Biodegradation of tert-butyl alcohol (TBA), and treatment of TBA contaminated wastewater by aerobic granules

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Biodegradation of Tert-Butyl Alcohol (TBA), and Treatment of TBA Contaminated Wastewater by Aerobic Granules

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ABSTRACT

The focus of this study is the biodegradation of *tert*-butyl alcohol (TBA) and the complete biological removal of TBA from contaminated water using aerobic granulation system. Aerobic granulation is a novel biological wastewater treatment technology which has several benefits as compared to the conventional activated sludge systems. Aerobic granules are dense and compact microbial aggregates with good settling capabilities, good bioactivity, larger biomass retention, and good resistance to chemical toxicity. TBA was chosen as a model compound in this study because of its high recalcitrance and inhibitory characteristics. The aims of this study were to evaluate the utility of the sequencing batch reactor (SBR) to acclimate microorganisms towards biological removal of TBA, to cultivate TBA-degrading aerobic granules in SBR using TBA as sole carbon and energy source, to isolate and characterize TBA-degrading pure microbial cultures, and to elucidate a TBA degradation mechanism of aerobic granules achieved via microbial interaction.

For these purposes, two sequencing batch reactors (SBRs), designated as reactor One (R1) and reactor Two (R2), were initially inoculated with non-acclimated activated sludge and fed with TBA as the sole carbon source. Start-of-cycle TBA concentrations at both R1 and R2 reactors were initially set at 100 mg L^{-1} with a cycle time of 24 h and a volumetric exchange ratio of 50% to maintain a TBA loading rate of not more than $100 \text{ mg L}^{-1} \text{ d}^{-1}$. Step increases in TBA loading rates up to $600 \text{ mg L}^{-1} \text{ d}^{-1}$ were achieved by employing two different strategies. In Reactor R1, the start-of-cycle TBA concentration was first raised to 150 mg L^{-1} on day 90, and the cycle time was then reduced from 24 h to 12, 8 and 6 h on days 100, 121 and 199, respectively. In Reactor R2, the start-of-cycle TBA concentration was raised to 150 mg L^{-1} on day 90, and then to 300, 450, and 600 mg L^{-1} on days 100, 121 and 199, respectively. These acclimation strategies favored the retention of increasingly higher densities of well-adapted microbial populations in both reactors. The

increases in TBA loading finally produced better settling biomass and higher biomass concentrations with higher specific TBA biodegradation rates. TBA was completely removed from the system and the effluent TBA concentrations were consistently below the detection limit of 25 $\mu\text{g L}^{-1}$.

The use of progressively shorter cycle times in Reactor R1 created favorable selection pressures that fostered the self-immobilization of the microorganisms in the aerobic granules which first appeared on day 125. However, the aerobic granules were also formed in the Reactor R2 about 180 days after the reactor start-up. The granules had a clearly defined shape and appearance, settled significantly faster than the suspended sludge in the reactor and eventually became the dominant species of biomass in the reactor. Specific TBA biodegradation rates in the granules followed the Haldane model for substrate inhibition, and peaked at 14 $\text{mg TBA g VSS}^{-1} \text{ h}^{-1}$ at a TBA concentration of 300 mg L^{-1} of Reactor R1 granules and 17 $\text{mg TBA g VSS}^{-1} \text{ h}^{-1}$ at a TBA concentration of 320 mg L^{-1} of Reactor R2 granules.

Denaturing gradient gel electrophoresis (DGGE) was employed to produce genetic fingerprints that could provide information on the composition and diversity of microbial communities in the stabilized TBA-degrading microbial consortia from Reactor R1 and R2, respectively. DGGE analysis of PCR-amplified 16S rRNA gene fragments from nucleic acids extracted from the aerobic granules over a 4-week period between days 220 and 247 revealed the presence of a highly stable microbial community with members only belonging to the α , β and δ subdivisions of Proteobacteria and the Cytophaga-Havobacteria-Bacteroides (CFB) group. This is probably a result of the selection pressures exerted by the SBR operation or the specificity required for TBA biodegradation, which would favor the enrichment of certain bacterial species to the detriment of others.

Two TBA-degrading microbial pure cultures were isolated from the aerobic granules by using TBA as sole carbon and energy source. It was found that the

strains TBA-R1-001 and TBA-R2-005 were phenotypically and phylogenetically different from each other, and belonged to the genera *Bacillus* and *Methylobacterium*, respectively. The 16S rRNA gene sequence analysis revealed that strain TBA-R2-005 was closely related to *Methylobacterium organophilum* str. XX with a sequence identity of 99.7%. The strain TBA-R1-001 was closely related to *Bacillus megaterium* IAM 13418 with a sequence identity of 99.8%. The culture based (e.g. plating and colony formation counting) and culture independent (e.g. PCR-DGGE) methods confirmed that the strain TBA-R2-005 was a predominant bacterium which survived in the TBA-degrading aerobic granules and played roles in TBA biodegradation. Although the DGGE method did not show TBA-R1-001 as a predominant bacterium in aerobic granules, the abundance in number, coupled with TBA degradation capability implied that this strain also played an important functional role in aerobic granule treating TBA. It is the first time that *Bacillus megaterium* and *Methylobacterium organophilum* species are found to use TBA as sole carbon and energy source.

The survey of total culturable TBA-degrading bacteria (using TBA as sole carbon and energy source) and the total culturable heterotrophic bacteria (including TBA-degrading and non-TBA-degrading heterotrophic bacteria) from aerobic granules showed that the number of heterotrophic bacteria were at least 10-fold as high as the TBA-degrading bacteria. TBA-degrading bacteria were those microbes using TBA as sole carbon and energy source. Heterotrophic bacteria were microbes including TBA-degrading bacteria as well as non-TBA-degrading bacteria. Since the aerobic granules were fed with TBA as sole carbon and energy source, the results indicated that a large proportion of the culturable heterotrophic bacteria could survive in granules but may not be directly involved in the TBA biodegradation. It was found that a novel non-enzyme direct attacking TBA biodegradation mechanism, designated as microbial driven Fenton reaction, might be employed by bacteria residing in aerobic granules to break down TBA. Hydroxyl radical, a reactive oxygen species, was potentially generated through the microbial driven Fenton

reaction, and played an important role in the novel TBA biodegradation mechanism.

It was detected that extracellular H_2O_2 accumulation and iron-reducing capacity of aerobic granules were functions associated with TBA utilization. The two elements are the pre-requirements for hydroxyl radical generation through Fenton reaction. In addition, the TBA-degrading aerobic granules could also digest polyvinyl alcohol, a recalcitrant polymer, when TBA was supplied as a carbon source. The extracellular redox capability of aerobic granules was also detected. All the evidence supported the biological hydroxyl radical generation procedure as a favorite in the TBA-degrading aerobic granulation system.

Publications

The following papers have been published or accepted for publication:

Tay, S.T.-L., W.-Q. Zhuang, and J.-H. Tay. (2005) Start-up, microbial community analysis and formation of aerobic granules in a *tert*-butyl alcohol degrading sequencing batch reactor. *Environmental Science and Technology* 39: 5774-5780.

Zhuang, W.-Q., J.-H. Tay, S. Yi, and S.T.-L. Tay. (2005) Microbial adaptation to biodegradation of *tert*-butyl alcohol in a sequencing batch reactor. *Journal of Biotechnology* 118: 45-53.

The following paper has been accepted for oral presentation:

Tay, S.T.-L., W.-Q. Zhuang, and J.-H. Tay. (2005) Aerobically grown microbial granules for *tert*-butyl alcohol biodegradation. 4th IWA Activated Sludge Population Dynamics Specialist Conference: microbial population dynamics in biological wastewater treatment. University of Queensland, Brisbane, QLD, AUSTRALIA.

The following journal papers are currently submitted for review:

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Zhuang, W.-Q., J.-H. Tay, and S.T.-L. Tay. Isolation, identification and characterization of two *tert*-butyl alcohol (TBA) degrading bacteria isolated from

TBA-degrading aerobic granules (Submitted to Applied Microbiology and Biotechnology).

Zhuang, W.-Q., J.-H. Tay, and S.T.-L. Tay. Novel aerobic granules mediated *tert*-Butyl alcohol (TBA) biodegradation mechanism – microbial driven FENTON reactions (Submitted to Journal of Biotechnology).

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List of Acronyms

3-CCA	Coumarin-3-carboxylic acid
7-OHCCA	7-hydroxy-coumarin-carboxylic
APHA	American Public Health Association
CLSM	Confocal Laser-Scanning Microscopy
CMTR	Completely Mixed Tank Reactor
COD	Chemical oxygen demand
DETAPAC	Diethylenetriaminepentacetic acid
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
DPD	N,N-diethyl-1,4-phenylenediammonium sulfate
EPS	Extra-cellular Polysaccharides
ETBE	<i>Ethyl tert-butyl Ether</i>
FISH	Fluorescent in situ Hybridization
GAC	Granular Activated Carbon
HRT	Hydraulic Retention Time
MSM	Mineral Salt Medium
MTBE	<i>Methyl tert-Butyl Ether</i>
p-NDA	Dimethylnitrosoaniline
PCR	Polymerase Chain Reaction
POD	Horseradish peroxidase
PVA	Polyvinyl Alcohol
SBR	Sequencing batch reactor
SEM	Scanning Electron Microscopy
SOUR	Specific Oxygen Utilization Rate
SPME	Solid Phase MicroExtraction
SRT	Sludge Retention Time
SVI	Settling Volume Index
TAME	<i>tert-Amyl Methyl Ether</i>
TBA	<i>tert-Butyl Alcohol</i>
TBF	<i>tert-Butyl Formate</i>
UASB	Upflow anaerobic sludge blanket
USEPA	United States Environmental Protection Agency
VOC	Volatile Organic Compound

List of Symbols

K_{fw}	Fuel-water partition coefficient
K_i	Inhibition constant (mg L ⁻¹)
K_m	Half-saturation constant (mg L ⁻¹)
K_{oc}	Soil organic partition coefficient
K_s	Half-saturation constant (mg L ⁻¹)
V_{max}	The maximum specific substrate degradation rates (mg g-VSS ⁻¹ hr ⁻¹)
μ_{max}	The maximum specific growth rate (h ⁻¹)

Chapter One

Introduction

1.1 Background

Tert-butyl alcohol (TBA) is an anthropogenic organic compound which has been used for more than thirty years. TBA is directly added to fuels, often in association with methyl *tert*-butyl ether (MTBE), as an octane index enhancer to reduce vehicle emissions (Piveteau et al. 2001). In contrast to MTBE, which is almost exclusively used as a fuel oxygenate, TBA is also widely used as a solvent and an intermediate in many industrial processes. For example, TBA is used in the manufacture of plastics, resin polymers, perfumes, paint removers, insecticides and pharmaceutical products.

Because of TBA's widespread use as well as its possible accumulation as an intermediate of MTBE biodegradation, TBA is often present as a contaminant in groundwater that is impacted, for instance, by spills of oxygenated fuels. TBA concentrations of up to 160 mg L^{-1} have been measured in groundwater plumes in the vicinity of a chemical plant (Day and Gulliver 2003).

TBA's ubiquity in the environment poses a health concern as it is a known toxin and animal carcinogen (Cirvello et al. 1995; Bradley et al. 2002; Schmidt et al. 2004). A drinking water action level of $12 \text{ } \mu\text{g L}^{-1}$ has been set in California as a safeguard against potential human toxicity (Kane et al. 2001). TBA is highly soluble and is not readily retarded by adsorption to the aquifer solids in groundwater flow systems. TBA has a low Henry's Law constant and a low fuel–water partition coefficient (K_{fw}), and its physicochemical characteristics render TBA unsuitable for treatment by conventional remediation technologies, such as air stripping or adsorption (Zwank et al. 2002).

Biological treatment could be a viable alternative since TBA is known to be biodegradable under oxic conditions (Day and Gulliver 2003). In addition, several studies on pure bacterial cultures reported that TBA could be biodegraded or cometabolized by bacteria using MTBE or other petroleum hydrocarbons as sole carbon source (Fayolle et al. 2001; Piveteau et al. 2001).

However, the *tert*-butyl structure of the TBA molecule, with three methyl groups attached to a tertiary carbon, tends to make it more resistant to biological degradation than less complex gasoline components such as benzene and toluene. Moreover, studies involving laboratory microcosms and cell suspensions have found that TBA could accumulate as the rate-limiting intermediate in MTBE biodegradation (Hatzinger et al. 2001; François et al. 2003).

In many cases, TBA metabolizes more slowly than MTBE, and in some cases, TBA biodegradation is possible only at reduced MTBE concentrations (Salanitro et al. 1994; Steffan et al. 1997; Fayolle et al. 2001). Moreover, the enzyme systems involved in TBA biodegradation have not been adequately elucidated. Some studies proposed that TBA was degraded by the same enzyme which bacteria used to degrade MTBE (Fayolle et al. 2001). However, functional enzymes have not yet been isolated and studied in detail.

Many questions are still unclear in the TBA biodegradation area. Only a few TBA-degrading pure microbial cultures have been isolated so far and this might be one of the reasons because it limits the investigation, evaluation and collection of useful physiological information. Hence, understanding the roles of the bacteria in the TBA biodegradation is limited. Nevertheless, from the engineering point of view, bioremediation of TBA/MTBE contaminated water is possible because several studies have demonstrated MTBE/TBA biodegradation in bioreactors (Morrison et al. 2002; Vainberg et al. 2002; Wilson et al. 2002; Zein et al. 2004).

Biodegradation of MTBE and TBA under aerobic conditions has been investigated in several bioreactor systems such as simple stirred tank reactors, fluidized bed bioreactors, attached growth reactors and membrane bioreactors (Morrison et al. 2002; Vainberg et al. 2002; Wilson et al. 2002; Zein et al. 2004). Such studies are driven by the need for ex-situ treatment at sites where groundwater extraction is required to halt the migration of contaminant plumes. However, not all the above mentioned bioreactor systems work very efficiently in TBA removal. One striking conclusion from these reactor studies is that a high biomass concentration is necessary for effective contaminant degradation. Unfortunately, the reason for high biomass concentration demand remains unclear.

High biomass concentrations can be easily achieved in aerobic granulation wastewater treatment systems. Aerobic granulation can be regarded as an aggregation of biomass that results from the self-immobilization of microorganisms under suitable selection pressures. The resulting compact biomass aggregates, or sludge granules, contain the necessary physiological capabilities to break down the undesired pollutants in wastewater. Compared with conventional aerobic wastewater treatment systems, the granulation system offers several advantages such as a denser and stronger sludge structure, high biomass settleability and retention, high degrading ability, high volumetric conversion capacity, and high organic loading rate (Peng et al. 1999; Beun et al. 1999; Tay et al. 2002a). Many studies have shown the feasibility of applying this technology to treat different types of wastewater (Beun et al. 1999; Beun et al. 2002; Tay et al. 2001a; Moy et al. 2002; Jiang et al. 2002 and 2004a).

Generally, aerobic granulation is considered to be able to supply a relatively favorite microniche for microorganisms under variable conditions. The dense structure of aerobic granules gives rise to a substrate concentration gradient because of diffusion resistance (Jiang et al. 2004a&b). The mechanism protects microorganisms in aerobic granules by diluting the toxic chemical below some

threshold value to avoid inhibition and allow continued microbial activity and substrate utilization (Jiang et al. 2004a&b).

In addition, aggregation of activated sludge cells into aerobic granules helps establish syntrophic relationships and potentially facilitates horizontal gene transfer, interspecies substrate exchange and removal of metabolic product. Because of these characteristics, aerobic granules have been shown to achieve high organic loading rates with simple substrates such as glucose and phenol (Moy et al. 2002; Jiang et al. 2004a). To date, aerobic granules have been tested with easily degradable substrates, for example, glucose, acetate, and ethanol. However, it is currently still unknown whether the aerobic granules can be formed by using recalcitrant compounds and the performance of aerobic granules on removal of these recalcitrant compounds.

1.2 Objectives

The main objectives of this study are:

1. To evaluate the utility of using a sequencing batch reactor for biodegradation of TBA and to study the formation of aerobic granules in SBR using TBA as sole carbon and energy source;
2. To study the dominant microbial populations within TBA-degrading granules using conventional and advanced 16S rRNA based molecular microbiology methods;
3. To investigate microbial driven Fenton reaction occurring in TBA-degrading aerobic granules and to study the reaction as a possible TBA biodegradation mechanism achieved via microbial interactions.

This work will be useful in understanding how sequencing batch reactors and

aerobic granules can be exploited for effective biodegradation of TBA and other recalcitrant chemicals. The current study could also contribute to a better understanding of the microbial community of these aerobic granules and provide information useful for manipulation of the aerobic granulation process and optimization of microbial community for biodegradation of recalcitrant compounds.

1.3 Organization of the thesis

Chapter 2 is a detailed literature review and discussion on aerobic granulation as well as TBA biodegradation. Chapter 3 describes the formation and properties of aerobic granules using TBA as sole carbon and energy source. Dominant microbial populations in TBA-degrading aerobic granules were studied with denaturing gradient gel electrophoresis (DGGE) and other molecular microbiology methods. Chapter 4 describes two pure microbial cultures isolated from TBA-degrading granules using TBA as sole carbon and energy source. Chapter 5 focuses on evaluating the feasibility of one of the TBA biodegradation mechanisms, microbial driven Fenton reactions TBA breakdown, archived by different bacteria population's interactions. Chapter 6 summarizes some important conclusions from the experimental results and outlines some recommendations for future work.

Chapter Two

Literature Review

The background information related to this study will be reviewed in this chapter. The chapter contains two main parts. In the first part, *tert*-butyl alcohol (TBA) biodegradation is reviewed. In the second part, the topic of aerobic granulation is addressed.

2.1 Microbial Degradation of Methyl *tert*-Butyl Ether (MTBE) and *tert*-Butyl Alcohol (TBA)

Tert-butyl alcohol (TBA) is a tertiary alcohol which has been used for more than thirty years as a solvent, an intermediate in many industrial processes, as well as a fuel oxygenate. However, it started to gain wide attention in recent years because of its widespread use as well as its potential human toxicity. Researchers studied the environmental contamination and biodegradation of methyl *tert*-butyl ether (MTBE), an extensively used fuel oxygenate, had also noticed the importance of TBA as a stable intermediate of MTBE degradation. However, up-to-date, there is still quite limited information addressed on the contamination, transportation, and degradation of TBA in the environments. In most published reports, TBA was studied associated with MTBE because it is the first stable intermediate of MTBE degradation.

2.1.1 Fuel oxygenates and associated environmental concerns

Fuel oxygenates include ethers (e.g., methyl *tert*-butyl ether [MTBE], ethyl *tert*-butyl ether [ETBE], *tert*-amyl methyl ether [TAME]), and alcohols (e.g.,

tert-butyl alcohol [TBA], methanol and ethanol). The chemical structures of these oxygenates are shown in Figure 2-1. These oxygenates are commonly added to gasoline to enhance octane index and improve combustion efficiency. Ethers have been generally preferred to alcohols which can be corrosive to some materials used in engines and can also lead to problems of phase separation when mixed with hydrocarbons (Vautrain 1999).

MTBE, probably the most commonly used oxygenate, was first added to gasoline to enhance the octane rating, in the 1970s when alkyl lead additives were discontinued in the United States. In 1979, MTBE was approved by the USEPA to be used as an octane booster to replace tetraethyl lead in gasoline (Fayolle et al. 2001). In an effort to reduce the environmental consequences associated with fuel combustion, the 1990 Clean Air Act Amendments required that oxygenates be added to reformulated gasoline in order to reduce tailpipe carbon monoxide and other smog forming emissions (Deeb et al. 2000a). Since then, the production and use of MTBE has increased dramatically.

In 1995, MTBE was the third most produced organic chemical in the United States, with an annual production of approximately 8 million tons. World MTBE-production capacity was approximately 25 million tons in 1999 (Fayolle et al. 2001). When used as a fuel oxygenate, MTBE is added to gasoline at a concentration ranging from 11-15% by volume. Although MTBE, TAME and ETBE exhibit similar properties as octane enhancers (Table 2-1), MTBE is the dominant fuel oxygenate mainly because of its low production cost and ease of blending and handling (Fayolle et al. 2001; USEPA 1998). In 1999, MTBE accounted for approximately 85% of all oxygenates used in the United States (approximately 15 billion liters per year) which also represented almost 60% of the global MTBE demand (Eskew and Geisler, 2004).

TBA is a key intermediate in degradation of several ether-based fuel oxygenates,

MTBE

TAME

ETBE

TBA

Ethanol

Figure 2-1 The chemical structures of some oxygenates.

Table 2-1. Physicochemical characterizations of different oxygenates.

Substance	MTBE	TAME	ETBE	TBA	TBF ^a	Ethanol
CAS No.	1634-04-4	994-05-8	637-92-3	75-65-0	762-75-4	64-17-5
Commercially available	Yes	Yes	Yes	Yes	Yes	Yes
Molecular weight [g/mol]	88.15	102.18	102.18	74.12	102.13	46.07
Boiling point [°C]	55.2	86.3	67.0	82.4	83.0	78.5
Density [kg/L]	0.744	0.77	0.73	0.791	0.879	0.79
Water solubility [g/L]	48	12	12	Complete	Complete	complete
Henry's law constant, K_H [atm·m ³ /mol]	0.0059	0.052	0.109	0.00014	0.0059	0.0021-0.0026
Log Kow	1.24	--	--	0.35	1	--
Log Koc	1.05	--	--	0.41	1.91	--
Fuel-water partition coefficient, K_{fw}	16	--	--	0.24	--	--
Air-fuel partition coefficient, K_{ag}	0.0015	--	--	0.0024	--	--

a: TBF refers to *tert*-butyl formate

such as methyl *tert*-butyl ether [MTBE] and ethyl *tert*-butyl ether [ETBE]. However, besides being a by-product of MTBE biodegradation, TBA may also be present as an impurity in gasoline and this may contribute to elevated TBA concentrations in groundwater at contaminated sites. In a study of New Jersey gasoline, it was found that TBA was present in five out of six samples at up to 11% of MTBE concentration. The authors (Kramer and Douthit, 2000) concluded that TBA was added intentionally to the fuel since TBA impurities in MTBE should be below 2%. TBA was also found in seven gasoline samples from Switzerland, but at lower concentrations which could be explained as MTBE impurities (Zwank et al. 2002).

In both studies, aqueous phase concentrations of TBA in batch equilibrium experiments reached the high milligram to low gram per liter range. For Germany, the use of TBA in mixtures with methanol as fuel oxygenate has been reported (Schmidt et al. 2004). The formation of branched alcohols such as isopropanol and *sec*-butanol in gasoline due to oxidative transformations of olefins has been recently shown (Schmidt et al. 2004). This investigation did not include TBA but presumably TBA could be formed via the same mechanism. In contrast to MTBE, which is almost exclusively used as a fuel oxygenate, TBA is also a widely used solvent and intermediate in industrial processes (Clark 2002). TBA has been included into the U.S. high production volume (HPV) chemicals list by USEPA since 1990 [http://www.epa.gov/opptintr/chemrtk/hpv_1990.pdf]. The U.S. high production volume (HPV) chemicals are those which are manufactured in or imported into the United States in amounts equal to or greater than one million pounds per year.

The wide usage of MTBE has resulted in the contamination of different environments, especially groundwater (Fiorenza et al. 2002). Squillace et al. (1996) sampled a large number of wells, springs, and groundwaters in urban and rural areas from 1993 to 1994. At a reporting level of $0.2 \mu\text{g L}^{-1}$, MTBE was detected in 27% of 210 wells and springs sampled in eight urban areas and 1.3% of 549 wells sampled in 21 rural areas. It was also found in 1% of 412 deeper groundwater samples from

major aquifers in nine areas. In addition, a study of volatile organic compounds (VOCs) in ambient groundwater in U.S. was conducted by the United States Geological Survey (USGS) National Water Quality Assessment Program (NAWQA) (Squillace et al. 1999). The NAWQA found that MTBE was detected at a frequency of 16.9% in wells in urban areas and 3.4% in wells in rural areas. Of 60 VOCs, only trichloromethane was detected with greater frequency.

An important case of contamination by MTBE occurred in 1996 in the city of Santa Monica (California, USA), where high levels of MTBE (up to $600 \mu\text{g L}^{-1}$) were detected in drinking water wells, causing the closure of half of the municipal water supply (USEPA 1999) and requiring the expensive importation of untainted water from outside the city. Detectable levels of MTBE have also been found in some rivers in Germany (Achten and Puttman 2000). Now, MTBE is the second most commonly detected VOC (volatile organic compound) in water next to trichloromethane (USEPA 1999).

Other non-MTBE oxygenates, including ETBE, TAME and TBA, together make up to 8% of United States oxygenates market. There are virtually no data on the environmental behavior of these oxygenates, due primarily to difficulties in delineating their extent in the environment, lack of systematic analytical procedures for their determination as a group, and lack of regulatory requirement for their analysis. Shih et al. (2004) recently reported a study on evaluation of the impact of fuel hydrocarbons and oxygenates on groundwater resources in the United States. The authors measured these compounds' occurrence, distribution, and spatial extent in groundwater beneath leaking underground fuel tank (LUFT) facilities, and analyzed data from over 7200 monitoring wells in 868 LUFT sites. It was found that TBA has the greatest site maximum (geometric mean) groundwater concentration among the chemicals analyzed. The results also indicated that MTBE posed the greatest potential for groundwater resources contamination, followed by TBA and benzene.

From the above-mentioned reports, the high frequency of detection of MTBE and TBA in groundwater samples is rather surprising, as these compounds have only recently been widely used as gasoline additives. It has been reported that MTBE and TBA may enter groundwater in the same manner as other gasoline components, for example, leaking underground storage tanks and pipelines, accidental spills, releases at refueling station, manufacturing and storage facilities, and inappropriate disposal practices (Stocking et al. 2000; Fayolle et al. 2001; Fischer 2003). Both MTBE and TBA are highly water soluble with low Henry's law constant. They dissolve readily in water, and partition into water rather than air. A dimensionless Henry's law constant of 0.05 or greater indicates a volatile compound, while a compound with a lower Henry's law constant prefers the water phase to the gas phase.

Furthermore, the K_{oc} of MTBE is lower than that of benzene. In a sand with an organic carbon content of 0.1%, only 8% of the MTBE would be sorbed to the aquifer material, but 40% of the benzene would be sorbed (Squillace et al. 1999). Compared with MTBE, TBA has a lower Henry's law constant as well as a lower K_{oc} value. A common finding at gasoline spill sites is that MTBE usually migrates ahead of the gasoline components such as benzene, toluene, ethylbenzene, and xylenes (BTEX). This happens both because MTBE has greater mobility and also because it is less subject to biodegradation during transport. Similar considerations hold true for TBA.

MTBE is known to exert toxic effects on aquatic organisms. After inhalation, MTBE is distributed to brain, kidney and liver, where it is primarily metabolized into TBA and formaldehyde. Studies of chronic exposure of rats and mice to MTBE suggest that it is carcinogenic (Belpoggi et al. 1998; Bird et al. 1997). However, the potential carcinogenic effect of MTBE on humans remains a matter of debate (Hartley et al. 1999). The taste and odor of MTBE are a major issue because consumers can detect the oxygenate in contaminated water at very low levels, with thresholds ranging from $2.5 \mu\text{g L}^{-1}$. Based on taste and odor concerns, the USEPA's Office of Water has established a drinking-water advisory level of $20\text{--}40 \mu\text{g L}^{-1}$ as guidance (USEPA

1999). This level is also supposed to provide a large margin of caution against any potential health effects.

In contrast to MTBE, TBA is a known animal carcinogen and exhibits human toxicity (Cirvello et al. 1995). TBA is a main metabolic intermediate during the MTBE and ETBE biodegradation. Because of these reasons, a drinking water action level for TBA of $12 \mu\text{g L}^{-1}$ has recently been set in California (Kane et al. 2001). However, difficulties on the analysis of this compound at trace levels had led to a lack of attention to TBA contamination. Nevertheless, some field investigations have already reported the presence of TBA at accidental spill or leakage sites (Kolhatkar et al. 2002; Kramer and Douthit, 2000; Landmeyer et al. 1998 & 2001; Zwank et al. 2002), sometimes at concentrations higher than MTBE.

2.1.2 Removal of MTBE and TBA contamination

There are several remediation options for dealing with MTBE and TBA contamination. These compounds can be removed from water by pump and treat methods that pass groundwater over activated carbon, and by air sparging (Deeb et al. 2000a & 2003; Stocking et al. 2000). However, it should be pointed out that although physical methods such as air-stripping and granular activated carbon (GAC) adsorption are currently used for *ex situ* treatment of MTBE and TBA contaminated water, these technologies actually just transfer the contaminants from one phase to another. The ultimate destruction or disposal of the contaminants would still need to be addressed. In addition, MTBE and TBA's relatively low Henry's law constant and poor sorption onto organic matter render these methods less efficient in treating the compounds as compared to other volatile organic compounds such as benzene or toluene (Fayolle et al. 2001; Deeb et al. 2000a; Stocking et al. 2000). This means that significantly higher air to water ratios or GAC concentrations are required to achieve the required removal efficiency, which eventually increases the water treatment cost.

Unlike the air-stripping and GAC technologies, advanced oxidation is a destructive process which converts MTBE and/or TBA through chemical transformation. Moreover, it provides a promising treatment option for MTBE in the relatively high concentrations between 0.1 and 80 mg L⁻¹ compared with less than 0.1 mg L⁻¹ of GAC technologies (Cater et al. 2000). The published advanced oxidation technologies applied for MTBE remediation included ozone/H₂O₂ combinations (Safarzadeh-Amiri, 2001), ozone/UV combinations (Graham et al. 2004), UV/H₂O₂ combinations (Cater et al. 2000), ultrasonic irradiation (Selli et al. 2005) and ultrasonic irradiation/ozone combinations (Kang and Hoffman 1998). Although all these technologies could effectively destruct MTBE, major by-products were sometimes generated during the treatment process including *tert*-butyl formate (TBF), TBA, acetone, methyl acetate, and aldehyde. Hence, the by-product formation and

relatively high treatment cost are the major concerns of these advanced oxidation technologies in the real application.

Bioremediation is another innovative treatment process that is gaining wide interest. The attractiveness of biologically treating MTBE and TBA lies in the cost-effective as well as the potential for the destruction of the contaminants rather than simply transferring the contaminants from one medium to another. Since MTBE and TBA are relatively novel pollutants in the environment, there are few studies conducted to evaluate its biodegradability. Early studies suggested that MTBE is resistant to microbial attack under both aerobic and anaerobic conditions (Fujiwara et al. 1984; Jensen and Arvin, 1990; Suflita and Mormile, 1993). Fujiwara et al. (1984) did not observe aerobic degradation of 100 mg L^{-1} MTBE by activated sludge. Jensen and Arvin (1990) reported that 10 mg L^{-1} MTBE did not aerobically biodegrade with an activated sludge inoculum. A recent field study concluded that natural attenuation of MTBE was very slow and in some cases undetectable (Fayolle et al. 2001). In fact, MTBE has been used as a conservative tracer to evaluate the natural attenuation of benzene and toluene at a site polluted by gasoline (Fayolle et al. 2001). Results showed that the half-life for MTBE in most natural groundwater systems was at least 2 years in contrast to 2 to 3 months for BTEX (benzene, toluene, ethylbenzene and xylenes) (Schirmer et al. 1998).

The recalcitrance of MTBE has been attributed to its chemical structure, with the ether bond and tertiary carbon atom responsible for the difficulty in biodegradation. Suflita and Mormile (1993) determined that oxygenates with a tertiary or quaternary carbon atom were more recalcitrant than unbranched or slightly branched, related chemicals (e.g. butyl methyl ether was degraded in aquifer sediments or bacterial cultures). However, recent laboratory studies have reported the ability of several bacterial and fungal cultures from various environmental sources to degrade MTBE under anaerobic or aerobic conditions either as the sole source of carbon and energy (Eweis et al. 1998; Mo et al. 1997; Fortin and Deshuiese, 1999a&b; Fortin et al. 2001;

Hanson et al. 1999; Erickson 2001; Feris et al. 2004) or cometabolically (Hardison et al. 1997; Steffan et al. 1997; Garnier et al. 1999; Magar et al. 2002; Smith et al. 2004).

Similarly, TBA has also long been recognized as a non-biodegradable compound because of its tertiary carbon chemical structure. Only until recently, TBA's biodegradability was revealed by associated studies of MTBE biodegradation because TBA was generally considered to be a stable dead-end intermediate during MTBE biodegradation. Generally, TBA biodegradation is even more difficult as compared to MTBE, especially when these two compounds co-existed in the contaminated water. On the other hand, several studies reported that TBA was biodegraded faster than MTBE and no TBA accumulation was noticed during MTBE biodegradation (Fayolle et al. 2001; Schmidt et al., 2004). These inconsistencies in biodegradation behavior have led researchers to postulate that some bacteria might use the same enzyme to attack both compounds (e.g. cytochrome P450), but other bacteria might use different enzymes to attack MTBE and TBA. However, little is known about the bacterial enzymes used to attack MTBE and TBA. Nevertheless, the several successful cases of biodegradation of MTBE and TBA in laboratory and field experiments point to the feasibility of bioremediation to treat oxygenate contaminated waters.

It is important to understand the advantages and limitations of TBA and MTBE biodegradation before designing and applying biological treatment techniques. Thus far, several laboratory experiments and field studies have demonstrated MTBE biodegradation under both aerobic and anoxic conditions. For example, Yeh and Novak (1994) evaluated the biodegradation of MTBE in soils from three different sites under various anaerobic and anoxic conditions. These researchers reported that the biodegradation of MTBE was observed under methanogenic conditions in a soil with low organic carbon content. The coexistence of easily degraded organic compounds was shown to inhibit MTBE degradation.

However, a lack of anaerobic MTBE biodegradation has also been reported by

some researchers (Bradley et al. 1999; Puig-Grajales et al. 2000). Laboratory microcosm studies also revealed that MTBE could be biodegraded under denitrification, sulfidogenic, Fe(III) reducing, and Mn(IV) reducing conditions. Unfortunately, TBA biodegradation under similar conditions has not been well elucidated. The biodegradation of TBA under denitrification conditions was studied by Yeh and Novak (1994), and the results showed that TBA could be biodegraded after a very long acclimation period. TBA biodegradation under denitrification conditions was further confirmed by Bradley et al. (2001a&b, 2002). On the other hand, other studies reported failure to detect MTBE/TBA biodegradation under denitrification conditions (Borden et al. 1997; Somsamak et al. 2001).

The biodegradation of TBA under sulfidogenic conditions has not been clearly demonstrated yet. Somsamak et al. (2001) found a stoichiometric accumulation of TBA when MTBE was biodegraded under sulfidogenic conditions. This finding suggested that TBA biodegradation was the rate-limiting step in the overall removal of MTBE under sulfidogenic conditions. TBA biodegradability under Fe(III) and Mn(IV) reducing conditions is inconclusive because of conflicting results from several microcosm studies. Finneran and Lovely (2001) reported the occurrence of TBA mineralization without a lag phase whereas MTBE biodegradation required an adaptation period of 250 days. However, the identity of the terminal electron acceptor in the sediments was not established due to the co-existence of sulfate and Fe(III). Bradley et al. (2002) suggested that TBA degradation in the Finneran and Lovely (2001) study was more likely attributable to sulfidogenic activity.

MTBE could also be biodegraded under methanogenic conditions (Wilson et al. 2000). TBA was found as a dead-end product of MTBE biodegradation in several methanogenic microcosms (Kolhatkar et al. 2002; Schmidt et al. 2004). TBA biodegradation under methanogenic conditions has not been demonstrated, and TBA is considered to be an accumulating dead-end product of MTBE degradation under such conditions. (Schmidt et al. 2004).

To understand the mechanisms of the biodegradation of MTBE/TBA by bacteria, it is better to obtain pure bacterial cultures which could use the target compound as sole carbon and energy source. The physiological and biochemical characteristics of these bacteria could be studied in detail and the knowledge on the bacteria could be employed to properly design suitable bioremediation strategies. In the last decade, researchers tried many ways to isolate MTBE- as well as TBA-degrading bacteria in order to study their characteristics. However, so far, only limited mixed and pure microbial cultures from a number of environmental sources have been shown to aerobically metabolize MTBE and TBA under laboratory conditions (Table 2-2). The reasons why MTBE- and TBA-degrading bacteria are so difficult to isolate are still not clear. Nevertheless, the information accumulated from the several successful studies showed that MTBE as well as TBA could be used as sole carbon and energy source and be completely mineralized to H₂O and carbon dioxide.

Table 2-2 Degradation of MTBE and TBA with microbial consortia and pure cultures.

Consortium/Strain	Substrate	Detection Method	MTBE degradation rate [mg/g·h]	TBA accumulation	TBA degradation rate [mg/g·h]	Reference
BC-1 [C] ^a	MTBE	GC, ¹⁴ C	34.4	Yes	14.0	Salanitro et al. 1994
Mixed Culture [C]	MTBE	GC				Park and Cowan 1997
<i>Methylobacterium mesophilicum</i> [P]	MTBE/TBA	GC, ¹⁴ C	28% over 2 weeks	NR	NR	Mo et al. 1997
<i>Rhodococcus</i> sp. [P]	MTBE/TBA	GC, ¹⁴ C				
<i>Arthrobacter rilicis</i> [P]	MTBE/TBA	GC, ¹⁴ C				
<i>Rubrivivax gelatinosus</i> PM1 [P]	MTBE	GC, ¹⁴ C	50.2	No	Unknown	Hanson et al. 1999
Methylotrophic bacterium CIP I-205 (<i>Burkholderia cepacia</i>) [P]	TBA	GC	No	-	35.6	Piveteau et al. 2001
Mixed culture F [C]	MTBE	GC, ¹⁴ C	7.1-52.9	Yes	Similar with MTBE	Fortin et al. 2001
<i>Hydrogenophaga flava</i> ENV 735 [P]	MTBE/TBA	GC, ¹⁴ C	121.6	Yes	Slow	Steffan et al. 2000
<i>Mycobacterium austroafricanum</i> IFP 2012 [P]	MTBE/TBA	GC	23.8-52.9	Yes	17.8-22.2	François et al. 2002, 2003
Mixed culture [C]	MTBE/TBA	GC	0.21	No	3.1	Kharoune et al. 2002
Cometabolic						
ENV425 (<i>Nocardia</i> strain)	Propane	GC, ¹⁴ C	12.2	Yes	3.6	Steffan et al. 1997
ENV421	Propane		24.3	Yes	5.3	
ENV420	Propane		ND	ND	Unknown	
<i>Mycobacterium vaccae</i> JOB5 [P]	Propane	GC	64.5	Yes	23.1	Smith et al. 2003a&b
<i>Mycobacterium vaccae</i> JOB5 [P]	Alkanes	GC	45.0-55.5	Yes	Slow	Johnson et al. 2004

<i>Xanthobacter</i>	Alkanes	GC	134.9	ND	Slow	Hyman and O'Reilly 1999
<i>Pseudomonas mendocina</i>			10.6			
<i>Alcaligenes eutrophus</i>			39.7			
Filamentous fungus <i>Graphium sp.</i>	Butane	GC	0.93	Yes	ND	Hardison et al. 1997
<i>Pseudomonas aeruginosa</i>	Pentane	GC	10.3	NR	2.1	Garnier et al. 1999
<i>Arthrobacter</i>	Butane	GC, ^{14}C	17.9	Yes	0.0044	Liu et al. 2001
<i>Rhodococcus rubber</i> IFP2007	Ethanol	GC	37.0	Yes	not degrade	Hernandez-Perez et al. 2001

a: [C] represents microbial consortium or mixed culture; [P] represents pure microbial culture.

The first report of a culture capable of MTBE degradation involved a heterotrophic microbial consortium, designated as BC-1 (Salanitro et al. 1994). This culture was enriched from an activated sludge unit treating industrial chemicals. BC-1 consisted of at least 6 different species, was maintained on MTBE in a chemostat with cell recycle and was shown to degrade up to 200 mg-MTBE L⁻¹ at a rate up to 34 mg g-cells⁻¹ h⁻¹. While this culture was able to utilize MTBE as a primary growth substrate, growth rate (0.01 per day) and cellular yields (0.21-0.28 g-cells (g-MTBE)⁻¹) were relatively low. Although this culture could use MTBE as sole carbon source, the transient accumulation of *tert*-butyl alcohol (TBA) was observed. TBA could be further degraded by the consortium, and the TBA degradation rate was slower than the MTBE degradation rate. The abilities of several cultures related to BC-1 to degrade MTBE were later reported (Deeb et al. 2000a; Fayolle et al. 2001). These cultures included consortia designated BC-4 and MC-100, and a pure culture, designated as SC-100, which was identified as a *Rhodococcus* sp.

A mixed culture enriched from petroleum refinery activated sludge was also shown to degrade both MTBE and TBA, and to utilize both as sole carbon and energy source (Park and Cowan 1997). The maximum specific growth rates of the culture on MTBE were 0.36-1.44 d⁻¹ and the cellular yields were 0.33-0.43 mg cells-COD mg⁻¹ substrate-COD at 30°C.

A number of microorganisms have been reported to grow on MTBE and/or TBA as sole carbon and energy source. These microorganisms use oxygen as electron acceptor when they degrade MTBE or TBA. So far, there is no report on pure cultures using other electron acceptors with MTBE and TBA as sole carbon and energy source. Mo et al. (1997) isolated three pure cultures, *Methylobacterium mesophilicum* (ATCC 700107), *Arthrobacter ilicis* (ATCC 700109) and a *Rhodococcus* sp. (ATCC 700108), using MTBE as the sole carbon source and energy source. This was the first report of pure microbial strains able to grow on MTBE as

sole carbon and energy source. The three cultures were shown to biotransform 29% of 200 mg L⁻¹ MTBE in less than 2 weeks with 8% of the MTBE mineralized to CO₂ by the end of the first week (5×10⁵ to 5×10⁷ CFU mL⁻¹). MTBE degradation rates decreased significantly in the presence of other carbon sources including TBA, TBF, iso-propanol, acetone and pyruvate. Slow growth rates on MTBE were observed leading the researchers to conclude that MTBE was either a poor growth substrate or that one of its metabolites might inhibit culture growth.

Hanson et al. (1999) isolated a gram-negative bacterium, PM-1, from an MTBE-fed compost biofilter. This bacterial strain is probably the most intensively studied of MTBE-degrading microorganisms. PM-1 was able to degrade MTBE at concentrations as high as 500 mg L⁻¹. In laboratory conditions, about 50% of the carbon from MTBE was recovered as CO₂ with a cellular yield of 0.18 g-cells g⁻¹ MTBE (Bruns et al. 2001). Deeb et al. (2001) identified PM-1 as a *Rubrivirex* sp. strain and showed that it could simultaneously degrade benzene and MTBE by two different pathways after growth on MTBE. No TBA accumulated during growth on MTBE, and it was suggested that two different monooxygenases were involved in MTBE and in TBA degradation. Unfortunately, the TBA biodegradation rate of strain *Rubrivirex* sp. PM-1 has not yet been reported to-date.

Once strain PM-1 was identified and characterized, researchers could employ molecular microbiological techniques (e.g. PCR) to study the potential function and behavior of closely-related microorganisms involved in the biological remediation of MTBE contamination. Autochthonous bacteria identical to PM-1 were found in several MTBE contaminated groundwater sites (Hristova et al. 2003; Kane et al. 2001). Phylogenetic analysis based on 16S rRNA gene sequences of these bacteria showed very high similarity to that of PM-1. Subsequent microcosm studies showed an increase in cell density of PM-1-like microorganisms with increased MTBE removal (Hristova et al. 2003). In addition, similar PM-1-like microorganisms were linked to MTBE degradation in a field-scale bioaugmentation study by using

realtime-PCR methods. The results provided evidence of the feasibility for using this group of bacteria for *in situ* bioremediation of MTBE contaminated groundwater. TBA accumulation was not detected when a mixed culture containing PM-1 similar bacteria degraded MTBE as sole carbon source (Sedran et al. 2002).

Besides strain *Rubrivirex* sp. PM-1, there are several MTBE-degrading strains which have been successfully isolated and characterized. Goodfellow et al. (2004) isolated several strains from the MTBE-degrading mixed culture MC-100, and identified one of these isolates as belonging to genus *Rhodococcus*. Another *Rhodococcus ruber* strain (formerly called *Gordonia terrae*) was also found to degrade MTBE (Hernandez-Perez et al. 2001). Steffan et al. (2000) also isolated a new strain, *Hydrogenophaga flava* ENV735, which grew slowly on MTBE alone but rapidly on MTBE or TBA in the presence of 100 mg L⁻¹ yeast extract. This strain degraded MTBE up to concentrations of 3 g MTBE L⁻¹. TBA was produced and transiently accumulated in the culture medium. Cell yields of 0.20 to 0.26 g cells g⁻¹ MTBE were obtained on MTBE plus 100 mg L⁻¹ of yeast extract.

Different substrate inhibitors were applied to investigate the MTBE and TBA biodegradation pathways and to identify the enzymes involved (Steffan et al. 2000). The results showed that MTBE and TBA degradation were inhibited by different inhibitors, indicating that separate genes controlled MTBE and TBA degradation in this microorganism. TBA degradation was inhibited by low concentrations of formaldehyde, a possible intermediate of MTBE biodegradation. This was consistent with observations that TBA degradation commenced only after MTBE concentrations decreased to insignificant levels.

Compared with studies on MTBE-degrading microorganisms, there are only limited studies on TBA-degrading microorganisms. Piveteau et al. (2001) determined that strain CIP I-2052 could use TBA as a sole carbon and energy source, but could not degrade MTBE. Phylogenetic analysis placed strain CIP I-2052 as a

close relative of *Burkholderia cepacia* within the β -Proteobacteria. TBA degradation by this strain was stimulated by trace levels of cobalt. The utilization of formate and methanol as sole carbon and energy source revealed the methylotrophic nature of this strain.

François et al. (2002) isolated a TBA-degrading bacterial strain, *Mycobacterium austroafricanum* IFP 2012, from TBA enriched activated sludge. In contrast with strain CIP I-2052, strain IFP 2012 utilized a broad range of substrates as sole carbon and energy source, including many fuel oxygenates (e.g. MTBE, TBA, TAME, ETBE, TAA, methanol, and formate). TBA was initially accumulated when strain *M. austroafricanum* IFP 2012 grew on MTBE as sole carbon and energy source. The cell density started to increase only when MTBE concentration decreased to very low level and TBA started to be degraded. The results suggested potentially different kinds of gene regulation mechanisms and different enzymes were employed by this bacterial strain to attack MTBE and TBA.

Studies on the cometabolic degradation of MTBE have reported the ability of mixed and pure cultures to degrade MTBE to TBA or to CO₂ following growth on either alkanes or aromatic compounds. Hardison et al. (1997) reported the ability of a filamentous fungus, *Graphium* sp. strain ATCC 58400, to partially degrade MTBE to TBA following growth on propane or *n*-butane. Steffan et al. (1997) reported the ability of several propane-grown pure cultures to cometabolize MTBE. One strain, designated ENV425, was shown to mineralize MTBE. Stoichiometric amounts of TBA were produced during the biodegradation of MTBE. In fact, in nearly every case, TBA degradation did not occur until MTBE was completely removed from the reactors.

In addition, MTBE cometabolism was also reported with a benzene-grown culture designated PEL-B201 (Deeb et al. 2000b). PEL-B201 was shown to degrade approximately 3.4 mg L⁻¹ of MTBE in 12 hours following growth on benzene (2×10^8

cells). MTBE degradation was severely inhibited in the presence of benzene at concentrations as low as 0.15 mg L^{-1} presumably due to competitive inhibition. In the presence of 0.3 mg L^{-1} of benzene, the time required to degrade 3.4 mg L^{-1} of MTBE increased from 12 to 50 hours. Accordingly, increasing concentrations of MTBE were shown to inhibit benzene degradation. Preliminary results from this study suggested that MTBE degradation can also take place by cultures grown on cyclohexanone, *o*-xylene or camphor.

MTBE and TBA biodegradation pathways have been proposed from pure culture studies (Church et al. 2000; Steffan et al. 1997). Among all the published documents, MTBE was always found to be degraded through TBA as the key intermediate no matter whether the microorganisms used MTBE as sole carbon and energy source or via cometabolism (Figure 2-2). Although *tert*-butoxymethanol has been proposed as an intermediate in MTBE-degrading, it has yet to be demonstrated.

The work of Steffan et al. (1997) still provides the most detailed information on the degradation pathway of TBA. 2-Methyl-2-hydroxyl-1-propanol (MHP) and 2-hydroxyisobutyric acid (HIBA) were identified as degradation intermediates of TBA. The authors suggested three possible pathways for further degradation, as shown in Figure 2-2. Key intermediates of these pathways are (i) methacrylic acid, (ii) 2,3-dihydroxyl-2-methyl propionic acid and lactic acid, (iii) isopropanol, acetone, hydroxyacetone, and pyruvic acid. The strains grew on all of these intermediates albeit slowly.

Church et al. (2000) proposed the acetone pathway for strain PM-1, although no TBA degradation intermediates were detected. The formation of HIBA has recently been confirmed by François et al. (2002) for another pure culture, *M. austroafricanum* IFP2012. Although the authors could not detect any further degradation intermediates, they suggested the acetone pathway because the same protein patterns were found in whole cells growing on MTBE, TBA or HIBA on the one hand and IPA

Figure 2-2 Proposed MTBE and TBA biodegradation pathway (Steffan et al. 1997).

or acetone on the other hand. Piveteau et al. (2001) proposed the pathway involving lactate for a methylotrophic bacterium able to degrade TBA but not MTBE. This strain was not able to grow on the intermediates in the acetone pathway (e.g. isopropanol, acetone, hydroxyacetone) or on methacrylic acid. No information is available on MTBE and TBA biodegradation pathways under anoxic and anaerobic conditions because of lack of MTBE- and TBA-degrading pure cultures.

In summary, both MTBE and TBA could be biodegraded under aerobic conditions. The intermediates of MTBE and TBA biodegradation pathways are nontoxic or less toxic than MTBE and TBA themselves. While biological removal of TBA has been investigated in reactor systems such as simple stirred tank reactors, fluidized bed bioreactors, attached growth reactors and membrane bioreactors (Acuna-Askar et al. 2000; Morrison et al. 2002; Stringfellow and Oh 2002; Vainberg et al. 2002; Wilson et al. 2001, 2002a&b; Zein et al. 2004), these studies have been performed with TBA, not as sole substrate, but in co-mixture with MTBE. As TBA is a common co-contaminant with MTBE and a product of MTBE biodegradation, such studies are driven by the need for ex-situ treatment at sites where groundwater extraction is required to halt the migration of contaminant plumes. One striking conclusion from these reactor studies is that a high biomass concentration is necessary for effective contaminant degradation. Considering the advantages of aerobic granulation (e.g., high biomass retention, resistance to toxic compounds, and flexible operation mode), this technology can potentially be applied to treat TBA contaminated waters.

2.2 Aerobic Granules

Aerobic granulation is a biological wastewater treatment technology which was developed in recent years (Morgenroth et al. 1997; Peng et al. 1999; Beun et al. 1999; Tay et al. 2001a). It was first reported in 1997 by Morgenroth and his colleagues (1997). From year 1998 to 2001, another three groups reported successful cultivation of aerobic granules in sequencing batch reactors (SBRs) using different kinds of carbon sources (Peng et al. 1999; Beun et al. 1999, 2000; Tay et al. 2001a). Since then, the aerobic granulation technology has attracted increased research interest (Tay et al. 2005; Liu et al. 2005a & b; Hu et al. 2005; Qin et al. 2004a & b; Arrojo et al. 2004; Tsuneda et al. 2003; Jang et al. 2003; Beun et al. 2002 & 2001).

Aerobic granules can be regarded as compact and dense microbial aggregates with a spherical outer shape. Figure 2-3 shows several aerobic granules cultivated by different research groups. Aerobic granules consist largely of bacteria and other microorganisms. Compared with the most widely used conventional aerobic activated sludge systems, the granulation systems offer many advantages such as a denser and stronger sludge structure, higher biomass settling capability and retention, higher volumetric biodegradation capability, higher volumetric conversion capacity (Peng et al. 1999; Beun et al. 1999, Tay et al. 2001a,b,c; 2002a).

2.2.1 Cultivation of aerobic granules

For years, researchers have tried to understand the granulation process, and thus to generate an optimized strategy to cultivate granules in aerobic conditions. The areas investigated can be divided into two main categories. The first deals with the operational aspects of the process. The optimized operational conditions and reactor configurations have been investigated to ensure the granulation. The other research

Figure 2-3 Typical aerobic granules cultured in SBRs fed with ethanol or acetate. [A] adapted from Beun et al. (1999); [B] adapted from Beun et al. (2002); [C] adapted from Qin et al. (2004b); [D] adapted from Liu et al. (2004c).

category deals with the microbiological aspects of granulation and tries to explain why and how the granulation happened. It should be noted that both research areas are not carried out absolutely separately. Indeed, they share information with each other, and together contribute to make the aerobic granulation technology controllable and applicable.

As a matter of fact, from microbiological aspects, the concept of microbial self-immobilization to form aggregates is not a new idea. Microbial aggregation is known to favor bacterial adaptation, survival, and many other important physiological functions under a variety of environmental conditions (Bossier and Verstraete 1996; O'Toole et al. 2000; Rickard et al. 2003). The well known microbial floc in the activated sludge system is a type of bacterial aggregation. Granular sludge or granules represent another form of microbial aggregation. Granular sludge cultivated under anaerobic conditions has been exploited since the 1970's and is now widely applied in upflow anaerobic sludge blanket (UASB) bioreactors (Lettinga et al. 1984). Under anaerobic conditions, it was commonly hypothesized that the specific syntrophic bacterial interactions in the methanogenic systems are the main driving force for sludge granulation (Schmidt and Ahring 1996).

Granulation under aerobic conditions is a more recent phenomenon. Unfortunately, in contrast with anaerobic phenomena, the mechanisms of bacterial aggregation under aerobic conditions have not yet been elucidated. Aerobic bacterial aggregation is a very complex phenomenon and diverse aerobic bacteria can form different types of aggregates under aerobic conditions (Stoodley et al. 2002). The commonly observed aerobic bacterial aggregation in nature is called biofilm which has been intensively studied in the last two decades. Biofilm based technology has also been used in biological wastewater treatment. In some aspects, aerobic granulation could be recognized as a special type of biofilm which is formed without supporting carrier (Tay et al. 2002a). Consequently, studies on formation of aerobic granules can leverage on our existing knowledge of biofilm formation.

Operational conditions and reactor configuration parameters used in some aerobic granulation studies were summarized in Table 2-3. These operational conditions are very typical and representative, and are still widely employed by different research groups in studying aerobic granules nowadays. Beun et al. (1999) proposed a hypothesis for aerobic granules formed in sequencing batch. It was based on general process of biofilm formation. They believed that suspended growing cells, flocs, and granules can concomitantly exist in biological wastewater treatment systems. However, in the conventional system (e.g. activated sludge system), suspended cells and flocs could grow faster than granules, thus finally outcompete granules. If the accumulation of suspended cells or flocs could be avoided, then superior granules can be achieved. Beun et al. (2002) demonstrated their hypothesis in the following study by using sequencing batch airlift reactors and short settling time. The different sludge morphologies were shown in Figure 2-4.

Although Beun et al. (1999) first proposed a competition-based hypothesis for aerobic granulation, there was no direct evidence to show how the granules formed from activated sludge. To date, almost all reported aerobic granulation studies relied on activated sludge as seeding sludge. From the micro-point of view, granule formation has to be a gradual process from seeded sludge to matured aerobic granules. Tay et al. (2001a) first used different microscopic techniques to investigate aerobic granule formation from seeded activated sludge collected from a wastewater treatment plant. In their case, two sequencing batch reactors (SBRs) were used to cultivate aerobic granules. Sodium acetate and glucose were supplied as sole carbon source for each SBR. The results showed that the seeded activated sludge had a very loose and irregular structure, dominated by filamentous bacteria, as shown in Figure 2-5a and -5b. After approximately one week cultivation, compact microbial aggregates began to be observed in both reactors. Rod- and cocci-shaped bacteria gradually replaced the filamentous bacteria and became dominant in the acetate-fed reactor.

Table 2-3 Reactor configuration and operational parameters for aerobic granule cultivation.

	Morgenroth et al. 1997	Peng et al. 1999	Beun et al. 1999	Beun et al. 2002	Tay et al. 2002a
Inoculum	Municipal WWT Sludge	Bulking activated sludge	Standard SBR Sludge	Nutrient removing WWT activated sludge	Acetate acclimated sludge
Reactor type & volume	Column, 31.4 L	SBR, 5 L	Column 2.25-2.5 L	Airlift, 3.01 L	Column, 2.3 L
Configuration (cm)	Φ20×100H	---	Φ5.6×150H	Φ6.25 (riser: Φ4×90)	Φ6×80H
Mixing & aeration	Bubble air diffusers	Stirring at 400 rpm	Fine bubble aerator	Fine bubble aerator	Fine bubble aerator
Substrate	Molasses	Sodium acetate	Ethanol	Sodium acetate	Sodium acetate
Superficial gas velocity (m/s)		0.083	0.014-0.041	0.022*	0.024
Dissolve oxygen (mg/L)	2	3.5-4	----	----	----
HRT (hr)	8	8	6.75-8	5.6	8
SRT (day)	≈ HRT	20	0.6-5.8		<15
Cycling time (hr)	4	4	3-4	3	4
Volumetric loading rate (kg-COD/M ³ ·d)	2.9*	1.5	2.5-7.5	2.5	6.0
Adding influent (min)	13	30	2	2	5
Aeration (min)	220	150	177-237	170	225
Settling (min)	1	45	2	3	5
Withdrawal (min)	6	15	1	5	5
Picture of granules				NR

Figure 2-4. The aerobic granule formation process reported by Beun et al. (2002).

In contrast, the filamentous bacteria still prevailed in the glucose-fed reactor. The granular sludge with clear round outer shape was formed in both reactors two weeks after reactor start-up, as shown in Figure 2-5c and -5d.

The filamentous bacteria were almost completely not seen in acetate-fed aerobic granules under microscope. In contrast, glucose-fed granules were still dominated by filamentous bacteria, at least, on the surface of granules (Figure 2-5e and -5f). It is interesting because that result agreed with previous reports of the activated sludge process that glucose enhanced the growth of filamentous bacteria (Chudoba 1985). After operation for three weeks, aerobic granules matured in both reactors. At this stage, both glucose- and acetate-fed granules had a very regular round-shaped outer surface. The mean sizes of glucose- and acetate-fed aerobic granules were 2.4 and 1.1 mm, respectively. Compared to acetate-fed granules, glucose-fed granules had a fluffy outer surface because of the predominance of filamentous bacteria. Scanning electron microscope (SEM) observations further revealed that the glucose-fed mature aerobic granules indeed had a filamentous dominant outer surface, while the acetate-fed aerobic granules had a very compact microstructure in which cells were tightly linked together and rod shape bacteria were predominant. Aerobic granulation appears to be a gradual process involving the progression from seeded sludge to compact aggregates, further to granular sludge and finally to mature granules.

Figure 2-5. The aerobic granule formation from seed sludge was observed using light microscopy and scanning electron microscopy as reported by Tay et al. (2001a). Seeding activated sludge under light microscopy (a) and SEM (b); aerobic granule formed using glucose (c) and acetate (d) as sole carbon source; and surface of the glucose granule (e) and acetate granule (f).

2.2.2 Characteristics of aerobic granules

The sludge or flocs in conventional activated sludge systems have a loose, irregular and sometimes fluffy structure (Tay et al. 2001a). The settling ability of these types of sludge is an important consideration for effective wastewater treatment. Aerobic granulation technology is able to overcome the poor settling sludge commonly encountered in activated sludge system (Beun et al. 1999; 2000; Tay et al. 2001; 2002; Arrojo et al. 2004). Besides, aerobic granules have many other superior characteristics, for example, regular shape with good separation from liquor phase, strong and integrated three dimensional structure; tolerance to high concentration of toxic compounds, high biomass retention in bioreactor, and ability to handle high organic loading rates (Morgenroth et al. 1997; Tay et al. 2002a; Jiang et al. 2002; 2004; Moy et al. 2002).

Under microscopy, aerobic granules are clearly distinguished from activated sludge and sludge flocs. As shown in Figure 2-4, aerobic granules have a well defined shape, nearly spherical, with a clear boundary (Peng et al. 1999; Tay et al. 2001a; Zhu and Wilderer 2003). In addition, the particle size is often used as a very important factor to distinguish aerobic granules from activated sludge flocs. In all reported aerobic granulation studies, the mean particle sizes of aerobic granules range from 0.2 to 5 mm in diameter (Beun et al. 1999; 2002, Tay et al. 2001a-c; Arrojo et al. 2004). However, the normal activated sludge flocs' mean particle sizes are generally smaller than 0.15 mm in diameter. This obvious difference in size can be clearly differentiated by the naked eyes.

The settling property of biomass is also an important factor which is used to distinguish aerobic granules from normal activated sludge flocs. The settling ability is usually determined by two characterization parameters, SVI and settling velocity. The sludge volume index (SVI) is commonly used to indicate the compactness of sludge. Normally, aerobic granules may have SVI value less than 90 mL g⁻¹ SS, or

sometimes, less than $60 \text{ mL g}^{-1} \text{ SS}$ (Jiang et al. 2002; Tay et al. 2005). Such values indicate aerobic granules have very compact structure compared with conventional activated sludge. The sludge settling velocity is crucial to determine how fast the sludge could settle. The settling velocity is generally as high as 30 to 70 m/h for aerobic granules (Liu and Tay 2004). This is comparable with that of the anaerobic granules, but is at least three folds higher than that of activated sludge flocs with typical settling velocity of 8 to 10 m/h. The high settling velocities of aerobic granules lead to more biomass retention in the reactor and this can enhance the overall performance and stability of the bioreactor. A high concentration of retained biomass also ensures a faster degradation of pollutants. Aerobic granules have very low SVI and very high settling velocity, and consequently better settling ability compared with conventional activated sludge.

Another characteristic, which has been widely discussed, is specific gravity. It has been accepted that the specific gravity is correlated with the structure of aerobic granules. The specific gravity of aerobic granules typically ranged from 1.004 to 1.065 (Etterer and Wilderer 2001; Tay et al. 2001a; Yang et al. 2004a&b). Aerobic granules with smaller sizes tend to be more compact compared to larger aerobic granules (Toh et al. 2003; Yang et al. 2004a). Compact structures of aerobic granules and higher specific gravities give aerobic granules better settling property compared with activated sludge.

2.2.3 Factors affecting aerobic granulation

The microbial granulation process could be affected by several operational parameters as well as microbial characteristics. To obtain stable aerobic granules, a number of factors may be satisfied in granulation process.

2.2.3.1 Seed sludge

The type of seed sludge is known to influence the formation and properties of anaerobic granulation process (Liu and Tay 2004; Tay et al. 2001b&c). However, up-to-date, there is no direct evidence to show that sludge characteristics can affect the aerobic granulation process as much as it affects the anaerobic granulation process. Aerobic granules could be formed from broad types of seed sludge, for example, acclimated and non-acclimated activated sludge (Tay et al. 2001a; Jiang et al. 2004). It is no doubt that high quality seed sludge would significantly increase the aerobic granules formation process as well as enhance the stability of formed aerobic granules. The important factors that determine the quality of seed sludge for aerobic granulation generally include settling ability, sludge hydrophobicity and surface charge density, and microbial activity.

2.2.3.2 Reactor configuration

Reactor configuration can have important impacts on the flow pattern of liquid, substrate distribution, which in turn can influence the microbial aggregation in the reactor. So far, in almost all reported studies, aerobic granules were only successfully cultivated using sequencing batch reactors (SBRs) (Beun et al. 1999; Liu and Tay 2002). Table 2-3 (page 32) summarized information from several published aerobic granulation studies. Conventional activated sludge system uses a so called

completely mixed tank reactor (CMTR). It is known that column-type upflow reactor and CMTR have very different hydrodynamic behaviors in terms of the interactions between flow pattern and microbial aggregates. In the column type reactor, the upflow of liquid or aeration could generate a more homogenous circular flow and localized vortexing along the reactor's axis. Under such conditions, microbial aggregates may be subject to a constant hydraulic attrition. The circular flow apparently forces the microbial aggregates to adapt a regular granular shape that has a minimum surface free energy (Liu and Tay 2004).

In a column-type upflow reactor, a high ratio of reactor height to diameter (H/D) can ensure a longer circular flow trajectory which in turn provides a more effective hydraulic attrition to microbial aggregates. However, in CMTRs microbial aggregates stochastically move with dispersed flow in all directions. Thus, microbial aggregates are subject to varying localized hydrodynamic shear force, upflow trajectories and random collisions. Under such circumstances, only flocs with irregular shape and size instead of regular granules occasionally form (Liu and Tay 2002). For practical applications, the SBR should have a high H/D ratio to improve selection of granules by the difference in settling velocity (Beun et al. 1999).

2.2.3.3 Substrate composition

Aerobic granules have been successfully cultivated with a variety of substrates, including glucose, acetate, ethanol, phenol, synthetic wastewater, and real municipal wastewater (Beun et al. 1999; Peng et al. 1999; Tay et al. 2001a, 2003b; Moy et al. 2002; Jiang et al. 2002; Schwarzenbeck et al. 2004). All these reported substrates are easy to degrade and can supply enough energy to support microbial growth and maintain other physiological functions. It seems that once the substrate composition could supply enough energy for microorganisms, it may not be treated as an essential parameter in aerobic granulation process. It is important because it means that

aerobic granules could be used to treat very broad contaminants or very broad types of wastewater. In addition, aerobic granules have been also cultivated for nitrifying purpose using inorganic carbon source (Tay et al. 2002b; Tsuneda et al. 2003). These nitrifying granules showed an excellent nitrification capability.

More recently, aerobic granules were also successfully developed in laboratory-scale SBR for treating particulate organic matter rich wastewater (Schwarzenbeck et al. 2003). Unfortunately, there is no study using recalcitrant compounds to culture the aerobic granules yet. Recalcitrant compounds are resistant to biodegradation and, most of time, are not good substrates supporting growth even though they could be degraded by some bacteria. To culture aerobic granules using recalcitrant compounds may require special strategies and operational conditions to overcome negative effects of recalcitrant compounds. If it could be demonstrated that aerobic granules could be cultivated by using recalcitrant compounds, it might greatly expand the future application areas of this novel biological wastewater treatment technology.

It needs to be pointed out that although substrate composition may not affect granulation process, it did affect the granules morphology and microbial diversity. Generally, granules' microstructure and microbial communities' diversity appeared to be related to the type of carbon source (Yi et al, 2003; Yang et al. 2005). Filamentous bacteria have been shown to dominate the outer surface of glucose-fed aerobic granules (Tay et al. 2001a). In contrast, rod- and cocci-shape bacteria dominated the acetate-fed granules and gave rise to very compact granule structures. The effect of different substrates may be more crucial when the aerobic granulation system is used to treat real wastewater because such wastewater may also contain very broad and unspecific contaminants.

2.2.3.4 Organic loading rate

Organic loading rate (OLR) is an essential parameter involved in anaerobic granulation process, and it was found that higher OLR favored the formation of anaerobic granules in UASB systems. In contrast, aerobic granules can be formed under a very wide OLR range, from 2.5 to 15 kg chemical oxygen demand (COD) $\text{m}^{-3} \text{day}^{-1}$ (Moy et al. 2002; Liu et al. 2003). It revealed that aerobic granulation is not as sensitive as anaerobic granules to the OLR.

Similar with substrate composition effects, OLR is not a sensitive parameter in aerobic granule formation but did have impacts on the morphology and physiological characteristics of granules. When the OLR increased from 3 to 9 kg-COD $\text{m}^{-3} \text{day}^{-1}$, mean granular size also increased from approximately 1.6 to 1.9 mm (Liu et al. 2003a). In addition, higher OLR may cause a relatively weakened structure which significantly reduced the strength of the aerobic granule. Such effects were also noticed in anaerobic granules as well as biofilm systems (Liu and Tay, 2004). The reason is higher OLR encourages the growth rate of bacteria, eventually increasing the chance of bacteria detaching from granules or biofilms, and hence reduces the strength of the three-dimensional structure of the microbial community (Liu et al. 2003a). Besides these effects, higher OLR did not significantly impact the dry biomass density in reactors, specific gravity, and sludge volume index (SVI) (Liu et al. 2003a; Tay et al. 2004).

2.2.3.5 Hydraulic retention time

In almost all biological wastewater treatment systems, hydraulic retention time (HRT) is a crucial operational parameter. Accumulating results show that HRT is also a very important operational parameter which is directly related with aerobic granules formation (Yang et al. 2004b; Liu and Tay 2004; Tay et al. 2005). For

example, Tay et al. (2002a) investigated the HRT effects on the nitrifying granule formation in SBR systems using different HRTs ranging from 6 to 48 hours. Nitrifying granules only formed under 12, 16 and 24 hours HRT, but no granules were observed in reactors using 6 or 48 hours HRT. The authors postulated that a reasonably short HRT stimulated microbial activity, enhanced the production of cell polysaccharides and improved the cell hydrophobicity which overall benefited granulation process.

In SBR system with a fixed volumetric exchange ratio, HRT is associated with a parameter called washout frequency. A suitable washout frequency may encourage the growth of relatively large microbial aggregates to form granules; at the same time, it suppresses the growth of suspended growth bacteria because of frequent washout of the suspended bacteria. This washout eventually minimizes the competition between large cell aggregates and suspended bacteria for substrate, nutrients, oxygen, and living capacity. However, if the SBRs were running at an extremely short cycle time (less than 3 h-cycle), like the case Tay et al. (2002a) reported, sludge loss occurred through hydraulic washout because bacterial growth was not rapid enough to compensate the washout. Finally, a complete washout of sludge blanket occurred and led to a failure of microbial granulation. Thus, the conclusion is that the HRT should be short enough to inhibit the suspended growth, but long enough for aggregated microbial growth and accumulation.

2.2.3.6 Hydrodynamic shear force

The effects of hydrodynamic shear force on aerobic granulation were studied in the column-type sequential batch aerobic blanket reactors (Tay et al. 2001c). Hydrodynamic turbulence caused by upflow aeration served as the main shear force in the systems. Evidence showed that a high shear force favors the formation of aerobic granules and granule stability (Tay et al. 2001c). It was found that aerobic

granules could be formed only above a threshold shear force in terms of superficial upflow air velocity above 1.2 cm s^{-1} . The regular-shape, round, and compact aerobic granules were developed at higher hydrodynamic shear force (Tay et al. 2001a, c). The granule density and strength were also proportionally related to the shear force applied (Tay et al. 2003a). These observations may imply that the structure of aerobic granules is mainly determined by the hydrodynamic shear force present in a bioreactor. It was found that the shear force has a positive effect on the production of polysaccharide, biomass activity, and hydrophobicity of cell surface and specific gravity of granules. The polysaccharide and hydrophobicity effects will be discussed in the following sections.

2.2.3.7 Settling time

The crucial importance of settling time on granules formation has been recognized since the first studies on aerobic granules were reported (Beun et al. 1999; Tay et al. 2001a). Settling time is a unique operational parameter involved in SBR system. In a classical SBR operation cycle, the biomass is settled at each end of every cycle, just before the treated water discharged. Generally, settling time plays a role like cutoff selection to choose the biomass with certain settling capability. Suspended bacteria and poor settling ability biomass could be washed out by the discharged effluent. This results in a preferable condition for the superior growth of aerobic granules because granules have higher gravity and better settling ability to enhance biomass retains in the reactors. Thus, the settling time acts as a major hydraulic selection pressure on microbial community.

Qin et al. (2004a&b) reported that aerobic granules were successfully cultivated and became dominant only in the SBR operated at a settling time of 5 min. Mixtures of aerobic granules and suspended sludge were observed in the SBRs operated at settling times of 20, 15, and 10 min. The production of extracellular polysaccharides

was stimulated and the cell surface hydrophobicity improved significantly at short settling times. In addition, it was reported that the mature aerobic granules tend to settle within 1 min, leaving a clear supernatant in the reactor (Tay et al. 2001a). Granules with excellent settling properties are essential for the effective functioning of biological wastewater treatment systems.

2.2.3.8 Feast-and-Famine regime

Starvation period may play an important role in granulation. Bacteria in SBR system suffer the so-called feast and famine regime as substrate concentration is continuously decreasing from initial to the end within a SBR cycle. Thus, the aeration period of the operation actually consists of two phases: a degradation phase in which the substrate is depleted to a minimum, followed by an aerobic starvation phase in which the external substrate is no longer available. Previous studies on the response of bacteria to starvation have demonstrated that, under starvation conditions, bacteria became more hydrophobic which, in turn, facilitated microbial adhesion and aggregation (Bossier and Verstraete 1996). In addition, cells in starved colonies were found to form connecting fibrils which, in turn, strengthened cell-to-cell interaction and communication. Consequently, starvation-induced changes in the characteristics of the cell surface seem to favor the formation of strong microbial aggregates.

In the case of aerobic granulation, Tay et al. (2001a) proposed that the periodical starvation in the sequencing batch reactor would play a role in triggering changes in the cell surface and metabolism and lead to stronger microbial aggregates. Under such circumstances, microbial aggregates would become increasingly dense with the cycling operation of SBR. Therefore, the microbial structure becomes more and more compact as the result of periodical starvation in SBR system. Recently, McSwain et al. (2004) developed an operation strategy to enhance aerobic granulation

by intermittent feeding strategy. A feast-famine cycle or pulse feeding of the SBR favored the formation of compact and dense aerobic granules. Although the periodical starvation in SBR is important for microbial aggregation, the contribution of other operation conditions should not be neglected.

2.2.3.9 Extra-cellular polysaccharide (EPS) and hydrophobicity

Extra-cellular polysaccharides are a type of bio-materials synthesized by bacteria themselves under different cultivation conditions (Sutherland 1985 and 1999). Extra-cellular polysaccharides can mediate cohesion and adhesion of cells, formation of 3D matrix structure, participate in microbial physiological activities, and play an important role in maintaining the structural integrity of the heterotrophic granular matrix (Costerton et al. 1995; Stoodley et al. 2002). The importance of EPS to the bacterial granulation process has been studied in activated flocs, biofilms, anaerobic granules as well as aerobic granules (Liu et al. 2004a). The accumulation and importance of EPS in all these microbial ecosystems have been demonstrated intensively. However, it should be pointed out that EPS content in granular sludge was significantly higher than other types of bacteria aggregations (e.g. activated sludge and biofilm) (Tay et al. 2001a).

Presence of EPS can help to stabilize bacterial membrane structure and may also serve as a protective barrier for bacteria to harsh environmental conditions (Jiang et al. 2004b). Experimental evidences from aerobic granulation studies have shown that selection pressures, including hydrodynamic shear force, settling time, and feast-and-famine regime, were the main driving forces to bacteria to synthesize more EPS for granulation. Unfortunately, it is not known if such high EPS content in aerobic granule was a trigger or symptom of granulation.

Cell surface hydrophobicity is an important affinity force in cell

self-immobilization and attachment processes (Prescott et al. 1999; Liu et al. 2003b). The role of cell surface hydrophobicity in the formation of aerobic granules has not been clear. Liu et al. (2004b&d) have linked the formation of heterotrophic and nitrifying granules to the cell surface hydrophobicity. The hydrophobicity of granular sludge was nearly twofold higher than that of conventional bioflocs. A high shear force or hydraulic selection pressure imposed on microorganisms resulted in a significant increase in the cell surface hydrophobicity, while the cell surface hydrophobicity seemed not to be sensitive to the changes in the organic concentrations or loading rates in the range of 500 to 3000 mg-COD L⁻¹.

2.2.3.10 Other factors

Several studies also reported that aerobic granule formation was affected by other operational parameters, operational strategies, or chemicals.

In the conventional activated sludge system and other aerobic wastewater treatment system, dissolved oxygen concentration (DO) is an important parameter with significant impacts on treatment efficiency. It is easy to understand because aerobic bacteria normally have relatively higher activities at DO concentrations higher than 2 mg L⁻¹. So, it is no surprise that aerobic granules are generally cultivated at DO concentration higher than 2.0 mg L⁻¹. However, Peng et al. (1999) reported that aerobic granules could also form at DO concentrations as low as 0.7 to 1.0 mg L⁻¹ in a SBR. It seems that aerobic granules formation could be achieved within a relatively wide DO concentration range.

It has been known from anaerobic granulation studies that Ca²⁺ ion could significantly increase the anaerobic granules formation as well as enhance the stability of formed anaerobic granules. Jiang et al. (2003) reported that 100 mg L⁻¹ Ca²⁺ could accelerate the aerobic granules formation from 32 days to 16 days after the

reactor startup. In addition, the Ca^{2+} amended aerobic granules showed good settling ability, better strength, and higher EPS content compared with non- Ca^{2+} amended granules. This result reinforced the hypothesis that Ca^{2+} ion served as a bridge to promote bacterial aggregation because Ca^{2+} could bind with negatively charged materials like cell membrane and EPS.

Recently, it was found that free ammonia in the culture medium could depress the aerobic granulation (Yang et al. 2004a). The authors investigated the inhibitory effect of free ammonia on aerobic granulation in a SBR fed with acetate as the sole carbon source. Aerobic granules formed only when the free ammonia concentration was less than 23.5 mg L^{-1} . It was found that high free ammonia concentration resulted in a significant decrease of cell hydrophobicity and also repressed the production of extracellular polysaccharides. Changes in hydrophobicity and polysaccharide production were likely responsible for the failure of aerobic granulation at high free ammonia concentrations.

2.2.4 The hypothesis of aerobic granules formation and microstructure

Aerobic granulation is a very complex process which might involve numerous factors interacting to finally result in the formation of aerobic granules. The hypothesis for aerobic granulation has been raised from intensive studies (Beun et al, 1999; Liu and Tay 2002a). It is agreed that a number of conditions have to be met and the physical, chemical, and biological forces contributing to granulation need to be viewed in combination for bacteria to form aerobic granules.

In general, aerobic granulation is a dynamic process and involves several stages, as proposed by Beun et al. (1999) and Tay et al. (2001a). In the first stage, bacterial connection and aggregation seems very important. Filamentous fungal pellets and bacterial cell aggregates served as an immobilization matrix in which bacteria could grow out to colonies. The factors involved in this step are hydrodynamics, diffusion mass transfer, gravity, thermodynamic effects, and cell mobility.

In stage two, the cell aggregates are continuously growing to larger size, during which period some of them may disintegrate. However, the bacterial colonies still could remain in the reactor because they are already large enough to settle sufficiently fast. The colonies and aggregates may further grow out to granules. Some attractive forces, including Van der Waals forces, opposite charge attraction, surface tension and hydrophobicity, are believed to play important roles to maintain these aggregates.

In the third stage, maturation of bacterial cells' aggregates through production of extracellular polymer, growth of cellular clusters, metabolic change, environment-induced genetic effects that facilitate the cell-cell interaction and result in a highly organized microbial structure.

In the final stage, shaping of the steady state three-dimensional structure of

microbial aggregate by hydrodynamic shear forces might be involved.

2.2.4.1 Microbial structure

As early as 1999, Peng et al. (1999) reported that aerobic granules may be involved a three-level structure. The authors observed aerobic granules cultivated in a SBR fed with acetate as sole carbon and energy source. They reported that the granules are organized as follows: the first level is about 0.5 to 5 μm thick containing living cells, lysed cells and debris, and influent solid particles. The second level is about 5 to 50 μm thick containing bacterial microcolonies encapsulated in polymer matrix. And the third level is made up of numerous individual particles and microcolonies enmeshed in exopolymers. The size of these granules is around 0.3 to 0.5 mm.

On the other hand, a layered structure of aerobic granules also from a SBR system feeding glucose as sole carbon source was reported by Tay et al. (2002b). Fluorescent *in situ* hybridization (FISH) methods combined with confocal laser-scanning microscopy (CLSM) was used to examine the various layers within the granules. It was found that aerobic granules contained general by four layers. The outside layer was mainly occupied by aerobic bacteria. Strictly aerobic ammonium-oxidizing bacteria, *Nitrosomonas* spp., were detected and dominant in this layer. The layer was situated at a depth of 70 to 100 μm from the edge of granules. A dense polysaccharide layer was observed at a depth of 400 μm from surface. Following was an anaerobic layer, in which obligate anaerobic bacteria *Bacteroides* spp. was detected. This layer was situated at a depth of 800 to 900 μm from the surface of granules. Finally, a dead cell layer was detected at a depth of 800 to 1000 μm from the surface of the granules. This may imply that it is the accumulation of polysaccharide at upper layer blocking the oxygen and nutrient sources transfer.

In addition, channels and pores were also found in the granules. Tay et al. (2002b) used 0.1 μm diameter size fluorescent microsphere standards to detect the channels and pores in the granules. The results showed that these fluorescent beads penetrated to as deep as 900 μm , and the strongest fluorescence densities were detected at the depth of 300 to 500 μm . The results indicated that the most of channels in aerobic granules could extend to 300 to 500 μm depth into the granules. It was believed that channels and pores could help to overcome the diffusion limitation within the granules. As discussed by the authors, the layered structure develops due to diffusion limitation. Since the oxygen and substrate in wastewater can only diffuse for a certain distance, therefore, strictly aerobic microbes stay at the outer layer of granules. The presence of channels and pores can overcome a portion of diffusion limitation. However, when these channels and pores are blocked by polysaccharides, a strictly anaerobic microbial layer can be formed. When the channels and pores are completely clogged, the dead cells dominate the centre core.

Liu et al. (2004c) observed a mushroomlike structure of aerobic granules developed at high substrate N/COD ratios. The nitrifying population was mainly located at a depth of 70 to 100 μm from the surface of the granule (Tay et al. 2004c). Research has shown that biofilms of mixed bacterial communities can form thick layers consisting of differentiated mushroomlike structures (Costerton et al. 1981) that are similar to structures observed in aerobic granules. There is strong evidence that bacteria sense and move towards nutrients (Prescott et al. 1999). It has been demonstrated that biofilms can form the mushroomlike structures by simply changing the diffusion rate; that is, the biofilm structure is largely determined by nutrient concentration (Wimpenny and Colasanti 1997). Since nitrifying bacteria are slow-growing, the mushroomlike structures seem to result from the demand of nitrifying population for nutrients. These structures improve the access of the nitrifying population to nutrients. As Watnick and Kolter (2000) noted, in mixed biofilms, bacteria distribute themselves according to who can survive best in the particular microenvironment and the high complexity of the resulting microbial

Chapter Two

community appears to be beneficial to its stability. Consequently, the distribution of different microbial populations in a granule may have an effect on its stability.

2.2.5 Applications of aerobic granulation technology

As an emerging novel wastewater treatment biotechnology, the application potential is one of the greatest concerns on this biotechnology. Many studies have been conducted and reported on successful application of this novel biotechnology to treat different types of wastewater.

2.2.5.1 Treatment of high-strength organic wastewater

As discussed above, aerobic granules have been cultivated using synthesized wastewater containing different types of sole carbon and energy source. This points out the versatility of this technology. However, more attractive features of aerobic granulation system is that this system could tolerate and handle very high strength COD containing wastewater compared with conventional activated sludge system. The feasibility of applying aerobic granulation technology for the treatment of high-strength organic wastewaters was demonstrated by Moy et al. (2002), who examined the ability of aerobic granules to sustain high organic loading rates by introducing step increases in organic loading only after the COD removal efficiencies had stabilized at values of >89% for at least 2 weeks. Aerobic granules cultivated this way on glucose were exposed to organic loading rates that were gradually raised from 6.0 to 9.0, 12.0, and 15.0 kg COD m⁻³ day. Aerobic granules were able to sustain the maximum organic loading rate of 15.0 kg COD m⁻³ day while removing more than 92% of the COD.

Besides this, aerobic granulation system could retain high amounts of biomass because of the compact and dense structure of the granules. Biomass concentrations as high as 6.0 to 12.0 g L⁻¹ have been obtained in SBRs operated with a volumetric exchange ratio of 50% (Tay et al. 2002a&c). High biomass concentration is believed to generate benefits such as ease of degradation of recalcitrant compounds, high volumetric biodegradation rates, and smaller reactor footprints.

2.2.5.2 Treatment of phenolic wastewater

Phenolic compounds are a group of toxic compounds which are widely used in many industrial applications. Thus, phenol-contaminated industrial wastewaters is a general problem in wastewater treatment plant because of the toxicity of these compounds to the bacteria. High concentration phenolic compounds kill microorganisms and eventually cause bioreactor failure. It is very attractive that aerobic granulation system seems very suitable to be applied in phenolic compounds containing wastewater treatment. Jiang et al. (2002, 2004a) investigated the feasibility of treating phenol-containing wastewater with aerobic granular sludge. Granular sludge is less susceptible to toxicity of phenol because much of the biomass in the granules is not exposed to the same high concentration as present in the bulk wastewater. The phenol-degrading aerobic granules displayed an excellent ability to degrade phenol (Jiang et al. 2002, 2004a). For an influent phenol concentration of 500 mg L^{-1} , a stable effluent phenol concentration of less than 0.2 mg L^{-1} was achieved in the aerobic granular sludge reactor (Jiang et al. 2002, 2004a). The high tolerance of aerobic granules to phenol can be exploited in developing compact high-rate treatment systems for wastewaters loaded with a high concentration of phenol. Aerobic granules may be proven to be powerful bioagents for removing other inhibitory and toxic organic compounds from high-strength industrial wastewaters.

2.2.5.3 Removal of nitrogen and phosphate

Beun et al. (2001) studied nitrogen removal in a sequencing batch airlift reactor (SBAR) under conditions of decreased dissolved oxygen (DO) concentrations. A simulation model was developed to describe and evaluate the effects of several process conditions in the SBAR on N-removal performance. It has been shown that nitrification, denitrification, and removal of chemical oxygen demand (COD) can

occur simultaneously in a granular sludge SBR. DO concentration was found to have a significant effect on the efficiency of denitrification by microbial granules and a certain level of mixing was necessary for ensuring sufficiency of mass transfer between the liquid and granules during denitrification.

Yang et al. (2004a) investigated the simultaneous removal of organics and nitrogen by aerobic granules. Heterotrophic, nitrifying, and denitrifying populations were shown to be successfully coexisting in microbial granules. Increased substrate N/COD (N refers to nitrogen) ratios led to significant shifts among these three populations within the granules. Coexistence of heterotrophic and nitrifying populations in aerobic granules was also observed by Jang et al. (2003). Enhanced activities of nitrifying and denitrifying populations were achieved in granules developed at high substrate N/COD ratios; however, the heterotrophic populations in granules tended to decrease with the increase of substrate N/COD ratio. In addition, the effects of dissolved oxygen concentrations on N removal were also reported (Beun et al. 2001; Yang et al. 2003). It appears that complete organics and nitrogen removal can be efficiently and stably achieved in a single granules-based SBR.

Lin et al. (2003) successfully developed phosphorus-accumulating microbial granules in SBRs operated at substrate P/COD ratios ranging from 1/100 to 10/100 by weight. The soluble COD and $\text{PO}_4\text{-P}$ profiles showed that the granules had typical P-accumulating characteristics, with uptake of soluble organic carbon and the concomitant release of phosphate in the anaerobic stage, followed by rapid phosphate uptake in the aerobic stage. The size of P-accumulating granules exhibited a decreasing trend with increasing of substrate P/COD ratio. The structure of the granules became more compact and dense as the substrate P/COD ratio increased. The P uptake by granules was in the range of 1.9% to 9.3% by weight, or comparable to that of the conventional enhanced biological phosphorus removal (EBPR) processes. These results will certainly spur the further development of novel granule-based EBPR technologies.

2.2.6 Summary

Aerobic granulation is a complex process with many crucial factors involved. Unfortunately, not all of these factors are clearly understood up-to-date. Nevertheless, previous studies on the aerobic granulation process have offered a general guidance to cultivate aerobic granules in SBR systems. The structure-function and biodegradability studies of aerobic granules point to the potential of this novel biotechnology for wastewater treatment.

So far, studies of aerobic granules have involved easily degradable substrates. There is little or no information on how aerobic granules would respond to recalcitrant compounds. *Tert*-butyl alcohol (TBA) has long been recognized as a recalcitrant compound. Only in recent years, the biodegradability of TBA has been reported. TBA is also a carcinogenic pollutant whose presence can pose serious environmental impacts. This compound was picked as a model compound to study if it can serve as a substrate for degradation by aerobic granules.

Chapter Three

Cultivation of Aerobic TBA-Degrading Granules in Sequencing Batch Reactors

3.1 Introduction

Tert-butyl alcohol (TBA) is widely used as a solvent and an intermediate in many industrial processes. Because of its widespread use as well as its possible accumulation as an intermediate of methyl *tert*-butyl ether biodegradation, TBA is often present as a contaminant in groundwater by the spill of oxygenated fuels. TBA concentrations of up to 160 mg L⁻¹ have been measured in groundwater plumes in the vicinity of a chemical plant (Day and Gulliver 2003).

Aerobic biodegradation of TBA has been investigated in bioreactor systems such as simple stirred tank reactors, fluidized bed bioreactors, attached growth reactors and membrane bioreactors (Morrison et al. 2002; Vainberg et al. 2002; Wilson et al. 2002a; Zein et al. 2004). Such studies are driven by the need for ex-situ treatment at sites where groundwater extraction is required to halt the migration of contaminant plumes. One striking conclusion from these studies is that a high biomass concentration is necessary for effective contaminant degradation.

The main objective of this study is therefore to evaluate the utility of using a sequencing batch reactor (SBR) for complete biodegradation of TBA as sole carbon source; and to cultivate aerobic granules under the investigated conditions. This work will be useful in understanding how SBRs as well as aerobic granules can be exploited for effective biodegradation of TBA and other recalcitrant chemicals.

3.2 Materials and Methods

3.2.1 Reactor operation

Two sequencing batch reactors (SBRs) (10 cm diameter) with a working volume of 2.0 L were inoculated with fresh activated sludge from a municipal wastewater treatment plant to give an initial biomass concentration of approximately 2900 mg VSS L⁻¹ (VSS stands for volatile suspended solids). The two reactors were designated as Reactor One (R1) and Reactor Two (R2) regarding to the operational conditions applied. In general, R1 has shorter cycle time and R2 has higher start-of-cycle TBA concentrations. Both reactors were maintained at 25°C in a temperature control room. TBA was fed as the sole carbon and energy source with a synthetic wastewater with the following composition (per liter): 0.8 g of NaNO₃, 0.8 g of Na₂HPO₄, 0.3 g of KH₂PO₄, 0.4 g of MgCl₂·6H₂O, 0.1 g of CaCl₂, 1.5 mg of CoSO₄, 1.0 mg of CuSO₄, and 5.0 mg of FeCl₃ (Piveteau et al. 2001). Effluent was discharged at a volumetric exchange ratio of 50%. Fine air bubbles for aeration were supplied through a dispenser at the reactor bottom at an airflow rate of 0.4 to 0.5 L min⁻¹ to maintain a dissolved oxygen concentration above 4 mg L⁻¹.

The reactor experiments were carried out in five phases, with each subsequent phase utilizing a higher TBA loading rate (Table 3-1). In Phase 1, both reactors were initially operated sequentially in 24 h cycles (5 min of influent filling, 23 h 20-30 min of aeration, 20-30 min of settling, and 5 min of effluent withdrawal) to give a hydraulic residence time (HRT) of 48 h. Because TBA was not completely degraded within each cycle in Phase 1, the amount of TBA in the influent was adjusted to maintain a TBA concentration of 100 mg L⁻¹ in the reactor at the start of each cycle. By day 81, both reactors were able to completely remove TBA within each cycle. Hence, the influent TBA concentration was fixed at 200 mg L⁻¹ to give a TBA concentration of 100 mg L⁻¹ in the reactors at the start of each cycle. The reactors were then tested for their ability to handle higher TBA loadings. This was generally achieved by introducing incremental reductions in cycle time in R1 and step-wise increases in influent TBA concentration in R2.

Table 3-1. Operational conditions of reactors R1 and R2.

	Phase 1		Phase 2		Phase 3		Phase 4		Phase 5	
Time period	Days 0-89		Days 90-99		Days 100-120		Days 121-150		Days 151---	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
Cycle time (h)	24		24		12	24	8	24	6	24
SRT^a (d)	< 10		20-30		20-30		20-30		20-30	
HRT^b (h)	48		48		24	48	16	48	12	48
Start-of-cycle TBA conc. (mg L⁻¹)	100		150		150	300	150	450	150	600
TBA loading rate (mg L⁻¹ d⁻¹)	100		150		300		450		600	

a: SRT – Sludge Retention Time

b: HRT – Hydraulic Retention Time

In R1, the influent TBA concentration was raised to 300 mg L^{-1} on day 90 (Phase 2), and the cycle time was reduced from 24 h to 12, 8 and 6 h on days 100 (Phase 3), 121 (Phase 4) and 199 (Phase 5), respectively. The TBA loading rate in Phase 1 did not exceed $100 \text{ mg L}^{-1} \text{ d}^{-1}$, while the TBA loading rates in Phases 2, 3, 4 and 5 were 150, 300, 450 and $600 \text{ mg L}^{-1} \text{ d}^{-1}$, respectively.

In R2, the influent TBA concentration was raised to 300 mg L^{-1} on day 90 (Phase 2), and the influent TBA concentration was increased from 300 mg L^{-1} to 600, 900 and 1200 on days 100 (Phase 3), 121 (Phase 4) and 199 (Phase 5), respectively. The cycle time was maintained at 24 h. The TBA loading rate in Phase 1 was below $100 \text{ mg L}^{-1} \text{ d}^{-1}$, while the TBA loading rates in Phases 2, 3, 4, and 5 were 150, 300, 450 and $600 \text{ mg L}^{-1} \text{ d}^{-1}$, respectively.

3.2.2 Analytical methods

pH, volatile suspended solids (VSS), sludge volume index (SVI) and dissolved oxygen (DO) were measured periodically using standard methods (APHA, 1998). Biomass size was measured with a laser particle size analysis system (Malvern Mastersizer 2600) (Jiang et al. 2004a). Biomass samples were fixed and dried, then viewed with a scanning electron microscope (Stereoscan 420, Leica Cambridge Instruments), as described previously (Zhuang et al. 2002). A direct injection gas chromatograph method (Steffan et al. 1997) was used to determine TBA concentrations. All liquid samples were collected and analyzed immediately. Cell-free aqueous samples ($1\text{--}3 \text{ }\mu\text{L}$) were automatically injected into a Hewlett Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a DB624 column (Hewlett Packard, $30 \text{ m} \times 0.53 \text{ mm} \times 3 \text{ }\mu\text{m}$). The oven temperature was held at 60°C for 3 min and was then ramped to 120°C at $40^\circ\text{C min}^{-1}$, which was held for 3 min, and finally raised to 200°C at $40^\circ\text{C min}^{-1}$. TBA peaks and concentration values were determined using Chemstation software (Hewlett Packard). A solid phase microextraction (SPME) method (Cassada et al. 2000) with a detection limit of $25 \text{ }\mu\text{g L}^{-1}$ was employed to assay TBA concentrations

below 1.0 mg L^{-1} .

TBA biodegradation rates were determined by incubating biomass samples at predefined TBA concentrations (100 mg L^{-1} for time profile measurements and from 20 to 1200 mg L^{-1} for biodegradation kinetics study). The mineral salts (MS) medium used in the batch incubations had the following composition (per liter): 0.4 g of NaNO_3 , 0.4 g of Na_2HPO_4 , 0.15 g of KH_2PO_4 , 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g of CaCl_2 , 1.0 mg of CoSO_4 , 1.0 mg of CuSO_4 , and 3.0 mg of FeCl_3 . For each batch, 100 mL of biomass was harvested from the reactor during the aeration period, washed twice, resuspended in 100 mL of fresh MS medium and transferred into a 500 mL reagent bottle with screw cap. Abiotic controls contained 2.9 g L^{-1} sodium azide. Bottles were shaken in the dark at 25°C on an orbital shaker at 200 rpm and assayed periodically. All experiments were performed in triplicate. Biomass samples were then centrifuged and washed and returned to the reactor. A kinetic analysis of the biodegradation data was performed for stabilized biomass samples from Phase 5 (on days 230 and 231) using Haldane's formula for an inhibitory substrate $V = V_{\max} S / [K_s + S + (S^2/K_i)]$ where V and $V_{\max}$ are the specific and the theoretical maximum specific substrate biodegradation rates ($\text{mg TBA g VSS}^{-1} \text{ h}^{-1}$) respectively, and S , K_s and K_i are the substrate concentration, half-saturation constant and inhibition constant (mg TBA L^{-1}), respectively (Jiang et al. 2002).

3.2.3 DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE)

Genomic DNA of enriched TBA-degrading biomass was extracted based on a protocol described previously (Kowalchuk et al. 1997). Approximately 200-300 mg (wet weight) of biomass was harvested in duplicate during the aeration stage of the SBR cycle and used immediately for DNA extraction. This involved bead beating followed by extraction with saturated phenol (pH 8.0), phenol-chloroform (1:1) and chloroform:isoamyl alcohol (24:1). The extracted DNA was precipitated overnight with a sodium acetate-ethanol mix at -20°C and dissolved in sterile

deionized water. Extracted DNA samples were stored in a -20°C freezer before use.

PCR primers P2 (5'-ATTACCGCGGCTGCTGG-3') and P3 (5'-CGCCCCGCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGAGGCAGCAG-3', containing 40 bp of GC clamp) were used to amplify the variable V3 region of bacterial 16S rRNA gene (corresponding to positions 341 to 534 in the *Escherichia coli* sequence) (Muyzer et al. 1993). Touchdown PCR was performed (Watanabe et al. 1998) with a Mastercycler Gradient thermal cycler (Eppendorf AG, Hamburg, Germany) using a 100 μL (total volume) mixture containing 2.5 U of Taq DNA polymerase (Promega, USA), 10 μL of 10 $\times$ Thermophilic DNA polymerase Buffer B, 8 μL of 25 mM MgCl_2 , 200 mM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 2 μL of the DNA extract (concentration 100 ng μL^{-1}). Successful PCR was confirmed by electrophoresis of amplicons through a 2.0% agarose gel in TAE buffer stained with ethidium bromide.

The PCR-amplified fragments were separated by DGGE using a DCode system universal mutation detection system (Bio-Rad Laboratories, USA) as described previously (Muyzer et al. 1993). A 25 mL 40% to 65% urea-formamide denaturant gradient gel [10% (w/v) acrylamide solution (40% acrylamide and bis acrylamide, 37.5:1 stock solution, Bio-Rad Laboratories) in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM $\text{Na}_2\text{-EDTA}$, pH 8.0)] was covered by a 4 mL acrylamide stacking gel (10%) without denaturant. 40 μL of PCR amplicons from DNA of biomass samples were loaded with 20 μL loading dye in each well. The gel was placed in TAE buffer and run at 40 V at 60°C for 30 min and then at 85 V at 60°C for 15 h. After electrophoresis, the gel was stained with ethidium bromide for 30 min, and viewed and photographed with an EDAS 290 gel imaging system (Kodak, USA). Selected DNA bands were aseptically excised and re-amplified by PCR several times as described previously (Watanabe et al. 1998) in order to obtain partial sequence information with the ABI PRISM BigDye Terminator Cycle Sequencing

ready-reaction kit (version 3.0) and the ABI model 310A sequencer (Applied Biosystems, Perkin-Elmer), according to manufacturer instructions. The partial sequences were compiled and aligned using BioEdit software and analyzed with BLAST and other algorithms as described previously (Zhuang et al. 2002).

3.3 Results

3.3.1 Reactor performance

Reactor R1 was initially seeded with fresh activated sludge from a municipal wastewater treatment plant. The starting sludge had a biomass concentration of 2910 mg VSS L⁻¹, a mean biomass size of 0.15 mm and an SVI value of 124 mL g⁻¹. TBA concentrations in the effluent persisted at levels of approximately 85 mg L⁻¹ during the first 30 days of operation in Phase 1, and this was accompanied by sharp declines in biomass concentration and biomass size as well as a deterioration in settling ability (Figures 3-1 and 3-2). However, improvements in biomass characteristics and reactor performance became noticeable after this initial period. TBA concentrations in the effluent declined steadily from day 33 and were below detection (< 25 µg L⁻¹) by day 90. Biomass concentrations reached their lowest level of 1100 mg VSS L⁻¹ on day 20, but demonstrated an increasing trend thereafter, rising to 1570 mg VSS L⁻¹ at the end of Phase 1. Similar patterns were also observed in mean biomass size and SVI during this period. Mean biomass size was as low as 0.07 mm on day 31, but increased to 0.13 mm on day 90. SVI exceeded 300 mL g⁻¹ for several days from day 21, but improved to below 150 mL g⁻¹ from day 90.

After Reactor R1 biomass was able to completely remove the input TBA within each operating cycle, step increases in TBA loading were subsequently introduced. The microbial consortium in the reactor was able to sustain the increases in TBA

Figure 3-1. Time profiles of start-of-cycle TBA concentrations (O), effluent TBA concentrations (Δ) and biomass concentrations (VSS) (□) of Reactor R1.

Figure 3-2. Time profiles of mean biomass size (O) and sludge volume index (SVI) (Δ) of Reactor R1.

loading without any drop in effluent quality as effluent TBA concentrations remained below detection. The increases in TBA loading were accompanied by corresponding increases in biomass concentration and biomass size and improvements in biomass settling ability. The biomass concentration, biomass size and SVI improved to eventually stabilize at 4540 mg VSS L⁻¹, 0.32 mm and 57 mL g⁻¹, respectively, at the end of reactor operation (Figures 3-1 and 3-2).

Granular sludge aggregates or aerobic granules were first observed in the Reactor R2 during Phase 4, approximately 125 days from the start of reactor operation. The onset of aerobic granules occurred shortly after the cycle time was reduced from 12 h to 8 h, and triggered a jump in mean biomass size to above 0.15 mm as well as a marked reduction in SVI to below 80 mL g⁻¹. These improvements in settling characteristics led to greater retention of biomass in the reactor and concomitant increases in biomass concentrations.

Reactor R2's starting sludge had a biomass concentration of 2900 mg VSS L⁻¹, a mean biomass size of 0.14 mm and an SVI value of 120 mL g⁻¹. TBA concentrations in the effluent persisted at levels of approximately 80 mg L⁻¹ during the first 30 days of Phase 1, and this was also accompanied by sharp declines in biomass concentration and biomass size as well as a deterioration in settling ability as the biomass struggled to adapt to the TBA feed (Figures 3-3 and 3-4). Similar with the Reactor R1, the improvements in biomass characteristics and reactor performance were noticeable after this initially difficult period. TBA concentrations in the effluent declined steadily from day 35 and were below detection (< 25 µg L⁻¹) by day 96. Biomass concentrations reached their lowest level of 1120 mg VSS L⁻¹ on day 39, but demonstrated an increasing trend thereafter, rising to 1500 mg VSS L⁻¹ at the end of Phase 1. Similar patterns were also observed in mean biomass size and SVI during this period. Mean biomass size was as low as 0.09 mm on day 51, but increased to 0.11 mm on day 90. SVI reached 290 mL g⁻¹ on day 31, but improved to below 130 mL g⁻¹ from day 90.

The TBA loading into the Reactor R2 was increased by employing a strategy that involved step increases in the start-of-cycle TBA concentrations while keeping the

Figure 3-3. Time profiles of start-of-cycle TBA concentrations (O), effluent TBA concentrations (Δ) and biomass concentrations (VSS) (□) of Reactor R2.

Figure 3-4. Time profiles of mean biomass size (O) and sludge volume index (SVI) (Δ) of Reactor R2.

HRT constant. Overall, the changes produced a six-fold increase in TBA loading. Effluent TBA concentrations fluctuated within the first 2 to 3 cycles after each step increase in influent TBA concentration, but fell below detection immediately after. The increases in TBA loading were generally accompanied by corresponding increases in biomass concentration and biomass size and improvements in biomass settling ability, except during two occasions in Phases 4 and 5. On the first occasion, the biomass size decreased from 0.22 to 0.19 mm between days 120 and 200, but without any corresponding deterioration in biomass concentration or SVI. On the second occasion, both biomass concentration and biomass size decreased after the start-of-cycle TBA concentration was increased at the beginning of Phase 5, contributing to a decline in settling ability. Nevertheless, the reactor recovered from this initial disturbance, and the biomass concentration, biomass size and SVI improved to reach 5210 mg VSS L⁻¹, 0.23 mm and 90 mL g⁻¹, respectively, at the end of Phase 5.

Aerobic granules were first observed in the Reactor R2 towards the end of Phase 4, approximately 180 days from the start of reactor operation. The granules had a clearly defined shape and appearance, and settled significantly faster than the suspended sludge in the Reactor R2. Although there were declines in biomass concentration, biomass size and settling ability when the start-of-cycle TBA concentration was raised to 600 mg L⁻¹ at the beginning of Phase 5, these were short-lived as the granules quickly grew to displace the suspended flocs as the dominant form of biomass in the Reactor R2 (Figure 3-5). The incursion of aerobic granules resulted in better settling and lower SVI values, and this in turn led to better biomass retention in the reactor.

3.3.2 TBA biodegradation rates

Specific TBA biodegradation rates of Reactor R1 were measured by harvesting reactor biomass periodically and incubating in batch cultures with 100 mg L⁻¹ TBA. Specific TBA biodegradation rates were insignificant during the first 30 days, but followed an increasing trend thereafter, to reach 2.2 mg TBA g VSS⁻¹ h⁻¹ on day 94

Figure 3-5. Scanning electron microscopy images of (a) R2 biomass on day 3; (b) R1 biomass on day 228; and (c) R2 biomass on day 228. (Scale bar = 150 μm)

and 8.7 mg TBA g VSS⁻¹ h⁻¹ on day 152, stabilizing at this level until the end of the reactor operation (Figure 3-6). The ability of the stabilized biomass in Phase 5 to degrade TBA was evaluated by sampling reactor biomass on days 240 to 244 and monitoring TBA disappearance over time with batch cultures incubated at different TBA concentrations. The specific TBA biodegradation rate increased with TBA concentration from 0 to 300 mg L⁻¹ to peak at 14 mg TBA g VSS⁻¹ h⁻¹, and showed a moderate decline with further increases in TBA concentration. The Haldane equation was used to fit the biodegradation data, with calculated kinetic parameters of $V_{\max} = 18 \pm 1$ mg TBA g VSS⁻¹ h⁻¹, $K_s = 47 \pm 7$ mg L⁻¹ and $K_i = 1861 \pm 297$ mg L⁻¹ (Figure 3-7).

Specific TBA biodegradation rates of Reactor R2 were also measured by harvesting reactor biomass periodically and incubating in batch cultures with 100 mg L⁻¹ TBA. Specific TBA biodegradation rates were insignificant during the first 20 days, but followed an increasing trend thereafter, to reach 3.6 mg TBA g VSS⁻¹ h⁻¹ on day 94, 6.0 mg TBA g VSS⁻¹ h⁻¹ on day 120, and 7.4 mg TBA g VSS⁻¹ h⁻¹ at the end of Phase 5 (Figure 3-8). Specific TBA biodegradation rates showed slight reductions briefly near the beginning of Phases 4 and 5 when the start-of-cycle TBA concentrations were increased (Figure 3-8), but recovered soon after.

The ability of the stabilized biomass in Phase 5 to degrade TBA was evaluated by sampling reactor biomass on days 240 to 244 and monitoring TBA disappearance over time with batch cultures incubated at different TBA concentrations. The specific TBA biodegradation rate increased with TBA concentration from 33 to 320 mg L⁻¹ to peak at 17 mg TBA g VSS⁻¹ h⁻¹, and showed a mild decline with further increases in TBA concentration. There was a good fit between the biodegradation data and the Haldane equation, with calculated kinetic parameters of $V_{\max} = 25 \pm 3$ mg TBA g VSS⁻¹ h⁻¹, $K_s = 161 \pm 10$ mg L⁻¹ and $K_i = 3314 \pm 386$ mg L⁻¹ (Figure 3-9).

Figure 3-6. Time profile of specific TBA biodegradation rates of Reactor R1 (error bars denote standard deviations of triplicate measurements).

Figure 3-7. Specific TBA biodegradation rates of Reactor R1 aerobic granules at different TBA concentrations.

Figure 3-8. Time profile of specific TBA biodegradation rates of Reactor R2 (error bars denote standard deviations of triplicate measurements)

Figure 3-9. Specific TBA biodegradation rates of Reactor R2 aerobic granules at different TBA concentrations.

3.3.3 Detection of dominant species by DGGE

DGGE was employed to produce genetic fingerprints that could provide information on the composition and diversity of microbial communities in the stabilized TBA-degrading microbial consortium. DNA was extracted from both R1 and R2 reactors' biomass that were sampled once every few days, from day 220 to day 247 in Phase 5. PCR amplification and DGGE analysis of community 16S rRNA genes were performed in replicate on pooled aliquots of extracted nucleic acids. Identical fingerprints were obtained for replicate samples. Figure 3-10 shows representative DGGE profiles of amplified 16S rRNA genes from Reactor R1 biomass. At least ten different bands were detected on the polyacrylamide gel. The fingerprint patterns were similar for the different biomass samples, indicating a highly stable microbial community.

Biomass characteristics and reactor performance also showed little variation during this period. Recovered bands were reamplified by PCR and run on a DGGE gel to confirm purity. This was repeated several times to obtain a pure DNA product for sequencing. Partial 16S rRNA gene sequences of close to 200 nucleotides were successfully obtained from nine different DGGE bands (TBA101 to TBA109 in Figure 3-10 and Table 3-2). The majority of the bacterial sequences grouped with members of Proteobacteria, with four in the β subdivision, two in the α subdivision and one in the δ subdivision. The remaining two sequences clustered with the Cytophaga-Flavobacteria-Bacteroides (CFB) group.

Figure 3-11 shows representative DGGE profiles of amplified 16S rRNA genes from Reactor R2 biomass. Bacterial primers also produced at least ten different bands on the polyacrylamide gel. The fingerprint patterns were similar for different biomass samples too, indicating a highly stable microbial community residing in R2 granules. Biomass characteristics and reactor performance of R2 also showed little variation during the period. Selected DGGE bands were excised for DNA sequence.

Figure 3-10. Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA genes from aerobic TBA-degrading granules in Reactor R1.

Figure 3-11. Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA genes from aerobic TBA-degrading granules in Reactor R2.

Table 3-2. Sequence analysis of DGGE bands from TBA-degrading biomass in Reactor R1.

Band	Sequence length (bases)	Closest relatives (Accession number)	Identity (%)	Phylogenetic Division
TBA101	196	Uncultured delta proteobacterium clone 7A_6 (AY251224) <i>Acidobacterium capsulatum</i> phylum TM6 (X97099)	92.2 90.8	δ -proteobacteria
TBA102	169	Uncultured alpha proteobacterium clone C46.2IPG (AF431185) <i>Bradyrhizobium</i> sp. SJSal (AF352563)	98.3 95.9	α -proteobacteria
TBA103	188	Uncultured eubacterium WJGRT-60 (AF175606)	97.9	CFB group
TBA104	195	Elbe River Snow isolate Iso18 (AF150698)	100.0	β -proteobacteria
TBA105	169	<i>Sphingomonas</i> sp. G296-14 (AF395037)	98.8	α -proteobacteria
TBA106	188	Uncultured bacterium clone Ebpr7 (AF255646) Uncultured bacterium clone DGGE band 21 (AY437414)	100.0 98.4	CFB group
TBA107	194	Phenanthrene-degrading bacterium 90 (AY177371) <i>Roseateles depolymerans</i> strain 61B2 DSM11814 (AB003626)	96.4 95.9	β -proteobacteria
TBA108	193	Beta proteobacterium HS5/S24542 (AY337603) MTBE-degrading bacterium PM1 (AF176594)	99.5 93.8	β -proteobacteria
TBA109	194	<i>Denitromonas aromaticus</i> (AB049763) <i>Azoarcus denitrificans</i> Td-3 (L33693)	99.5 93.8	β -proteobacteria

Table 3-3. Sequence analysis of DGGGE bands from TBA-degrading biomass in Reactor R2.

Band	Sequence length (bases)	Closest relatives (Accession number)	Identity (%)	Phylogenetic Division
TBA201	168	Uncultured alpha proteobacterium clone C46.21PG (AF431185) <i>Bradyrhizobium</i> sp. Sjsa1 (AF352563)	98.3 95.9	α -proteobacteria
TBA202	188	Uncultured bacterium clone Ebpr7 (AF255646) Uncultured bacterium clone DGGGE band 21 (AY437414)	100.0 98.4	CFB group
TBA203	194	Phenanthrene-degrading bacterium 90 (AY177371) <i>Roseateles depolymerans</i> strain 61B2 DSM11814 (AB003626)	96.4 95.9	β -proteobacteria
TBA204	168	<i>Methylobacterium organophilum</i> (D32226) <i>Methylobacterium extorquens</i> (AY460189)	100.0 100.0	α -proteobacteria
TBA205	194	<i>Denitromonas aromaticus</i> (AB049763) <i>Thiobacillus aquaesulis</i> (U58019)	99.5 93.8	β -proteobacteria
TBA206	192	Uncultured bacterium PHOS-HE08 (AF314448)	98.0	β -proteobacteria
TBA207	169	Uncultured alpha proteobacterium clone C32.15SM (AF431150) uncultured eubacterium WD233 (AJ292595)	97.1 97.7	α -proteobacteria

Partial 16S rRNA gene sequences of close to 200 nucleotides were successfully obtained from seven different DGGE bands (TBA201 to TBA207 in Figure 3-11) and compared with nucleotide sequences from GenBank (Table 3-3). All but one of the bacterial sequences grouped with members of Proteobacteria, with three sequences each in the α and β subdivisions. The remaining sequence clustered with the Cytophaga-Flavobacteria-Bacteroides (CFB) group.

Figure 3-11 shows a comparison of representative DGGE profiles of R1 and R2 reactors. It is obvious that the DGGE fingerprints in R1 and R2 were different from each other, indicating that different communities were separately established in the two reactors. DGGE bands could be observed that were unique to each reactor. However, some community members were found to be present in both reactors. Four co-migrating DGGE bands belonging to the α -Proteobacteria, β -Proteobacteria and CFB groups were detected in both reactors (Figure 3-12 and Table 3-4). Three of the four DGGE co-migrating bands had partial 16S rRNA gene sequences that were at least 98% identical to those of other known bacterial strains. DGGE bands R1-1 and R2-1 clustered with α -Proteobacteria and were closely related with the genus *Bradyrhizobium* in the Rhizobium-Agrobacterium group. DGGE bands R1-3 and R2-3 belonged to β -Proteobacteria and were closely related to *Roseateles depolymerans*. This is a recently reported genus and the type culture was isolated and named because it could degrade biopolymers such as polyhexamethylene carbonate (PHC) (Suyama *et al.* 1999).

Figure 3-12. Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA genes from aerobic TBA-degrading granules in R1 (lanes 1-3 from left) and R2 (lanes 4-6 from left). Co-migrating bands were sequenced to confirm their close relatedness and are labeled as shown.

Chapter Three

Table 3-4. Identities of co-migrating denaturing gradient gel electrophoresis bands from R1 and R2.

Band	Number of bases	Closest relative (Accession number)	Identity (%)	Phylogenetic division
R1-1/R2-1	169/168	Uncultured alpha proteobacterium clone C46.21PG (AF431185)	98.3	α -Proteobacteria
		<i>Bradyrhizobium</i> sp. SJSa1 (AF352563)	95.9	
R1-2/R2-2	188/188	Uncultured bacterium clone Ebpr7 (AF255646)	100.0	CFB group
		Uncultured bacterium clone DGGE band 21 (AY437414)	98.4	
R1-3/R2-3	194/194	Phenanthrene-degrading bacterium 90 (AY177371)	96.4	β -Proteobacteria
		<i>Roseateles depolymerans</i> strain 61B2 DSM11814 (AB003626)	95.9	
R1-4/R2-4	194/194	<i>Denitromonas aromaticus</i> (AB049763)	99.5	β -Proteobacteria
		<i>Azoarcus denitrificans</i> Td-3 (L33693)	93.8	

3.4 Discussion

3.4.1 Cultivation of aerobic TBA-degrading granules

This study demonstrated that it was possible to form aerobic granules in the SBR for biodegradation of TBA as sole carbon and energy source. The periodic and sequential variations in operating conditions in the SBR can select for robust microbial communities with good settling, high substrate removal efficiencies and the ability to withstand unplanned fluctuations or disturbances (Irvine and Ketchum Jr, 1989; Wilderer et al. 2001). Aerobically grown granular sludge is a recent innovation in SBR technology. These aerobic granules are self-immobilized spherical microbial aggregates that possess a strong compact microbial structure and excellent settling ability (Morgenroth et al. 1997; Beun et al. 1999; Tay et al. 2001a; Jiang et al. 2002; Moy et al. 2002; Jiang et al. 2004a; Liu et al. 2004a; Pan et al. 2004).

As a matter of fact, any reactor system that can exert the appropriate combination of microbial and hydraulic selection pressures should conceivably be capable of achieving granule formation (Angenent et al. 2004). However, it should be pointed out that granulation should not be expected to occur automatically. Successful granule formation may depend, for instance, on the acclimation procedure. The basis for aerobic granule formation is a continuous selection for sludge particles cultivated in the SBR such that dispersed and less dense particles are washed out, while components that are denser are retained in the system. Conditions considered important for granule formation and maintenance include short settling times, high shear stresses, moderate hydraulic selection pressures and the presence of feast-famine cycles (Tay et al. 2001a&b; Pan et al. 2004).

In the current study, a moderate selection pressure was first applied during

start-up by operating the SBR with a cycle time of 24 h and a low start-of-cycle TBA concentration of 100 mg L⁻¹. This served to encourage the growth of biomass and avoid biomass loss as a result of hydraulic washout, and is of greater significance in this study since microorganisms that grow on relatively recalcitrant xenobiotics are usually considered slow growers (Fayolle et al. 2001). Although some biomass was initially lost from the SBRs, the use of a 24 h cycle time was validated by the subsequent recovery in biomass concentration from day 20 to 40 of reactor operation.

A shorter starting cycle time would likely have caused severe biomass loss that could not be compensated for by the growth of new biomass. In the case of Reactor R1, progressively shorter cycle times were only employed in the later phases of reactor operation after significant increases in biomass concentration were achieved in the preceding phase. By adopting a careful operating strategy of step reductions in cycle time, adequate selection pressures were brought to bear that primed the biomass to adapt to the correspondingly higher TBA loading rates without suffering any deterioration in effluent quality.

In aerobic biofilms, increasing the rate of substrate loading will generally give rise to thicker biofilms with decreased densities (Park et al. 1998). However, the formation of less compact biofilms as a consequence of higher substrate loading did not materialize in the aerobic granulation process. Rather, the higher TBA loading rates in the current study produced biomass with larger mean sizes, but with lower SVI values and better settling abilities.

The operational flexibility of the SBR to use shorter cycle times or higher influent TBA concentrations as a means of increasing TBA loading rates creates a higher frequency of volumetric exchange, which in turn exerts a higher hydraulic selection pressure on the biomass such that more compact forms of biomass with better settling characteristics are retained in the reactor while less compact forms are

washed out. Consequently, aerobic granules first appeared in the reactor not long after the cycle time was shortened to 8 h. Granulation was probably triggered when the hydraulic selection pressure exceeded a certain critical threshold as a result of the reduction in cycle time.

In addition to cycle time, substrate concentration could also affect the granulation process by selecting and enriching different bacterial species and influencing the size, settling ability and bioactivity of the granules (Tay and Yan, 1996; Moy et al. 2002). A progressively higher start-of-cycle TBA concentration was only introduced in each of the later phases in the operation of Reactor R2 after complete TBA removal was accompanied by a substantial increase in biomass concentration and a significant improvement in biomass settling ability in the preceding phase.

A carefully-timed step increases in substrate loading operational strategy was adopted. Adequate selection pressures were brought to bear that primed the biomass to adapt to the higher TBA concentrations without suffering any deterioration in either biomass or effluent quality. Still, it was surprising that aerobic granules could form in Reactor R2 under a 24 hour-cycle. Because it has been reported unsuccessful aerobic granulation in 24 hour-cycle bioreactors using acetate or glucose as sole carbon and energy source.

The reason of granulation in Reactor R2 has not yet been understood. It was probably because of the sole carbon and energy source used in this study. TBA is a recalcitrant compound. When bacteria use TBA as sole carbon and energy source, the yield efficiency is always low, resulting in a relatively slow growth rate. Slow growing bacteria were preferred in the aerobic granules because these bacteria were demonstrated to increase the stability of aerobic granules (Liu et al. 2004c). Thus, it is still feasible that aerobic granules could be formed under 24 hour-cycle time using TBA as sole carbon and energy source.

3.4.2 Seeding sludge selection

Activated sludge from a municipal plant was chosen as the inoculum in this study because of its typically high degree of microbial diversity, which improved the likelihood of selection of specific bacterial strains with the desired catabolic capability of TBA degradation. Activated sludge has been used in previous studies in which enrichment cultures were developed using MTBE and/or TBA as substrate (Salanitro et al. 1994; Schmidt et al. 2004). The sludge inoculum in the study came from an activated sludge plant that treated combined municipal and industrial wastewaters.

As the SBRs were inoculated with fresh non-acclimated activated sludge, a relatively long acclimation period of about thirty days was required before TBA concentrations in the effluent started to decrease. An adequate adaptation duration is an essential requirement for the biodegradation of toxic or recalcitrant compounds. Adaptation has been defined as a change in the microbial community that increases the rate of transformation of a target compound as a result of exposure to the compound. Such adaptation of natural microbial communities to new substrates may arise as a result of a combination of different mechanisms, such as gene transfer or mutation, enzyme induction and population changes (Spain and Van Veld 1983).

There is evidence to suggest that genes involved in the degradation of ether fuels might be transferred horizontally between bacteria belonging to the *Mycobacterium/Rhodococcus* group (Beguin et al. 2003). Although the extent to which each of these adaptive mechanisms contributes to the development of TBA-degrading granules has yet to be determined, what is clear is that such adaptation can select for individuals or groups of microorganisms best suited to tolerate and degrade the xenobiotic of interest, and these will eventually sweep over and dominate the microbial community.

Changes in biodegradation rates may also be mediated by exposing the microbial communities to perturbations or other forms of manipulation. In engineered systems such as bioreactors, the ability of microbial communities to adapt may be influenced by a variety of factors such as the amount and quality of the inoculum as well as the environmental conditions under which the adaptation is to be achieved.

3.4.3 TBA biodegradation rates of aerobic granules

Aerobic granules harvested from R1 in Phase 5 possessed a maximum specific TBA biodegradation rate of $13.8 \text{ mg TBA g VSS}^{-1} \text{ h}^{-1}$. Biomass harvested from R2 on day 240 possessed a maximum specific TBA biodegradation rate of $16.8 \text{ mg TBA g VSS}^{-1} \text{ h}^{-1}$. These are comparable to the TBA biodegradation rates of $14.0 \text{ mg TBA g VSS}^{-1} \text{ h}^{-1}$ reported for an MTBE-degrading consortium and to the TBA biodegradation rates of between 1.1 to $35.5 \text{ mg TBA g VSS}^{-1} \text{ h}^{-1}$ reported for several bacterial isolates (Salanitro et al. 1994; Steffan et al. 1997; Garnier et al. 1999; Kharoune et al. 2001; Piveteau et al. 2001; François et al. 2003).

TBA biodegradation kinetics in the aerobic granules followed the Haldane model for substrate inhibition. However, the high K_i value (R1) of $1861 \text{ mg TBA L}^{-1}$ and K_i (R2) of $3314 \text{ mg TBA L}^{-1}$ indicated that the inhibitory effects of TBA toxicity were not severe as high specific TBA biodegradation rates were still achieved at high TBA concentrations. This is consistent with an earlier finding that the compact granular structure sheltered microorganisms in the granules against the toxicity effects of high phenol concentrations in the surrounding milieu (Jiang et al. 2002).

By comparing the kinetic parameters for R1 and R2, it is found that $V_{\max}$ in the two reactors had comparable values of 18.2 and $24.9 \text{ mg TBA g VSS}^{-1} \text{ h}^{-1}$, respectively. However, the K_s and K_i values estimated from two reactors showed significant differences. The biomass in R1 has relatively lower values than R2 for K_s and K_i . Usually, the value of $K_s/V_{\max}$ is used to indicate the affinity of the

biomass to the substrate. As mentioned earlier, Reactor R1 and R2 have comparable values of $V_{\max}$, therefore, the difference in K_s values actually reflected the different affinity of biomass to the TBA.

The K_s value in Reactor R1 granules is approximately 47.4, which is more than three times lower than for R2. This indicated that Reactor R1 granules have a higher substrate affinity compared to Reactor R2 granules. On the other hand, Reactor R2 granules have a higher K_i value indicated that granules from Reactor R2 could perform biodegradation at higher concentrations before substrate inhibition becomes dominant.

The difference in kinetic parameters indicated that the granules in Reactor R1 and R2 have a different degradation behavior towards TBA. This different degradation behavior in fact was the result of the strategies under which the reactors were running. Since granules in Reactor R1 was exposed to a relatively lower start-of-cycle TBA concentration, so it is not surprising that the granules from Reactor R1 have a relatively higher affinity to the substrate. It also explained the reason that granules from Reactor R2 have theoretically higher resistance to the inhibitory effects of TBA toxicity.

3.4.4 Predominant microbial populations in the TBA-degrading aerobic granules

DGGE analysis of PCR-amplified 16S rRNA genes from nucleic acids extracted from the aerobic granules sampled from both Reactor R1 and R2 over a four-week period in Phase 5 revealed the presence of a highly stable microbial community, albeit with a remarkably low degree of phylogenetic diversity with members belonging within only the Proteobacteria and CFB groups (Tables 3-2 and 3-3). This is probably a result of the selection pressures exerted by the SBR operation, which would favor the enrichment of certain bacterial species to the detriment of

others. Previously isolated TBA-degrading bacteria have only been found in the Proteobacteria and high G+C groups (Mo et al. 1997; Deeb et al. 2000a; Steffan et al. 2000; Piveteau et al. 2001; François et al. 2002; François et al. 2003; Schmidt et al. 2004) and it is not clear if the low diversity of isolates documented thus far is due to TBA's recalcitrance or if the enzymatic pathways for TBA degradation are confined within certain tight phylogenetic clusters.

DGGE band TBA102 clustered with the Rhizobium-Agrobacterium group of α Proteobacteria, while DGGE bands TBA105 and TBA108 were most closely related to *Sphingomonas* sp. strain G269-14 in the α Proteobacteria subdivision and to *Rubrivivax gelatinosus* strain PM1 in the β Proteobacteria subdivision, respectively. Bacteria in these groups are known to degrade MTBE and utilize complex organic compounds for carbon and energy (Hanson et al. 1999; Christner et al. 2001; Pruden et al. 2001 and 2003; Zein et al. 2004). DGGE bands TBA201 and TBA207 clustered with α Proteobacteria and were closely related to bacterial strains belonging to the Rhizobium-Agrobacterium group.

This group was found to dominate the microbial community in a pilot-scale biofilm reactor degrading MTBE and contains many members capable of utilizing complex organic compounds for carbon and energy (Zein et al. 2004). Members of this group are also known to synthesize a variety of cell-surface carbohydrates, some of which may provide functions important to symbiosis and protection against environmental stresses (Louch and Miller, 2001). DGGE band TBA204 also clustered within the α Proteobacteria subdivision and was most closely related to *Methylobacterium organophilum*.

Methylobacterium is a pink-pigmented facultative methylotrophic genus that has been isolated using MTBE as sole carbon and energy source (Mo et al. 1997). Members of the *Methylobacterium* genus can degrade many toxic and recalcitrant xenobiotic compounds such as trinitrotoluene and dichloromethane (Vannelli et al.

1998; Aken et al. 2004), are able to produce extracellular polysaccharides and storage biopolymers (Madison and Huisman, 1999), and have been found in abundance in environmental biofilms (Kelley et al. 2004). In addition, the biomass from both reactors also possessed several DGGE bands that comigrated together as shown in Figure 3-12. DGGE bands R1-1 and R2-1 clustered with α -Proteobacteria and were closely related with the genus *Bradyrhizobium* in the Rhizobium-Agrobacterium group. Members from this group have been reported as dominant community members in a pilot-scale biofilm reactor degrading MTBE (Zein et al. 2004). Many members from genus *Bradyrhizobium* have very broad substrate utilization ability probably because of a very powerful and unspecific cytochrome P450 enzyme system (Kelly et al. 2004). Interestingly enough, the P450 enzyme is thought to play a role in the degradation of MTBE and TBA, and the presence of *Bradyrhizobium* in the TBA-degrading aerobic granules is certainly consistent with this hypothesis.

DGGE bands R1-3 and R2-3 belonged to β -Proteobacteria and were closely related to *Roseateles depolymerans*. This is a recently reported genus and the type culture was isolated and named because it could degrade biopolymers such as polyhexamethylene carbonate (PHC) (Suyama et al. 1999). The ability to accumulate PHB granules is a trait common to many species in this taxon. Bacteria grown in SBR systems are known to accumulate storage biopolymers as an energy source to counteract physiological stresses imposed by feast-famine regimes. Hence, microorganisms that can store and degrade these biopolymers should survive and thrive in the reactors in this study.

3.4.5 Summary

This study demonstrates the successful application of SBRs for complete TBA biodegradation and successful cultivation aerobic granules using TBA as sole carbon and energy source. Compact, well settling aerobic granules were formed in the two

SBRs for TBA biodegradation. A moderate selection pressure was initially applied to encourage the growth and retention of biomass and avoid biomass loss as a result of hydraulic washout. Two acclimation strategies were used that involved step decreases in cycle time or step increases in start-of-cycle TBA concentrations only after complete TBA removal and improvements in biomass concentration and biomass settling ability were achieved. The operational flexibility of the SBR was exploited to create appropriate selection pressures that led to the eventual formation of compact self-immobilized microbial granules with excellent settling characteristics and high specific TBA biodegradation rates. These adaptations toward granule formation broadens the benefits of using the SBR to target the biodegradation of toxic or recalcitrant chemicals. From a practical perspective, refinements such as the use of better inocula (Tay et al. 2004a) could be investigated to improve and accelerate microbial acclimation to TBA biodegradation and boost SBR performance.

Chapter Four

Isolation and Characterization of Two *tert*-Butyl Alcohol (TBA)-Degrading Pure Microbial Cultures

4.1 Introduction

It has been documented that both MTBE- and TBA-degrading bacteria are difficult to isolate from mixed cultures enriched by these compounds as sole carbon and energy resources (Fortin et al. 2001). Although the exact reasons behind such difficulties have not yet been identified, a common agreement is that both MTBE and TBA are recalcitrant compounds which may not serve as good substrates for supporting bacteria growth (Schmidt et al. 2004). To date, only a handful of isolates have been documented which are able to make a living with TBA as a sole carbon and energy source (Mo et al. 1997; Deeb et al. 2000a&b; Steffan et al. 2000; Piveteau et al. 2001; François et al. 2002&2003; Schmidt et al. 2004).

All these isolates belonged to the proteobacteria and high G+C bacteria groups. One of the MTBE-degrading pure cultures, *Rubrivivax gelatinosus* PM1, has been intensively studied during the last 5 years (Hanson et al. 1999; Deeb et al. 2000a, 2001; Piveteau et al. 2001). This strain was initially isolated by using MTBE as sole carbon and energy source, however, it is not reported whether it could also use TBA as sole carbon source. In addition, autochthonous bacteria identical to PM1 were found in several MTBE contaminated groundwater sites (Hristova et al. 2001, 2003; Kane et al. 2001). To study the MTBE- and TBA-degrading pure cultures could provide physiological and biochemical information, such as growth rate, yield efficiency, degradation kinetics and bacterial genetics. Such information is useful

for better understanding of the biodegradation process and can eventually be applied to design and optimize suitable biotreatment strategies.

The TBA-degrading aerobic granules were successfully cultivated in two SBR systems as discussed in Chapter Three. Molecular analysis based on 16S rRNA genes revealed a stable microbial community residing in the TBA-degrading aerobic granules. However, it is not possible to know the functions and biochemical characteristics of these bacteria from only the molecular study. Therefore, the main objective of the current chapter is to isolate and characterize, from the TBA-degrading aerobic granules, pure microbial cultures which could use TBA as sole carbon and energy source. The study described the biochemical and physiological characteristics of these isolates and probed the interactions between these isolates. Reasons for their survival and persistence in aerobic granules were postulated. This work enhances the current knowledge on TBA biodegradation and TBA-degrading microorganisms.

4.2 Materials and Methods

4.2.1 Reactor operation

TBA-degrading aerobic granules were cultivated in two sequencing batch reactors (SBRs) R1 and R2 from non-conditioned activated sludge seed as described in chapter three. The reactors were operated at a loading rate of $0.6 \text{ kg TBA m}^{-3} \text{ day}^{-1}$ and reached steady state after 8 months of operation under different operational strategies. The different operational conditions for Reactors R1 and R2, respectively, were introduced in Chapter 3. Aerobic granules with mean sizes ranged from 0.2 to 0.3 mm were observed in both reactors at steady state. The steady state granules were harvested for both cultivation and ribosomal-based experiments.

4.2.2 Bacterial isolation, growth, and maintenance

Twenty milliliters (20 mL) of TBA-degrading granular sludge was aseptically collected during the aeration stage of SBR operation from Reactors R1 and R2. Samples were firstly disrupted with a mortar and bacterial cells were detached from granules and flocs using a beadbeating method as described previously (Tay et al. 2002c). Separated cells were washed three times with sterilized PBS (Phosphate Buffer Saline) and resuspended in 20 mL sterilized PBS buffer. The supernatant was then diluted with PBS buffer (10^1 to 10^8 dilutions), and 100 μL of each dilution was spread onto a mineral salts (MS) medium-based agar plate solidified with 1.5% Bacto Agar.

The MS medium had the following composition (per liter): 0.4 g of NaNO_3 , 0.4 g of Na_2HPO_4 , 0.15 g of KH_2PO_4 , 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g of CaCl_2 , 1.0 mg of CoSO_4 , 1.0 mg of CuSO_4 , and 3.0 mg of FeCl_3 . Trace elements and vitamins were added as previously described (Cote and Gherna 1994). TBA (4 mM) was added to the medium as sole carbon and energy source. The plates were stored in a desiccator

and incubated in a 25°C incubator. A small piece of filter paper soaked with TBA (approximately 200 µL) was put into a desiccator and changed periodically. Plates were monitored over a 4-week incubation period, during which colonies were picked and restreaked onto fresh MS agar plates with TBA as sole carbon source. Strains TBA-R1-001 and TBA-R2-005 were obtained after several successful transfers. Purity was confirmed by streaking onto R2A agar plates as well as microscopic examination. Purified cultures were preserved in R2A broth with 15% sterilized glycerol at -80°C.

Heterotrophic bacteria were enumerated by spreading 100 µL of each dilution described above onto R2A agar plates and incubated in a 25°C incubator. Colony forming units (CFU) were counted during a 4-week period.

Methanol and/or formate are considered to be potential key intermediates of TBA degradation according to proposed TBA cometabolism pathways (Steffan et al. 1997). Hence, pure cultures of strains TBA-R1-001 and TBA-R2-005 were tested on methanol, formate, as well as TBA as sole carbon and energy source in MS liquid medium supplied with vitamin solution. Cultures of strain TBA-R1-001 grew well on methanol and formate in liquid medium in conventional shake flasks. The OD₆₀₀ of TBA-R1-001 in replicate shake flasks increased from 0.12 to 0.69 after 9 days of incubation at 30 °C with shaking at 250 rpm.

Cultures of strain TBA-R2-005 also grew well in shake flasks with methanol and formate as sole carbon and energy source. Final OD₆₀₀ values ranged from 0.5 to 1.0 (equivalent to 150 to 300 mg L⁻¹ dry biomass weight) from initial OD₆₀₀ values of less than 0.1 after 3 days of incubation at 30 °C with shaking at 250 rpm. However, strains TBA-R1-001 and TBA-R2-005 could not grow well on TBA in liquid medium in conventional shake flasks. Therefore, extra aeration was supplied by pumping.

Air-filter-sterilized air continuously pumped into the shake flasks through a

diffuser to enhance oxygen transfer, and to maintain a dissolved oxygen concentration higher than 6 mg L⁻¹. Strain TBA-R2-005 grew well and degraded TBA efficiently under this aerated culture condition. Strain TBA-R1-001 could degrade TBA under aeration conditions, but it showed a complex growth and degradation profile.

Cell suspensions were filtered through a 0.2 µm filter and the filter and cells were dried to a constant weight for 24 h at 80°C for dry biomass weight measurements (Onysko et al. 2000).

4.2.3 Morphological, biochemical, and physiological analyses

Gram-stain was carried out using a BD gram-staining kit according to the manufacturer's instructions (BD, USA). A non-staining Gram-stain method (Buck 1982) was also performed to validate the results. Cultures were observed with light microscopy and scanning electron microscopy (SEM) as described previously (Zhuang et al. 2002).

Enzyme profiles and carbon substrate utilization characteristics were determined using the API ZYM and API 20E assays according to the manufacturer's instructions (BioMérieux, France). In addition, both strains were cultivated on MS liquid medium supplemented with various carbon sources added to a final concentration as follows: formate (8 mM), formaldehyde (10 mM), methanol (150 mM), sodium acetate (3.0 mM), ethanol (2.2 mM), acetone (2.0 mM), iso-propanol (3.5 mM), and glycerol (3.0 mM). These compounds were either potential intermediates at downstream TBA degradation pathway, or substrate for methylotrophic bacteria. All experiments were performed with conventional shake flasks. Cultures were incubated in a 30°C incubator with shaking. Growth was considered to have occurred when the observed OD₆₀₀ value exceeded twice the initial value.

4.2.4 Analytical methods

A direct injection gas chromatograph method (Steffan et al. 1997) was used to determine TBA concentrations. All liquid samples were collected and analyzed immediately. Cell-free aqueous samples (1-3 μL) were automatically injected into a Hewlett Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a DB624 column (Hewlett Packard, 30 m $\times$ 0.53 mm $\times$ 3 μm). The oven temperature was held at 60°C for 3 min and was then ramped to 120°C at 40°C min⁻¹, which was held for 3 min, and finally raised to 200°C at 40°C min⁻¹. TBA peaks and concentration values were determined using Chemstation software (Hewlett Packard).

4.2.5 16S rRNA gene sequencing and phylogenetic analyses

A whole cell direct lysis PCR method was used for amplification of 16S rRNA genes. The nearly full length 16S rRNA gene was amplified by PCR with forward primer Eubac27F and reverse primer Universal 1492R (Lane, 1991). The PCR products were purified with Promega PCR and DNA purification kit according to manufacturer's instructions (Promega, USA). 16S rRNA gene sequences were obtained in both directions for both strains using the ABI model 310A DNA sequencer (Applied Biosystems, Perkin-Elmer, USA) and the ABI PRISM BigDye terminator Cycle Sequencing ready-reaction kit (Applied Biosystems, Perkin-Elmer, USA). The partial sequences were compiled and aligned using BioEdit software and analyzed with BLAST and other algorithms as described previously (Zhuang et al. 2002).

4.2.6 PCR and denaturing gradient gel electrophoresis (DGGE)

Genomic DNA of enriched TBA-degrading biomass was extracted based on a protocol described by Kowalchuk et al. (1997). Same biomass sampled for serial

dilution described above was used for DNA extraction also. Extracted DNA samples were stored in a -20°C freezer before use.

PCR primers P2 and P3 (containing 40 bp of GC clamp) were used to amplify the variable V3 region of the bacterial 16S rRNA gene (corresponding to positions 341 to 534 in the *Escherichia coli* sequence) (Muyzer et al. 1993). Touchdown PCR was performed (Watanabe et al. 1998) with a Mastercycler Gradient thermal cycler (Eppendorf AG, Hamburg, Germany) using a 100 μL (total volume) mixture containing 2.5 U of Taq DNA polymerase (Promega, USA), 10 μL of 10 $\times$ Thermophilic DNA polymerase Buffer B, 8 μL of 25 mM MgCl_2 , 200 mM of each deoxynucleotide triphosphate, 20 pmol of each primer, and 2 μL of the DNA extract (concentration 100 ng μL^{-1}). Successful PCR was confirmed by electrophoresis of amplicons through a 2.0% agarose gel in TAE buffer stained with ethidium bromide.

The PCR-amplified fragments were separated by DGGE using a DCode universal mutation detection system (Bio-Rad Laboratories, USA) as described previously (Muyzer et al. 1993). A 25 mL 40% to 65% urea-formamide denaturant gradient gel [10% (w/v) acrylamide solution (40% acrylamide and bis acrylamide, 37.5:1 stock solution, Bio-Rad Laboratories) in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM $\text{Na}_2\text{-EDTA}$, pH 8.0)] was covered by a 4 mL acrylamide stacking gel (10%) without denaturant. 40 μL of PCR amplicons from DNA of biomass samples were loaded with 20 μL loading dye in each well. The gel was placed in TAE buffer and run at 40 V at 60°C for 30 min and then at 85 V at 60°C for 15 h. After electrophoresis, the gel was stained with ethidium bromide for 30 min, and viewed and photographed with an EDAS 290 gel imaging system (Kodak, USA). Selected DNA bands were aseptically excised and re-amplified by PCR several times as described previously (Watanabe et al. 1998) in order to obtain partial 16S rRNA gene sequence information. The partial sequences were compiled and aligned using BioEdit software and analyzed with BLAST.

4.2.7 Oxygen utilization rate of resting cells

Methanol- and TBA-grown cells were aseptically harvested at late-exponential phase, washed three times with MS liquid medium, and resuspended in oxygen saturated MS liquid medium (pH 8.0) to give a final OD₆₀₀ of 0.1±0.01. To determine the oxygen utilization rates of isolates, a DO meter with Clark-type polarographic oxygen electrodes (YSI 5300A, YSI Incorporated, Yellow Springs, OH, USA) was used to measure the oxygen concentrations. Reaction volume was 3 mL. Reaction was started by adding methanol (3.1 mM) or TBA (1.35 mM) as substrate to the solution. Endogenous respiration was determined in the same manner, but with carbon source added. Experiments were performed at 25°C and at least in triplicate.

4.2.8 TBA biodegradation by mixed culture of TBA-R1-001 and TBA-R2-005

Suspension cultures of strains TBA-R1-001 (glycerol-fed) and TBA-R2-005 (methanol-fed) were harvested by centrifugation, washed three times with room temperature PBS, re-suspended in fresh MS liquid medium, and equally mixed (236 and 212 mg-dry weight L⁻¹, respectively) to give a final 300 mL of mixed cell suspension which was transferred into a 1 L size glass bottle. Aeration was supplied through dispenser at the bottom of the bottle. TBA was added as sole carbon and energy source at a concentration of 150 mg L⁻¹. Total organic carbon (TOC) concentrations were measured as described previously (Jiang et al. 2004a). Control experiments were performed in the same manner, but with single-species cell suspensions. In addition, conventional shake flasks were also used to grow the two-species mixed culture.

4.2.9 N,N-dimethyl-*p*-nitrosoaniline (*p*-NDA) reduction

Stock *p*-NDA solution was prepared by first dissolving *p*-NDA (Aldrich, USA) in milliQ-H₂O to give a concentration of 4.0 mM and then filtered through a 0.2 µm

nylon filter for sterilization. To measure the *p*-NDA bleaching, 1 mL of the stock solution was added into 100 mL MS liquid medium supplied with TBA (1.4 mM) as sole carbon and energy source. Strains TBA-R1-001 or TBA-R2-005 was added into the MS medium to give a final concentration of approximately OD₆₀₀ 0.50±0.03. Control experiments were performed at the same manner except no carbon source was added to the medium. All experiments were performed in dark in bottles covered with aluminum foil. *p*-NDA concentration was measuring by filtering the culture medium through a 0.2 µm nylon filter and then measured the absorbance of the filtrate at 440 nm wave length using a Du 640B UV/VIS spectrophotometer (Beckman Coulter, USA).

4.2.10 H₂O₂ concentration measurement

Hydrogen peroxide concentrations in the culture medium were determined using a previously described spectrophotometric assay based on the horseradish peroxidase-catalyzed oxidation of N,N-diethyl-*p*-phenylenediamine (DPD) (Bader et al. 1988) except the photometric cell of 1 cm pathlength was used. Methanol-grown TBA-R2-005 cells were aseptically harvested at late-exponential phase, washed twice with 0.05 M phosphate buffer (pH 8.0), and resuspended in the same buffer to give a dry biomass weight of 1.0±0.15 g L⁻¹. Sterilized air was continuously pumped into the cell suspension. Either methanol or TBA was used as sole carbon substrate.

To eliminate the effects of trace iron, the phosphate buffer was treated by CHELEX 100 (Sigma, USA) before use. To measure the H₂O₂ concentration, samples were filtered through a 0.2 µm filter, diluted accordingly, and then mixed with enzyme stock solution and DPD solution. The absorption spectrum (551 nm) was taken 1 min after the addition of the enzyme. Positive control experiments were performed at the same manner using known concentrations of H₂O₂ solutions as standard. MS liquid medium without inoculant was also used as blank control. MS liquid medium without carbon source was used as negative control.

4.3 Results

4.3.1 TBA-degrading bacteria isolation

Two pure microbial cultures, designated as TBA-R1-001 and TBA-R2-005, were respectively isolated from reactors R1 and R2 by using TBA as sole carbon and energy source. Serially-diluted biomass samples were prepared and spread-plated onto MS agar plates. Visible colonies (diameter > 1 mm) with different morphotypes were observed to form on MS plates with 10^5 -fold and lower dilutions of biomass inocula from both reactors R1 and R2 after 4 weeks of incubation. Colonies from the 10^4 - and 10^5 -fold dilutions were picked and transferred onto fresh MS agar plates supplied with TBA as sole carbon and energy source. After 2 to 3 repeated transfers, only two distinct colonies still has the capability to grow on MS agar plate with TBA as sole carbon and energy source. The other colonies were unable to grow after the first transfer to a new MS agar plate.

The first of the two surviving colonies was light brown round shaped and grew to a diameter of 3 mm after 4 days of incubation on MS agar plates. On R2A agar plates, the strain could form colonies with diameter of 3 mm within 2 days. The fast growing light brown colonies originated only from reactor R1. These colonies appeared on MS agar plates inoculated with 10^5 -fold dilution of R1 biomass.

The second colony was pink, round-shaped, and grew to a diameter of 1 mm after at least 7 days of incubation on MS agar plates supplied TBA as sole carbon and energy source. Even on R2A agar plates, this strain needed 4 to 5 days to form colonies with 1 mm diameter. The pink colonies were observed on MS agar plates inoculated with a 10^4 -fold dilution of reactor R1 and with a 10^5 -fold dilution of biomass from reactor R2 after 6 to 7 days of incubation. The pink colonies were individually picked and transferred to fresh agar plates until pure. The strains from

these colonies have identical 16S rRNA gene sequences and were identified as a *Methylobacterium* species based on both 16S rRNA gene sequence analysis and physiological and biochemical tests. Thus, a candidate strain TBA-R2-005 was selected to represent the isolates from the pink colonies.

4.3.2 Identification and characterization of strain TBA-R1-001

Strain TBA-R1-001 appeared only on 10^5 -fold dilution MS agar plates inoculated with serial-diluted biomass from reactor R1. Total culturable heterotrophic bacterial counts were 6.8×10^9 and 4.2×10^9 cells (g dry biomass)⁻¹ in reactors R1 and R2, respectively. The abundance of strain TBA-R1-001 was estimated to be 5.2×10^8 (R1) cells (g dry biomass)⁻¹. So, strain TBA-R1-001 accounted for 7.6% of the culturable heterotrophic bacteria in reactor R1.

Strain TBA-R1-001 is a rod-shaped, Gram positive, endospore forming (center), non-motile, catalase positive, and oxidase negative bacterium (Figure 4-1). Strain TBA-R1-001 grew rapidly on R2A agar plates, and could grow on several other tested agar plates, including tryptic soy agar, nutrient agar, plate counting agar, and LB agar. Cells of TBA-R1-001 were between 1 and 4 μm in length and were more than 1 μm in diameter when grown with glycerol (3.0 mM) as sole carbon source in liquid medium at 30°C.

The bacterium was tested to grow well on glycerol, ethanol, acetate, pyruvate, succinate, glucose, and fructose. Growth was best with glycerol. No vitamin solution was necessary when strain TBA-R1-001 was cultured in liquid medium using these carbon sources. The strain could also grow poorly on iso-propanol; OD₆₀₀ of cell suspension only increased from 0.11 to 0.18 in an 8 days incubation period. Vitamin solution did not enhance the growth of the isolate with iso-propanol as carbon source. This strain could also grow on C₁ compounds such as methanol, formaldehyde and formate. The bacterium formed colonies (> 3.0 mm in diameter)

Figure 4-1 Phase contrast micrograph of strain TBA-R1-001.

on MS agar plates supplied with TBA as sole carbon and energy source within 4 days. However, cultivation of strain TBA-R1-001 in shake flasks supplied with TBA as sole carbon source and energy source was not successful.

The following characteristics, tested using API ZYM and API 20E, are positive for strain TBA-R1-001: alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase (weak), trypsin, α -chymotrypsin, β -galactosidase, citrate utilization (weak), naphthol-AS-BI-phosphohydrolase, glucose, rhamnose, melibiose, amygdalin (weak), arabinose, NO_3 to NO_2 .

The following characteristics are negative for strain TBA-R1-001: esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, mannitol, inositol, sorbitol, sucrose.

Phylogenetic analysis based on almost full 16S rRNA gene sequence placed strain TBA-R1-001 with other members of the genus *Bacillus* (Table 4-1). According to the 16S rRNA gene sequence identity matrix (1269 bp), the closest relatives to *Bacillus megaterium* strain TBA-R1-001 are *Bacillus megaterium* IAM 13418 (T), *B. simplex* DSM 1321 (T), *B. cohnii* DSM 6307 (T), and *B. circulans* IAM 12462 (T), with sequence identities of 99.8, 99.2, 96.4, 95.7%, respectively.

4.3.3 Identification and characterization of strain TBA-R2-005

Based on its occurrence on the 10^4 - and 10^5 -fold dilution plates from reactors R1 and R2, respectively, strain TBA-R2-005 was estimated to present in the TBA-degrading microbial consortia at a concentration of 3.4×10^7 (R1) and 1.8×10^8 (R2) cells (g dry biomass)⁻¹, respectively. As the total heterotrophic bacterial counts

Table 4-1 Identity matrix based on 16S rRNA gene sequences for strain TBA-R1-001 and close relatives.

	Name	16S rRNA gene sequence identities (%)											
		1	2	3	4	5	6	7	8	9	10	11	12
1	<i>B. megaterium</i> str. TBA-R1-001												
2	<i>B. megaterium</i> IAM13418 (T)	99.8											
3	<i>B. benzoenorans</i> DSM5391 (T)	95.6	95.5										
4	<i>B. circulans</i> IAM12462 (T)	95.7	95.8	97.3									
5	<i>B. psychrosaccharolyticus</i> ATCC23296 (T)	95.5	95.5	95.4	94.9								
6	<i>B. simplex</i> DSM1321 (T)	98.2	98.1	95.8	95.4	95.4							
7	<i>B. cohnii</i> DSM6307 (T)	96.4	96.4	95.3	95.3	94.9	96.4						
8	<i>B. fastidiosus</i> DSM91 (T)	94.6	94.6	93.3	93.1	93.7	94.6	94.8					
9	<i>B. azotoformans</i> DSM1046 (T)	93.3	93.3	93.5	93.2	92.1	92.8	93.3	91.6				
10	<i>B. pumilus</i> NCDO1766 (T)	95.1	95.0	95.2	94.3	94.1	95.0	95.2	93.5	92.7			
11	<i>B. licheniformis</i> DSM13 (T)	93.8	94.0	94.3	93.8	92.8	94.0	94.2	93.4	92.8	96.7		
12	<i>B. subtilis</i> NCDO1769 (T)	93.8	94.0	94.0	93.6	93.1	94.0	94.1	93.8	92.9	97.3	98.6	

of reactor R1 and R2 were 6.8×10^9 and 4.2×10^9 cells (g dry biomass)⁻¹, respectively, strain TBA-R2-005 accounted for 0.5% and 4.3% of the total culturable heterotrophic bacteria in reactors R1 and R2, respectively.

Strain TBA-R2-005 is a pink-pigmented, rod-shaped, Gram negative, non-sporulating, non-motile, catalase positive, oxidase positive and forms rosette like aggregates in liquid medium (Figure 4-2). Aggregates of cells of strain TBA-R2-005 were also observed in samples prepared for SEM observation. Cells of TBA-R2-005 were between 2 and 4 μm in length and were less than 1 μm in diameter when grown with methanol as carbon source in liquid medium at 30°C.

The bacterium was tested for growth on different C₁ carbon sources, including methanol, formaldehyde, and formate. Growth on C₁ compounds is a defining attribute of methylotrophic bacteria. The bacterium could also grow on other carbon source including acetate, pyruvate, succinate, fumarate, glucose (poor), fructose, as well as on R2A agar, nutrient agar, and tryptic soy agar. No vitamin solution was necessary when strain TBA-R2-005 was cultivated in liquid medium using the above-mentioned carbon sources.

The bacterium also formed colonies (> 1.0 mm in diameter) on MS agar plates supplied with ethanol as sole carbon and energy source, but no growth was observed in MS liquid medium supplemented with ethanol. With TBA as sole carbon and energy source, vitamin solution was necessary to support the growth of strain TBA-R2-005. TBA-R2-005 formed pink colonies on MS agar plates with TBA as sole carbon and energy source without vitamin supplement. However, vitamin solution was necessary for cultivation of strain TBA-R2-005 in MS medium with TBA as sole carbon and energy source. Under such culture condition, TBA biodegradation as well as cell growth were not observed if the medium had no vitamin supplement.

Figure 4-2 **A** Phase contrast micrograph and **B** scanning electron micrograph of strain TBA-R2-005

The following characteristics, tested using API ZYM and API 20E, are positive for strain TBA-R2-005: alkaline phosphatase, esterase, esterase lipase (week), leucine arylamidase, valine arylamidase (week), acid phosphatase, glucose, rhamnose (week), naphthol-AS-BI-phosphohydrolase, arabinose.

The following characteristics are negative for strain TBA-R2-005: lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase, mannitol, inositol, sorbitol, sucrose, melibiose, amygdalin.

Phylogenetic analysis based on almost full 16S rRNA gene sequence placed strain TBA-R2-005 with other members of the genus *Methylobacterium* (Table 4-2). According to the 16S rRNA gene sequence identity matrix (1379 bp), the closest relatives to *Methylobacterium* sp. strain TBA-R2-005 are *M. organophilum* strain XX, *M. organophilum* [D32226], *M. extorquens*, *M. podarium* and *M. rhodium*, with sequence identities of 99.7, 99.7, 97.1, 96.9, 96.3%, respectively.

4.3.4 TBA biodegradation profiles of strains TBA-R1-001 and TBA-R2-005

When TBA was supplied as sole carbon and energy source, the growth as well as biodegradation profiles of strain TBA-R1-001 were different from strain TBA-R2-005. Strain TBA-R1-001 had a long lag phase when exposed to TBA as sole carbon source no matter whether the cells were previously grown on TBA or another carbon source. The lag phase was normally 6 to 18 hours depending on the initial carbon source used. During the lag phase, the cells number, in terms of OD₆₀₀, was only slightly decreased. TBA concentration decreases were comparable with those in the killed control as shown in Figure 4-3. After the lag phase, TBA

Table 4-2 Identity matrix based on 16S rRNA gene sequences for strain TBA-R2-005 and close relatives.

	Name	16S rRNA gene sequence identities (%)								
		1	2	3	4	5	6	7	8	9
1	<i>M. organophilum</i> str. TBA-R2-005									
2	<i>M. organophilum</i> str. XX JCM 2833 (T)	99.7								
3	<i>M. organophilum</i> [D32226]	99.7	100							
4	<i>M. extorquens</i> ATCC 43645 (T)	97.1	97.1	97.1						
5	<i>M. extorquens</i> JCM 2802 (T)	96.4	96.4	96.4	99.3					
6	<i>M. mesophilicum</i> JCM 2829 (T)	96.1	96.3	96.3	94.9	94.8				
7	<i>M. podarium</i> [AY468364]	96.9	96.9	96.9	98.6	98.3	94.8			
8	<i>M. rhodinum</i> JCM 2811 (T)	96.3	96.3	96.3	97.8	97.6	95.3	97.8		
9	<i>M. radiotolerans</i> JCM 2831 (T)	96.2	96.5	96.5	95.1	95.0	98.0	94.7	95.6

Figure 4-3 TBA removal by strain TBA-R1-001 from the TBA-fed cells (*closed circles ●*), glycerol-fed cells (*open circles ○*), and negative control (*open triangles Δ*). Negative control experiments were performed as same manner except killed cells of TBA-R1-001 were inoculated.

started to be degraded by strain TBA-R1-01; at the same time, the OD₆₀₀ showed a sharp decrease (data not shown).

It was observed that cells of strain TBA-R1-001 attached to the inner surface of the bottle and formed attached biofilms. It was difficult to measure cell density under these conditions. The mean TBA degradation rate at this time was approximately 18 mg-TBA h⁻¹ L⁻¹.

When grown on TBA as sole carbon and energy source, strain TBA-R2-005 completely degraded the TBA with no detectable accumulation of intermediates as measured by direct injection GC/FID method. Robust growth of strain TBA-R2-005 was associated with decrease in TBA concentration (Figure 4-4). The average growth rate was 0.046±0.002 h⁻¹, with an average yield of 0.29±0.04 g-dry weight g⁻¹-TBA.

4.3.5 PCR-DGGE analysis

DGGE was employed to produce genetic fingerprints that could provide information on the composition and diversity of microbial communities in the stabilized TBA-degrading microbial consortium. DGGE bands from reactors R1 and R2 did not co-migrate with the band associated with strain TBA-R1-001. It probably means strain TBA-R1-001 was not a dominant bacterium in the TBA-degrading aerobic granules.

On the other hand, Figure 4-5 shows representative DGGE profiles of amplified 16S rRNA genes from the R2 biomass and *M. organophilum* str. TBA-R2-005. The band associated with strain TBA-R2-005 co-migrated with band TBA204. No band was noticed to co-migrate with TBA-R2-005 in R1 sludge. Recovered band TBA204 was reamplified by PCR and run on a DGGE gel to confirm purity. This was repeated several times to obtain a pure DNA product for sequencing.

Figure 4-4 Growth (*open circles* ○) and TBA utilization (*open squares* □) during incubation of strain TBA-R2-005 in MS liquid medium with vitamins supplied. Negative control was performed as the same manner except no bacterial cells were inoculated (*closed symbols* ●, ■). As a semi-volatile compound, TBA could be slowly air stripped as shown a decreasing of TBA concentration in negative control experiments. The Arrows indicates replenishment of TBA into the culture medium.

Figure 4-5 Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA genes from [A] strain TBA-R2-005 and [B] Reactor R2 aerobic granules. Strain TBA-R1-001 has no co-migrate bands from either R1 granules or R2 granules.

Partial 16S rRNA gene sequence of 168 nucleotides was obtained from the band TBA204. Comparison with nucleotide sequences from GenBank indicated that TBA204 was identical to a taxonomically unidentified member of the α -Proteobacteria, and was 100% identical to *M. organophilum* and *M. extorquens*. The sequence of band TBA204 was also 100% identical to the 16S rRNA sequence of strain TBA-R2-005.

4.3.6 Oxygen utilization profiles of strain TBA-R2-005

Cell suspensions of strain TBA-R2-005 grown on methanol could degrade TBA without a lag phase. Similarly, cells grown on TBA as sole carbon source also could degrade methanol without lag phase. A significant difference between methanol- and TBA-associated oxygen utilization was that TBA was only degraded under high concentrations of dissolved oxygen, as indicated in Table 4-3. In contrast, methanol was degraded by both methanol- and TBA-grown cells at comparable rates under both high and low initial DO concentrations. Cells of strain TBA-R2-005 that were grown on R2A medium showed no immediate methanol- and TBA-associated oxygen utilization. Further study showed that strain TBA-R2-005 started to utilize methanol within 1 hour after exposure to this compound as sole carbon source, and that this strain took several days to re-adapt to TBA as sole carbon source.

Accurate determinations of O₂ utilization for strain TBA-R1-001 were difficult to obtain because it was not possible to obtain cell suspensions on TBA as sole carbon and energy source. If the TBA fed cells were detached from the biofilm, washed, and resuspended, they did not use TBA immediately. TBA-R1-001 cells grew in suspension with glycerol as sole carbon and energy source were unable to utilize TBA immediately. A long lag phase was required before strain TBA-R1-001 started to degrade TBA. Another difficulty encountered was that strain TBA-R1-001 preferred to attach to the inner surfaces of the culture vessels rather than grow in suspension.

Table 4-3 Oxygen utilization rate of strain TBA-R2-005 resting cells by using methanol and TBA as sole carbon substrate.

	Oxygen utilization rate (mg min ⁻¹ g ⁻¹ dry weight)				
	Methanol		TBA		Negative Control
	High DO ^a	Low DO ^b	High DO	Low DO	
Methanol-grown cells	9.7	8.9	1.1	0.1	0.1
TBA-grown cells	7.3	7.8	1.3	0.1	0.1
R2A-grown cells	0.1	ND ^c	0.1	ND	0.1

a: initial DO equal to saturated oxygen concentration

b: initial DO was 5 mg L⁻¹ or less.

c: ND means non-detected

4.3.7 TBA biodegradation by mixed culture of strain TBA-R1-001 and TBA-R2-005

When strains TBA-R1-001 and TBA-R2-005 were mixed together and inoculated into MS liquid medium, the mixed culture was found to efficiently degrade TBA as sole carbon and energy source. As shown in Figure 4-6, the TBA removal rate of the mixed culture was significantly higher as compared to the individual monocultures. In addition, it was found that the dual-species culture could degrade TBA in shake flasks without additional aeration. It was mentioned previously that neither TBA-R1-001 nor TBA-R2-005 could degrade TBA in shake flasks. The dual-species mixed culture could degrade TBA in shake flasks suggesting that commensalistic interactions could be at play to facilitate this result. TBA abiotic loss (e.g. evaporation) was negligible under shake flasks conditions as measured before.

4.3.8 *p*-NDA reduction

p-NDA has long been used as a probe for detecting reactive oxygen species (ROS) in biological systems. These ROS, such as hydroxyl radical can react and bleach the *p*-NDA from bright yellow to colorless. Strain TBA-R2-005 degraded TBA more rapidly at higher DO concentration which potentially caused ROS generation as byproducts of metabolic and respiration activity. *p*-NDA was bleached by strain TBA-R2-005 incubated with either methanol or TBA as sole carbon and energy source (Figure 4-7). No bleaching of *p*-NDA was detected in control experiments, showing that the *p*-NDA bleaching observed was directly associated with the metabolic activity of strain TBA-R2-005. In addition, pH value of the culture medium decreased significantly from 7.72 to 4.85 during the bleaching of *p*-NDA.

Strain TBA-R1-001 could also bleach *p*-NDA when incubated with TBA as sole carbon and energy source. The *p*-NDA bleaching curve of this strain is shown in Figure 4-8. There was a slight decrease in *p*-NDA concentration in the control

Figure 4-6 TBA biodegradation in shake flasks by a dual-species mixed culture (close circles ●), strain TBA-R1-001 alone (close triangles ▲), and strain TBA-R2-005 alone (open squares □).

Figure 4-7 Bleaching of *p*-NDA during growth of TBA-R2-005 on methanol (triangles Δ) and TBA (circles $\circ$) as sole carbon and energy source. Negative control (squares $\square$) was performed in the same manner except killed cells of TBA-R2-005 were used as inoculant.

Figure 4-8 Bleaching of *p*-NDA during growth of TBA-R1-001 on TBA (closed circles $\bullet$) as sole carbon and energy source. Negative control (closed squares $\blacksquare$) was performed at the same manner except kill cells of TBA-R1-001 were used as inoculant.

experiment with any carbon source. pH value of the culture medium decreased from 7.96 to 4.73 also during the *p*-NDA bleaching by strain TBA-R1-001.

4.3.9 H₂O₂ concentration measurements

H₂O₂ is an essential metabolic byproduct of aerobic organisms including bacteria. Aerobic bacteria constantly generate H₂O₂ as a byproduct of aerobic respiration and metabolism. However, H₂O₂ is usually generated intracellular and dispersed immediately after it generated. TBA biodegradation by strain TBA-R2-005 occurred primarily under conditions of higher dissolved oxygen concentration. Aeration was also necessary for strain TBA-R1-001 to degrade TBA. But, strain TBA-R1-001 did not accumulate H₂O₂ during TBA biodegradation. However, H₂O₂ accumulated in iron-free phosphate buffer during methanol and TBA degradation by strain TBA-R2-005. H₂O₂ produced by strain TBA-R2-005 was directly associated with metabolic activities as shown in Figure 4-9.

Figure 4-9 Hydrogen peroxide accumulated extracellular during strain TBA-R2-005 growth on methanol (triangles Δ) and TBA (circles $\circ$) as sole carbon and energy source. Rectangle symbols ($\square$) represent negative control.

4.4 Discussion

4.4.1 Isolation of TBA-degrading strains

The direct isolation method is often employed to obtain dominant, functionally important bacterial members from a microbial community (Watanabe et al. 1998; Zhuang et al. 2002; Jiang et al. 2004a). Although TBA-degrading strains have previously been reported (Mo et al. 1997; Piveteau et al. 2001), the ecological information on these strains was missing. In the current study, strain TBA-R1-001 and TBA-R2-005 were directly isolated from TBA-degrading aerobic granules cultivated in two SBR systems which were operated under 600 mg L⁻¹ d⁻¹ TBA loading and 6 h-cycle (Reactor R1) and 24 h-cycle (Reactor R2) cycle times, respectively.

Strains TBA-R1-001 was estimated to constitute 7.6% of total culturable heterotrophic bacteria from Reactor R1 granules. Strain TBA-R2-005 constituted 4.3% of total culturable heterotrophic bacteria from Reactor R2 granules. DGGE analysis of 16S rRNA gene fragments revealed that one dominant DGGE band (TBA204) from Reactor R2, which clustered within the α -Proteobacteria subdivision and was most closely related to *Methylobacterium organophilum*, had 100% sequence identity with strain TBA-R2-005.

The results from culture independent as well as culture based methods showed that strain TBA-R2-005 was a dominant bacterium in Reactor R2 granules. Strain TBA-R1-001 had a high cell abundance in Reactor R1 granules but was not detected in DGGE fingerprints of community DNA from R1 biomass. The abundance of TBA-R1-001 relative to the total culturable heterotrophic bacteria in Reactor R1 granules (7.6%) was higher than the corresponding abundance of strain TBA-R2-005 in Reactor R2 granules (4.3%).

It should be pointed out that culturable bacteria constitute an estimated 0.1 to 10% of the total bacteria in an ecosystem (Amann et al. 1995), and the DGGE method has a detect limitation generally accepted as 1% of total bacteria in any ecosystem (Muyzer et al. 1993; Muyzer and Smalla 1998). So, it should not be surprising that strain TBA-R1-001 could not be detected by the DGGE in Reactor R1 granules, although this strain was dominant in the culturable population. Taken together, the culture based and culture-independent results indicated that strains TBA-R1-001 and TBA-R2-005 were dominant members of the TBA-degrading microbial community residing in the SBR systems and played an important functional role in TBA biodegradation.

4.4.2 Phylogenetic analysis of TBA-degrading strains

Phylogenetic analysis based on 16S rRNA gene sequence placed strain TBA-R2-005 in the *Methylobacterium* genus. The closest relative to strain TBA-R2-005 was *M. organophilum*, with a sequence identity of 99.7%. *M. organophilum* as well as other members from the genus *Methylobacterium* are well known for their capability to assimilate one-carbon compounds (Patt et al. 1974; Patel et al. 1978; Wolf and Hanson 1978; Machlin and Hanson 1988). Almost all members from this *Methylobacterium* genus are pink-pigmented facultatively methylotrophic (PPFM) bacteria which are capable of growth on C₁ compounds (including formate, formaldehyde, and methanol) as well as on a wide range of multi-carbon growth substrates. These bacteria are also able to produce extracellular polysaccharides and storage biopolymers (Ackermann and Babel 1997; Madison and Huisman 1999), and have been found in abundance in environmental biofilms (Kelley et al. 2004).

Strain TBA-R1-001 belongs to the species *Bacillus megaterium*, and its almost full length 16S rRNA gene sequence is 99.8% identical to that of the type culture,

Bacillus megaterium IAM 13418 (Table 4-1). From the Phylogenetic point of view, the genus *Bacillus* is a very large and complex genus with many members. The most famous and intensively studied *Bacillus* species is probably *Bacillus subtilis*. Some members from this genus showed very broad substrate utilization ability, especially the capability of petroleum hydrocarbon biodegradation (Zhuang et al. 2002). This might be the first report that *Bacillus megaterium* can use TBA as well as C₁ compounds as sole carbon and energy source.

Both *B. megaterium* str. TBA-R1-001 and *M. organophilum* str. TBA-R2-005 can use C₁ compounds as sole carbon and energy source. This is a typical feature of a group of bacteria called methylotrophic bacteria (Hanson and Hanson 1996). In the previous studies, two TBA-degrading pure cultures, *Methylobacterium* sp. and *Burkholderia* sp. were also characterized as methylotrophic (Mo et al. 1997; Piveteau et al. 2001). It seems reasonable that bacteria that use TBA or MTBE as sole carbon and energy source might be methylotrophic. Although the pathway for TBA biodegradation has not yet been established (François et al. 2003), propanol, ethanol, methanol and/or formate are thought to be the potential intermediates of TBA biodegradation (Steffan et al. 1997). Formate or methanol should be the first intermediate after the TBA molecule is broken down. Thus, bacteria that can degrade TBA as sole carbon source may have a competitive advantage if these bacteria have the capability to further oxidize the intermediates of TBA degradation. Then, formate or methanol could be used by these bacteria for growth and energy generation.

As early as 1978, Patel and coworkers (1978) reported that methanol dehydrogenase (MDH) from *M. organophilum* str. XX could attack TBA in vitro, even though the degradation rate was less than 10% of that with methanol as substrate. This might be the only report of enzymatic attack of TBA in vitro. However, it was not clear if *M. organophilum* str. XX could degrade TBA in vivo. Although MDH from strain XX could use TBA in vitro, it may not necessarily mean that the same

enzyme could attack TBA in vivo. In fact, MDH from strain XX also used ethanol and propanol in vitro, but strain XX could not grow on ethanol and propanol as sole carbon and energy source (Patel et al. 1978).

In the current study, it was proven that *M. organophilum* str. TBA-R2-005 could use TBA as sole carbon and energy source. Resting cells of methanol-grown strain TBA-R2-005 could degrade TBA without a lag phase, as shown in Table 4-3, indicating that TBA-degrading enzymes were expressed when this strain used methanol as substrate. In addition, TBA-grown cells of strain TBA-R2-005 could degrade methanol without lag phase and at rates comparable to those of methanol-grown cells. It makes sense that MDH is the enzyme that strain TBA-R2-005 used to attack both TBA and methanol. However, in contrast with its methanol degradation profile, strain TBA-R2-005 degraded TBA only at a relatively higher DO concentration. This probably means that strain TBA-R2-005 required other factors to cooperate in order to efficiently degrade TBA.

Cells grown on R2A medium did not immediately use either methanol or TBA. This reinforced the notion that MDH might play an important role in TBA biodegradation in this strain. Strain TBA-R2-005 grown on R2A medium needed a relative long lag phase (several days) to re-adapt to TBA as compared to re-adaptation to methanol (less than 1 hour) as carbon source. This suggested that TBA degradability could be induced but the gene regulation and enzyme expression of the first step of TBA degradation was more complex than just methanol degradation. All these lines of evidence showed that MDH was involved in the TBA biodegradation of strain TBA-R2-005 and potentially played an important role to support this strain's growth on TBA as sole carbon and energy source. However, it is not clear if the enzyme was directly involved in TBA attack. This topic is currently under investigation by other research group.

Strain TBA-R1-001 has more than 99.7% 16S rRNA gene identity with *Bacillus*

megaterium IAM 13418^(T). It is well known that *B. megaterium* carries the gene encoding the cytochrome P-450 BM3 monooxygenase. P-450 monooxygenase has been proposed as an MTBE- and TBA-attacking enzyme (Fulco 1991; Steffan et al. 1997), although there is still a lack of in vitro data to support the hypothesis. Nevertheless, the current study showed that *B. megaterium* str. TBA-R1-001 could use TBA as sole carbon and energy source, as well as the P-450 test for this isolate was positive. The special growth profile when the isolate used TBA as sole carbon and energy source revealed that the expression of the TBA-attacking enzyme might be associated with the adhesion or aggregation of this strain. This special feature has never been reported before. It might be the reason that the strain could stay in reactor R1 and finally persisted in aerobic granules.

4.4.3 TBA degradation by a dual-species mixed culture

When the two pure cultures were inoculated together to form a dual-species mixed culture, the TBA biodegradation profile was changed. The TBA removal rate was significantly increased as compared to the single species pure culture. The results revealed that a microbial interaction might be involved in the dual-species mixed culture system when they were using TBA as sole carbon and energy source. TBA is a recalcitrant compound; its tertiary structure prevents the enzymatic attack of the molecule. The first step of TBA degradation is the rate limiting step and the downstream pathway compounds of TBA degradation are easily used substrates for bacteria.

Strains TBA-R1-001 and TBA-R2-005 could use C₁ compounds as sole carbon and energy source. Strain TBA-R2-005 used formate and methanol more efficiently than TBA-R1-001, while strain TBA-R1-001 grew more efficiently on the iso-propanol. Strain TBA-R2-005 showed poor growth on ethanol and on iso-propenol in liquid medium. The two strains therefore exhibited different substrate utilization behaviors towards the C₁ compounds as well as other

multi-carbon alcohols. It could be postulated that each isolate in the mixed culture might use the favorite intermediate to support their functions and growth. Besides the higher TBA removal rate, TBA biodegradation could also be achieved in shake flasks. On the other hand, mono cultures only degraded TBA under a high dissolved oxygen environment created by forced aeration. It is currently not clear why the pure cultures only degraded TBA under high DO concentrations, nor why the dual-species mixed culture system could degrade the TBA in shake flasks where the DO levels were significantly lower. The results indicated that the interactions between these two cultures could help to overcome the high DO requirement. Other researchers had also observed that mixed cultures were more efficient than mono culture at MTBE and/or TBA biodegradation (Fortin et al. 2001).

It should be pointed out that while *Methylobacterium organophilum* and *Bacillus megaterium* have been studied for several decades, the biochemical features of these genera are still not very well understood. In recent years, it was found that members of the *Methylobacterium* genus can degrade many other toxic and recalcitrant xenobiotic compounds such as trinitrotoluene [TNT] (Aken et al. 2004) and MTBE (Mo et al. 1997). The current study is the first that to document the ability of *Methylobacterium* to use TBA as sole carbon and energy source. Although the ability of strain TBA-R2-005 to reduce TNT was not investigated, it was found in the current study that strain TBA-R2-005 reduced *p*-NDA during growth on methanol and TBA as sole carbon and energy source.

Strain TBA-R1-001 could also reduce *p*-NDA when incubated with TBA or glycerol as sole carbon and energy source. Bleaching of *p*-NDA was associated with metabolic activity (Figure 4-8). In addition, strain TBA-R2-005 extracellularly accumulated certain levels of H₂O₂ when it grew in a high DO concentration environment. It is still not clear the relationship between TBA biodegradation, extracellular redox activity, H₂O₂ production and dissolved oxygen concentrations to strain TBA-R2-005.

4.4.4 Summary

In summary, two TBA-degrading bacteria isolated from TBA-degrading aerobic granules by the direct isolation method were discussed in this chapter. The characteristics of strain *Bacillus megaterium* str. TBA-R1-001 and strain *Methylobacterium organophilum* str. TBA-R2-005 extend the knowledge of TBA-degrading bacteria. Both isolates may have a potential for application in the bioremediation of TBA contaminated wastewater. Since *Bacillus* and *Methylobacterium* bacteria are common and widespread in a variety of environments, their involvement in natural attenuation of toxic compounds may be of ecological importance.

Chapter Five

Mechanism of *tert*-Butyl Alcohol (TBA)

Biodegradation by TBA-degrading Aerobic Granules

5.1 Introduction

TBA has long been recognized as a recalcitrant compound because the *tert*-butyl structure of this compound blocks the TBA molecule from enzymatic attack. However, several studies on isolation and identification of TBA-degrading microorganisms have recently been reported (Mo et al. 1997; Schmidt et al. 2004). This dissertation also describes the cultivation of TBA-degrading aerobic granules and the isolation of two bacterial strains using TBA as sole carbon and energy source. To date, only a handful of TBA-degrading strains have been isolated (Schmidt et al. 2004). The first step of the TBA degradation pathway was postulated to involve the attack by alkane monooxygenase at one of the methyl groups to form TBF (Steffan et al. 1997). Cytochrome P-450 has also been implicated in TBA biodegradation. Unfortunately, functional enzymes have not yet been isolated and purified with detectable *in vitro* activities from these TBA-degrading bacteria (Hardison et al. 1997).

Beside biodegradation, TBA can also be degraded by pure chemical methods. The hydroxyl radical ($\text{HO}^\bullet$) is a very active reactive oxygen species (ROS) (Cater et al. 2000; Hammel et al. 2002) that could nonspecifically react with organic compounds via electron extraction or addition mechanisms (Gabriel et al. 2004). There have been several recent reports on treating MTBE/TBA contaminated water with hydroxyl

radical-based methods, for example, UV/O₃ system or Fenton reaction (Garoma and Gurol, 2004; Xu et al. 2004; Burbano et al. 2005). The chemical formation of hydroxyl radical through Fenton reaction is as follow:

The basis of such TBA treatment methods stems from the well-established notion that TBA is a hydroxyl radical (HO[•]) scavenger. It reacts with HO[•] to form TBF and subsequently breaks down to form several byproducts, including acetone, methanol, formaldehyde, formate and acetate (Garoma and Gurol 2004).

Certain wood decay fungi are known to employ HO[•] as an active oxidant to non-enzymatically attack wood cell wall (Hammel et al. 2002). In other biological systems, HO[•] generated by Fenton reaction was also discovered and found to play an important role in the DNA damage (Touati 2000). The pure chemical HO[•] generation system requires low pH (<4) condition. However, it has been demonstrated that in a biological system containing ferrous iron and hydrogen peroxide, HO[•] could potentially be formed even under physiological conditions (McCormick et al, 1998; Juven and Pierson 1996). For instance, white rot fungus could reduce ferric iron and secrete H₂O₂, and eventually generate HO[•] to degrade wood cell walls. This has been demonstrated by a pure fungal culture under aerobic conditions (Hammel et al. 2002). However, there is currently no report on pure bacterial cultures or even mixed bacterial cultures that can achieve such reactions under aerobic conditions.

Recently, Jiang et al. (2004a) studied the pure cultures isolated from phenol-degrading aerobic granules which are cultivated and formed by using phenol as sole carbon and energy source. The authors concluded that some bacteria with high phenol degradability may play an important role in phenol removal function; however, some other bacteria with very limited phenol degradability but with high auto- and co-aggregation capability may enhance the granules formation and stability.

In addition, Møller et al. (1998) demonstrated the existence of metabolic interactions in mixed culture biofilms. Such metabolic interactions have been well studied in anaerobic granules.

However, in aerobic systems (e.g. aerobic granules and biofilm), the information is quite limited. A single aerobic granule potentially contains a high diversity of aerobic, facultative as well as strictly anaerobic microorganisms (Tay et al. 2003b; Yi et al. 2003). TBA-degrading aerobic granules have been shown by DGGE analysis to have a diverse microbial population. However, only two TBA-degrading strains have been directly isolated from TBA-degrading aerobic granules. The total culturable heterotrophic bacteria numbers were at least 10-fold higher than the number of TBA-degrading bacteria.

The apparent differences between TBA-degrading bacteria and heterotrophic bacteria raised a question on how these heterotrophic bacteria survived in an ecological system in which TBA was supplied as sole carbon and energy source. During the TBA biodegradation, the first step of TBA biodegradation is the limiting step because downstream pathway intermediates are easily used substrates (Steffan et al. 1997). It makes sense that some heterotrophic bacteria can live on TBA-degrading intermediates without directly attacking TBA by themselves.

The concern is if and how these bacteria contribute in the first step of TBA degradation. Since microbial interactions have been demonstrated (Møller et al. 1998; Jiang et al. 2004a), it was hypothesized that the TBA biodegradation by TBA-degrading aerobic granules may be partially contributed by an indirect attacking pathway; a pathway relying not on direct enzyme attack, pure chemical reactions achieved via different functional microbial populations' interactions. The microbial driven Fenton reaction potentially proves or confirms the hypothesis. The main objective of this study is therefore to study the potential and feasibility of TBA-degrading aerobic granules-driven Fenton reactions.

5.2 Materials and Method

5.2.1 Reactor operation and TBA-degrading microbial consortium enrichment

TBA-degrading aerobic granules were cultivated in a 2 L working volume column-type SBR (10 cm diameter and 28 cm working height) as previously described in Chapter 3. TBA was fed as sole carbon and energy source with a synthetic wastewater (MSM-a medium without mineral salts and vitamins solution). The reactor was finally maintained at $600 \text{ mg L}^{-1} \text{ d}^{-1}$ TBA loading rate. Aerobic granules were collected from SBR and immediately used for experimental assays.

5.2.2 Culture medium and chemical reagents

TBA (>99%), ferrous citrate, potassium phosphate, diethylenetriaminepentacetic acid (DETAPAC), potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), horseradish peroxidase (POD), N,N-diethyl-1,4-phenylenediammonium sulfate (DPD), 30% hydrogen peroxide (H_2O_2), catalase, Chelex100, and polyvinyl alcohol (PVA) were obtained from Sigma. Dimethylnitrosoaniline (*p*-NDA) and coumarin-3-carboxylic acid (3-CCA) were obtained from Aldrich. 7-hydroxy-coumarin-carboxylic acid (7-OHCCA) was purchased from Molecular Probes (Invitrogen Singapore Pte Ltd, Singapore).

The culture medium (MSM-b medium) used in batch studies had the following compositions (per liter, pH 7.6-7.8): 0.4 g of NaNO_3 , 0.4 g of Na_2HPO_4 , 0.15 g of KH_2PO_4 , 0.2 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g of CaCl_2 , 2.5 mg FeCl_3 , 1.0 mg of CoSO_4 , and 0.5 mg of CuSO_4 . In some cases, ferric citrate was used to replace FeCl_3 . No other mineral salts and vitamin solutions were added.

5.2.3 Hydrogen peroxide (H_2O_2) generation

H_2O_2 concentrations were determined using a previously described spectrophotometric assay based on the horseradish peroxidase (POD)-catalyzed colorimetric method (Bader et al. 1988). A cobalt-bicarbonate spectrophotometric method (Masschelen et al. 1977) was also used to validate the H_2O_2 concentration results measured by the first method. The cobalt-bicarbonate method was suitable for H_2O_2 concentrations ranging from 0.02 to 0.2 mg L^{-1} and gave essentially identical results as the POD method. The POD method was more sensitive and it could detect lower H_2O_2 concentrations. Standard H_2O_2 concentrations were prepared by diluting H_2O_2 (30%) with Milli-Q H_2O to the required concentrations. The concentrations were calibrated by measuring absorption of H_2O_2 at 240 nm ($\epsilon = 40.0 \text{ M}^{-1} \text{ cm}^{-1}$) as described by Bader et al. (1988).

Aerobic TBA-degrading granules were harvested from the reactor during aeration stage, washed three times using Milli-Q H_2O , and resuspended using MSM-b medium. Batch studies were performed in 500 mL acid washed reagent bottles with 150 to 200 mL reaction volume. TBA was supplied for sole carbon and energy source. Experiments were started after the addition of TBA into the medium. To measure the H_2O_2 concentration, 3 mL sample was withdrawn, quickly passed through a 0.2- μm filter, and the filtrate was immediately mixed with POD and DPD solutions. The absorption spectrum (551 nm) was taken 1 minute after the addition of POD. Control experiments were performed in the same manner except no TBA was added to the final solution. Dry biomass concentration (VSS) and dissolved oxygen concentration (DO) were determined in accordance with the standard methods as described previously (APHA, 1998).

Ferrous iron reacts with H_2O_2 according to the Fenton reaction to form Fe^{3+} . Ferrous iron (Fe^{2+}) was detected in TBA-degrading aerobic granules as a precipitate form, although only ferric iron (Fe^{3+}) was added into the culture medium. So, it was

interesting to note the change in H_2O_2 concentrations once ferrous iron was eliminated from aerobic granules. If higher H_2O_2 concentration could be achieved, it probably means that the ferrous iron reacts with H_2O_2 in the aerobic granulation system and it will be evidence of aerobic granule-driven Fenton reaction.

Unfortunately, there was no applicable method which could extract iron from aerobic granules without disrupting the granules' structure and metabolic activities. Using iron chelators is an alternative way because once iron is sequestered by chelators, it will not effectively react with H_2O_2 . DETAPAC is a commonly used ferrous iron chelator that forms a bright yellow Fe(II) complex and prevents Fe(II) from reacting with H_2O_2 (Yoshimura et al. 1992). However, when DETAPAC was added to a solution of aerobic granules, the medium gradually turned bright yellow. The bright yellow complex interfered with H_2O_2 measurements using either the POD or cobalt-bicarbonate method. So, it was not possible to measure whether higher H_2O_2 concentration could be achieved once the iron is removed from aerobic granules.

5.2.4 Iron reducing capability by aerobic TBA-degrading granules

The concentrations of total (including 0.5 N HCl acid extractable) and dissolved ferrous iron were determined by the phenanthroline based on colorimetric method (APHA, 1998). The content of dissolved ferrous iron was determined in suspension upon filtration through a nylon membrane filter with 0.2- μm pores. The solution was immediately mixed with phenanthroline stock solution and acetate-ammonium buffer at a volume ratio of 2:1:5 with vigorous stirring. The samples were then properly diluted with milliQ- H_2O and the colors developed were measured within 5 minutes using Beckman DU 6400 UV/visible spectrophotometer at 510 nm wave length. To determine the total ferrous iron, it was first extracted from the sample for 15 minutes by adding 1 N HCl (1:1 mixing). The content of total ferrous iron was then measured as the same manner as described above. To establish the calibration

curve, serial dilutions of a standard ferrous iron solution with a concentration of 1000 mg L⁻¹ was used and prepared by dissolving 0.355 g FeCl₂·4H₂O in 100 mL of 0.5 N HCl, .

TBA-degrading granules were harvested during aeration stage of SBR cycle, washed three times with milliQ-H₂O, and resuspended in a 50 mM phosphate buffer (PSB). Prior to testing for iron-reducing capability, the granules were aerated for at least 2 hours in PSB containing no carbon source to oxidize any carryover Fe(II). After aeration, total ferrous iron concentration decreased more than 92% compared with its concentration in fresh granules. The iron-reducing capability of aerobic granules was tested under both anaerobic and aerobic conditions.

For the anaerobic conditions, granules were inoculated with N₂ gas purged MSM-b medium (final DO less than 0.5 ppm) in a 150 mL serum bottle with a Teflon coated stopper without headspace. Ferric citrate, as an extra iron source, was added to medium with final concentration of 15 mM. Serum bottles were gently shaking on an orbital shaker and all experiments were performed under room temperature. Dissolved ferrous iron and total ferrous iron concentrations were periodically measured as described above. 10 mL sample (a mixture of liquid and granules) was collected for the analysis of total ferrous iron concentrations in each time; and 5 mL supernatant was collected for the analysis of dissolved ferrous iron. Sampling was performed in a Bactron Anaerobic/Environmental Chamber (Sheldon Manufacturing Inc, Cornelius, OR, USA).

As for the aerobic conditions, experiments were performed in a 500 mL reagent bottle with aeration. No ferric citrate was added to the culture medium under the aerobic condition experiments. Hence, the batch study results could reflect the real situation in the bioreactors. In both anaerobic and aerobic experiments, TBA was added as carbon and energy source. However, citrate may be used by some of microorganisms from aerobic granules as carbon and energy source. Except for

TBA and citrate, no other carbon source was added to the medium. All the experiments were performed in triplicate.

5.2.5 Extracellular redox activity of TBA-degrading aerobic granules

Extracellular redox activity was crucial to $\text{HO}^\bullet$ producing function of white root fungus and was essential for iron reducing activities (Stahl et al. 1995; Stahl and Aust 1995; Guillén et al. 1997; Qi and Jellison, 2004). $\text{Fe}(\text{CN})_6^{3-}$ is a large molecule which does not penetrate into cells and is widely used in extracellular redox activity measurements. Aerobic granules were harvested and washed as mentioned above. Resuspended aerobic granules were inoculated in a 500 mL reagent bottle with 100 mM phosphate buffer (pH 8.2) to a final volume of 200 mL. TBA was used as sole carbon and energy source. $\text{Fe}(\text{CN})_6^{3-}$ stock solution was carefully added to the final concentrations of 1.0-1.5 mM. Fine air bubbles were supplied through a diffuser at the bottom of the bottle. The experiments were performed in the dark by bottles covered with aluminum foil and conducted at room temperature. DO was maintained higher than 5.0 mg L⁻¹. $\text{Fe}(\text{CN})_6^{3-}$ concentration was measured by a modified spectrophotometric method described by Bonner (1955) using a Beckman DU640 UV/Visible spectrophotometer at a wavelength of 420 nm.

5.2.6 Hydroxyl radical ($\text{HO}^\bullet$) detection by *p*-NDA and 3-CCA

p-NDA is used in many studies to demonstrate the existence of $\text{HO}^\bullet$ and other reactive oxygen species (Imlay et al. 1988). Stock *p*-NDA solution was prepared by first dissolving *p*-NDA in milliQ-H₂O to give a concentration of 4.5 mM and then filtered through a 0.2 µm nylon filter. To measure bleaching of *p*-NDA, 1 mL of the stock solution was added to 100 mL MSM-b medium supplied with TBA (1.4 mM) as sole carbon and energy source. TBA-degrading granules were inoculated into the medium to give a final concentration of approximately 3.85 g L⁻¹ dry biomass weight. Control experiments were performed in the same manner except no carbon source was

added to the medium. All experiments were performed in the dark by flasks covered with aluminum foil. *p*-NDA concentration was measured by first filtering the culture medium through a 0.2 μm nylon filter and then the absorbance of the filtrate at 440 nm wave length was measured using a Beckman DU640 UV/Visible spectrophotometer. *p*-NDA concentration was linearly correlated with OD_{440} over a range of 3 to 60 μM .

Hydroxylation of coumarin-3-carboxylic acid (3-CCA) produces one major fluorescent substance, 7-hydroxy-coumarin-carboxylic acid (7-OHCCA), which has previously been used for the specific detection of hydroxyl radicals generated by fungi (Tornberg and Olsson, 2002). A stock solution (5 mM) of 3-CCA was prepared by dissolving 3-CCA in K_2HPO_4 buffer (40 mM, pH 9) at 50°C overnight. 7-OHCCA, as standard, was dissolved in K_2HPO_4 buffer (40 mM, pH 9). The relative fluorescence was measured with a Perkin-Elmer LS 50B luminescence spectrometer. The resulting values are presented in this study as fluorescence units (FU).

5.2.7 Cometabolic Polyvinyl alcohol (PVA) and 2,4-dichlorophenol (2,4-DCP) by aerobic granules

Experiments were performed using TBA-degrading granules in 500 mL reagent bottles with 200 mL MSM-b medium under aerobic conditions. TBA with either PVA or 2,4-DCP was added as substrates. In control experiments, TBA was omitted so that either PVA or 2,4-DCP was the sole substrate in MSM-b medium.

Quantitative determination of PVA concentration in liquid cultures was assessed by spectrophotometric analysis after the addition of boric acid and iodine solutions to the filtered culture medium according to the procedure described by Finley (1961). PVA formed a green complex with iodine in the presence of boric acid. The wave length of maximum spectrophotometric absorbance of the complex is 690 nm.

The 2,4-DCP concentration was also measured using spectrophotometric methods described by Mercer et al. (1996). Measurements were conducted under 510 nm wave length.

5.2.8 Analytical method

pH, volatile suspended solids (VSS), sludge volume index (SVI) and dissolved oxygen (DO) were measured periodically using standard methods (APHA, 1998).

A direct injection gas chromatograph method (Steffan et al. 1997) was used to determine TBA concentrations. All liquid samples were collected and analyzed immediately. Cell-free aqueous samples (1-3 μL) were automatically injected into a Hewlett Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a DB624 column (Hewlett Packard, 30 m $\times$ 0.53 mm $\times$ 3 μm). The oven temperature was held at 60°C for 3 minutes and was then ramped to 120°C at 40°C min⁻¹, which was held for 3 minutes, and finally raised to 200°C at 40°C min⁻¹. The TBA peaks and concentration values were determined using Chemstation software (Hewlett Packard).

5.3 Results

5.3.1 H₂O₂ generation of TBA-degrading aerobic granules

Under aerobic conditions, hydrogen peroxide (H₂O₂) was generated and accumulated extracellularly by TBA-degrading aerobic granules incubated with TBA as sole carbon and energy source (Figure 5-1). H₂O₂ concentrations increased in association with TBA degradation and accumulated to stabilize at $1.17 \pm 0.04 \text{ mg L}^{-1}$ within the first 60 minutes. This level was well below the concentration (34 mg L^{-1}) considered to be toxic to microbial cells (Imlay et al. 1988; McKinzi and DiChristina, 1999). In the control experiments where no carbon source was supplied, H₂O₂ accumulation was not detected (Figure 5-1). The results indicated that H₂O₂ was generated in association with microbial metabolic activity.

Extracellular H₂O₂ concentrations were also affected by biomass concentrations and dissolved oxygen concentrations, as shown in Figure 5-2 A and B. When biomass concentration was lower than 500 mg L^{-1} , no extracellular H₂O₂ accumulation was detected using POD and cobalt-bicarbonate methods. Extracellular H₂O₂ concentrations generally increased with the increase of biomass concentrations stabled at $1.22 \pm 0.14 \text{ mg L}^{-1}$. However, when the biomass concentration increased from 8.3 to 10.4 g L^{-1} , the H₂O₂ concentration further increased to $2.59 \pm 0.17 \text{ mg L}^{-1}$. In addition, dissolved oxygen concentrations also affected H₂O₂ accumulation (Figure 5-2 B). The results showed that higher dissolved oxygen concentrations promoted H₂O₂ production and accumulation compared with lower dissolved oxygen concentrations.

Figure 5-1. Hydrogen peroxide concentration detected in MS medium supplied TBA as sole carbon and energy source ($\square$), and control experiment without carbon source ($\triangle$). The increase of H_2O_2 concentration is associated with TBA concentrations decreases ($\circ$).

Figure 5-2 Hydrogen peroxide production as a function of [A] biomass concentrations; and [B] dissolved oxygen concentrations.

5.3.2 Ferric iron reduction capability of aerobic granules

Ferric citrate was used to assess iron-reducing capabilities of the TBA-degrading aerobic granules. The granules showed iron-reducing capability under both aerobic and anaerobic conditions, but with different profiles. Under anaerobic conditions, total Fe^{2+} (including precipitate form and soluble form) started to increase almost immediately but at a very low rate (Figure 5-3). The rate increased significantly after 1 to 1.5 hours of incubation under anaerobic conditions. In addition, the percentage of soluble ferrous iron was more than 50% of the total detected ferrous iron. In contrast, under aerobic conditions, ferrous-iron concentration increases were detected after at least 45 minutes of incubating the aerobic granules. Only 5.6% of total ferrous iron was detected in soluble form while 94.4% was in precipitate form under aerobic conditions. It needs to be pointed out that most of the ferrous iron was in a precipitate form under aerobic conditions and by using freshly collected aerobic granules from bioreactors the results agreed with the assay results. Table 5-1 summarizes the iron concentration from both anaerobic and aerobic conditions after 6 hours of incubation.

5.3.3 Extracellular redox activity of TBA-degrading aerobic granules

Ferricyanide (1.5 mM) could be extracellularly reduced by TBA-degrading aerobic granules when TBA was used as sole carbon and energy source (Figure 5-4). In a two-hour period, 0.81 mM ferricyanide was reduced by 4.45 g L^{-1} biomass (dry weight), which gave an average reduction rate of $1.52 \text{ nM} [\text{min} \cdot \text{g-biomass}]^{-1}$. After starvation for 24 hours the granules were used as a control and these granules did not show significant ferricyanide reduction as shown in Figure 5-4. Furthermore, pH value was significantly decreased during ferricyanide reduction even though phosphate buffer was used in these experiments. Because some bacteria were reported to degrade ferricyanide and used it as a nitrogen source, experiments were

Figure 5-3. Aerobic granules iron reduction capability assayed under anaerobic conditions. Total ferrous iron concentrations (O) increased almost immediately after the experiment started and reached its peak after 6 hours of incubation.

Table 5-1 Final Fe(II) concentration in aerobic and anaerobic conditions

	Aerobic condition (mM)	Anaerobic condition (mM)
Total Fe(II)	2.58±0.18	9.45±0.12
Soluble Fe(II)	0.148±0.071	4.91±0.22

Figure 5-4. Ferricyanide reduction by TBA-degrading aerobic granules with TBA as sole carbon and energy source (●), and negative control without carbon source (○).

performed to test whether ferricyanide was reduced or degraded by aerobic granules (Ebbs 2004). Diluted H_2O_2 solution (10 mM) was slowly added to 0.2- μm filtered supernatant to give a final H_2O_2 concentration of 2.0 mM. Ferrous cyanide could be re-oxidized back to ferricyanide by this method and an average of 87% reduction of ferricyanide was observed.

5.3.4 Active oxygen species tests

p-NDA bleaching assay was used to measure primarily active oxygen species, singlet oxygen and hydroxyl radicals. TBA-degrading aerobic granules can efficiently bleach 40 μM *p*-NDA in 30 minutes compared with the control experiments as shown in Figure 5-5.

To confirm that hydroxyl radical was produced by aerobic granules under aerobic conditions, 3-CCA assay was performed. The purchased 7-OHCCA in a K_2HPO_4 buffer (40 mM, pH 9) exhibited an emission maximum at 444 nm and an excitation maximum at 388 nm. Thus, the emission was measured at 444 nm in all experiments. The fluorescence reached at 113 fluorescent units (FU) after the granules were incubated with TBA as sole carbon and energy source for 24 hours. The fluorescence in control experiments without carbon source was only 18 FU. The fluorescence was stable at approximately 171 FU after 3 days of culturing and no further increase was noticed even though TBA was replenished into culture medium.

5.3.5 PVA co-metabolism by TBA-degrading aerobic granules

PVA was degraded by TBA-degrading aerobic granules when cultured under aerobic conditions (Figure 5-6). TBA-degrading aerobic granules did not degrade PVA alone as no PVA degradation occurred in control experiments without supplying TBA as carbon and energy source. In the current study, the initial TBA concentration

Figure 5-5 *p*-NDA bleaching by TBA-degrading aerobic granules with TBA as sole carbon and energy source (□), and negative control which is granules without any carbon resources (■).

Figure 5-6 TBA-degrading aerobic granule can degrade PVA (△) once TBA (□) was supplied as carbon source. Negative control was performed in the same manner with PVA (▲) but no TBA was supplied.

was 12 times higher than the PVA concentration. With the TBA degraded by aerobic granules, the ratio of TBA to PVA was decreased. It was noticed that PVA degradation rate was slow at the initial stage when TBA concentrations were still high (several times higher than PVA concentration). However, PVA degradation rate increased when the TBA to PVA ratio decreased to 4 and less. After 24 hours, more than 50% of PVA was degraded by granules.

2,4-DCP was degraded by TBA-degrading aerobic granules when TBA was supplied as carbon and energy source (Figure 5-7). Similarly with PVA co-metabolism, the granules did not degrade 2,4-DCP in control experiments which were not supplied with TBA. No further identification of 2,4-DCP metabolisms was attempted.

5.3.6 TBA-biodegradation inhibitors

Several chemicals were found to reduce the TBA removal efficiency (Table 5-2). These chemicals showed different inhibition efficiency on TBA removal from less than 10% to almost total inhibition. It should be pointed out that the inhibition mechanisms of different chemicals might be different. Even for a single chemical, the inhibition mechanism might involve many aspects. Thus, the current study does not attempt to investigate the detailed inhibition mechanisms of each individual chemical.

Figure 5-7 TBA-degrading aerobic granule can degrade 2,4-DCP ($\triangle$) once TBA ($\square$) was supplied as carbon source. Negative control was performed in the same manner with 2,4-DCP ($\blacktriangle$) but without TBA.

Table 5-2. Inhibitor effects to TBA biodegradation

Chemical/Reagent	TBA removal efficiency (%)
TBA alone	Control experiment (100%)
Sodium Acetate ^a	Totally inhibited (0%)
NH ₄ NO ₃	Totally inhibited (0%)
mannitol	43.2±9.7%
benzoate	36.8±12.4%

a: The initial inhibitor concentration was 100 mg L⁻¹, respectively.

5.4 Discussion

5.4.1 Aerobic granules mediated Fenton reaction

The current study addresses the possibility that Fenton-like reactions are driven by interactions of different bacteria residing in TBA-degrading aerobic granules and the Fenton-like reactions may possibly play an important role in TBA biodegradation. Extracellular hydrogen peroxide generation and iron-reducing capability of TBA-degrading aerobic granules were measured and the possibility of $\text{HO}^\bullet$ generated by Fenton-like reaction was addressed in this study. This is the first time that microbial-driven Fenton-like reaction was studied in an aerobic granulation system.

As shown in equation (1), Fenton or Fenton-like reactions require at least two elements; H_2O_2 and reduced metal elements. The Fenton-like reactions in biological systems have been studied for a long time. It has been well documented that $\text{HO}^\bullet$ could be generated into intracellular cells by the Fenton-like reactions (Imlay, 2003). The toxicity of these intracellular $\text{HO}^\bullet$ to the cells has also been extensively studied (Touati, 2000). However, information on microbial-driven Fenton-like reactions in extracellular environments is still very limited.

To date, most studies on extracellular Fenton-like reactions in biological system have focused on the white- and brown-rot fungi. These fungal species could generate extracellular H_2O_2 by various pathways and also reduce ferric iron to ferrous iron. Hence, $\text{HO}^\bullet$ may be generated by Fenton-like reactions. Because of such $\text{HO}^\bullet$ generation capability, these fungi possess very broad substrate utilization ability, including some recalcitrant xenobiotics (e.g. dichlorophenol, PolyEthelene Glycol, and so on) (Cameron et al. 2000).

In addition, both H_2O_2 generation and iron-reduction had been demonstrated in

pure fungal cultures under aerobic conditions (Tornberg and Olsson, 2002). Compared with fungal-driven extracellular Fenton reactions, reports on bacteria-driven extracellular Fenton reactions are quite limited, though the occurrence of Fenton reactions within bacterial cells had been well studied and documented. The lack of information on extracellular Fenton reaction driven by pure bacterial cultures may be attributed to the special requirements of Fenton reaction whereby Fe^{2+} and H_2O_2 must be both present.

So far, there are no reports on pure bacterial cultures which could secrete H_2O_2 and reduce ferric iron under aerobic conditions. This is not surprising because the conditions required for H_2O_2 generation and iron-reduction generally do not agree with each other. As mentioned above, H_2O_2 is an essential metabolic byproduct of aerobic bacteria. The aerobic bacteria constantly generate H_2O_2 as a byproduct of aerobic respiration and metabolism (González-Flecha and Demple, 1995; Messner and Imlay, 1999), as not all H_2O_2 is generated by respiration (Seaver and Imlay, 2004). In vitro studies have shown that H_2O_2 can be formed by the adventitious oxidation of redox enzymes by molecular oxygen. Many reactive oxygen species (ROS), including H_2O_2 , superoxide anion radical (O_2^-), and the highly reactive hydroxyl radicals ($\text{HO}^\bullet$), are generated continuously in cells grown under aerobic conditions. These ROS derive from sequential univalent reductions of molecular oxygen catalyzed by several membrane-associated respiratory chain enzymes.

It has been reported that flavins, quinones and metal centers of these respiration enzymes were all univalent electron carriers of sufficiently low redox potential to react with molecular oxygen to ROS. Unfortunately, there are presently limited data that firmly establishes the amount of H_2O_2 that is formed as a byproduct of metabolism. It was reported that some *E. coli* spp. generate H_2O_2 associated with their metabolic activity (Imlay, 2003). Exponential phase growing cells generated H_2O_2 efficiently, while stationary phase cells almost did not generate H_2O_2 . In another case, *Shewanella putrefaciens* str. 200 could generate and accumulate

extracellular H_2O_2 to approximately 1 mg L^{-1} under aerobic conditions (McKinzi and DiChristina, 1999).

It was observed that H_2O_2 was produced and accumulated in the extracellular culture medium at approximately 1.2 mg L^{-1} level when the aerobic granules were using TBA as sole carbon and energy source. In contrast, no H_2O_2 accumulation was detected in the control experiments in which no carbon source was added, as shown in Figure 5-1. In addition, there was no H_2O_2 concentration increase in the culture medium without aerobic granules. The initial H_2O_2 concentrations in the culture medium were probably caused by autoclave of the medium as the scenario. It can be postulated from the results that the H_2O_2 production and accumulation was a physiological function associated with metabolic activity of TBA-degrading bacteria residing in aerobic granules when they degraded TBA. The physiological and biochemical reasons for the extracellular H_2O_2 production and accumulation by these bacteria are still not very clear. Some reasons are discussed in the following section.

Instead of nonspecifically generated by respiration enzymes, H_2O_2 is also purposely produced by some microorganisms, although the physiological function has not been very clearly established. Some of the well documented H_2O_2 secretion bacteria includes *Enterococcus faecalis*, *Shewanella putrefaciens* str. 200, *Streptococcus pyogenes*, *Streptococcus sanguis*, *Streptococcus gordonii*, *Lactobacilli* spp. (Imlay, 2003; Huycke and Moore, 2002; McKinzi and DiChristina, 1999). The general pathway of these bacteria to generate H_2O_2 includes producing O_2^- first and then the O_2^- reacts with oxygen dismutase to generate H_2O_2 .

However, in contrast to H_2O_2 generation, iron-reduction may happen only in anaerobic conditions when some iron-reducing bacteria use iron as an electron acceptor. Very diverse iron-reducing bacteria have been isolated from different environments (Lovley, 1993). Some facultative bacteria can also use iron as electron acceptor when oxygen concentration is limited, for example *Shewanella putrefaciens*

(DiChristina and DeLong, 1994; DiChristina, 1992). Iron-reducing bacteria were found in activated sludge flocs as well as in some biofilm-based bioreactors operated in aerobic conditions (Nielsen et al. 2002). It has been found that strictly anaerobic bacteria survived in aerobic granules cultivated by glucose as carbon source (Tay et al. 2002c). In addition, aerobic, facultative, denitrifying bacteria have been found living in aerobic granules and they play important functional roles (Kim et al. 2004). Hence, it is not surprising that iron-reducing bacteria can thrive and survive in aerobic granules. Aerobic granules can reduce iron under both aerobic and anaerobic conditions, but with different profiles as mentioned above (Table 5-1).

Under aerobic condition, there was an obvious lag phase before ferrous iron concentration starting to increase. However, under anaerobic condition, ferrous iron concentration started to increase almost immediately although the initial rate was quite low. The difference reinforced the finding that iron-reducing bacteria not only survived but also played their functions in aerobic granules. In recent years, some natural occurred or bacteria synthesized compounds were found to function as electron shuttling compounds and to significantly contribute to the iron-reducing activity (Lovly et al. 1993; Newman and Kolter, 2000). These compounds, including quinones or containing quinone moieties, antibiotics, and cytochrome c, could serve as redox shuttle to transfer electrons to solid phase ferric iron surface and enhance the iron-reducing activity (Hernandez and Newman, 2001; Newman and Kolter, 2000).

Hydroxyl radical might be therefore generated as a result of interactions between different microorganisms residing in the aerobic granules, for example, iron-reducing bacteria and H_2O_2 secretion bacteria. This feature of aerobic granules can potentially be employed to degrade other recalcitrant xenobiotics. Polyvinyl alcohol was tested in this study. It was found that PVA was not degraded in control experiments where no TBA was supplied as carbon source. It means that the microbial consortium in aerobic granules cannot degrade and use PVA as carbon and

energy source.

When TBA was added as a carbon source, PVA was also degraded. Although the possibility that TBA induced some necessary enzymes to PVA biodegradation cannot be omitted, the result showed a high possibility that $\text{HO}^\bullet$ generated during TBA biodegradation and nonspecifically destroyed PVA. Some other results also indicated that $\text{HO}^\bullet$ was generated during TBA biodegradation and played an important role in destroying the TBA structure. For example, when DETAPAC was added to the culture medium, TBA degradation rate decreased approximately 23 percentage as compared to control experiments. DETAPAC could block ferrous iron to react with H_2O_2 and generate $\text{HO}^\bullet$. Hence, these results also support the hypothesis.

To date, only a single report has addressed the application of the bacterial driven Fenton reaction to degrade a recalcitrant compound – pentachlorophenol (McKinzi and DiChristina, 1999). In the study, an aerobic/anaerobic shifting operational strategy was used to achieve H_2O_2 generation and iron-reduction. It can be expected that microbial driven Fenton reaction could be employed in many aspects of wastewater treatment if such reaction can be achieved and controlled easily. Aerobic granulation system, as a novel biological water treatment system, is a suitable medium to achieve such microbial driven Fenton reaction. Because of the compact structure of aerobic granules, bacterial interactions may be easier to happen in these granules. Different functions of microorganisms may thrive and survive in aerobic granules and meanwhile play an important role to benefit metabolism and stable granular structure as found in phenol-degrading aerobic granules. In addition, both H_2O_2 generation and iron-reduction can be achieved at same time in a single reactor without complex operation strategies.

5.4.2 Summary

This study evaluated the possibility that TBA-degrading aerobic granules

generated $\text{HO}^\bullet$ and employed this active oxygen species to breakdown TBA. The results showed that H_2O_2 accumulated in the culture medium during the granules degrading TBA as sole carbon and energy source. The accumulated H_2O_2 concentrations were found to positively relate with granular sludge concentrations as well as dissolved oxygen concentrations. Ferric iron (Fe^{3+}) in culture medium could be reduced by granules to form ferrous iron (Fe^{2+}) even under aeration conditions. Both H_2O_2 generation and ferric iron (Fe^{3+}) reduction were functions related with metabolic activities because they were not detected from control experiments which were not supplied with carbon source.

In addition, cometabolic degradation of PVA and 2,4-DCP supported that $\text{HO}^\bullet$ was potentially generated by TBA-degrading aerobic granules. Aerobic granules driven Fenton reaction could be potentially employed to treat broad recalcitrant compounds. This will significantly expand the future application areas of this novel wastewater treatment biotechnology. For better understanding and controlling this special function, it is important to know whether the H_2O_2 generation is a special feature of TBA-degrading aerobic granules or a common character of granular type sludges. It may be important to exploit the particular favorite conditions for the hydroxyl radical generation by aerobic granules. These questions may be answered through investigating microbial populations residing in aerobic granules and the different populations' functions as well as interactions.

Chapter Six

Conclusions and Recommendations

6.1 Conclusions

As a novel biological wastewater treatment technology, aerobic granulation system has gained wide attention and extensive studies in the recent years. To date, almost all successful aerobic granulation processes were started from activated sludge as seeds. Compared with the conventional activated sludge flocs, the advantages of aerobic granules include denser and compact micro-structure, better settling capability, larger biomass retention in the reactor, higher volumetric biodegradation rate, and good resistance for high organic loading rate.

Using *tert*-butyl alcohol (TBA) as a model compound, this study demonstrates for the first time that aerobic granules can be cultivated by recalcitrant compounds. These aerobic granules can totally and efficiently remove the contaminants from wastewater. TBA was completely removed from the contaminated water by aerobic granules in sequencing batch reactors in the current study. The formation process of TBA-degrading aerobic granules, predominant microbial populations of the TBA-degrading granules, culturable TBA-degrading bacteria, and one of the TBA biodegradation mechanisms of aerobic granules have been systematically investigated through monitoring the reactors performances as well as employing traditional cultivation and advanced molecular microbiology methods.

Aerobic granules could be formed by using TBA as sole carbon and energy sources. However, properly designed enrichment and reactor operational conditions

have to be fulfilled in order to maintain sufficient biomass as well as supply necessary selection pressures for the granulation process. Two different cultivation strategies were successfully employed in the study to cultivate TBA-degrading granules.

Aerobic granules formed faster in Reactor R1 which had a short cycle time and lower start-of-cycle TBA concentrations. Granules also formed in Reactor R2 which had longer cycle time and higher start-of-cycle TBA concentrations, although the mean granular size and SVI of R2 granules were not as good as R1 granules. Nevertheless, aerobic granules in both Reactor R1 and R2 have good settling ability and relatively higher TBA removal rate compared with published data. Furthermore, aerobic granules could completely remove TBA from contaminated water to a non-detected level.

The study expanded the knowledge on the aerobic granules cultivation on recalcitrant and toxic compounds. In addition, it reinforced that selection pressures were one of the main driven forces in the aerobic granules formation. Finally, the results revealed that biological removal of TBA from contaminated water by the aerobic granulation system was feasible.

Molecular microbiology method (e.g. DGGE) was used to study the predominant microbial populations in the stable TBA-degrading aerobic granules. The results showed a relatively narrow microbial diversity with members only belonging to the α , β and δ subdivisions of Proteobacteria and the Cytophaga-Flavobacteria-Bacterioides (CFM) group. This is probably a result of the selection pressures exerted by the SBR operation or the specificity required for TBA biodegradation, which would favor the enrichment of certain bacterial species to the detriment of others.

Traditional culture based methods were employed to study the functional microbial strains from TBA-degrading aerobic granules. Only two microbial pure cultures, *Bacillus megaterium* str. TBA-R1-001 and *Methylobacterium organophilum*

str. TBA-R2-005, were successfully isolated from aerobic granules by using TBA as sole carbon and energy source. Although it is the first time that TBA was found to serve as sole carbon and energy source for the two microbial strains, the type cultures of *M. organophilum* and *B. megaterium* were actually isolated several decades ago. Apparently, these microorganisms could employ their enzyme systems to degrade TBA efficiently and use TBA as carbon source. In addition, the two species of microorganisms (*M. organophilum* and *B. megaterium*), especially the *M. organophilum* species, were found to be ubiquitous in the natural environments. The result shows that biological TBA contaminated wastewater treatment can be an alternative bioremediation strategy for the future application.

A non-enzyme direct attacking TBA biodegradation mechanism, which is achieved via microbial driven Fenton reaction, was proposed and evaluated in the study. The results provided evidence to support that microbial driven Fenton reaction was employed by TBA-degrading aerobic granules to break down TBA. The mechanism could be one of the possible explanations to the question of why culturable heterotrophic bacteria numbers in the TBA-degrading aerobic granules were significantly higher than the number of bacteria using TBA as sole carbon and energy source. It also explains that higher dissolved oxygen concentration as well as relatively high biomass concentration could enhance TBA removal efficiency because the two parameters both encouraged the microbial driven Fenton reaction. In addition, it is worth pointing out that hydroxyl radical generated via Fenton reaction is a very active oxygen species. It could nonspecifically react with almost all organic compounds. Hence, aerobic granules possessing such hydroxyl radical generation capabilities could be potentially employed to treat a broad range of recalcitrant compounds. It will significantly expand the future application areas of this novel wastewater treatment biotechnology.

Comprehensive TBA biodegradation studies were conducted in this study to understand the microbial transformation and biodegradation of TBA in SBR

bioreactors by aerobic granules. This study also serves to address the potential of biodegradation as a treatment alternative in the removal of TBA from contaminated wastewaters. TBA-degrading cultures have been developed and studied to the point that it can now be anticipated that carefully designed biological treatment systems can be a cost effective alternative technology to remove TBA to effluent concentrations that will meet regulatory requirements. The isolation of pure microbial cultures from the aerobic granules is helpful for understanding the biochemical and molecular biological aspect of TBA biodegradation. Non-enzyme direct attacking TBA biodegradation mechanism of aerobic granules enhances the knowledge of microbial interactions within a mixed culture biosystem and provides future application potential of the aerobic granulation system to degrade difficult compounds via microbial driven Fenton reactions.

6.2 Recommendations for Further Study

There are several areas where extensions to this work can be considered for further study.

To optimize the operational parameters for aerobic granule systems to treat TBA.

The aerobic granulation system could be used for TBA bioremediation; however, the start-up times of several months were excessively long for an engineering application. It is important to understand how to quickly start-up an aerobic granule system so that such systems could be efficiently employed in a real application in the future. Crucial parameters, such as cycle time, seed sludge characteristics, and substrate feeding strategies, should be addressed to achieve the goal. In addition, temperature is also a very crucial parameter which will significantly affect the microbial activity. Optimizing the aerobic granules system lower than 15 °C could be considered because the groundwater temperatures are normally at this range or even lower.

To discover the enzyme system of the two TBA-degrading pure microbial cultures employed to attack TBA as substrate.

To date, the enzymology of TBA biodegradation is still not fully understood. With the two pure TBA-degrading bacteria isolated from this study, it is possible to study the TBA-degrading enzyme systems in more detail. It will be indeed important to understand what enzyme that the bacteria used to attack TBA, and how the bacteria regulated their enzyme systems to use TBA as sole carbon and energy source. Most importantly, whether the gene encoding the enzyme can be self-transferred into different types of bacteria? If the information can be obtained, bioremediation strategies will be properly designed for both *in situ* and *ex situ* TBA removal, and the TBA biodegradation efficiency could be greatly enhanced.

To discover the microbial driven Fenton reaction of aerobic granules cultivated by other carbon source.

It is interesting to know whether the microbial driven Fenton reaction is a specific degradation mechanism for TBA or a common feature of aerobic granules. Other types of aerobic granules cultivated by, for example, acetate should be examined for Fenton reactions. If it is a common feature of aerobic granules, why and how the microbial populations in aerobic granules process such Fenton reaction needs to be understood. In addition, it will be useful to optimize the Fenton reaction within the aerobic granule to enhance the degradation efficiency as well as protect microbial cells from the damage of the Fenton reactions.

To evaluate the broader applicability of aerobic granule systems to degrade emerging environmental contaminants.

Globalization and associated increases in human, along with advancements in measuring and monitoring techniques, are among reasons emerging contaminants have become subject to focus in recent years. The emerging environmental contaminants (EECs) could be a challenging issue for aerobic granular systems applicability. EECs are sometimes very recalcitrant xenobiotics and present in environmental samples at very low concentrations (from mg L^{-1} to $\mu\text{g L}^{-1}$ level). To effectively remove the EECs as well as maintain aerobic granular structure and function could be a main objective of research studies. The operational conditions and bioreactor configurations may be significantly different compared with current settings in this study.

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