Development and analysis of two bioaugmentation approaches: a volatile organic compound bioaugmentation case and a pentachlorophenol bioaugmentation case

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NANYANG
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BIOAUGMENTATION APPROACHES: A VOLATILE
ORGANIC COMPOUND BIOAUGMENTATION CASE
AND A PENTACHLOROPHENOL BIOAUGMENTATION
CASE**

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SCHOOL OF BIOLOGICAL SCIENCES

2016

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List of Abbreviation

ALDH2	mitochondrial aldehyde dehydrogenase 2
AnMBR	Anaerobic Membrane Bioreactor
CO ₂	carbon dioxide
COD	chemical oxygen demand
CP	chlorophenol
DCP	2,4-dichlorophenol
DCPIP	2,6-Dichlorophenolindophenol
DGGE	Denaturing Gradient Gel Electrophoresis
DNAN	2,4-Dinitroanisole
DSM	Desulfitobacterium frappieri medium
FBBR	fluidized bed biofilm reactors
FDH	formaldehyde dehydrogenase
FISH	Fluorescent in situ hybridization
HPLC	High-Performance Liquid Chromatography
MATH	microbial adhesion to hydrocarbons
MBTH	3-methyl-2-benzothiazolinone hydrazone hydrochloride
MCL	middle chain length
MSM	mineral salt medium
NES	Non <i>E.coli</i> Strain
NTU	Nephelometric Turbidity Unit
OD	Optical density
OH	hydroxyl
P450s	cytochrome P450 enzymes
PAH	polycyclic aromatic hydrocarbon
PCP	pentachlorophenol
PCR	Polymerase Chain Reaction
PID	a photo-ionization detector
PNP	p-nitrophenol

qPCR	real-time quantitative PCR
RG	Risk Group
SAMBRs	Submerged anaerobic membrane bioreactors
SWF	Synthetic Wastewater Feed Medium
TAE	Tris-acetate-EDTA
TCP	trichlorophenol
TeCP	tetrachlorophenol
TPPB	two-phase partitioning bioreactor
TSS	Total suspended solids
UASB	up-flow anaerobic sludge blanket reactors
UV	ultraviolet
VFA	volatile fatty acids
VOCs	volatile organic compounds
VSS	volatile suspended solid

Abstract

Bioaugmentation (i.e. introduction of microorganism with specific metabolic capacity that can enhance biological processes) as a cost-effective method for remediation of environmental pollutants, has gained popularity over the decades. One of the most challenging concerns in the use of this technology is the sustainability of the bioaugmentation system. In this project, we aimed to develop two co-culture bioaugmentation systems to achieve satisfactory efficiency and sustainability for two biotechnological applications (one aerobic and the other, anaerobic), using differing approaches.

In the first approach, members of the bioaugmentative consortium to be developed were drawn from a previously analyzed pool of isolates from a two-liquid phase partitioning bioreactors (TPPBs) handling volatile organic compound (VOC) removal. After multidimensional characterization of 26 candidate strains, one final consortium with outstanding VOCs' removal capacity was formulated by 12 out of the 26 strains. Two methods of testing the bioaugmentation efficiency were developed after this consortium was formulated. In the first method, the consortium's removal efficiency was tested within an enclosed environment in an air purification device, which reaches 100% removal of formaldehyde. The second method is colorimetric in nature, measuring formaldehyde concentration dissolved in aqueous medium. The bioaugmentative consortium's formaldehyde removal efficiency can be maintained within the range of 80%-100% over at least 16 days under settings allowing for random environmental perturbation. Microbial interaction among strains in this formulation of co-culture was briefly assessed under 2-strain co-culture setting.

In the second approach, activated sludge from local wastewater treatment plant was used as the start-up seed of anaerobic bioreactors and bioaugmentative consortia were developed to remove refractory compounds that may negatively impact the anaerobic wastewater treatment process. The composition of the consortia were left uncharacterized while development was going on. After acclimatization of the activated sludge to

pentachlorophenol (PCP) and its metabolic intermediate separately over one year, each bioaugmentative consortium and several mixtures' PCP removal efficiency and biogas production were tested. The potential bioaugmentative sludges were put through a dilution scheme and heat treatment to reduce the complexity of the consortia for more regulated usage in the future. Several mixtures were made with the subcultures of these simplified consortia. Their bioaugmentation efficiency was tested by High-Performance Liquid Chromatography (HPLC), and one mixture of the subculture revealed 76.1% of PCP removal.

In the process of developing bioaugmentative consortia, relevant microbial dynamics were preliminarily reviewed through denaturing gradient gel electrophoresis (DGGE), but found not to be appropriate or sufficient in depth to reveal useful information. Two recent advances in techniques – microfluidic q-PCR and pyrosequencing – were preliminarily explored for application in microbial population tracking and bacterial interaction investigation.

Chapter 1. General Introduction

1.1 Environmental Contaminants

With the world's population gradually but continually expanding, the demand for living resources is, inevitably, also increasing. In order to meet these demand, there is a rapid expansion of industries to produce food, vehicles, medicine, personal care products, and construction materials, to list just a few (Kamaludeen, Arunkumar, Avudainayagam, & Ramasamy, 2003). The rising agriculture and manufacturing industries have been releasing a wide range of xenobiotic compounds to the surrounding soils, water bodies and air, making it difficult to maintain the quality of the environment in which we live in. Global attention has been focused on ways to sustain and preserve the environment from such deterioration. Although this endeavour sometimes involves engineering methods of physico-chemical nature, in many cases, it is made easier by involving biotechnology (Kumar, Bisht, Joshi, & Dhewa, 2011).

1.2 Bioremediation of Environmental Contaminants

Bioremediation is a term used to describe a technology that utilizes the metabolic potential of living things, in most cases microorganisms, to clean up contaminated environments (Mohan et al., 2009). Among the several existing ways known to be applied to remove pollutants from the environment, bioremediation has several advantages: (i) it is a natural process, (ii) it is cost effective, (iii) it brings about the destruction or removal of toxic chemicals from the environment and not merely separates them out, and (iv) it expends less energy when compared to other technologies (Kumar et al., 2011).

1.2.1 In Situ versus Ex Situ Bioremediation

Based on the site at which bioremediation is designed to take place, it can be classified into two categories: in situ bioremediation, and ex situ bioremediation. The former means the remediation process is accomplished on site, and there is no necessity to transport the contaminated material e.g. soils or water to another site to complete the remediation process. On the other hand, ex situ bioremediation involves relocation of target samples to

achieve bioremediation, for example, excavation of contaminated oil or pumping of groundwater to be channeled to reactor facilities and treatment plants.

Most often, we will find that in situ bioremediation is applied to the degradation of contaminants in saturated soils and groundwater suffering from widespread pollution. It has an advantage in terms of cost, since no relocation of contaminated sample is required. However, the major difficulties in the implementation of in situ bioremediation appear to be related to the delivery of appropriate electron acceptors (e.g. oxygen, nitrate and sulfate), the bioavailability of substrate (e.g. hydrocarbon), and the presence and expression of appropriate microbial catabolic capabilities which can meet the needs of the bioremediation (Hu & Chan, 2015).

In contrast, ex situ treatment of contaminated soil and water are commonly operated in bioreactors after they are pumped up from a contaminated site and transported to the destination facilities. Bioremediation in reactors involves the processing of contaminated material e.g. soil, sediment, air, sludge or water through engineered containment systems, which contains microbial communities capable of degrading target contaminants (Alvarez & Polti, 2014).

In general, the rate and extent of biodegradation are greater in a bioreactor system conducted ex situ than in systems operated in situ, as the contained environment of bioreactor is controllable and hence more manageable and predictable. In this project, two cases of bioremediation, one operating as a hybrid of the two types of bioremediation, and the other primarily as ex situ bioremediation, would be examined.

1.2.2 Bioremediation through Natural Attenuation

Microorganisms are the foundational and major contributors driving the sequence of chemical transformations occurring in bioremediation processes. Left alone, common pollutants in the environment can be degraded to a certain extent by microorganisms already present in the natural environment. This is referred to as natural attenuation. However, relying only on natural attenuation for bioremediation is often time-consuming,

for two reasons. Firstly, microorganisms with the relevant metabolic capability may take time to build up their respective critical mass and modulate the level of biochemical responses to achieve satisfactory performance. Secondly, the native microbial community may not immediately contain strains capable of degrading the target pollutant and would have to wait out sufficient generations in order for appropriate variants to evolve (Mitchell & Gu, 2010).

1.2.3 Biostimulation and Bioaugmentation

In order to circumvent this drawback of natural attenuation (i.e. requiring greater expenditure of time to attain significant pollutant removal), two approaches are generally applied to enhance the microorganisms' rate of biodegradation of pollutants: biostimulation and bioaugmentation. Biostimulation refers to the addition of nutrients or electron acceptors to the system in order to stimulate the metabolic activity of the indigenous microorganisms. This approach works well under scenarios in which metabolically capable strains are already present within the microbial community residing in the vicinity of the contaminated materials. Bioaugmentation, on the other hand, refers to the introduction of specific microorganism(s) with the desired functional capabilities into bioremediation settings. These inoculated microorganisms may work with the indigenous community in the system to enhance the pollutant degradation rate (El Fantroussi & Agathos, 2005). The focus of this thesis is on the approach of bioaugmentation. In the two cases of study presented in this thesis, the addition of relevant microorganisms in order to achieve a better performance in biodegradation were considered.

1.3 Bioaugmentation Application – Current Status

1.3.1 Bioaugmentation Approaches

Many successful bioaugmentation applications have been reported over the past few decades of research. These include various cases adopting the approach of introducing a culture of pure microbial strain into the target system. A specific strain, usually fairly well characterized and tested within laboratory settings for the desired biological activities, is inoculated after pre-adaptation to enhance bioremediation. For example, the carbon

tetrachloride-degrading strain *Pseudomonas stutzeri* KC was applied to an aquifer contaminated by nitrate and carbon tetrachloride with considerable success (Michael et al., 2002). Likewise, the pentachlorophenol biodegrading strain *Desulfitobacterium hafniense* has been effectively applied to a methanogenic fixed-film reactor (Lanthier, Juteau, Lepine, Beaudet, & Villemur, 2005).

In contrast to the use of a specific strain of characterized functions and properties discussed above, the approach of introducing pre-adapted consortia has also seen much success. The complexity expected of microbial communities often makes it challenging to specify their exact community structures. Hence if a microbial community is acclimatized for a target pollutant and then used as a bioaugmentative agent, it is often simply referred to as a consortium and qualified by its acclimatization history. In a case involving anaerobic biodegradation of ground water contaminated by Benzene-Toluene-Ethylbenzene-Xylene-Ethanol mixtures, effective bioaugmentation was achieved through the use of two different methanogenic consortia (Da Silva & Alvarez, 2004).

While the above two approaches have been the mainstay in the field of bioremediation, in recent years, a number of researchers have started to explore formulating a mixture of several strains of metabolic relevance to generate a defined bioaugmentative consortium. Sprocati and co-workers, for instance, managed to enhance the bioremediation process of soil co-contaminated by diesel oil and heavy metal by introducing metabolically tailored microbial consortia consisting of ten to twelve strains (Sprocati et al., 2012).

1.3.2 Challenges in Application of Bioaugmentation

The concept of bioaugmentation may be attractive, but in practice, many challenges remain and much work is still required to bring about a more reliable application.

1.3.2.1 Failed Cases of Bioaugmentation

It is noteworthy that among the bioaugmentation attempts executed, there were as many failed cases being reported in literature as there were successful ones (El Fantroussi &

Agathos, 2005). In principle, the approach of applying specific and characterized strains provide greater certainty and control to the bioremediation process than the popular alternative of an uncharacterized but pre-adapted consortium. However, more often than not, microorganisms which were observed to be capable of degrading pollutants in pure-culture settings failed to function when inoculated into contaminated environments. In one study, for example, *Pseudomonas* strains capable of mineralizing 2,4-dichlorophenol (DCP) and *p*-nitrophenol (PNP) in culture media prepared in the laboratory did not grow or metabolize DCP when inoculated into lake waters (Goldstein , Mallory, & Alexander, 1985). One of the more recent reports on the failure of bioaugmentation describes how Fu et al (Fu, Fan, Liu, Liu, & Liu, 2009) massively inoculated a series of biological aerated filters with a heterotrophic nitrifying bacterium, *Lutimonas* sp. H10, which not only did not enhance the ammonia removal process, but in fact led to a major nitrification breakdown.

Other than clear cases of lack of bioremediation improvement after bioaugmentation, in some applications, despite a significant increase in bioreactor performance observed soon after bioaugmentation, the positive effects could only be maintained for a short time after inoculation. In a study involving phosphate removal, after enhancement of phosphate removal as a result of bioaugmentation to the sludge, the added strains were found to have almost disappeared from the reactor after 16 hydraulic retention time (Patureau et al., 2001). The sustainability of the inoculated strains therefore appears to be another common hurdle in the application of bioaugmentation.

1.3.2.2 Factors Leading to Bioaugmentation Failure

Failure of bioaugmentation may be attributed to a number of factors. In a recent review, Herrero and Stuckey (2015) (Herrero & Stuckey, 2015), summarized some of them: (i) nutrient status may be growth-limiting for bioaugmentative strains e.g. influent may have too low a level of specific substrates; (ii) inhibitory substances e.g. toxic compounds may be present in the system to be treated, which (iii) may be manifestation of antagonistic effects by other microorganisms e.g. through production of antibiotics or bacteriocins; (iv)

bacteriophages are present which may cannibalize the bioaugmentative strains; or (v) operating conditions of the bioreactors may be adverse for the bioaugmentative strains e.g. low temperatures.

Evidence so far suggests that the problem with unsuccessful bioaugmentation first lies with the strain selection criteria. In most strain selection process, only the strains' degradation capacity is assessed. However, in applications, other traits equally important for the success of the bioaugmentation (related to the factors mentioned above) were often unconsidered. Thompson et al. (2005) suggested the single standard selection criteria (metabolic capacity) and poor knowledge of the composition and population dynamics of source communities are responsible for most of the bioaugmentation failures.

Conventional strain selection for bioaugmentation is usually based on biodegradation ability alone, and in most cases, tested when the strain is in the pure culture form. The reason for bioaugmentation failures of these strains when applied in the target environment could be due to negligence to consider the ecological context of such environment. The massive addition of bacteria intended to induce a biological activity could result in undesirable effects that disturb the ecosystem equilibrium. In addition, the ability to survive in the target environment, ability to prevail in the fluctuating conditions (e.g. moisture, nutrients, redox, pH and osmotic factors), and competition for nutrients with indigenous microorganisms would contribute greatly to the fate of inoculated strains (Laura, Hung, & Jack, 1993). It is suggested that future strategies for bioaugmentation should include providing the added free-living bacteria with protection from those environmental disturbances and competitive disadvantages (Bouchez et al., 2000). On the other hand, there are many who argued strongly that selection of suitable microorganisms for bioaugmentation should, in the first place, be from a pool of strains which are known to survive for a long time in a specific target habitat, and are therefore more likely to bring about continuous degradation (Thompson et al., 2005).

1.4 Co-culture Bioaugmentation versus Single-Culture Bioaugmentation

It can be surmised from the above discussion that resilience to various environmental disturbances is an important factor to consider when strategizing for a bioaugmentation approach. In this respect, co-culture bioaugmentation may be more effective than a single-strain bioaugmentation as greater resilience may be attained through the following aspects.

1.4.1 Zymogenous versus Autochthonous Strategies of Microorganisms

“Zymogenous” is a term applied to microorganisms that exist mostly in the resting phase, but when stimulated by available substrates specific to its metabolism, will demonstrate brief periods of high activity, and then return to the resting phase after substrates are exhausted. Most species from the genus *Pseudomonas*, for example, employ the zymogenous metabolic strategy. The other type, referred to as “autochthonous” is defined by microorganisms that demonstrate slow but continuous metabolic activity, and *Arthrobacter* is a good example (Hausner, Starek, & Bathe, 2009). It is therefore logical to envisage that a zymogenous strain would be a suitable bioaugmentation candidate when the system is expected to encounter pollutants (specific “substrates”) in occasional fluxes. When the contaminant is present, the zymogenous strain which has remained dormant will explode in activity and growth rate, and then its active subpopulation rapidly diminishes when the contaminant is completely degraded. An autochthonous candidate, on the other hand, is suitable when a low but continuous stream of substrates is expected to be present, allowing degradation of contaminants to be performed slowly but steadily in a sustained manner (Thompson et al., 2005).

Many researchers have studied bioaugmentation using microorganisms belonging to either type of metabolic strategists. For example, Beskoski and co-workers (Beskoski et al., 2011) have claimed to achieve a high degree of biodegradation of total petroleum hydrocarbon through the use of a primarily zymogenous microbial consortium. In another study, zymogenous microorganisms isolated from soil was found to demonstrate considerably high bioremediation potential in phenanthrene and methyl phenanthrenes biodegradation (Solevic et al., 2011). At the other end of the spectrum, other researchers have managed to assemble autochthonous microbial consortia with bioaugmentative effects successfully.

For example, enhanced phytoremediation of salt-marsh sediments contaminated with cadmium was achieved through an autochthonous consortium developed by Teixeira et al (Teixeira, Almeida, Nunes da Silva, Bordalo, & Mucha, 2014) to complement the plants' root system.

Based on these backgrounds, it can be speculated that if one consortium contains both types of microorganisms, this consortium may be able to maintain the population in low-contaminant environmental conditions and yet efficiently carry out biodegradation when high fluxes of contaminants flood the community. When the contaminant flushes in, the zymogenous microorganisms will be “woken up” and carry out the necessary metabolism. When the level runs low, the zymogenous subpopulation will turn quiescent, but the autochthonous subpopulation will keep up a continuous remediation of the low levels of target compound still remaining, or maintain an overall activity through use of substrates generally present.

1.4.2 Cooperative Metabolism

In bioaugmentation using a single strain, the target output is such that metabolism of the contaminant is brought to completion (i.e. mineralized) by a single strain. In bioaugmentation involving co-cultures, there is an additional option of allowing strains of different metabolic traits to work cooperatively and in sequence to bring about a similar outcome. A growing number of empirical studies have demonstrated positive correlation between diversity and stability within a community (Ives & Carpenter, 2007). One of the reasons is that interconnected metabolic pathways provide resilience, while multiple members possessing similar metabolic pathways give rise to redundancy which further contributes to overall robustness. The community is not dependent on the specific contaminant alone for carbon or energy, and a subpopulation of it can utilize intermediates of the pathway instead. With more variety in biodegradation pathways available that can be adopted, co-culture bioaugmentation are less likely to be affected by the common reasons for failure mentioned in section 1.3.2.2, including low substrate concentration, inhibitory substances, antagonistic effects and predation by bacteriophages.

1.5 Exploring Co-culture Bioaugmentation

Considering these potential advantages of co-culture bioaugmentation over single culture bioaugmentation, co-culture bioaugmentation would be explored in this study. To date, how the most effective strains for a bioaugmentative co-culture should be selected remains an unresolved question. As it is a complex issue, the solution is more likely to be a hybrid manifestation of various schools of thoughts depending on the context of the application, than a clear-cut approach.

1.5.1 Construct of the Thesis

In this study, two cases of bioaugmentation involving different applications of bioremediation (detailed in Chapters 2 and 3) have been designed for co-culture bioaugmentation. In the first case, the consortium would be constructed by taking bacterial strains belonging to a community possessing steady bioremediation activity, and from which significant groups have already been isolated and identified to the genus level through an earlier study. In the second case, several consortia would be acclimatized for specific bioremediation purposes even as their members' identity remained unknown, and they were applied singly or in combination to the target application. Their performances in the intended system would be assessed and the effective consortium selected and analyzed as needed. In order to evaluate these two bioaugmentation systems for their biodegradation efficiency, this study also attempted to develop or modify some assays for application to these two specific cases and methods for investigating the interaction between the strains occurring in the consortia. Finally, some preliminary work is presented that involve the development of more sophisticated analysis that can bring about better microbial population tracking for future studies (refer to Chapter 4).

1.5.1.1 Two Settings of Biological Processes for Application of Bioaugmentation

Biological processes can occur within the spectrum of aerobic to anaerobic conditions, ranging from highly aerated circumstances, to environments completely devoid of oxygen. Exactly where a process is positioned along that spectrum depends on the main metabolic

output desired and the characteristic of the microbial strains that are able to carry out the biochemical reactions for the processes. If the microorganisms require atmospheric oxygen to function, aerobic conditions are essential. On the other hand, for efficient anaerobic pathways to occur, conditions must be anoxic and oxygen may even be inhibitory to the development of anaerobic cultures (Vidali, 2001). For the two bioremediation applications we have chosen to study, one of them would need to be set up for running in an aerobic condition and the other would need to be set up for an anaerobic condition.

In the first case involving an aerobic setting, the desired outcome is the removal of volatile organic compounds (VOCs) such as hexane and formaldehyde from contaminated air by passing them through a microbial culture (the process is sometimes referred to as biofiltration). The strains that would be used to develop the bioaugmentative consortia in this study originated from a highly aerated two-phase partitioning bioreactor (TPPB) designed for hexane removal. It had been seeded from a microbial community isolated from oil-contaminated soil and acclimatized by hexane, and then allowed to run for more than a year with a stable feed of hexane (Ng, 2014). The hexane biodegradation was carried out through catabolic pathways involving alkane monooxygenase and/or cytochrome c in the first step, which require molecular oxygen. (Lee, Kim, Cho, Ahn, & Hwang, 2010; van Beilen, Li, Duetz, Smits, & Witholt, 2003) Therefore, it would be expected that the bioaugmentative consortia developed from such a source would have to operate similarly in an aerobic environment.

In the second case, the desired outcome of bioaugmentation is the removal of recalcitrant compounds such as pentachlorophenol (PCP) from an anaerobic wastewater treatment system. Activated sludge from a local wastewater treatment plant was used as the start-up seed of the anaerobic bioreactor, which was later challenged with PCP and experienced a significant drop in performance. The development of the bioaugmentative consortia would be based on this community of activated sludge origin, which likely contains populations of bacteria, archaea, fungi, and protozoa (Kim & Speece, 2002). The anaerobic prokaryotic subpopulation of this community plays a major role in the anaerobic digestion and hence

the bioaugmentative consortia would have to be developed to be compatible with the anaerobic setting.

1.5.1.2 Two Approaches to Develop Bioaugmentative Co-cultures

To the bioremediation applications discussed above, two bioaugmentation approaches and tracks of studies were respectively developed.

In the first approach – applied to the aerobic biofiltration of VOCs – we picked significant representative groups of strains, which had been isolated and sequenced for identification from a previously stably maintained TPPB showing established hexane removal activities, to formulate a consortium that could enhance VOC removal in an air-purification device. The consortium's bioaugmentation capacity would then be tested by two methods: chamber air testing and colorimetric liquid testing. After the co-culture has been successfully formulated, the fitness and performance sustainability of the co-culture would also be checked, by challenging it in a setting that would be exposed to environmental conditions that allow perturbations. Following this, interaction between selected strains would be briefly surveyed to identify possible relationships.

In the second approach – applied to the biodegradation of PCP in anaerobic waste water treatment – bioaugmentative formulation would be acquired by strategically acclimatizing indigenous microbial community without attempting to make specific strain identification. Consortia from the sludge in an anaerobic wastewater treatment system would be acclimatized to the target pollutant and its intermediate metabolites separately, in order to develop several bioaugmentative co-cultures. These would be tested for bioremediation efficacy without characterization of the community as it was expected to be highly complex. Some simplification of the consortia would be carried out through serial dilution. The variously “simplified” co-cultures would be mixed if necessary to form new consortia, checking if the combination has in anyway enhanced the efficiency. Strains would only be isolated from the effective bioaugmentative consortium and characterized in the future as the last stage of the bigger project managed by our laboratory.

Figure 1.1 Aims, approaches and expected outcomes of bioaugmentation approach 1 (upper) and 2 (lower).

1.5.2 Survey of Microbial Dynamics Relevant to Bioaugmentation

As conveyed in section 1.3.2.2, the focus of bioaugmentation research nowadays should not be only on the metabolic capacity of the microbial consortia taking part in environmental bioprocesses, but also on how these respond and relate to bioprocess efficiency. After formulating the consortia and testing their bioaugmentation efficiency, ideally, we would want to look at the dynamics of the co-culture community across time, as this may bring about a better understanding of the interplays of microorganisms that influence the efficiency of biological processes. Ultimately, the integration of such knowledge from varied types of bioprocess may provide valuable information that can be used to make better selections of co-cultures for bioaugmentation. However, methods for analysis of community dynamics range from low to high resolution with their respective advantages and disadvantages. The methodologies of choice are therefore very much dependent on the context and needs.

In the past few decades, many molecular biological tools have been developed to study bacterial community structure to replace the time-consuming and highly biased traditional cultivation methods to study the community structure of microorganisms. Many of these

methods involve the use of specific nucleic acid probes coupled to fluorescent dyes, to identify and/or quantify the population of certain type of strain in a community (Hoshino & Inagaki, 2012). Fluorescent in situ hybridization (FISH) and real-time quantitative PCR (qPCR) belong to this category. Since the methods are dependent on probes (primers with specificity to target groups), what can be analyzed is constrained by the predictability of the identities of target groups and prior knowledge of the genera or species in the community. Furthermore, for sufficient reach of the probes to the microorganisms' chromosomes without background interference – for instance, when the samples are attached to soil and sludge particles – it would be necessary to perform time-consuming and laborious cell isolation before the tests (Frischer, Danforth, Healy, & Saunders, 2000; Kallmeyer, Smith, Spivack, & D'Hondt, 2008; Lunau, Lemke, Walther, Martens - Habben, & Simon, 2005).

The alternatives to probe-based community analytical methods are a number of Polymerase Chain Reaction (PCR)-based fingerprinting or sequencing techniques. Such methods make use of universal primers targeting ribosomal RNA genes, which allows analyses of microbial communities to occur without any prior knowledge of the species in the community, and hence are suitable for unidentified environmental samples (Chaudhary, Brabcova, Buriankova, & Rulik, 2013). Methods that fall into this category include construction of 16S rDNA library (followed by DNA sequencing) and PCR-Denaturing Gradient Gel Electrophoresis (DGGE). The former requires numerous steps in cloning, and the subsequent sampling for sequencing is often constrained in coverage and unsuitable for monitoring over a dynamic time course (Dahllöf, 2002). The latter is a genetic fingerprinting technique introduced into microbial ecology by (Muyzer, de Waal, & Uitterlinden, 1993) and is based on the separation of PCR amplicons of 16S rDNA of the same size but different sequences on an electrophoresis gel containing DNA denaturing agent. It can profile the genetic diversity of a microbial community from a specific environment and allows simultaneous analysis of multiple samples. DGGE is thus a useful method for monitoring shifts in community structure over time for diverse purposes. For instance, it has been used to evaluate microbial diversity during food fermentation

(Ercolini, 2004), and to monitor community shifts in a polycyclic aromatic hydrocarbon (PAH) polluted soil (Jousset, Lara, Nikolausz, Harms, & Chatzinotas, 2010).

The PCR-based methods require extraction of total DNA from the microbial community beforehand, and suffer from the drawback that the quantitative data may be subject to biases from DNA extraction and amplification. In recent years, the development of next-generation sequencing techniques such as pyrosequencing has somewhat removed this bias due to amplification (Desai, Pathak, & Madamwar, 2010). And the state of art of bioinformatics tools such as metagenomics and metatranscriptomics technologies were started to be applied for microbial communities' analysis in recent years. (Bikel et al., 2015; G. Zhang et al., 2016) Other than identifying the abundance and diversity of microbial community in a large scale with great sampling depth, the metagenomic can additionally identify and analyze the gene content and predict functional potential of proteins encoded in the genomes of the microbial community. As for metatranscriptomic analysis, it allows the identification of expressed transcripts in the microbiome. The transcript numbers can also be used to compare the gene expression profiles between microbial communities. (David et al., 2014; Franzosa et al., 2014) However, it is relatively expensive (particularly if analysis over multiple time points is desirable) and generates large volume of data that often pose challenges in standardization and interpretation to bring about significant conclusions (Yilmaz et al., 2011). The scale of such analysis will itself constitute an extensive project.

In this study, the technique of DGGE would be applied first due to its universality and suitability for initial survey and profiling across time. The limitations often associated with the DGGE process, e.g. microheterogeneity of 16S rDNA leading to multiple bands (Muyzer & Smalla, 1998) and overestimation or underestimation of diversity (Casamayor, Pedros-Alio, Muyzer, & Amann, 2002) would be checked during application and moderated accordingly. This would also give us the means of considering other higher-resolution community analytical methods such as pyrosequencing and development of more useful techniques for analysis across time in the future.

1.5.3 Objectives

In summary, the objectives of this study are:

1. To develop two types of bioaugmentative co-cultures for different bioremediation applications using approaches deemed appropriate for each application.
2. To assess the efficacy of bioaugmentative co-cultures and validate them in application.
3. To preliminarily analyze some of the bacterial interactions and changes in community structure relevant to the bioaugmentative co-cultures in order to make recommendation for future studies that could refine these approaches to bioaugmentation.

Through these approaches for forming and analyzing the bioaugmentation consortia, bacterial interplay or community dynamics and their influence on bioaugmentation efficiency and sustainability, we hope to gain more insight about effective formulation of bioaugmentative co-culture and identify the limitations in current methodology to address the complexity involved.

Chapter 2. Co-culture Bioaugmentation for Biofiltration of Volatile Organic Compounds

2.1 Introduction

2.1.1 Volatile Organic Compounds

Volatile Organic Compounds (VOCs) refer to a group of organic compounds which are characterized by high vapour pressure, and thus will vaporize readily into the atmosphere (Kennes & Veiga, 2013). Included in this category of compounds are, for example, low molecular weight solvents such as acetone, methanol and formaldehyde, aliphatic hydrocarbons such as hexane and octane, and aromatic hydrocarbons such as benzene, pyrene and toluene. VOCs can be released both by natural physical activities (e.g. volcanic eruption and fires) and anthropogenic activities (e.g. manufacturing of paints, adhesives, gasoline, plastics and many other products) (Toscano et al., 2011).

In general, VOCs' concentrations are higher indoors than outdoors because of the closed nature and restricted volumes characteristic of indoor spaces. Not surprisingly, indoor micro-environment is the largest contributor to the total daily VOC inhalation exposure in urban populations (Adgate et al., 2004; De Bruin et al., 2008), since most urban dwellers spend on average around 90% of their time indoors (Klepeis et al., 2001; Schweizer et al., 2007). With the commonly encountered level of exposure to VOCs nowadays, health hazards are greatly increased. For instance, total cancer risk is found to be associated with exposure to VOCs in addition to other air pollutants such as carbon monoxide, sulfur dioxide, and particulate matters (PM 2.5 and PM 10) (Kampa & Castanas, 2008). The potential health risks associated with VOC exposure have provoked both concerns toward environmental pollution of the air by VOCs, as well as interest in advancing removal technologies for this class of pollutants.

There are several physico-chemical methods which can bring about removal of VOCs, such as condensation (Buonicore, 1992), photo-Fenton oxidation technology (Punzi, Mattiasson, & Jonstrup, 2012) and activated carbon adsorptions (Wang, Hines, & Farrier, 1979). The shortcoming of such methods is high cost and low efficiency and efficacy when

dealing with large volumes of contaminated sample with low concentration of pollutants (Zhou, 2011). In contrast, biological treatment methods are more effective in eliminating low to moderate VOC concentrations (1 to 1000 ppm), which is relevant for most industrial and indoor residential exposures (Kim, 2004). These methods primarily involve the use of microorganisms to degrade pollutants present in the air stream into carbon dioxide, water, inorganic compounds, and biomass. The configuration of the removal set-up can take various forms, resulting in systems such as bioscrubbers, biofilters and biotrickling filters (Shareefdeen & Singh, 2005). Hence biological removal of VOCs is also commonly referred to as biofiltration. Removal of VOCs may be performed at the industrial level, e.g. from effluent air of manufacturing plants, or at the domestic level, e.g. as part of the functions of indoor air-purification device.

2.1.2 Domestic Air-Purification Device

Many types of indoor air-purification products exist in the market, with various designs for domestic or office use and adopting different approaches for removing major classes of pollutants from the air. The most common ones are those making use of surface emission trap technology or photocatalytic oxidation, while the ones exploiting biological activities are less frequently marketed (Piazza, Lee, & Hayes, 2006).

Air-purification devices making use of surface emission trap technology adapt filter-type components, e.g. adsorbent sheets or cartridges, into the designs for the cleaning of polluted air. The application of adsorbent components directly at the source of the emissions can result in an immediate stop or reduction of the emitting pollutants and this is the main advantage of the technology. However, the types of pollutants that can be removed depend on the adsorbent material. For VOC removal, activated carbon will be required in the filtration component, but the efficacy of the device will drop as surfaces of the activated carbon filters rapidly reach saturation with adsorbed VOCs. (Markowicz & Larsson, 2012).

Devices adopting photocatalytic oxidation technology, on the other hand, get rid of VOCs through the production of hydroxyl (OH) radicals when specific material surfaces are irradiated by ultraviolet (UV) light. The strong oxidative power of OH radicals is

responsible for the conversion of VOCs to less harmful compounds. The designs of existing devices in the market have therefore incorporated components that carry photocatalyst-coated materials and light sources that produce the UV spectrum of light. (Destailats et al., 2012).

Although devices incorporating physico-chemical methods such as adsorption and photo-oxidation are available for removal of VOCs in domestic settings, as mentioned in section 2.1.1, these methods are actually not efficient at handling VOCs that involve steady exposure to low to moderate concentration of pollutants (Zerbonia, Spivey, Agarwal, Damle, & Sanford, 1995), i.e. under most residential contexts. From this perspective, if VOCs are the main target pollutants, air-purification devices exploiting biological processes are more desirable than the available selection of products. However, such devices are uncommon and the concept is only beginning to gain popularity in recent years due to the subpopulation of “green” consumers with a preference for products perceived to be more environmentally friendly. The simplest types of such devices incorporate pollutant-degrading enzymes into water, through which air is passed through. Utilization of microorganisms directly, as often done in biofiltration reactors under industrial settings, is rarely adopted in devices for residential use.

Since 2010, Company X (the identity is not revealed due to non-disclosure agreement) has marketed a biologically driven air-purification device that includes a component carrying microbial culture (refer to section 2.3.3.1). The formulation of microbial culture was able to remove a test VOC – formaldehyde – up to 60-70%. To the best of our knowledge, this was the only air-purification device in the market that utilizes microorganisms directly to tackle VOC removal. At the start of this project, our laboratory was commissioned by Company X to develop a formulation of microorganisms that could enhance the VOC removal capability beyond what the original formulation is able to. We therefore chose to explore one of the bioaugmentation strategies on this application.

2.1.3 Volatile Organic Compounds Relevant to This Study

Company X chose to use formaldehyde as the test VOC in their initial assessment of the air-purification device because this is a common standard used by other manufacturers. However, it is also interested to determine and enhance the range of VOCs the device is able to handle beyond formaldehyde, such as aliphatic or aromatic hydrocarbons. As the VOC examined for biological removal in our laboratory's past studies was hexane (refer to section 2.1.4) which falls into one of the categories, we proposed to target this compound first, in parallel with assessment of formaldehyde removal. Toluene and benzene were peripherally included as they are also common VOCs that are checked when indoor air quality is monitored by regulatory authorities (Adgate et al., 2004). The average concentrations indoor and health effects of these four VOCs are listed in Table 2.1.

The VOCs that are relevant to this study are therefore: hexane, formaldehyde, toluene and benzene. Hexane is a linear 6-carbon hydrocarbon molecule which is often present in glues used in parts of buildings, furniture and electronic products. Thus low level emissions of hexane can be expected to occur from such sources within indoor environments. Likewise, formaldehyde can serve as alternative solvent base in these classes of materials and contribute to indoor emissions of VOCs. Unlike hexane which is highly hydrophobic, formaldehyde is hydrophilic and readily dissolves into water. Toluene and benzene are used as solvents in synthetic fragrances, cosmetics, plastic materials, inks, and cleaning agents. They are not linear hydrocarbons (as hexane is), but are aromatic, i.e. having a benzene ring as part of their structures. Hence the VOCs assessed in this study are distributed across a few chemical groups with varying characteristics (Shin & Jo, 2012).

Table 2.1 Average indoor concentration and their health effects of the four VOCs from literature.

Compound	Indoor concentration (ppb) Average	Health effects	References
Hexane	1.25	Damage to the central nervous system	(Shin & Jo, 2012)
	16.3		(Weisel et al., 2005)
Formaldehyde	26.9	Respiratory disorders, irritation of the eyes	(Edwards, Jurvelin, Koistinen, Saarela, & Jantunen, 2001)
Toluene	2.69	Neurological effect	(Weisel et al., 2005)
Benzene	0.87	Immunological disorders, leukemia, neurological effect	(Weisel et al., 2005) (Edwards et al., 2001)

As in Singapore particularly, indoor hexane, benzene, and toluene levels are significantly higher than in Europe according to one study that compared the VOCs concentration in office building between Singapore and Europe (Zuraimi et al., 2006). As stated in this paper, the concentration range of three target compounds of our study in Singapore are as follows: 4.4–114.5 ($\mu\text{g m}^{-3}$) for hexane, 32.4–167.5 ($\mu\text{g m}^{-3}$) for benzene and 112.9–1641.8 ($\mu\text{g m}^{-3}$) for toluene. Compare with the average concentration provided in Table 2.1 (with concentration converted to $\mu\text{g m}^{-3}$), 4.4 ($\mu\text{g m}^{-3}$) for hexane, 2.9 ($\mu\text{g m}^{-3}$) for benzene and 10.1 ($\mu\text{g m}^{-3}$) for toluene, they are all lower than the concentration ranges found in Singapore. Therefore, there surely are demands for indoor air treatment in Singapore.

Although we didn't find any references stating that the formaldehyde concentration in Singapore are specifically higher than standard, formaldehyde exposure in most newly renovated buildings can do harm to human health, since they are abundantly released by new furniture, glue and painting. (Lazenby, Hinwood, Callan, & Franklin, 2012) Therefore, as long as there are renovation work taking place in Singapore, there would be a need for formaldehyde removal for better indoor air quality. (Liu, Mason, Guo, Krebs, & Roache,

2015)

2.1.4 Two Phase Partitioning Bioreactors for Removal of Hexane

Our laboratory has undertaken a project in the past involving bioremediation of hexane. The source microbial community used to initiate the project was sampled from a patch of petroleum-contaminated soil in a car park in Singapore (Ng, 2014). Since petroleum and machine oils associated with cars are expected to carry some of the other target VOCs for our current application, we considered this community to be an appropriate source to formulate the bioaugmentative co-cultures necessary for the air-purification device.

In the hexane bioremediation project, the petroleum-contaminated soil sample was first subjected to acclimatization in mineral salt medium (MSM) with hexane as the sole carbon source and then transferred for longer term maintenance in a two phase partitioning bioreactor (TPPB). This consists of an aqueous phase with nutrients (MSM in this case) within which microorganisms reside, and a water-immiscible non-aqueous liquid phase (silicone oil in this case) which more readily solubilize the hydrophobic target pollutant (hexane in this case). This biphasic design of TPPB increased bioavailability of the VOCs compared to the monophasic aqueous counterpart (Muñoz, Arriaga, Hernández, Guieysse, & Revah, 2006). In our hands, the TPPB allowed an accumulation of biomass that was two orders of magnitude higher than in the monophasic control. The TPPB was operated with a daily feed of hexane for over a year, and it was found that hexane removal efficiency increased gradually up to week 24 and thereafter maintained a steady high level, up to and beyond the full one year. The community within the TPPB was analyzed with a combination of culture-dependent (isolation on solid media) and culture-independent (DGGE and 16S rDNA clone library) methods (Ng, 2014).

Unlike most of the complex microbial community analyzed from environments, a tally of the molecularly-determined composition of the TPPB community against the identities of its culturable subpopulation revealed that the majority of the members are culturable with respect to the cultivation conditions applied in our studies. They could either be isolated in rich media, or minimal media with hexane (as carbon source). Figure 2.1 shows the data

from such a combination of analysis, for the period of weeks 32-40 when the TPPB had reached and maintained the high hexane removal efficacy. A total of 23 genera identified are listed in the phylogenetic tree where 9 genera (boxed in magenta) had multiple species isolated and 6 genera (boxed in yellow) had only one species identified in culture. The remaining 8 genera found (boxed in green) had no culturable isolates sampled from the TPPB (Ng, 2014).

The fact that the majority of the TPPB community members identified was culturable provided an advantage in terms of formulating bioaugmentative co-cultures. Each strain could be cultured independently and then systematically mixed for co-culture after sufficient biomass has accumulated. Our bioaugmentation strategy for the application to the air-purification device was therefore aligned to this observation, and exploits the strains isolated during this week 32-40 period when the community has clearly reached a balance in terms of optimal function (maintenance of high hexane removal efficiency).

Figure 2.1 Phylogenetic tree of the 16S rDNA sequences of the strains present in the TPPB at Week 32-40 which exhibited steady removal of hexane. The closest type species identified are presented (left) and the relative abundance indicated (right). Strains with the closest identity to the type species boxed in magenta (multiple species found) and yellow (only one species found) are culturable, while those boxed in green are identified through 16S rDNA clone library and no culturable counterpart was isolated.

2.2 Materials and Methods

2.2.1 Strain Cultivation

2.2.1.1 MSM Medium and MSM-Glucose Medium

The MSM used in this experiment was adapted from (Muñoz et al., 2006). This MSM contains the following (g/l): KH_2PO_4 , 1; K_2HPO_4 , 2; NH_4Cl , 0.75; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; CaCl_2 , 0.018; and 1 ml/l of a trace element solution containing (g/l unless otherwise stated): 6.76 ml/l of concentrated HCl , $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 1.5; H_3BO_3 , 0.06; $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.12; ZnCl_2 , 0.07; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.025; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.015; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$,

0.025; EDTA, 5.2. The final pH of the medium was 7. MSM agar plates were made of 15 g Bacto Agar (BD Difco) in every litre of MSM medium.

MSM-glucose medium contains 1 g of glucose dissolved in every litre of MSM media. MSM-glucose agar plates were made of 15 g Bacto Agar (BD Difco) in every litre of MSM-glucose medium.

2.2.1.2 LB Medium

The LB medium used in this experiment contains 10 g/l Bacto-tryptone, 5 g/l yeast extract and 10 g/l NaCl. The final pH was adjusted to 7.5 with NaOH. LB agar plates were made of 15 g of Agar (BD Difco) in every litre of LB medium.

2.2.1.3 Single Strain Substrate Testing

2.2.1.3.1 On Agar Plates

The 26 strains from the 12 genera were streaked from permanent vials which are kept in storage at -80°C on MSM, MSM-glucose and LB agar plates. MSM-glucose and LB plates were incubated at 37°C in a standing incubator (MRC), and MSM plates were incubated at room temperature in a desiccator (inner diameter 210 mm), perfused with hexane by injecting 1 ml of hexane onto 5 g of activated carbon placed within the dessicator. Observation was taken after 3 days of incubation.

2.2.1.3.2 In Liquid Culture

In liquid, the 26 strains were inoculated in MSM, MSM-glucose and LB medium from the same permanent vials. For MSM cultivation, these isolates were grown in 12 ml glass culture tube (16×125 mm, HACH) containing 4 ml MSM medium and 400 µl silicone oil (poly(dimethylsiloxane) 200® fluid viscosity 20cSt, Sigma Aldrich), supplemented with 8 µl of hexane (hereafter referred to as “MSM-silicone oil-hexane” cultivation system). For MSM-glucose, these isolates were grown in the same glass tube with 4 ml MSM-glucose medium and 400 µl silicone oil. For LB cultivation, isolates were grown in those tubes

containing 4 ml of LB medium and 400 µl silicone oil. Glass culture tubes were incubated at 37°C with shaking at 250 rpm speed. Their Nephelometric Turbidity Unit (NTU) were monitored using a Turbidimeter (HACH 2100AN) on a daily basis for 7 days.

2.2.2 2,6-Dichlorophenolindophenol (DCPIP) test

2.2.2.1 Cell Suspensions Preparation

In making cell suspensions for DCPIP experiment, colonies were picked from respective streaked plates (preferred medium) using sterile inoculation loops and inoculated in MSM. Washing of cells was performed three times (suspending of cells in MSM, centrifuging at 4500 rpm for 10 minutes, removal of supernatant, resuspending of cells with fresh MSM) for colonies picked from streaked LB agar plates.

Optical density (OD) of stock cell suspensions was taken using Shimadzu UV Spectrophotometer UV-1800 at wavelength of 600 nm (hereafter referred to as OD₆₀₀). Stock cell suspensions were then diluted using fresh MSM to OD₆₀₀ = 1.

2.2.2.2 Hexane and Glucose as Carbon Source

In this experiment, each strain was tested with both hexane and glucose as their sole carbon source respectively and a negative control with no carbon source. The following chemicals and reagents were used: 99% hexane (Merck); DCPIP (MP Biomedicals, LLP, France), 150 µM. Non *E.coli* Strain (NES) cell suspensions of OD₆₀₀=0.1 were used.

Negative control set contains 3.6 ml of MSM, 400 µl of DCPIP (1500 µM), 400 µl of silicon oil, and 8 µl of hexane. Hexane set contains 3.2 ml of MSM, 400 µl of DCPIP (1500 µM), 400 µl of silicon oil, 8 µl of hexane and 400 µl of respective cell suspension at OD₆₀₀ = 1. MSM-glucose set contains 3.2 ml of MSM-glucose 0.125% media, 400 µl of DCPIP (1500 µM), 400 µl of silicon oil, and 400 µl of respective cell suspension at OD₆₀₀ = 1.

2.2.2.3 Incubation and Sample Taking

After inoculation, tubes were incubated in shaking incubator (Certomat® IS Sartorius Stadim Biotech) at 30 °C and at a shaking speed of 170 rpm. All tubes were allowed 30 minutes in the shaking incubator prior to the measurement of 1st sample (designated at time = 0 hours). A 96-well plate (Greiner®) was used to take 200 µl samples from each tube. Infinite® 200 Pro (Tecan) was used with for measurement of the absorbance of the samples at 600 nm.

2.2.3 *alkB*, CYP 153 and FDH Gene Test

PCR were performed in a final volume of 50 µl PCR reaction mixture containing 5 µM each of the forward and reverse primers (Table 2.2), 200 µM 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, Röthlisberger, Witholt, & van Beilen, 1999). 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 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 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™ 100 bp DNA Ladder Plus (Fermentas) was used. The gels were stained by ethidium and photographed using filtered UV illumination by Chemi-genuis Bio imaging system (Syngene).

It is considered as positive results when bands are visible to the naked eye under the UV illumination and negative results when there was no band visible to the naked eye in the same condition.

Table 2.2 *alkB* and CYP153 primers sets used in this experiment, their melting temperature, target size and their reference. (The CYP 153 PCR was conduct by Ng (2014)).

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			

A list of GD-FDH protein sequences was downloaded from GenBank in FASTA format, including *Pseudovibrio* sp. FO-BEG1, *Nodularia spumigena* CCY9414, *Alphaproteobacterium* BAL199, Rhodobacterales *bacterium* HTCC2255, *Loktanella vestfoldensis* SKA53, *Roseobacter* sp. MED193, *Sulfitobacter* sp. NAS-14.1, and *Mesorhizobium loti* MAFF303099. These protein sequence were aligned using “clustal W”, and the degenerate primer was designed from conserved sequences. The forward and reserve primer were designed as 5'-CNACNGGNRTNGGNGCNGTN-3' and 5'-GNYTNGGNGSNATHGGNYTN-3' respectively.

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 95°C for 30 seconds, annealing at 59 °C for 1 minute and elongation at 72 °C for 1 minute. The final extension step was set at 72 °C for 10 minutes.

2.2.4 Cell Surface Hydrophobicity and Emulsification Activity Test

2.2.4.1 Cell Surface Hydrophobicity

This test is modified from (Pembrey, Marshall, & Schneider, 1999), bacterial cultures from the monophasic (which means to have one single phase of liquid, in this case, it's aqueous phase) and the biphasic systems (means to have two liquid phases, in this case, the culture contains both aqueous phase MSM medium and oil phase) 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: $[(OD_{\text{before}} - OD_{\text{after}}) / OD_{\text{before}}] \times 100$.

2.2.4.2 Emulsification Activity Test

The protocol to determine emulsifier activity was adapted from (Soares, Guieysse, Delgado, & Mattiasson, 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.

2.2.5 Growth on Formaldehyde, Toluene and Benzene as Sole Carbon Source

For formaldehyde, toluene and benzene the substrate test was done on the plate to get a quick screening of the 26 strains before the actual removal test on the device. The amount of VOCs used for infusion in desiccators was as follow: 1000 µl of formaldehyde; 500 µl of benzene; and 500 µl of toluene. The decision to add a lesser amount of benzene (as compared to hexane) was based on the higher toxicity levels of benzene. This toxicity has been shown in the (Horikoshi, Antranikian, Bull, Robb, & Stetter, 2011).

After incubation for 7 days, the Petri dishes were set on a black background, light from a localized source is projected to the Petri dish for observation. If the colonies are visible to the naked eye, then it considered as positive growth in Table 2.8; if there were no visible colony to the naked eye, then it's considered as negative growth.

2.2.6 Formulation of Bioaugmentative Co-cultures through Mixing of Selected Strains

2.2.6.1 Inoculation and Operating conditions of TPPB for Cultivation of Bioaugmentative Co-cultures

The 26 strains were first inoculated individually in 4 ml “MSM-silicone oil-hexane” system for liquid cultivation. On day 1, all strains were streaked and incubated on LB or MSM agar according to their growth preference as shown in Table 2.5. On day 4, the indicated strains (refer Appendix 1) making up a co-culture was picked and inoculated together in the 4ml MSM-silicone oil-hexane cultivation system to generate the co-culture in glass culture tubes. The co-cultures were incubated at 37 °C with shaking at 250 rpm speed. On the same day, various hexane degraders identified through the characterization round of study (refer section 2.3.1) were streaked on MSM plate and incubated with hexane perfusion. On day 7, hexane degraders were inoculated into the non-hexane degrading co-cultures which were being cultivated since day 4 and further cultivated at 37 °C with shaking at 250 rpm speed.

On day 12, TPPBs were prepared by inoculating the co-culture (made on day 7) into the double sidearm (Celstir) bioreactors, and topping up to the volume of 450 ml MSM and 50 ml silicon oil. Hexane was added at a volumetric load of 100 g/m³/d, which was equivalent to 76 µl for this TPPB set up. Hexane was added twice a day (38 µl each time) within the first week and daily afterwards. The bioreactors were incubated at room temperature (23 to 25 °C) and mechanically stirred at 150 rpm.

2.2.6.2 Sampling and Maintenance of Co-Culture in TPPB

Co-culture sampling from TPPB was scheduled as follows (after the inoculation on day 12): on days 13, 15, 17, 19, 21, 26, 27, 28, 29, 30, 33, 34, 35, 36, 37, 40, 43, 47, 50, 54, 57, 61, 64. In the sampling step, aqueous and organic samples of 20 ml and 5 ml respectively, were removed, and then analyzed immediately or stored at -20 °C for further analysis. Equivalent volume of fresh MSM and silicone oil were added back into the bioreactors after sampling.

The pH of the four bioreactors was monitored using a pH indicator strip ranging from pH 0 to 14 (Merck). When the pH of the bioreactor fell below 5, the old MSM was replaced with fresh MSM. 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 re-suspended in 200 ml of MSM and re-introduced into the bioreactors. This ensured that the cells were not removed during the media change process.

2.2.7 Chamber Air Testing

2.2.7.1 The Testing Chamber

The structure and mechanical design of the air-purification device merchandized by Company X is introduced in section 2.3.3.1. Henceforth, this device will be referred to simply as “Air-purifier A” for reason of confidentiality. The VOC removal efficiency of the Air-purifier A was tested within an enclosed chamber which consists of an open-top cubic container (chamber) with a pair of rubber gloves mounted through and sealed to two holes on its side wall. The container was made of plastic (mainly polypropylene) and its total volume was approximately 1m³. A closed system was created using this container by covering the top with a large glass plate and sealing the sides with plasticine clay and masking tape.

2.2.7.2 Basic Procedure of the Chamber Air Testing

Within the chamber system for testing VOC removal efficiency of Air-purifier A, the following items were placed: a small scale electric fan, a Petri dish, a 200 µl micropipette (with a tip attached), a photo-ionization detector (PID) (RAE system, San Jose, USA), and the Air-purifier A.

Manipulation of the components within the closed chamber was then carried out through the gloves. The small scale electric fan was intended to promote air circulation inside the container and the PID was for measurement of the VOC level within the chamber. The Petri dish was used to contain the liquid VOC from which vaporization was allowed to occur, aided by the fan’s circulation of the chamber air. When the vaporization has reached the maximum level, the Petri dish lid with plasticine sealant would be put on to prevent further release of the VOC to the chamber air while changes in the VOC level were being measured.

Figure 2.2 Illustration of the testing chamber layout: volume of the testing chamber (upper) and layout of the testing chamber (lower).

2.2.7.3 Testing of VOCs Level to Acquire Internal Control

The internal control experiments were meant to establish the basal VOC level resulting from possible emission of components in the chamber i.e. electric fan, Petri dish, gloves and PID. All of those components were placed in the testing chamber, then the chamber was sealed, and VOCs reading were recorded by PID over 120 minutes at the interval of 3 seconds.

2.2.7.4 Profiling of Vaporization and Saturation of the 4 VOCs

To obtain the saturation concentration of the 4 VOCs and their natural reduction profile, the electric fan, Petri dish, 200 µl pipette, a sealed bottle of each VOC and PID were positioned in the testing chamber. After sealing, the PID was turned on and 200 µl of

respective VOC was dispensed into the Petri dish. The electric fan was turned on to facilitate VOCs' vaporization, which was monitored by the PID. After saturation was reached, the electric fan was turned off, and the Petri dish was sealed with its lid to prevent further evaporation. The PID were then allowed to continue to record the VOC level for the following 100 minutes.

To check the proportion of VOCs removed by component C in Air-purifier A, the testing system was set up with electric fan, Petri dish, 200 μ l pipette, PID and Air-purifier A without bacteria infused, sealed inside the chamber. Air-purifier A without bacteria was prepared as described: 300 g porous matrix B (autoclaved and dried) was soaked in 300 ml sterile ddH₂O and then aliquoted into 4 cartridges for installation in Air-purifier A and component C was fitted in each cartridge. The Air-purifier A was placed in a zip-lock bag and a vacuum pump was used to removal the air inside the zip-lock bag to prevent VOC dilution when the bag was open (which would result in releasing "external" air). Instead of dispensing 200 μ l of VOCs, 50ul was dispensed, and Air-purifier A was switched on and run for more than 60 minutes, and the VOCs' concentration were recorded at 3 second interval.

The percentage of formaldehyde reduction or removal was calculated by the following equation: percentage reduction = (Starting concentration- Current concentration)/Starting concentration $\times$ 100%.

2.2.7.5 Testing of VOCs Removal by Bioaugmentative Cultures

To check the proportion of formaldehyde removed by Air-purifier A with bioaugmentative cultures integrated, the testing system was set up with electric fan, Petri dish, 200 μ l pipette, PID and Air-purifier A with bacteria infused in its matrix B, positioned inside the chamber. Air-purifier A with bacteria was prepared as described: bacteria co-culture was diluted to cell density of 3×10^5 with sterile ddH₂O in a volume of 300 ml, 300 g porous matrix B (autoclaved and dried) was soaked in diluted co-culture and then was kept at 4 °C overnight. On the following day (testing day) the pre-prepared porous matrix B were

aliquoted into 4 cartridges for installation in Air-purifier A and component C was fitted in each cartridge. The Air-purifier A was placed in a zip-lock bag and a vacuum pump was used to remove the air inside the zip-lock bag to prevent VOC dilution when the bag was open (which would result in releasing “external” air). 50 µl of VOCs were dispensed, and Air-purifier A was switched on and run for more than 60 minutes, and the VOCs’ concentration was recorded at 3 second interval. Duplicate experiments were carried out for every co-culture sample.

2.2.8 Colorimetric Liquid Testing of Formaldehyde Removal

2.2.8.1 Colorimetric Liquid Testing on Liquid Samples

Formaldehyde concentration in the culture supernatant was determined using a colorimetric reaction with FeCl_3 and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) as reported previously (Yamazaki, Tsugawa, & Sode, 2001). To 200 µl of sample containing up to 10 ppm of formaldehyde, 40 µl of 0.4% MBTH in 2 N HCl was added. After 20 min of incubation at room temperature, 10 µl of 1% (w/v) FeCl_3 was added. After 10 min of incubation at room temperature, 250 µl of acetone was added. The final solution was incubated for 20 min at room temperature for the color to stabilize. The absorbance at 670 nm was measured using a spectrophotometer (Tecan).

2.2.8.2 Colorimetric Optimization Experiment

Formaldehyde with different concentration in MSM media was prepared. One set of the concentration series was mixed with 1:1 volume of MSM media, and another mixed with 1:1 volume of NES 140 culture. The final volume of each sample is 200 µl. The final formaldehyde concentration of each sample includes: 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm. These were incubated for different period of time, including 0 min, 30 min, 60 min and 120 min. After incubation, triplicate samples were subject to colorimetric measurement as described in section 2.2.8.1.

2.2.8.3 Quantification of Formaldehyde Removal under Colorimetric Liquid Setting for Comparison with Chamber Air Setting

In addition to the components set up in the chamber testing (section 2.2.7.2), one more item was included into the chamber – a beaker of water to capture the soluble formaldehyde concentration from the air within the chamber. The testing of formaldehyde removal within the chamber was carried out as described in section 2.2.7.2. Instead of measuring the formaldehyde gas level using PID, the liquids accumulated within the porous matrix B cartridge (either water for control, or the bioaugmentative co-cultures tested) and the beaker of water were collected and assayed using the colorimetric method as described in section 2.2.8.1.

2.2.9 Drop Plate Experiment for Interaction Survey

In this experiment, each strain was first streaked on agar plates of their preferred media for 3 days as describe in section 2.2.1.3.1 to accumulate biomass. Cell suspensions were later made of these colonies. The strain chosen to serve as the background lawn ($OD_{600} = 0.25$) was spread on MSM agar plates to form a lawn, while 10 μ l cell suspensions of the other 11 strains ($OD_{600} = 0.25$) were dropped in triplicates onto that lawn. One set of plates without the lawn but with the individual strains dropped on to the surface in triplicates similarly were prepared as a control set. These two sets of plates were then incubated in desiccator infused with 1 ml hexane. Plate pictures were taken using Bio-Rad Molecular Imager® Gel Doc™ XR+ Imaging System with the software Quantity One version 4.6.9 (Bio-Rad 1D Analysis Software, The Discovery Series™, USA). Observation of the lawns and the drops was taken after 7 days of incubation.

2.2.10 DGGE

2.2.10.1 Cell Lysis

Cell pellets were first collected by centrifuging 4 ml of cultures at 12,000 g for 8 minutes and the supernatant carefully and completely aspirated. 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. After that, 3.6 mg of lysozyme (Sigma-Aldrich) was added and the mixture was incubated at 37 °C for one hour to hydrolyze 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.

2.2.10.2 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 µl 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 µl 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.

2.2.10.3 Primer

Table 2.3 Primers that were used to target the bacterial 16S rDNA V3 region for DGGE.

Primer	Sequence (5'→3')	T _a (°C)	Size of product (bp)	Reference
V3f- GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	55- 65	233	(Muyzer et al., 1993)
V3r	ATT ACC GCG GCR GCT GG			

2.2.10.4 Conditions for PCR

The PCR condition for 16S rDNA V3 region of the bacterial communities for DGGE was modified from (Muyzer et al., 1993). The programme consisted of an initial cycle of denaturation of the genomic DNA at 94 °C for 3 minutes. This is then followed by 20 cycles of touchdown (denaturation at 94 °C for 1 minute, annealing starting at 65 °C, with reduction of 0.5 °C per cycle down to 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.

2.2.10.5 Gel Electrophoresis and visualization

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. The PCR amplicons of size 233 bp were checked by electrophoresis 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 then separated in an 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).

2.3 Results and Discussion

2.3.1 Selection of Strains from TPPB Source Community

As described in the earlier Introduction section, the hexane biofiltration TPPBs which our laboratory had previously maintained for more than a year was able to sustain a high rate of hexane removal from week 24 onwards. From the analysis of community structure of week 32-40 (Figure 2.1), out of the 23 genera identified by molecular means, 15 genera were found to be culturable (Ng, 2014). For the remaining 8 genera, no culturable isolates were sampled from the TPPB during the course of the previous study.

One school of thoughts regarding bioaugmentation strategy advocates that microbial strains suitable for bioaugmentation ought to be selected from a pool of strains which are known to survive together for a long time in a specific target habitat, because they are more likely to bring about continuous degradation under similar context (Thompson et al., 2005). Since the TPPB community comprising of these groups of bacteria have been shown to be compatible to each other and sustainable with respect to hexane biofiltration (Ng, 2014), it

fitted the bill as the “source” pool for related bioaugmentation. We may infer that: (a) there is sufficient metabolic redundancy within this community to contribute to robustness of hexane degradation, (b) there is sufficient diversity to allow differentiation of roles and/or cooperation and (c) the relationship between such complementary groups has equilibrated to a stage that can withstand some environmental perturbation. For the purpose of formulating a bioaugmentative co-culture, we needed to strike the balance between retaining a level of complexity that embraces the above, and yet reducing to a composition that is manageable from the operational point of view. Furthermore, the suitability of the bioaugmentative culture to the intended application, i.e. as a component of Air-purifier A (refer 2.3.3.1) designed and marketed by Company X, has to be kept in mind. Hence we needed to select strains which are representative and yet practicable to use from the TPPB pool. Although the subpopulation of non-culturable bacterial groups no doubt also contributes to the complexity and possibly stability of the TPPB community, we focused on the culturable component for this reason of practicality.

As a start, we considered the fact that Air-purifier A is intended for domestic and office use. Therefore, out of the 15 genera of culturable strains, *Mycobacterium*, *Burkholderia* and *Gordonia* were excluded from our choice due to safety consideration. Based on the search in Risk Group (RG) Classification in Infectious Agents Database under American Biological Safety Association ("Infectious Agents Database under American Biological Safety Association," 2015) (<http://www.absa.org/index.html>), many strains in the above mentioned three genera belong to the category of RG 2, which means they are associated with human disease of mild to moderate severity. Although preventative or therapeutic interventions are available for the RG 2 group of microorganisms, considering the risk of exposure of household air-purification devices to the general population, strains belonging to RG 2 and above were deemed unsuitable to be included in the bioaugmentative co-culture.

The strains being considered after this basis of elimination are presented in Table 2.4. Of these, 6 genera (*Achromobacter*, *Chryseobacterium*, *Variovorax*, *Microbacterium*, *Rhodococcus* and *Sphingomonas*) had more than one species identified from the TPPB

community, while from each of the remaining genera, only one species was identified. One logical way of formulating a “core” version of the community to be used as bioaugmentative co-culture is to pick a representative strain from each genus, provided the strain is able to fulfill its role in the “core” function. It may also be possible to reduce the number of genera if the functional redundancy level is very high within the TPPB community. A series of tests that may be relevant for the target application (VOC removal in air-purification device) were therefore run to characterize these strains.

Table 2.4 The 26 strains being evaluated for suitability in bioaugmentation of VOC removal in Air Purifier A: their family, genus, species and strain designation used in this thesis.

Family	Genus	Species	Strain designation
Alcaligenaceae	<i>Achromobacter</i>	<i>xylosoxidans</i>	NES 28
		<i>insolitus</i>	NES 61
		<i>spanius</i>	NES 146
Burkholderiaceae	<i>Cupriavidus</i>	<i>oxalaticus</i>	NES 57
Caulobacteraceae	<i>Caulobacter</i>	<i>segnis</i>	NES 43
Comamonadaceae	<i>Variovorax</i>	<i>paradoxus</i>	NES 42
		<i>boronicumulans</i>	NES 89
Flavobacteriaceae	<i>Chryseobacterium</i>	<i>indolgenes</i>	NES 27
		<i>formosense</i>	NES 32
		<i>pallidum</i>	NES 140
		<i>hominis</i>	NES 141
		<i>gleum</i>	NES 144
Flavobacteriaceae	<i>Flavobacterium</i>	<i>lindanitolerans</i>	NES 36
Microbacteriaceae	<i>Microbacterium</i>	<i>laevaniformans</i>	NES 29
		<i>aoyamense</i>	NES 35
		<i>paraoxydans</i>	NES 56
		<i>maritypicum</i>	NES 60
Nocardiaceae	<i>Rhodococcus</i>	<i>aetherivorans</i>	NES 30
		<i>phenolicus</i>	NES 54
		<i>ruber</i>	NES 73
		<i>wratislaviensis</i>	NES 99
Sphingobacteriaceae	<i>Pedobacter</i>	<i>koreensis</i>	NES 75
	<i>Mucilaginibacter</i>	<i>ximonensis</i>	NES 151
Sphingomonadaceae	<i>Sphingomonas</i>	<i>yabuuchiae</i>	NES 59
		<i>echinoides</i>	NES 152
Xanthomonadaceae	<i>Nevskia</i>	<i>romosa</i>	NES 97

2.3.1.1 Growth on Rich and Minimal Media

In the application of bioaugmentation, one of the most basic requirement is that there are reliable means to increase the biomass of the strains to quantities that can be inoculated

into the bioremediation system. The TPPB community from which the strains have been isolated have been cultured and maintained in a minimal medium (MSM) carrying hexane as the only carbon source. Hence, to isolate strains from this community, MSM supplemented with hexane was naturally used as one of the culture media (Ng, 2014). This would select for microorganisms which are able to mineralize hexane in isolation – we shall refer to this group as “hexane degraders”. However, it was also expected that the community would consist of microorganisms which are not hexane-degrader per se, but rely on the metabolites (intermediates) transformed by the hexane degraders to grow (Freilich et al., 2011). To capture this group, the TPPB community had also been concurrently isolated on rich LB medium and MSM with glucose as the sole carbon source (Ng, 2014). Since the purpose back then was to collect as many culturable isolates as practicable, each isolate’s growth profile in all three types of media have not been determined. However, knowing this would be necessary to determine the growth conditions to bring the bioaugmentative strains up to the quantity suitable for application, as well as characterize the nutritional preference of the strains for optimal biomass accumulation. It would also help us identify potential cooperative relationship.

Hence, the 26 strains’ ability to grow on LB and MSM media (supplemented with hexane or glucose) were tested. The results are presented in Table 2.5. Each strain was incubated in the three types of media in both their liquid and agar forms. For MSM with hexane, the biphasic “MSM-silicone oil-hexane” liquid cultivation system was utilized to maximize the bioavailability of hexane for the bacterial strains. The growth of each of the strain in liquid media was observed and recorded through turbidimetric measurement (expressed in the unit of NTU – refer section 2.2.1.3.2) for 7 days. An increase in turbidity of a liquid culture by less than 5-fold (considered not significant) after 7 days was indicated as “-” while increases beyond that (usually beyond 10-fold) were indicated as “+”. For growth of colonies on agar plates, an indication of “+” means that there are visible colonies on agar plates by the end of 7 days, while “-” means there were no colony growth within the period of observation.

Table 2.5 Observation of growth of the 26 strains in LB and MSM media in both solid (agar plate) and liquid form. For liquid culture: “-” represents biomass increases by fold between 0 to 5, “+” represents biomass increases by fold above 10. For agar plates: “+” represent multiple colonies observed, “-” represents no colony growth observed. Data shown have been replicated over 3 independent experiments.

Genus	NES No.	LB		MSM+Hexane		MSM+Glucose	
		Solid	Liquid	Solid	Liquid	Solid	Liquid
<i>Achromobacter</i>	28	+	+	-	-	+	+
	61	+	+	-	-	+	+
	146	+	+	-	-	+	+
<i>Cupriavidus</i>	57	+	+	-	-	+	+
<i>Caulobacter</i>	43	+	+	-	-	+	+
<i>Variovorax</i>	42	+	+	-	-	+	+
	89	+	+	-	-	+	+
<i>Chryseobacterium</i>	27	+	+	-	-	+	+
	32	+	+	-	-	+	+
	140	-	-	+	+	-	-
	141	-	-	+	+	-	-
	144	+	+	-	-	+	+
<i>Flavobacterium</i>	36	+	+	-	-	+	+
<i>Microbacterium</i>	29	+	+	-	-	-	-
	35	+	+	-	-	-	-
	56	+	+	-	-	-	-
	60	+	+	-	-	-	-
<i>Rhodococcus</i>	30	+	+	+	-	+	+
	54	+	+	+	-	+	+
	73	+	+	+	-	+	+
	99	+	+	+	-	+	+
<i>Pedobacter</i>	75	+	+	-	-	-	-
<i>Mucilaginibacter</i>	151	+	+	-	-	-	-
<i>Sphingomonas</i>	59	+	+	-	-	+	+
	152	+	+	-	-	+	+
<i>Nevskia</i>	97	-	-	+	+	+	+

The overall data showed that all the strains under our consideration can be reproducibly cultivated at least in one medium or other, gaining biomass to allow for further

experimentation. Since LB is a rich medium, it would be expected to support most, if not all strains' growth. Indeed, almost all strains gained significant biomass over 7 days whether in liquid or on agar. The exceptions are NES 97, NES 140 and NES 141. These three strains were also the only ones capable of growing on MSM with hexane, both in liquid and on agar. The rest of the strains were either completely unable to increase in quantity using hexane as the only carbon source, or in the case of *Rhodococcus* strains, only showed growth on agar. NES 97 was further able to accumulate significant biomass in MSM with glucose, but NES140 and NES141 could not. The preference of NES 97, NES 140 and NES 141 for hexane as the carbon source contrasts starkly with the rest of the strains, which clearly prefer LB and/or the simple sugar – glucose – as the sole carbon source. These 3 strains are therefore considered “hexane degraders”, with NES 140 and NES 141 (both of which belong to the genus *Chryseobacterium*) being more “specialist” in nature than NES 97 (belonging to the genus *Nevskia*).

Both of *Nevskia* and *Rhodococcus* were commonly found in soil (Röttig et al., 2016; Shivaji, Reddy, Aduri, Kutty, & Ravensschlag, 2004; Weon et al., 2008). And under both of the genera there are strains being reported to be able to degrade alkanes including hexane, many researches have been done on *Rhodococcus* for its biodegradation related mechanism (Cappelletti et al., 2015; Laczi et al., 2015; Stancu, 2015). For *Nevskia*, there are papers examined the assimilation of alkanes, for example in paper (Leandro et al., 2012) three strains named as *Nevskia ramosa*, *Nevskia soli* and *Nevskia terrae* were all found to be able to assimilated alkanes with chain length varying between C8 and C20 and other carbon sources such as sugars, organic acids, alcohols and amino acids, but growth was better with alkanes than with other carbon sources. For *Chryseobacterium*, it was also a genus commonly isolated in soil (Chaudhary & Kim, 2016), strain *Chryseobacterium nepalense* sp. nov. was found able to assimilate L-rhamnose, D-maltose, glycogen, D-glucose, salicin, L-arabinose, and L-histidine; but not trisodium citrate, D-mannose, D-mannitol, N-acetyl glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, and phenylacetic acid. Some of the strains like as our strain, was found not able to assimilate glucose, for example *Chryseobacterium arachidis* LMG 27813^T and *Chryseobacterium geocarposphaera* LMG 27811^T in paper (Wang et al., 2016).

It could be noted that the ability to grow on LB does not necessarily correlate with ability to use glucose under minimal media conditions. The genera *Microbacterium*, *Pedobacter* and *Mucilaginibacter*, were able to grow in LB but unable to do so in MSM with glucose. Furthermore, differences between biomass accumulation in liquid and on agar media of the same nutrient base exist, such as for *Rhodococcus* strains as highlighted previously – growth was detected on MSM agar with hexane, but not in the corresponding liquid medium. However, a closer examination of the cultures suggested this could be due to limitation in detection by the turbidimeter, as *Rhodococcus* strains appeared to cluster and aggregate on the silicone-oil interphase of the biphasic culture system. This rendered a low turbidimetric reading overall, because it was not homogeneously suspended in the aqueous portion of the liquid culture. Cell aggregation of hydrophobic strain *Rhodococcus erythropolis* has sometimes been reported in conditions of salinity (Liu, Chang, & Liu, 2009). Under saline conditions, the biodegradation was detected along with the cell aggregate formation during the cultivation. In our case, when cultivated in the mineral salt-based environment of MSM, the *Rhodococcus* strains would be expected to form aggregates as they increase in biomass too. The fact that *Rhodococcus* could grow on hexane under agar cultivation suggests that it is metabolically capable of degrading hexane, but the aggregative property made detection of its growth in liquid challenging. As such, we have also categorized *Rhodococcus* strains as “hexane degraders”, but less “specialized” since they grow equally well on LB and MSM with glucose.

There are papers reported studies on strains that degrading agar which are often isolated from marine environment and occasionally from other sources, for example *Alteromonadaceae* sp. (Kwak et al., 2012), *Microbulbifer agarilyticus* sp. & *Microbulbifer thermotolerans* sp. (Miyazaki et al., 2008) and *Paenibacillus* sp., *Pseudomonas* sp., & *Klebsiella* sp. (Song et al., 2015). Particularly, there is one paper reporting an agar-degrading *Rhodococcus* sp. isolated from printing and dyeing wastewater, and one extracellular agarase was purified and characterized in this study (Feng & Li, 2013). So it is possible that the growth of the 4 *Rhodococcus* strains in our collection on the MSM plates was due to agar degradation. This possibility cannot be completely ruled out based on the current data available, however, on the other hand, the cell aggregation in the HACH

tube was also clearly visible by naked eyes in repeated experiments. And the aggregated clusters were very tight, even vigorous agitation by vortex machine cannot break apart the aggregation. In turbidimeter, light source emitted light in front of the HACH tube, and the turbidity (NTU) is calculated based on the amount of scattered light (by the particle in the media) at an angle of 90°. During the experiment, when the culture tube of *Rhodococcus* strain was vortexed and placed in the turbidimeter, the reading keeps fluctuating until around 10s later, when the turbidity reading become stable but in a very low level, therefore the data in table 2.5 showed all *Rhodococcus* negative growth in MSM + Hexane cultivation system. In conclusion, the *Rhodococcus* strains were able to grow in MSM+ Hexane cultivation system, but due to the constrain of the measurement instrument, their biomass increase cannot be accurately manifested in the data set.

A survey of each genus with multiple species revealed that most species within the same genus exhibited similar growth profiles across the different media. The only exception is the genus *Chryseobacterium*, where NES 140 and NES 141 manifested contrasting growth preference from the rest of the *Chryseobacterium* species. This implies that in most cases, the diversity within a genus in the source TPPB community was not significant for this set of properties assessed.

As expected, not all the strain isolated from the hexane degrading bioreactor can metabolize hexane sufficiently to bring about growth. Other than the reasons presented earlier as to why some strains isolated from TPPB are not able to utilize hexane as the substrate (i.e. they rely on metabolic intermediates released by hexane degraders), another possibility lies in their different degrees of tolerance to hexane (Mukhopadhyay, 2015). The level of hexane used in the experiment may be tolerable to some strains, but too toxic for other strains. In the TPPB community, the hexane concentration can be reduced after biodegradation by the strains which can metabolize hexane rapidly, allowing strains with less tolerance to survive. This level of cooperative “protection” is not offered in single strain cultures presented in Table 2.5.

Although turbidity indirectly reflects the accumulation of biomass in the cultures, it is unable to reveal whether the cell in the culture is at least metabolically active in transforming hexane to some extent, if the process does not result in biomass increase. In order to check if the strains are capable of some level of hexane conversion when it is the only carbon source available, DCPIP reduction assay was performed.

2.3.1.2 DCPIP Reduction Assay

DCPIP is an electron acceptor that is blue in its oxidized form (with a peak absorbance at 600 nm) and colorless in its reduced form (Yoshida et al., 2001). The principle of DCPIP reduction assay is based on the phenomenon that during the microbial oxidation of carbon sources, electrons are transferred to electron acceptors such as O₂, nitrates and sulphate.

In all the major metabolic pathways in cells, redox reactions and specific redox couples cycle between oxidized and reduced states, helping to regulate the flow of metabolites through these pathways. (Bidoia, 2010). DCPIP and its reduced form serves as an intermediate during the reaction, reaching an equilibrium eventually based on the flux of metabolism.

Hence, by incorporating an electron acceptor such as DCPIP to the culture medium, the extent or current state of oxidation reaction occurring within the culture can be manifested by the color change of the solution as it captures the electrons produced in the process (Adriano Pinto Mariano et al., 2009). This method is widely used as an indicator to assess the ability of the microorganism to utilize a specific substrate. For example, Mariano et al. (2008) used DCPIP to conduct a hydrocarbon biodegradability test to assess some culture's ability to degrade commercial diesel. Cormarck and Fraile (1997) was able to confirm one *Acinetobacter* strain's n-hexadecane degradation capability by the color change of DCPIP 12 h after inoculation with the substrate.

2.3.1.2.1 DCPIP Reduction Assay with Hexane as Carbon Source

Fixed quantities of the 26 bacterial strains cultivated in their preferred media were harvested and introduced into MSM supplemented with hexane. The percentage of DCPIP reduced (observed as decolorization of the blue reagent), corresponding to the relative level of metabolic oxidation of hexane by each strain, is presented in Figure 2.3.

Figure 2.3 Percentage of level of DCPIP reduced when the 26 strains were cultivated with hexane as sole carbon source. Duplicate experiments are done, average and standard deviation indicated.

The profiles of DCPIP reduction in the presence of hexane appeared to be consistent with the hexane degraders identified through growth tests (refer Table 2.5) presented in the earlier section. The 2 strains from the genus *Chryseobacterium* – NES 140, NES 141 – and NES 97 from the genus *Nevskia* showed the most reduction (30-40 %) in DCPIP, and all 4 strains of *Rhodococcus* showed moderate levels (10-15 %) of DCPIP reduction as would be expected of hexane degraders. The former group's high metabolic activities in response to hexane as the substrate is consistent with the data that these strains have a strong preference to grow on media using hexane as the carbon source, while the latter group's moderate level of metabolic activities matches our characterization that these hexane degraders are “less specialized”, based on their growth profiles in the various media (refer Table 2.5).

The rest of the strains did not show significant capability to catalyze the oxidization of hexane in this given experimental condition using DCPIP reduction assay. Hexane catabolism is initiated by an initial step of hexane oxidation. A bacterium may be able to initially transform hexane through oxidation but unable to complete the entire sequence of catabolism due to the lack of the necessary enzymes, which can be compensated for if others with metabolic capacity are present. In such instance, some level of DCPIP reduction in this assay may be detected despite the bacterium's inability to grow on media with hexane as the only carbon source on their own in the earlier test (refer Table 2.5). Hence, from the data above, we infer that the lack of growth of all these strains in MSM supplemented with hexane stems from a total lack of even the initial step of hexane catabolism, and not due to their inability to move on to the downstream stages of the biochemical pathway.

2.3.1.2.2 DCPIP Reduction Assay with Glucose as Carbon Source

Glucose is one of the basic and easily utilizable carbon source for most microorganisms. The growth assay presented earlier (refer Table 2.5), however, revealed that some of the strains were unable to grow on minimal media supplemented with glucose. The DCPIP reduction assay was therefore also conducted in MSM with glucose. The results are presented in Figure 2.4 below.

The group of hexane degraders showing strong preference for growth on hexane (NES 97, NES 140 and NES 141) showed a moderate level of DCPIP reduction (10-15%), while the less specialized hexane degrader group (the 4 *Rhodococcus* strains – NES 30, NES 54, NES 73 and NES 99) generally showed a higher level of DCPIP reduction (25-40%). This trend matches NES 97 and the *Rhodococcus* strains' general comparative growth profiles on MSM with hexane versus glucose. For NES 140 and NES 141, however, which were shown to be unable to grow on MSM with glucose (Table 2.5), metabolic utilization of glucose could be detected by this DCPIP reduction assay. This implies that the usage of glucose by NES 140 and NES141 is at a rate too low to manifest growth within the 7-day observation period conducted for the growth media test. For NES 97, NES 140 and NES

141, compared to their high DCPIP reduction level when hexane was used as the substrate (Figure 2.3), these lower glucose catabolic activities reinforced our assessment that these strains preferred using hexane as the carbon source instead of glucose. Likewise, the higher DCPIP reduction in glucose medium shown by the *Rhodococcus* strains NES 30, NES 54, NES73 and NES 99 (Figure 2.4) compared to their moderate DCPIP reduction in hexane medium (Figure 2.3) gave us the confidence that these are indeed less specialized hexane degraders.

Figure 2.4 Percentage of level of DCPIP reduced when the 26 strains were cultivated with glucose as sole carbon source. Duplicate experiments are done, average and standard deviation indicated.

The genera of *Microbacterium*, *Pedobacter* and *Mucilaginibacter* appeared to be unable to metabolize glucose, based on this DCPIP reduction assay. The results are again consistent with the lack of their growth on MSM supplemented with glucose, shown previously (Table 2.5). The lack of growth (i.e. biomass accumulation) of a bacterial strain on minimal media such as MSM could be due to either its inability to metabolize the carbon source (glucose) provided, or its need for essential nutrients which are not supplied in the MSM base (e.g. specific amino acid or vitamin), or both. The results suggested that for this group of bacteria, the reason is likely to be the former, since a simple metabolic processing of glucose not leading to biomass accumulation was sufficiently detected through DCPIP

reduction (although we cannot dismiss the possibility that the “essential nutrient” needed for metabolic processing of glucose was lacking).

The rest of the strains in the series, which are all capable of growing on MSM supplemented with glucose, showed correspondingly high level of DCPIP reduction, as expected.

2.3.1.3 PCR of *alkB*, CYP 153, and FDH and Gene

Based on previous experiment on the strain growth (section 2.3.1.1) and metabolism (section 2.3.1.2), we managed to get a list of strains that are able to assimilate hexane as sole carbon source. However, in this project, we were not selecting single strain which are able to degrade hexane completely to CO₂ and H₂O. We were looking for strains who were able to participate even partially in the entire pathway. Since our main objective of the study was to formulate a consortium that has diversity in metabolic characteristics. In both growth medium test and DCPIP assay, only strains who completely degrades hexane gets positive results. Although some of the strains were not able to complete the whole process, they might still be able to contribute while cultivated in a community, given that there was complementary strain which would finished the rest of the pathway. In this section, some of the key genes involved in certain steps of the pathway were chosen for a brief survey in their distribution among the candidate strains. Even they were found to be present in strains doesn't necessarily mean the strain must be able to fulfill the function of the enzyme translated by the target genes, since gene translation is another factor that affects the final results. This experiment was carry out purely for gauging the potential of these strains, and getting a brief picture of the genetic composition of the strains.

PCR of *alkB*, CYP153, and FDH was conducted to screen for the distribution of putative catabolic genes carried by the 26 strains, as an additional characterization of their potential in a community exercising cooperative metabolism. These three genes known to be involved in the biodegradation pathways of the two main VOCs to be assessed – hexane and formaldehyde – were chosen for this PCR screening. The presence of homologues to the genes *alkB* and CYP153 were checked to for possible involvement in hexane

catabolism, and that of the formaldehyde dehydrogenase (FDH) gene was checked for involvement in formaldehyde degradation.

2.3.1.3.1 *alkB*, CYP 153 and FDH Genes in VOC Bioremediation

Biochemical studies of alkane biodegradation have shown that there are two main classes of enzymes that are involved in the first step of the hexane degradation process. The first is particulate alkane hydroxylases (pAHs), and among this class, *alkB* is the most studied gene (Rojo, 2010). Many researchers have been using the presence of the *alkB* gene to gauge bacterial strains' potential to degrade MCL (middle chain length) alkanes. For example, it was found in *Pseudomonas putida* GPO1 which degrades C₃ to C₁₂ n-alkanes (Baptist, Gholson, & Coon, 1963), *Geobacillus sp.* strain MH-1 which is a thermophilic strain that degrades alkanes with different length chains in crude oil (Liu et al., 2009), as well as many other strains in *Mycobacterium*, *Rhodococcus* and *Proteobacteria* (van Beilen et al., 2006).

Another class of enzyme clearly unrelated to PAHs is cytochrome P450 enzymes (P450s). These alkane-hydroxylating P450s were found in mammalian liver (Bernhardt, 2006), yeasts and in some bacteria (van Beilen et al., 2006). Recent evidence, using highly degenerate PCR primers, showed that many isolates selected for their ability to grow on C₅-C₁₀ alkanes contain CYP153 enzymes from cytochrome P450 family (van Beilen & Funhoff, 2007).

The presence of *alkB* gene or CYP153 in a bacterial strain's genome may indicate it possess the ability to mineralize hexane, or that it may potentially contribute to the overall hexane biodegradation process by contributing some initial steps. It is likely that the hexane degrading characteristics shown in the previous two sections (section 2.3.1.1 and section 2.3.1.2) are due to the *alkB* or CYP153 expression, but in addition, other sub-players may be identified.

Formaldehyde is omnipresent in the environment, and due to its potentially lethal effects, most cells, both prokaryotic and eukaryotic, commonly have systems for formaldehyde

oxidation or detoxication (Barber & Donohue, 1998; W. Zhang et al., 2013). The initial step in these systems is to oxidize formaldehyde to formate, which is then further oxidized to CO₂ and H₂O (Mashford & Jones, 1982). One of the most studied dehydrogenases involved in this step is the medium chain alcohol dehydrogenase family. (Eklund et al., 1981; Jenkins & Tanner, 2006; Sanghani, Robinson, Bosron, & Hurley, 2002) One example is this formaldehyde dehydrogenase (FDH) that catalyzes the oxidation of formaldehyde with NAD⁺ as an electron acceptor and exists in a wide variety of organisms from *Escherichia coli* to humans (Lasse Uotila & Koivusalo, 1974; L Uotila & Koivusalo, 1989).

Figure 2.5 Common formaldehyde metabolism pathways in cells. Figure adapted from paper (Dorokhov, Shindyapina, Sheshukova, & Komarova, 2015).

The PCR testing in this section is not enough to get a complete picture of the distribution of all the hexane & formaldehyde biodegradation related gene among the strains. There are many genes other than *alkB*, *CYP153*, and *FDH* involved in the hexane and formaldehyde degradation process, the three genes we picked are just some of the most commonly studied

gene in one step of the complete biodegradation pathway. Since in this experiment, our aim was not to do the complete genetic profile of all related genes with our strains.

Another reason that only FDH was tested among all the genes in formaldehyde degradation pathway is that at this stage formaldehyde degradation was not our priority. Moreover, formaldehyde is a common environmental contaminant found in many industrial and medical products (Gonzalez et al., 2006), as well as being endogenously produced in all living organisms as a result of metabolism (methionine, histamine, methanol, and methylamine), spontaneous dissociation of 5,10-methylene tetrahydrofolate, or oxidative demethylation of DNA and RNA (Aas et al., 2003; Falnes, Johansen, & Seeberg, 2002; Hanson, Gage, & Shachar-Hill, 2000). To prevent the lethal and mutagenic effects of formaldehyde, various repair mechanisms have evolved in living organisms. Detoxification of formaldehyde can be carried out by many enzymes, for example, formaldehyde dismutase, methylformate synthase, or glutathione-independent formaldehyde dehydrogenase (Bystrykh et al., 1993; Ito, Takahashi, Yoshimoto, & Tsuru, 1994; Sakai, Murdanoto, Sembiring, Tani, & Kato, 1995). However, these enzymes have been found in only a limited number of organisms, whereas a glutathione-dependent repair system appears to be widespread in nature and has been found in many prokaryotes (except for archaea) and all eukaryotes (Hanson et al., 2000; Harms, Ras, Reijnders, Van Spanning, & Stouthamer, 1996; W.-H. Lee, Wheatley, Benedict, Huang, & Lee, 1986; Lasse Uotila & Koivusalo, 1979). Therefore, the glutathione-dependent formaldehyde dehydrogenase (FDH) was chosen for the basis screening.

Overall, we couldn't not conclude that FDH negative strains do not have the potential to contribute to formaldehyde removal, we could only conclude that FDH positive strains have the potential to degrade formaldehyde.

2.3.1.3.2 Results of Screening for *alkB*, CYP 153 and FDH genes

The *alkB* gene as one of the key gene involved in first step of the hexane degradation process was PCR tested on the 26 strains, using primers adapted from (Smits et al., 1999). The results showed that only the strains NES 30, NES 73, NES 97, NES 140 and NES 141 have strong positive bands. The results are consistent with the growth experiment results observed in section 2.3.1.1 and the DCPIP experiment in section 2.3.1.2.

The CYP 153 gene as the second important gene in alkane-hydroxylating was also PCR tested using primers adapted from (Amouric et al., 2010). This series of PCR of CYP 153 gene was conducted in confirmation of and in addition to the work by Ng (2014). Many strains showed positive presence of CYP 153 gene product, including NES 27, NES 30, NES 54, NES 59, NES 73, NES 97, NES 99 NES 140 NES 141 and NES 152. Among these 10 strains, NES 27, NES 59 and NES 152 did not show growth in either liquid or agar media supplemented with hexane, and no DCPIP reduction was observed. The PCR of CYP 153 was conducted as a potential metabolic capacity checking, for possible involvement of the strain in certain pathway. No growth on hexane despite the presence of putative CYP 153 genes could either imply that the strains did not express the gene under the condition of growth or assay, or that the gene was expressed but was insufficient to complete the whole process of oxidization of hexane to CO₂ due to the lack of genes handling subsequent steps in the metabolic pathway.

Table 2.6 PCR results of *alkB* CYP153 and FHD on the genomic DNA of the 26 strains: “-” indicates no amplification observed on gel electrophoresis under the condition describe in section 2.2.3, “+” indicates positive band observed on gel electrophoresis with target band size under the same condition as described in section 2.2.3.

Genus	Strain designation	<i>alkB</i>	CYP153	FDH
<i>Achromobacter</i>	NES 28	-	-	-
	NES 61	-	-	-
	NES 146	-	-	-
<i>Cupriavidus</i>	NES 57	-	-	-
<i>Caulobacter</i>	NES 43	-	-	-
<i>Variovorax</i>	NES 42	-	-	+
	NES 89	-	-	+
<i>Chryseobacterium</i>	NES 27	-	+	-
	NES 32	-	-	-
	NES 140	+	+	+
	NES 141	+	+	+
	NES 144	-	-	-
<i>Flavobacterium</i>	NES 36	-	-	-
<i>Microbacterium</i>	NES 29	-	-	-
	NES 35	-	-	-
	NES 56	-	-	-
	NES 60	-	-	-
	NES 30	+	+	-
<i>Rhodococcus</i>	NES 54	-	+	-
	NES 73	+	+	-
	NES 99	-	+	-
<i>Pedobacter</i>	NES 75	-	-	-
<i>Mucilaginibacter</i>	NES 151	-	-	-
<i>Sphingomonas</i>	NES 59	-	+	-
	NES 152	-	+	-
<i>Nevskia</i>	NES 97	+	+	+

It should be noted that for the strains NES 30, NES 73, NES 97, NES 140, and NES 141 the presence of both *alkB* and CYP 153 genes in their genomes were picked up by the PCR screening. The hexane-degrading traits for strains NES 54 and NES 99 could therefore be

inferred to be due to the function of the CYP 153 gene, while those of NES 30, NES 73, NES 97, NES 140 and NES141 would be due to the dual actions of the gene products of CYP153 and *alkB*. This appears to support the DCPIP data, whereby NES 97, NES 140, and NES 141 showed 30-40% DCPIP reduction and NES 30 and NES 73 showed 10-15% DCPIP reduction – the former group which contains both gene showed higher DCPIP reduction compared to the latter group. These two gene may both contribute to the hexane removal.

For FDH gene, we designed a FDH degenerate primer from 8 microbial species, including *Pseudovibrio* sp. FO-BEG1, *Nodularia spumigena* CCY9414, *Alphaproteobacterium* BAL199, Rhodobacterales *bacterium* HTCC2255, *Loktanella vestfoldensis* SKA53, *Roseobacter* sp. MED193, *Sulfitobacter* sp. NAS-14.1, and *Mesorhizobium loti* MAFF303099. The FDH primers' sequence can be referred in section 2.2.3. The forward and reserve primer were designed as 5'-CNACNGGNRTNGGNGCNGTN-3' and 5'GNYTNGGNGSNATHGGNYTN-3' respectively. The results showed that among the 26 strains, 5 of them are positive in the FDH gene PCR, including NES 42, NES 89, NES 97, NES 140 and NES 141. Among them 97 showed strong positive and NES 42 showed weak positive. Although none growth of either strain was observed on the MSM plates infused with formaldehyde in section 2.3.1.5, the presence of the FDH gene indicated the potential of these strain to degrade formaldehyde. As illustrated in section 2.3.1.2.2, the lack of growth my due to lack to essential nutrients on the MSM agar plates.

Although the assay results provided strong evidence for possession of the potential to express key enzymes, the actual gene expression level is another factor we need to bear in mind. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins, otherwise in non-protein coding genes such as transfer RNA (tRNA) or small nuclear RNA (snRNA) genes, the product is a functional RNA.(Alberts et al., 2013)

In prokaryotic organisms, their DNA floats freely within the cell cytoplasm where during protein synthesizing, the processes of transcription (DNA to RNA) and translation (RNA

to protein) occur almost simultaneously. When the resulting protein is no longer needed, transcription stops. Thus, the regulation of transcription is the primary method to control what type of protein and how much of each protein is expressed in a prokaryotic cell. All of the subsequent steps occur automatically. When more protein is required, more transcription occurs. Unlike in eukaryotes, where regulatory processes occurring after mRNA is made (post-transcriptional, translational and protein degradation regulation) plays an important role (Silva & Vogel, 2016), in prokaryotic cells, the control of gene expression is mostly at the transcriptional level. ("Prokaryotic versus Eukaryotic Gene Expression," 2016)

Bacteria are typically exposed to an ever-changing environment in which nutrient availability may increase or decrease radically. Bacteria respond to such variations in their environment by altering their gene expression pattern; thus, they express different enzymes depending on the carbon sources and other nutrients available to them. In bacteria, this sort of genetic regulation is mediated at the level of transcription.(Ralston, 2008) Therefore, the possession of potential to express key enzymes does not equal to the ability to degrade certain components.

Decades of biochemical and structural studies have mapped out a comprehensive mechanism for prokaryotic translation. There are many studies focused on the structural and kinetic mechanisms of prokaryotic translation.(Agirrezabala & Frank, 2009; J. Chen, Tsai, O'Leary, Petrov, & Puglisi, 2012; Dunkle & Cate, 2010; Frank & Gonzalez Jr, 2010; Milón, Maracci, Filonava, Gualerzi, & Rodnina, 2012; Rodnina & Wintermeyer, 2001). And for now, the express of the key enzyme encoding gene are beyond the scope of this study.

2.3.1.4 Cell Surface Hydrophobicity and Emulsification Activity Test

Other than the growth, metabolic and genetic profiling of the 26 strains, the cell surface hydrophobicity of each strain and the emulsification activity of the liquid medium each strain was cultured in were also determined. It has been previously established that the

community that had developed within the TBBP with hexane as sole carbon source were influenced by the non-aqueous phase of liquid (silicone oil) introduced into the system to increase the bioavailability of hexane. Many strains that were isolated from this community have been altered in these two traits, which would be important for their growth and metabolism in TPPB (Ng, 2014).

Cell surface hydrophobicity was determined using a modified procedure of the microbial adhesion to hydrocarbons (MATH) technique as described by (Pembrey et al., 1999). The result 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 assay employed to monitor the strains' ability to accumulate emulsification activity in its culture media was based on a method extensively used by many others to indirectly detect for the presence of free biosurfactants in microbial cultures (Fleck, Bicca, & Ayub, 2000; Soares et al., 2003). Cell-free supernatants from different samples were used to mix with of hexane and the emulsions formed were measured by optical density to indicate emulsification activity.

Table 2.7 Cell surface hydrophobicity (as MATH %) of strains cultivated with hexane or glucose as the sole carbon source in MSM. “-” represents MATH % between 1 to 10, “+” represents MATH % between 10 to 20, “++” represents MATH % above 20. Emulsification activity of strains cultivated with hexane or glucose as the sole carbon source, in MSM. “-” represents weak activity, “+” represents medium activity, “++” presents strong activity.

Genus	NES No.	Cell surface hydrophobicity (as MATH %)		Emulsification activity	
		Hexane	Glucose	Hexane	Glucose
<i>Achromobacter</i>	28	-	-	-	-
	61	-	-	-	-
	146	-	-	-	-
<i>Cupriavidus</i>	57	-	-	-	+
<i>Caulobacter</i>	43	-	-	-	-
<i>Variovorax</i>	42	++	-	+	-
	89	++	-	+	-
<i>Chryseobacterium</i>	27	+	-	+	-
	32	+	-	+	-
	140	++	+	+	-
	141	++	+	+	-
	144	+	-	+	-
<i>Flavobacterium</i>	36	++	-	+	-
<i>Microbacterium</i>	29	-	-	+	-
	35	-	-	+	-
	56	-	-	+	-
	60	-	-	+	-
<i>Rhodococcus</i>	30	++	-	++	-
	54	++	-	++	-
	73	++	-	++	-
	99	++	-	++	-
<i>Pedobacter</i>	75	-	-	-	-
<i>Mucilaginibacter</i>	151	-	-	-	-
<i>Sphingomonas</i>	59	-	-	-	+
	152	-	-	-	+
<i>Nevskia</i>	97	+	-	++	-

In general, strains able to grow with hexane as the sole carbon source (NES 97, NES 140, NES 141 and the 4 *Rhodococcus* strains NES 30, NES 54, NES 73 and NES 99) exhibited

greater cell surface hydrophobicity and emulsification activity in hexane-supplemented media. Two non-hexane degraders, *Variovorax* strains NES 42 and NES 89, were also found to have these traits enhanced when cultured in the presence of hexane. These strains were earlier found to be carrying FDH genes (refer Table 2.6). However, in media supplemented with glucose, overall, the cell surface hydrophobicity and emulsification activity of all these strains remained lower, inferring that these traits were induced from the need to metabolize the highly hydrophobic hexane – greater cell surface hydrophobicity and emulsification activity enable the strains to associate more closely with the substrate hexane. The more readily bio-accessible hydrophilic glucose did not require development of higher cell surface hydrophobicity.

The *Microbacterium* strains (NES 29, 35, 56, and 60) showed negative surface hydrophobicity and positive emulsification activity in the results. They did not grow with either hexane or glucose supplemented medium, they can only be cultivated by LB (in section 2.3.1.1). In PCR experiment (section 2.3.1.3.2), they were found out to be negative in all three gene tested. Based on the testing results in section 2.3.1.4, they were not hydrophobic but with some emulsification activity, which means the strain itself is hydrophilic but they probably were producing biosurfactant in the MSM + hexane cultivation system.

Emulsions are commonly observed when liquid hydrocarbons and water are mixed during bioremediation or fermentation (Atlas & Bartha, 1992). These emulsions dramatically increase the area of the oil-water interface, thereby enhancing bioavailability. For a dispersion of one liquid in another to be stable enough to be classified as an emulsion, a third component, such as a surfactant, must be present to stabilize the system. (Dorobantu, Yeung, Foght, & Gray, 2004) Fine solid particles such as silica beads can also stabilize emulsions if they attach at the interface between the oil and the water to prevent droplets from coalescing (Briggs, 1921; Yan, Gray, & Masliyah, 2001).

If a bacterium is positive in emulsification activity, there might be two mechanisms involved as mentioned above. Firstly, emulsification may be associated with the surface

properties of the cells, as a result of attachment to the oil-water interface by general hydrophobic interactions rather than specific recognition of the substrate(Cooper & KOSARIC, 1982; Van Loosdrecht, Lyklema, Norde, Schraa, & Zehnder, 1987), in this case, these bacterial cells behave as fine solid particles at interfaces. Secondly, bacteria can produce surfactants or emulsifiers that stabilize emulsions(Allen, Francy, Duston, Thomas, & Ward, 1992; Cooper & KOSARIC, 1982), some microorganisms can emulsify hydrocarbons even in the absence of cell growth or uptake of hydrocarbons (Rosenberg & Rosenberg, 1985). Similar observations have been reported by other authors that both hydrophilic and hydrophobic bacteria produce surface active compounds when grown on glucose or hexadecane(Bouchez Naïtali, Rakatozafy, Marchal, Leveau, & Vandecasteele, 1999; Neu & Poralla, 1990; Vasileva-Tonkova, Galabova, Stoimenova, & Lalchev, 2008). Therefore, in our case, these *Microbacterium* strains are likely to be hydrophilic but with the capacity to produce biosurfactant.

Looking simply from the point of view of distribution of bacteria in a two-liquid-phase system according to their cell surface hydrophobicity and ability to emulsify, we can see that some strains will stay very much in the aqueous phase, while others may adhere to emulsions of oil-in-water. One speculation is that such a distribution may be important to maintain a stable and sustainable bacterial community(Owsianiak et al., 2009). While formulating the bioaugmentative co-culture, this aspect will need to be kept in mind.

2.3.1.5 Growth on Formaldehyde, Toluene and Benzene as Sole Carbon Source

Other than hexane and formaldehyde as the main targets for assessing microbial removal of VOCs, there are two other VOCs (toluene and benzene) that we raised as within our radar of assessment (refer section 2.1.3). Although the presence of FDH gene was screened by PCR, the ability of the strains to mineralize formaldehyde as the sole carbon source that leads to biomass accumulation has not been tested. For toluene and benzene, although they are not our primary targets and only serve to provide supplementary information about the bioaugmentative approach, it would be desirable to have a preliminary idea how prevalent toluene/benzene catabolic activities may be amongst our collection. Therefore, one quick

round of screening of these 26 strains' ability to grow on mineral media supplemented with formaldehyde, toluene or benzene was performed in independent triplicates.

Surprisingly, none of the 26 strains showed growth on MSM agar infused with formaldehyde, indicating that although formaldehyde catabolic potentials are present amongst the strains, gaseous formaldehyde itself is not a substrate that supports growth well. On the other hand, the majority of the 26 strains showed growth when infused in toluene or benzene when there is no alternative carbon source available, and these are even more prevalent than growth on the substrate hexane in which they have been acclimatized for over one year. This observation, coupled with the fact that the bacterial community from which these strains were derived had not been exposed to toluene or benzene in the TPPB for over half a year, suggests that the native environment (oil-contaminated soil in a car park) of the community has sufficient toluene and benzene to maintain the presence of these strains, and the toluene/benzene catabolic capacity is not detrimental to hexane biodegradation per se (or else the strains would have been competed out). The observation gave us some confidence that the 26 strains have a diversity of VOC metabolic pathways to provide some robustness in function in the eventual application in Air purifier A.

Table 2.8 Growth observation of the 26 strains on MSM plate with formaldehyde, toluene or benzene infused desiccators respectively. “-” indicates negative growth, “+” indicates positive growth.

Genus	NES No.	Formaldehyde	Toluene	Benzene
<i>Achromobacter</i>	28	-	+	+
	61	-	-	-
	146	-	-	-
<i>Cupriavidus</i>	57	-	+	+
<i>Caulobacter</i>	43	-	+	+
<i>Variovorax</i>	42	-	+	+
	89	-	-	-
<i>Chryseobacterium</i>	27	-	-	-
	32	-	+	+
	140	-	+	+
	141	-	+	+
	144	-	-	-
<i>Flavobacterium</i>	36	-	-	-
<i>Microbacterium</i>	29	-	+	-
	35	-	+	-
	56	-	+	-
	60	-	+	+
<i>Rhodococcus</i>	30	-	+	+
	54	-	+	+
	73	-	+	+
	99	-	+	+
<i>Pedobacter</i>	75	-	+	+
<i>Mucilaginibacter</i>	151	-	-	-
<i>Sphingomonas</i>	59	-	+	+
	152	-	-	-
<i>Nevskia</i>	97	-	+	+

2.3.1.6 Overview of the Characteristics of the 26 Strains

The following figure of clustering analysis of the 26 strains is based on an agglomerative hierarchical clustering. The previously tested 18 characteristics include LB-Solid, LB-

Liquid, MSM-Hexane-Solid, MSM-Hexane-Liquid, MSM-Glucose-Solid, MSM-Glucose-Liquid, DCPIP-Hexane, DCPIP-Glucose, alkB, CYP153, FDH, Cell surface hydrophobicity (as MATH%)-Hexane & Glucose, Emulsification Activity-Hexane & Glucose, Formaldehyde, Toluene, and Benzene. These total 18 features formed an 18-dimensional hyper plane. The Euclidean distance is the metric for distance comparison. We can see that when the height is around 5.2, there will be 5 distinct clusters formed, meaning 5 different types of strain can be classified based on these 18 tested features.

Figure 2.6: cluster dendrogram of the 26 strains being testing, height represent Euclidean distance for comparison.

In general, the spread of the strains' characteristics showed diversity between different genera. Reviewing results from section 2.3.1.1 to section 2.3.1.4 together, the 3 strains NES 97, NES 140 and NES 141 were found to exhibit clear evidence of their ability to utilize hexane as carbon source, with greater preference and efficiency in degrading hexane over other substrates, e.g. glucose or even the rich medium LB with an array of organics. Therefore, for ease of further discussion and analysis, these 3 strains shall be referred to as "hexane degrader specialists". The *Rhodococcus* strains (all four of them in this study) were also found to be able to grow on hexane (Table 2.5) and reduce DCPIP to some extent

with hexane as the substrate (Figure 2.3), and hence showed that they are capable of metabolizing hexane, albeit at a more moderate level. In contrast to the hexane degrader specialists, however, the *Rhodococcus* strains were also able to grow well when supplemented with glucose or when in rich media (Table 2.5). Thus these strains shall be referred to as “hexane degrader non-specialists”. The remaining strains all exhibited growth on LB or minimal media supplemented with glucose (Table 2.5), which enable them to be cultured in laboratory for experiments, but presented a distinct lack of hexane catabolism. A few of them, such as the *Chryseobacterium* strain NES 27 and the *Sphingomonas* strains NES 59 and NES 152, showed genetic potential with the presence of CYP153 homologues in their genomes (Table 2.6) and could possibly still contribute to specific stage of the overall metabolism turned over by the whole community. Most of the 26 strain showed their ability to grow on toluene plates, except for two *Achromobacter* (NES 61 and NES 146), one *Variovorax* (NES 89), two of *Chryseobacterium* (NES 27 and NES 144), the only *Flavobacterium* (NES 36), the only *Mucilaginibacter* (NES 151) and one of the *Sphingomonas* (NES 152). For benzene, other than the above mentioned strains, three more *Microbacterium* showed negative results, including NES 29, NES 35 and NES 56 (Table 2.8). Some strains (NES 42, NES 89, NES 140 and NES 141) showed positive result in FDH gene PCR, which indicates possible contribution in the initial oxidation stage of formaldehyde degradation (Table 2.6).

In the next stage of this project, we would formulate an efficient microbial consortium that works on the Air-purifier A. Although maintaining the greater diversity of the source community from the TPPB would more closely replicate the equilibrium and sustainability attained, for the practical application of formulating a bioaugmentative co-culture, a reduction in component strains would be desirable. The balance has to be such that the selected component strains are still able to collectively represent the “core” attributes of metabolic capacity and robustness existent in the source community. Therefore, we attempted to formulate a “simplified” microbial consortium which will work well on the target application, and which could allow further investigation into the factors or dynamics that influence the bioaugmentation process.

In consideration of all the 26 strain's profile, although we couldn't find any information about which strains belong to zymogenous or autochthonous in literature, there are some observation in those experiments indicating that some of the strain e.g. NES 54 are likely to be autochthonous, as these organisms that do not show a remarkable increase in numbers in response to the addition of an available carbon source and do not rush to reproduce just because of a sudden influx of nutrients (also referred as K-strategists) (Sigee, 2005), and some of the strain e.g. NES 97, NES 140 (for hexane) and NES 42, NES 59 (for glucose) are likely to be zymogenous, as these organisms exhibit a tremendous increase in numbers in response to the addition of available carbon sources and they can take advantage of any sudden increase of nutrients in their environment which enable them to reproduce quickly and abundantly (also referred as r-strategists) (Sigee, 2005). The diversity of the strains provides higher chance of them falling in both category, thus potentially enhanced the sustainability of the consortia as hypothesized in section 1.4.

Based on this hypothesis, in the following section: 2.3.2.1, when we make combination of strains to formulate culture for Air-purifier A, we include both hexane degrader specialist (which matches the characteristic of zymogenous) and hexane degrader non-specialist for many sets of consortia (which matches the characteristic of autochthonous).

2.3.2 Formulation of Bioaugmentative Consortia

2.3.2.1 Growth Profile and Compatibility of Consortia Formulated for Assessment of their Ability to Mineralize Target Compound Hexane

Based the above section 2.3.1.6, we have gained an overview that these 26 strains have diverse characteristics. Not all of the 26 strains demonstrated evidence of degradation of any of the 4 VOCs, but the potential collaborative metabolism might be present among those strains. They might be contributing in other ways, for example, producing metabolic intermediates that could join the pathway to productive outcomes. Such contribution has been reported before, for example, in one paper polycyclic aromatic hydrocarbons (PAH) 6 individual strains were found capable of co-metabolic degradation of PAH in a range varying among strains. Khan et al. (2013) observed that complete and high rate of 2,4-

Dinitroanisole (DNAN) degradation was achieved with no accumulation of intermediates, when *Pseudomonas* sp. strain FK357 and *Rhodococcus imtechensis* strain RKJ300 was co-culture. The study clearly demonstrates that complete degradation of DNAN occurs as a result of the metabolic cooperative activity of two members within a bacterial consortium. Similarly, some of the strains among the 26 can form groups of co-metabolism pairs or groups.

As compared to the full community in TPPB, consortia formed by selecting from the representative 26 strains can help reduce the complexity of the community, yet may still represent the functional structure of the full community in some way as we anticipated. However, it is also possible that the simplified consortium loses the essential members that are responsible for the community stability and result in a failure of bioaugmentation process as a consortium. For example, among the failure reason stated in section 1.3.2.2, antagonistic effect was listed as a potential cause of a failed bioaugmentation.

When this project was initiated, Company X has commissioned our laboratory to develop a co-culture of microorganisms for the improvement of their product which included a component carrying the company's proprietary microbial culture. For reason of licensing confidentiality, this microbial culture will be referred to as "Formula Original". Formula Original has never been tested for hexane removal and hence was included as a control for reference. In this section, in order to formulate an optimal consortium for Air-purifier A, different combination of the 26 strains was co-cultured to formulate various consortia, and their growth curve was profiled. The growth on biomass was used as an indicator of mineralization of hexane, since the only carbon source provided in the culture are hexane. Summary of different groups of consortia formulated under various rationale has been summarized and presented as Table 2.9. The exact collection of strains within each formulation, however, are organized in Appendix 1.

Before this experiment were embarked on, a few general projections were made about the possible profile of a simplified consortium designed to mimic a complex community. The first scenario is that the set of strains worked well as a community and achieved complete

mineralization of hexane as a whole, in such a way that collective growth is sustainably supported. Some of the strains may partner each other in cooperative metabolism, and promoted greater efficiency than a single-strain pure culture. A second scenario is that only the specialists work significantly and primarily, and others in the community are competed out after some time. A third possibility is that some of the strains (e.g. the specialist) may exhibit antagonistic effects on other strains; hence the mineralization capacity is compromised, resulting in a community not capable of degrading hexane. In the fourth situation, the mineralization of the consortium is not compromised, and performs exactly as a single strain specialist does. Indeed, after performing the experiment involving the 68 culture combinations (refer Table 2.9 and Appendix 1) it was possible to categorize the growth profiles into a few main types. Hence, for the ease of discussion, Figure 2.6 showed the 4 typical profiles that surfaced. In Appendix 1, the type of profile presented by each numbered culture/co-culture is listed. However, in this section, the discussion will centre around the bulk groups listed in Table 2.9.

Figure 2.7 OD₆₀₀ of the consortium cultivated in TPPB with hexane as sole carbon source for over 80 Days. Shown are representative curves from 2 or 3 independent experiments. Formula Original represents the formula used by company X previously. Bioreactor Community represents the sample taken from the TPPB where all the strains used were isolated from. Type A, B, C and D were 4 type of representative growth profile of all the consortia tested. (refer Table 2.9)

In general, for all the consortium which gained biomass, we clearly observed the typical growth curve of bacterial (or other microorganisms, as protozoa, microalgae or yeasts) batch culture, which consists of four phases: lag phase, log phase (exponential phase), stationary phase, and death phase. Our observation spans 80 days, and would stop a considerable period after the stationary phase has been reached, as non-typical carbon source utilization often kicks in after exponential phase.

As shown in Figure 2.7, Type A represents culture that cannot grow with hexane as sole carbon source at all. Type B represents culture that grow well at the beginning, but cannot sustain the biomass over the long run. Type C represents a situation whereby the culture picks up the growth only very gradually over the time but is be able to sustain the biomass when it reaches stationary phase. Type D represents the case whereby the culture is able to pick up the growth immediately after being cultured with hexane and can sustain a high level of biomass over a prolonged period. As a first reference, the source TPPB community belongs to type D profile. When Formula Original was eventually tested in our hands for hexane utilization, it was found that it falls into the type A profile.

Initially, the three of the hexane degrader specialist were cultured on their own to test their growth profile. As observed (Table 2.9), all single culture formed with hexane degrader specialist (from culture 1 to culture 3), belong to type B and type C profile. For NES 140 and NES 141, the growth was slow but sustained (Type C profile). It appeared that it takes some time for *alkB* and CYP153 gene to start functioning in these strains. For NES 97, the growth was fast but lasted only short term (Type B profile). One possible reason might be that some intermediates accumulate during its growth inhibits further growth of NES 97.

From culture 8 to culture 19, each 3 hexane degrader specialist and each 4 hexane degrader non-specialist was mixed to formulate 12 type of 2-strain co-culture, when NES 140 and NES 141 were co-cultured with each of the non-specialist, type C of profiles were observed (Table 2.9). When NES 97 was c-cultured with each of the non-specialist, type B of profiles were observed. However, none of them attained the desirable type D profile. Their growth

type remains the same as the when the specialist was cultured alone, which means the addition of any non-specialist did not make a difference in their overall metabolism.

From culture 20 to 22, each 3 of specialist was co-cultured with another specialist to form a 2-strain co-culture (Table 2.9). When two of them were from the same genus, antagonism effect was observed, for example, in the NES 140 & NES 141 co-culture, type C of profile was observed, indicating their metabolism of hexane were inhibited at the first stage. When the 2 of them were from different genera (NES 140 & NES 97 and NES 141 & NES 97), no antagonism effect was observed, and the profile belongs to type B.

From culture 23 to culture 30, 2 of the hexane degrader specialist group (NES 140+NES 97, and NES 141+ NES97) which we have earlier established to not exhibit antagonism, were co-cultured with each of the *Rhodococcus* strain, which are all hexane degrader non-specialists (Table 2.9). All of them exhibited profile type B. This means the addition of *Rhodococcus* strain did not change the growth traits of the culture significantly, and there are no negative consequence.

From culture 31 to culture 38, some new strain was added to these earlier combinations. They are the strains NES 57, NES 43, NES 36, NES 75 and NES 151, which are unique within their respective genera from the TPPB community. This tests their compatibility with the hexane degrader specialist groups (NES 140+NES 97, and NES 141+ NES97) and different *Rhodococcus* strains (Table 2.9). Among them, 2 of the co-culture were observed to hold type D profile. They were both with NES 54 included in the co-culture, indicating NES 54 might be an indispensable strain for an ideal co-culture in our consortium.

From culture 39 to culture 42, different species of genera that contains more than one strain were added to culture 31 to culture 34 (NES 140 set) to formulate co-cultures. Similarly, culture 43 to culture 46, was formulated with different strains of those genera to culture 35 to culture 38 (NES 141 set) (refer Table 2.9). Among these consortia, some falls into type B, some falls into type C, and the rest falls into type B. There was no clear pattern identified that can be associated with the addition of certain strain to make a conclusion.

From culture 47 to culture 49, for each of the representative strain used in the consortium, most of them were chosen from the benzene/toluene degraders from their genera (Table 2.9). For example, NES 28, NES 42, NES 32, NES 60, NES 59 were all with benzene and toluene metabolic capacity (section 2.3.1.5). NES 151 was excluded from the consortium, since this strain did not show any potential to contribute VOCs removal based on its property profile (refer Appendix 1). All three of the consortia were observed with type D profile, which meet the standard of characteristic that we are looking for.

Finally, as a confirmatory set of controls, cultures 50 to culture 68 were run. All these single-strain cultures formed with non-hexane degraders indeed presented type A profile (no growth) as expected.

Among all the co-cultures tested, two 8-strain cultures, and three 13-strain cultures also showed type D profile. Among these 8 type D co-cultures, culture 47 to culture 49 were from the same group and form with the same strain selecting principle which consists of 2 of hexane degrader specialists (NES 97 and NES 140), one hexane degrader non-specialist (NES 54), and 9 representative strains from other genera (including *Achromobacter*, *Cupriavidus*, *Caulobacter*, *Variovorax*, *Flavobacterium*, *Microbacterium*, *Pedobacter*, *Sphingomona*, *Sphingomonas*). In comparison, the 8-strain cultures were not as inclusive, and the 13-strain cultures were not as consistent, because they are from different co-culture group with various types of growth profile, while for culture 47 to 49, all of them demonstrated a type D profile. There might be some reason behind this (e.g. functional redundancy and synergism among those strains).

Table 2.9 Cultures tested on their biomass growth profile. Members in each culture listed in this table can be found in Appendix 1. In “Description” row, the similarity/category of culture or the rationale of the concoction was described. In “Type(s) of profiles” row, each profile type of the culture was listed with the differentiating strain in the brackets. In “Remarks” row, the cultures’ growth characteristic was briefly stated.

Culture No.	Description	Type(s) of profiles	Remarks
1 - 3	Hexane degrader specialist on their own	B (97) and C (140, 141)	Growth slow but sustained for 140/141, fast but short term for 97
4 - 7	Hexane degrader non-specialist and how it fares in larger TPPB liquid culture	C	Growth slow but sustained for all culture
8 -19	Specialist with non-specialist	B (97) C (140, 141)	Growth slow but sustained for 140/141+ <i>Rhodococcus</i> , fast but short term for 97+ <i>Rhodococcus</i>
20 - 22	Combination of specialist from same or different genera	B (140, 141) C (140, 141)	Growth slow but sustained when from same genus, fast short term when from different genera
23 - 30	The above “working combination” with different <i>Rhodococcus</i> strains	B	Fast but short term for all culture
31 - 38	Unique members from the relevant genera added and compatibility tested with different <i>Rhodococcus</i> strains	B (30, 99) C (73, 99) D (54)	Fast but short term when 30/99 in culture, Growth slow but sustained when 73/99 in culture; Fast and long term when 54 in culture
39 - 42	Trying out different species of genera with multiple species NES140 set	B (29) C (146, 144,56) D (61,89, 32, 35,60, 152)	No obvious pattern observed
43 - 46	As above but NES141 set	B (28, 27, 56) C (146, 144, 29, 35, D (60,)	No obvious pattern observed
47 - 49	Consortia 1-3	D	Fast and long term for all culture
50 - 68	Single-strian culture of non-hexane degraders	A	Unable to increase in biomass for all culture

2.3.3 Testing of VOC Removal by Bioaugmentative Consortia in Air-purifier A

The 3 consortia selected through comparison of various combinations of strains, presented in the previous section, showed that they possess the capacity to utilize hexane as the sole carbon source to bring about a collective accumulation in biomass for their respective communities, and in a sustained manner. A few of the strains included within these 3 consortia have been earlier shown to be capable of growing on MSM plates with benzene or toluene as substrate (refer Table 2.5), and some of the strain showed potential to degrade formaldehyde as they carried FDH gene homologues (refer Table 2.6). Based on these evidences, it was reasonable for us to predict that the 3 co-cultures selected have the potential to biodegrade our 4 target VOCs (hexane, formaldehyde, toluene, and benzene), and could serve as the bioaugmentative consortia in the air-purification device developed by Company X (refer section 2.3.3.1). The following section describes the studies conducted to check if the consortia are capable of reducing the level of VOCs in the testing chamber (about 1 m³ in volume) when installed in the air purification device. The performance of the respective co-cultures would then be assessed and compared to pick out the most effective bioaugmentative consortium for application.

2.3.3.1 Structure and Operational Features of Air-purifier A

Company X has commissioned our laboratory to develop a co-culture of microorganisms which could surpass the performance of Formula Original in its air-purification device referred to as “Air-purifier A” throughout this study for reason of licensing confidentiality. The device is small scale (can be placed on a table like a medium-sized flower pot) and thus suitable for use in offices and households. The co-cultures formulated through our studies would be substituted in the place of Formula Original to allow their VOC removal activities to be examined in the context of Air-purifier A operation.

The schematics of Air-purifier A structure is illustrated in Figure 2.8. A small-scale fan and a light are mounted on top of the device while a cylindrical structure in the middle

holds cartridges composed of porous Matrix B and biological components C. The cartridges holding Matrix B also carries the bacterial consortium Formula Original. The wicks dangling from the cartridge are designed to absorb water from the reservoir below by capillary effect to support the persistence of bacteria held among the porous matrix B.

Figure 2.8 Illustration of the structure of Air-purifier A used for testing of the consortium’s bioaugmentation efficiency. It contains a fan and a light on the top, cartridge for porous matrix B and other biological component C in the middle, and a tank of water in the bottom, with four wicks connecting the cartridges and water tank.

When Air-purifier A is switched on, the fan starts to work, promoting air circulation through the entire product, including the space over Matrix B and biological components C. The component C is constant, and does not serve as a variable in this experiment (refer 2.2.7.2 for the operation during testing). Air-purifier A uses sterile Matrix B to act as a scaffold for the microbial co-culture to adhere to, which is prepared by soaking in the bacterial culture, so that bacteria can penetrate into and attach onto the small pore surfaces inside Matrix B.

Air-purifier A with Formula Original is able to remove formaldehyde by approximately 60-70% over one hour, according to previous tests conducted by Company X. Its ability to remove 3 other VOCs – hexane, toluene and benzene – were completely unknown at the point that the commissioned project was first undertaken.

2.3.3.2 Chamber Set up for the in-air VOC Removal Test

In evaluating the performance of air purification devices, many studies used testing chamber to determine the removal efficiency of target pollutants. The size of testing chamber varies from several liters to room size.

In one instance, for example, Markowicz and Larsson (2012) conducted the experiment in a 2.6-litre plastic box, with 1-cm diameter hole to allow for sampling of air through a tube before and after the experiment for a GC-MS analysis. In another case, the air-purifier's ability to remove organic matter and floating germ in indoor air (Kim et al., 2006) was measured using an 800 l-capacity chamber. The air-purifying device was put into the chamber before it was sealed and organic materials were injected into it. For the evaluation of sterilizing capability of floating germs of the air-purifying device, an air sampler (M.A.Q.S II, OXOID Co.) was used. The air sample analysis was performed later. There is another case in which target pollutants were introduced at a constant rate into a 20 m³ environmental chamber that was ventilated at a constant air exchange rate of 1/h. The air purification unit was installed on an external ductwork loop and operated in recirculation mode, by re-introducing the air processed by the air cleaner into the chamber. Samples of air were collected simultaneously upstream and downstream of the cleaner at different times for analysis (Destailats et al., 2012).

In our case, the testing chamber is approximately 1000 litre capacity as stated in section 2.2.7.1; the chamber volume is similar with the second case (Kim et al., 2006) mentioned above. One thing different from the above mentioned cases is that VOC concentration in our study was monitored in real time within the tightly sealed chamber throughout the entire operation, at an interval of every 3 seconds. The concentration of VOCs in the air of the sealed chamber was recorded by a device called photo-ionization detector (PID) (RAE system, San Jose, USA), which can typically measure VOCs and other gases in the sub-ppb to 10000 ppm range. The PID is an efficient and inexpensive detector for gas and vapor, producing instantaneous readings and is able to operate continuously. These hand-

held, battery-operated detectors are widely used in military, industrial, and confined working facilities for safety (Smith, Lepage, Harrer, & Brochu, 2007).

2.3.3.3 Internal Control

Since the testing chamber was self-constructed in our laboratory, and no prior quality control data were available, it was important to check if the chamber met two essential criteria. First, it needed to be ensured that VOCs are minimally emitted by the materials within the chamber during the test. Second, the natural decrease in VOCs concentration enclosed in the chamber, e.g. due to adsorption onto surfaces within the chamber, had to be within an acceptable range during the test duration, so that we could be confident that the reductions in VOC level were attributable to Air-purifier A function.

Figure 2.9 Total VOC concentration detected over 120 minute within the sealed testing chamber, containing tools and equipment as normally required in experiments.

The total VOC emission of components in a sealed chamber was monitored over 120 min. This basal level of total VOC emitted remained within 0.225 ppm, which was negligible in comparison to the concentration of the four VOCs tested at saturation or near saturation

i.e. at least 10 ppm (refer section 2.1.3). This affirmed that the level of VOCs internally emitted by the chamber and equipment is insignificant relative to the actual testing conditions for VOC removal.

2.3.3.4 Profiling of Vaporization and Saturation of Hexane, Formaldehyde, Toluene and Benzene

In this set of experiments, we aimed to obtain data on the changes in VOC concentration during and after evaporation in the sealed chamber. The saturating VOC concentration attainable in the testing chamber was monitored using the method describe in the method section 2.2.7.4.

Figure 2.10 Changes in formaldehyde concentration over 250 minutes. At 145 minutes, the evaporation facilitated by fan was stopped. Thereafter, formaldehyde concentration remaining in the chamber was monitored for another 100 minutes.

In first set of profiling, formaldehyde was found to be slow to reach saturation in comparison with other three VOCs. The saturation of formaldehyde in the chamber took about 150 minutes (reaching approximately 40 ppm). Then the evaporation process was stopped and the natural decrease of formaldehyde over a further 100 minute was recorded.

A 6.3% of reduction in concentration was observed after this 100-minute duration (Figure 2.10).

The profile indicated that formaldehyde takes quite some time to saturate in the enclosed chamber. For the practicality of future experiment, a set timing allowing for vaporization of formaldehyde to occur to a sufficiently significant level for testing was maintained, instead of waiting for it to reach saturation. Since we only have one set of testing chamber and equipment for the testing, shortening the testing time reasonably for each set of sample would allow more samples to be assessed in a single day. Hence, in the second set of formaldehyde profiling (refer section 2.3.3.4), we decided to stop the evaporation process when the formaldehyde concentration reaches 10 ppm to simulate the experimental conditions later adopted, and observed that the concentration gradually dropped 17.9% over 60 minutes (Figure 2.11).

Figure 2.11 Profiles of evaporation and saturation of the 4 VOCs inside the chamber: (a) hexane: evaporation was stopped within one minute (b) formaldehyde: evaporation was stopped concentration reaches about 8 ppm (c) toluene: evaporation was stopped within one minute (d) benzene: evaporation was stopped within one minute.

In contrast, for hexane, only a few seconds after the evaporation process started, the hexane concentration reached about 37 ppm (saturation). Then the active evaporation process was stopped and a gradual 3.5% of reduction in hexane concentration could be observed over the next 60 min (Figure 2.11).

For toluene, similarly to hexane, only after a few minutes of evaporation, the concentration reached about 220 ppm and maintained this concentration. Then the natural decrease in toluene level over 100 minute was recorded, and a reduction of 25.6 % was observed after 60 min (Figure 2.11).

In the case of benzene, it did not reach saturation as quickly as hexane, but was significantly faster than the formaldehyde to reach saturation. Only a few minutes before the evaporation process was stopped, the benzene concentration reached about 300 ppm and maintained this concentration. The benzene concentration naturally decreased within the chamber over the further 100 minutes recorded, showing a 7.9% of reduction in concentration within 60 min (Figure 2.11).

Based on the observations mentioned above, we would conclude that hexane, benzene and toluene would reach saturation concentration quickly in the chamber. However, formaldehyde takes quite some time to attain saturation. Therefore, the starting concentration of the respective VOCs to assess the removal efficiency of Air-purifier A was established as follows: testing for the removal of hexane, toluene or benzene would start at their saturation concentration in the chamber; formaldehyde would start at around 10 ppm. Although differing degree of natural reduction of VOCs occurred in the chamber setting, there would still be significant quantity of VOCs remaining to allow for the removal by the air-purifier.

2.3.3.5 Removal of VOCs by Biological Component C

After the starting concentration of each VOCs were decided, the next piece of information required for our analysis was the extent to which the biological component C could remove the various VOCs. Although this is a constant factor that is a part of Air-purifier A, the contribution of biological component C has to be subtracted from the overall VOC removal in order to more appropriately assess the VOC removal capacity of the bacterial consortia formulated by our laboratory.

The VOCs were first allowed to vaporize to attain the desired starting condition in the chamber as established in the previous section. Following this, Air-purifier A composed of only biological component C (i.e. no bacterial cultures in Matrix B) was switched on. The VOC concentrations dropped and the percentage of decrease are presented in Figure 2.12. The natural reduction of each VOC in the absence of Air-purifier were also measured as a

control. The difference between the two would reflect the contribution of C to the removal of VOC.

Figure 2.12 Percentage of removal of VOCs in chamber with and without Air Purifier with C only. Duplicate experiments are done, average and standard deviation indicated.

The results (Figure 2.12) showed that indeed Air-purifier A with component C alone could remove the 4 VOCs in the chamber to various extent. However, compensating for the natural reduction in the absence of Air-purifier A, it was clear that the percentage of removal ranged within the 20% mark. The levels of VOCs remaining after component C activity shall be used as the baseline when calculating the removal of VOCs by Air-purifier A carrying various bacterial co-cultures to be tested.

2.3.3.6 Removal of 4 VOCs by Formulated Bioaugmentative Consortia

In the section, we started to test the Air-purifier A's removal capacity as a completed unit with different bacterial sample being installed in the unit. Explain how this set of data is worked out based on control values obtained in previous subsection.

Figure 2.13 Removal percentage of 4 VOCs in testing chamber by hexane cultivated consortia (subtracted the natural decrease of the VOCs and the removal by component C only) over 1 hour. Duplicate experiments are done, average and standard deviation indicated. Need to add original formula data

The efficacy of VOC removal through the activities of control and test co-cultures under air-purifier settings were tested accordingly in the chamber test conditions established earlier. The controls include Formula Original and the source community from TPPB (refer Bioreactor Community in Figure 2.13) in its full complexity (Week 40 sample) and these were used as a reference for comparison against the 3 formulated bioaugmentative Consortia (1, 2 and 3 from section 2.3.2) in Air Purifier A. The percentage removal refers to the final concentration of VOCs in the chamber subtracted by the VOC level in the presence of Air-Purifier carrying only component C, and worked as a percentage against the latter, and presented in Figure 2.13. For hexane, Formula Original itself was unable to provide significant removal, which was consistent with the growth profile exhibited in Figure 2.7 (section 2.3.2). Consortium 2 manifested the highest level of VOC removal

among the 3 formulated bioaugmentative consortia, at almost the same degree of efficacy as that by the source Bioreactor Community under the Air-purifier A setting. For formaldehyde, all 3 consortia outperformed the bioreactor community, and amongst them, Consortium 2 achieved the highest formaldehyde removal. For toluene, the performance varies dramatically, and again, Consortium 2 achieved the highest removal. For benzene, none of the consortia showed significant removal after subtracting component C's contribution. Overall, Consortium 2 appeared to be the best performing consortium, and proved to be a great value-add over Formula Original. This was not only in terms of formaldehyde removal which was previously tested for Formula Original but is now further enhanced by Consortia 2, but also in terms of the additional VOCs that it can remove, which for Formula Original, was really fairly limited to formaldehyde and could not handle hexane, toluene or benzene.

However, considering that all the strains were isolated from a hexane degrading community, the removal efficiency would have been expected to be better. This could be due to the structural design of the Air-purifier (refer section 2.3.3.1), which is purely aqueous based, whereas the original consortium degrades hexane in a TPPB, which has oil phase as a buffer and delivering medium. Without the biphasic arrangement to improve bioavailability of hexane to Consortia 2 in Air-purifier A, the hexane catabolic potential might not have been applied to the fullest. A case in point would be that even the Air-purifier A carrying the source TPPB community maintained a similarly low level of hexane removal efficacy.

For toluene, about 20-25% greater removal were achieved through the use of various bioaugmentative consortia (refer Figure 2.13). In the previous section (section 2.3.1.5), some of the strains showed ability to grow on MSM plate with toluene as the sole carbon source. The removal capacity could be due to the metabolism or adsorption of the toluene, but the exact mechanism is not within the scope of this thesis.

For benzene, no improvement on its removal was observed, compared with “Air-purifier with C” only (refer Figure 2.13). Although in previous section, some of the strains showed

ability to grow on MSM plate with benzene as the sole carbon source, in the gaseous benzene removal assay, none of the co-cultures appeared to be contributing to the benzene removal. There are several possible reasons. The first is that like hexane, and perhaps even more severely so, the hydrophobicity of benzene impairs their contact with bacteria strains. The second is that, unlike the hexane set of data, these strains were not being cultured by the exact substrate (benzene) to attain greater biomass for experimentation with benzene, so the metabolic capacity might now have been turned on within 1 hour (testing duration). The third reason could be that the concentration used in this study was too toxic for the microbes concerned.

Considering hexane, toluene and benzene's growth profile on MSM plates, it is surprising that among three of the hydrophobic VOCs, toluene removal was best achieved by Air-purifier A. There might be several reasons for the prevalent growth with benzene and toluene on MSM plates. Firstly, these strains were originally derived from oil contaminated soil in a car park (benzene and toluene are petroleum components). It is possibly that the strains' benzene and toluene metabolism pathways were triggered during the substrate testing when incubated in benzene/toluene infused environment. Secondly, since the testing was only carried out on agar plates, cannot rule out the possibility of agar degradation as mentioned in point 5, further cultivation testing would be performed if intent to find out more. However, this section is a supplementary part of this study, it did go further on the testing.

Figure 2.14 showed the real time removal of formaldehyde within the testing chamber. Consortium 2 showed 100% removal of formaldehyde at a rate much greater than the Formula Original, achieving 100% removal as early as 20 min. Interestingly, this "simplified" or "core"-formulated consortium was even observed to perform better than the source Bioreactor Community. This was a pleasant surprise as the consortium was formulated and selected based on characteristics positive for a hexane degrading functionality, yet it demonstrated higher formaldehyde removal efficiency than the hexane removal efficiency. However, that would be expected to be primarily due to the fact that the Air purifier is a water-based system which is more suitable for removing hydrophilic

chemicals such as formaldehyde, than hydrophobic ones such as hexane, or even toluene or benzene. Nevertheless, the fact still holds that the bacterial strain composition functioned in favor of formaldehyde removal.

Figure 2.14 Percentage removal of formaldehyde by Air Purifier A with Bioreactor Community, Formula Original or Consortium 2 in the chamber over the time course of 60 min. Shown are representative curves from 2 or 3 independent experiments.

Initially the main target of this project was to formulate a consortium which was capable of degrading hexane to a satisfactory extend for indoor air purification, and formaldehyde was one of the three other common indoor VOCs that was tested alongside with hexane (the main target VOC). The focus was shifted to formaldehyde (from this section onwards) after since the culture was found out unexpectedly to be more efficient in treating formaldehyde in the given experiment setting.

It is surprising to find out the 100% of formaldehyde removal was achieved with consortium 2 in Air-purifier A in considering the negative growth of strain on MSM plates infused with formaldehyde in section 2.3.1.5. There might be several reasons for this phenomenon. Firstly, the removal performance tested were not 100% resulted from VOCs' biodegradation. And in section 2.3.4.3 (comparison between two assays for formaldehyde removal), it has been found out that other components of the device played a substantial

role in facilitating formaldehyde removal. Secondly, some of the formaldehyde removal might be due to adsorption by, for example, extracellular polysaccharide of the bacteria, and this adsorption can be the first step of biodegradation.

2.3.3.7 Maintenance of Bacterial Co-Culture in the Air-purifier A System

We noted that Air-purifier A uses sterile matrix B to act as a scaffold for Formula Original, and the bacterial co-culture would not need to compete with “indigenous community” at this stage of testing. However, as stated in the introduction, one of the important features of successful bioaugmentation is its resilience and robustness in the presence of environment fluctuation – in other words, sustainable removal efficiency. So, after testing the removal efficiency of the Air-Purifier A at a stage when the bacterial co-culture was freshly introduced, its sustainability after preparation was checked as well.

There are two scenarios about the sustainability of the co-culture testing. In first scenario, sustainability was tested on the consortium level, meaning the consortium continued to be cultivated in the TPPB, and samples are being taken for formaldehyde removal test every two or three days between day 0 - day 16 (for Consortium 2) and day 0 - day18 (for Formula Original). Day 0 was set as the date on which formaldehyde removal peak is reached base on the previous results; there we aim to observe the removal efficiency change during about 2 weeks after the peak. In second scenario, sustainability was test on the environmental perturbation level, after the culture was integrated in the Air Purifier A, it was allowed to stand at room temperature in an open room to mimic the actual usage setting for more than 2 weeks, during the same time frame, its formaldehyde removal efficiency was tested every two or three days during the testing period.

Figure 2.15 Formaldehyde removal of the bioreactor days: (a) samples freshly taken at different date from the TPPB before each test (b) same sample being installed in the Air-Purifier A and tested on different dates. Duplicate experiments are done, average and standard deviation indicated.

As illustrated in Figure 2.15, in first scenario (a), the removal efficiency of Consortium 2 fluctuated between 80% and 90% during day 0-16. In second scenario (b), the results showed that although at day 0 the removal efficiency is 83.9%, it was able to increase more than 95% after day 10, and further maintained above 95% removal up to day 16.

The preliminary data showed that the bacteria's formaldehyde removal capacity is not decreased with time (during day 0 -day 16) although the condition in air-purifier is much less controlled than in batch cultivation setting. The results indicated that this consortium's formaldehyde removal efficiency stabilized within certain range.

Using the chamber testing set-up, the process of acquiring one set of experimental data on VOC removal efficiency of a bioaugmentative consortium (i.e. one VOC with one Air-Purifier bioaugmentative sample) required about 2 hours (including sample preparation, device setting up, testing chamber purging and sealing) to test each consortium sample's removal efficiency (refer section 2.2.7.2). Moreover, there was only one set of apparatus in our lab, therefore only one sample can be tested at each time point. Thus, it would be

useful if a quicker and more convenient method to assess formaldehyde removal efficiency could be developed for future investigation requiring more permutation of conditions.

2.3.4 Development and Optimization of Colorimetric Testing

A colorimetric method for determining the concentration of soluble formaldehyde level has previously been developed. According to the Yamazaki et al. (2001) the detection range of formaldehyde concentration is 0.2 ppm to 400 ppm using this method. Since this is within the range as was generated in the test chamber for monitoring the formaldehyde concentration in the presence of Air-Purifier A in section 2.3.3.4, the method was deemed a potential alternative allowing greater depth in analysis.

Colorimetric method has several advantages over the previous methods: it takes less time for each assays with higher throughput, each well in the 96-well plates holds one sample, while each chamber can only test with one sample at each time (refer section 2.2.8). This method gives the potential to do more samples in each day, allowing more parameter to be manipulated and more time points to be tested in the future if such need arises. Here we aimed to develop and verify this method as an alternative and substitute of formaldehyde removal assays in the chamber testing set up.

2.3.4.1 Optimization of Several Parameters for Measurement of Formaldehyde

Considering that the peak of absorbance of a cell suspension to determine cell density is 600 nm, which overlaps with the absorbance of the colorimetric reagent (FeCl_3 & MBTH, refer section 2.2.8), the colorimetric reading might suffer interference from light scattering by the bacterial cells in suspension. One way to overcome the potential interference was to spin the cell down to remove it from the solution carrying colorimetric factors before measuring the optical density. We tested whether this spinning procedure would be necessary to obtain reliable data.

Figure 2.16 Percentage of formaldehyde remaining with 4 different starting concentrations: 6.25 pm, 12.5 pm, 25 pm, and 50 pm. Triplicate reading are taken, and standard deviation indicated.

The data shown above (Figure 2.16) is the percentage formaldehyde remaining after incubation with pure NES 140 culture for four length of time (0 min, 30 min, 60 min and 150 min) with 4 different starting concentrations. As shown in the above figure, in the non-spin set, the percentage of formaldehyde remaining is steadily decreasing as time goes by, however in the spin set, the percentage formaldehyde remaining fluctuates intensively with much greater standard deviation. In this spinning set, the two higher concentration sets did not show reliable decrease of formaldehyde when the incubation times goes by. The non-spin set showed more reliable readings, especially for the 12.5 ppm and 6.25 ppm concentration sets.

Spinning might cause the dissolved formaldehyde to evaporate form the liquid culture, thus resulting higher fluctuation of formaldehyde concentration among various samples. During the experiment, after spinning, duplicated sample sets were taken from Eppendorf tubes to the 96-well plate one by one for incubation with and thereafter testing of the OD readings. The samples taken later might have formaldehyde dissolved back to the liquid culture, whereas the sample taken earlier might have formaldehyde escaped while the Eppendorf tube was open for sample dispensing. Therefore, caused a higher standard deviation then the non-spin group.

Figure 2.17 OD 670 reading of the “Control (w/o microbes)” set and “with NES 140” culture set with 4 different starting concentrations after 3 incubation durations without spin. Triplicate readings are taken, and standard deviation indicated.

The above figure (Figure 2.17) showed the OD reading of negative control set (without any microbes) and the positive control set with four starting concentration and three incubation durations. As the data demonstrate, all positive sets showed lower OD reading (thus lower formaldehyde concentration). As incubation time increases, the gaps between the positive control sets and negative control sets also increases, indicating more formaldehyde being removed.

Table 2.10 Quade test with post-hoc statistics. *F* means *F* statistics, num df stands for numerator degree of freedom, den df stands for denominator degree of freedom. *Italic number indicates the value is lower than 0.05 and being statistically significant.*

	30 min			60 min			150 min		
<i>F</i>	6.75			5.5			8.5		
num df	3			3			3		
den df	6			6			6		
<i>p-value</i>	0.024			0.037			0.014		
	50 ppm	25 ppm	12.5 ppm	50 ppm	25 ppm	12.5 ppm	50 ppm	25 ppm	12.5 ppm
25 ppm	1	-	-	0.660	-	-	0.151	-	-
12.5 ppm	0.065	0.065	-	0.044	0.082	-	0.003	0.024	-
6.25 ppm	0.009	0.009	0.184	0.013	0.024	0.390	0.012	0.104	0.315

The statistical analysis of the result in Figure 2.17 was performed using Quade test, and the result is stated in the above table (Quade, 1979). According to the analysis, all three time-groups have *p* values < 0.05, which means there are significant (95% confidence interval) difference among the four initial formaldehyde concentrations for each time-group. Following by this conclusion, post-hoc paired tests were employed to identify the optimal parameters. According to the *p*-values of the paired comparisons, if the incubation time is 30 min, then the initial concentration set as 6.25 ppm would be significantly better than 25 ppm and 50 ppm; if the incubation time is 60 min, then the initial concentration set as 6.25 ppm and 12.5 ppm would be significantly better than 50 ppm; if the incubation time is 150 min, then the initial concentration set as 25 ppm and 50 ppm would be significantly better than 6.25 ppm and 12.5 ppm.

In consideration of time needed for each experiment, and the actual formaldehyde concentration in indoor environment, the final experiment parameter was set as follows: 12.5 ppm is the initial concentration and 60 min is the incubation time for future testing on various consortia.

2.3.4.2 Colorimetric Testing on the Consortium Samples

After validating the colorimetric methods and determined the parameters, we proceeded to test the formaldehyde removal with consortia samples. We would like to compare this method with the previous chamber testing, to see if the removal efficiency data obtained

from the two assays was consistent. If not, further investigate would be needed to determine what causes the inconsistency in order to exploit the respective methods appropriately.

Figure 2.18 (a) Percentage of formaldehyde removal by Air-Purifier A over the days shown using chamber testing set-up (b) Percentage of formaldehyde removal percentage of consortia in different date in MSM media by colorimetric method

The consortium taken from the same date range of cultivation for the TPPB (different batch) was tested by both methods, and their results were compared. The gap observed between the chamber test result and the colorimetric were likely due to the formaldehyde removal function contributed by the other biological component C, water tank, and even porous matrix B. As shown in Figure 2.18, in chamber test, the formaldehyde removal fluctuates between 85% and 100%, however in the colorimetric testing the formaldehyde removal fluctuates between 35% and 50%. Chamber test method measures a combination of removal effect, including, and the other biological component C, water tank, porous matrix B (increase the exposure of the bacteria to the formaldehyde, so more interaction surface for bacteria to direct contact with formaldehyde). Whereas the colorimetric methods only measure the removal effect of dissolved formaldehyde in solution by the bacteria residing within it.

2.3.4.3 Comparison between Two Assays for Formaldehyde Removal (Colorimetric Testing combined with the Chamber Testing)

After the formaldehyde removal data was obtained for the colorimetric method, and the result showed a shortfall of percentage of formaldehyde removal in the latter, which could be attributed to other components of the Air-purifier A. We planned an experiment to use the colorimetric method to analyze what happened in the chamber, by testing the formaldehyde concentration both in air and water inside the testing chamber by both PID and colorimetric method.

In this experiment, the setting was slightly differently designed from the one for chamber testing. One set of beaker with water was place inside the chamber (outside Air-purifier A). Two sets of the cartridge inside the Air-purifier A was soaked with Formula Original and the other two sets were soaked with Consortium 2. The experimental procedure for measurement of formaldehyde level that followed was identical to that in the chamber testing for VOCs removal (section 2.2.7.2 and section 2.2.7.5).

“Inside Air-purifier with Formula Original” refers to liquid sample collected from cartridges with Formula Original after one round of chamber testing. “Inside Air-purifier with Consortium 2” refers to liquid sample collected from cartridges with Consortium 2 after chamber testing. “Inside control Air-purifier” refers to water sample collected from the water tanks at the bottom of Air-purifier A. “Outside Air-purifier” refers as the water sample in the beaker outside Purifier A. All these liquid sample are subject to colorimetric experiment and the results are shown in Figure 2.19.

Figure 2.19 Formaldehyde concentration in different liquid sample within the testing chamber. Triplicate reading are taken, and standard deviation indicated.

In the figure above (Figure 2.19), liquid sample from “Inside Air-purifier with Consortium 2” has a much lower formaldehyde level when compared with sample from “Inside Air-purifier with Formula Original”, even though they were placed in the exact same environment and exposed to sample level of formaldehyde. The lower concentration in the liquid sample indicates that the soluble formaldehyde has been biodegraded more by the Consortium 2 than the Formula Original.

“Inside control Air-purifier” has about 25% lower formaldehyde level compared with the sample “Outside Air-purifier”. The formaldehyde dissolved in the water reservoir was removed by the bacteria (the bacteria might possibly be travelling down through the wick from the cartridge).

In comparison with “Inside Air-purifier with Consortium 2” and “Inside control Air-purifier”, we can see about 2.9 ppm of formaldehyde was dissolved and remaining in the water reservoir inside the “Air purifier A” during this one hour, and in “Inside Air-purifier with Consortium 2” the formaldehyde is only 0.06 ppm, so around 98% of formaldehyde enter the cartridge was removed by the combination of Consortium 2, porous matrix B and biological component C.

This combined removal efficiency is close to the chamber testing result of PID data. Therefore, it suggests strongly that the Air-purifier A's removal efficiency was achieved by the collective contribution of several components of the device (consortium, porous matrix B, biological component C, and water reservoir). While the colorimetric removal testing is only achieved by bacteria strains either by biodegradation or bio-absorption. The colorimetric experiment addresses the need to study the bacterial contribution alone, which agrees with the primary focus of this project.

Table 2.11 Quade test with post-hoc statistics of the formaldehyde concentration in different liquid sample within the testing chamber. Italic number indicates the value is lower than 0.05 and being statistically significant.

	Inside Air-purifier with Formula Original	Inside Air-purifier with Consortium 2	Inside control Air-purifier
Inside Air-purifier with Consortium 2	0.1066	-	-
Inside control Air- purifier	0.1066	<i>0.009</i>	-
Outside Air-purifier	<i>0.009</i>	<i>0.0013</i>	0.1066

The statistical analysis was performed using Quade test as well, and the result is stated in the above table (Quade, 1979). According to the analysis, these four samples formaldehyde concentration have a p values of 0.0060 (which is < 0.05), that means there are significant (95% confidence interval) difference among them. Post-hoc paired tests were employed to evaluate the difference among each sample, the concentration of “Outside Air-purifier” is significantly higher than the rest of the samples expect for “Inside control Air-purifier”, which means although there might be small amount of microbes migrated from the cartridge to the water tank, the removal contribution of these microbes are not as significant as the consortium installed in the cartridge (in both “Inside Air-purifier with Formula Original” and “Inside Air-purifier with Consortium 2” samples), since they have apparently much higher biomass.

After validating the colorimetric for testing formaldehyde removal in solution, we would use this method for microbial interaction studies in the next section.

2.3.5 Preliminary Survey of the Co-Culture Interaction

2.3.5.1 Overview

Microbial community is a complex system, and the interactions between each member of the community is a complicated network. There are many factors involved in the dynamic interactions, including metabolic interactions (e.g., syntrophic relationships, competition for substrates), and quorum sensing (production of growth promoting factors or antibiotics). All these factors are essential to comprehensively investigate the networks of interspecies interactions in a stable community and to understand the mechanisms controlling the stability of microbial communities. (Kato, Haruta, Cui, Ishii, & Igarashi, 2008)

Some researchers have studied the interaction within simple co-cultures. For example, Kato et al. (2008) conducted a research on a simple and stable community consisting of four strains of microorganisms. All possible combination of strains was investigated in order to reveal the effects among them. There are suppressive effects observed between two strains, also there are indirect effect among three of the strains. The extinction of one strain is indirectly prevented by another strain through an indirect effect. The result indicated that in addition to direct metabolite interactions, certain kind of indirect relationships could have a great impact on microbial community structure in the natural environment.

There are also reports on diversity–stability relationships within a community. In an ecological field, diversity–stability relationships (more diverse communities enhance stability) have been hotly debated (Ives & Carpenter, 2007; McCann, 2000). As one of the reasons, it was hypothesized that a complex array of interactions acts as buffers against dramatic population explosion or extinction. In the communities in this study, it appears that the effects of one strain on the other strain tend to be weakened when third-party strain(s) are present. This type of buffering interaction (suppression of advantageous strains

and promotion of disadvantageous strains) would prevent population explosion or extinction, which induce collapse of community structure.

In the larger purpose of our study, to investigate the consortium as a system required examination of interactions between strains, which may reveal each strain's function/role in this consortium. However, it will be highly complex to study the interaction between a co-culture consisting of 12 strains. The complete part of the study may be consisting of 2-strain consortium, 3-strain consortium, up to the 12-strain consortium. There would be thousands of combination among them. At this stage, we have focused on only studying about 33 combinations among them, they are the combination of three hexane degrader specialists with each rest of 11 strains. Since these 3 specialists are the main contributors for hexane degradation within the co-culture, it would be a good preliminary work to start with their interaction with other stains. Although we didn't study all of the direct and indirect interactions happening within the community, we hoping by revealing part of the interactions among all will take us one step closer to the bigger picture.

2.3.5.2 Agar Plate Interaction Study

The purpose of this agar-plate interaction study is to investigate the strains' relationship with regards to effect on growth with each other. In order to do so, a bacterial lawn was generated by spreading the bacterial suspension over the MSM plate. Following that, one 10 µl aliquot of the counterpart culture would be dropped on the plate. On the control plates, the counterpart culture was dropped on the plate without the growth of the lawn. After 6 days of incubation in in the hexane infused desiccator, the plates were taken out and compared for the effect on growth between the lawn strain and dropped strain (refer section 2.2.9).

The drop plate experiment is used to observe effect between two strains when they are growing in direct contact. The method was modified from (Smith, Des Etages, & Snyder, 2004); which used this lawn experiment to study the antagonism and synergism by a

diffusible factor between yeasts and bacteria. Hence this novel method allows us to use the bacteria lawn to assess the interaction between 2 strains of bacteria.

Three of the strains, NES 54, NES 97, and NES 140, were used to create bacterial lawn to study effect with other strains during metabolizing hexane. Because only in MSM plates incubated with hexane infusion, will provide environment for these strains enter the hexane metabolism pathway. Only three of the strains were able grow on MSM plate, thus form a bacterial lawn. The enhancement and inhibition phenomenon and the effects observed in this experiments are highly reproducible among 3 replicates.

Table 2.12 The effect of strain interaction on MSM plates observed between strains NES 54 and rest 11 strains, when incubated with hexane infusion. Shown are representative results from 2 or 3 independent experiments.

Effect	Strains	Control plate	Lawn plate
Lawn Strain	54
Enhanced growth	/		
Inhibited growth	42
	97
	140
No effect	28
	32
	36
	43
	57
	59

Effect	Strains	Control plate	Lawn plate
	60
	75

In this set of experiment (Table 2.12), interaction of strain NES 54 and all other 11 strains was surveyed. NES 54 inhibited the growth of NES 42, NES 97 and NES 140, as there are blank holes on the lawn being observed. For, rest of the strains, there are no effect being observed.

Table 2.13 The effect of strain interaction on MSM plates observed between strains NES 97 and rest 11 strains, when incubated with hexane infusion. Shown are representative results from 2 or 3 independent experiments.

Effect	Strains	Control plate	Lawn plate
Lawn strain	97
Enhanced growth	32
Inhibited growth	54
	57
No effect	140
	28
	36
	42
	43
	59
	60

Effect	Strains	Control plate	Lawn plate
	75

In this set of experiment (Table 2.13), interaction of strain NES 97 and all other 11 strains was surveyed. NES 97 was found to inhibit the growth of strain NES 54, NES 57, and NES 140, probably due to substrates competition. It was also found that enhanced growth of NES 32 occurred on the lawn, compared to the control plate. For the remaining strains, no effect was observed.

Table 2.14 The effect of strain interaction on MSM plates observed between strains NES 140 and rest 11 strains, when incubated with hexane infusion. Shown are representative results from 2 or 3 independent experiments.

Effect	Strains	Control plate	Lawn plate
Lawn Strain	140
	32
	36
Enhanced growth	42
	57
	59
	28
Inhibited growth	54
	97
	43
No effect	60

Effect	Strains	Control plate	Lawn plate
	75

In this set of experiment (Table 2.14), interaction of strain NES 140 and all other 11 strains was surveyed. NES 140 was found to inhibit the growth of strain NES 28, NES 54, and NES 97, probably due to substrates competition. There is some enhancement effect being observed in this experiment in NES 32, NES 36, NES 42, NES 57, and NES 59. In these sets, for NES 32 and NES 36, in control plates, growth was not observed in full. For NES 42, NES 57 and NES 59, there were growth as well in the control plate, but much more robust in the lawn plate. There are blank holes on the lawn being observed when culture NES 28 was plated together with NES 140 lawn. However, for NES 97, NES 140, there is reduced growth instead of blank hole being observed.

In the substrates testing, among these 12 strain, only NES 54, NES 97 and NES 140 was able to show growth on MSM plates after 3 days' incubation in section 2.3.1.1 however in this section, after 7 days of incubation, more strains were found to be able to grow on MSM control plates – NES 42 and NES 59. Considering that NES 42 is positive in FDH PCR, NES 59 is positive in CYP 153 PCR (refer Table 2.6), it is possible that these strain are capable of mineralizing hexane, but at a much slower rate comparatively, possibly belonging to the autochthonous (K-strategists) group.

Table 2.15 Summary of the interaction effect between strains. “N” indicates non-effect, “I” indicates inhibitory effect, “E” indicates enhancement effect.

Base strain on lawn plate	Strains (NES)											
	28	32	36	42	43	54	57	59	60	75	97	140
54	N	N	N	I	N	-	N	N	N	N	I	I
97	N	E	N	N	N	I	I	N	N	N	-	I
140	I	E	E	E	N	I	E	E	N	N	I	-

The above table (Table 2.15) summarizes the effect between, 3 “hexane degrading strains” and other strains. Much unpredicted phenomena have been discovered in this experiment. Their interaction between is very diversified, a lot more complex than imagine.

2.3.5.3 Liquid Co-Culture Interaction Study

In this liquid co-culture interaction study, NES 97 and NES 140 were used to form 2-strain co-culture with the rest of strains. They were cultivated with hexane as carbon source, and their formaldehyde removal was tested by colorimetric methods.

Figure 2.20 2-strains co-culture interaction experiment: strain NES 97 and the rest 11 strains. Grey bars represent formaldehyde removal of single strain culture; White bars represent formaldehyde removal of 2-strain co-culture.

In this set, NES 97 was mixed with all other 11 strains. In single strain set, NES 97 showed the highest removal efficiency, and NES 140 showed the second highest (refer Figure 2.20). This is consistent with the FDH PCR results in section 2.3.1.3.2, NES 97 showed strong positive results and NES 140 showed positive results. The formaldehyde removal capacities of the strains are quite likely to be resulted by FDH activity with the microbes.

Compare with single NES 97 strain’s formaldehyde removal. NES 32 and NES 36 might cause inhibition of formaldehyde removal, since the removal efficiency of co-culture are

much lower than the NES 97 single culture. Some of the strains slightly improved NES 97's activity when they are co-cultured, including NES 28, NES 42, NES 43 and NES 59.

Beside, NES 97 showed slightly decreased formaldehyde activity when co-cultured with NES 140 instead of adding up the removal efficiency, indicating they might be competing for substrate when co-cultured.

Figure 2.21 Two-strains co-culture interaction experiment: strain NES 140 and the rest 11 strains. White bars represent formaldehyde removal of single strain culture; grey bars represent formaldehyde removal of two-strain culture.

In this set, NES 140 was mixed with all other 11 strain. Almost all strain co-culture showed higher formaldehyde removal efficiency compare to NES 140 single strain, expect for NES 42 which showed approximately same removal efficiency (refer Figure 2.21). NES 97 showed decrease formaldehyde activity by about 12 % when co-cultured with NES 140. All other 10 strains showed increased activity when co-cultured with NES 140.

Table 2.16 Summarize of the interaction effect between strains. “N” indicates non-effect, “I” indicates inhibitory effect, “E” indicates enhancement effect.

Base strain in co-culture	Strains (NES)											
	28	32	36	42	43	54	57	59	60	75	97	140
97	E	I	I	E	E	N	N	E	N	N	-	N
140	E	E	E	N	E	E	E	E	E	E	I	-

The Table 2.16 summarizes the effect between 2-strain consortia in their formaldehyde removal. When compare the results between “on agar plate interaction study” and “in liquid co-culture interaction study”, the effect observed between each strain are not exactly the same. Considering the complexity of the microbial interaction and different categories of interaction involved with the culture, the culturing environment are quietly possibly affecting the result of interaction between the strains, for example, in liquid culture, signaling molecules or metabolic intermediates are easily diffused in liquid culture.

The enhancement effect can be resulted from metabolic cooperation of substrates, for example, Jobson et al. (1979) reported that sulfate-reducing bacteria (previously found not be able to grow on oils under anaerobic conditions) was found able to grow on the residues from the aerobic bacterial degradation of some oils. The growth of the sulfate-reducing bacteria was a result of biodegradation of metabolic intermediates of the aerobic bacterial culture.

In literature searching for antagonism effect between bacteria, other than competition for substrates (paper Role of competition for substrate in bacterial antagonism in the gut), most antagonism effect of bacterial was discovered in relation to pathogen, for example, one paper reported that (Jayanth, Jeyasekaran, & Shakila, 2002) some strains (e.g. *Alteromonas*) isolated from marine environment were found exhibited a wide antibacterial spectrum against human pathogens associated with seafood such as *Staphylococcus aureus*, *Salmonella typhi* and *Vibrio cholerae*. However, we believe these effect can be occurring between environmental strains as well, just not as many was studied and discovered yet.

Still, there is still a lot information remaining unknown inside the consortiums, for example, what is the populations' ration inside the consortium after these days of cultivation, what are the reasons for these population shift. In chapter 4, we trailed to develop a method for tracing population change within the co-culture to continue this study as the first step.

2.3.6 Testing on DGGE's Suitability for Population Monitoring

2.3.6.1 DGGE Method for Microbial Community Analysis

DGGE is one of the most commonly used methods among the culture-independent fingerprinting techniques. The denaturing agent causes DNA to melt at different stage, and as a result the melting DNA spreads over the gel to give a fingerprint of the community.

The fragments separated by DGGE can be excised, cloned and sequenced for identification. (Chaudhary et al., 2013). Moreover, it is a sensitive method, according to (Muyzer et al., 1993); it's possible to identify constituents that represent only 1% of the total community. Therefore, even the non-dominant microbial communities in sample are detectable.

However, there are also certain limitations associated with the DGGE process. These include requiring very high technical expertise during the experiment, and formation of primer dimmers, and improper staining (Chaudhary et al., 2013).

Moreover, the band on DGGE gel may not necessarily represent only one particular strain, and one particular strain' 16s rDNA amplicon may not only form one band on the DGGE gel. The reason responsible for multiple bands for single specie's sample is micro-heterogeneity, which is defined as slight differences in structure between essentially identical molecules, present in some strains' rRNA encoding genes (Muyzer & Smalla, 1998) and could subsequently lead to an overestimation of community diversity (Casamayor et al., 2002). The reason of different DNA sequences share the same motilities in the gel electrophoresis is identical GC consents between the DNAs. Therefore, when some gene shared the same GC content, one band may actually represent more than one species (Gelsomino, Keijzer-Wolters , Cacco, & van Elsas, 1999).

2.3.6.2 Preliminary Test with DGGE

In the application of this particular case, we are searching for a tool test the population dynamic within the microbial community. And the DGGE method would be tested to check whether it meet the requirement of tracking each strain's population change when cultivation in a consortium. Since in our simplified consortia, there are only 12 strains, they may or may not fall in the technical limitations of the DGGE (e.g multiple bands for one strain or shared band position with different strain) mentioned in the above section.

In this experiment, PCR amplicons are mixed to form a ladder, and the ladder and each individual amplicons are run under the same condition, so that each band's identity in the ladder can be referred by the individual amplicons on the same gel. And the overlap of bands and multiple bands from single strain would be identified from there.

Figure 2.22 DGGE of PCR product of gDNA from 12 strains and three identical ladder (mixture of all samples). The number above each lane indicates the PCR product is from which NES strain. "Triplicate Mix" represents three replicate of ladder.

Figure 2.23 Identity designated as NES strains of the bands on the mixed sample.

As shown in Figure 2.22 and Figure 2.23, the results indicate that gDNA sample from strain NES 42 and NES 60 yield two bands on different lanes. In the contrary, some of the different strains share the same band position, for example NES 32 and NES 43; NES 36, NES 42 and NES 60, NES 97 and NES 140, NES 30 and NES 54.

If each of the bands from the 12 strains would settle at different position of the ladder, then it would be an excellent method for population dynamics analysis. However, the results indicate the opposite. The multiple bands from one strain's gDNA and overlap bands from multiple strains' DNA defected out purpose of utilizing DGGE as the technique to relatively quantify and monitor each strain's population change over time.

Overall, the DGGE technique is not appropriate for our purpose of monitoring a community constituent with limited number of known strains it can only serve as a method to reflect a brief impression of the microbial diversity of the community.

2.3.7 Future Work

2.3.7.1 Main Achievements and Conclusions of the Project

In this chapter, we managed to develop a microbial consortium with 12 strains, which is highly efficient in removing indoor air pollutant formaldehyde (refer section 0). And this consortium demonstrated some sustainability in formaldehyde removal within changing environment (refer section 2.3.3.7). During the removal efficiency testing, we customized a colorimetric assay for dissolved formaldehyde concentration measurement (refer section 2.3.4). With the development of this colorimetric method, testing time and sample throughput are substantially enhanced. More combinations of consortium can be tested for their formaldehyde removal efficiency.

We also explored preliminarily the interaction between different strains during when they are co-cultured (refer section 2.3.5). It opens up more possibilities in future experiment, for example, maybe the strain inhibit NES 140 can be excluded for the co-culture and see the performance. Sometimes indirect interaction is also important. Perhaps, interaction within smaller group of co-culture can be studied.

It would be important to grasping the characteristic features of complex microbial communities to acquire insight into direct and indirect relationships among the members, in addition to the metabolic properties. Investigation of population change within those consortia might reveal important insights.

Therefore, we test the feasibility of use DGGE to track the 12 strains' population dynamics within the community, the results showed some strain has multiple bands on the gel, some strain has overlapped band positive with other strain (refer section 2.3.6). It was found not to be an appropriate method to fulfill this requirement.

2.3.7.2 Main Focus of the Future Work for this Project

In order to utilize a suitable technology for microbial dynamics in this system, we considered to employ digital PCR method in future work. Unlike real time quantitative PCR, digital PCR is a new technology that allows absolute quantification of target genes without any standards. The PCR reaction was conducted in micro chambers. The number of fluorescent chambers was counted which is either positive or negative (binary output). The amount of target DNA is estimated from the number of chambers containing amplification products on the basis of Poisson statistics, which can predict how input DNA is distributed into all small chambers. (Day, Dear, & McCaughan, 2013; Sedlak & Jerome, 2014)

There are a few commercial digital PCR platforms available in the market, such as QX200™ Droplet Digital PCR System (Bio-Rad), QuantStudio®3D Digital PCR System (Life Technologies), etc. There are slight differences between these platforms, for example, some generate nanolitre-volume droplet as the amplification reaction venue, some partition DNAs into nanolitre-volume reaction wells in a chip.

As part of the project, we conceptualized a system to test each strain's abundance within the community using one customized digital PCR system from our collaborator (Gong et al, personal communication; refer section 4.3.1). This instrument presents one significant difference from the commercial platform. The latter mostly requires pre-extraction of gDNA before loading the sample to the platform, but Gong et al's micro-fluidic qPCR device enables on-chip extraction of DNA. This implies that bacterial samples can be loaded onto the chip in cellular form and then its gDNA extracted directly on the chip through a series of automated fluid handling steps. The advantage we could expect from this feature include allowing the quantity of component strains of a co-culture to be digitally counted as a single gets trapped in a single well that would be subject to qPCR.

In chapter 4, this new technology that we are currently developing will be further elaborated on. With this method, it is hoped that in-depth investigation of the strains' population dynamics and interaction may be investigated.

Chapter 3. Co-culture Bioaugmentation for Removal of Pentachlorophenol in Anaerobic Membrane Bioreactors

3.1 Introduction

3.1.1 Wastewater and Wastewater Treatment

Wastewater is defined as water that has been used by households, industries, and commercial establishments, which is no longer useful and may contain contaminants. It is comprised of water from household sinks, washing machines and kitchen appliances, as well as water flushed from toilets, and therefore contains a combination of nutrients and chemicals. Industrial contributions to wastewater include carbon, nitrogen, and phosphorous nutrients, as well as other chemicals depending on the specific industry, which may sometimes be toxic and/or recalcitrant (US EPA, 2004).

When left untreated, these nutrients and chemicals enter natural water systems where they cause harm to the environment and human health (Emily, 2010). Environmental problems such as disruption of ecosystem balance and contamination of water bodies occur when wastewater treatment plants do not have the capacity to treat all of the wastewater that they collect, or when they fail to adequately treat wastewater due to function “upsets”. Today, adequate wastewater treatment is a major factor that contributes to freshwater quality and health of community. Hence, it is necessary to treat wastewater effectively to ensure the sustainable development of the society. Continual efforts are being made to enhance the efficiency of wastewater treatment systems worldwide (Malik, Hsu, Johnson, & de Sherbinin, 2015).

Wastewater treatment refers to a process whereby wastewater is converted into an effluent that can be either returned to the water cycle with minimal environmental issues, or safely reused. There are many ways to treat wastewater, for example, chemical treatment (oxidative processes), physical treatments (adsorption) and biological treatments (bio-transformation by microorganisms), which include both aerobic (e.g. activated sludge treatment, trickling filtration) and anaerobic (e.g. anaerobic digestion) methods (Samer, 2015).

Physical and chemical methods of wastewater removal are effective only if the effluent volume is small, which limits the use of these methods to small-scale removal. Another limiting factor of these methods is cost. This is true even for physico-chemical wastewater treatment in laboratory-scale studies, and thus have proven to be even more unsuitable to be used by large-scale industry (Robinson, McMullan, Marchant, & Nigam, 2001). The significant advantages that biological method has, over and above the other methods in terms of cost-effectiveness in treating large volumes of wastewater, have made it the mainstream approach towards wastewater treatment worldwide, including both aerobic and anaerobic methods.

3.1.2 Aerobic and Anaerobic Wastewater Treatment

Aerobic wastewater treatment is a process where bacteria degrade organic matter and other pollutants involved in various production systems through the utilization of oxygen. The degradation of organic matter is achieved in the presence of oxygen as it serves the role of an electron acceptor while organic materials are converted to carbon dioxide. Generally, large amounts of microbial biomass, i.e. sludge, are formed in such wastewater treatment processes. (Lee & Welander, 1996) Anaerobic digestion, on the other hand, involves the degradation and stabilization of organic materials in the absence of oxygen by microorganisms and leads to the formation of biogas (a mixture of carbon dioxide and methane) and also accumulation of sludge (Kelleher et al., 2002). The rate of microbial biomass accumulation in anaerobic processes is considerably lower compared to their aerobic counterparts, partly due to the channeling of carbon atoms to the formation of biogas, which is generated by bacteria drawing organic compounds from the biodegradable material in the wastewater. The majority of the biogas produced is methane, which can be stored and used for energy production (Lee & Welander, 1996). (Ghosh & Pohland, 1974; van Starckenburg, 1997).

Aerobic biological processes have been commonly used in the treatment of organic wastewaters for achieving high degree of treatment efficiency over 100 years, while in anaerobic treatment, considerable progress has been achieved in anaerobic biotechnology

for waste treatment based on the concept of resource recovery and utilization while still achieving the objective of pollution control over recent decades (Seghezzo, Zeeman, van Lier, Hamelers, & Lettinga, 1998; Yeoh, 1995).

Both treatment methods are capable of achieving high organic removal. In general, aerobic systems are suitable for the treatment of low strength wastewaters (biodegradable COD concentrations less than 1000 mg/l) while anaerobic systems are suitable for the treatment of high strength wastewaters (biodegradable COD concentrations about or over 4000 mg/l), which is the case in our application (refer section 3.3.1) (Chan, Chong, Law, & Hassell, 2009).

3.1.3 Some Advantages of Anaerobic Membrane Bioreactor (AnMBR)

One of the limiting factor of traditional anaerobic treatment was the intrinsic slow growth rate of the anaerobic microorganisms, which results in a large reactor volume necessary for wastewater treatment by anaerobic digestion. One advanced solutions to improve the efficiency of anaerobic treatment is to integrate anaerobic wastewater treatment reactor with membrane filtration process to form an anaerobic membrane bioreactor system.

In this system, the membrane filtration process can separate the treated wastewater from the anaerobic biomass and, at the same time, concentrate the biomass in the anaerobic bioreactor to a desired level. Thus, sludge (which contains the bulk of microbial biomass carrying out wastewater treatment processes) can be effectively retained inside the reactor due to the presence of the membrane, providing optimal conditions for organic matter degradation, and leading to cleaner effluent (Ozgun et al., 2013). These progresses and the potential of the AnMBR as an energy recovery technology have stimulated increased research interests in AnMBRs. (Chang, 2014)

3.1.4 Submerged Anaerobic Membrane Bioreactors (SAMBRs)

SAMBR is one type of AnMBRs, whereby the membrane can be immersed in the reactor tank itself. Anaerobic digestion of municipal wastewater in submerged anaerobic membrane bioreactors (SAMBRs) has attracted increasing attention in recent years due to

much progress in reducing hydraulic retention time, and thus reducing system size and increasing economic viability (Stuckey, 2012). Like other AnMBRs, the membrane in a SAMBR can prevent the slow-growing microorganisms from being washed out of the reactor, and thus increase the survival of bioaugmenters (Baraldi, Damianovic, Manfio, Foresti, & Vazoller, 2008). In our study, the bioaugmentative culture developed would be used in SAMBRs eventually.

3.1.5 Functions of Microorganisms Involved in Anaerobic Digestion And their Structure Dynamics During the Process

3.1.5.1 Function of Microorganisms Involved in Anaerobic Digestion

There are generally four stages of microbial metabolism in the anaerobic digestion process – hydrolysis, acidogenesis (fermentation), acetogenesis and methanogenesis (Lozano, Mendoza, de Arango, & Monroy, 2009). In these stages, various groups of microorganisms are functioning and cooperating with each other to achieve effective organic material utilization and methane production.

In the first stage, hydrolysis of complex carbohydrates or organic macromolecules to simpler molecules such as glucose occurs through the collective actions of a diverse array of microorganisms. Specialized groups of microorganisms may come into play here, depending on the starting substrates present. According to the pyrosequencing data from a full-scale anaerobic reactor, for instance, (Guo et al., 2015), bacteria from the genus *Halothermothrix* were predicted to be the main hydrolyzer of polymers to monomers which contribute to the dissolution of particulate materials in the sludge during this first stage.

Subsequently, some members in the sludge would bring the process into the acidogenic stage, whereby VFA, CO₂ and H₂ are produced as a result. The Bacteroidaceae family is one such group of bacteria, generally playing the important role in hydrolyzing and fermenting organic materials with the result of producing organic acids during the anaerobic digestion process. (Traversi, Villa, Lorenzi, Degan, & Gilli, 2012). Many members from the Firmicutes phylum were found to be able to degrade various VFAs,

taking on the metabolic steps that follow (Garcia-Peña, Parameswaran, Kang, Canul-Chan, & Krajmalnik-Brown, 2011).

At the third stage, the acetogenic group of bacteria starts to convert the products from the previous stage to acetate. Bacteria participating in this stage of anaerobic digestion have been found to be from many different genera. According to (Ragsdale & Pierce, 2008), there are more than 20 bacterial genera that include over a hundred of acetogenic species reported to date, including *Moorella*, *Sporomusa*, *Clostridium* and *Ruminococcus* to list just a few. (Drake, Gößner, & Daniel, 2008)

The last stage of anaerobic digestion is when methane production occurs. In this step, methanogens are the key microorganisms driving the process. Based on the study by Guo et al. (2015), *Methanosaeta*, *Methanospirillum*, *Methanosarcina*, *Methanoculleus* and *Methanoregula* are the important methanogenic genera that are found in the several full-scale anaerobic digestion sludges sampled from Beijing, China. Two of the genera are known to use acetate as substrate for methanogenesis: *Methanosaeta* and *Methanosarcina*. It has been established that *Methanosaeta* is a specialist that uses only acetate, whereas *Methanosarcina*, in contrast, is a generalist which can make use of a variety of other carbon sources besides acetate, such as methanol and methylamine, as well as hydrogen and carbon dioxide, for methane production (Liu & Whitman, 2008). *Proteobacteria* are also important microbes in methanogenesis process because most of *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria* are well-known glucose, propionate, butyrate, and acetate-utilizing microbial communities (Ariesyady, Ito, & Okabe, 2007).

3.1.5.2 Microbial Structure in Anaerobic Wastewater Treatment

Anaerobic Wastewater Treatment technology has become enormously popular in the past two decades, and knowledge of microbiological aspects of the technology has also accumulated significantly. Great advances have been made in elucidating the diversity of both cultured and uncultured microbes in anaerobic digestion processes (Narihiro & Sekiguchi, 2007).

As illustrated in the above section (section 3.1.5.1), there is great diversity of microorganisms involved in the various steps of the anaerobic digestion process. It is of great importance to study these main players in the process in order to understand and manipulate the process for better performance.

Great advances have been made in elucidating the diversity of both cultured and uncultured microbes in anaerobic digestion processes (Narihiro & Sekiguchi, 2007), since anaerobic wastewater treatment technology has become more and more popular in the past two decades, and knowledge of microbiological aspects of the technology has also accumulated significantly.

Previously, the microbial community composition was mostly being characterized by 16S rRNA gene cloning-based analyses of the microbial community. For example, in paper (Roest et al., 2005), the microbial structure was characterized by DGGE fingerprinting, and via cloning and sequencing of dominant amplicons from the bacterial and archaeal patterns. Recently, the metagenomic sequencing has been a popular method used to characterize microbial community structure of the anaerobic digestion sludge from wastewater treatment plant. For example, Guo et al. (2015) found that anaerobic digestion sludge contains highly complex microbial communities, which play crucial roles in sludge treatment. Taxonomic analysis indicated that overall bacteria were dominant whereas a considerable abundance of archaea were also detected in the anaerobic digestion sludge. Key microorganisms and related pathways involved in methanogenesis were further revealed as well.

In the study, we would use DGGE first to get an overall impression of the microbial dynamics during the acclimation process (refer section 3.3.2) and for the future, consider adopting a pyrosequencing method for more in-depth investigation (refer section 4.3.2).

3.1.6 Anaerobic Digestion of Wastewater Carrying Toxic Compounds

Domestic wastewater may contain remnants of pharmaceutical products and their metabolic intermediates due to widespread usage in the human population. Depending on

the origin of the industrial waste stream, they may also contain inhibitory or toxic substances such as ammonia, sulfide, heavy metals, and refractory organics. Considering that the primary workhorse in wastewater treatment processes are microorganisms, the inhibitory and toxic effects of these substances on the microbial community can have a spectrum of influence on the output of the treatment plant. Accumulation of these substances may therefore lead to what is commonly referred to as “reactor upset”. This may manifest in anaerobic wastewater treatment processes in the forms of reduced biogas production, altered biogas methane content, and inability to degrade organic matters further (Chen, Cheng, & Creamer, 2008). Hence, remediation of inhibitory or toxic compounds in wastewater is essential for maintaining the efficiency of wastewater treatment processes.

3.1.7 Pentachlorophenol (PCP) Biodegradation

3.1.7.1 Prevalence and Toxicity of PCP

PCP is one of the inhibitory substances that may be found in the wastewater from industrial sources. It is commonly used in wood manufacturing industries as a preservative and as a pesticide in agriculture (Bevenue & Beckman, 1967). The accumulation of PCP in water bodies and soil is often due to improper practice of discharging industrial wastes. PCP is considered as “recalcitrant” (Hattemer-Frey & Travis, 1989) due to its high toxicity and high chemical stability (Kitunen, Valo, & Salkinoja-Salonen, 1987).

In humans, exposure to PCP may result in adverse health effects, such as kidney and liver intoxication, thyroid hormone imbalance and the possibility of cancer (Orton, Lutz, Kloas, & Routledge, 2009; Park et al., 2007; van den Berg, 1990). Considering the significant health hazard of PCP and its generally inhibitory effects to microorganisms that are driving the various stages of anaerobic digestion, it is imperative that PCP be removed from wastewater from industrial sources, before discharging the effluent back to the environment.

Although there is specific requirement on PCP level in Singapore’s drinking water regulated by the Environmental Public Health (EPH) (Quality of Piped Drinking Water) Regulations (“Singapore Drinking Water Quality (July 2015 - June 2016) ”, 2016),

Singapore is not particularly polluted by PCP. However, in nearby country China, there are problems with PCP pollution in several major water bodies, such as Tai Lake and Yangtze River. (Yi Chen et al., 2016; Cui et al., 2017) Therefore, China could be the potential market of successful remediation technology of PCP.

3.1.7.2 Alternative Methods to Remove PCP

There are a variety of ways to remove PCP from contaminated materials. For example, Xue and Wang (2008) reported that PCP in aqueous solution can be removed by γ -irradiation treatment. In this case, the degradation efficiency was 100%, i.e. complete detoxification of a 75 mg/l PCP solution could be achieved, but requires an irradiation procedure at the dose of 15 kGy. Compared to such physical/chemical technologies which are not economical when carried out at the scale of wastewater volume flow, many researchers raised the use of bioremediation as the more cost-effective and desirable option (Kao, Chen, & Su, 2001; Kao & Wang, 2000).

3.1.7.3 Current state of the PCP Bioaugmentation Research

3.1.7.3.1 PCP Biodegradation Pathway

Microbial degradation of PCP has been studied in both aerobic and anaerobic systems over the past decades. While aerobic PCP biodegradation are more extensively examined (McAllister, Lee, & Trevors, 1996), a considerable number of researchers have nevertheless actively investigated anaerobic PCP degradation in aquatic, sludge, and soil environment (Abed et al., 2002; Litchfield & Rao, 1998; McAllister et al., 1996; Tartakovsky, Manuel, Beaumier, Greer, & Guiot, 2001).

Reductive dechlorination has been suggested to be the primary PCP biodegradation mechanism, which is defined as the replacement of a chlorine atom with hydrogen, releasing hydrogen chloride as the by-product. (Tartakovsky et al., 2001; Vallecillo, Garcia-Encina, & Pena, 1999). Under this pathway, PCP undergoes biodegradation in a stepwise manner forming various metabolic intermediates at different level of the pathway, including tetrachlorophenol (TeCP), trichlorophenol(TCP), dichlorophenol (DCP) and

chlorophenol (CP). These metabolic intermediates are toxic and can be accumulated in the environment (Xie, Abrahamsson, Fogelqvist, & Josefsson, 1986). The aromatic ring has to be totally dechlorinated prior to ring cleavage, as shown in Figure 3.1. Essentially PCP needs to be sequentially removed of the chlorine groups until eventually, it is reduced to phenol. Further degradation of phenol results in the production of methane and carbon dioxide (CO₂) (Vallecillo et al., 1999). As there are many established pathways and bacterial strains known to be able to degrade phenol further to substrates that can be fed into the subsequent stages of anaerobic digestion (Powlowski et al., 1994; Karlsson et al., 1999; Santos et al., 2004), the focus of this project shall be on the biotransformation of PCP up to phenol, which poses greater challenge for anaerobic communities generally maintained in wastewater treatment systems. Knowing the reductive dehalogenation pathway of PCP allows us to consider bioaugmentation not only to target PCP itself, but also its metabolites, to better exploit the possibility of interspecies cooperative metabolism.

In contrast to the anaerobic scenario, there are comparatively more types of pathways that bacteria can adopt under aerobic conditions for biodegradation of PCP. Ring cleavage can occur either before or after removal of the chlorine substituents. As a result, aerobic biodegradation of PCP tends to give rise to a larger array of intermediates of varying toxicity (Reddy & Gold, 2000). However, for the purpose of this study, we would not be considering these as they are only applicable in the aerobic context.

Figure 3.1 General pathway of reductive dehalogenation for PCP during anaerobic biodegradation: TeCP, tetrachlorophenol; TCP, trichlorophenol; DCP, dichlorophenol; CP, chlorophenol; CO₂, carbon dioxide; CH₄, methane. Figures are adapted from (Field & Sierra-Alvarez, 2008).

3.1.7.3.2 Various Researches on Different Topics Related to PCP (and other Chlorinated Compounds) Biodegradation

There were researches previously conducted in relation to PCP bioremediation, for example, one case is reported on a PCP-degrading bacterium isolated and identified as *P. mendocina* NSYSU. This single strain was tested when PCP was its sole source of carbon

and energy and caused the complete removal of PCP, but in a batch biodegradation experiments, not in a continuously functioning bioreactor setting (Kao et al., 2004).

Several other works have reported on the anaerobic treatment of wastewaters containing chlorinated compounds by different bioreactors (Farhadian, Ducheze, Vachelard, & Larroche, 2008), including anaerobic bioreactors with immobilized biomass (Khodadoust, Wagner, Suidan, & Brenner, 1997; Lanthier et al., 2005), fluidized bed biofilm reactors (FBBR) (Mun, He, & Ng, 2008), up-flow anaerobic sludge blanket reactors (UASB) (Shen, Liu, & Feng, 2005), and membrane anaerobic bioreactors which have been recently presented as an effective technology to treat toxic compounds (Lopez, Monsalvo, Puyol, Mohedano, & Rodriguez, 2013; Singhanian, Christophe, Perchet, Troquet, & Larroche, 2012).

Regardless of the type of anaerobic reactor employed, the key (design and operation) to an efficient system is the development of a protocol for the acclimatization and maintenance of adequate biological activity (Speece, 1996). In this project, we aim to develop an efficient microbial consortium for PCP bioaugmentation for eventual application to SAMBRs.

Besides researches on biodegradation of PCP or other chlorinated compounds. There are also researcher working on developing other tools to facilitate PCP bioremediation. For example, in paper (Dercová, Sejáková, Skokanová, Barančíková, & Makovníková, 2007), researcher used organomineral complex as a trap to facilitate PCP bioremediation by real soil sample as it reduces the potential toxicity of PCP to microbial community by lowering its bioavailability.

3.1.7.3.3 Activated Sludge as the seeding for SAMBRs

Activated sludge process is a biological wastewater treatment method in which a mixture of wastewater and activated sludge is agitated and aerated. During the process, activated sludge is combined with wastewater to form a mixed liquor (Davis & Cornwell, 1985) and a part of the activated sludge will be eluted. To maintain a constant biomass in the tank,

some of the eluted activated sludge will be treated and returned to the mixing tank while the excess activated sludge will be disposed as waste sludge (Manual, 1977).

For several reasons, even though activated sludge is derived from an aerobic setting, it is considered a good source of seeding for anaerobic membrane bioreactors. First, a considerable amount of anaerobic methanogenic bacteria can be found in activated sludge. Second, it is easy to obtain large amounts of the sludge from the numerous aerobic activated sludge plants available since it is the mainstream mode of wastewater treatment. Finally, the activated sludge usually contains little sand and soil and is mainly composed of both aerobic and anaerobic biomass, hence requiring no additional step of enrichment or sieving (Hänel & Hemmings, 1988; Lens, De Poorter, Cronenberg, & Verstraete, 1995). Therefore, for the purpose of our study, it would be used for seeding our bioreactors too, and put through acclimatization with the target compound PCP and its intermediates.

3.1.8 The Main Objectives and Structure of this Research

The overall aim of our laboratory's research team aims to develop a direct bioaugmentation strategy by introducing a bacterial consortium to degrade various industrial and municipal wastewater pollutants within the SAMBR, including PCP.

The sub-project in this chapter was designed to contribute to the team's main project with two main objectives: firstly, to develop preliminary consortia that could be used to degrade organic matter and reduce PCP concentration in wastewater from activated sludge and secondly, to briefly survey the population dynamics in our batch scale bioreactor which may be applied in future phases of bioaugmentation work.

3.1.8.1 The Difference of this Approach with the Approach in Chapter 2

Past studies have shown that activated sludge system contains highly diverse and dynamic microbial consortia (Ibarbalz, Figuerola, & Erijman, 2013). The carbon sources available in the sludge for is also very diverse, since they originate from the various the organic matters present in the wastewater. Consequently, the microbial community residing in the

sludge could further maintain its already-significant diversity. In contrast, the TPPB reactor serving as the source community for the first bioaugmentation case presented in chapter 2 has been fed with hexane as the sole carbon source for nearly a year (refer section 2.1.4), which very likely selected against some groups, resulting in their elimination over the time. At the point of drawing on the TPPB community as a source of bioaugmentative strains, we were already dealing with a community that has been reduced in complexity to some extent.

Considering that we would expect the sludge community serving the anaerobic bioreactor to be much more diverse and complex, it would be impractical to follow the approach in chapter 2, i.e. isolating many culturable strains for re-formulating a bioaugmentative consortium. It is also well known that microorganisms contributing to some of the key stages e.g. acidogenesis and methanogenesis, are difficult to cultivate as pure culture, since they function better in syntrophic mode of existence. Hence a different approach leveraging on relationship already established would be developed.

3.1.8.2 Development of Preliminary Consortia for Wastewater Treatment with Pollutant PCP

In the first stage of our study, activated sludge would be put through acclimatization of the target compound PCP and its various intermediates (refer section 3.3.1). During this process, several metabolic indicators would be closely monitored. After acclimatization has proceeded until reductive dechlorination could be observed in all the reactors to different extent, the sludge samples from these bioreactors would be applied in the next stage of investigation and used as the bioaugmentative biomass in the test batch reactor cultures for assessment of bioremediative performance. Brief microbial dynamics monitoring utilizing DGGE would also be conducted in this process (refer section 3.3.2) in order to get an overview of how much of the microbial community composition has been changed during the acclimatization process.

In the second stage, we mixed some of the bioaugmentative consortia to test efficiency of PCP removal. Since in previous stage, these consortia were only exposed to intermediates. In this stage, we explored the formula of mixed consortia and conditions for future SAMBRs application. The acclimated sludge from individual bioreactors B, E, F and G (refer section 3.2.2.1) were used individually as consortia from single bioreactor for real application; and four mixtures of sludge were made from bioaugmentative consortia from different bioreactors: B and G (BG), E and G (EG), F and G (FG), and B, E, F and G (BEFG). Each consortium and their mixture were tested with two different concentrations for their PCP reducing and biogas production performance in this set of experiment. (refer section 3.3.3)

In the meanwhile, we also wanted to simplify the community and finalize the consortium for SAMBR inoculation. The bioaugmentative consortia from single bioreactor were subject to a dilutions scheme to simplify the consortium-, followed by heat treatment to see if by eliminating non-spore forming strains would enhance the overall PCP removal of the co-culture (refer section 3.3.4). The final candidate consortia for SAMBR were selected after the simplification process and serial test on their biomass growth and PCP reducing performance.

While we are developing bioaugmentative consortia for SAMBRs, we also want to break down the community for strain isolation, and characterization of the community. This part is the preliminary work of strain isolation and strain interaction study.

3.2 Materials and Methods

3.2.1 Cultivation Condition and Media

3.2.1.1 Anaerobic Culture Condition

All bacterial cultures were cultured in anaerobic condition in this chapter. Bacteria culture media stock was prepared with flushing of nitrogen gas. Culture media was sealed in serum bottles for storage. Bacterial cultures were contained in Hungate tubes (16 x 125mm), which were autoclaved and flushed with nitrogen prior to inoculation. All bacterial cultures were incubated at 35°C with 120 revolutions per min (rpm).

3.2.1.2 Chlorophenol Stock Solution

PCP stock solution was prepared by dissolving PCP in 0.5 M NaOH (20 g/l). PCP metabolic intermediates were prepared using the same method to the final concentration of 20 mM and 100 mM respectively. These include 20 mM 2,4,6-trichlorophenol (2,4,6-TCP), 20 mM 2,3-dichlorophenol (2,3-DCP), 20 mM 2,5-dichlorophenol (2,5-DCP), 100 mM 2-chlorophenol (2-CP), 100 mM 3-chlorophenol (3-CP), 100 mM 4-chlorophenol (4-CP) and 100 mM phenol.

3.2.1.3 Cultivation Media

Desulfitobacterium frappieri medium (DSM) ("Desulfitobacterium frappieri medium," 2013) media contains the following (g/l): KH_2PO_4 , 0.27; K_2HPO_4 , 0.35; NH_4Cl , 0.53; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.10; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.002; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.07; Trace element solution, 0.128 ml/l; Resazurin, 1 ml/l; Yeast Extract, 1; Na_2SO_3 , 0.63, Na pyruvate, 6.05.

Synthetic Wastewater Feed Medium (SWF) (Kargi, Eker, & Uygur, 2005) was prepared as 100X stock media. Stock media was then diluted with MilliQ water to 10x and 1x. 1x Synthetic Media was used in reactor feed for maintenance of anaerobic sequencing batch reactors. 1X Synthetic Media contains the following (g/l): Peptone, 0.2; Nutrient

Broth/Beef Extract, 0.14; Urea, 0.01; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.004; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; K_2HPO_4 , 0.011; NaCl , 0.007; NaHCO_3 300mg/l.

3.2.2 Acclimatization with Each Chemical in the Biodegradation Pathway

In this experiment, 5 anaerobic sequencing batch reactors were set up with added PCP & its intermediates for acclimatization of activated sludge. And metabolic indicators are monitored during the acclimatization process, including COD, VFA and PCP (and its metabolic intermediates) concentration.

3.2.2.1 Anaerobic Sequencing Batch Reactor Maintenance

Anaerobic sequencing batch reactors were set up with capacity stated in Table 3.1. Reactors were maintained with continuous stirring at 35 °C. 1X SWF Media containing variable concentration of toxic compound (Table 3.1) with pH adjusted to 7.2-7.5 were prepared for the respective reactors. Feeds were administered to the reactors under concurrent flushing with nitrogen gas to maintain anaerobic condition.

Table 3.1 Five anaerobic sequencing batch reactors with list of toxic compound added to each reactor.

Name of Bioreactor	Type of refractory compound	Concentration of refractory compound added in stepwise fashion (μM)	Capacity
A (Control)	NA	NA	2L
B	PCP	2 to ~20	2L
E	2,4,6-TCP & DCP (2,3-DCP; 2,5-DCP)	10 to ~240	1L
F	CP (2-CP; 3-CP; 4-CP)	10 to ~240	1L
G	Phenol	20 to ~1000	1L

3.2.2.2 Metabolic Indicators' Analytical Procedures

5 ml of effluent samples were obtained from each reactor. Samples were centrifuged on Eppendorf 5810R at 4000 rpm for 5 minutes. Supernatants were collected and aliquoted for the respective metabolic indicator testing.

3.2.2.2.1 COD

COD sample were prepared with 2.5 ml of supernatant obtained from sludge samples, 1.5 ml of standard potassium dichromate digestion solution and 3.5 ml of sulphuric acid. COD mixture was incubated at 150 °C heating block for 2 hours. Results were read out with UV spectrophotometer at 420 nm and 600 nm.

3.2.2.2.2 VFA

VFA samples were prepared with 0.9 ml of supernatant obtained from sludge samples in 10% formic acid. Samples were filtered through 0.2 µm PTFE filter and analyzed with Agilent 7890A gas chromatography. VFA methods was adapted from (Siedlecka et al., 2008).

3.2.2.2.3 High-Performance Liquid Chromatography (HPLC)

HPLC samples were prepared with 0.5 ml of supernatant obtained from sludge samples in 50% methanol. Samples were filtered through 0.2 µm PTFE filter and analyzed with Shimadzu CBM 20A HPLC. Mobile phase consisted of 0.1% acetic acid in gradient of 40-90% methanol in MiliQ water.

To measure the concentration of the refractory compound, HPLC (Shimadzu, Japan) featuring an Agilent Poroshell 120EC-C18 (4.6x100 mm, 2.7 µm particle size) column was utilized. Different standards were established to measure the compound of interest. For culture medium containing PCP, we used a PCP standard to measure the concentration of PCP. A "CP-mixed" standard, consisting PCP, TCP, DCP, MCP and Phenol, was used to measure metabolites of PCP.

3.2.3 Test Population Dynamics with DGGE

3.2.3.1 DNA Extraction

In this experiment, sludge samples obtained from bioreactor were left to settle for 5 minutes, 1.5 ml of supernatant was obtained. Samples were centrifuged at 14000 rpm for 2 minutes, supernatant was discarded and approximately 0.1 g dry weight of cell pellet was obtained. Cell lysis was performed with bead beating in 3 rounds of 20 seconds. Cell pellet

was resuspended in cell lysis mixture containing 567 µl of TE buffer, 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K. Mixture was incubated at 37 °C for 1 hour. Cellular debris was removed by the addition of 100 µl of 5M NaCl and 80 µl of CTAB/NaCl solution, mixture was incubated at 65 °C for 30 minutes. 800 µl of chloroform/isoamyl alcohol was added and centrifuged at 14000 rpm for 5 minutes. DNA was precipitated with 600 µl of isopropanol and centrifuged at 14000 rpm for 5 minutes. Supernatant was discarded. DNA pellet was washed with 70% ethanol and centrifuged at 14000 rpm for 5 minutes. Supernatant was discarded and DNA pellet was air dried for 30 minutes. DNA extracted was resuspended in 50 µl of TE buffer and stored at -20°C (Wilson, 1987).

3.2.3.2 PCR for the Extracted DNA Sample

DNA extracted from the sludge samples obtained from the reactors were amplified with PCR. PCR products were then used for DGGE experiment. Each PCR reaction was performed in 50 µl reaction volume. Standard PCR mixture consists of 1X buffer with Mg²⁺ (Thermoscientific), 200 µM of dNTP (Thermoscientific), 0.4 µM of forward and reverse primer (Table 3.2), 1U of Kapa Taq (Thermoscientific), 400 ng/µl of DNA template and top up to 50 µl with PCR grade water.

Table 3.2 Details of the primer used for DGGE.

Primer	Sequence
Forward	5' CGC CCG CCG CGC CCC CGC CCC CCG CCG CCG CCC CCG CCT ACG GGA GGC AGC AG 3'
Reverse	5' CCG TCA ATT CMT TTG AGT TT 3'

PCR reaction was operated with S1000 Thermal Cycler (Biorad) with initial denaturation (3 minutes, 95 °C), followed by 30 cycles of denaturation (1 minute, 94 °C), annealing (1 minute, 50 °C), extension (1 minute, 72 °C), and final extension (30 minutes, 72 °C).

3.2.3.3 DGGE of the PCR Amplified Sample

100% denaturant stock solution was prepared with 40% formamide and 7M urea. 10% polyacrylamide gel was prepared with 35-55% denaturing gradient. Gel was then submerged in Universal Mutation Detection System (Biorad) with 7 litre of 1X TAE buffer. Electrophoresis was performed at 150 V for 10 hours. The internal standard is the sample from reactor A taken on Week 17 just for reference to see how the DGGE bands changes with time in different reactors.

3.2.4 PCP Removal Test of the Acclimatized Sludge and their Mixture

Batch bioaugmentation experiments were conducted in serum bottles to select for the best bioaugmentative consortia to add to the SAMBRs. Anaerobic sludge maintained in the lab with SWF (COD~ 460 mg/l) was used as the target for bioaugmentation. The acclimatized sludge B, E, F and G were used individually as consortia from single bioreactor; four mixtures of sludge: B and G (BG), E and G (EG), F and G (FG), and B, E, F and G (BEFG) were also tested as mixed consortia. Two different concentrations were tested for each set of consortium or mixtures: 10% and 30% of the total volatile suspended solid (VSS) (refer section 3.3.3 or the definition of VSS). In the mixture the sludge from different sources had an equal VSS. These sample were tested for their PCP removal and biogas production performance at the PCP concentration of 13 μ M in batch culture condition. One No Aug set contains base activated sludge and 13 μ M PCP, and one Control set contains only base activated sludge were included and tested for comparison.

Before the experiment, all sludge bioaugmentative consortia were washed and resuspended in an anaerobic mineral medium (MSM media prepare with flushing of nitrogen gas). Then, certain amounts of consortia, base sludge and synthetic media were pipetted into a 125-ml serum bottle under a N₂ atmosphere to give a total volume of 25 ml containing 1500 mg/l VSS, 500 mg/l COD and 13 μ M PCP. The serum bottles were sealed and incubated at 35 °C and 120 rpm. The gas production during the process was monitored daily up to 8/9 days, and at certain intervals 200 μ l samples were drawn from each bottle for the analysis of PCP concentration.

3.2.5 Simplify the Community and Finalize the Consortium for SAMBR

3.2.5.1 Dilution and Heat Treatment of Bioaugmentative Consortia

In this experiment, sludge sample of each bioaugmentative consortium from single bioreactor was subject to 8 times of 10-fold serial dilution. 1 ml of sludge sample was diluted with 9 ml of DSM for each 10-fold dilution. And all these diluted set were incubated in Hungate tubes and observed for biomass growth. The cultures were kept in an incubator shaker (INFORS HT, Switzerland) at 35 °C and set to 120 rpm for at least 12 days. Turbidity was also measured every two days to observe the growth rate by a turbidimeter 2100N (Hach, USA).

4 ml of 10^{-5} diluted sample were obtained from previous step. Half (2 ml) of the obtained sludge was subjected to heat at 80 °C for 30 minutes to retain spore-forming bacteria in the sludge while the non-heated sludge was used to observe the effect of non-spore forming bacteria.

3.2.5.2 Growth Test on the Diluted and Heat-treated Bioaugmentative Consortia

10^{-5} diluted sample, both heat-treated and non-heat treated, were tested for their fold change in culture turbidity using turbidimeter 2100N (Hach, USA) as an indication of biomass growth. Undiluted sludge were also cultivated as the control set. Turbidity of all these samples were measured on day 12.

Subculture of the diluted consortia (both heat treated and non-heat treated sets). Other than the subculture of consortia from 4 bioreactors, 4 combinations of subculture, BG, EG, FG and BEFG were also included. These two sets of samples were incubated with DSM (medium) in Hungate tubes. Consortia tubes and its duplicates were kept in an incubator shaker at 35°C and set to 120 rpm.

3.2.5.3 SAMBRs Set-up, Operation Condition for PCP Removal Test of Subcultures of Bioaugmentative Consortia

Several SAMBRs (effective volume of 3.2 litre) equipped with a Kubota microfiltration

membrane module (filtration area 0.11 m²) was set up in the laboratory and operated at 35 °C (via a water bath) to treat the SWF. The subcultures of diluted and heat-treated bioaugmentative consortia (obtained from section 1323.2.5.1) and their mixture were applied to the aliquots of the sludge and effluent of SAMBRs. After start-up, the hydraulic retention time (HRT) was reduced gradually from 24 h, to 12 h, and further down to 6 h until the reactor reached a steady state. The sludge retention time in the reactor was controlled at 213 days to minimize sludge production via wasting 30 ml of mixed liquor suspended solids every 2 days. In order to avoid frequent membrane cleaning, the flux was maintained constant at 5 l/m²-h. The transmembrane pressure, pH and oxidation-reduction potential were continuously monitored with a digital pressure gauge and a pH probe. At one stage after the 5-month mark, 10 µM of PCP was injected into the SAMBR to simulate the situation of a wastewater treatment plant being contaminated by PCP. At Day 175, the sample from SAMBRs were taken for PCP removal testing by HPLC method described in section 3.2.2.2.3.

3.3 Results and Discussion

3.3.1 Acclimatization of Activated Sludge with PCP and Its Metabolic Intermediates

The catabolism of PCP under anaerobic conditions requires the process of reductive dehalogenation to take place until all chlorine has been removed from the aromatic ring to give rise of phenol, as illustrated in Figure 3.1 earlier. The intermediates of this process, therefore, are TeCP, TCP and variously substituted DCPs/CPs. If reductive dehalogenation cannot reach completion in a wastewater treatment system, the remaining toxicants – PCP and/or its intermediates – would likely inhibit the growth and activities of some of the microorganisms driving the downstream processes of anaerobic digestion. This would have the consequence of the wastewater treatment system releasing effluents that still retain a high level of organics, and a reduction in the quantity of biogas produced. The challenge in handling PCP biodegradation arise from the lack of specific enzymes for the initial few steps of reductive dehalogenation. Since metabolic cooperation between bacterial strains carrying enzymes targeting different steps of the PCP degradation pathway could be a viable scenario, multiple acclimatization process occurring in parallel was attempted. Instead of solely acclimatizing with PCP, the activated sludge was independently acclimatized with TCP, a mixture of DCPs, a mixture of CPs and phenol. The latter series of sludge would, in principle, be able to select for the subpopulations capable of handling the respective intermediates, and serve as potential bioaugmentative partners to the PCP-acclimatized sludge.

The acclimatization was carried out in batch reactors set up with the designated toxicant groups as detailed in Table 3.1 (refer section 3.2.2.1). During the acclimatization process, indicators reflecting wastewater processing output were closely monitored to assess the metabolic and bioremediation activities in each reactor. The indicators used in this context were COD (chemical oxygen demand), VFA (volatile fatty acids), methane production and toxicant (PCP and its metabolic intermediates) concentration.

COD is an indication of the amount of organic compound present in the sample. The desirable endpoint of a wastewater treatment process would be a significant reduction of

COD in the effluent observed. VFA are volatile fatty acid with carbon chain of 6 or less, produced during sludge fermentation. As outline earlier in section 3.1.5, during anaerobic digestion, organic compounds are converted in a successive manner by different groups of microbes, and the last stage involves the methanogens. VFA accumulation is commonly associated with the inhibition of methanogens. Thus, we should expect low level of VFA in the effluent of bioremediation process that went to completion, i.e. achieving methane production (Bitton, 2005). In an ideal situation, the process should show low levels of COD, VFA and PCP in the effluent and a high level of methane production. At the beginning of the acclimatization process, concentration of each toxicant group would be increased gradually to test the tolerance threshold (based on observation of the metabolic indicators) of the sludge. As the concentration increased, a point would arise whereby the anaerobic process clearly became compromised, and thereafter, the toxicant concentrations would be maintained and not increased further.

Figure 3.2 Metabolic indicators measured for bioreactor A (the control set) during 50 weeks (a) COD of both influent and effluent of reactor A (b) VFA accumulation of the effluent in reactor A (c) Methane production of reactor A. Data presented were from single sampling obtained between week 1 and week 50.

Reactor A served as the control which contained only the sludge under the operating condition adopted by our laboratory to simulate anaerobic wastewater treatment, with no toxic compounds added. Since this sludge was obtained from the municipal water treatment plant, it was expected to be able to remove most of the COD within the synthetic wastewater media formulated. As shown in Figure 3.2, the COD level in the effluent was significantly reduced from the influent level, affirming that the sludge in reactor A was able to remove most of the organic compounds fed into the sludge over the 50 weeks of observation. The effluent VFA level remained at below 1 mM and methane production was within the range of 400-600 ml.

Figure 3.3 Metabolic indicators measured for bioreactor B during 50 weeks (a) COD of both influent and effluent of reactor B (b) VFA accumulation of the effluent in reactor B (c) Methane production of reactor B. (d) PCP concentration in both influent and effluent of reactor B. Data presented were from single sampling obtained between week 1 and week 50.

Reactor B contained sludge which was laced with PCP, starting at Week 5 and gradually reaching a concentration of 40 μM on Week 8. At that point, it was clear that the bioprocessing capacity of the reactor has been “upset”, showing concurrent increases in effluent COD and VFA concentrations, and a decrease in methane production. A direct check of the effluent PCP concentration showed that indeed it has significantly accumulated in the bioreactor. This PCP concentration was maintained to acclimatize the

sludge community, and the bioreactor continued to be monitored. The non-productive state of wastewater processing continued up to Week 24, after which the system appeared to recover slightly in terms of methane production (with concurrent reduction in effluent VFA) but even at Week 40, the level remained at approximately 80 ml, still 5-6 fold lower than that of the control reactor (Figure 3.2). The restoration of COD removal never quite occurred even at Week 40. It seemed that sludge community have not managed to enrich for an effective PCP-degrading subpopulation, although the improvement in methane production (fairly low but sustained) suggested that some groups of microorganisms with possibly greater tolerance to PCP that could contribute towards that outcome have been selected.

The methane yield (per COD removed) was slightly increased over the PCP acclimatization process. Many factors can affect the methane yield (per COD removed), in practice the amount of methane recovered per gram of COD removed will probably be slightly less than the theoretical methane production of anaerobic process as some of the COD may be used as source of microbial growth, there are microbes that consume COD and compete with methane producing bacteria. The possible reason in our case is that biomass in the reactor were growing rapidly right after the inoculation, and by the end of the acclimatization the growth of the microbes reached a stationary phase. Therefore, more COD removed can be used for methanogenesis and resulted a slightly higher COD yield.

Figure 3.4 Metabolic indicators measured for bioreactor E during 50 weeks (a) COD of both influent and effluent of reactor E (b) VFA accumulation of the effluent in reactor E (c) Methane production of reactor E. (d) Concentration of PCP metabolic intermediates in both influent and effluent of reactor E. Data presented were from single sampling obtained between week 1 and week 50.

In a similar manner, Reactor E at Week 5 was injected with three intermediates: 2,4,6-TCP, 2,3-DCP, and 2,5-DCP and its metabolic indicators were monitored for over 50

weeks. COD removal was impaired when concentration of toxicants added at Week 8 reached 210 μM as shown in

Figure 3.4, after week 8, both COD and VFA concentrations in the effluent started to accumulate, and methane production remained low at below the 200 ml mark. The recovery of the system in terms of COD removal efficiency and methane production came about from Week 20 onwards, but only fully returned to the level comparable to the control Reactor A at Week 32. This also corresponded to the elimination of TCP and DCPs altogether (refer panel (d) of Figure 3.4). The various PCP metabolites decreased at different rates but eventually at Week 32, almost all of the TCP/DCPs have been metabolized.

Figure 3.5. Metabolic indicators measured for bioreactor F during 50 weeks (a) COD of both influent and effluent of reactor F (b) VFA accumulation of the effluent in reactor F (c) Methane production of reactor F. (d) Concentration of PCP metabolic intermediates in both influent and effluent of reactor F. Data presented were from single sampling obtained between week 1 and week 50.

Reactor F contained a mixture of 2-CP, 3-CP, 4-CP, which were gradually increased over Weeks 5 to 10 to reach 670 μM , at which point the negative effect it has on the bioreactor's

COD removal function became very apparent. Figure 3.5 showed that COD in effluent started to build up gradually since week 6. VFA concentration had also built up to a significant level by week 10. The adverse effect on methane production, however, was not significant, implying that a subgroup of methanogens may have been able to exploit some side processes not affected by VFA accumulation to generate methane. The recovery of the function of the system could be observed from Week 16 onwards, and this was reinforced by the fact that the concentration of one of the metabolites, 2-CP, in the effluent were rapidly decreasing, and were eliminated by Week 20. The remaining two toxicants, 3- and 4-CPs took longer but eventually also dropped to negligible level by Week 38.

Figure 3.6 Metabolic indicators measured for bioreactor G during 50 weeks (a) COD of both influent and effluent of reactor G (b) VFA accumulation of the effluent in reactor G (c) Methane production of reactor G. (d) Phenol concentration in both influent and effluent of reactor G. Data presented were from single sampling obtained between week 1 and week 50.

In Reactor G, which was acclimatized with phenol gradually to a concentration of 1230 μM by Week 10, a high degree of COD removal could be observed, comparable to that of the control Reactor A (compare Figure 3.6 to Figure 3.1). VFA accumulation was absent from the entire experiment. The phenol concentration in the influent in fact took some time

to build up despite the addition of over 1000 μM as early as Week 10, implying that fairly rapid means of removing phenol existed in the sludge community.

Overall, all the reactors were able to develop their microbial communities into one that were better able to handle the toxicants compared to the original sludge. Although the community in Reactor B, for example, could not entirely mitigate the inhibitory effects of PCP, some recovery in methane production manifested. For its metabolic intermediates, more promising acclimatized communities appeared to have been generated although the duration of time to bring about full recovery varied.

3.3.2 Preliminary Check of Community Dynamics Using DGGE

It is generally an accepted fact that bacterial community structure and diversity influences wastewater treatment output, stability and robustness. The changes in metabolic indicators observed during the acclimatization stages in Section 3.3.1 therefore imply that the community structure within these bioreactors have shifted. As the eventual purpose of the acclimatized sludge community was to serve as the source for developing bioaugmentative cultures, it would be good to check that there were indeed significant changes in the community make-up, to distinguish from the remote (but not unthinkable) possibility that the altered outcomes in these reactors were simply due to changes in gene expression with no effect on community composition per se.

Unlike the case of the TPPB source community, the sludge community is more complex with a large proportion of unculturable microorganisms. Hence the molecular means of survey was adopted, in the form of DGGE. Key time points/periods identified through monitoring the acclimatization process were selected to be put through DGGE analysis.

Figure 3.7 No toxic compound was added to reactor A, which is used as a control. Variable concentration of PCP was added to reactor B. Green arrows indicate the first event of gain band and red arrow indicate first event of lost band.

Reactor A was the control carrying no toxic compound. Therefore, acclimatization only occurs in response to the culture conditions and nutrients provided with the setup of the anaerobic sequencing batch reactor. The changes observed in reactor A are considered as the basis of acclimatization without toxicity induced. It was observed that a few new bands appeared during the acclimatization – band 2 and 4 appeared in week 20, band 5 and 6 appeared in week 28 after acclimatization. All four strains remained present up to the latest sampled culture. The sludge environment in our batch reactor have clearly been conducive for the growth and survival of these strains. These are expected to be the main workhorse serving basic functions in the sludge for anaerobic digestion over the four processes of hydrolysis, fermentation, acetogenesis and methanogenesis. To identify these strains, the bands could eventually be collected and the DNA extracted for further sequencing.

In reactor B, PCP had been added, and it had been exerting inhibitory effects on the microbial community. Different extents of acclimatization were expected to be reflected in DGGE profile (Figure 3.7). The presences of bands 1, 2, 4 and 5 seemed similar to those in reactor A. These bands appeared in the initial set up when concentration of PCP was low. With increasing concentration of PCP added to reactor B, further appearance of bands 8, 10 and 13 were observed, which could be due to an increase in the proportion of strains that possess surviving traits in environment with PCP, i.e having tolerance to and/or catabolic capability towards PCP.

Figure 3.8 2, 4, 6-trichlorophenol, 2, 3-dichlorophenol and 2,5-dichlorophenol were added to reactor E, 2-monochlorophenol, 3-monochlorophenol, 4-monochlorophenol were added to reactor F and phenol was added to reactor G. Green arrows indicate the first event of gain band and red arrow indicate first event of lost band.

In both reactor E and reactor F, the DGGE tested between week 20 to 35 and week 15 to 25 respectively of acclimatization revealed active changes in microbial community in response to increasing concentration of toxicants added (Figure 3.8). Some of the bands appeared during the process and some of the bands disappeared during the process, indicating dynamic microbial population shifts.

In reactor G, a total of 5 gains of bands were observed, and no loss of bands event (Figure 3.8). Reactor G shows that the rapid changes in microbial community by gaining 5 bands in the span of 10 weeks were beneficial to its metabolic activities, since the phenol removal were kept effective during the acclimatization. Increasing complexity of the microbial community in reactor G may be a crucial factor that contributed to the successful acclimatization to phenol. The presence of difference species provides greater diversity in biochemical pathways available that are able to carry out biodegradation to completion successfully.

Similarities between the banding patterns generated by PCR-DGGE of the samples during acclimatization were analyzed using the Pearson correlation coefficient and the results were displayed graphically as a dendrogram. The clustering algorithms used to calculate the dendrograms was an unweighted pair group method with arithmetic averages (UPGMA). The cluster analysis and dendrogram generation were carried out by using the Bionumerics software (Applied Maths, Austin, Texas, USA).

Figure 3.9 UPGMA dendrogram of DGGE profiles of amplified 16S rDNA of the total bacterial community from sludge obtained at different acclimatizing stages using BioNumerics7.6 software. The cluster tree images are aligned alongside (a) clustering of samples from reactor A (b) clustering of samples from reactor B (c) Clustering of samples from reactor E (d) Clustering of samples from reactor F (e) Clustering of samples from reactor G.

The similarities between the DGGE patterns produced from fingerprints of the various amplicons for each reactor was calculated and visualized as a clustered tree to evaluate the dynamics within the groups of bacteria. Clear differences in bacterial community structure

were observed from each point sampled during the acclimatization process, which indicates that the added PCP and its intermediates are the driving force for bacterial selection and adaptation.

Shannon-Wiener diversity calculation: The Shannon-Wiener index of diversity was used to determine the diversity of communities sampled from each bioreactor from different weeks. This index was calculated by the following equation: Shannon-Wiener index using Bionumerics software (Applied Maths, Austin, Texas, USA).

$$H = - \sum_{i=1}^s (P_i * \ln P_i)$$

Table 3.3 Shannon-Wiener diversity index of each sample calculated using software BioNumerics7.6.

Reac tor A	Shannon diversity Index	Reac tor B	Shannon diversity Index	Reac tor E	Shannon diversity Index	Reac tor F	Shannon diversity Index	Reac torG	Shannon diversity Index
W0	0.818	W0	0.6847	W5	0.8164	W5	0.8024	W5	0.6
W7	0.8447	W7	0.8929	W20	0.9756	W15	0.8237	W10	0.8711
W12	0.9226	W12	0.8782	W35	0.8325	W25	0.7851	W15	0.8948
W20	0.765	W20	0.8184						
W28	1.0078	W28	0.9105						
W34	0.7389	W34	0.9643						

In the present study, the Shannon-Wiener index of diversity was applied to 16S rDNA genes from bioreactor communities that were separated on denaturing gels according to their sequence heterogeneity. Using both the total number of DGGE bands and their relative intensities, it was possible to calculate the bacterial diversity index. This would in theory reflect on sample diversity without the need for cultivation. As suggested in Figure 3.7 and Figure 3.8, some of the bands appeared and some of the bands disappeared during the acclimatization. In general, in all bioreactors, there were increase in diversity observed

within the first few weeks after the sludge acclimatization, thereafter, the diversity starts to fluctuate in reaction to the different chemicals in the influent.

This DGGE experiments gave a brief overview of the microbial dynamics during the acclimatization process using PCP and its metabolic intermediates (TCP, DCPs, CPs and phenol). Changes in the microbial community can range from biochemical changes in the indigenous community to changes in proportion of the selected subpopulation (Lederberg, 1949; Lin et al., 2009). These changes can lead to gain of functions such as tolerance and degradation of toxic compound. The process of acclimatization will also eliminate microorganisms that lack the ability to adapt to the environment. The data obtained from DGGE analysis gave us the confidence that sufficient changes has occurred in the composition of the consortia in the various reactors as a result of acclimatization. Based on the dynamics revealed by DGGE, the sludge for Reactor B at Week 30, that for Reactor E at Week 35, for Reactor F at Week 25 and Reactor G at Week 10 would be used in the later experiments requiring bioaugmentative consortia from the acclimatized Reactor sludges.

3.3.2.1 Pyrosequencing

Although we managed to profile the dynamics of community structure during the acclimatization process from the DGGE results, the information collected is far from detailed and in-depth. Hence a side-project in our laboratory has been set up to explore the possibility of using a next generation sequencing technology: pyrosequencing to get a profile of the ecological content of our anaerobic bioreactor.

3.3.3 PCP Removal and Biogas Production Test of the Sludge from each Reactor and their Mixture

In this section, the acclimatized sludges from each bioreactor from section 3.3.1 were tested for their biodegradation capacity of PCP under different conditions, and they will be referred to as bioaugmentative sludge, as the microbial consortia within the sludge are expected to be able to augment the performance of the reactors. Two different quantity

based on the VSS (volatile suspended solid) of bioaugmentative sludges would be used to assess their suitability in future applications.

Total suspended solids (TSS) refers to the concentration of suspended (non-soluble) solids in the sludge, including silt, clay, plankton, organic wastes, and inorganic precipitates. VSS is the loss on ignition of TSS, representing the non-ash part of the total suspended solids, and it relates closely to biomass growth (Queenan, Burton, & Bechir, 1996). Here we use the VSS to quantify the biomass added for each experiment. In the bioaugmentative consortia from single bioreactor experiment, 10% VSS and 30% VSS from each reactor were added to the anaerobic wastewater treatment sludge maintained in our laboratory and used to seed the SAMBR in the eventual application. These augmented sludge samples were then tested for their efficacy to remove PCP from starting concentration of 13 μM , then analyzed for PCP concentration over 14 days.

Figure 3.10 Upper figure: PCP concentrations in bioaugmented reactors with different amount and source of bioaugmentative sludges from day 0 to day 14. The error bars represent the range of the duplicate measurements. Lower figure: Cumulative PCP removal display of the same data of the upper figure, the error bars represent the range of the duplicate measurements.

The PCP concentration detected in the samples over several time points are presented in Figure 3.10. Comparing the data sets from day 0 and day 3, it could be noted that reactors augmented with E showed significant PCP removal, in particular the ones augmented with 30% of sludge E – as much as 30% of the PCP had been removed within the short span of 3 days. Sludges augmented with 30% G also exhibited significant degree of PCP removal. All other samples showed slight (and insignificant) decrease in the PCP concentration during the 3 days.

After Day 6, sludges augmented with F also presented a significant decrease in PCP concentration. Those augmented with 30% E and 30% G continued to show a steady level of PCP reduction throughout these 14 days. By the end of this experiment, the 30% E augmented sludge has achieved the highest PCP removal (41.4%). The sludges augmented with B (both 10% and 30%) showed no significant PCP removal, even though it was acclimatized directly with PCP itself.

Figure 3.11 Cumulative biogas production (as the ratio relative to the controls V_t/V_c , V_t stands for Volume testing, and V_c stands for Volume control) in batch reactors bioaugmented with A) 10% acclimatized sludge, and B) 30% acclimatized sludge. The error bars represent the range of the duplicate measurements.

Figure 3.11 shows the cumulative biogas production ratio in batch reactors bioaugmented with the acclimatized sludge. The No Aug. group represent the sample tested without any bioaugmentative consortium, and the Ctrl group represents the sample tested without any PCP added in the activated sludge. For reactors supplemented with 10% bioaugmentative

sludge, the E-augmented sample produced the most biogas among all the reactors, although the restoration of methane production was unable to reach the control level in the absence of PCP. The next highest biogas producer was reactors augmented with G. Similarly in the 30% bioaugmented sludge set of data, over the 9 days observed for biogas production, reactors augmented with 30% acclimatized sludge E produced more biogas than any other bioreactors. The biogas production in reactors augmented with sludge B, either at 10% or 30% of the total VSS, was not significantly different from the non-bioaugmented control, implying that sludge B provides limited bioaugmentation effect with respect to bio-gas production capacity.

Since one motivation of acclimatizing with intermediates of PCP degradation pathway was so that bioaugmentation based on metabolic cooperation may potentially be applied, two or more of the bioaugmentative sludges were mixed. Similar to the single sludge bioaugmentation procedure, 10% VSS and 30% VSS of the mixtures were tested for their PCP removal performance, under the same test condition as before, with PCP removal monitored over 14 days.

Figure 3.12 Upper figure: PCP concentrations in bioaugmented reactors with different amount and combination of bioaugmentative sludges from day 0 today 14. The error bars represent the range of the duplicate measurements. Lower figure: Cumulative PCP removal display of the same data of the upper figure, the error bars represent the range of the duplicate measurements.

The PCP degradation profile (Figure 3.12) was similar to what had been observed previously in bioaugmentation with a single sludge. The reactors augmented with EG

exhibited the highest PCP removal among all. In particular, the ones augmented with 30% EG showed as much as 43.5% removal of PCP by the end of 14 days. Reactors augmented with 30% BG and 30% BEFG also showed significant and a promising degree of PCP removal. The reactors augmented with FG and without any bioaugmentative consortium were not significantly altered in PCP removal compared to the non-augmented control.

Figure 3.13 Cumulative biogas production (as the ratio relative to the controls V_t/V_c V_t stands for Volume testing, and V_c stands for Volume control) in batch reactors bioaugmented with A) 10% multiple sludges, and B) 30% multiple sludges. The error bars represent the range of the duplicate measurements.

Bioaugmentation by mixed sludges were further assessed using biogas production as the monitoring parameter, similar to the single sludge augmentation series of experiments. It was observed that bioreactors augmented with the same sludge mixture but at higher concentrations (30% of bioaugmentative consortia) produced more biogas than the sludge with lower amount of consortia (10%) for all mixtures. Among all the augmented reactors, the ones augmented with 30% EG were the best to maintain comparable biogas production efficiency as the control without PCP inhibition, it achieved the same level of gas production as the control set, by the end of Day 8.

The statistical analysis was performed using Quade test to compare whether there is statistically significant difference among four of the best performing consortia, including

30% E, 30% G, 30% BG and 30% EG, in terms of both PCP removal and biogas production, and the result is stated in the above table.(Quade, 1979) According to the analysis, although 30% EG group showed the highest in PCP removal percentage, the statistical analysis result (p -value = 0.6014) did not suggest significant difference between the four most efficient consortia (including 30% E, 30% G, 30% BG, and the 30% EG). Similarly, in gas production, the p -value = 0.7002 did not suggest significant difference between these four sample as well.

It is possible that the relatively poor performance observed in B was due to the complicated degradation steps of PCP anaerobic biodegradation. But the rest of the consortia are all degrading PCP at this section (section 3.3.3), so the biodegradation processes these sample undergo would be the same, and the four best performing consortia (30% E, 30% G, 30% BG, and the 30% EG) still out-performed the 30% B consortium. Extending the acclimatization time in section 3.3.1 might help increase the PCP removal efficiency of the consortium B, but it's also very difficult to arbitrarily set a time frame for PCP and keep "fairness" for the rest intermediates.

In this experiment, we found that the 4 above mention consortia out-performed others in term of both PCP removal and biogas production. It is interesting to note that they worked better than bioaugmentative sludge B, which was a consortium directly acclimatized by PCP (refer Section 3.3.1). This implies that selecting for a complete PCP-degrading subpopulation directly may be more challenging than a peripheral strategy of mixing independent consortia acclimatized to the metabolic intermediates (e.g. TCP, DCPs and CPs) of the recalcitrant compound (PCP). Interesting, although sludge B on its own were unable to enhance PCP removal to the reactor sludge, combining it with the sludge G had a synergistic effect, achieving higher bioaugmentation than what G could bring about on its own. Considering that G was acclimatized with phenol, which is a metabolite several steps downstream in the biodegradation of PCP, we may infer that the synergy created are likely to be not just due to sequential enhancement of each step of the metabolic pathway. Instead, it may have to do with the diversity (more bacterial strains are able to degrade phenol) juxtaposing to create more permutation of viable metabolic cooperation routes.

3.3.4 Simplify the Community and Finalize the Consortium for SAMBR

The ultimate desirable outcome from this study would be the development of a bioaugmentative consortium that could be maintained and reproducibly applied. The common failings of consortia-based bioaugmentation are that, while they have a higher frequency of success compared to pure-culture bioaugmentation because of the robustness accorded by the community diversity, the very complexity also subject it to lesser predictability. In the previous section, the sludges were applied without much treatment and some showed considerable bioaugmentative potential. However, if they are to be applied at a larger scale or multiply, there has to be some means of reliably increasing the bioaugmentative biomass. Sludge may be maintained in bioreactors set to conditions that maintain steady state for the community, but that tends to be dynamic. If a core consortium less the particulate impurities and complicating factors from a sludge could be developed, it may be more conducive to seed an anaerobic wastewater treatment system requiring bioaugmentation from such a more “controlled” consortium. To calibrate the possible balance between a sufficiently complex and therefore robust bioaugmentative community against the core collection allowing sustained co-culture growth and augmentative functions, a simplification procedure through serial dilution was attempted.

Figure 3.14 illustrates the general steps involved in this attempt. The first step involved the 10-fold serial dilution of the sludge samples from the acclimatized reactors B, E, F, and G, up to 10^8 dilution, in triplicates. Each round of dilution would have reduced the complexity to some extent, but the replicates are likely to diverge in performance by nature of aliquot heterogeneity of a non-homogenous complex community. In the second stage, the replicates of each dilution would then be checked for the bioaugmentative activity and the dilution last retaining the activity in all three replicates would be noted. Using this dilution of consortia, a heat treatment alternative would be tested, as some groups of bacteria involved in anaerobic processes are known to be heat resistant (Juteau, Beaudet, McSween, Lépine, & Bisailon, 1995 136; Villemur, 2013 2013) The resultant consortia and their combinations would then be tested for growth on and removal efficiency of PCP.

Figure 3.14 Flow of steps to make final consortia for SMABRs.

Functionally important population being diluted out in this approach is one of the concerns, therefore, we only dilute to the minimum concentration that all three replicated samples would grow with DSM with PCP, to make sure the diluted consortia do possess basic function of PCP tolerance/degradation capacity. This selection standard lessened the possibility of sifting out the functionally important strains after the screening.

3.3.4.1 Dilution and Heat Treatment of Bioaugmentative Consortia

The sludges from acclimatized reactors B, E, F and G at the Weeks identified to be suitable for bioaugmentation (refer section 3.3.1 and 3.2.2) were serially diluted in triplicates and their growth in DSM with PCP were checked. Only if growth was observed in all 3 replicates of a particular dilution would be documented as a “+” in Table 3.4. The data show that 10^5 fold is most likely the threshold dilution that captures the majority of the core groups of microorganisms necessary for PCP removal under anaerobic context.

Table 3.4 Growth of bioaugmentative consortia on PCP after serial dilution. “+” represents positive for all 3 replicates in growth on PCP, “-” represents negative in any one of the triplicates growth on PCP.

Sludge	Dilution							
	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
B	+	+	+	+	+	+	-	-
E	+	+	+	+	+	-	-	-
F	+	+	+	+	+	+	-	-
G	+	+	+	+	+	-	-	-

This 10⁵ dilution cultures were then put through heat treatment as described in section 3.2.5.1. Spore-forming bacteria refers to bacteria that are able to form spores and hence can withstand extreme environmental conditions (e.g. heat). This in a way enhances the sustainability of the bioaugmentation process, which is one of the characteristic that we are looking for in the project. Another important reason for eliminate the complexity of the community based on their spore-forming ability is that recent papers have shown that several spore-forming bacteria are able to degrade PCP and its metabolites via the reductive dehalogenation pathway and *desulfitobacterium hafniense* PCP-1 is one of them (Madsen & Aamand, 1992; Villemur, 2013; X. Zhang & Wiegel, 1990). Even with heat treatment, spore-forming bacteria PCP-1 is still able to carry out its degradation function (Juteau et al., 1995). This additional parameter provides alternative clue to select potential spore-forming bacteria that can degrade PCP and its metabolites.

Half volumes of each diluted sludge sample were subjected to heat at 80°C for 30 minutes and the subsequent growth in DSM in the presence of PCP were monitored. The data are shown in Figure 3.15.

Figure 3.15 Fold change in culture turbidity as growth indicator of undiluted sludge, 10⁻⁵ dilution of non-heat treated and 10⁻⁵ dilution of heat treated cultures in DSM supplemented with PCP. The error bars represent the range of the duplicate measurements.

In the growth test of the 10⁻⁵ dilution sample (both heated and non-heated), diluted bioaugmentative consortia from reactors B, E, and F was found to have good tolerance (shows growth even in PCP), but not for the diluted consortia from reactors G. The latter observation was noted, despite that fact that it actually has high PCP removal and retention of methane production, particularly in combination with others (refer section 3.3.3). Diluted consortia from reactors F has considerable heat-resistance subpopulation compared to others. The mixture of BG, EG, and BEFG was found to have potential to be applied on SAMBR. The data here in relation of the earlier results (refer section 3.3.3) underscores the importance to consider bioaugmentation not only from the perspective of PCP removal (bioremediation) but also with respect to the retention of main function of the bioreactors (COD removal and biogas production). If we relied only on this set of data to screen out potential bioaugmentative consortia, G would have missed our mark due to its apparent low level of “work done on PCP biodegradation”. However, as evidenced from actual performance testing in a situation closer to the final application (anaerobic wastewater

treatment resulting in biogas production) in Figure 3.13, G might turn out to be an effective candidate to be mixed in for bioaugmentation application.

The 10^5 dilution consortia will be referred to as sub-cultures (SC) in subsequent experiments where they were applied to aliquots of the sludge and effluent of SAMBR contaminated with PCP.

3.3.4.2 PCP Removal Test on the Subcultures of Diluted and Heat-treated Bioaugmentation Consortia

As a part of the larger research programme, our laboratory has in operation several SAMBRs for over more than 6 months (Xiao, Yaohari, De Araujo, Sze, & Stuckey, 2016), the settings of which is described in section 3.2.5.3. At one stage after the 5-month mark, $10\text{ }\mu\text{M}$ of PCP was injected into the SAMBR to simulate the situation of a wastewater treatment plant being contaminated by PCP (e.g. from an industrial wastewater stream). The SAMBRs went into an “upset” state similar to when Reactor B was initially spiked with PCP for acclimatization (Figure 3.3). The bioaugmentation SCs, either singly or in combination, were applied to aliquots of the sludge and effluent from this SAMBR to check their compatibility with this wastewater treatment system. Results are as shown in Figure 3.16.

Figure 3.16 Percentage of reduction in PCP concentration for SCs of both heated and non-heated sets. SC stands for subculture. The error bars represent the range of the duplicate measurements.

For formaldehyde removal of both “SC non-heated only” and “SC non-heated + heated” samples, the statistical analysis was performed using student’s-t test to compare whether their differences are significant or not, according to the statistical results, there are three samples showed significant difference between the “SC non-heated only” and the “SC non-heated + heated” sets, including F, FG, and BEFG.

Table 3.5 student's-t test result of the percentage PCP reduction between "SC non-heated only" and "SC non-heated + heated" samples. Italic number indicates the value is lower than 0.05 and being statistically significant.

	Difference of means	Std Err	t	p-value
B	0.5	0.730	0.685	0.553
E	-2.3	1.033	-2.227	0.114
F	-4.5	0.821	-5.484	<i>0.006</i>
G	-1.5	0.981	-1.528	0.222
BG	2.7	1.079	2.503	0.113
EG	4.6	2.277	2.020	0.130
FG	-22.4	3.078	-7.278	<i>0.007</i>
BEFG	-17.2	2.263	-7.599	<i>0.002</i>

The reduction in PCP concentration between the non-heated set and the mixture of non-heated plus heated subcultures from previous section was compared in Figure 3.16. Among the consortia from single bioreactor, the SC from bioreactor E showed highest PCP removal for both non-heated sample and the mixture. The bioaugmentative consortium from reactor E was found to be most effective in removal PCP previously in section 3.3.3. It can be concluded that simplification through dilution and heat treatment did not eliminate the PCP bioremediating microbes. In all the SC from single bioreactor, there were no significant difference in PCP removal being observed between the non-heat treated set and the complete consortia (non-heated mixed with heated), indicating the PCP microbes in most of the SC may consist of a large spore-forming population. The only exception is the SC of bioreactor F, which resulted a slight increase in PCP removal after being mixed with the heat-treated counterpart.

After mixing with SC from bioreactor G, the PCP removal efficiency of the SC containing B or E have an increase in general. This result is consistent with the finding in section 3.3.3 (refer Figure 3.12). After mixing with SC from bioreactor G, the PCP removal efficiency of SC from F showed a significant increase in performance in the heat-treated and non-heat treatment mixture. It may be that the microorganism which contribute to the synergistic effect include spore-forming strains that are resistant to heat.

Among all the single or mixed SC from various bioreactors, the mixed subculture of bioaugmentative consortia from BEFG was found to be most effective in removing PCP, for both non-heated group and the mixture of non-heated and heated group. The highest removal of PCP is reaching 76.1 %; and this consortium may qualify to be the seed for future application.

3.4 Summary

In this chapter, we attempted to develop bioaugmentative consortia that are effective in alleviating the negative effects of PCP in an anaerobic wastewater treatment system. Activated sludge was acclimatized to PCP and its intermediates, and their metabolism was closely monitored, through parameters such as influent/effluent COD, VFA and toxicant concentration, as well as methane production. Some sludges exhibited the relevant catabolic properties after more than half a year of acclimatization, and they were tested, either in isolation or in combination, for PCP removal and bio-gas production. There were clear differences in the efficacy of PCP removal and bio-gas production between bioaugmentative sludges acclimatized by different intermediates. One of the samples were observed to be capable of removing up to 43.5 % of PCP during the wastewater treatment process.

In order to develop more controlled bioaugmentative consortia than crude acclimatized sludges, serial dilution and heat-treatment were carried out to partially simplify the microbial community in these consortia. The 10^5 -fold dilution subcultures selected and characterized in this process were applied to effluents of a PCP-contaminated SAMBR. The BEFG mixture including both non heat-treated and heat-treated subcultures was found to be able to remove about 75% of PCP in this context. This bioaugmentative consortia would be a candidate for SAMBR inoculation.

The only microbial dynamic study carried out here was based on DGGE, but our laboratory is also exploring the use of the new generation sequencing methods. In chapter 4, our preliminary attempts at adapting the method will be presented. This involved the use of

16SrRNA V3-V4 hypervariable region of prokaryotes for the detection of bacteria and archaea within our anaerobic bioreactor, using an Illumina MiSeq sequencer (Kiyohara et al., 2012).

Chapter 4. Preliminary Work for Studying Microbial Dynamics in Bioaugmentation Process

4.1 Introduction

The objectives of this project include the development of co-culture bioaugmentation for two biotechnological applications. The two systems that could enhance the target biological processes were generated successfully for pollutant remediation using metabolic capacity of microorganisms. As a natural follow up to this outcome, several lines of future work can be envisaged and undertaken whereby the microbial dynamics and interactions can be examined in greater depth.

4.1.1 Approach One: Bioaugmentative Co-culture for Aerobic VOC Removal

In chapter two, we described the process and success of developing a microbial consortium with 12 culturable strains, which is validated to be highly efficient in removing one of the indoor air pollutant formaldehyde. In formulating this consortium, we used a hypothesized standard for choosing strains, which is to select to with the purpose to represent diverse characteristics. We speculate that diverse characteristics of the strains reflecting different metabolic traits and have co-existed in the same environment over a long period of time are more likely to work co-operatively to metabolize the target compound. Hence, out of the 109 identified isolates from the TPPB source community, we chose strains from different genus, and tested many of their metabolic characteristics to finalize the composition. For genus carrying several species within the TPPB community, we screened their compatibility with other members in order to eliminate excessive redundancy and to allow narrowing down to the minimalistic choice within a genus.

The co-cultures selected based on this hypothesis were formulated and one of them succeeded in vastly improving formaldehyde removal efficiency as compared to the proprietary microbial culture used in Air Purifier A as marketed by Company X. This outcome supported our rationale in this bioaugmentative strategy. Moreover, this consortium demonstrated some sustainability in formaldehyde removal within changing

environment. Two scenarios about the sustainability of the co-culture were tested in the first approach. They showed maintenance of stable function of formaldehyde removal efficiency within a test duration of about 16 days when exposed in an open environment. This proved that the consortium selected based on our criteria do demonstrated certain level of sustainability in bioaugmentation performance.

We also explored preliminarily the interaction between different strains when they are co-cultured (section 2.3.5.3). Some of the microbial interaction data has been obtained with the customized colorimetric method. More combinations of consortium can be tested for their formaldehyde removal efficiency using this method. The changes in community structure within these consortia will be one important piece of information for elucidating the interactions occurring within the consortium. Therefore, we planned to develop a micro-fluidic method for future microbial dynamics study, and the method development and preliminary results will be illustrated in following sections.

4.1.2 Bioaugmentation for PCP Degradation in Anaerobic System

In the second system, the approach to formulate an efficient bioaugmentation consortium is clearly different from the first approach outlined above. Here, there was no available pool of culturable strains that could be used to formulate controlled consortia. Moreover, by the nature the anaerobic digestion process is extremely complex, requiring diverse groups of microorganisms to work in sequence, and many of these are known to be difficult to cultivate. We considered the high toxicity of the starting target PCP and started to acclimatize the strains with intermediates along the pathways of the target pollutant.

The activated sludge was acclimatized to PCP and its intermediates. After a certain period of acclimatization, these sludges and their mixture were tested for PCP removal performance in different condition. There are differences in PCP removal between sludge being acclimatized by different intermediates. After a few round of dilution and mixture, one of the consortia formulated was found to be able to remove about 75% of PCP in the influent, which is a significant achievement in this project. Studies on these sludges and their optimal condition for best performance in PCP removal in bigger scale wastewater

treatment reactors or plants would be considered in the future experiments within our laboratory.

After carrying out DGGE analysis, the appearance and disappearance of some band were noted and associated with the PCP bioaugmentation performance. In future, pursuing the identity of these members would provide important clues for microbial dynamics studies.

It became apparent during the course of this study that the most critical analysis to follow ought to be the study of microbial interaction in the anaerobic microbial bioaugmentation system. In this chapter we shall present some of the preliminary results of our exploration.

4.1.3 Objective of this Chapter

In the previous chapters, we mainly employed DGGE analysis to capture the microbial community dynamics crudely to monitor the bioaugmentation process. For approach one (section 2.3.6), we tested the suitability of DGGE for microbial population analysis for the 12-strain consortium. It was found to be inappropriate for microbial population monitoring in this setting. For approach two (section 3.3.2), some strains' dynamics was found to be coincident with bioreactors' change in performance.

As mentioned in in section 2.3.7.2, the micro-fluidic q-PCR, compared to DGGE, has more potential in determining the identity and quantity of strains in consortia. This platform, once developed, will be a great tool to explore the population change within any consortium formulated by various combination of the 12 selected strains. Therefore, future investigation of the microbial interaction would be greatly promoted by utilizing of this method. It is hoped that the results of microbial interaction study would reveal more about what is happening within the successful consortium.

Another technology we considered to further our causes is pyrosequencing, which could provide in-depth sequencing and data analyses at a much extensive level than traditional molecular biological techniques (refer section 3.3.2.1). Within a single run of pyrosequencing, hundreds of thousands of sequence reads can be obtained and more

sequence information data can be generated. It also permits a better coverage of individual sample, increasing the chances of detecting rare species. With sample as complex as the activated sludge in approach two, one pyrosequencing of each sample could potentially cover a large proportion of information regarding microbial species within the sample. With these information, we would be able to associated the particular bioreactor condition change with the population dynamics, and hence use this information to fine tune the bioreactor operation condition.

Overall, the micro-fluidic method is better suited to investigate interaction among a limited group of bacteria of known identity, which is the scenario presented in the first approach. In contrast, the pyrosequencing method is more suitable for getting an overall picture of a complex community where the exact members are mostly not known. This corresponds to the situation we encounter in anaerobic bioreactors in the second approach. These two technology are the entrance to our future work and still under development stage; here we present the concept and approach designed for microfluidic method of study community population dynamics and demonstrated some preliminary data obtained from the pyrosequencing method.

4.2 Material and Methods

4.2.1 Microfluidic qPCR

4.2.1.1 Bacterial Strains and Genomic DNA Used

The genomic DNA (gDNA) of these strains were extracted by suspending cell pellets in 144 µl of 10 mM Tris 2 mM EDTA with 1% Triton X-100, and lysed by beat beating using Mini-Beadbeater-1 (BioSpec). Subsequently, 3.6 mg of lysozyme (Sigma-Aldrich) was added and the mixture was incubated for 1 hour at 37°C; 0.056 mg of Proteinase K (Macherey Nagel) was further added and followed by 56°C incubation (Thermomixer Eppendorf) for 18 hours. Macherey-Nagel Nucleospin Kit was used for extraction of DNA from the cell lysate, following the manufacturer's protocol. The extracted DNA was measured by Nanodrop Spectrophotometer (BioFrontirer Technology) at 260 nm.

4.2.1.2 Primer and Probe Design

One set of universal primer and probe (which amplifies all the 12 strains) and 12 set of specific primer and probe (which only amplifies its' target strain) were designed from most conservative region and most variable region respectively. Primers were checked for cross-reactivity, self-dimerization, and hairpin formation and potential secondary structure formation using OligoAnalyzer. Primer and probe sequences are given in Table 4.2 in section 4.3.1.1.

4.2.1.3 Endpoint PCR

Endpoint PCR testing both universal primer and specific primer were conducted with different annealing temperature, as presented in the Results section. A 10 µl PCR reaction volume contained 1X PCR buffer (Fermentus), 2.5 mM MgCl₂, 0.25 µM of each dNTPs, 0.5 µM of each primer (AIT biotech), 0.5 U of Taq Polymerase (Fermentus), and 2.5 ng of template DNA. Cycling was performed with initial denaturation for 3 min at 90°C, 35 cycles with 30s of denaturing at 95°C, 20s of annealing at different temperature including 55°C, 57°C, 59°C, 61°C, 63°C, 65°C, 60s elongation at 72°C, and final elongation for 10 min at 72°C. Presence of products was confirmed on 3% agarose gel followed by ethidium bromide staining.

4.2.1.4 Q-PCR

Quantitative PCR reactions were performed on Roche LightCycler 480 system. All reactions were performed in duplicates and with non-template controls on each plate to rule out the reagent contamination. 10 µl reaction system contains: 1X probe master mix (LightCycler® 480 Probes Master, Roche), 0.5 µM of each primer (AIT biotech), and 0.1 µM of probe (AIT biotech), template DNA 2.5x 10⁻³ng. The final concentration of gDNA template was 2.5 × 10⁻⁴ng/µl, because this is the expected concentration of single genome in each well of the micro-fluidic chip.

Each qPCR consisted of the following steps: 10 min of initial pre-incubation at 95°C to activate the polymerase, followed by 55 cycles of 10 s of denaturation at 95°C, 30 s of

annealing at 63°C, and 1 s of elongation at 72°C. Analysis was done on LightCycler480 Software release 1.5.0.

4.2.2 Pyrosequencing

Specific forward and reverse primers complementary to the upstream and downstream of the V3-V4 region of interest were designed with Illumina overhang adapters, and used to amplify the templates from genomic DNA. A subsequent limited-cycle amplification step was performed to attach multiplexing indices and Illumina adapter sequences to the amplicons. The sequence information of the locus-specific primers and the overhang adapters used are provided in Table 4.1.

Table 4.1 Sequence of 16S Amplicon PCR primers used for V3-V4 amplification. Locus-specific sequences are in bold (V3-V4), Illumina overhang adapter sequence in italics.

16S PCR primer	Sequence (5'- 3')
Forward	AATGATACGGCGACCA CCGAGATCTACACTCTTTCCCTACACGACGCTCTT <i>CCGATCTCCTACGGG AGGCAGCAG</i>
Reverse	CAAGCAGAAGACGGC ATACGAGATNNNNN GTGACTGGAGTTCAGACGTG <i>TGCTCTTCCGATCT GACTACNVGGGTATCTAATCC</i>

Amplicons were subsequently quantified by qPCR using the KAPA library quantification kit and pooled in equimolar concentrations into a single library. Library QC was then carried out on Agilent Bioanalyzer 2100 DNA 1000 chips. The pooled library was subsequently denatured and added to the Illumina MiSeq sequencing reagent cartridge and loaded into the MiSeq instrument. Cluster generation and sequencing was performed on the MiSeq according to specifications and generated paired-end reads of 250bp each. Data was then analyzed using the MiSeq Reporter Metagenomics Workflow and the Illumina BaseSpace 16S Metagenomics App.

4.3 Preliminary Results

4.3.1 Development of Microfluidic Q-PCR for Future Study of Microbial Dynamic in our Application

Previously, a high-throughput microfluidic poly-(dimethylsiloxane) biochip has been developed by (Zhang, Gong, Zeng, & Sze, 2013) in our laboratory which is used for investigating the association of a common bacterial pathogen, *Pseudomonas aeruginosa*, to single host human lung epithelial A549 cells, and revealed an adherence distribution that has not been previously reported.

Here we aimed to modify this platform to investigate microbial population dynamics in bioaugmentation process. Because this microfluidic system enables isolation of a single cell within each one micro-well and to further purify the cell's DNA on-chip followed by qPCR, we intend to make use of a biochip structured with more than 1000 micro-well and divided into several independent sections.

Single cells from a co-culture may be loaded by one-step vacuum-driven micro-fluidics and each section is subject to qPCR for quantification of one specific strain. The collective counts tallied from all sections will allow us to derive the relative composition of the co-culture under study. In this section, we describe the first stage of our development of the micro-fluidic quantitative PCR method for co-culture population monitoring.

4.3.1.1 Primer and Probe Design

In order to quantify specific strains within a co-culture carrying these 12 strains, a two-dye qPCR system was conceptualized. Firstly, to count the total number of bacterial cells (occupancy of micro-wells on the chip), a bacterial universal primers and probe (Yakima yellow as reporter, TAMRA as quencher, which gives out yellow fluorescence upon amplification) would have to be designed to amplify all the strains. Secondly, to count specific strains among this population, primers and probes (FAM will be used as reporter, TAMRA as quencher, which gives out green fluorescence upon amplification) specific to each member of the consortium would have to be designed. Primers and probes were therefore designed using sequences obtained in 16s rDNA sequencing by methods stated in section 4.2.1.2.

Table 4.2 Primer and Probe sequences for 12 specific strains and one set of universal Primer and Probe used in micro-fluidic experiment.

Strain No	Primer/Probe	Sequence 5'-3'
NES 28	Forward Primer	TAATACGTAGGGTGCAAGCG
	Reverse Primer	ATTCCACCTCCCTCTGACAC
	Probe	FAM-TTCTTTCCGAACCGCCTGCG-TAMRA
NES32	Forward Primer	GGCCCTATGGGTTGTAAACTT
	Reverse Primer	GTTAGCCGGTGCTTATTCGT
	Probe	FAM-CCTTCAGCTTTCCACACGTGGAAA-TAMRA
NES 36	Forward Primer	CAAGACTGAACCAACCATGC
	Reverse Primer	ACGGAGTTAGCCGATCCTTA
	Probe	FAM-AGGACCGTCATCCTGCCCCG-TAMRA
NES 42	Forward Primer	TAATACGTAGGGTGCAAGCG
	Reverse Primer	CAGCAATGCAGTCACAGATG
	Probe	FAM-TACCGCCTGCGCACGCTTTA-TAMRA
NES 43	Forward Primer	ACGCTGTAAACGATGAGTGC
	Reverse Primer	CCCGTCAATTCCTTTGAGTT
	Probe	FAM-CGTCACCGACAGGCATGCCT-TAMRA
NES 54	Forward Primer	GTAACACGTGGGTGATCTGC
	Reverse Primer	TGCACCAGTAAACCTTTCCA
	Probe	FAM-TGCAGCCTCTGGTCATATCCGG-TAMRA
NES 57	Forward Primer	ACATGCCACTAACGAAGCAG
	Reverse Primer	ATCTCACGACACGAGCTGAC
	Probe	FAM-CCACTTTCCTTTTCGGGCACC-TAMRA
NES 59	Forward Primer	ACGTAGGCGGCTTTGTAAGT
	Reverse Primer	CCACTGACCTCTCCTGGATT
	Probe	FAM-TGCCTTTGAGACTGCATCGCTTG-TAMRA
NES 60	Forward Primer	GGCGTAAAGAGCTTCGTAGG
	Reverse Primer	CTGATATCTGCGCATTCCAC
	Probe	FAM-TCAACCTCCGGCCTGCAGTG-TAMRA
NES 75	Forward Primer	CGTTATCCGGATTTATTGGG
	Reverse Primer	TCCATTCAAGCCCATCAGTA
	Probe	FAM-AGGCCGCCTACGCACCCTTT-TAMRA
NES 97	Forward Primer	GATCAGTCACACCGGAAGT
	Reverse Primer	GGTATTGCTGGATCAGGCTT

Strain No	Primer/Probe	Sequence 5'-3'
NES 140	Probe	FAM-CCGGACTCCTACGGGAGGCA-TAMRA
	Forward Primer	GGCCCTATGGGTTGTAAACTT
	Reverse Primer	GTTAGCCGGTGCTTATTCGT
	Probe	FAM-CCCTCACGAGGGTAGGTTTATCCCTG-TAMRA
Universal primers	Forward Primer	CCTACGGGDGGCWGCA
	Reverse Primer	GGACTACHVGGGTMTCTAATC
	Probe	Yakima Yellow-CAGCAGCCGCGGTA-TAMRA

4.3.1.2 PCR Results

As described in section 4.3.1.1, in order to quantify the population of each strain within the consortium, each strain must be identified by specific primer/probe set when the sample contains a mixture of gDNA from all the 12 strains. A universal primer/probe set capable of amplifying all the 12 strains must be present to serve as a positive control. The universal primer and probe set and one of the specific primer and probe set are ultimately meant to be loaded together in one of the sections in the micro-fluidic chip after the gDNA extraction, and quantified through different fluorophores. Hence qPCR for both primer/probe sets would have to be conducted at the same annealing temperature. We therefore first needed to determine the common annealing temperature range using end-point PCR.

Universal primer set was first used to PCR-amplify the 12 strains' genomic DNA. 4 temperatures were tested: 59°C, 61°C, 63°C, and 65°C, as shown in Table 4.3. It was found that at T_m 59°C and T_m 61°C, all 12 strains could be amplified. When the annealing temperature increases to 63°C, NES 36 could not be amplified. When the temperature increases to 65°C, many strains failed to be amplified, including NES 36, NES 42, NES 43, NES 75, NES 97 and NES 140 (refer Table 4.3).

Specific primer to each strain at different temperature was also tested at the annealing temperatures shown in the above table. At annealing temperature of 57°C and 59°C, all the 12 strains can be amplified by its own specific primer set. At annealing temperature of 63°C and 65°C, NES 75 failed to be amplified (refer Table 4.3).

Based on the data obtained, 61°C and 63°C were further considered as the annealing temperatures to test in qPCR on the chip. Although when annealing temperature was set at 61°C, both specific primer and universal primer can amplify their target strains, when specific primer (NES 28 & NES 140) sets were tested on all 12 strains' gDNA, non-specific amplification of specific primer was observed (data not shown). 63°C for annealing temperature was decided over 61°C, because higher annealing temperature tends to reduce the non-specific binding of each specific primer, thus increase the primer specificity.

Table 4.3 Results of endpoint PCR of universal primer set and 12 specific primer set at different annealing temperature. “+” indicated band being observed on agarose gel after staining, “-” indicated band not being observed on agarose gel after staining.

Strain/Tm	Universal primer				Specific Primer			
	59°C	61°C	63°C	65°C	57°C	59°C	63°C	65°C
NES 28	+	+	+	+	+	+	+	+
NES 32	+	+	+	+	+	+	+	+
NES 36	+	+	-	-	+	+	+	+
NES 42	+	+	+	-	+	+	+	+
NES 43	+	+	+	-	+	+	+	+
NES 54	+	+	+	-	+	+	+	+
NES 57	+	+	+	+	+	+	+	+
NES 59	+	+	+	+	+	+	+	+
NES 60	+	+	+	+	+	+	+	+
NES 75	+	+	+	-	+	+	-	-
NES 97	+	+	+	-	+	+	+	+
NES 140	+	+	+	-	+	+	+	+

4.3.1.3 Q-PCR Results

At this stage, the specificity of 5 primer and probe sets (NES 36, NES 54, NES 59, NES 75, and NES 140) was tested using conventional qPCR system (the micro-fluidic platform will only be used after the early optimization stage using conventional qPCR system) as shown in Figure 4.1. NES 36, NES 54, and NES 75’s primer and probe sets showed high specificity, meaning only their target strain was amplified under this experimental condition, all the rest sequencing from other strain remained negative in amplification. However, primers and probe sets of NES 59 and NES 140 showed cross-amplification with several of the non-specific strains, indicating that better primers/probes would have to be designed.

Figure 4.1 Amplification curve of primer and probe sets (a) NES 36 (b) NES 54 (c) NES 75 with 12 strains' 16S rDNA, at annealing temperature of 63°C.

4.3.1.4 Conclusions

In micro-fluidic experiment, one universal primer/probe set for quantification of the total bacterial population and 12 specific primer/probe sets for each member of the consortium were designed. Preliminary testing of these primers using endpoint PCR was conducted to determine the PCR annealing temperature for all primer/probe sets, because ultimately, the qPCR will be performed at one-step in one micro-fluidic plate. The testing result of these primer sets leads to the decision of the annealing temperature at 63°C. In the qPCR testing of the specific primer sets of 5 strains, 3 of the primer/probe sets showed satisfactory

specificity towards their target strains, and the other 2 showed cross amplification with non-target strains.

4.3.1.5 Future Work and Expected Outcome

Development of this method is currently still ongoing, the specificity test of the rest 7 probe and primer sets would be continued. And those primer and probe sets failed the specificity test would be redesigned and undergone another round of specificity checking until they can differentiate their target strain with rest of the strains in the q-PCR testing.

Once both universal and specific primer and probe sets were finalized, we would start to validate our design on the micro-fluidic platform. Pure strain testing would be the first step, in this step, single strain culture would be loaded to the chip and followed by the on-chip DNA extraction and q-PCR. Ideally, positive wells should be identical for universal and specific primer and probe sets. Thereafter, mixed strains with known ratio would be used to test the accuracy of the platform. Actual sample would be tested followed by the above described validation steps.

An important analytical component in our undertaking of bioaugmentation by co-culture is a reliable method for monitoring of co-culture population shifts, so that the correlation with its biodegradative performance may be tracked in future. This tool, once developed, could help us greatly in formulating a sustainable co-culture bioaugmentation to achieve better performance.

4.3.2 Trial Pyrosequencing of Microbial Community in SAMBR

In this study, we are adapting a method, which uses a universal primer based on the V3-V4 hypervariable region of prokaryotic 16S rDNA for the detection of bacteria and archaea within our anaerobic bioreactor using an Illumina MiSeq next-generation sequencer. (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014)

4.3.2.1 Results

To start, one activated sludge sample in SAMBR from before PCP addition was tested by pyrosequencing method described in section 4.2.2. As shown in the following figure, the inner pie represents the dominant phylum that has been identified. The community consists of 69.11% *Euryarchaeota*, 14.19% of *Firmicutes*, 12.1% of *Synergistetes*, and the rest as listed in the below figure. The outer pie shows the dominant class that has been identified. This result would be a reference for sample later collected on different data with different condition (refer Figure 4.2).

Figure 4.2 Microbial community composition in activated sludge before PCP addition (at Phylum and Genus levels) revealed by 16S rRNA sequencing on Illumina MiSeq platform

4.3.2.2 Conclusions

In this preliminary pyro-sequencing experiment, the protocol and method was established, and first sample of the sludge was tested and the community composition of activated sludge prior to the acclimatization was mapped out. In future, this method would be used for samples from different bioreactor during different stage to correlate the performance to the community structure.

4.3.2.3 Future Work and Expected Outcome

The obtained sequencing data also allows the determination of the functional potential of the community; these data when properly analyzed can lead to discovery of novel enzymatic functions, microorganisms and genes that may be used for other bioremediation.(Lovley, 2003; Russell et al., 2011)

Despite the powerful capacity to describe the gene content and potential functions encoded in sequenced genomes, the pyrosequencing approach has a very limited role in revealing gene expression level during the bioaugmentation. Therefore, metatranscriptomics technology can be applied in comparison studies. A typical metatranscriptomic experiment involves the isolation of the total RNA from the sample followed by RNA enrichment. After that, the RNA is fragmented to smaller pieces followed by cDNA synthesis using reverse transcriptase and random hexamers or oligo (dT) primers. From there on, the 5' and/or 3' ends of the cDNA are repaired and adapters are ligated, followed by library cleanup, amplification and quantification and finally the library is sequenced.(Bikel et al., 2015) The metatranscriptomics data could be paired with community's genomics data obtained pyrosequencing methods to analyze if the transcript abundance is reflecting changes in community composition and bioaugmentation performance.

Chapter 5. Conclusion

In conclusion this study, two co-culture bioaugmentation systems have been successfully developed and their performances have been investigated under two approaches in the duration of this project. At the meantime, their microbial dynamics have been preliminarily reviewed, and new assays for more depth microbial dynamics tracking and microbial interaction study were partly developed and being continued.

In the first approach, we managed to formulate a microbial consortium for a domestic indoor air purifier was formulated, which is able to achieve 100% of formaldehyde removal in the testing enclosed chamber as a combination effort of both microbes and other components of the purifier. The members of the consortium were previously isolated from a two-liquid phase partitioning bioreactors (TPPBs), and were identified to genus level. After multidimensional characteristic checking of 26 candidate strains, the final consortium with outstanding VOCs' removal capacity was formulated by 12 of the 26 strains. Two methods of testing the bioaugmentation efficiency have been developed after this simplified consortium was formulated. Other than the before mentioned enclosed environment testing, there is a colorimetric experiment, which measures formaldehyde dissolved in MSM (mineral salt media). And the consortium formulated showed up to 50% of dissolved formaldehyde removal using this assay. In sustainability testing, the consortium was found to be able to maintain 80%-100% formaldehyde over at least 16 days in the air purification device under conditions subject to random environment perturbation. Microbial interaction among strains has also been briefly observed assessed under 2 strain co-culture settings.

In the second approach, one bioaugmentative consortium has been formulated as the seed for future application in SAMBRs, which is aiming for targeting bioaugmentation of PCP contaminated wastewater. Activated sludge from local wastewater treatment plant was used as the start-up seed of bioaugmentation for developing the bioaugmentative consortium. The microbial composition of the identity of the consortium remained unknown uncharacterized during the process. After acclimation of the activated sludge to

PCP and its metabolic intermediate separately over one year, each bioaugmentative consortium and several mixtures' PCP removal and biogas production was tested, followed by a dilution scheme and heat treatment to simplify the community composition of the consortia before the final consortium was formulated. Several mixtures were made with the subcultures of these simplified consortia. Their bioaugmentation efficiency was assayed through High-Performance Liquid Chromatography (HPLC)., one mixture of the bioaugmentative subculture revealed about 76.1% of PCP removal in lab scale SAMBRs, a rate significantly higher than the separately acclimatized culture. And this culture has shall be been selected as the bioaugmentative seed for future runs of SAMBRs.

In summary, the major findings of this study were listed as follows:

- 100% of formaldehyde removal achieved with the formulated aerobic consortia based on the strain selection standard we hypothesized in section 2.3.2.1
- The co-culture formulated were found to possess sustainability of formaldehyde removal performance in open environment within 16 days of observation
- One protocol for measuring soluble formaldehyde concentration was customized and validated for future study
- Different patterns of interactions (e.g. growth enhancement and inhibition) were observed during the brief 2-strain co-culture assay
- DGGE method was found not able to fulfil the need for microbial community analysis in future study, micro-fluidic q-PCR method was under customization as the substitution.
- 76.1% of PCP removal achieved with the acclimatized and simplified anaerobic consortium during the wastewater treatment process
- DGGE method was utilized to track community evolution and the information obtained from this method was limited, thus pyrosequencing method was partially developed for future study

Overall, for 1st approach, the advantages are: the strains were isolated from a functioning bioreactor which reduces the time for acclimatization; the composition of the finalized co-

culture was relatively simple for controlled microbial interaction study in future. Meanwhile, it also required a great amount of work to be done before hand, including, acclimatization, strain isolation, identification and characterization. Therefore, it might not be applicable for many of the bioaugmentation project. It can be possible to miss out some functionally important strain during the strain selection process, since not all the strains isolated were selected as candidate strains.

For 2nd approach, it could be time consuming to acclimatize the microbial community to a new chemical from the very beginning, especially for anaerobic sludge, it takes as much as 50 weeks to finish the initial acclimatization process in our case. However, it also increases the chance to obtain a consortium being efficient in removing target component. In regarding for microbial interaction study, in this system, even after several steps of simplification, the community composition was still relatively complex.

Endeavors to study microbial dynamics were also made in both approach with the purpose to learn how the microbial population dynamics evolve, and in which way these changes can be related to bioprocess efficiency. DGGE (Denaturing gradient gel electrophoresis) has been found not to be appropriate for strain population tracking in approach one. Although it was used for preliminary survey microbial dynamics of the consortium in approach two, and some changes in community composition were found to be coincident with bioaugmentation performance change, it was not sufficient to reveal the complete information of the complex microbial community. Two assays for future studies in bacterial interaction investigation were under exploration, for microbe population tracking, including microfluidics q-PCR and pyrosequencing. In addition, metatranscriptomics technology can be applied in comparison studies, the metatranscriptomics could be paired with community's genomics data obtained by micro-fluidic q-PCR and pyrosequencing methods to analyze if the transcript abundance is reflecting changes in community composition as described in section 4.3.2.3. These work shall bring the conclusions of this study to a further level enabling more reliable bioaugmentation practice in future applications.

Appendix 1

Strains	Genus		<i>Achromobacter</i>			<i>Cupriavidus</i>	<i>Caulobacter</i>	<i>Variovorax</i>		<i>Chryseobacterium</i>					<i>Flavobacterium</i>	<i>Microbacterium</i>				<i>Rhodococcus</i>				<i>Pedobacter</i>	<i>Mucilaginibacter</i>	<i>Sphingomonas</i>		<i>Nevskia</i>
	NES No.		28	61	146	57	43	42	89	27	32	140	141	144	36	29	35	56	60	30	54	73	99	75	151	59	152	97
Properties	LB	Solid	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
		Liquid	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	MSM + Hexane	Solid	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+
		Liquid	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	MSM + Glucose	Solid	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	+
		Liquid	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	+
	<i>alkB</i>		-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+
	CYP153		-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+	+
	FDH		-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Cell surface hydrophobicity (as MATH)	Hexane	-	-	-	-	-	++	++	+	+	++	++	+	++	-	-	-	-	++	++	++	++	-	-	-	-	+
		Glucose	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Emulsification activity	Hexane	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	++	++	++	++	-	-	-	-	-	++
		Glucose	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	Formaldehyde		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Toluene		+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+	-	+
	Benzene		+	-	-	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	-	+	-	+
Culture No.	Type of Profile (refer Figure 2.7)	Strain(s) included in Formulation																										
1	C																											
2	C																											

Strains	Genus		<i>Achromobacter</i>			<i>Cupriavidus</i>	<i>Caulobacter</i>	<i>Variovorax</i>		<i>Chryseobacterium</i>					<i>Flavobacterium</i>	<i>Microbacterium</i>				<i>Rhodococcus</i>				<i>Pedobacter</i>	<i>Mucilaginibacter</i>	<i>Sphingomonas</i>		<i>Nevskia</i>
	NES No.		28	61	146	57	43	42	89	27	32	140	141	144	36	29	35	56	60	30	54	73	99	75	151	59	152	97
Culture No.	Type of Profile (refer Figure 2.7)	Strain(s) included in Formulation																										
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23	B																											
24	B																											
25	B																											
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27	B																											

Strains	Genus		<i>Achromobacter</i>			<i>Cupriavidus</i>	<i>Caulobacter</i>	<i>Variovorax</i>		<i>Chryseobacterium</i>					<i>Flavobacterium</i>	<i>Microbacterium</i>				<i>Rhodococcus</i>				<i>Pedobacter</i>	<i>Mucilaginibacter</i>	<i>Sphingomonas</i>		<i>Nevskia</i>
	NES No.		28	61	146	57	43	42	89	27	32	140	141	144	36	29	35	56	60	30	54	73	99	75	151	59	152	97
Culture No.	Type of Profile (refer Figure 2.7)	Strain(s) included in Formulation																										
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45	B																											
46	D																											
47	D																											
48	D																											
49	D																											
50	A																											
51	A																											
52	A																											

Strains	Genus		<i>Achromobacter</i>			<i>Cupriavidus</i>	<i>Caulobacter</i>	<i>Variovorax</i>		<i>Chryseobacterium</i>					<i>Flavobacterium</i>		<i>Microbacterium</i>				<i>Rhodococcus</i>				<i>Pedobacter</i>	<i>Mucilaginibacter</i>	<i>Sphingomonas</i>	<i>Nevskia</i>	
	NES No.		28	61	146	57	43	42	89	27	32	140	141	144	36	29	35	56	60	30	54	73	99	75	151	59	152	97	
Culture No.	Type of Profile (refer Figure 2.7)	Strain(s) included in Formulation																											
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