity Pearson product-moment correlation coefficients for DGGE profiles (A) monthly from 0 to 52 weeks and (B) weekly from 0 to 12 weeks. The samples were collected from the MPBs (MPB1 and MPB2) and the aqueous phase (TPPB1 and TPPB2) and interfacial fractions (TPPB1-IF and TPPB2-IF) of TPPBs. Percent change is defined as $100 - \text{\%similarity}$ (Marzorati et al, 2008). The rates of change (Δt) values (insets) were calculated as the average and the standard deviation of the respective change % values across weekly ($\Delta t_{1\text{week}}$) or at every 4 weeks ($\Delta t_{4\text{week}}$) interval. Values and errors bars represent means and standard deviation based on duplicate DGGE experiments.

3.3.2.2.2 Community structures in comparison to initial microbial consortium

Other than looking at the dynamic change of the bacterial profiles across time, it is also important to analyze how the bacterial composition in both types of bioreactors had diverged across time with respect to the initial microbiota. In this analysis, the DGGE profiles of all time points were compared to the profile of the initial microbiota (i.e. the 0th week culture).

Figure 3.10 shows the bioreactors bacterial communities' degree of divergence from the original inocula across time. Bacterial composition of both types of bioreactors diverged from the initial microbiota rapidly during the first 12 weeks of operation to as low as 21-25 % of similarity for the TPPBs (aqueous phase) and 42-50 % for the MPBs. Interestingly, the IFs of TPPBs were more similar to the initial inocula, at the 50-70 % range. The frequent rises and falls in their similarity indices in all three types of samples imply that the communities were alternately becoming more and less similar to the 0th week consortia during this period, rather than “maintaining” the degree of differences throughout the 12 weeks.

After the 12 weeks, the similarity indices increased and eventually stabilized. At the stabilized state, the MPBs and the aqueous phase of the TPPBs appeared to have swung back to being more similar to the original community structure than their 12-week dynamic states. The TPPB IFs, on the other hand, have converged with their aqueous counterparts, showing equivalent similarity indices.

It should be noted that although the consortia was inoculated into the aqueous phase of the bioreactors at 0th week, as early as 2nd week, the IFs of the TPPBs already showed a similarity of approximately 50% with the initial microbiota. This suggests that there was a significant subpopulation of bacteria in the initial consortia that has an affinity for the NAL (as discussed in Section 2.4.2 on high density of microbes in IF), in order to generate such an IF-associated subpopulation within the short time frame.

Figure 3.10 Similarity indices between the initial consortium and each bioreactor's consortia across time. DGGE profiles of the samples from the MPBs (MPB1 and MPB2) and the aqueous phase (TPPB1 and TPPB2) and interfacial fractions (TPPB1-IF and TPPB2-IF) of TPPBs were compared to the initial consortium across time. The dashed line denotes the 12th week point, before which (from 2 to 12 weeks) data are presented as weekly and after which data are presented as monthly. Mean data from duplicate experiments are shown with their respective standard error.

3.3.2.2.3 Diversity richness of bacterial communities in bioreactors

The range-weighted richness index (Rr) of each DGGE profile was calculated based on the number of bands and the related percentage of denaturing gradient (Section 3.2.7.2) as described by Marzorati et al (2008). The Rr values were then plotted across time (Figure 3.11) and used to follow the changes in diversity richness in the bioreactors' bacterial consortia.

The Rr analysis showed that all of the communities experienced a drop in their diversity richness during the first 12 weeks, which explained the decrease in similarity between the microbial communities and the initial microbiota during this period (Figure 3.10). Similar drop in Rr has been reported by Beecroft et al (2012) and Lee and Cho (2008) where it was further suggested that selective pressures promote a reduction in the number of species.

However, after Week 12, the Rr of the respective bioreactors began to increase as they stabilized to their equilibrium community states. Since the selection pressure which has presumably resulted in the reduction in Rr during the first 12 weeks continued to exist beyond this period, this increase in diversity richness could no longer be simply dismissed as due to selection pressure. Also, during this period, there appears to be large differences between the duplicate pairs (i.e. TPPB1 vs TPPB2, MPB1 vs MPB2) with respect to this parameter, Rr. A divergence in Rr between duplicate samples suggests that diversity richness is sensitive to small differences existing in replicates of complex systems (Roelke et al, 2003). As a result of this divergence, it was not possible to identify significant trends in Rr when TPPBs and MPBs were compared against each other.

According to the classification by Marzorati et al (2008), Rr values of less than 10 is considered to be low in richness, usually attributed to adverse or restrictive environments on microbial growth. Hence, even though there were changes in Rr across the 52 weeks, they were all within the “low” diversity range, even at the point of initial inoculum. This is perhaps not surprising as the usage of hexane as the sole carbon source in both the acclimatization procedure and during bioreactor cultivation would have created a highly selective environment, reducing diversity richness. The presence of NAL in the TPPBs apparently did not “ease” the selective pressure sufficiently to push the diversity richness indices of the TPPBs higher. Considering that the TPPBs’ microbial consortia displayed higher hexane removal efficiency than the MPBs (Figure 2.9 and 2.17), yet their Rr did not differ significantly, we could not attribute the differential efficiency to diversity richness. Instead, the types of bacteria (specific taxa) or the proportion of bacteria species that make up the consortia may be the basis of improvements in hexane biodegradative capacity in the TPPBs over the MPBs.

Figure 3.11 Diversity richness (Range-weighted richness) of bacterial consortia in the bioreactors. The diversity richness of the MPBs (MPB1 and MPB2) and the aqueous phase (TPPB1 and TPPB2) and interfacial fractions (TPPB1-IF and TPPB2-IF) of TPPBs was calculated based on the number of bands in the DGGE profiles and the corresponding percentage of denaturing gradient used. The dashed line denotes the 12th week point, before which (from 2 to 12 weeks) data are presented as weekly and after which data are presented as monthly. Mean data from duplicate experiments are shown with their respective standard error.

3.3.2.2.4 Functional organization of bacterial communities in bioreactors

It has been speculated that bacterial species, which are able to mineralise hexane or its intermediates more efficiently and possess certain intrinsic characteristics that aid them to survive in the bioreactors, would probably be enriched and present in greater proportions. The nature of distribution of such specialized microorganisms over others (i.e. are they evenly distributed in number, or is there a bias for the few specialized microorganisms?) can be described by the parameter referred to as functional organization (Fo). To derive the values of Fo, we plotted the Pareto-Lorenz (P-L) evenness curves (Section 3.2.7.2) which are based on the cumulative numbers of bands and their cumulative intensities (Mertens et al, 2005) for the bacterial DGGE profiles from the TPPBs and the MPBs. A representative plot based on TPPB1 DGGE profiles at various time points is shown (Figure 3.12). This type of plot reflects the functionality of microbial communities (Marzorati et al, 2008; Wittebolle et al, 2008). A 45° diagonal line on the P-L plot corresponds to a perfect evenness

situation (all members are present in equal quantity) and is usually included in the plots as a reference line (as in Figure 3.12). Translated to DGGE terms, it represents the scenario where all bands in the DGGE lane showed similar intensities.

From the P-L curves, the F_o values were derived as described in Section 3.2.7.2, and presented in Figure 3.13. These F_o values reflect the relation between the structure of a microbial community and its functional redundancy (Lubarsky et al, 2012; Wittebolle et al, 2008). The F_o values of the bacterial communities in all the bioreactors are in the range considered to be of “medium F_o ” (Marzorati et al, 2008; Wittebolle et al, 2008). Conceptually, it refers to a balanced community, where there is the presence of certain dominant species in high proportion while the majority of the species is present in lower proportions. Such a community is believed to be able to deal with changing environmental conditions while preserving its functionality e.g. hexane degradation in the case of our bioreactors (Marzorati et al, 2008). The “dominant species” as described by the F_o analysis refer to bacterial groups which formed the prominent bands in the DGGE profiles. This motivated us to decipher the identities of the bacterial taxa represented by these prominent bands (Section 3.3.4).

Figure 3.12 Pareto-Lorenz curve generated with DGGE profiles of aqueous phase TPPB1 community as an illustrative example. Each curve is generated from one DGGE profile from the specified week's (w) consortium in TPPB1. The functional organization (F_o) values refer to the cumulative species abundances (represented by the cumulative normalized intensities of DGGE bands on the y-axis) of the total population which corresponded to 20 % of

the bands, i.e. where the curve cuts the vertical line at 0.2. In this figure, the Fo of two of the curves among the data set are interpolated using black arrows as examples.

Figure 3.13 Functional organization (Fo) values of the samples collected from the MPBs (MPB1 and MPB2) and the aqueous phase (TPPB1 and TPPB2) and interfacial fractions (TPPB1-IF and TPPB2-IF) of TPPBs over 52 weeks. The dashed line denotes the 12th week point, before which (from 2 to 12 weeks) data are presented as weekly and after which data are presented as monthly. Mean data from duplicate experiments are shown with their respective standard error.

3.3.2.2.5 Cluster analysis, multi-dimensional scaling (MDS) and principle component analysis (PCA) of DGGE profiles

To further analyze the DGGE data obtained for microbial community structures in the bioreactors across time, Pearson correlation coefficients were processed between pairs of DGGE profiles and the cluster method was applied to compare the banding patterns from the bioreactors across time. A dendrogram based on UPGMA clustering was created to determine the hierarchy of similarity between the different microbial communities (Figure 3.14A). The dendrogram showed that samples from TPPBs and MPBs formed separate clusters, indicating that the banding patterns have greater similarity within each types of bioreactor – as would be consistent with microbial communities adapting to operating

conditions common to either type of bioreactors. However, beyond that, only a limited level of information could be drawn from the dendrogram, as they often oversimplify the similarity matrix, resulting in overestimated hierarchical structures being presented (Boon et al, 2002).

As such, alternative methods such as MDS and PCA were also applied to the data. MDS and PCA are both ordination analysis methods which are commonly applied to reduce complex DGGE patterns of the bioreactors to points in a 2 or 3-dimensional space, where the distance between points are linked to their relatedness (Boon et al, 2002; Schafer et al, 2001; van Hannen et al, 1999). MDS replaces the clustering step of the dendrogram and present the relatedness between the samples, whereas PCA analyzes the data directly by computing binary band-matching matrices (Boon et al, 2002) based on the DGGE band profiles of the bioreactors. Hence, a combination of MDS and PCA analyses would likely reveal relationships that are more meaningful for interpretation of the microbial community dynamics.

The MDS plot (Figure 3.14B) demonstrated that the majority of the earlier week samples (4-12 weeks) form a loose cluster (circled with red dashed line) near to the initial inoculum (the purple dot). Across time, the microbial communities within each bioreactor continued to diverge away as observed from the increasing distance between the samples from later weeks and the initial inoculum. The divergence in the microbial communities resulted in the formation of separate clusters by individual bioreactors during the stabilized stage in the MDS plot. Similarly, it was also observed in the PCA plot (Figure 3.14C) that the earlier weeks' samples were loosely clustered close to the initial inoculum while the samples from individual bioreactors during the stabilized stage formed separate clusters further way from each other. Hence it appears that each bioreactor eventually developed into a microbial community with a DGGE band profile set that was fairly unique to itself. The closer relatedness between the samples from the same bioreactor during this “stabilized” stationary phase as compared to those in the exponential growth phase (i.e. up to 12 weeks) corroborates well with the community dynamics data shown in the earlier Section 3.3.2.2.1 which revealed lower rates of change occurring during the stabilization stage (Figure 3.9). We could now be confident that not only were there less

changes between one time point to the next, the structures of the microbial communities within the respective bioreactors were more or less stabilized after 12 weeks of operation.

The closer proximity between the two TPPBs ' clusters, as opposed to the further distance between the two MPB clusters (observed in both MDS and PCA plots) reflects that the microbial communities between duplicate TPPBs were more similar than between that of the MPBs. Within the individual TPPBs, we noted further that samples from both the aqueous and interfacial fractions were located close to one another (Figure 3.14B and C). This implies that the monophasic environment for hexane utilization has a greater tendency to spur divergence (from the same inoculum community) than the biphasic one. This point shall be re-visited in later discussions.

Figure 3.14 Cluster and dimensional analyses of DGGE profiles. Cluster analysis based on UPGMA (A), MDS(B) and PCA (C) performed using data sets derived from the DGGE profiles of the bioreactors. The metric scale of the dendrogram in (A) denotes percentage of similarity while the distance between two points in (B) and (C) reflect similarity of the bacterial DGGE profiles. The early weeks which clustered near to the initial inoculum were circled in red dashed lines. Data points are labelled as Bioreactor_Week, e.g. MPB2_48W.

3.3.2.2.6 Similarity comparison between sets of bioreactors

After the comparison of overall trends through the use of the parameters described so far, we further scrutinized the similarities between specific sets of data based on several main queries.

3.3.2.2.6.1 Between aqueous phase of TPPBs and MPBs

Figure 3.15 shows the divergence in the bacterial community structures of the TPPBs and the MPBs compared pairwise. A drop in the similarity indices in DGGE profiles between the bioreactors could be observed as early as 4th week of the operation. The similarities then rebounded to the mid range up to 28th week, and then continued to diverge. The similarities indices of both TPPBs compared to MPB1 were, on the average, lower compared to MPB2 after 12 weeks.

Specifically looking at the similarities indices of 28th and 44th weeks, i.e. the data points used for hexane degradation efficiency study (Figure 2.9 in Section 2.3.3.1.5), TPPB bacterial communities were found to be 40-59 % similar to MPBs at Week 28 and then dropped to 17-47 % similarity at 44th week. This increased microbial divergence between the TPPBs and the MPBs at 44th week compared to 28th week correlated with to the greater difference in the hexane degradation efficiency between the two types of bioreactors at 44th week (Figure 2.9 and 2.17).

Specific comparison of the DGGE profiles of 28th and 44th weeks' samples within each bioreactor was also done (Figure 3.16). The TPPB set of comparison revealed a similarity of 65-73 %, implying that the bacterial compositions had changed considerably during this period of time (Figure 3.23). As for the MPBs, they showed similarity of about 81- 85 %. These data are in agreement with the data presented earlier that the specific hexane degradation rates of the MPBs consortia did not change significantly from Weeks 28 to 44, while those of the TPPBs improved by at least 16-fold (Figure 2.9 and 2.17).

Figure 3.15 Degree of similarity between community structures of consortia in TPPBs and MPBs over time. DGGE profiles of bacterial communities from the aqueous phase of TPPBs (TPPB1 and TPPB2) were compared pair-wise against monophasic bioreactors (MPB1 and MPB2) based on Pearson product-moment correlation coefficients. The dashed line denotes the 12th week point, before which (from 2 to 12 weeks) data are presented as weekly and after which data are presented as monthly. Mean data from duplicate experiments are shown with their respective standard error. (*) indicates 28th and 44th week where hexane degradation rates of the MPBs and aqueous phase of TPPBs were examined as shown in Figure 2.9.

Figure 3.16 Degree of similarity between the 28th and 44th week communities in each bioreactor. The 28th and 44th week DGGE profiles of bacterial communities in the aqueous phase of TPPB1 (A), TPPB2 (B), MPB1 (C) and MPB2 (D) were compared. Mean data from duplicate experiments are shown with their respective standard error.

3.3.2.2.6.2 Between aqueous phase and IF of TPPBs

The accumulation of biomass at the IF of the TPPBs (Section 2.3.3.2.1-2.3.3.2.3) and the differences in the hexane degradation efficiency (Figure 2.17 under Section 2.3.3.2.4) prompted us to compare the DGGE profiles of bacterial communities within the aqueous phase and the IF of the TPPBs.

An overall trend of increasing similarity (Figure 3.17) across time was observed, and then remained stable from 16th week onwards. Such increase in similarity suggests that a larger subpopulation of bacteria from the aqueous phase may have migrated to the IF over time, resulting in a convergence of community structures in these two fractions within the same TPPB. Looking specifically at the 44th week, 78- 88 % of similarities were observed (Figure 3.17). In earlier studies, differences in the specific hexane degradation rates of the two fractions have been noted (Figure 2.17), which may be due to this degree of variations in community structures between the two fractions of TPPBs.

Figure 3.17 Degree of similarity between community structures of consortia in the aqueous phase and the interfacial fraction of TPPBs across time. Bacterial DGGE profiles of the aqueous phase (TPPB1 and TPPB2) and interfacial fractions (TPPB1-IF and TPPB2-IF) of the TPPBs at each of the time points were compared. The dashed line denotes the 12th week point, before which (from 2 to 12 weeks) data are presented as weekly and after which data are presented as monthly. Mean data from duplicate experiments are shown with their respective standard error. (*) indicates 44th week where hexane degradation rates of the interfacial fractions of TPPBs were examined as shown in Figure 2.17.

In conclusion, a survey of the bacterial community dynamics based on DGGE analysis revealed that there were no clear correlation between the functional efficiency of the TPPBs and the MPBs and their consortia' gross parameters such as community dynamics, diversity richness and functional organization, or even the degree of similarity between specific pairs of consortia. Thus, we would need to identify the bacterium or groups of bacteria that have contributed to the difference in community structures and resulted in the improvement in the hexane degradation.

3.3.3 Identification of bacterial taxa present in bioreactors

In order to discern the bacterial groups that have contributed to differential hexane degradation efficiency, we made use of both culture-based and culture-independent approaches. The culture-based methods allowed for the isolation of culturable microorganisms, while the culture-independent methods based on 16S rDNA sequence diversity allows the phylogenetic assignment of bacteria present in the microbial consortia to be made.

3.3.3.1 16S rDNA clone library of bioreactor inoculum (0th week sample)

The sensitivity of 16S rDNA clone library analyses, with respect to understanding the community diversity and phylogenetic make-up, is higher than that of the DGGE technique (van Elsas & Boersma, 2011).

Since the bioreactors were operating under aseptic conditions, all bacterial members found within the bioreactors would have originated from the initial consortium. In this study, in order to determine the composition of the original inoculum for comparison with the later weeks' samples, the full-length 16S rDNA amplicon library was generated using the genomic DNA preparation of the 0th week microbial consortium. This would provide the best spread of strains for identification and referencing, to better understand the community dynamics within all bioreactors. After ligation and transformation of the recombinant library into the *E. coli* host (Section 3.2.4), a total of 200 colonies were screened and out of these, 168 clones were identified to be carrying the correctly sized inserts of 1.5 kb (corresponding to the size of a full-length 16S rDNA). These clones were given the pre-fix "C" followed by their numerical designation (e.g. C12) and put through the process of identification along with the BP series of strains described in Section 3.3.3.2.

3.3.3.2 Culturable isolates sampled from bioreactors

One readily available source of culturable isolates from the bioreactors was the colonies grown on the agar plates as culturable cell count analyses were conducted weekly (Section 2.3.3.1.4 and 2.3.3.2.3).

It has been recognized that classification by colony morphotypes do not allow clear distinction of strains/species/genus to be made. However, depending on the context of the community, some useful information may be gleaned. In this instance, the various colony morphotypes (Figure 3.5) were used to aid in choosing the culturable isolates to be sampled – the number of isolates of each morphotype to be picked for molecular identification (Table 3.5) was based on the observable relative abundance (Figure 3.6), so that within the very limited sampling that we have taken, as to determine the diversity of the bioreactor consortia and to make meaningful interpretation.

The collection of these isolates was from two major phases of the bioreactors' biomass development as discussed in Section 2.3.3: exponential phase (0 to 12th week) and the stationary growth phase (12 weeks onwards).

Table 3.5 Number of colonies of each morphotype sampled from the bioreactors

Colonies on MSM agar infused with hexane were classified into five colony morphotypes, namely Halo, Flower-shaped, Orange, Small and Medium.

Colony morphotype	Halo	Flower-shaped	Orange	Small	Medium	Sum
MPB	4	4	12	7	3	30
TPPB	4	2	12	14	2	34
TPPB1-IF	2	3	25	11	4	45
Total	10	9	49	32	9	109

These isolates were first checked by PCR using bacterial (Tbf/Tbr) and fungal (Tff/Tfr) specific primer sets (shown in Table 3.1). Amplicons were observed only with the use of bacterial specific primers, indicating that all 109 isolates were bacteria, probably because the fungal population is too low in abundance (Figure 3.4 under Section 3.3.1.3) or are too fastidious, only able to

grow in selective medium. These isolates were given the pre-fix “BP” followed by their numerical designation (e.g. BP34), and put through identification as the C-series of clones.

3.3.3.3 Molecular identification of clones/isolates based on strategy combining PCR-ARDRA and 16S rDNA sequencing

In many cases when working with unidentifiable environmental isolates, sequencing of the full-length 16S rDNA allows their phylogenetic affiliation to be determined, and based on this, each unique 16S rDNA sequence was assigned a molecular operational taxonomic unit (MOTU) identity (Blaxter, 2004). However, to sequence all isolates can be costly (Drancourt et al, 2000). To determine the molecular identity of a total of 277 16S rDNA sequences (of the 168 clones and 109 culturable isolates), we first applied the ARDRA method (Section 3.2.6.1) to categorize the isolates into different groups based on the molecular fingerprints generated. The use of ARDRA, complemented with the sequencing of full-length 16S rDNA sequence of strategically selected clones/isolates was able to lead to taxonomic identification more cost-efficiently (Cook & Meyers, 2003; Jose & Jebakumar, 2012; Zeng et al, 2007).

The 16S rDNA of the 168 clones and the 109 isolates (in total 277 strains) were therefore characterized by ARDRA using two restriction enzymes, *HaeIII* and *RsaI*, each enzyme digest generating one molecular profile so that a database of 2 ARDRA fingerprints per isolate could be built up. Isolates with the same pair of molecular fingerprints were classified under the same ARDRA pattern group (PG) (Figure 3.18 showing 2 representative PGs, 41 and 43). A total of 55 such PGs were identified (Table 3.6, 1st column) and the number of strains belonging to each PG shown (Table 3.6, 2nd column).

Figure 3.18 Full-length 16S rDNA ARDRA fingerprints of representative pattern groups, PG41 (A) and PG43 (B). The full-length 16S rDNA PCR-amplified fragments were digested by *HaeIII* and *RsaI* respectively and subsequently electrophoresed through 2.5% agarose gel along with the molecular weight marker, 100bp DNA Ladder Plus (Fermantas). Isolates exhibiting the same fingerprints for each of the two restriction digests were classified under the same ARDRA pattern group (PG).

3.3.3.3.1 Identification of selected clones and isolates by 16S rDNA sequencing

Since ARDRA alone can only group strains of the same genus based on their molecular fingerprints, but provides no information on the actual identity of the bacteria (Sklarz et al, 2009), the 16S rDNA of at least one representative clones/isolates from each ARDRA PG were sequenced so that a preliminary association between taxonomic identity and molecular fingerprints could be established. This was also necessary as the taxonomic resolution level of ARDRA differs from community to community and we need to establish this relationship within our system.

The sequence data set comprising of the full-length 16S rDNA of (i) one representative isolate from each of the 55 ARDRA PGs and a further 48 isolates randomly picked from (ii) several of the more populous PGs and (iii) a spread of different colony morphotypes (refer Section 3.3.3.2) were submitted to the Ribosomal Database Project (RDP-II) release 10.31 (Cole et al, 2009) to obtain

matches for the closest-related type strains. Alignments and matches were performed and referenced against the collection of type strains available in the RDP when deriving taxonomic assignments for the isolates, instead of matching against similar sequences from other non-type strains. This is because type strains are better characterized and thus could provide a more reliable identification (Timke et al, 2005). The sequence similarities of the isolates to the closest-matched type strains are listed in Table 3.6. Taxonomic identification to the species, genus, family/ order/ class and phylum levels were set at $\geq 99\%$, $\geq 95\%$, $\geq 90\%$ and $\geq 80\%$ respectively, similar to the criteria used by Bosshard et al (2003) and Giongo et al (2010).

Evaluation of the 16S rDNA-sequencing results revealed that all of the 101 sequenced members have $\geq 95\%$ similarity to that of previously characterized bacterial type strains in the RDP database, allowing them to be identified up to the genus level. Amongst them, 49 isolates (49 %) showed sequence matches with $\geq 99\%$ similarity, indicating that these members could be identified to the species level. Phylogenetic analyses were further performed on these sequences to show the diversity among the sequenced members within each genus (Figure 3.20) and in relation to other genera (Figure 3.21).

3.3.3.3.2 ARDRA profiles and 16S rDNA-based taxonomic identification

The ARDRA data set consisting of 55 PGs (Table 3.6, first column), into which the 277 samples of 16S rDNA were grouped as discussed in Section 3.3.3.3, showed that more than one isolate/clone (Table 3.6, 2nd column) have been classified into PGs 2, 3, 7, 8, 10, 11, 13, 14, 15, 16, 18, 21, 22, 23, 24, 30, 31, 32, 33, 34, 35, 39, 40, 41, 42, 43, 44, 45, 49 and 52. From some of these PGs, several more isolates have been selected to be sequenced, e.g. in total 5 out of the 13 isolates belonging to PG 2 were sequenced. Comparing the taxonomic identities of the sequenced isolates within such PGs showed that only one genus (but may be more than one species) can be found within one PG (Table 3.6, 5th column for PGs 2, 3, 7, 10, 11, 13, 15, 16, 21, 22, 23, 24, 31, 34, 35, 41, 42, 43, 49 and 52). Conversely, isolates and clones belonging to different genera were always grouped into different PGs. It was also noted from observation across all

PGs and their taxonomic identities that one genus may be represented by more than one PG (Table 3.6, shaded boxes), sometimes allowing differentiation at the species level and hence a slightly higher resolution of diversity within one genus.

This associative analysis of PCR-ARDRA groupings to their taxonomic identities based on full-length 16S rDNA sequences was able to show that the ARDRA approach was able to reliably discriminate a total of 109 isolates and clones at the genus level, and even for some of them down to the species level. Hence, the remaining 176 unsequenced isolates and clones, which were classified into the 55 PGs, were inferred to be of the same genus as the sequenced members of the respective PGs in Table 3.6. This information forms the basis of the analysis of community structures and dynamics discussed in the following Section 3.3.4 and 3.3.5.

Table 3.6 Pattern Groups (PGs) generated from ARDRA of 16S rDNA library clones of the 0th week microbial consortium and the culturable isolates sampled from the bioreactors. The clones and isolates were assigned to the PG based on the restriction enzyme digest profiles generated by *Hae*III and *Rsa*I. Each clone (given a unique C number) and each isolate (given a unique BP number) were picked randomly within its PG to be representatives and sequenced for their full-length 16S rDNA. The isolates' colony morphotype (CM) classifications are indicated as: Halo (H), Flower-shaped (F), Orange (O), Small (S) and Medium (M). In the case of the clones, they are labeled as Unknown (U) in the CM column. Δ and ^ represent bacteria isolated from the aqueous phase and interfacial fraction of TPPBs respectively while ▫ stands for bacteria from MPBs. These sequences were compared with the type species in the RDP database. Accession numbers of the closest type-species matches and their similarity score (%) are shown. Boxes which are shaded indicate that these are genera found to be represented by more than 1 PG. “*” indicates that the PG group has been found to carry sequenced members with closest matches to more than one type species of the same genus.

PG	No. of samples	Designation of strains	CM	Closest type species and Accession number	Score (%)
Class: Actinobacteria (198 clones and isolates)					
1	1	BP108 ^Δ	S	<i>Gordonia amicalis</i> (T); IEGM; AF101418	99.9
*2	13	BP5 ^Δ	S	<i>Gordonia humi</i> (T); CC-12301; FN561544	98.0
		BP82 [▫]	F	<i>Gordonia humi</i> (T); CC-12301; FN561544	100
		BP92 [▫]	F	<i>Gordonia humi</i> (T); CC-12301; FN561544	100
		BP87 ^Δ	F	<i>Gordonia polyisoprenivorans</i> (T); DSM 44302; Y18310	99.3
		BP90 ^Δ	F	<i>Gordonia polyisoprenivorans</i> (T);	99.2

				DSM 44302; Y18310	
3	1	BP37 [^]	O	<i>Gordonia polyisoprenivorans</i> (T); DSM 44302; Y18310	96.6
	1	C127	U	<i>Gordonia polyisoprenivorans</i> (T); DSM 44302; Y18310	99.3
4	1	BP64 ^Δ	M	<i>Gordonia polyisoprenivorans</i> (T); DSM 44302; Y18310	98.2
5	1	BP96 ^Δ	F	<i>Gordonia polyisoprenivorans</i> (T); DSM 44302; Y18310	99.2
6	1	C78	U	<i>Phycococcus ginsenosidimutans</i> (T); BXN5-13; EU332824	97.7
7	4	C79	U	<i>Lapillicoccus jejuensis</i> (T); type strain: R-Ac013; AM398397	98.6
		C150	U	<i>Lapillicoccus jejuensis</i> (T); type strain: R-Ac013; AM398397	98.2
8	3	C18	U	<i>Leifsonia xyli</i> (T); JCM 9733; AB016985	99.0
9	1	BP9 ^Δ	S	<i>Microbacterium aoyamense</i> (T); KV- 492; AB234028	97.6
10	1	BP3 ^Δ	S	<i>Microbacterium laevaniformans</i> (T); DSM 20140; Y17234	98.0
	1	C2	U	<i>Microbacterium laevaniformans</i> (T); DSM 20140; Y17234	97.7
*11	1	BP30 [^]	S	<i>Microbacterium paraoxydans</i> (T); CF36; AJ491806	97.8
	2	C4	U	<i>Microbacterium paraoxydans</i> (T); CF36; AJ491806	99.7
		C183	U	<i>Microbacterium resistens</i> (T); DMMZ 1710; Y14699	97.5
12	1	BP34 [□]	S	<i>Microbacterium maritopicum</i> (T); type strain: DSM 12512; AJ853910	97.4
*13	33	BP15 [□]	O	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	95.0
		BP48 [□]	S	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	95.3
		BP69 ^Δ	S	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.9
		BP79 [^]	S	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.6
		BP21 [^]	O	<i>Mycobacterium canariasense</i> (T); 502329; AY255478	99.6
		BP99 [^]	F	<i>Mycobacterium canariasense</i> (T); 502329; AY255478	99.3
		BP22 [^]	S	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.7
	14	C45	U	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.7
14	3	BP65 ^Δ	S	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.8
15	4	BP42 ^Δ	O	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.8
	1	C156	U	<i>Mycobacterium cosmeticum</i> (T); LTA- 388; AY449728	99.1

16	1	BP75 [^]	O	<i>Mycobacterium cosmeticum</i> (T); LTA-388; AY449728	99.8
	8	C12	U	<i>Mycobacterium cosmeticum</i> (T); LTA-388; AY449728	99.0
	1	C119	U	<i>Mycobacterium cosmeticum</i> (T); LTA-388; AY449728	96.8
17	1	C75	U	<i>Mycobacterium cosmeticum</i> (T); LTA-388; AY449728	99.6
18	6	C34	U	<i>Mycobacterium flavescens</i> (T); ATCC 14474.; X52932	97.5
19	1	C62	U	<i>Mycobacterium rufum</i> (T); JS14; AY943385	99.0
20	1	C74	U	<i>Mycobacterium rufum</i> (T); JS14; AY943385	99.1
21	6	C104	U	<i>Saxeibacter lacteus</i> (T); type strain: DLS-10; AM778124	97.7
		C113	U	<i>Saxeibacter lacteus</i> (T); type strain: DLS-10; AM778124	97.3
*22	4	BP4 ^Δ	O	<i>Rhodococcus aetherivorans</i> (T); 10bc312; AF447391	98.3
		BP47 [^]	O	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.6
*23	1	BP71 [□]	O	<i>Rhodococcus aetherivorans</i> (T); 10bc312; AF447391	99.8
	37	C114	U	<i>Rhodococcus aetherivorans</i> (T); 10bc312; AF447391	98.9
		C16	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.8
		C64	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.6
		C67	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.4
		C77	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	98.5
		C105	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.0
		C108	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	98.5
		C117	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	98.9
		C160	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.4
*24	4	BP26 [□]	O	<i>Rhodococcus phenolicus</i> (T); G2P; AY533293	98.5
	34	C1	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.6
25	1	BP73 [□]	O	<i>Rhodococcus wratislaviensis</i> (T); NCIMB 13082 type strain; NR_026524	98.4
26	1	C14	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.4
27	1	C46	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	95.1
28	1	C172	U	<i>Rhodococcus ruber</i> (T); type strain: DSM43338; X80625	99.4
29	1	C20	U	<i>Propionimonas paludicola</i> (T); Wd;	98.1

				AB078858	
Class: Deinococci (2 clones)					
30	2	C10	U	<i>Deinococcus aerius</i> (T); TR0125; AB087288	90.5
Class: Flavobacteria (26 clones and isolates)					
31	4	BP6 [^]	S	<i>Chryseobacterium formosense</i> (T); CC-H3-2; AY315443	98.0
	1	C8	U	<i>Chryseobacterium formosense</i> (T); CC-H3-2; AY315443	97.9
32	2	BP66 ^Δ	S	<i>Chryseobacterium hominis</i> (T); type strain: NF802; AM261868	99.3
33	2	BP107 [□]	S	<i>Chryseobacterium hominis</i> (T); type strain: NF802; AM261868	99.2
*34	1	BP1 ^Δ	S	<i>Chryseobacterium indologenes</i> (T); LMG 8337; AM232813	98.5
	12	C6	U	<i>Chryseobacterium gleum</i> (T); CCUG 14555; AM232812	98.0
		C35	U	<i>Chryseobacterium gleum</i> (T); CCUG 14555; AM232812	97.9
		C102	U	<i>Chryseobacterium indologenes</i> (T); LMG 8337; AM232813	98.6
35	3	BP104 ^Δ	H	<i>Chryseobacterium pallidum</i> (T); type strain: 26-3St2b; AM232809	99.1
		BP105 ^Δ	H	<i>Chryseobacterium pallidum</i> (T); type strain: 26-3St2b; AM232809	99.1
36	1	BP10 ^Δ	S	<i>Flavobacterium lindanitolerans</i> (T); IP10; EF424395	95.3
Class: Alphaproteobacteria (10 clones and isolates)					
37	1	BP17 ^Δ	O	<i>Caulobacter segnis</i> (T); MBIC2835; AB023427	98.6
38	1	BP33 ^Δ	O	<i>Sphingomonas yabuuchiae</i> (T); GTC868; AB071955	97.0
39	6	C28	U	<i>Sphingomonas echinoides</i> (T); ATCC 14820T; AB021370	99.1
40	2	C3	U	<i>Rhizobium tubonense</i> (T); CCBAU 85046; EU256434	96.9
Class: Betaproteobacteria (29 clones and isolates)					
*41	2	BP2 ^Δ	S	<i>Achromobacter xylosoxidans</i> (T); type strain: DSM 10346; Y14908	98.8
		BP35 [□]	S	<i>Achromobacter xylosoxidans</i> (T); type strain: DSM 10346; Y14908	98.5
	3	C40	U	<i>Achromobacter insolitus</i> (T); LMG 6003; AY170847	99.0
		C146	U	<i>Achromobacter insolitus</i> (T); LMG 6003; AY170847	98.7
*42	5	C5	U	<i>Achromobacter spanius</i> (T); LMG 5911; AY170848	99.1

		C170	U	<i>Achromobacter spanius</i> (T); LMG 5911; AY170848	99.4
43	2	BP29 ^Δ	S	<i>Burkholderia arboris</i> (T); R-24201; AM747630	98.3
		BP32 [□]	S	<i>Burkholderia arboris</i> (T); R-24201; AM747630	99.0
44	4	C11	U	<i>Burkholderia fungorum</i> (T); LMG 16225; AF215705	99.6
45	2	C42	U	<i>Burkholderia fungorum</i> (T); LMG 16225; AF215705	99.2
46	1	BP31 ^Δ	S	<i>Cupriavidus oxalaticus</i> (T); DSM 1105; NR_025018	97.6
47	1	BP39 ^Δ	S	<i>Variovorax boronicumulans</i> (T); BAM-48; AB300597	99.1
48	1	BP63 ^Δ	H	<i>Variovorax boronicumulans</i> (T); BAM-48; AB300597	97.5
49	5	BP16 ^Δ	M	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	99.0
		BP18 ^Δ	M	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	99.0
		BP27 [□]	H	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	99.1
		BP67 ^Δ	H	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	98.8
	1	C23	U	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	98.7
50	1	BP7 ^Δ	H	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	97.9
51	1	BP23 [□]	H	<i>Variovorax paradoxus</i> (T); DSM 66; AJ420329	97.4
Class: γ-proteobacteria (3 isolates)					
*52	3	BP68 ^Δ	H	<i>Nevskia ramosa</i> (T); Soe1; AJ001010	99.3
		BP80 ^Δ	H	<i>Nevskia ramosa</i> (T); Soe1; AJ001010	98.5
Class: Sphingobacteria (3 clones and isolates)					
53	1	BP94 [□]	F	<i>Mucilaginibacter ximonensis</i> (T); XM-003; EU729366	95.2
54	1	BP46 [□]	S	<i>Pedobacter koreensis</i> (T); WPCB189; DQ092871	96.4
55	1	C137	U	<i>Siphonobacter aquaeclarae</i> (T); P2; FJ177421	98.8

3.3.3.3.3 Association of colony morphotypes to taxonomic groups

Based on the above taxonomic assignment, colony morphotypes were re-examined to see if any of them uniquely represented a taxonomic group (Figure 3.19). It was noted that (i) as expected, more than one genera could be found within each colony morphotype – the morphotype group S were most heterogeneous (9 genera), followed by O (4 genera), then F and M (both 3 genera), while the H morphotype encompassed only 2 genera: *Variovorax* and *Nevskia*. It was also noted that (ii) some genera, such as *Chryseobacterium*, *Mycobacterium* and *Gordonia*, exhibited varied colony morphotypes. For example, isolates of the genus *Mycobacterium* were found to be distributed among the F, O, S and M morphotypes.

These observations confirmed that for the microbial communities in our system, the dynamics shown earlier based on colony morphotype distribution profiles (Figure 3.6) could indeed only be used as a preliminary and crude survey. Instead, the identities of the clones/isolates obtained from the above work (Section 3.3.3.3.1 and 3.3.3.3.2) shall be complemented with DGGE profiles (Section 3.3.4) to provide a better picture of the microbial dynamics in a later section (Section 3.3.5).

Figure 3.19 Distribution of genera within each colony morphotype. After the 109 bacterial isolates have been assigned taxonomic identities to the level of genus based on their 16S rDNA (either sequenced directly or associated through ARDRA PGs) and their distributions among the five colony morphotypes – Halo, Flower-shaped, Orange, Small and Medium – are shown.

3.3.3.4 Phylogenetic relationships of bacteria in initial inoculum and bioreactor communities

In order to establish the relationship between the clones derived from the initial inoculum and the culturable isolates sampled from the bioreactors, the sequences of 16S rRNA genes of all the type species which had been surfaced as “closest matches” (Table 3.6) were mapped for their phylogenetic relationship to each other (Figure 3.20A). In total, clones affiliated to 23 genera of bacteria consisting of 44 species have been identified, which is considered to be of moderate to high diversity, compared to what is typically found in soils (Nacke et al, 2011). Of these genera, 8 of them belonged to the class Actinobacteria, comprising of gram positive bacteria which are G-C rich.

The relative abundance of the clones affiliated to these genera within the 0th week 16S rRNA library clones were then examined and compared to the set of isolates sampled from the bioreactors (Figure 3.20B). Of the 23 genera, 9 genera (Figure 3.20A, boxed in purple) have been commonly found in the bioreactors across time and 8 genera (boxed in green) have been identified exclusively from the 168 clones of the 0th week clone library. The remaining 6 genera (boxed in yellow) have been identified exclusively from the isolates sampled from the bioreactors. This is only a cursory survey of the distribution of genera between the initial inoculum and the bioreactor communities during operation, and should not be used for in-depth interpretation as the data were based on a fairly limited sampling of isolates.

Figure 3.20 (A) Phylogenetic tree of the 16S rDNA sequences of the type species of the bacteria genera identified as listed in Table 3.5 and (B) abundance of different bacteria genera from the different bioreactors. The tree was constructed using in MEGA5 (Tamura et al, 2011). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Number next to the species name refers to the Genbank accession number. *Dietzia maris* X79290 was used as outgroup. A total of 23 genera identified were listed in the phylogenetic tree where 9 genera (boxed in purple) had been commonly found in the bioreactors across time and 8 genera (boxed in green) had been identified from 168 clones of 0th week clone library. The remaining 6 genera (boxed in yellow) had been identified from the isolates cultured from TPPBs (aqueous phase and IF) and MPBs. The abundance of different bacteria genera (as percentage) in 0th week clone library, and in different bioreactors across time, plotted on the right, had been identified based on PCR-ARDRA and 16S rDNA sequencing of representative clones/isolates.

3.3.3.5 Intra-genus diversity of clones/isolates

The phylogenetic relationship of the sequenced clones/isolates within each genus was also presented (Figure 3.21). The evolutionary (horizontal) distance between each strain of the same genus would provide an indication of the diversity in their 16S rDNA sequences and the degree of novelty of these sequences in comparison to the type species.

The clones and isolates which belonged to the same genus but different PGs formed separate clusters in the phylogenetic trees. Among these clusters, some members were found to have identical 16S rRNA sequence, and therefore were designated the same MOTU (refer designation such as G1, G2, My1, My2 etc).

Figure 3.21 Phylogenetic analysis of the full-length 16S rDNA sequences of the clones and isolates. The full-length 16S rDNA sequenced clones and isolates under different genera, (A) *Gordonia*, (B) *Mycobacterium*, (C) *Rhodococcus*, (D) *Microbacterium*, (E) *Lapillicoccus*, (F) *Saxeibacter*, (G) *Chryseobacterium*, (H) *Sphingomonas*, (I) *Achromobacter*, (J) *Burkholderia*, (K) *Variovorax* and (L) *Nevskia* were plotted separately. The trees were constructed using Neighbor-Joining algorithm (Saitou & Nei, 1987) with 5,000 bootstraps via MEGA5 (Tamura et al, 2011). The bootstrap values are shown next to the branches in percentage (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Number next to the type species name refers to the Genbank accession number. These clones and isolates under the same branch in the phylogenetic tree were grouped together in a cluster and given their Molecular Operational Taxonomic Unit (MOTU) designation e.g. G1, My1.

3.3.4 Association of DGGE profiles to MOTU identities

The DGGE profiles of the 16S rDNA hypervariable V3 regions of the bacterial consortia presented earlier allowed the temporal trends within the MPBs and the two fractions of the TPPBs to be analyzed (Section 3.3.2.2), based on the parameters suggested by Marzorati et al (2008) and Wittebolle et al (2008). However, there were no discernible features with respect to these community-based parameters that could explain the differential hexane degradation efficiency between the TPPBs and the MPBs (discussed in Section 3.3.2). Clearly, the identities of the resident bacteria within the bioreactor communities needed to be examined.

Thus, we have worked to derive the taxonomic affiliation of the 277 clones/isolates from the 16S rDNA clone library (of the initial inoculum) and the culturable isolates (of the bioreactors during operation) (Section 3.3.3). However, from the perspective of temporal analysis of complex communities, the sampling size of 277 was admittedly deficient and limited. If the identities of bacteria could be associated to specific band positions on a DGGE gel, it may be possible to leverage on the extensive sampling provided by DGGE profiles of the bioreactors' bacterial communities across time, to reach further conclusion about their development and dynamics. In this section, the association process and the resulting data shall be presented.

3.3.4.1 Construction of reference DGGE ladder using MOTU-designated clones/isolates

The first step in this attempt was to determine the DGGE profiles of all unique MOTUs originating from the 0th week 16S rDNA clone library (i.e. the C-series strains) and the bioreactors' culturable subpopulations (i.e. the BP-series strains). The genomic DNA of the respective strains were extracted (Section 3.2.1), the 16S rDNA amplicons obtained, and put through DGGE (Section 3.2.2.3 and 3.2.7). All of the 47 clones and most of the 54 isolates exhibited DGGE profiles carrying a single prominent band, while a few of the isolates carried multiple bands (indicated with * in Table 3.7). The latter observation was

not surprising due to the multiplicity and heterogeneity of rRNA gene operons in the genomes of some bacterial species (Kang et al, 2010). All of these bands (whether they occurred singly or as multiples) were mapped according to their relative positions on the DGGE gel (Figure 3.22A). The clone collection generated 20 unique band positions (serially numbered as c-1, 2,...) while the isolate collection generated 29 (serially numbered as i-1, 2,...), which were physically pooled to generate Reference Ladder of Clones (LC) and Reference Ladder of Isolates (LI), respectively (Figure 3.22B). Several of the bands in LC and LI mapped to the same positions, e.g. c-1/i-2, c-2/ i-4. By merging the members of LC and LI, a complete reference ladder (LCI) mapping all 36 unique band positions that have known association to MOTU identities could be generated (Figure 3.22C). The origins of these band positions (serially numbered as Band 1, 2,...) with reference to their MOTU designation and classification (e.g. PG) according to systems used in previous experimental contexts are listed in Table 3.7.

A survey of Table 3.7 shows that 20 of the Band positions, e.g. Band 1, and 3, could be uniquely associated to a single genus. Of these 20 Bands, it was noted that 4 were associated to more than one MOTU. It was further noted that those MOTUs which were within the same genus but distant enough to belong to different ARDRA PGs could be distinguished by DGGE, i.e. taking up different band positions. For example, the MOTUs (C4, C5, C6, C8) belonging to PG31 and PG34, but all affiliated to the genus *Chryseobacterium*, took up the positions of Band 4 and Band 2 respectively. Band positions with these properties (“single” genus) would allow association of DGGE profiles to the genus level without complications. On the other hand, there were cases whereby some MOTUs that belong to different genera and different PGs, in fact profiled to the same Band position. Examples are PGs that belong to the genera *Achromobacter* (PG41) and *Mycobacterium* (PG20) both of which took up the position of Band 12. DGGE profiles displaying these Band positions (“composite” genera) would therefore have to be interpreted with sufficient prudence and caution.

Figure 3.22 (A) DGGE gel of unique band and multiple bands' examples respectively, from the clone library set (with C number) and the culturable isolate set (with BP number) with the band profiles superimposed to form the hypothetical reference ladder, (B) The comparison of the band pattern of clone (LC) and isolate (LI) ladder and (C) the construction of the combined ladder (LCI). The combined ladder comprised of 36 bands spanning over 23 genera. It is formed by pooling the PCR products of *16S rDNA* V3 region of culturable bacteria and clones which formed unique band positions. Origins of the bands and their phylogenetic affiliations are presented in Table 3.7.

Table 3.7 List of clones and isolates and its associated DGGE band position on the combined ladder. The band positions of the clones from 0th week clone library and the culturable isolates with their respective ARDRA groups (PGs) and phylogenetic affiliation on the combined ladder, LCI. Such isolates and clones had also formed similar band positions on the ladder.

	Clones			Culturable isolates							
Band	LC	Isolate	PG	LI	Isolate	PG	MOTU Designation	Phylogenetic affiliation			
1				i-1	BP46	PG54*	Pe1 [#]	<i>Pedobacter</i>			
2	c-1	C102 C6 C35	PG34	i-2	BP2	PG41*	A5	<i>Achromobacter</i>			
					BP1	PG34	C8	<i>Chryseobacterium</i>			
							C5				
							C6				
3				i-3	BP2	PG41*	A5	<i>Achromobacter</i>			
4	c-2	C8	PG31	i-4	BP6	PG31	C4	<i>Chryseobacterium</i>			
5				i-5	BP46	PG54*	Pe1 [#]	<i>Pedobacter</i>			
6				i-6	BP10	PG36	F1 [#]	<i>Flavobacterium</i>			
					BP39	PG47	V1	<i>Variovorax</i>			
					BP63	PG48	V2				
					BP16	PG49	V4				
					BP18		V5				
					BP27		V3				
					c-3	C23	PG49	BP67		V6	
								BP7	PG50	V7	
				BP23	PG51	V8					
	7				i-7	BP46	PG54*	Pe1 [#]	<i>Pedobacter</i>		
BP104, BP105						PG35	C3	<i>Chryseobacterium</i>			
8	c-4	C137	PG55				Si1 [#]	<i>Siphonobacter</i>			
9				i-8	BP68 , BP80	PG52	N1	<i>Nevskia</i>			
10				i-9	BP10	PG36*	F1 [#]	<i>Flavobacterium</i>			
11				i-10	BP66	PG32	C1	<i>Chryseobacterium</i>			
					BP107	PG33	C2				
12	c-5	C146	PG41				A1	<i>Achromobacter</i>			
		C74	PG20				My13	<i>Mycobacterium</i>			
13				i-11	BP35	PG41*	A6	<i>Achromobacter</i>			
					BP29	PG43	B3	<i>Burkholderia</i>			
					BP32		B4				
					BP31	PG46*	Cu1 [#]	<i>Cupriavidus</i>			
14	c-6	C10	PG30				D1 [#]	<i>Deinococcus</i>			
		C28	PG39				Sh1	<i>Sphingomonas</i>			
15				i-12	BP35	PG41*	A6	<i>Achromobacter</i>			
					BP17	PG37	Ca1 [#]	<i>Caulobacter</i>			
16				i-13	BP35	PG41*	A6	<i>Achromobacter</i>			
					BP31	PG37*	Cu1 [#]	<i>Cuprivadus</i>			
17	c-7	C18	PG8				Le1 [#]	<i>Leifsonia</i>			
18				i-14	BP33	PG38*	Sh2	<i>Sphingomonas</i>			
	c-8	C170	PG42				A2	<i>Achromobacter</i>			
		C11	PG44				B2	<i>Burkholderia</i>			
		C3	PG40				Rh1 [#]	<i>Rhizobium</i>			
19				i-15	BP33	PG38*	Sh2	<i>Sphingomonas</i>			
	c-9	C40	PG41				A4	<i>Achromobacter</i>			
20	c-10	C5	PG40	i-16	BP2	PG41*	A5	<i>Achromobacter</i>			
21				i-17	BP35	PG41*	A6	<i>Achromobacter</i>			
					BP9	PG9	M2	<i>Microbacterium</i>			

22	c-11	C42	PG45				B1	<i>Burkholderia</i>	
23	c-12	C78	PG6				Ph1 [#]	<i>Phycicoccus</i>	
				i-18	BP35	PG41*	A6	<i>Achromobacter</i>	
24	c-13	C2	PG10	i-19	BP3	PG10	M1	<i>Microbacterium</i>	
		C4				BP30	PG11		M5
					BP34	PG12	M4		
		C183	PG11				M3		
25	c-14	C79	PG7				L1	<i>Lapillicoccus</i>	
		C150			L2				
26				i-20	BP5	PG2	G6	<i>Gordonia</i>	
					BP82, BP92		G7		
					BP87, BP90		G4		
27				i-21	BP65	PG14*	My6	<i>Mycobacterium</i>	
					BP42	PG15*	My7		
					BP75	PG16*	My10		
					BP15	PG13*	My17		
					BP21		My14		
					BP48		My16		
					BP69, BP79		My15		
					BP99		My9		
					BP22		My4		
					28				
BP42	PG15*	My7							
BP75	PG16*	My10							
BP15	PG13*	My17							
BP21		My14							
BP48		My16							
BP69, BP79		My15							
BP99		My9							
BP22		My4							
		c-15	C45	PG13					
	C156		PG15	My3					
	C12		PG16	My2					
	C119			My8					
	C75			PG17	My1				
	C62		PG19	My12					
	C20		PG29	Pr1 [#]					
29	c-16	C113	PG21				S2	<i>Saxeibacter</i>	
		C114	PG23				R10	<i>Rhodococcus</i>	
				i-23	BP4	PG22*	R3	<i>Rhodococcus</i>	
BP47					R4				
BP71					PG23*	R5			
30				i-24	BP94	PG53*	Mu1 [#]		<i>Mucilaginibacter</i>
					BP4	PG22	R3		<i>Rhodococcus</i>
					BP47		R4		
					BP71	PG23*	R5		
					BP26	PG24*	R9		
	c-17	C16, C64, C67	PG23				R13		
C77							R1		
C105							R11		

		C108					R2	
		C117						
		C160						
		C1	PG24				R15	
		C14	PG26				R14	
		C46	PG27				R16	
		C172	PG28				R6	
31				i-25	BP26	PG24*	R9	<i>Rhodococcus</i>
			BP94		PG53*	Mu1 [#]	<i>Mucilaginibacter</i>	
32				i-26	BP108	PG1*	G5	<i>Gordonia</i>
			BP96		PG5*	G3		
33	c-18	C127	PG3	i-27	BP37	PG3	G1	<i>Gordonia</i>
					BP108	PG1	G5	
					BP96	PG5*	G3	
34				i-28	BP73	PG25	R8	<i>Rhodococcus</i>
	c-19	C34	PG18					My11
35	c-20	C104	PG21				S1	<i>Saxeibacter</i>
36				i-29	BP64	PG4	G2	<i>Gordonia</i>

* Isolates which showed multiple DGGE bands.

[#] For genus which contain only 1 clone/isolate, such clone/isolate is automatically assigned Molecular Operational Taxonomic Unit (MOTU) designation e.g. Pr1, Mu1.

3.3.4.1.1 Coverage of bioreactor consortia by reference DGGE ladder

The reference ladders were checked for the extent of coverage of bands within the DGGE profiles that displayed temporal development of the bacterial communities in the bioreactors (Figure 3.7 and 3.8).

Figure 3.23 shows the DGGE profiles of the initial inoculum (0th week microbial consortia) compared against LC, LI and LCI. A Band Distribution (BD) profile is also presented, which was derived by mapping all the band positions that have surfaced during the entire temporal DGGE analysis (Figure 3.7 and 3.8) into a single lane. This represents the complete “diversity” as manifested through DGGE banding.

Some bands in the 0th week profile were not represented within the LC (Figure 3.23A, red arrows), implying that the sampling of clones (based on which the LC was constructed) of the 16S rDNA clone library of the 0th week inoculum was not sufficient to fully cover its diversity. These were, however, adequately covered by the LCI (the contribution of which originated partly from the LI). There were additional bands in the LCI which did not correspond to the 0th week profile (Figure 3.23A, blue arrows). These bands have their origins in the collection of culturable isolates, which suggests that they were bacterial groups which were possibly present in very low abundance in the initial consortia but were enriched within the bioreactor conditions over time. Comparison of the LI and the BD profiles revealed bands which were not found within the LI but were covered by LCI (Figure 3.23A, orange arrows). This again demonstrated the inadequacy of the small sampling of culturable isolates to reflect the full diversity of the bioreactor communities, and affirmed our strategy of using complementary techniques to expand the coverage. Figure 3.23B attempts to illustrate this by presenting the percentage of coverage achieved by the respective reference ladders (rows) over the band positions found in the DGGE profiles of various sample sets (columns). Neither the LC nor the LI could provide sufficient coverage (ranging between 49- 82 %), but their combined profile of the LCI were able to improve this significantly (88- 100 %).

Several bands in the BD profile (originating from DGGE profiles of the bioreactors during operation but not the initial inoculum) were not represented in the LCI (Figure 3.23A, green arrows), which accounted for the less than 100 % coverage under TPPBs, TPPB-IFs and MPBs in Figure 3.23B.

3.3.4.2 Identification of MOTU corresponding to excised DGGE bands

To bring about a more complete association of band positions to MOTU, the bands found on BD which were not covered by the LCI were excised from the respective DGGE gels and sequenced (Section 3.2.7.3). Only 4 out of the 6 bands were successfully put through DNA sequencing. The results are shown in Table 3.8. Due to the fact that only the V3 region of the 16S rDNA was run on DGGE, the sequence obtained were too short to bring about phylogenetic assignment to the genus level, except for GE2. The others were given taxonomic identity up to the level of order.

3.3.5 Analysis of community dynamics at genus level resolution

The complete association of the band positions on BD to the phylogenetic affiliation at the level of genus (except GE1, 3 and 4) is presented in Figure 3.24. The bioreactors' bacterial community dynamics shall be re-examined and considered through the use of this set of information.

Table 3.8 Bacterial taxa identified from DGGE bands. Bands at positions marked by blue-colored arrows on the BD profile in Fig 3.7 and 3.8 has been excised, the DNA extracted and sequenced. Around 200 bp of the 16S rDNA V3 region from the excised gel band were submitted to RDP database. Each sequenced band is given a unique band ID. The closest RDP sequence, together accession number, and the similarity score (in percentage) are shown. These sequences were also classified using RDP taxonomic classifier using a bootstrap value of 50% as the classifier threshold.

DGGE band	Closest relative and Accession number	Similarity score (%)	RDP taxonomic Classifier							
			Phylum ^a	RDP confidence estimate (%)	Class ^a	RDP confidence estimate (%)	Order ^a	RDP confidence estimate (%)	Genus ^a	RDP confidence estimate (%)
GE1	uncultured Gordonia sp.; CG97; JN5411	87.9	Actinobacteria	80	Actinobacteria	80	Actinomycetales	80		
GE2	Variovorax sp. MN 36.2; AJ313017	96.9	Proteobacteria	100	β-proteobacteria	100	Burkholderiales	100	Variovorax	98
GE3	uncultured Mycobacterium bacterium; 4S_2d01; FJ382305	83.6	Actinobacteria	82	Actinobacteria	82	Actinomycetales	81		
GE4	Streptomyces sp. S75; JX007992	79.8	Actinobacteria	97	Actinobacteria	97	Actinomycetales	96		

^a Only assignments with a confidence level above 80% are listed.

Band	Phylogenetic affiliation (MOTU number)
1	<i>Pedobacter</i> Pe1 ^S
2	<i>Achromobacter</i> A5 ^S , <i>Chryseobacterium</i> C5,6,7,8 ^S
3	<i>Achromobacter</i> A5 ^S
4	<i>Chryseobacterium</i> C4 ^S
5	<i>Pedobacter</i> Pe1 ^S
6	<i>Flavobacterium</i> F1 ^S , <i>Variovorax</i> V1 ^S , 2 ^H , 4 ^M , 5 ^M , 3 ^H , 6 ^H , 7 ^H , 8 ^H
7	<i>Pedobacter</i> Pe1 ^S , <i>Chryseobacterium</i> C3 ^H
8	<i>Siphonobacter</i> Si1
9	<i>Nevskia</i> N1 ^H
GE1	Actinomycetales
10	<i>Flavobacterium</i> F1 ^S
11	<i>Chryseobacterium</i> C1 ^S , 2 ^S
12	<i>Achromobacter</i> A1, <i>Mycobacterium</i> My13
GE2	<i>Variovorax</i>
13	<i>Achromobacter</i> A6 ^S , <i>Burkholderia</i> B3 ^S , 4 ^S , <i>Cupriavidus</i> Cu1 ^S
14	<i>Deinococcus</i> D1, <i>Sphingomonas</i> Sp1
15	<i>Achromobacter</i> A6 ^S , <i>Caulobacter</i> Ca1 ^O
16	<i>Achromobacter</i> A6 ^S , <i>Cupriavidus</i> Cu1 ^S
17	<i>Leifsonia</i> Le1
18	<i>Sphingomonas</i> Sp2 ^O , <i>Achromobacter</i> A2, <i>Burkholderia</i> B2, <i>Rhizobium</i> Rh1
19	<i>Achromobacter</i> A4, <i>Sphingomonas</i> Sp2 ^O
20	<i>Achromobacter</i> A3,5 ^S
21	<i>Achromobacter</i> A6 ^S , <i>Microbacterium</i> M2 ^S
22	<i>Burkholderia</i> B1
23	<i>Phycococcus</i> Ph1, <i>Achromobacter</i> A6 ^S
24	<i>Microbacterium</i> M1 ^S , 3, 4 ^S , 5 ^S
25	<i>Lapillicoccus</i> L1,L2
26	<i>Gordonia</i> G4 ^F , G6 ^F , G7 ^F
GE3	Actinomycetales
27	<i>Mycobacterium</i> My 4 ^S , 6 ^S , 7 ^O , 9 ^F , 10 ^O , 14 ^O , 15 ^O , 16 ^S , 17 ^O
28	<i>Mycobacterium</i> My1, 2, 3, 4 ^S , 5, 6 ^S , 7 ^O , 8, 9 ^F , 10 ^O , 12, 14 ^O , 15 ^S , 16 ^S , 17 ^O , <i>Propioniceimonas</i> Pr1
29	<i>Saxeibacter</i> S2, <i>Rhodococcus</i> R3 ^O , 4 ^O , 5 ^O , 10
30	<i>Rhodococcus</i> R1, 2, 3 ^O , 4 ^O , 5 ^O , 7, 6, 9 ^O , 11- 16 <i>Mucilaginibacter</i> Mu1 ^F
31	<i>Rhodococcus</i> R9 ^O , <i>Mucilaginibacter</i> Mu1 ^F
32	<i>Gordonia</i> G3 ^F , 5 ^S
33	<i>Gordonia</i> G1 ^O , 3 ^F , 5 ^S
GE4	Actinomycetales
34	<i>Rhodococcus</i> R8 ^O , <i>Mycobacterium</i> My11
35	<i>Saxeibacter</i> S1
36	<i>Gordonia</i> G2 ^M

Figure 3.24 Complete compilation covering all the bands observed in the DGGE profiles in this study and their phylogenetic affiliation (substantiated by MOTU identification). This combined ladder and the associated information in the table have been consolidated from the studies described and discussed in Section 3.3.4.1 and 3.3.4.2. Superscripts in red refer to the colony morphotypes associated with each MOTU, as Halo (H), Flower-shaped (F), Orange (O), Small (S) and Medium (M).

3.3.5.1 Selection of Prominent Bands (PB)

The band intensity of DGGE profiles is known to correlate at least semi-quantitatively with the abundance of the corresponding bacterial taxa in the microbial consortium. As the technique of DGGE carries with it some inherent bias and limitations with respect to such quantitative assessment (Heuer et al, 1997; Heuer & Smalla, 1997; Wittebolle et al, 2008), we decided to focus on those bands that have, at some point or other in the temporal analysis gained a level of prominence (defined as having a band intensity >10 % of the total band intensities within a DGGE profile). These Prominent Bands (PBs) are presented in Table 3.9, along with information on the sample sets from which their PB status was noted. The GEs which were originally selected to be excised and sequenced based on their prominent intensity were not given separate PB designations, but are presented in the same Table according to their relative positions on the BD profile.

Table 3.9 List of prominent DGGE bands in 0th week inoculum and microbial communities in MPB, TPPB and TPPB-IF. The inferred phylogenetic affiliation and MOTU designation are based on studies described earlier in section 3.3.4. The colony morphotypes exhibited by the culturable isolates were also listed. Superscripts in red refer to the colony morphotypes associated with each MOTU, as Halo (H), Flower-shaped (F), Orange (O), Small (S) and Medium (M). Ticks indicated the dominance of such bands in the different fractions and types of bioreactors.

Prominent bands (PB) or GE no.	0 th week inoculum	Bioreactor communities across time			Band no. on combined ladder LCI	Phylogenetic affiliation and MOTU number
		TPPB	TPPB-IF	MPB		
PB1	√	√	√		2	<i>Achromobacter</i> A5 ^S , <i>Chryseobacterium</i> C5, 6, 8 ^S
PB2	√				4	<i>Chryseobacterium</i> C4 ^S
PB3		√	√	√	5	<i>Pedobacter</i> Pe1 ^S
PB4	√	√	√	√	6	<i>Flavobacterium</i> F1 ^S , <i>Variovorax</i> V1 ^S , 2 ^H , 4 ^M , 5 ^M , 3 ^H , 6 ^H , 7 ^H , 8 ^H
PB5	√				8	<i>Siphonobacter</i> Si1
GE1		√	√	√	-	Actinomycetales
GE2		√	√		-	Variovorax
PB6		√	√		15	<i>Achromobacter</i> A6 ^S , <i>Caulobacter</i> Ca1 ^O
PB7	√			√	20	<i>Achromobacter</i> A3,5 ^S
PB8	√	√	√	√	21	<i>Achromobacter</i> A6 ^S , <i>Microbacterium</i> M2 ^S
GE3		√	√		-	Actinomycetales

PB9	√	√	√		28	<i>Mycobacterium</i> My1, 2, 3, 4 ^S , 5, 6 ^S , 7 ^O , 8, 9 ^F , 10 ^O , 12, 14 ^O , 15 ^S , 16 ^S , 17 ^O , <i>Propionicimonas</i> Pr1
PB10	√				29	<i>Saxeibacter</i> S2, <i>Rhodococcus</i> R3 ^O , 4 ^O , 5 ^O , 10
PB11	√			√	30	<i>Rhodococcus</i> R1, 2, 3 ^O , 4 ^O , 5 ^O , 7, 6, 9 ^O , 11- 16 <i>Mucilaginibacter</i> Mu1 ^F
PB12	√	√	√	√	33	<i>Gordonia</i> G1 ^O , 3 ^F , 5 ^S
GE4				√	-	Actinomycetales
PB13		√	√	√	34	<i>Rhodococcus</i> R8 ^O , <i>Mycobacterium</i> My11
PB14		√	√	√	36	<i>Gordonia</i> G2 ^M

3.3.5.2 Dynamics of key genera in bacterial communities of bioreactors

It was possible to quantitate the intensities of the PBs in relation to the total intensity of the bands in that DGGE profile, and plot their relative abundance across time, as shown in Figure 3.25. Note that the “single” and “composite” columns in Figure 3.25A refer to what was discussed in Section 3.3.4.1, where it was highlighted that some of the bands in the LCI have “single” genus assigned while others have “composite” (i.e. more than one) genera converging on a single band position. Hence, the “composite” column would have the respective color blocks defined by more than one genus (as aligned to the phylogenetic tree in Figure 3.25A). Based on this analysis scheme, we also specifically scrutinized the distribution of bacterial genera within the TPPBs and the MPBs for the 28th and 44th weeks (Figure 3.26), over which time the specific hexane degradation rates of the communities in the TPPBs have developed to one with higher efficiency. As it may sometimes be necessary to refer to the phylogenetic relationship between the various bacterial groups, the complete list of MOTU assignation organized according to the taxonomic rank is presented as Table 3.10. For a more meaningful interpretation of the results, we have also applied MDS and PCA methods to study the distribution of key genera that had been identified in the bioreactors across time (Figure 3.27). In the subsequent sections, different aspects of the dynamics shall be discussed with reference to these Figures and Table and with correlation to various earlier data.

Figure 3.25 Distribution of the dominant bacteria present in MPB1 (A), MBP2 (B), TPPB1(C), TPPB2 (D), TPPB1-IF (E) and TPPB2-IF (F) over 52 weeks. The identities of these bacteria were listed earlier in Table 3.9 and their relative abundance (in percentage) was obtained from the measurement of the band intensities using Gel Compar II. Majority of these isolates were able to separate into single genus while four bands remained composite, unable to determine the relative abundance of individual bacteria genus in the bioreactors.

Figure 3.26 Distribution of the dominant bacteria present in MPB1 (A), MBP2 (B), TPPB1(C), TPPB2 (D), TPPB1-IF (E) and TPPB2-IF (F) in 28th and 44th week. The relative abundance of each bacteria had been shown earlier in Figure 3.24.

Figure 3.27 MDS and PCA analysis using relative abundance of key bacterial genera in each bioreactor. The MDS(A) and PCA (B) were first plotted for samples over the whole time course of 52 weeks. Subsequently, MDS (C) and PCA (D) were plotted for samples from 16 to 52 weeks of bioreactor operation, i.e. omitting the samples up to week 12. Data points are labelled as Bioreactor_Week, e.g. MPB2_48W.

Table 3.10 Summary of the different bacteria identified in the bioreactors. These clones and isolates had been identified and grouped based on a combination of techniques such as ARDRA, colony morphotypes, CM, (if any), full-length *16S rDNA* sequencing and DGGE band position. The MOTU designations of these clones and isolates as shown earlier in the phylogenetic trees in Figure 3.20 were listed. In the DGGE column, the band position of the isolates and clones corresponding to the combined ladder shown in Figure 3.11 and Table 3.8 were listed. The bacteria identified via sequencing of the excised bands were also included. The identities of the strains used for study in Chapter 4 were listed.

Sequenced clone/ isolate/ band	Closest type species	CM	MOTU Designation	DGGE (Band position)	Designation of Strains used for study in Chapter 4
Class: Actinobacteria					
Genus: <i>Gordonia</i>					
BP37	<i>G. polyisoprenivorans</i>	O	G1	33	<i>Gordonia</i> G1
C127	<i>G. polyisoprenivorans</i>	-	G1	33	
BP96	<i>G. polyisoprenivorans</i>	F	G3	32, 33	<i>Gordonia</i> G3
BP108	<i>G. amicalis</i>	S	G5	32, 33	<i>Gordonia</i> G5
BP87	<i>G. polyisoprenivorans</i>	F	G4	26	<i>Gordonia</i> G4
BP90	<i>G. polyisoprenivorans</i>	F	G4	26	
BP5	<i>G. humi</i>	S	G6	26	<i>Gordonia</i> G6
BP82	<i>G. humi</i>	F	G7	26	<i>Gordonia</i> G7
BP92	<i>G. humi</i>	F	G7	26	
BP64	<i>G. polyisoprenivorans</i>	M	G2	36	<i>Gordonia</i> G2
GE1	Actinomycetales (uncultured <i>Gordonia</i> sp.)	-	-	GE1	Actinomycetales
GE3	Actinomycetales (uncultured <i>Mycobacterium</i> sp.)	-	-	GE3	Actinomycetales
Genus: <i>Mycobacterium</i>					
C75	<i>M. cosmeticum</i>	-	My1	28	<i>Mycobacterium</i> My1
C12	<i>M. cosmeticum</i>	-	My2	28	<i>Mycobacterium</i> My2
C156	<i>M. cosmeticum</i>	-	My3	28	<i>Mycobacterium</i> My3
BP22	<i>M. cosmeticum</i>	S	My4	27, 28	<i>Mycobacterium</i> My4
C45	<i>M. cosmeticum</i>	-	My5	28	<i>Mycobacterium</i> My5
BP65	<i>M. cosmeticum</i>	S	My6	27, 28	<i>Mycobacterium</i> My6
BP42	<i>M. cosmeticum</i>	O	My7	27, 28	<i>Mycobacterium</i> My7
C119	<i>M. cosmeticum</i>	-	My8	28	<i>Mycobacterium</i> My8
BP99	<i>M. canariasisense</i>	F	My9	27, 28	<i>Mycobacterium</i> My9
BP75	<i>M. cosmeticum</i>	O	My10	27, 28	<i>Mycobacterium</i> My10
C62	<i>M. rufum</i>	-	My12	28	<i>Mycobacterium</i> My12
BP21	<i>M. canariasisense</i>	O	My14	27, 28	<i>Mycobacterium</i> My14
BP69	<i>M. cosmeticum</i>	S	My15	27, 28	<i>Mycobacterium</i> My15
BP79	<i>M. cosmeticum</i>	S	My15	27, 28	
BP48	<i>M. cosmeticum</i>	S	My16	27, 28	<i>Mycobacterium</i> My16
BP15	<i>M. cosmeticum</i>	O	My17	27, 28	<i>Mycobacterium</i> My17
C34	<i>M. flavescens</i>	-	My11	34	<i>Mycobacterium</i> My11
C74	<i>M. rufum</i>	-	My13	12	<i>Mycobacterium</i> My13
Genus: <i>Rhodococcus</i>					
C77	<i>R. ruber</i>	-	R1	30	<i>Rhodococcus</i> R1
C108	<i>R. ruber</i>	-	R2	30	<i>Rhodococcus</i> R2

BP4	<i>R.aetherivorans</i>	O	R3	29, 30	<i>Rhodococcus</i> R3
BP47	<i>R. ruber</i>	O	R4	29, 30	<i>Rhodococcus</i> R4
BP71	<i>R. aetherivorans</i>	O	R5	29, 30	<i>Rhodococcus</i> R5
C172	<i>R. ruber</i>	-	R6	30	<i>Rhodococcus</i> R6
C117	<i>R. ruber</i>	-	R7	30	<i>Rhodococcus</i> R7
C114	<i>R. aetherivorans</i>	-	R10	29	<i>Rhodococcus</i> R10
C105	<i>R. ruber</i>	-	R11	30	<i>Rhodococcus</i> R11
C160	<i>R. ruber</i>	-	R12	30	<i>Rhodococcus</i> R12
C16	<i>R. ruber</i>	-	R13	30	<i>Rhodococcus</i> R13
C64	<i>R. ruber</i>	-	R13	30	
C67	<i>R. ruber</i>	-	R13	30	
C14	<i>R. ruber</i>	-	R14	30	<i>Rhodococcus</i> R14
C1	<i>R. ruber</i>	-	R15	30	<i>Rhodococcus</i> R15
C46	<i>R. ruber</i>	-	R16	30	<i>Rhodococcus</i> R16
BP26	<i>R. phenolicus</i>	O	R9	30,31	<i>Rhodococcus</i> R9
BP73	<i>R. wratislaviensis</i>	O	R8	34	<i>Rhodococcus</i> R8
GE4	Actinomycetales (<i>Streptomyces</i> sp.)	-	-	GE4	<i>Streptomyces</i> St1
Genus: <i>Microbacterium</i>					
BP9	<i>M. aoyamense</i>	S	M2	21	<i>Microbacterium</i> M2
BP3	<i>M. laevaniformans</i>	S	M1	24	<i>Microbacterium</i> M1
C2	<i>M. laevaniformans</i>	-	M1	24	
C183	<i>M. resistens</i>	-	M3	24	<i>Microbacterium</i> M3
BP34	<i>M. maritypicum</i>	S	M4	24	<i>Microbacterium</i> M4
BP30	<i>M. paraoxydans</i>	S	M5	24	<i>Microbacterium</i> M5
C4	<i>M. paraoxydans</i>	-	M5	24	
Genus: <i>Leifsonia</i>					
C18	<i>L. xyli</i>	-	Le1	17	<i>Leifsonia</i> Le1
Genus: <i>Lapillicoccus</i>					
C79	<i>L. jejuensis</i>	-	L1	25	<i>Lapillicoccus</i> L1
C150	<i>L. jejuensis</i>	-	L2	25	<i>Lapillicoccus</i> L2
Genus: <i>Phycicoccus</i>					
C78	<i>P. ginsenosidimutans</i>	-	Ph1	23	<i>Phycicoccus</i> Ph1
Genus: <i>Saxeibacter</i>					
C104	<i>S. lacteus</i>	-	S1	35	<i>Saxeibacter</i> S1
C113	<i>S. lacteus</i>	-	S2	29	<i>Saxeibacter</i> S2
Genus: <i>Propionicimonas</i>					
C20	<i>P. paludicola</i>	-	Pr1	28	<i>Propionicimonas</i> Pr1
Class: Deinococci					
Genus: <i>Deinococcus</i>					
C10	<i>D. aerius</i>	-	De1	14	<i>Deinococcus</i> De1
Class: Flavobacteria					
Genus: <i>Chryseobacterium</i>					
BP66	<i>C. hominis</i>	S	C1	11	<i>Chryseobacterium</i> C1
BP107	<i>C. hominis</i>	S	C2	11	<i>Chryseobacterium</i> C2
BP104	<i>C. pallidum</i>	H	C3	7	<i>Chryseobacterium</i> C3
BP105	<i>C. pallidum</i>	H	C3	7	
BP6	<i>C. formosense</i>	S	C4	4	<i>Chryseobacterium</i> C4
C8	<i>C. formosense</i>	-	C4	4	
C6	<i>C. gleum</i>	-	C5	2	<i>Chryseobacterium</i> C5
C35	<i>C. gleum</i>	-	C6	2	<i>Chryseobacterium</i> C6

C102	<i>C. indologenes</i>	-	C7	2	<i>Chryseobacterium</i> C7
BP1	<i>C. indologenes</i>	S	C8	2	<i>Chryseobacterium</i> C8
Genus: <i>Flavobacterium</i>					
BP10	<i>F. lindanitolerans</i>	S	F1	6, 10	<i>Flavobacterium</i> F1
Class: <i>Sphingobacteria</i>					
Genus: <i>Mucilaginibacter</i>					
BP94	<i>M. ximonensis</i>	F	Mu1	30, 31	<i>Mucilaginibacter</i> Mu1
Genus: <i>Pedobacter</i>					
BP46	<i>P. koreensis</i>	S	Pe1	1, 5, 7	<i>Pedobacter</i> Pe1
Genus: <i>Siphonobacter</i>					
C137	<i>S. aquaeclarae</i>	-	Si1	8	<i>Siphonobacter</i> Si1
Class: α-proteobacteria					
Genus: <i>Caulobacter</i>					
BP17	<i>C. segnis</i>	O	Ca1	15	<i>Caulobacter</i> Ca1
Genus: <i>Sphingomonas</i>					
C28	<i>S. echinoides</i>	-	Sp1	14	<i>Sphingomonas</i> Sp1
BP33	<i>S. yabuuchiae</i>	O	Sp2	18,19	<i>Sphingomonas</i> Sp2
Genus: <i>Rhizobium</i>					
C3	<i>R. tubonense</i>	-	Rh1	18	<i>Rhizobium</i> Rh1
Class: β-proteobacteria					
Genus: <i>Achromobacter</i>					
BP2	<i>A. xylosoxidans</i>	S	A5	2, 3, 20	<i>Achromobacter</i> A5
C146	<i>A. insolitus</i>	-	A1	12	<i>Achromobacter</i> A1
C170	<i>A. spanius</i>	-	A2	18	<i>Achromobacter</i> A2
C5	<i>A. spanius</i>	-	A3	20	<i>Achromobacter</i> A3
C40	<i>A. insolitus</i>	-	A4	19	<i>Achromobacter</i> A4
BP35	<i>A. xylosoxidans</i>	S	A6	13, 15,16, 21, 23	<i>Achromobacter</i> A6
Genus: <i>Burkholderia</i>					
C42	<i>B. fungorum</i>	-	B1	22	<i>Burkholderia</i> B1
C11	<i>B. fungorum</i>	-	B2	18	<i>Burkholderia</i> B2
BP29	<i>B. arboris</i>	S	B3	13	<i>Burkholderia</i> B3
BP32	<i>B. arboris</i>	S	B4	13	<i>Burkholderia</i> B4
Genus: <i>Cupriavidus</i>					
BP31	<i>C. oxalaticus</i>	S	Cu1	16	<i>Cupriavidus</i> Cu1
Genus: <i>Variovorax</i>					
BP39	<i>V. boronicumulans</i>	S	V1	6	<i>Variovorax</i> V1
BP63	<i>V. boronicumulans</i>	H	V2	6	<i>Variovorax</i> V2
BP16	<i>V. paradoxus</i>	M	V4	6	<i>Variovorax</i> V4
BP18	<i>V. paradoxus</i>	M	V5	6	<i>Variovorax</i> V5
BP27	<i>V. paradoxus</i>	H	V3	6	<i>Variovorax</i> V3
BP67	<i>V. paradoxus</i>	H	V6	6	<i>Variovorax</i> V6
C23	<i>V. paradoxus</i>	-	V6	6	
BP7	<i>V. paradoxus</i>	H	V7	6	<i>Variovorax</i> V7

BP23	<i>V. paradoxus</i>	H	V8	6	<i>Variovorax</i> V8
GE2	<i>Variovorax</i> sp.	-	-	GE2	<i>Variovorax</i> V9
Class: γ-proteobacteria					
Genus: <i>Nevskia</i>					
BP68	<i>N. ramosa</i>	H	N1	9	<i>Nevskia</i> N1
BP80	<i>N. soli</i>	H	N1	9	

3.4 Discussion and Conclusions

3.4.1 Dynamics of the key genera distribution as analyzed by MDS and PCA

The MDS and PCA (Figure 3.27A and B) computed based on the relative abundance of key bacterial genera in the bioreactors across 52 weeks (Section 3.3.5.2) revealed two distinct clades (circled with red dashed lines) formed by the TPPBs and MPBs samples during the stationary phase. The community make-up as defined by the key genera in the TPPBs was therefore distinct from that of the MPBs. The tighter clustering within each clade (each type of bioreactors) exhibited by this analysis was unlike the 4 loose clusters formed by each of the TPPB and MPB communities in the earlier MDS and PCA analysis performed based on DGGE band profiles (Figure 3.14, Section 3.3.2.2.5). This suggests that the distribution of key genera at the stationary phase could be more conserved within each type of bioreactors than reflected by DGGE bands. On the other hand, the exponential phase (0 to 12 weeks) samples were fairly distant from the two tight clades of the stationary phase samples (note the outlying points), similar to what was observed in the DGGE profile-based MDS and PCA (Figure 3.14, Section 3.3.2.2.5).

To further evaluate at a higher resolution the level of relatedness in distribution of key genera between individual bioreactors during the stationary phase shown in Figure 3.27A and B (and not masked due to the comparatively distant exponential phase communities), a second set of MDS and PCA analysis were performed using the community profiles for the stationary phase (12 weeks onwards) only (Figure 3.27C and D). As expected, the new MDS plot (Figure 3.27C) showed obvious dissimilarity in the clustering from the first plot (i.e. Figure 3.27A)—the MPB1 and MPB2 have now separated into two distinct clusters while the aqueous and interfacial fractions of both TPPBs remained tightly interspersed within one cluster. (Note: the dissimilarity between Figure 3.27 A and C is due to the fact that MDS analysis is dependent on the relatedness between each and every data point, hence removal of a data set will affect the overall relatedness plot of the remaining data sets.) This implies that as far as the distribution of key genera was concerned, greater divergence existed between the two MPBs than between the two TPPBs. On the other hand, PCA plots (Figure

3.27B and D) showed tight clusters for both TPPB and MPB communities during stationary phase. Since PCA plots were based on the first 3 principle components (i.e. 3 most dominant genera) of each data set, we interpreted this to mean that for the MPBs, although there were divergence in the distribution of key genera overall between the duplicate bioreactors, the 3 most dominant genera remained conserved between them. For the TPPB microbial communities, in both MDS and PCA plots, datapoints from the aqueous and interfacial fractions of the duplicate bioreactors were closely positioned to one another, forming a single tight cluster (Figure 3.27C and D). We speculate that the stability in hexane feed stream within the biphasic environment of TPPBs could have promoted conservation and reproducibility in the microbial compositions. This would make TPPB desirable for industrial operations, as consistent biodegradative performance could be more reliably achieved.

Having covered the overall relatedness through these dimensional analysis, we will discuss, in the following sections, specific changes in the composition of key bacterial genera across time as observed in the biomass development of the bioreactor (Figure 2.5) – firstly the exponential phase (2 to 12 weeks), followed by the stationary phase (12 weeks onwards).

3.4.2 Dynamics of key subpopulations during exponential growth phase

Analysis was made by dissecting the time course into the two stages as observed in the biomass development of the bioreactor (Figure 2.5) – the exponential phase (2 to 12 weeks) and the stationary phase (12 weeks onwards). We shall focus on the exponential phase of microbial community development in this section.

It was apparent from various parameters used to qualify the nature of bacterial communities' dynamics based on DGGE densitometric profiles (Section 3.3.2) that the exponential phase was unstable with respect to community structure and fraught with changes. The community dynamics based on defined key genera (Figure 3.25) also reflected this trend of the period. The aqueous phase of the TPPBs, for example, experienced a transient surge in

Chryseobacterium C5-8 subpopulation which quickly receded, followed by a wave of increase of the *Mycobacterium* My1-10, 12, 14-17 subpopulation. Their IF counterparts were holding a significant proportion of *Variovorax* GE3 and rapidly building up the *Chryseobacterium* C5-8 subpopulation. The MPBs, on the other hand, were clearly developing a different bias of subpopulations from the TPPBs, the most apparent being the transient dominance of the Actinomycetales GE1 and GE2, and the gradual build up of the *Rhodococcus* R1-7, 10-16 subpopulations. The latter had a significant presence in the initial inoculum but saw a rapid decline within the TPPB communities, which rebounded back to significance within the MPBs.

Although there were commonalities between the duplicate sets of bacterial communities, there were also clear divergence even at this stage. For example, MPB2 has a dominance of *Variovorax* V1-8 subpopulation which was not found in MPB1, while TPPB2 showed a more sustained prominence of the composite subpopulation of *Caulobacter* Ca1, *Achromobacter* A6 and *Variovorax* V1-8 which was not seen in TPPB1. These divergence appeared to have an influence on the composition of the subsequent period corresponding to the stationary phase (which was maintaining greater stability).

3.4.3 Dynamics of key subpopulation during stationary growth phase

The stationary phase occurring after 12th week could in fact be dissected into two sub-periods: the stable phase of 12th -32nd weeks and the “building up” phase from 32nd week onwards. The phrase “building up” was used because there were no rapid increase in biomass (cell density) (Figure 2.5), yet clearly protein synthesis (Figure 2.6) and metabolic activities (Figure 2.7) were showing an increase, albeit not as rapidly as in the exponential phase. This was also the period over which the hexane degradation efficiency of the TPPB communities have managed to increase (44th week) compared to the stable period (28th week) (Figure 2.9). For the ease of comparison, the dissection of differential community structures between these two sub-periods shall be done using these two representative time points first, and thereafter, the comparison shall be made using the whole of stationary phase.

Comparison between Week 28 and Week 44 community structures

Unlike the TPPBs, the communities within the MPBs did not improve in hexane degradation efficiency over 28th to 44th weeks. A comparison of the respective time points (Figure 3.26), however, showed that the MPBs have developed changes in community structures as well, but these changes were simply not able to bring about increased biodegradation efficiency. In the case of the TPPBs, the most prominent difference between the pair of time points that was common to both TPPBs appeared to be the growing dominance of the composite subpopulation of *Microbacterium* M2 and *Achromobacter* A6 in the aqueous phase fraction and the *Mycobacterium* My1-10, 12, 14-17 subpopulation in the IFs, displayed by the 44th weeks' communities. Beyond that commonality, the IF of TPPB1 displayed similar trend as its aqueous phase community, but the IF of TPPB2 diverged, showing only a slight gain in the *Chryseobacterium* C4 subpopulation. This divergence could possibly account for the differentials observed between the 2 TPPBs' hexane degradation efficiency at 44th week (Figure 2.17).

Comparison between aqueous phase of TPPBs and MPBs

At stationary phase, the most apparent differences between the community structures of aqueous phase TPPBs and MPBs were the dominance of the *Mycobacterium* My1-10, 12, 14-17 subpopulation in the TPPBs in contrast to that of the *Rhodococcus* R1-7, 10-16 subpopulation in the MPBs. There was also a more prominent presence of the *Gordonia* G1, G3, G5 subpopulation in the MPBs than in the TPPBs. Beyond that, it was not possible to discern further significant differentials because there were divergence between the duplicate pairs of bioreactors, making comparison across the types of bioreactors difficult. The divergence, however, suggests to us that microbial communities have alternative solutions to gaining similar functions, a concept generally referred to as functional resilience and redundancy (Girvan et al, 2005).

Comparison between IF and aqueous phase of TPPBs

It was clear from a first browse of the respective TPPB and TPPB-IF pairs that the IF communities were a subpopulation of the aqueous phase counterparts.

Ironically, it was possible to reach this conclusion because of the divergence between the duplicate pairs i.e. TPPB1 versus TPPB2. No clear bias of specific bacterial groups within the IFs could be discerned, and it may well be the absence of certain groups from the IFs that were contributing to the functional differentials of the two fractions. For example, in TPPB1, there was an absence of the *Variovorax* V1-8 subpopulation, at least during Week 20-32. In any case, it was interesting to note that minor differences in ratio of the various groups could have resulted in different properties of the IF communities from the aqueous phase communities.

3.4.4 Probable key bacterial genera involved in the hexane degradation

Collectively, the TPPBs and TPPB-IFs at the stationary phase (from 12 weeks onwards) showed comparable high abundance of *Gordonia* G2, *Mycobacterium* My1-10, 11-12, 14-17, *Rhodococcus* R8, *Caulobacter* Ca1, *Achromobacter* A6 and *Variovorax* V1-8 (Figure 3.25 and Table 3.12). This was not observed in the MPBs and instead they showed greater abundance of *Gordonia* G1, 3, 5 and *Rhodococcus* R1-7, 10-16 subpopulation. The differing subsets of abundant bacterial groups corroborate well with the distinct separation of the two types of bioreactors observed in the MDS and PCA plots (Figure 3.27). The separate clusters of MPB1 from MPB2 in the MDS plot also suggested that the bacterial subpopulation between the two duplicate bioreactors diverged across time. A closer look at the dominant bacterial profiles of MPB1 and MPB2 indeed revealed that the MPB1 showed higher abundance of *Gordonia* G1, 3, 5, *Achromobacter* A5, *Pedobacter* Pe1 than MPB2, while MPB2 itself showed higher abundance of *Mycobacterium* My11 and *Rhodococcus* R8 than MPB1.

Literature review conducted on the various dominant bacterial genera identified above brought to our attention that *Mycobacterium*, *Gordonia* and *Rhodococcus* (which dominated in both TPPBs and MPBs in our study), have often been reported to be good degraders of organic compounds including alkanes, or be involved in oil-degradation in hydrocarbon-contaminated sites (Arenskotter et al, 2004; Bell et al, 1998; Cesário et al, 1997; Kibazohi et al, 2004; Larkin et al, 2005; Lee & Cho, 2008; Shen et al, 2009). However, to date,

no study has been performed which specifically examined their growth and properties relevant to biological activities in the biphasic system. To fill this knowledge gap, we next made use of the isolates obtained in our study which belong to these genera for characterization and analysis in the next chapter (Chapter 4).

3.4.5 Conclusions

In summary, analysis of the community dynamics of the MPBs and the aqueous phase and IF of the TPPBs were able to highlight several trends that may be correlated to or cross-referenced to the observations made in Chapter 2. These are presented in the summary table (Table 3.11) below. In addition, our combined culture-based and culture-independent approach has allowed us a more in-depth glimpse into the differentials between sets of communities. The dominance or bias of key genera or subpopulations in specific sets of bacterial communities would allow us to examine their respective contribution to the functionality of the communities. We have summarized our observations in this respect into Table 3.12. This shall be further explored in the following chapter.

Table 3.11 Summary of the microbial communities' dynamics across 52 weeks. This summary is based on the changes in the microbial communities of the bioreactors as shown by DGGE.

	TPPBs			MPBs		
Parameters/ Time course	0-12	12-32	32-52	0-12	12-32	32-52
Microbial growth	Exponential increase	Stationary phase		Exponential increase	Gradual increase	
Protein synthesis	Exponential increase	Stationary phase	Increase	Exponential increase	Stationary phase	Increase
Microbial activities	Exponential increase	Stationary phase	Increase	Exponential increase	Stationary phase	Increase
Specific Hexane degradation rate	-	Low	Increase	-	Low, maintained	
Total Hexane degradation rate	-	Low	Increase	-	Low, maintained	
Rate of change (Rr)	Increase from 0 to 4 weeks and decrease	Low, Maintained	Increase	Higher	Low, Maintained	Increase
Similarity with 0 th week microbial consortium	Decrease from 0 to 4 weeks and increase	Maintained		Decrease from 0 to 4 weeks and increase	Maintained	Decrease
Diversity Richness (Dr)	Decrease from 0 to 4 weeks and increase	Maintained		Decrease from 0 to 4 weeks and increase	Maintained	
Functional organization	Higher	Maintained		Higher	Maintained	
Similarity between TPPBs and MPBs	Decrease from 0 to 4 weeks and increase	Maintained	Decrease	-		

Table 3.12 Summary of the abundance of dominant bacteria present in the microbial communities across time. These bacteria corresponded to the densest bands on the microbial profiles of the bioreactors respectively. The colony morphotypes exhibited by the culturable isolates were also listed. Superscripts in red refer to the colony morphotypes associated with each MOTU, as Halo (H), Flower-shaped (F), Orange (O), Small (S) and Medium (M). ✓ and X indicated the presence and absence of such bacteria. ✓✓ indicated greater abundance of the particular group of bacteria when compared between the time intervals stipulated. “D” indicated the highest abundance of such bacteria within the time interval in the monophasic bioreactors (MPBs) and the aqueous phase and interfacial fractions of TPPBs (TPPB and TPPB-IF).

Bacteria	Time course (number of weeks)						
	0 th	4 to 12			12 to 52		
		MPB	TPPB	TPPB-IF	MPB	TPPB	TPPB-IF
<i>Gordonia</i> G1 ^{O,3} ^{F,5} ^S	✓	✓	✓	✓	✓✓	✓	✓
<i>Gordonia</i> G2 ^M	✓	X	✓ (TPPB1 only)	✓	X	✓✓	✓✓
Actinomycetales GE1	X	✓✓	✓✓	✓✓	✓	✓	✓
Actinomycetales GE2	X	✓✓	✓✓	✓✓	✓	✓	✓
<i>Mycobacterium</i> My1, 2, 3, 4 ^{S,5} , 6 ^{S,7} , 8, 9 ^F , 10 ^{O,12} , 14 ^O , 15 ^S , 16 ^S , 17 ^O	✓	✓	✓ D	✓ D	✓✓	✓✓ D	✓✓ D
<i>Mycobacterium</i> My11, <i>Rhodococcus</i> R8 ^O	✓	✓(MPB2 only)	✓	✓✓	✓✓	✓✓	✓
<i>Rhodococcus</i> R1, 2, 3 ^O , 4 ^O , 5 ^O , 7, 6, 9 ^O , 10- 16	✓ D	✓ D	✓✓	✓✓	✓✓ D	✓	✓
<i>Rhodococcus</i> R9, <i>Mucilaginibacter</i> Mu1 ^F	✓	X	✓✓	✓✓	✓	✓	✓
Actinomycetales GE4	X	✓ (MPB1 only)	X	X	✓✓	✓	✓
<i>Microbacterium</i> M2 ^S and <i>Achromobacter</i> A6 ^S	✓	✓	✓	✓	✓✓	✓✓	✓✓
<i>Saxeibacter</i> S2	✓	X	X	✓	✓	✓ (TPPB1 only)	✓ (TPPB1 only)
<i>Propionicimonas</i> Pr1	✓	✓✓ (MPB1 only)	X	✓	✓ (MPB1 only)	✓ (TPPB1 only)	✓ (TPPB1 only)
<i>Chryseobacterium</i> C4 ^S	✓	X	✓✓	✓✓	✓✓	✓	✓
<i>Chryseobacterium</i>	✓	✓✓	✓	✓	✓	X	X

C5, 6, 8 ^S		(MPB1 only)		(TPPB1 only)	(MPB1 only)		
<i>Flavobacterium</i> F1 ^S	√	√√	√√	√√	√	√	√
<i>Pedobacter</i> Pe1 ^S	√	√	√√	√√	√√	√	√
<i>Siphonobacter</i> Si1	√	√ (MPB2 only)	X	X	√√	√ (TPPB1 only)	√
<i>Caulobacter</i> Ca1 ^O , <i>Achromobacter</i> A6 ^S	√	√ (MPB2 only)	√√	√	√√	√	√√
<i>Achromobacter</i> A5 ^S	√	√ (MPB1 only)	X	X	√√ (MPB1 only)	√ (TPPB1 only)	√
<i>Achromobacter</i> A3	√	X	X	X	√	√	√
<i>Variovorax</i> V1 ^S , 2 ^H , 4 ^M , 5 ^M , 3 ^H , 6 ^H , 7 ^H , 8 ^H	√	√√	√√	√	√	√	√√
<i>Variovorax</i> GE3	X	X	√√	√√	X	√	√

Chapter 4: Characterization of Hexane Degraders Isolated From Bioreactors

4.1 Introduction

In Chapter 2, we showed that the microbial consortium in the TPPBs and the MPBs were able to degrade and utilize hexane, leading to an increase in the biomass within both types of bioreactors across time, but significantly more so in the TPPBs. The biomass accumulation can be conceived to be due to the metabolic activities of numerous strains which utilize hexane as the carbon source, working singly or together through metabolic cooperation, or a combination of both. The microbial community analysis of the consortia in the bioreactors across time in Chapter 3 revealed that there were bacterial groups that were dominant within both types of bioreactors, as well as those that had a clearly biased presence in one type of bioreactors or the other. Due to our two-pronged approach that did not rely solely on culture-independent methods but also involved a significant proportion of culture-based components, culturable isolates belonging to these dominant bacterial groups were available for further characterization and studies. The cultivation mode (growth on solid media using hexane as the sole carbon source) used in obtaining these strains (Section 2.3.3.1.4, 2.3.3.2.3 and 3.3.3.2) would be expected to select for those which have metabolic capacity to degrade hexane on its own. The characterization of these strains with respect to features relevant to hexane degradation in the TPPB context shall be the focus of this chapter.

Several studies have attempted to examine microbe-microbe and microbe-hydrocarbon interactions by extrapolating from detailed laboratory studies using isolates which had been sampled from hydrocarbon-contaminated environments. These included examination of functional and physiological properties of the isolates such as the oil emulsification abilities and the types of hydrocarbon accession modes (Bouchez-Naitali et al, 1999; Van Hamme et al, 2003). Attempts have also been made to construct co-cultures containing several well-defined strains in an effort to identify specific interactions that may be important

in biodegradative settings (Komukai-Nakamura et al, 1996). In this study, we undertook to dissect the roles of these strains from various genera by looking at their hexane utilization modes, growth, cell surface hydrophobicity and emulsification properties (biosurfactant production), and particularly with an interest on the influences that may be exerted by the NAL phase (silicone oil).

4.1.1 Alkane hydroxylase systems for hexane catabolism

The aerobic condition under which our bioreactors were operating (constant stirring for aeration) ensured that the hexane biodegradation would be through the aerobic mode of hexane catabolism. The mineralization of hexane under aerobic conditions is typically carried out via the alkane monooxygenase pathway (Lee et al, 2010; van Beilen et al, 2003) as previously discussed in Section 1.4.2. There are several classes of alkane hydroxylases (AHs), which are enzymes involved in the critical initiation step of activation of n-alkanes to 1-alkanols, as illustrated earlier (Figure 1.2). Among these, only a few of them (Table 4.1) have been found to be involved in the oxidation of medium-chain-length (MCL) n-alkanes (C5-C11, which the C6 hexane comes under).

The *alkB* gene encodes a class of membrane-bound non-heme iron monooxygenase (Rojo, 2010) and the presence of the gene has been commonly assessed to determine the degradation potential of microorganisms for MCL alkanes. An example of the better-characterised *alkB* system is that of *Pseudomonas putida* GPo1, a strain able to grow on C3 to C12 n-alkanes (Baptist et al, 1963). The *alkB* gene had also been reported to be present in alkane-degrading Actinobacteria (G-C rich Gram-positive bacteria) such as *Gordonia*, *Mycobacterium* and *Rhodococcus* (Amouric et al, 2010) and Proteobacteria such as *Burkholderia* (van Beilen et al, 2006). The other major group of alkane hydroxylase relevant to the oxidation of n-alkanes up to C16 is the CYP153 family of the cytochrome P450 monooxygenases. This enzyme is generally thought to be present in alkane-degrading eubacteria lacking the integral membrane hydroxylases, In some bacterial species, however, it has been found to co-exist with the *alkB* system (van Beilen et al, 2006).

The occurrence of more than one alkane oxidation system in one bacterium is fairly common in alkane-degrading bacteria such as *Acinetobacter borkumensis* and *Mycobacterium abscessus* HXN-1200 (van Beilen et al, 2006). The presence of multiple genes has been suggested to contribute to a wider substrate range and better environmental adaptation of the alkane degraders (Wang et al, 2010).

Table 4.1 Enzyme systems involved in the degradation of medium-chain alkanes.
The information was adopted from (van Beilen et al, 2003).

Enzyme class	Compositions and co-factors	Found to be present in
<i>alkB</i> -related alkane hydroxylases and subsequent pathway enzymes (shown in Fig 1.2)	Membrane hydroxylase and bi-nuclear iron rubredoxin and iron rubredoxin reductase and FAD, NADH	<i>Acinetobacter</i> , <i>Alcanivorax</i> , <i>Burkholderia</i> , <i>Mycobacterium</i> , <i>Pseudomonas</i> , <i>Rhodococcus</i> etc.
Bacterial P450 oxygenase systems (CYP153, class I)	P450 oxygenase and P450 heme ferredoxin and iron-sulfur ferredoxin reductase and FAD, NADH	<i>R. rhodochrous</i> 7E1C <i>Acinetobacter</i> sp. EB104

4.1.2 Physico-chemical properties that enhance hexane biodegradation

Prior to metabolizing the hexane, microorganisms will first have to access this carbon source. As previously discussed, to facilitate the uptake of this hydrophobic hydrocarbon, the microorganisms could also alter their cell wall/composition thereby increasing its hydrophobicity to enable it to adhere to the hydrocarbon droplets (Hernández & Muñoz Torre, 2011; MacLeod & Daugulis, 2005). Alternatively, the microorganisms may produce biosurfactants to disperse the hydrocarbon into oil-in-water emulsion. This increases the interfacial areas, enhancing the uptake of the carbon source into the cells (Van Hamme et al, 2006).

4.1.2.1 Cell surface hydrophobicity

Direct contact of the microbial cells with the hydrophobic hydrocarbon droplets has been reported in various studies where cell surface hydrophobicity (CSH) is commonly recognized as a factor responsible for such adhesion (Chakraborty et al, 2010; Hori et al, 2008; Obuekwe et al, 2009). One clear outcome of cellular adhesion to hydrophobic substrates is the direct uptake of the substrate into the bacterial cells for biodegradation. A study performed by Obuekwe et al (2009) was further able to establish a strong correlation between CSH and hydrocarbon degradation for isolates grown in monocultures.

The CSH of a microorganism has been found to depend on its cell wall/membrane composition (i.e lipids and proteins), which in turn is affected by the microbial activity and the environmental conditions (Hernández & Muñoz Torre, 2011). Changes in CSH for specific bacterial strains grown in the presence of hydrocarbons have been well discussed in several studies (Dorobantu et al, 2004; Hori et al, 2009; Hori et al, 2008). In relation to TPPBs, a study reported by Hernández and Muñoz Torre (2011) examined the effect of silicone oil on the CSH of several *P. aeruginosa* and *P. putida* strains grown in monoculture TPPBs during hexane and toluene biodegradation respectively. However, no conclusive correlation could be drawn due to the lack of significant changes of the parameters monitored. Recently, however, a role of microbial CSH was clearly demonstrated in a TPPB system, using consortia with differing levels of hydrophobicity but the study was unable to discern the contribution of specific strains in the consortia as the latter was completely uncharacterized (Hernández et al, 2012).

4.1.2.2 Biosurfactant production

Biosurfactants are mainly produced and secreted by microorganisms into the medium to facilitate the transport and translocation of hydrophobic substrates into the cells (Bognolo, 1999), although in some cases they may also be used as part of virulence strategy (Van Hamme et al, 2006). They refer to a class of amphipathic molecules consisting of both polar (e.g. a sugar or peptide) and non-

polar (e.g. fatty acid chain) moieties (Hommel, 1990). This structural feature of the biosurfactants allows them to form micelles that accumulate at the interface of two immiscible liquids such as water and hexane, thereby reducing surface tension and facilitating the emulsification of the hydrophobic compounds (Chandran & Das, 2010). One of the better known biosurfactants is the Emulsan, secreted by *Acinetobacter calcoaceticus* when growing on ethanol or alkanes (Marin et al, 1996). Others include rhamnolipids, produced by *Pseudomonas aeruginosa* when being cultivated on hydrocarbons (Singh et al, 2007).

Analysis of hydrocarbon contaminated sites have often revealed the presence of high concentration of biosurfactants which were associated with the presence of microorganisms such as *Rhodococcus* and *Norcadia* (Van Hamme et al, 2003). In the case of TPPBs, only two studies examining biosurfactant effects directly have been reported. One of them involved the species *Pseudomonas oleovorans* (Sakunthala et al, 2013) and the other *Rhodococcus erythropolis* (Boon et al, 2002). An increase in the NAL/aqueous interfacial area due to the action of the biosurfactants was presented as the basis of the increases in mass transfer in both TPPB systems.

4.1.3 Characterization of hexane degraders isolated from bioreactors

The hexane-degrading bacterial strains isolated from the bioreactors very likely possess certain characteristics that aid its better growth in the bioreactors and/or allowed them to contribute to the overall hexane utilization by the whole community. To dissect their roles, we attempted to characterize them with respect to properties that would be relevant to hexane biodegradation.

Firstly, their hexane utilization modes were determined by checking for the presence of the two key classes of AH genes (Section 4.3.1). Their growth in the presence and absence of silicone oil and alternative carbon source (glucose) were then examined (Section 4.3.2 and 4.3.2.1). Finally, CSH status (Section 4.3.3.1) and the emulsification properties (Section 4.3.3.2) of the bacterial strains were scrutinized.

4.2 Materials and methods

4.2.1 Culture medium

The culture media used in this experiment were MSM and MSM supplemented with 0.1 % glucose (MSM-glucose). MSM liquid medium and agar were prepared as described in Section 2.2.1. MSM-glucose liquid medium and agar was similarly prepared, but with addition of 0.1 % (w/v) of glucose into MSM.

4.2.2 Two phase (Biphasic) and monophasic liquid culture system

Cultivation of bacterial strains in the two phase (biphasic) liquid system was carried out in a mixture formulated at a volumetric ratio of 10 parts aqueous phase (MSM) to 1 part silicone oil (poly(dimethylsiloxane) 200® fluid viscosity 20 cSt (Sigma Aldrich). Cultivation in the monophasic control was carried out in a volume of MSM equivalent to the total volume of the biphasic system.

4.2.3 Bacterial culture conditions

A sub-group of strains presented in Table 3.10, which were from the collection of culturable isolates, has been selected for further study. They are listed in Table 4.2 below. These bacterial strains were streaked from permanent vials kept in storage at -80 °C onto MSM agar plates and incubated at room temperature in a desiccator infused with 1 ml of hexane for seven days.

For the study on the bacterial growth in liquid medium (Section 4.2.4), cell surface hydrophobicity (Section 4.2.5.1) and emulsification activity (Section 4.2.5.2), these strains were grown in a 16x 100mm glass culture tube (HACH) containing 4 ml of MSM medium supplemented with hexane (8 µl) or glucose (0.1 %), with or without 400 µl silicone oil.

For culturing of strains with surfactants, the strains were grown in MSM medium supplemented with 0.002% *Bacillus subtilis* surfactin (Sigma Aldrich) or

with 50% (v/v) of cell-free supernatants obtained from *Mycobacterium* My16 after 7 days of growth. Such bacterium has been found to be high surfactant producer (Figure 4.9).

Table 4.2 Strains used in this study. These 49 strains have been selected from the 109 culturable isolates (refer BP series) sampled from the bioreactors on the basis of unique MOTU identities as determined through 16S rDNA sequences (refer Fig 3.8 and Table 3.9).

Class	Strain
Actinobacteria	<i>Gordonia</i> G1, G2, G3, G4, G5, G6, G7
	<i>Mycobacterium</i> My4, My6, My7, My9, My10, My14, My15, My16, My17
	<i>Rhodococcus</i> R3, R4, R5, R8, R9
	<i>Microbacterium</i> M1, M2, M4, M5
Flavobacteria	<i>Chryseobacterium</i> C1, C2, C3, C4, C8
	<i>Flavobacterium</i> F1
Shingobacteria	<i>Mucilaginibacter</i> Mu1
	<i>Pedobacter</i> Pe1
α -proteobacteria	<i>Caulobacter</i> Ca1
	<i>Sphingomonas</i> Sp2
β -proteobacteria	<i>Achromobacter</i> A5, A6
	<i>Burkholderia</i> B3, B4
	<i>Cupriavidus</i> Cu1
	<i>Variovorax</i> V1, V2, V3, V4, V5, V6, V7, V8
γ -proteobacteria	<i>Nevskia</i> N1

4.2.4 Determination of biomass in monophasic and biphasic cultures

The biomass of bacterial cultures grown in monophasic system was determined by measurement of OD₆₀₀ during the initial inoculation and after 7 days of growth at 25 °C with constant shaking at 200 rpm.

For cultures grown in biphasic system, the total biomass after 7 days was determined by centrifuging the entire volume of bacterial culture (i.e. both phases) at 16,000 g for 10 minutes at room temperature and resuspending the pellet in the same volume of fresh MSM medium, before taking OD₆₀₀ measurement.

To determine the distribution of biomass in the aqueous phase and the IF of the biphasic system, the aqueous phase was first pipetted out for measurement of OD₆₀₀. Such aliquot was then returned to the bulk of biphasic culture and the

entire volume was centrifuged at 16,000 *g* for 10 minutes at room temperature. After resuspending the pellet in the same volume of fresh MSM medium, OD₆₀₀ was again measured. The difference between the turbidity of the aqueous phase before and after centrifugation was used to calculate the percentage distribution of bacteria in the IF: $(\text{Total OD}_{600} - \text{aqueous phase OD}_{600}) / \text{Total OD}_{600} \times 100$. Negative controls for this series of experiments included (i) the respective bacterial strains in MSM with no carbon source and (ii) the respective MSM media as dictated by the experiments without inoculation of bacteria.

The fold change in biomass after 7 days of growth was calculated by the following formula: Fold change = Final OD₆₀₀ / initial OD₆₀₀. The fold change of ≤ 1 indicated that the bacteria did not grow with hexane. These growth studies of the individual strains were carried in duplicate tubes in two independent experiments.

4.2.5 Detection of *alkB* and *CYP153* by PCR

PCR were performed in a final volume of 50 μ l PCR reaction mixture containing 5 μ M each of the forward and reverse primers (Table 4.2), 200 μ M each of deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 1 U Taq DNA polymerase (Fermentas), 1x Taq buffer with KCl (Fermentas), 1.5 mM MgCl₂ (Fermentas) and sterile distilled water. For *alkB* and *CYP153* gene amplification, one colony of each strain was directly added into the reaction mixture using a sterile pipette tip to serve as a source of DNA template. PCR was performed using a Thermocycler (Eppendorf).

The PCR condition for *alkB* amplification was adapted from Smits et al (1999). The programme consisted of an initial cycle of denaturation of template DNA at 95 °C for 10 minutes. This was then followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute and elongation at 72°C for 1 minute. The final extension step was set at 72°C for 10 minutes.

The PCR condition for *CYP153* amplification was adapted from Alonso-Gutierrez et al (2011) and involved an initial cycle of denaturation of the

genomic DNA at 95 °C for 5 minutes, followed by a touchdown thermal profile protocol during the first 10 cycles of step, where the annealing temperature was decreased by 1 °C per cycle from 65°C to 60 °C followed by 20 addition cycles at 60°C of annealing temperature.

The PCR products were electrophoresed using 1.0 % (w/v) agarose gel for 45 minutes at 90 V in 1X TBE buffer. The DNA size marker, GeneRuler™ 100bp DNA Ladder Plus (Fermentas) was used. The ethidium bromide stained gels were photographed using filtered ultraviolet (UV) illumination by Chemi-genuis Bio imaging system (Syngene).

Table 4.3 Primers used to test alkane hydroxylases

Target group	Primer	Sequence (5'→3')	T _a (°C)	Size of product (bp)	Ref
<i>alkB</i>	<i>alkBf</i>	AAYAGAGCTCAYGARYTRGGTCAYAAG	60	550	Smits et al (1999)
	<i>alkBr</i>	GTGGAATTCGCRTGRTGRTCIGARTG			
CYP153	P450f	GTSGGCGGCAACGACACSAC	60	339	Amouric et al (2010)
	P450r	GCASCGGTGGATGCCGAAGCCRAA			

4.2.6 Analysis of the physico-chemical properties

4.2.6.1 Cell hydrophobicity assay

Cell surface hydrophobicity was determined using a modified procedure of microbial adhesion to hydrocarbons (MATH) technique as described by Pembrey et al (1999). Bacterial cultures from the monophasic and the biphasic systems were first centrifuged at 16,000 g for 10 minutes at room temperature and resuspended in the same volume of fresh MSM. An aliquot of 1 ml of the resuspended culture was mixed with 200 µl of hexadecane in an eppendorf tube. The mixture was vortexed at full speed for 2 minutes and left to stand for 5 minutes to allow phase separation. Optical density of the aqueous phase fraction was measured at 400 nm before and after incubation with hexadecane. MATH was expressed as a percentage, calculated according to the formula:

$$[(OD_{\text{before}} - OD_{\text{after}}) / OD_{\text{before}}] \times 100$$

In the case of the TPPBs and MPBs, the microbial cultures were sampled from the aqueous phase of the TPPBs and the MPBs weekly over a period of 52 weeks as described in Section 2.2.5. MATH was determined as described above.

4.2.6.2 Emulsification measurement

The protocol to determine emulsifier activity was adapted from Soares et al (2003). The monophasic and the biphasic systems were centrifuged at 16,000 g for 10 minutes to obtain the aqueous phase supernatants. The supernatants were filtered through a 0.2 µm membrane filter (Minisart NML) to remove any cells or particulates. Then 3 ml of the cell-free supernatants were mixed with 750 µl of hexane and vortexed at high speed for 2 minutes. The emulsions formed were allowed to settle for 10 minutes after which the optical density was measured at 540 nm.

In the case of the TPPBs and monophasic bioreactors, the supernatants were obtained through centrifugation of the aqueous phase of the weekly samples collected from the bioreactors (Section 2.2.5).

4.3 Results

4.3.1 Types of alkane hydroxylase genes present in hexane degrader

Bacterial strains, which are able to grow in the presence of hexane (as the only carbon source), are found to possess either one or more of the functional genes encoding for AH (Berthe-Corthi & Fetzner, 2002). Hence, each of the bacterial strains being studied (Table 4.2) was tested for the presence of the genes *alkB* and CYP153, which encode the two classes of AH known to be able to handle hexane catabolism, using PCR with primers specific for the genes. The results are presented as part of Figure 4.1.

Strains possessing a single type of AH

Bacterial strains which carried either the *alkB* (43 % of the strains tested) or the CYP153 (12 %) gene were identified. Examples include *Chryseobacterium* C1, 2, 4 and 8 which carried only the *alkB* gene and *Gordonia* G1 which carried the CYP153 gene instead.

Strains possessing both types of AH

It appeared that the coexistence of both *alkB* and CYP153 in one bacterium is fairly common (45%) among the bacterial strains in our collection. For example, the presence of both AHs was observed in *Gordonia* G2 to 7 and *Rhodococcus* R4 and 9. However, it was also noted that not all *Gordonia* and *Rhodococcus* strains carried both *alkB* and CYP153, i.e. this is not a trait that is common to all species within these genera.

These observations were similar to what has been documented by (van Beilen et al, 2006) previously. The different AHs apparently possessed overlapping substrate ranges or induction patterns (van Beilen & Funhoff, 2007). Bacterial strains with multiple AHs have also been shown to be able to grow on a wide range of alkanes (van Beilen et al, 2006), increasing their applications in bioremediation.

Figure 4.1 Presence of alkane hydroxylase genes and growth on hexane as the sole carbon source on solid and liquid (monophasic and biphasic) cultivation systems.

Strains were checked for the presence of alkane hydroxylase(s) via PCR using gene-specific primers. For growth on solid medium, strains were streaked on MSM agar plates and incubated with hexane. For growth in liquid medium, strains were inoculated into MSM medium supplemented with hexane under biphasic and monophasic cultivation, i.e. with (+) and without (-) silicone oil (10% v/v) respectively. Changes in biomass were checked after 7 days. The dotted lines at the 4- and 8-fold marks demarcate the range for poor, medium, and good growth. Values and errors bars represent means and standard deviation of duplicate experiments.

^aY indicates presence of PCR products of the expected size range.

^bGrowth on MSM plates incubated with hexane in desiccators. +++ good growth, ++ medium growth, + scanty growth.

4.3.2 Growth of bacteria with hexane as sole carbon source

Although all the strains in this study were isolated based on growth on MSM agar supplemented with hexane, their levels of growth have yet to be compared. In addition, cultivation of the individual strains in the liquid medium was not carried out previously. As growth (i.e. biomass increase) could be used as a quick indicator of bacterial hexane mineralization/assimilation rate, we cultivated the test strains on both solid and liquid media (MSM base) and supplied hexane as the sole carbon source. For the latter, both monophasic and biphasic (i.e. with 10% silicone oil, similarly to the TPPBs) modes of cultivation were carried out, to mimic the fundamental difference between the environments of the MPB and the TPPB. The data are presented in Figure 4.1, and aligned with the AH gene status for reference and comparison.

It was observed that on solid media (refer column “Growth” in Figure 4.1) all the strains under the class Actinobacteria (*Gordonia*, *Mycobacterium*, *Rhodococcus*) showed good to medium growth on solid media, with the exception of *Microbacterium* M1, 4 and 5. Strains belonging to the class Flavobacteria (*Chryseobacterium* and *Flavobacterium*), α - β - γ - proteobacteria (*Achromobacter*, *Burkholderia*, *Cupriavidus*, *Caulobacter*, *Nevskia*, *Sphingomonas* and *Variovorax*) and Shingobacteria (*Mucilaginibacter* and *Pedobacter*) showed only scanty to moderate growth.

Interestingly, most of those strains showing good growth on solid media were able to sustain moderate to high growth (> 4 fold increase in biomass) even in the monophasic liquid cultures (Figure 4.1, pink symbols) e.g. *Gordonia* G5 and *Mycobacterium* My9, while poor growth on solid media could likewise be correlated with low growth in monophasic liquid cultures e.g. *Mucilaginibacter* Mu1 and *Flavobacterium* F1. In the biphasic system (blue symbols), the majority (91%) of strains in both groups were enhanced in growth, albeit to different extent, which reinforces the belief that the biphasic system supports greater biomass accumulation than the monophasic one, given the same quantity of hexane as the carbon source. However, a few exceptions were observed, e.g. within the genus of *Gordonia*, many of the strains did not fall into this general trend.

No apparent correlation could be found between the AH genotypes and these growth properties. For instance, those strains with two types of AH genes did not necessarily exhibit high growth. The strains' ability to grow well even in monophasic system were not dictated by one type of AH or the other or both.

There is, however, a significant correlation of good growth of the bacterial strains with their dominance in the bioreactors (Table 3.12). This implies that the high growth rate based on hexane utilization exhibited by the bacterial strains were likely to be, at least in part, responsible for their prevalence in the bioreactor communities.

4.3.2.1 Influence of NAL on growth with hexane vs with glucose

The improved growth in the biphasic culture mode compared to the monophasic one has been attributed to the enhanced mass transfer of the hydrophobic substrate hexane brought about by the presence of the NAL phase (discussed in Section 1.5). However, it is unclear if the NAL has influence beyond this physico-chemical factor, i.e. it may have induced certain physiological changes in a bacterium which could bring about increased hexane metabolism. To test this possibility, we made use of an alternative carbon source, glucose, which is hydrophilic and therefore readily bioavailable and unaffected by the NAL-dependent mass transfer pathways. It is also a common substrate for most bacteria and hence differential metabolic potential would have less part to play in the outcome. The bacterial strains were cultivated in biphasic and monophasic modes similarly to the previous experiment, but with glucose instead of hexane serving as the only carbon source. The results are mapped in relation to those of the hexane set (shown in Figure 4.1) in Figure 4.2.

It is clear from an overall comparison between pairs of biphasic and monophasic data (Figure 4.2, closed and open symbols), that when hydrophilic substrate such as glucose (green symbols) was used, the differential in growth were insignificant, unlike in the case of hexane (blue and pink symbols). This suggests that the silicone oil itself has no direct biological effect on the bacterial strains, as far as glucose metabolism per se is concerned. However, it does not

exclude the possibility that silicone oil induces physiological changes e.g. increased CSH and/or emulsifier activities, but the changes were not factors that would manifest in relation to glucose metabolism. This scenario would be explored through experiments presented in the subsequent sections.

Figure 4.2 Growth of isolates in glucose or hexane with (+) and without (-) silicone oil (10% v/v) respectively. The representative isolates were cultured in MSM medium in the presence of 0.1% glucose or hexane (note that it is the same set of data as shown in Figure 4.2) as sole carbon source. The dotted lines at the 4- and 8-fold marks demarcate the ranges for poor, medium, and good growth. Values and errors bars represent means and standard deviation of duplicate experiments.

4.3.3 Physico-chemical properties of hexane degraders

Due to the hydrophobic nature of the main substrate, hexane, some changes in physico-chemical properties of the microbial communities in the bioreactors (whether TPPB or MPB) would be expected in order to increase its bioavailability for cell uptake. These include aspects of the hydrophobicity of microbial cell surfaces and the production of microbial biosurfactants. At the community level, these parameters have been monitored over the 52-week operation of the bioreactors, and confirmed to be of relevance, as shown in Figures 4.3 and 4.4.

The analysis of CSH (Figure 4.3) was conducted based on the standard MATH assay (Section 4.2.5.1), which is expressed as the percentage of microbial adherence to a hydrophobic reagent, n-hexadecane. A high percentage of adherence implies a greater affinity for the hydrophobic n-hexadecane due to higher surface hydrophobicity. The CSH reached a value as high as 50 % in the initial development of both types of bioreactors and then fluctuated between the 30- 50 % range over 12th to 32nd weeks. Thereafter, the MPBs maintained their CSH level but those of the TPPBs gradually decreased. This was surprising to us at first, until we realized that there was an enrichment of the more hydrophobic cells to the IFs (Section 2.3.3.2.3). Over time, the enrichment may have created an even greater bias of hydrophobic subpopulation in the IFs and the converse (i.e. bias of hydrophilic subpopulation) in the aqueous phase – which was the fraction being assayed for MATH. The involvement of changes in CSH within the microbial communities was therefore indisputable, and affirmed our motivation to investigate this property of the hexane degraders in this study (Section 4.3.3.1).

The assay (Section 4.2.5.2) employed to monitor the bioreactors' emulsification activity was based on a method extensively used by many others to indirectly detect for the presence of free biosurfactants in microbial cultures (Fleck et al, 2000; Soares et al, 2003). The overall emulsification activities of the bioreactors' microbial communities (Figure 4.4), in contrast to the CSH profiles, were clearly more superior within the TPPBs than the MPBs. Other than the common factor of hexane in both types of bioreactors, which is itself likely to

induce biosurfactant production, the TPPBs have, in addition, a hydrophobic NAL phase. This may have induced further production of biosurfactants. The possibility would be investigated in relation to the individual strains' biosurfactant producing capacity (Section 4.3.3.2).

Figure 4.3 Cell Surface Hydrophobicity (expressed as MATH %) of microbial communities in the aqueous phase of the bioreactors. Microbial adhesion to hydrocarbons (MATH) is expressed as the percentage of decrease in the absorbance of aqueous phase of the bioreactors as cells bind to the hydrophobic hexadecane. Values and errors bars represent means and standard deviation of measurements in duplicates.

Figure 4.4 Emulsification activity in the aqueous phase of the bioreactors. The absorbance (OD₅₄₀) of the emulsion generated by cell-free supernatant obtained from the aqueous phase of the bioreactors was measured. Values and errors bars represent means and standard deviation of measurements in duplicates.

4.3.3.1 Cell surface hydrophobicity (CSH)

4.3.3.1.1 Assessment based on standard MATH assay

In order to check our hypothesis that the more hydrophobic cells in the TPPB microbial consortia may have been enriched within the IF through cell adhesion to the NAL, we turned to test the samples collected from the IFs during the weekly samplings. These samples were first pelleted and treated with Triton X-100 to remove the residual silicone oil on the microorganisms before suspending them in fresh MSM medium for the CSH assay. The results were compared to the MATH % obtained from the aqueous phase consortia of the TPPBs and the MPBs. These samples from the IFs indeed showed higher MATH% values compared to its aqueous counterparts (Figure 4.5).

Figure 4.5 Cell Surface Hydrophobicity (as MATH %) of microbial communities from monophasic bioreactors (MPB) and the aqueous phase (TPPB) and interfacial fraction (TPPB-IF) of TPPBs. Mean data from duplicate experiments are shown with their respective standard error. Samples from the same week sampling were presented by same symbols across different bioreactors. The MATH values of interfacial fractions were compared with the aqueous phase of TPPBs and MPBs from the same week sampling respectively using 1-sided Student's *t*-Test. (*) indicates significant difference between both sets ($p < 0.05$).

We then proceeded to assess the CSH of the hexane degraders in this study using the same MATH assay. The data obtained from the monophasic and biphasic cultures supplemented with either hexane or glucose as the sole carbon source were presented in Figure 4.6.

It was first noted that the CSH for the majority of these strains was higher when grown in the monophasic system with hexane than with glucose (compare open symbols). The higher MATH values indicated that more microbial cells of these isolates become hydrophobic in the presence of hexane. Looking at the biphasic systems, the CSH of the microbial biomass from the aqueous phase (cross symbols) for some selected isolates such as *Gordonia* G1, 3 and 4 displayed very low percentage MATH, close to 0%. The CSH of the

corresponding total (aqueous +IF) biomass cultivated with hexane, however, were significantly higher, at approximately 20%. In another example, *Rhodococcus* R8 attained MATH values of 74% for the total biomass compared to its aqueous phase cultures of 0%. Most importantly, this CSH enhancement by the silicone oil was not observed in biphasic cultivation with glucose (compare open and closed green symbols). This indicates to us that it was not simply the presence of the NAL that had increased the CSH of the bacterial strains, but rather the combined effect of hexane and the NAL.

Collectively, the higher MATH values displayed by the total microbial biomass, along with the low MATH values exhibited by the biomass from the aqueous phase of the biphasic systems, confirmed that the hydrophobic bacterial subpopulations have been recruited into the IF, leaving those with lower CSH in the aqueous phase.

Figure 4.6 Cell surface hydrophobicity (as MATH %) of strains cultivated with hexane or glucose as the sole carbon source, in MSM with (+) and without (-) silicone oil. Microbial adhesion to hydrocarbons (MATH) is expressed as the percentage of decrease in the absorbance in the aqueous cultures as cells bind to the hydrophobic hexadecane phase and are removed. The dotted lines at the 10%- and 20%- marks demarcate the ranges for low, medium, and high CSH. Values and errors bars represent means and standard deviation of duplicate experiments.

4.3.3.1.2 Assessment based on biomass distribution between aqueous phase and IF

The MATH assay makes use of n-hexadecane as the hydrophobic phase. In our TPPB system, the NAL phase (silicone oil) is also hydrophobic, and the recruitment of hydrophobic subpopulations to the IF is in fact a similar phenomenon as occurs in the MATH assay. The adherence of bacterial cells to the silicone oil, however, may vary from their adherence to n-hexadecane. Visually, we could observe that some bacterial strains preferentially accumulate in the IF during biphasic cultivation (Figure 4.7A), while others distribute their biomass between the two fractions in a less biased manner (Figure 4.7B) or with a complete bias into the aqueous phase (Figure 4.7C).

Figure 4.7 Images showing the differences in the bacterial distribution in aqueous phase and interfacial fraction when grown in biphasic systems. The bacterial cultures shown are those of (A) *Gordonia* G6, (B) *Mycobacterium* My16 and (C) *Microbacterium* M2 grown with hexane in the biphasic system, exhibiting degree of partitioning into IF as (A) > (B) > (C).

This distribution of the bacterial biomass in the biphasic system would be another indication of CSH, and specifically, in the context of silicone oil as the hydrophobic phase. The percentages of biomass recruited to the IF were determined for all the hexane degrader strains cultivated in the biphasic systems supplemented with hexane or glucose (Figure 4.8). The majority of the strains, when grown in the presence of hexane (blue symbols), showed an increased CSH with respect to silicone oil adherence, but when grown with glucose (green

symbols), displayed CSH only in the “low” range. This again confirmed that it was not the influence of silicone oil per se that resulted in a change in the physiology of the bacterial cells, leading to a change in the CSH, but instead the combined presence of hexane in silicone oil.

Figure 4.8 Distribution of bacterial biomass in the interfacial fraction of the MSM-silicone oil biphasic system. The strains were cultured with hexane or glucose in the presence of 10% silicone oil. The dotted lines at the 10%- and 20%-marks demarcate the ranges for low, medium, and high CSH. Values and errors bars represent means and standard deviation of duplicate experiments.

4.3.3.2 Emulsification activity in relation to biosurfactant production

We next moved on to examine biosurfactant production by the hexane degraders. Again, this was carried out in the monophasic and biphasic cultivation modes using hexane or glucose as the sole carbon source. The emulsification activity assay was quantified through the measurement of optical density (Section 4.2.5.2) and the results are presented in Figure 4.9.

A subset of the strains displayed low level of biosurfactant production when grown on hexane in a monophasic context (Figure 4.9, pink symbols) while another subset apparently could produce significant emulsification activity under the same condition. In the presence of the NAL phase, i.e. under biphasic cultivation (blue symbols), the majority of strains from both subsets were able to enhance their biosurfactant production, implying an “induction” effect by the NAL. The enhanced level of emulsification activity in the biphasic context corroborated our earlier observation of the bioreactor samples across the 52-week operation (Figure 4.2) whereby the TPPBs showed higher activities compared to the MPBs. There were, however, a few exceptions, e.g. the few strains belonging to the genera *Achromobacter*, *Burkholderia* and *Cupriavidus* remained completely unable to emulsify, whether in the presence or absence of the NAL. The emulsification activity seemed to be induced by “needs” because when the hydrophilic glucose was the substrate (green symbols), the emulsification activity remained low (or null, in some cases) and unaffected by the presence of silicone oil in the biphasic systems (compare open and closed green symbols). Again, this reinforces to us the fact that the influence of NAL on these bacterial strains only manifests when there is relevance to the substrate.

The biosurfactants produced may play two roles: the first, to emulsify the hexane itself directly (into small micelles for dispersal in the aqueous phase), and the second – which applies only in the biphasic situation – to initiate and/or stabilize the water/NAL emulsion formed at the IF. To gain a deeper understanding of this, known surfactants such as Triton X-100 (chemical surfactant) and *Bacillus subtilis* surfactin (biological surfactant) were first dissolved in the aqueous MSM. This was then mixed with 10 % silicone oil mimicking the biphasic systems in our experiments. The IFs were formed in both

mixtures after slight vortexing (Figure 4.10A). Analysis of the emulsification activities of the aqueous MSM supplemented with surfactants (Triton X-100 or *B. subtilis* surfactin) before and after (Figure 4.10A) mixing with the silicone oil showed a reduction of 25 % and 83 % in activities respectively (Figure 4.10B). This was likely due to the fact that a proportion of the surfactant molecules present in the aqueous medium became “trapped” at the IF through involvement in stabilizing the aqueous/NAL emulsion, and the assay only detected the proportion that was “free” in the aqueous phase. The scenario most probably applies to the IF and aqueous phase of our biphasic systems as well. The actual quantity of biosurfactants produced (summation of the “IF-trapped” and “free” portion), therefore, may be higher than what could be realistically assayed (the “free” portion). Even then, the “free” emulsifiers assayed from the biphasic systems (in the TPPBs and the single strain cultures) were higher in quantity than those detected in the monophasic counterparts. The need to emulsify not only one hydrophobic compound (hexane) but an additional one (the NAL) in the biphasic context may be the underlying reason why biosurfactant production was further induced by the presence of the NAL.

Figure 4.9 Emulsification activity of strains cultivated with hexane or glucose as the sole carbon source, in MSM with (+) and without (-) silicone oil. The absorbance (OD_{540}) of the emulsion generated by cell-free supernatants obtained from the aqueous phase of the biphasic systems and monophasic were measured. The dotted lines at the 0.3- and 0.6-marks demarcate the ranges for poor, medium, and high emulsification activity. Values and errors bars represent means and standard deviation of duplicate experiments.

Figure 4.10 Formation of interfacial fraction (IF) by Triton X-100 and *Bacillus subtilis* surfactin. (A) The IFs were generated by adding 0.002% Triton X-100 (left) or 0.002% *Bacillus subtilis* surfactin (right) to the MSM-10% silicone oil mixture. Such IFs could be maintained even after one month at room temperature, indicating high stability. (B) Prior to this, the emulsification activities of both surfactants were assayed (“Before”). After the formation of the IF, the aqueous phase was aliquoted and assayed for the emulsification activities of the remaining free surfactants (“After”). The absorbance (OD₅₄₀) of the emulsion generated by mixing the aqueous MSM with hexane reflects the emulsification activity of the surfactants. Values and errors bars represent means and standard deviation of measurements in duplicate experiments.

We then considered the subset of strains that were unable to generate emulsions under all four circumstances tested. These strains most probably do not possess biosynthetic machinery/components necessary for the production or secretion of surfactant molecules. If they were concurrently low in CSH, this lack of means to circumvent the low bioavailability issue of the hexane could well account for their poor growth when cultivated on their own. However, in a community context such as within the bioreactors, these strains will be in interaction with a biosurfactant-producing subpopulation which presumably will steadily release emulsifiers into the medium. To simulate such a situation, we selected the low-biosurfactant-producing (Figure 4.9) and low-CSH (Figure 4.6) strains *Achromobacter* A5 and *Cuprivadus* Cu1 and cultivated them (Section 4.2.3) with the medium comprised of 50% (v/v) of cell-free supernatants obtained

from *Mycobacterium* My16 (high-biosurfactant producer, refer Figure 4.9) after 7 days of monophasic growth. A control using the known surfactant, *B.subtilis* surfactin, supplemented into the fresh medium was put through identical procedure as the *Mycobacterium* biosurfactant. The results showed that both strains, when grown with either of the surfactants, displayed significantly enhanced growth with hexane under monophasic cultivation mode (Figure 4.11). This demonstrates the probable reliance of non-biosurfactant-producing hexane degraders on the biosurfactant-producing members within the microbial community.

Figure 4.11 Growth of *Achromobacter* A5 and *Cuprivadus* Cu1 in the presence of surfactants. Both strains were grown in MSM medium system supplemented with hexane. In one sample, half of the volume of the medium was replaced with the cell-free supernatant obtained from the *Mycobacterium* My16 after 7 days of growth (+ sup My) and in another sample, MSM medium was supplemented with *Bacillus subtilis* surfactin (+surfactin). Controls of the strains grown in MSM medium with no surfactants added were included. The initial emulsification activities of both medium were 0.45. The growths of these isolates were measured by OD₆₀₀ after 7 days and the fold increase in biomass was calculated. Values and errors bars represent means and standard deviation of measurements in duplicate experiments.

4.4 Discussion and Conclusions

4.4.1 Association of biosurfactant production and cell surface hydrophobicity with biomass growth

Hexane utilization by a microorganism depends not only on its genetic capacity (i.e. presence of catabolic genes) but also factors that affect the bioavailability of hexane to the microorganism. Extrinsically, the addition of a hydrophobic NAL phase to generate a biphasic system (as in the operation of a TPPB) can bring about greater mass transfer of the hexane into the aqueous phase. Intrinsically, the hydrophobicity of the microorganism's cell surface can facilitate direct adherence to the substrate (hexane) and/or the NAL phase if the culture is biphasic. The presence of surfactants, either self-produced or provided by external sources, can further improve hexane bioavailability through the emulsification of hexane itself and the NAL, if the latter is present. In this study, these factors were investigated with respect to the array of culturable strains isolated from the TPPBs and the MPBs. All the strains were verified to carry genes indicative of their intrinsic hexane catabolic capacity (Section 4.3.1) and manifested growth as pure cultures to different extent using hexane as the sole exogenous carbon source (Section 4.3.2). The association between each strain's level of growth on hexane (Figure 4.1), CSH (Figures 4.3) and biosurfactant production (Figure 4.4) were summarized in Table 4.4, to help us gain an understanding of the possible roles these strains may play in hexane utilizing microbial communities. The impact of having an additional NAL phase in the system was also considered by comparing these properties across monophasic and biphasic cultures.

To maximize the information obtainable from these data, a multivariate approach was further employed to map the various strains in 3-D space based on their similarity in growth, cell surface hydrophobicity and emulsification production in monophasic and biphasic systems. MDS plot using direct quantitative data of these properties showed that five distinct clusters could be formed (Figure 4.12). Strains from the same genera may be distributed to different clusters, e.g. strains under *Variovorax* could be found in cluster 2, 3 and 4. From here, it was possible to classify the strains into five functional classes

based on the combination of characteristics shown with respect to these properties (Table 4.5). Strains in Group 1, for instance, have a profile of low growth, CSH and emulsifier activity regardless of the presence of the NAL, while those in Group 5 takes the other end of the spectrum, having high ratings in all properties, also regardless of the presence of the NAL. Groups 2, 3 and 4, in comparison, are clearly influenced by the presence of the NAL phase, but varying in the degree of “basal” and “induced (by NAL)” status.

The group to which each strain is categorized into is indicated under the “Group” column of Table 4.4. It is interesting to note that within some genera, the profiles of all the strains isolated from our bioreactor were of the same functional Group (e.g. Group 4 for *Mycobacterium*, Group 5 for *Rhodococcus*), whereas in others, such as the genus *Gordonia*, there was a heterogeneous spread of properties, spanning almost the full spectrum from Groups 1 to 5.

Figure 4.12 Multivariate analysis of the biomass growth, cell surface hydrophobicity and emulsification activities of isolates when grown in monophasic and biphasic system. The isolates were clustered into 5 groups (circled in red, labelled 1-5).

Table 4.4 Summary of the growth and physiochemical properties of the different strains when cultured with hexane under monophasic and biphasic conditions, i.e. with (+) and without (-) silicone oil (10% v/v) respectively. The poor/low, medium, good/high of growth, cell surface hydrophobicity (CSH) and emulsification activity (EA) were indicated by +, ++, +++, according to the 3-tiered ratings indicated in each of the earlier Figure 4.1, 4.3 and 4.4. Strains, which exhibited similar trend in growth and physiochemical properties, were classified together under group column as Group 1 to 5. Description of profiles of Group 1 to 5 is shown in Table 4.5.

Genus	Strain	Monophasic			Biphasic			Group
		Growth	CSH	EA	Growth	CSH	EA	
<i>Gordonia</i>	G1	+	+	+	++	++	++	2
	G2	+	+	+	+	+	+	1
	G3	+	+	+	++	++	++	2
	G4	+	+	+	++	++	++	2
	G5	+++	+++	++	+++	+++	+++	5
	G6	++	++	++	+++	+++	+++	4
	G7	+++	+++	++	+++	+++	+++	5
<i>Mycobacterium</i>	My4	++	++	++	+++	+++	+++	4
	My6	++	++	++	+++	+++	+++	4
	My7	++	++	++	+++	+++	+++	4
	My9	++	++	++	+++	+++	+++	4
	My10	++	++	++	+++	+++	+++	4
	My14	++	++	++	+++	+++	+++	4
	My15	++	++	++	+++	+++	+++	4
	My16	++	++	++	+++	+++	+++	4
	My17	++	++	++	+++	+++	+++	4
<i>Rhodococcus</i>	R3	+++	+++	++	+++	+++	+++	5
	R4	+++	+++	++	+++	+++	+++	5
	R5	+++	+++	++	+++	+++	+++	5
	R8	+++	+++	++	+++	+++	+++	5
	R9	+++	+++	++	+++	+++	+++	5
<i>Microbacterium</i>	M1	+	+	+	+	+	+	1
	M2	+	+	+	++	++	++	2
	M4	+	+	+	+	+	+	1
	M5	+	+	+	+	+	+	1
<i>Chryseobacterium</i>	C1	+	+	+	++	++	++	2
	C2	+	+	+	++	++	++	2
	C3	+	+	+	+++	+++	++	3
	C4	+	+	+	++	++	++	2
	C8	+	+	+	+++	+++	++	3
<i>Flavobacterium</i>	F1	+	+	+	++	++	++	2
<i>Mucilaginibacter</i>	Mu1	+	+	+	++	++	++	2
<i>Pedobacter</i>	Pe1	+	+	+	+	+	+	1
<i>Caulobacter</i>	Ca1	+	+	+	+	+	+	1
<i>Sphingomonas</i>	Sp2	+	+	+	+	+	+	1
<i>Achromobacter</i>	A5	+	+	+	+	+	+	1
	A6	+	+	+	+	+	+	1
<i>Burkholderia</i>	B3	+	+	+	+	+	+	1
	B4	+	+	+	+	+	+	1
<i>Cupriavidus</i>	Cu1	+	+	+	+	+	+	1
<i>Variovorax</i>	V1	+	+	+	++	++	++	2

	V2	+	+	+	++	++	++	2
	V3	++	++	+++	+++	+++	+++	4
	V4	+	+	+	+++	+++	++	3
	V5	+	+	+	++	++	++	2
	V6	+	+	+	++	++	++	2
	V7	+	+	+	++	++	++	2
	V8	++	++	++	+++	+++	+++	4
<i>Nevskia</i>	N1	++	++	++	+++	+++	+++	4

Table 4.5 Growth and physiochemical properties exhibited by the five groups. The poor/low, medium, good/high of growth, cell surface hydrophobicity (CSH) and emulsification activity were indicated by +, ++, +++ respectively.

Group	Monophasic			Biphasic		
	Growth	CSH	EA	Growth	CSH	EA
1	+	+	+	+	+	+
2	+	+	+	++	+++	++
3	+	+	+	+++	+++	++
4	++	++	++	+++	+++	+++
5	+++	+++	++	+++	+++	+++

4.4.2 Distribution of properties of hexane degraders in bioreactor communities

The majority of the good hexane-utilizers (in the context of pure cultures), categorized as Group 4 and 5, belonged to the class Actinobacteria. This is certainly consistent with the observation we made earlier about the dominance of this class within the MOTUs identified in our community analysis (Section 3.3.3.4 and 3.3.5.2). We also noted from the earlier community analysis at the resolution of genus level (Section 3.3.3.3 and further discussed in Section 3.4) that there was a biased dominance of *Mycobacterium* (Group 4) exclusively observed in the TPPBs and of *Rhodococcus* (Group 5) and *Gordonia* G1/3/5 (Groups 2/2/5) exclusively in the MPBs. Since Group 5 bacteria are high in CSH and emulsification activity even without the NAL (whereas Group 4 is “enhanced” in these properties only in the presence of NAL), this enrichment of the Group 5 bacteria within the monophasic environments of the MPBs is very much in line with general expectations.

What was at first surprising, however, was that the bias shown by the better hexane-degrading communities in 44th week TPPBs compared to the 28th week community in the aqueous phase fraction was in the subpopulation of *Microbacterium* M2 and *Achromobacter* A6, having the properties of Group 2 and Group 1 respectively (Figure 3.25). In the biphasic context of TPPB, a further enrichment of a subpopulation with higher CSH and emulsification capacity would be logically expected over the long term. The work by Hernandez et al (2012) and Muñoz et al (2013) have given clear demonstrations of how highly hydrophobic consortia could bring about greater biodegradative enhancement over mixed hydrophobic/hydrophilic or highly hydrophilic ones. Other authors have also advocated the use of more hydrophobic strains as the means of augmenting TPPB operations (MacLeod & Daugulis, 2005). Our data showing an increase in the more hydrophilic subpopulation (Group 1 and 2) in a “more efficient” community of the 44th week at first appeared contrary to this expectation. However, when the IF of the 44th week community was examined, a higher proportion of *Mycobacterium* My1-10, 12, 14-17 (Group 4, i.e. with higher CSH and emulsification capacity) could indeed be observed compared to

the 28th week. This underscores the importance of considering the shifts in community in the TPPBs as a whole (i.e. aqueous phase and IF), and not make conclusions based on the phenomenon in one phase only.

To better understand this, we mapped the temporal community dynamic profile resolved at the genus level presented earlier (Figure 3.25 under Section 3.3.5.2) to one that was resolved at the functional group classification level (i.e. according to Table 4.4 and discussed in section 4.4.1). Figure 4.13 shows the outcome of this mapping. The functional group dynamic profile showed distinct distribution of the five Groups within the MPBs compared to the two fractions (aqueous and IF) of TPPBs. The most prominent distinction is that for the hydrophobic subpopulation (Group 4 and 5), the MPBs appeared to favour the maintenance of a higher abundance of Group 5 bacteria over Group 4 bacteria across time. Our demonstration that Group 5 bacteria showed the best growth and possess high CSH and emulsifier activity even in the absence of NAL phase (summarized in Table 4.5) provides a good explanation for this trend of MPBs. On the other hand, TPPBs supported a drastic shift towards more Group 4 bacteria across time at the expense of a significant decline in Group 5 bacteria, exhibited by the dynamic profiles of both the aqueous phase and the IF. This was most likely due to the fact that the hydrophobic properties of Group 4 bacteria are further enhanced in the presence of the NAL (Table 4.5, compare Monophasic and Biphasic blocks under Group 4), and would thrive better in a biphasic system where it could maximize this advantage by straddling both fractions. In the case of the other functional groups, at stationary phase (i.e. after Week 12) their relative abundance as compared to the initial inoculum could be generalized across all bioreactors as (i) not significantly changed for Group 1 and 2 bacteria and (ii) further reduced for Group 3 bacteria.

To discern beyond what was apparent through the eye-balling of this data, we also applied MDS to the data set profiling relative abundance of the functional groups (Figure 4.14). Consistent with the previous two sets of plots generated based on DGGE band profiles (Figure 3.14B) and relative abundance of key genera (Figure 3.27A), the initial inoculum and the earlier 12 weeks (Figure 4.14, circled with red dashed line) were distributed further from their stationary phase counterparts' clusters. This means that even from the perspective of functional

group distribution, stabilization of communities in the bioreactors was reached only after 12 weeks. Also consistent with previous MDS plots, clustering within the TPPBs were tighter than within the MPBs, implying that the distribution of functional groups within TPPBs were more conserved, while there was greater divergence between the MPBs.

We next considered the distribution of key genera within each functional group through MDS analysis during the stabilized stage of Weeks 16-52 (Figure 4.15). Closely clustered points in this series of plots would imply that the specific bacterial subgroup making up a particular functional group in several samples were highly similar. The scattered points in the MDS plots of Group 1 and 2 (Figures 4.15A and B) suggest that there were in fact considerable variation in bacterial compositions making up Groups 1 and 2, not only amongst different bioreactors, but even within the same bioreactor across time. Since we know from earlier discussion that the relative abundance of Groups 1 and 2 did not change significantly over the course of the stationary phase (Figure 4.13), the trend shown by the MDS plot is reflective of functional redundancy i.e. one bacterial group was easily replaced by another which was capable of similar functions (Lubarsky et al, 2012; Wittebolle et al, 2008) within Group 1 and 2 communities. For Group 3 bacteria (Figure 4.15C), it appeared that the majority of the samples from all bioreactors fell within the same cluster (circled with red dashed line), and the data points are so closely overlapping that separate dots cannot be distinguished (note the multiple labels around a single dot). This is most likely due to the relatively low abundance of Group 3 bacteria across the bioreactors (Figure 4.13) resulting in too few variable and hence statistically unreliable analysis. The distribution profile of Group 4 bacteria (Figure 4.15D) showed that the majority of the samples from both aqueous and interfacial fractions of the TPPBs were concentrated in a tight but lengthened cluster (circled with red dashed line, most dots within are overlapping) implying conservation of at least a subgroup of Group 4 bacteria across both TPPBs and across time. Referencing back to Figure 3.24, the likely candidates were the subgroup *Mycobacterium* My1-10, 12, 14-17. Finally, two distinct tight clusters under MDS plot of Group 5 bacteria (Figure 4.15E, circled in red dashed lines) were discernible, one for each of the MPBs, while the TPPB data sets were

scattered and spread out. As discussed earlier, MPBs maintained a high abundance of Group 5 bacteria throughout (Figure 4.13). The MDS analysis suggests that most of the composition of this Group within each MPB did not change much across time.

Taken together, these data support the notion that specific distribution of varied functional groups of bacteria may be favourable under certain culture conditions. The presence of a community that is completely hydrophobic, as described in the recent studies (Hernández et al, 2012; Muñoz et al, 2013), may not always be desirable or viable in the long run, from the point of view of both functional redundancy (Lubarsky et al, 2012; Wittebolle et al, 2008) and optimal functional group distribution. Instead, it may be that a moderately specialized community (medium F_o , as in the case of our bioreactor communities) such as one which comprised of the most fitting species as the dominant members and other supporting species in lower proportions, could thrive better under biphasic circumstances. Examples of such community have indeed been acknowledged previously to have greater resilience, where it can maintain its functionality in the presence of changing environmental conditions (Marzorati et al, 2008). In our case, the variation in the distribution profiles of the functional groups between the TPPBs and MPBs had demonstrated the versatility of the microbial community to evolve and adapt for a long-term operation.

Figure 4.13 Relative abundance of different groups of bacteria present in MPB1 (A), MBP2 (B), TPPB1(C), TPPB2 (D), TPPB1-IF (E) and TPPB2-IF (F) over 52 weeks. The presence of these bacteria as stated in the list above in the microbial communities were deduced from the presence of its corresponding DGGE bands in the DGGE profiles of different bioreactors. For example, DGGE band of the different bioreactors which corresponded to LCI Band 32 of *Gordonia* G1, 3 and 5 were considered to be present in Group 2 and 5 while bands corresponding to LCI Band 24 of *Microbacterium* M1, 4 and 5 were considered to be present in Group 1. The panel on the right showed the dominance (grey box) in the abundance of the various bacteria in the different bioreactors across time.

		MPB		TPPB		TPPB-IF	
		1	2	1	2	1	2
Group 1	Achromobacter A5	■					
	Achromobacter A6			■	■	■	■
	Burkholderia B3, 4		■				
	Caulobacter Ca1			■	■	■	■
	Cupriavidus Cu1	■					
	Gordonia G2			■	■	■	■
	Microbacterium M1, 4, 5	■	■				
	Pedobacter Pe1	■	■				
	Sphingomonas Sp2		■				
Group 2	Chryseobacterium C1, 2	■			■		■
	Chryseobacterium C4			■	■	■	■
	Flavobacterium F1			■		■	
	Gordonia G1, 3	■	■				
	Gordonia G4			■	■	■	■
	Microbacterium M2			■	■	■	■
	Mucilaginibacter Mu1			■		■	
	Variovorax V1, 2, 5, 6, 7				■		■
Group 3	Chryseobacterium C3	■	■				
	Chryseobacterium C8	■					
	Variovorax V4				■		■
Group 4	Gordonia G6			■	■	■	■
	Mycobacterium My4, 6, 7, 9, 10, 14, 15, 16, 17			■	■	■	■
	Nevskia N1			■	■	■	■
	Variovorax V3,8				■		■
Group 5	Gordonia G5	■	■				
	Gordonia G7			■	■	■	■
	Rhodococcus R3, 4, 5	■	■				
	Rhodococcus R8		■				
	Rhodococcus R9			■		■

Figure 4.14 MDS plotted based on the relative abundance of the five functional groups of bacteria in the bioreactors across 52 weeks. Data points are labelled as Bioreactor_Week, e.g. MPB2_48W.

Figure 4.15 MDS plot of the bacterial abundance distribution in each functional group of TPPBs, TPPB-IFs and MPBs at stationary phase. The dynamics occurring among bacterial genera in Functional Group 1 (A), Group 2 (B), Group 3 (C), Group 4 (D) and Group 5 (E) in MPBs (1MPB1, MPB2) TPPBs (TPPB1, TPPB2) and TPPB-IFs (TPPB1-IF, TPPB2-IF) were shown. The cluster formed by different samples in each MDS plot were circled. Data points are labelled as Bioreactor_Week, e.g. MPB2_48W.

4.4.3 Perspective on hexane uptake within a microbial community

Our study of the properties of the isolates obtained from the bioreactors and in relation to their community dynamics have provided clues on the probable roles played by the bacterial groups in the microbial consortium. In particular, the functional grouping of bacteria into five Groups with defined properties allowed us to view the microbial interactions within a community with respect to hexane uptake in a different light from phylogenetic diversity. Figure 4.16 shows the proposed mechanisms that may be occurring when a bacterial community is grown in the presence of hexane in liquid cultures of a monophasic and a biphasic nature. The possible involvement of specific Groups of bacteria within each mechanistic component is depicted.

Biosurfactants are able to aid in the emulsification of the hydrophobic hexane, forming micelles which, in turn, could enhance the bioavailability of hexane to the bacterial cells. This may be produced by Group 4 and 5 bacteria in the absence of the NAL (indicated as silicone oil in the figure) and further reinforced by Groups 2 and 3 in the presence of NAL. The formation of hexane micelles will likely benefit not only the biosurfactant-producers themselves, but also poor biosurfactant-producing Group 1 bacteria, allowing them greater access to hexane for utilization than if they have been cultured on their own. This has been simulated earlier in Section 4.3.3.2 in which the additional surfactants such as from *B. subtilis* (surfactin) and *Mycobacterium* My16 were able to enhance the growth of the poor hexane-growers, *Achromobacter* A6 and *Cuprivadus* Cu1.

Other than producing biosurfactants, Groups 4 and 5 bacteria, by virtue of their high CSH, may interact directly with hexane. In the presence of the NAL, the hydrophobic shift in cell surfaces of Groups 2 and 3 bacteria will include them into this mechanistic niche. Such increased hydrophobicity has been reported in *Mycobacterium frederiksbergense* growing in the presence of anthracene as a result of changes in the cell membrane compositions in contact with hydrophobic substrates (Wick et al, 2003). Furthermore, in the presence of NAL, high CSH bacteria will be able to migrate to the interfacial region to take advantage of the high concentration of hexane accessible at the aqueous/NAL interface.

Collectively, the spectrum of properties represented by Groups 1 to 5 is able to bring about a more sustained and efficient hexane utilization process within a microbial community when cultured in a biphasic mode with the NAL.

Indeed, further work performed by others within our laboratory have been able to demonstrate the importance of the interplay of bacteria from different groups (Zou, 2012). The simulated microbial consortium, which comprised of various combinations of bacterial strains obtained from the bioreactors, showed enhanced growth, sustained hexane degradation capacity and altered CSH/emulsification activities compared to that of a single isolate in the presence of hexane. As these were dependent on the combination of strains, the interplay of multiple bacterial species in the microbial consortium was clearly involved in the enhancement of the biological activities relevant to hexane mineralization. More in-depth dissection of such interplay is in currently on-going within our laboratory.

Figure 4.16 Schematic representation of the proposed mechanisms occurring with hexane degradation in a microbial consortium. In a monophasic system provided with hexane as sole carbon source, the interaction would induce the microorganisms to produce surfactant to emulsify the hexane droplets, forming micelles. The microdroplets may then be encapsulated in the microbial cell surface, entering the microbial cells. Alternatively, the interaction with hexane induced the cell to change its membrane compositions, becoming more hydrophobic. This allowed for the hexane to pass through the cell membrane, entering the cells. In the presence of silicone oil in biphasic system, the surfactant production is enhanced and more microbial cells were induced to become hydrophobic. Hence, the uptake of hexane into the microbial cells was enhanced. Some of the microorganisms present in the microbial consortium may prefer to utilize alternative carbon source such as by-products of other microorganisms or are dependent on the surfactant –producing microorganisms to form micelles so that they can take into the cells.

Chapter 5: Summary and Conclusion

5.1 Accomplishments of this study

The TPPBs set up in this study, using a base medium of MSM and a NAL of silicone oil at 10% v/v and a daily feed of hexane, were able to support better microbial growth (Figure 2.5 and 2.8), protein synthesis (Figure 2.6) and activities (Figure 2.7) than the MPBs controls over the 52 weeks of operation. These results were comparable to other studies (Aldric & Thonart, 2008; Hernández et al, 2012; Muñoz et al, 2013) but this work was the first to have attempted an analysis over such a long period of time. Optical density (Figure 2.14) and culturable cell count (Figure 2.16) analyses were able to show that microorganisms were present at a fairly high cell density in the IFs of the TPPBs. Microscopic observation was able to localize these cells to the aqueous/NAL interface and the aqueous milieu of the emulsion (Figures 2.11 and 2.13). Hexane degradation rate analysis conducted on 28th and 44th week samples of the bioreactors revealed that, unlike the microbial communities in the MPBs, those in the aqueous phase and the IFs of TPPBs have developed into a state of higher hexane removal efficiency over time (Fig 2.17).

Based on the above context, we proceeded to analyse the microbial community structures over time. The overwhelming dominance of the bacterial population over those of the fungi and archaea was first established, allowing us to focus on the bacterial community in our study (Section 3.3.1.3). As temporal analysis would require a large sampling of isolates or DNA species for high resolution analysis, which was not cost-effective, an approach leveraging on both culture-based and culture-independent methods were used (Figure 3.1). Although no apparent differences could be discerned between the bacterial community structures within the MPBs and the TPPBs based on the parameters of dynamics (Dy), diversity richness (Rr) and functional organization (Fo) (Section 3.3.2.2.1, 3.3.2.2.3 and 3.3.2.2.4), molecular analysis was able to lead to identification of phylogenetic affiliation of key groups of bacteria. This was mapped to the DGGE profiles of the bioreactors, producing community structure profiles across the 52

weeks depicting relative abundance of the key genera (Figure 3.25). The bias and predominance of specific subpopulations of various genus compared across the MPBs, the TPPBs and the TPPB-IFs were noted (Table 3.12).

As a significant proportion of culture-based components existed in our community analysis approach, culturable isolates belonging to these dominant bacterial groups were available for further characterization and studies. Their possible roles in the hexane degrading microbial communities of the bioreactors were investigated by looking at their AH genes (Section 4.3.1), growth (Section 4.3.2), cell surface hydrophobicity (Section 4.3.3.1) and emulsification activities (Section 4.3.3.2). Relevance to the TPPB system was additionally considered by examining the influences of the NAL phase (silicone oil). As a result of these efforts, association between each strain's level of growth on hexane, CSH and biosurfactant production could be made (Table 4.4), giving rise to categorization of five types of functional profiles (Table 4.5). Referencing this to the community dynamics resolved at the genus level (Figure 3.25), the functional Groups' distribution across the bioreactors' bacterial communities was revealed (Figure 4.13). The highly similar distribution observed across all samples and all time implied that there could be distinct advantages associated with such a distribution of functional groups. Finally, a hexane uptake scenario presenting possible bacterial interactions within a microbial community carrying the five functional Groups of bacteria was proposed.

5.2 Future perspectives

The study of the dynamics of the microbial consortium in both bioreactors, together with the characterization study of the bacteria isolated from the bioreactors in monocultures, have allowed us to understand the relationship existing within a hexane-degrading microbial community. Currently, based on the repertoire of strains in the five functional Groups revealed through this study, we were able to select a series of bacterial strains to form a bacterial consortium used for removal of formaldehyde in an air-purifier setting. Other researchers have also tried to construct simplified consortia containing several well-defined strains

but these were meant for basic scientific enquiry (Van Hamme et al, 2003). Our work may be systematically developed to work out the formula of a high performance bacterial consortia for bioreactor inoculation.

Emulsion properties are affected by surfactant concentration, surface tension, oil globule coalescence and collision kinetics (Hori et al, 2008; Kang et al, 2008). Many researchers have shown that biosurfactant production (Van Hamme et al, 2006) and changes in CSH of microorganisms greatly influence coalescence-collision equilibria and kinetics (Dorobantu et al, 2004; Hori et al, 2009). Our study has shown that specific bacterial strains carry distinct profiles of CSH and biosurfactant production capacity. Hence varying the combination of them could result in vastly differing interfacial features. Conventionally, random emulsion formation in TPPBs has been discussed in engineering terms as undesirable (Morrish et al, 2008; Muñoz et al, 2006) and to be deliberately minimized. However, it is indisputable that increased interfacial surface area in TPPBs enhances substrate mass transfer (Muñoz et al, 2007b). Instead of achieving this through mechanical means which will incur high costs and deter pilot scale operation (Quijano et al, 2009), the biological generation of stable emulsion may be a solution. In processes other than TPPBs, the use of surfactants to exploit emulsions with desired properties for higher interfacial area had been adopted resulting in the need for less energy input (Singh et al, 2007). Our work suggests that we may take this one step further by manipulating the composition of microbial communities to achieve desirable emulsion dynamics.

5.3 Conclusions

In conclusion, our TPPB system has been shown to function better in terms of hexane degradation compared to the monophasic controls. The community analysis of this system conducted over a 52-week operation and in comparison with the control monophasic system has revealed that the presence of silicone oil can select for a subset of bacteria to thrive in the IF and induce in another subset of bacteria biosurfactant production and an increase in CSH. However, low biosurfactant producers and low CSH bacteria will be able to

benefit within a community context from the activities of other bacteria with higher functionality.

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