University of Alberta

DESULFURIZATION OF OXIDIZED BITUMEN USING MICROORGANISMS THROUGH PROCESS-BASED DIRECTED EVOLUTION

by

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Abstract

Many *Rhodococcus* species are capable of utilizing the 4S biodesulfurization pathway to convert dibenzothiophene (DBT) to 2-hydroxybiphenyl (2-HBP) and sulfite, the latter of which is partially incorporated into cell biomass. The first steps of the 4S pathway involve the oxidation of DBT into $DBTO_2$ for further conversion. It is hypothesized that using $DBTO_2$ as the direct substrate for desulfurization would result in faster growth rates and increased desulfurization activity. Process-based directed evolution strategies were performed, using $DBTO_2$ as a selective pressure, with three *Rhodococcus* species to adapt the cultures and select for mutants with increased desulfurization activity. Process-based directed evolution was performed through two strategies: (i) sequential transfers and (ii) self-cycling fermentation (SCF). A change in desulfurization activity for *Rhodococcus rhodochrous* IGTS8 was observed and indicated a change in sulfur management in the cell. *Rhodococcus erythropolis* EPWF developed a gain-of-function mutation that enabled it to desulfurize $DBTO_2$.

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Hope and Faith

Never give up

Ask for what you need

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List of Abbreviations

2-HBP	2-hydroxybiphenyl
C_{18}	Octadecane
DBT	Dibenzothiophene
DBTO	Dibenzothiophene sulfoxide
$DBTO_2$	Dibenzothiophene sulfone
DMFA	Dimethylformamide
HPBS	2-hydroxybiphenyl 2-sulfinic acid
MSM	Minimal salt media
NB	Nutrient Broth
SCF	Self-cycling fermentation
SFMM	Sulfate-free mineral media
TSB	Tryptic Soy Broth

1. CHAPTER 1 – INTRODUCTION TO DESULFURIZATION OF OXIDIZED BITUMEN USING MICROORGANISMS THROUGH PROCESS-BASED DIRECTED EVOLUTION

1.1 Background

The combustion of sulfur compounds in fossil fuels releases sulfur oxides to the air, which can have adverse effects on public health and the environment. Sulfur dioxide (SO_2) is the predominant form of sulfur oxide found in the atmosphere. Water present in the atmosphere rapidly converts sulfur trioxide and sulfur dioxide into sulfuric acid (H_2SO_4) and sulfurous acid (H_2SO_3), respectively. Both sulfuric acid and sulfurous acid contribute to acid rain. The World Bank Group (1999) discussed many of the adverse health, environmental and economic effects that sulfur dioxide pollution and acid rain can have. Public exposure to sulfur dioxide in the air can reduce lung function, increase respiratory symptoms and diseases, cause eye, nose and throat irritation and decrease life expectancy. Sulfur oxide emissions can also have adverse effects on vegetation. Plants exposed to high concentrations of sulfur dioxide have been shown to become less productive, lose their foliage and even die prematurely. Sulfurous and sulfuric acid can change the pH of aquatic environments, alter forest ecosystems, and degrade building materials. Finally, some of the sulfur dioxide in the atmosphere is converted to sulfate aerosols. Sulfate aerosols can affect pulmonary, respiratory and sensory functions. Sulfate aerosols also scatter light as it tries to pass through the atmosphere. This can lead to reduced visibility and haziness as well as global cooling. Furthermore, sulfate aerosols can also travel long distances from the source of the sulfur oxide release, highlighting the need for global regulations on sulfur oxide emissions.

Sulfur content in fossil fuels varies from 0.03 to 7.89wt% (Soleimani et al., 2007). Many countries have regulations on allowable sulfur content in fuels as a measure to reduce sulfur oxide emissions. Hydrodesulfurization is the main method utilized to remove sulfur from fuels. It utilizes hydrogen at high temperatures and pressures to remove the sulfur from the fuel compounds and produce hydrogen sulfide gas. Hydrodesulfurization has many environmental

disadvantages itself. For instance, the high pressures and temperatures required make it an energy demanding process. In addition, hydrogen sulfide gas is poisonous, corrosive, flammable and explosive. It is also heavier than air making it a bigger threat to public health. However the main disadvantage of hydrodesulfurization is that it is only so effective at removing sulfur from fuel as some sulfur containing compounds are recalcitrant to this process.

Bitumen, heavy crudes and residual oils have a higher percentage of sulfur than light crudes; they also contain a greater concentration of compounds recalcitrant to hydrodesulfurization. Therefore, these fuels have been underutilized as resources. However, as reserves of low sulfur-content crudes are being depleted, attention is turning towards the remaining high sulfur-content reserves. In order to reduce the sulfur content of these heavy fuels, more effective methods for desulfurization are required.

Biodesulfurization is a promising alternative to hydrodesulfurization. Biodesulfurization utilizes bacterial cells to remove sulfur from fuel and incorporate it to their biomass as it is required for cell growth. Biodesulfurization occurs under atmospheric pressure and room temperature making it a process with low energetic demand. However, energy input would still be required for mixing of the system. The byproducts of biodesulfurization are biomass and sulfate, both of which would need to be separated from the fuel. Many studies have looked at the feasibility of the biodesulfurization process and the main challenge remains the fairly low desulfurization activity of bacterial strains. Many genetic engineering and some directed evolution studies have been conducted to try and develop mutant strains with increased desulfurization activities. The difficulties often encountered in these studies is that they often results in point mutations or single enzyme modification (one genetic change in the cell) and it is difficult to know the impact that this change will have on the cell as a whole. A beneficial improvement to one step in desulfurization may not lead to a general improvement of desulfurization as a whole. Additionally, many of these changes will not be expressed under ideal process conditions. This leads to numerous mutants that do not exhibit desired growth or desulfurization activities. Process-

based directed evolution strategies, such as chemostat, have been utilized along with a selective pressure to promote an overall cell mutation that can thrive at the desired process conditions. In the present study, the use of sequential batches and self-cycling fermentations as drivers for adaptation of different species of *Rhodococcus* are investigated.

1.2 Objectives and scope of work

The objective of this study was to use process-based directed evolution to improve the biodesulfurization of dibenzothiophene sulfone ($DBTO_2$) using *Rhodococcus* species. Two process-based directed evolution strategies were utilized: (i) sequential transfers and (ii) self-cycling fermentation (SCF).

Hypothesis:

Using $DBTO_2$ as a substrate for the 4S pathway, instead of dibenzothiophene (DBT), will result in faster growth rates and increased desulfurization activity. The desulfurization activity can also be improved by utilizing process-based directed evolution strategies with $DBTO_2$ as a sole sulfur source. Process-based directed evolution in sequential transfers and self-cycling fermentation will promote an overall cell mutation with increased desulfurizationy.

Scope of work:

Three *Rhodococcus* species were utilized in this study; *Rhodococcus rhodochrous* IGTS8 (ATCC 53968), *Rhodococcus* K1bD, and *Rhodococcus erythropolis* EPWF. The first two species are known to desulfurize DBT through the 4S pathway, while the latter only desulfurizes aliphatic compounds.

Sequential transfers were conducted in sulfate-free mineral medium (SFMM) and mineral salt medium (MSM) for all three cultures with $DBTO_2$ added as a sulfur source (selective pressure). *R. rhodochrous* IGTS8 was also cycled in the SCF with SFMM and $DBTO_2$ added as the sulfur source (selective

pressure). Comparative analysis of desulfurization activity was performed for cultures exposed to $DBTO_2$ for different numbers of generations.

2. CHAPTER 2 – LITERATURE REVIEW ON DESULFURIZATION OF OXIDIZED BITUMEN USING MICROORGANISMS THROUGH PROCESS-BASED DIRECTED EVOLUTION

2.1 Introduction

Fossil fuel combustion releases sulfur oxides that pose a threat to the environment through air pollution and acid rain, as well as threaten public health and economy. Acid rain accelerates the deterioration of historical buildings, causes soil erosion and drastically lowers the pH of marine environments, endangering species (Soleimani et al., 2007). Sulfur oxides also form sulfate aerosols that can be transported into the human body and lead to respiratory and sensory illnesses (World Bank Group et al., 1999). As is the case with air pollution, these compounds are transported by air streams, which means that adverse effects can be observed thousands of kilometers away from the source of sulfur oxide pollution. International co-operation to reduce sulfur oxide emissions in the environment has been the focus of several agreements since 1979 (Soleimani et al., 2007).

2.2 Desulfurization of bitumen

2.2.1 Sulfur in bitumen

Most sulfur oxides are produced by burning fuel that contains sulfur (World Bank Group et al., 1999). In a desire to reduce these sulfur emissions more stringent regulations on sulfur content in fuels are being imposed. There is also an economic benefit to removing sulfur from fuels; as the sulfur content decreases, the value of the fuel increases. After carbon and hydrogen, sulfur is the third most abundant element in petroleum (Kropp and Fedorak, 1998; Ma, 2010; Soleimani et al., 2007) and is present in two main forms: inorganic and organic (Soleimani et al., 2007). Its average concentration in crude oil can vary from 0.03 wt.% to 7.89 wt.%. In general, crude oils of higher density, such as bitumen, contain a higher percent of sulfur (Gray, 2010; Kropp and Fedorak, 1998).

Bitumen, heavy crudes, and residual oils are underutilized as fuel resources due to their high viscosity and sulfur contents (3-6 wt.%); because of this, conventional hydrodesulfurization (HDS) processes are not cost effective in the upgrading of heavy oils (Shennan, 1996). Inorganic sulfur compounds such as elemental sulfur and hydrogen sulfide that are present in most crude oils are not present in bitumen as it has undergone extensive bacterial attack over thousands of years (Gray, 2010; Shennan, 1996). Sulfur occurs in two major forms in bitumen: organic sulfides (38%) and thiophenes (62%) (Gray, 2010). The most common form of condensed thiophenes are dibenzothiophene (DBT), benzothiophene (BT), and their alkylated derivatives (shown in **Figure 2-1**) (Kropp and Fedorak, 1998). These condensed thiophenes have elevated boiling points (*e.g.* 332ºC for dibenzothiophene) compared to the sulfides making it difficult to remove these compounds. This represents a significant barrier, as regulations on fuels are increasingly strict on allowed levels of sulfur. Additionally the average sulfur content in the remaining global crude oil is increasing; future refineries are going to have to process heavier sour crude while facing increased pressure to reduce sulfoxide and carbon dioxide emissions (Kilbane, 2006).

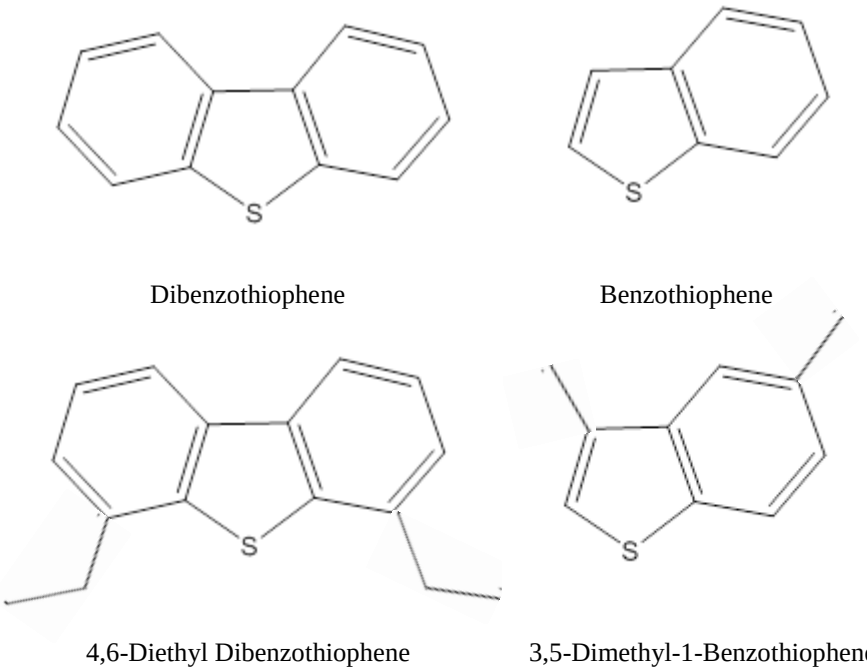

Figure 2-1 Dibenzothiophene (DBT), benzothiophene (BT) and an alkylated derivative of each

2.2.2 Current technologies for desulfurization

Current technologies of desulfurization, such as hydrodesulfurization (HDS) and desulfurization by ionic liquids, are only so effective at removing sulfur from the fuel.

HDS involves the reaction of the fuel and hydrogen in the presence of a catalyst (cobalt and molybdenum or nickel and molybdenum on an alumina base (Soleimani et al., 2007)) at elevated temperature and pressure to produce hydrogen sulfide and a reduced sulfur product (Gray et al., 1996). This method allows the removal of simple organic compounds such as aliphatic and alicyclic sulfur compounds in fuels, but the treatment of more complex organic sulfur compounds such as BT and DBT derivatives is a difficult task (Francisco et al., 2010). HDS occurs at temperatures ranging from 200-425ºC and pressures between 150-250 psi to achieve a minimum sulfur concentration of 15mg/kg

(Soleimani et al., 2007). The main disadvantages of HDS, as pointed out by Soleimani et al. (2007), are: (i) to desulfurize refractory sulfur compounds higher operating temperatures and pressures at longer residence times are required – leading to costly operation; (ii) many alkylated thiophenic compounds are considered to be recalcitrant to HDS; (iii) the addition of hydrogen in HDS can also result in the hydrogenation of olefins which reduces the value of the fuel – the fuel is therefore sent to a fluid catalytic cracking unit after HDS to increase its value; (iv) the increasing regulation on fuel sulfur content makes HDS a less viable option as the cost of the operation increases rapidly when trying to achieve lower sulfur concentrations; (v) the sulfur concentration in the remaining crude oil reserves is greater than in historically exploited reserves, making HDS a less viable option for future exploitation and treatment.

Desulfurization by ionic liquids involves the use of liquid organic salts to selectively extract sulfur compounds from fuels. It is seen as a clean and cheap process with mild operating conditions for extractive desulfurization. However the ionic liquid must have high capacity, a high solute distribution ratio and high selectivity (Francisco et al., 2010). Soleimani et al. (2007) also discuss a few disadvantages to ionic liquid desulfurization: (i) selection of ions is important as some ions can cause alkene polymerization in fuels; (ii) size of the ions can have a large impact on the selectivity of the liquid; (iii) solvent loss is inevitable as the recovery and recycling of ionic liquids is difficult.

2.3 Biodesulfurization of bitumen

Biodesulfurization is seen as an additional desulfurization technology that can be used alongside HDS or independently to remove sulfur from fuels. Biodesulfurization can remove sulfur from refractory organosulfur compounds like DBTs and BTs using microorganisms that can remove the sulfur to utilize it through their metabolism (Ma, 2010). Sulfur is required for cell growth and typically corresponds to 0.5-1% of the cell dry weight (Soleimani et al., 2007). Biodesulfurization requires lower capital cost as it operates at mild conditions of

atmospheric temperature and pressure and does not require molecular hydrogen (Alves and Paixão, 2011). It also produces far less carbon dioxide than HDS (Kilbane, 2006). Additionally, biocatalysts are often superior to chemical catalysts due to their high selectivity, and activity levels at ambient temperatures and mild pH values (Bornscheuer, 2001). However biodesulfurization also has several disadvantages (Soleimani et al., 2007); (i) some microorganisms utilize the carbon atoms in fuel as well as the sulfur atoms therefore reducing the value of the fuel; (ii) some compounds present in fuel and even some compounds produced by the microorganisms through desulfurization can be toxic to the cells and inhibit biodesulfurization; (iii) the desulfurization activity of wild type cells is not high enough to meet the requirements for the process to meet economic feasibility, therefore a recombinant cell with higher desulfurization activity needs to be developed; (iv) it has been proposed that a two-phase reactor would be needed to supply the microorganisms with water required for growth, however the size of reactor needed to allow sufficient phase contact is unimaginable.

Most studies suggest including biodesulfurization after HDS, however an alternative process could have biodesulfurization occur before HDS (Kilbane, 2006). This would allow the removal of most organosulfur compounds that are refractory to HDS before the HDS process making it more efficient. Many desulfurizing microorganisms – including *Rhodococci* (Folsom et al., 1999; Gallagher et al., 1993; Kilbane, 1990 b; Li et al., 2007; Ohshiro et al., 1996), *Microbacteria* (Li et al., 2005; Papizadeh et al., 2009), *Stenotrophomonads* (Papizadeh et al., 2011), *Pseudomonads* (Tang et al., 2012) and *Arthrobacters* (Seo et al., 2006) – have been studied for biodesulfurization. The desulfurization ability of some yeast (*Hansenula* spp.) and fungi (*Cunnighamella elegans*) has also been investigated (Crawford and Gupta, 1990; Stevens and Burgess, 1989).

2.3.1 Types of biodesulfurization

There are three main processes for biodesulfurization: destructive, anaerobic, and specific oxidative.

Destructive biodesulfurization utilizes both carbon and sulfur atoms from the fuel for cell metabolism. This undesirable breakage of carbon-carbon bonds deems this process destructive. Destructive biodesulfurization is useful in the biodegradation of environmental contaminants but, in the case of desulfurization of fuels, it results in a decrease in energy content and value of the fuel (Gallagher et al., 1993). Additionally, some aromatic sulfur-containing compounds are degraded into water-soluble products that exit the oil phase, also affecting the value of the fuel (Shennan, 1996). Furthermore, the target of most enzymatic attacks on sulfur-containing aromatic compounds (*e.g.* DBT) is not the thiophenic group but the aromatic ring itself (Kilbane, 1990 a). Therefore other aromatic compounds that do not contain sulfur can also be targeted, rendering this process non-specific for sulfur removal (Soleimani et al., 2007).

The microbial oxidative and destructive pathway for desulfurization of DBT is known as the Kodama pathway (Gallagher et al., 1993) (shown in **Figure 2-2**). The major end product of the Kodama pathway is 3-hydroxy-2-formyl-benzothiophene (HFBT), a sulfur-containing compound. In this case, sulfur is only metabolized by the microorganisms if DBT is completely degraded (Kilbane, 1990 a).

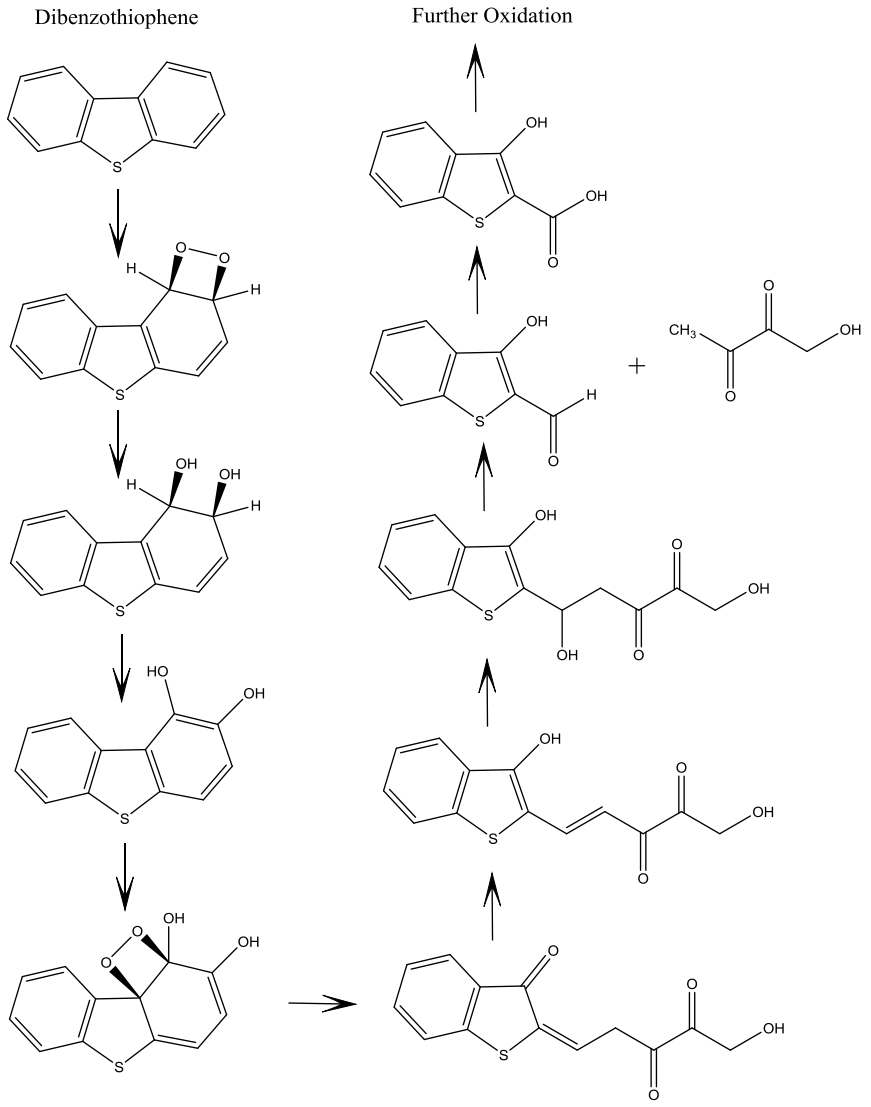

Figure 2-2 Kodama pathway for the biodesulfurization of dibenzothiophene (DBT) (figure adapted from Soleimani et al. (2007))

Specific oxidative desulfurization on the other hand involves a direct enzymatic attack on the sulfur-containing function. In the instance of DBT, the enzymatic attack occurs directly on the carbon-sulfur bond in the thiophenic ring of the molecule and not on the aromatic ring, as found in destructive desulfurization (Kilbane, 1990 a). The specific oxidative desulfurization pathway for DBT is commonly referred to as the 4S pathway and is shown in **Figure 2-3**. The end product of the 4S pathway, 2-hydroxybiphenyl (2-HBP), partitions back

into the oil phase preserving the fuel value (Ma, 2010). The sulfur removed from the DBT is made available for microbial utilization in the form of sulfite.

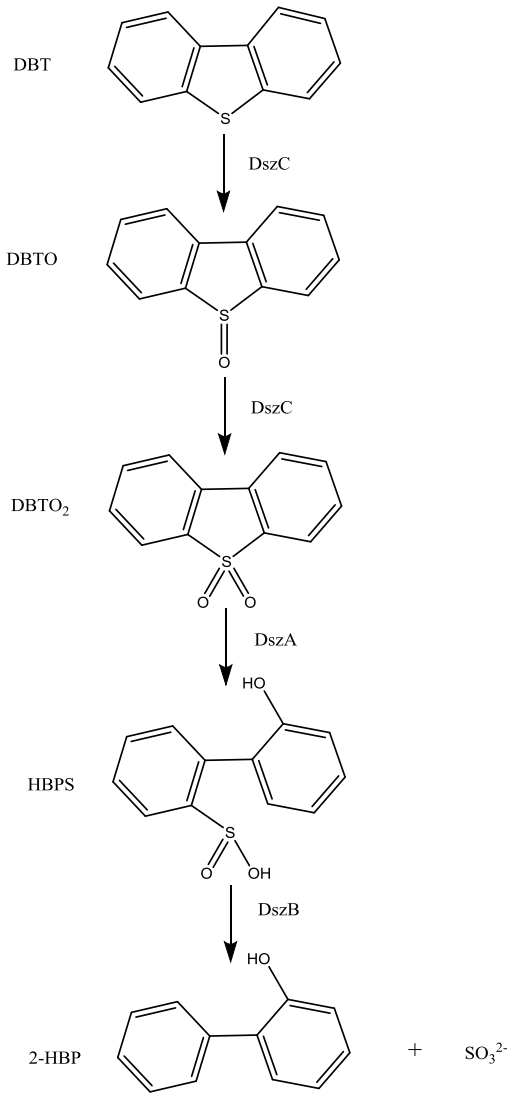

Figure 2-3 4S pathway for the desulfurization of DBT (adapted from Soleimani et al. (2007))

Anaerobic biodesulfurization is another process utilized for the desulfurization of fuels. However, anaerobic conditions are difficult to maintain and the desulfurization activity of the majority of isolated anaerobic strains has been reported to be insignificant (Armstrong et al., 1995). Additionally, a

reductant, such as hydrogen, would need to be supplied to the system and industrial safety measures would need to be in place for the capture and removal of byproducts such as H_2S. Despite these downsides, the oxidation of hydrocarbons to undesirable coloured gum-forming products under anaerobic conditions is minimal; an incentive for further research in this area (McFarland, 1999).

2.3.2 Biodesulfurization with *Rhodococcus*

The genus *Rhodococcus* has been found to successfully desulfurize DBT (a widely accepted model thiophenic compound) using the selective oxidative 4S pathway and has been the subject of many biodesulfurization improvement studies (Etemadifar et al., 2008; Li et al., 1996, 2007, 2008). *Rhodococcus* species have been found to desulfurize a wide range of sulfur-containing compounds and to exhibit deep desulfurization activity (Ma, 2010).

The genus *Rhodococcus* belongs to the phylum and class Actinobacteria, the order Actinomycetales and the family Nocardiaceae (Ma, 2010). *Rhodococci* have a great capability to remediate environmental contaminants as they possess a variety of plasmids (Kayser, 2002). *Rhodococci* are gram positive bacteria with inducible mycolic acid chains in their cell wall; these mycolic acid chains can allow *Rhodococci* to attach to the oil water interface of an aqueous-hydrocarbon system making them ideal candidates for the desulfurization of fuels. (Neu, 1996). Additionally, the DszC enzyme expressed by *Rhodococcus* and utilized in the 4S biodesulfurization pathway has a higher specific reaction rate for alkylated DBTs (Arensdorf et al., 2002) than DszC from other genera. This has further increased the interest in utilizing *Rhodococci* for the desulfurization of fuels.

2.3.3 *Rhodococcus* species utilized for biodesulfurization

Among the many *Rhodococcus* strains, *Rhodococcus rhodochrous* IGTS8 has been studied most extensively for biodesulfurization. *R. rhodochrous* IGTS8 is a Gram-positive rod-shaped bacterium approximately 0.5 µm long (Ma, 2010). *R. rhodochrous* IGTS8 was isolated by Kilbane (1990 b) and Energy Biosystems Corp. (EBC) for use in commercial microbial desulfurization. Having failed to find a naturally occurring organism with sulfur-specific metabolic activities, Kilbane (1990 b) grew a mixed bacterial culture (IGTS7) in a sulfur-limited continuous culture bioreactor subjected to a chemical mutagen. Two bacteria were isolated with strong desulfurization activities, *R. rhodochrous* IGTS8 and *Bacillus sphaericus* IGTS9; however other organisms that did not possess desulfurization activities were still able to grow. This indicated that the sulfur liberated from DBT by the desulfurizing organisms was made available to all bacteria present. One of the primary concerns that Kilbane had, was the stability of the desulfurization trait in *R. rhodochrous* IGTS8; previously isolated bacteria capable of desulfurizing organosulfur compounds lost their activity over time and could not be re-isolated. Kilbane (1990 b) grew *R. rhodochrous* IGTS8 on non-selective agar and in growth media under physiological stresses and found that the desulfurization trait was stable. Kilbane (1990 b) also found that the desulfurization trait in IGTS8 was inducible; some sulfur starvation or stress was required to observe the desulfurization trait.

Rhodococcus K1bD and *Rhodococcus erythropolis* EPWF have also been utilized in desulfurization studies (Kirkwood et al., 2005). *R. erythropolis EPWF* has been shown to oxidize aliphatic sulfides (like dibenzyl sulfide) but not dibenzothiophene (Kirkwood et al., 2005), making it an interesting candidate for evolution strategies. However, *R.* K1bD shows selective preference to degrading dibenzothiophene over simpler heterocyclic compounds (Kirkwood et al., 2005), which would be advantageous in the commercialization of the biodesulfurization process.

2.3.4 Biodesulfurization of DBT and $DBTO_2$ using the 4S pathway

The desulfurization of DBT using the 4S pathway (**Figure 2-3**) involves the consecutive oxidation of DBT to dibenzothiophene sulfoxide (DBTO), dibenzothiophene sulfone ($DBTO_2$) and finally 2-hydroxybiphenyl 2-sulfinic acid (HBPS). HBPS is then converted to 2-hydroxybiphenyl (2-HBP) and sulfite. This requires the use of four enzymes (Gray et al., 2003): (i) the oxidation of DBT into DBTO and then to $DBTO_2$ is catalyzed by the DBT monooxygenase DszC; (ii) the conversion of $DBTO_2$ to HBPS is catalyzed by the DBT-sulfone monooxygenase DszA; (iii) HBPS is catalyzed by the HBPS desulfinase DszB resulting in 2-HBP and sulfite; (iv) and the fourth enzyme is a flavin reductase enzyme (DszD) that is required to provide the reducing equivalents that are necessary to activate the molecular oxygen required by the flavin-dependent DszC and the DszA enzymes. The presence of DBTO is difficult to detect because it is readily converted to $DBTO_2$ through oxidation; in fact, the first oxidation step of DBT to DBTO is ten times slower than the second step, DBTO to $DBTO_2$ (Ma, 2010). DszA only oxidizes $DBTO_2$ and does not act on DBT or HBPS; however it has been shown to oxidize other sultones and sultines to form dihydroxybiphenyl (Ohshiro and Izumi, 2000). Isotopic labeling studies have shown that the oxygen utilized by the monoxygenase enzymes comes from molecular oxygen, indicating that an aerobic environment is required for the desulfurization of DBT through the 4S pathway (Ma, 2010). DszB can specifically cleave the carbon–sulfur bond of HBPS resulting in 2-HBP and a sulfite ion (Ma, 2010). Since the activity of DszB is the lowest among the enzymes utilized in the 4S pathway, this step is considered rate-limiting (Ma, 2010). In order to render microbial desulfurization feasible on an industrial scale, it is necessary to improve the activity of DszB. It has also been suggested that the overexpression of DszD, the flavin-reductase enzyme, can increase the overall rate of desulfurization (Gray et al., 2003). These improvements could be accomplished by utilizing genetic engineering strategies or directed evolution.

Experimental studies have also tested the relevance of using preliminary oxidizing steps for the desulfurization of DBT and starting the microbial desulfurization pathway at $DBTO_2$. $DBTO_2$ is more bioavailable to cells – it has a higher solubility (5.8mg/L) compared to DBT (1.0mg/L) at 25ºC (Seymour et al., 1997) – and is further down the 4S desulfurization pathway. This makes it an easier compound for bacteria to utilize as it requires fewer enzymes and cofactors for the biodesulfurization process. Experimental studies have tested the desulfurization results for growth with $DBTO_2$ versus DBT. Ohshiro et al. (1996) grew *R. erythropolis D-1* in cultures with DBT and with $DBTO_2$. They found that the cultures grown with DBT grew to a higher specific activity than those growing in the presence of $DBTO_2$. However, the cultures containing DBT reached a lower optical density value than the cultures containing $DBTO_2$. Additionally, Gallagher et al. (1993) found that growth was approximately the same with DBT and $DBTO_2$. Setti et al. (1994) and Wang and Krawiec (1996) both observed increased growth rates and increased desulfurization activity in *R. erythropolis* N1-36 and *Pseudomonas* sp., respectively, when grown in $DBTO_2$ versus DBT. They also observed an increased rate of production for 2-HBP with a greater final cell yield for growth with $DBTO_2$

2.3.5 Inhibitory and toxic compounds to biodesulfurization

Condensed thiophenes and their degradation products can have inhibitory effects on the biodegradation process or display toxicity towards the microorganisms used (Kropp and Fedorak, 1998). For example, Ohshiro et al. (1996) found that DBT concentrations higher than 3mM repressed the desulfurization activity of *R. erythropolis* D1. It is thus important to gain an understanding of the toxic or inhibitory limits of the organisms so that the biocatalysts can be improved and function at their optimum desulfurizing potential. Condensed thiophenes themselves can be toxic to the cells and are considered a major limitation to reaching high levels of biodesulfurization. Finding or engineering an organism capable of withstanding these toxic

concentrations is required for the implementation of biodesulfurization as an industrial process (Alves and Paixão, 2011).

The end products of the 4S pathway, 2-HBP and sulfite can also exhibit toxic and inhibitory effects on the cells and the degradation pathway. Ohshiro et al. (1996) showed that the presence of 2-HBP at 0.5mM inhibited 50% of the bacterial growth of *R. erythropolis* D1. They also found that the inhibitory effects of 2-HBP were only observed in cultures that had DBT as the sole sulfur source; cultures grown in the presence of 2-HBP with sulfate as the sole sulfur source did not experience significant inhibitory effects. This suggested that 2-HBP was inhibitory specifically to the 4S pathway. Additionally, Ohshiro et al. (1996) found that biphenyl was not inhibitory to the 4S pathway, indicating that the hydroxyl group on 2-HBP must be responsible for the inhibitory effects. As a result of high levels of biodesulfurization of DBT, high concentrations of 2-HBP will be formed. An effective biocatalyst must thus be able to maintain its desulfurization activity at greater 2-HBP concentrations.

Sulfite has also been shown to be inhibitory to cells (Kappler and Dahl, 2001). As it is an intermediate in the 4S pathway its accumulation in the cell is entirely possible (Aggarwal et al., 2012). It is still unclear how the cell utilizes and manages the accumulation of sulfite in the cell. However this is a key piece of information that, if understood, could provide further information on how to develop a biocatalyst with optimized desulfurization ability.

Sulfate has also been shown to accumulate in cultures that desulfurize DBT. This accumulation can also inhibit the 4S pathway (Gallagher et al., 1993; Yoshikawa et al., 2002). 5.0mM sodium sulfate added to a *Rhodococcus* culture containing DBT completely inhibited the 4S pathway so that no enzymatic activity was observed (Ohshiro et al., 1996). The presence of sodium sulfate at concentrations as low as 1.0mM was shown to inhibit the desulfurization of DBT by 72% compared to situations in which no sulfate was present (Ohshiro et al., 1996). However it has also been suggested that small concentrations of sulfate do not inhibit desulfurization, but may actually be required to improve desulfurization activity (Aggarwal et al., 2012; Kilbane, 1990 b). Figuring out

how the cells produce sulfate and the effect that sulfate has on the desulfurization pathway is another key aspect to developing a suitable biocatalyst.

2.3.6 Sulfur management after the 4S pathway

Aggarwal et al. (2012) proposed an addition to the 4S pathway that addresses the fate of the sulfite released from the desulfurization of DBT. They introduced two enzymes – sulfite reductase (SR) and sulfite oxidoreductase (SOR) –responsible for maintaining the balance of sulfite, sulfate and sulfide in the system. Aggarwal et al. (2012) proposed two possible hypotheses for how the cell manages its sulfur. One hypothesis proposed that SR would be utilized by the cell to convert sulfite into sulfide for biomass production. In this case, SOR would convert excess sulfite into sulfate to avoid any toxic effects associated with the accumulation of the former. The second hypothesis proposed that the cell would be unable to directly convert sulfite into sulfide by SR without first producing sulfate through SOR. This sulfate would then be recaptured by the cell and converted back into sulfite and then into sulfide via SR activity. **Figure 2-4** illustrates the functions of the SR and SOR enzymes as proposed by Aggarwal et al. (2012).

Using results from a previous study (Omori et al., 1995) along with a flux-based analysis, Aggarwal et al. developed a few conclusions on how changes in the SR and SOR can improve desulfurization activity: (i) SOR limits the desulfurization activity – genetic engineering to improve the copy number of SOR could be a solution; (ii) the production of sulfate by SOR also needs to be addressed; (iii) high concentration of sulfate produced through this scheme could have toxic effects on the cells – therefore sulfate would need to be continually removed from the system; and (iv) an increase in the expression of SOR along with a decrease in SR expression would starve the cells for sulfur, increasing the rate of desulfurization –however the balance of these activities would need to be determined. These improvements in the sulfur management of cells should be the focus of future genetic engineering or other improvement strategies, in part to

gain a full understanding of the sulfur management in the cells, and in part to develop a biocatalyst with increased desulfurization activity.

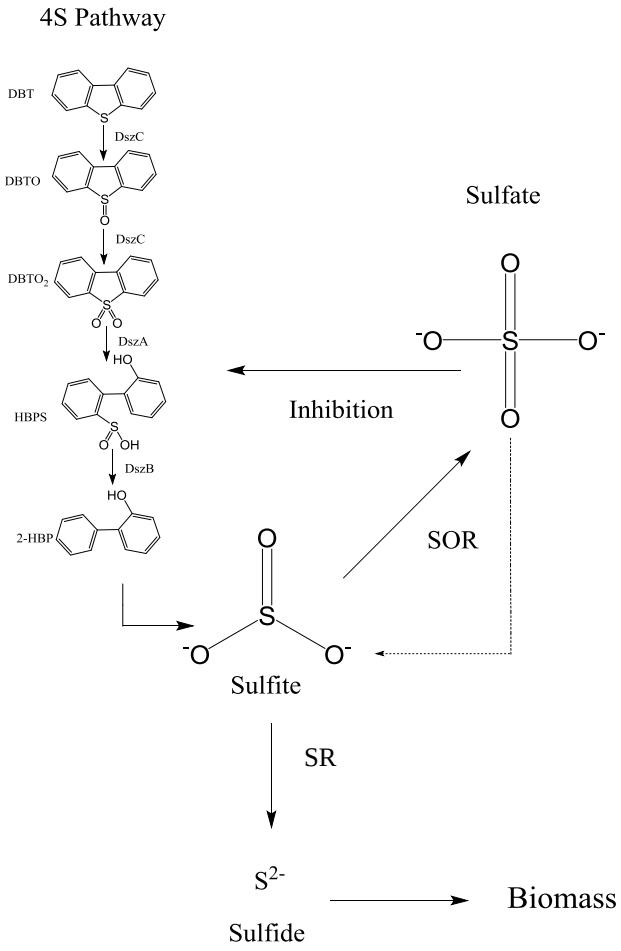

Figure 2-4 Additions to the 4S pathway as discussed by Aggarwal et al. (2012)

2.4 Improvement of biodesulfurization

2.4.1 Areas for biocatalyst improvement

Shennan (1996) discusses two disadvantages of biocatalysts that must be addressed in order for biodesulfurization to become a feasible process: (i) wild type cells only require 0.5-1% of elemental sulfur in their biomass, an excessive amount of cells would thus be required to sufficiently desulfurize the fuel; and (ii)

in order to account for the ions released through desulfurization, the media must have an appropriate buffering potential and inhibitory compounds must be removed. A successful biocatalyst would require a desulfurization activity ranging from 1.2 mmol DBT/g DCW/h to 3 mmol DBT/g DCW/h, where DCW refers to dry cell weight (Kilbane, 2006). Kilbane and Le Borgne (2004) demonstrated that *R. rhodochrous* IGTS8 has a desulfurization activity of only 72 µmol DBT/g DCW/h. To improve biocatalysis, the sulfur concentration required by the cell needs to be increased, or the way the cell manages its sulfur needs to be altered. Additionally, biocatalysts need to be developed to withstand higher concentrations of inhibitory compounds, as the removal of these compounds from the fuel is expensive and often considered not a viable option.

Furthermore, the substrate specificity of the biocatalyst should be broadened. Not only should the biocatalyst be able to remove sulfur from alkylated thiophenes, but it would be highly advantageous if it could remove nitrogen and metals from thiophenic compounds as well. Currently DszC shows oxidation activity on derivatives of DBT, but has not shown any activity on carbazole or dibenzofuran which have the same structure as DBT with the sulfur atom substituted by nitrogen and oxygen respectively (Ma, 2010). If the biocatalyst could be improved to remove other problematic compounds, like nitrogen and metals, the prospects of combining biodesulfurization with other treatments would render the process more viable.

Lastly, to further increase the large scale industrial feasibility of the biodesulfurization process, it might be advantageous to use a thermophilic bacteria (Gray et al, 2003). Gray et al. (2003) discussed some of the advantages that utilizing a thermophilic bacteria may have in an industrial setting: (i) improved enzymatic rates, (ii) decrease in contamination by other bacteria, and (iii) increased stability of the biocatalyst.

There are two main ways to improve biocatalyst; genetic modifications and directed evolution. Genetic modifications are typically site specific and are often unsuccessful due to the lack of information that specific changes in the cell can have on structural and mechanistic properties (Arnold and Volkov, 1999).

However, directed evolution acknowledges the complexity and inability to understand every aspect of the organism and aims to develop an overall mutation that increases the desired activity in cells.

2.4.2 Improvement of biodesulfurization through genetic modification

Genetic modifications in a cell can include changing the expression level of enzymes, modifying a promoter for a specific gene or group of genes, changing the expression of co-factors and rearranging or deleting genes. Some genetic modifications that have been done with *Rhodococcus* are outlined in Ma (2010). The disadvantage to genetic modification studies is that it is extremely difficult to determine the impact that a change or changes will have on the interactions that occur in the cell. The maximum desulfurization activity that has been achieved by a genetically modified *Rhodococcus* species is 250µmol/g DCW/h, which is well below the required commercialization activity range of 1.2mmol DBT/g DCW/h to 3mmol DBT/g DCW/h (Kilbane, 2006).

Li et al. (2007) determined that there was an overlap between the DszA enzyme and the DszB enzyme. They believed that this was responsible for a decrease in expression level of the DszB enzyme and lead to it being associated with the rate limiting step. They redesigned the Dsz operon and inserted it into *Rhodococcus erythropolis*. They were able to get an increase in desulfurization activity to about 120 µmol/g CDW/h which was 5-fold faster than the native Dsz operon.

In 2008, Li et al. furthered their understanding of the DszABC operon and determined that the levels of transcription and translation decreased according to the position of the genes in the operon, so that the DzsC enzyme had the lowest level of transcription and translation. They then rearranged the genes based on catalytic capabilities and expressed the new operon (DszBCA) in *R. erythropolis*. They were able to improve the desulfurization activity to 320 µmol/g CDW/h. However, only the expression of DszB and DszC was increased, while the expression levels of DszA decreased. This illustrates that is difficult to predict

how genetic modifications will affect the behavior of the cell as there are many unknown interactions that occur in the cell.

Kilbane (2006) concludes that a better understanding of host factors that contribute to the mechanism of the 4S pathway is required. Genetic manipulation experiments using alternative promoters or increased copy numbers of the genes have not been able to improve desulfurization activity to levels required for commercialization, indicating that host factors must play a significant role in the pathway. The native enzymes involved in the 4S desulfurization pathway are rate limiting factors which limit the improvements in desulfurization activity. To achieve the required biodesulfurization activity for commercial feasibility, alterations in the genes and regulatory sequences other than the dsz genes are needed (Kilbane, 2006).

2.4.3 Improvement of biodesulfurization through directed evolution

Directed evolution looks at improving enzyme performance and substrate specificity by applying a selective pressure on the microorganisms. Modifications result in a library of mutants in which the desired activity can be selected for. Biocatalysts need to be evolved to not only have increased activity, but also need to be able to perform under process conditions. Bornscheuer (2001) describes some of the many techniques have been utilized for directed evolution including mutagenesis, DNA shuffling, error prone PCR, staggered extension process and random priming recombination. One of the major challenges in directed evolution is the ability to compare the activity of the mutant library with a suitable assay system. Typical genetic libraries created from these techniques can have 10^4-10^6 mutants, therefore identification of mutants with the desired traits needs to be fast and accurate (Bornscheuer, 2001). Two main processes for the identification of desired mutants have been utilized: selection based systems and screening based systems. Selection based systems allow for a more rapid analysis of large libraries to identify organisms that exhibit the desired activity. Selection based systems are rare, but can deliver dramatic results as they only let colonies that carry the

desired improvement and growth rate survive. This also ensures that the desired activity does not interfere with cellular metabolism (Arnold and Volkov, 1999). Screening based systems can compare the differences in activity of the mutant library. Screening based systems are required when having the desired activity is not directly linked to cell survival (Arnold and Volkov, 1999). However, a mutant biocatalyst that has been identified to have higher activity through selection or screening based systems may not function well in process conditions. pH, temperature, and solvent stability are all factors that should also be evaluated in biocatalysts selection.

Chemostats have been utilized for process-based directed evolution strategies (Arensdorf et al., 2002). Chemostats can offer substrate limitation conditions with a constant environment that can promote adaptive mutations with increased growth rate and/or increased activity. Semi-continuous fermentations have also been utilized to select for mutants with faster growth rates (Zelder and Hauer, 2000). In semi-continuous fermentations part of the previous batch of cells is utilized as an inoculum for the current batch and so forth. This selects for cells with a faster growth rate (Zelder and Hauer, 2000). However it is important to note that an increase in growth rate does not always correspond to an increase in desired activity. Process-based directed evolution strategies utilized selection strategies that rely on a selective pressure for growth while operating under process conditions.

Continuous cell division in chemostats and in semi-continuous cultures promotes the continued generation of random mutants even at low mutation rates. If these mutants grow faster and/or utilize the resource better than the wild type organisms they will eventually take over the population (Arensdorf et al., 2002). Process-based directed evolution strategies are especially useful to develop gain-of-function mutations. Arensdorf et al. (2002) used a chemostat to develop gain-of-function mutants from a *Rhodococcus* species that were capable of desulfurizing thiophenes that the wild strain could not. They ran the chemostat for 240 days sequentially changing the sulfur compounds present and adjusting their concentrations to pressure the organisms into utilizing the only available sulfur

source and gradually selecting mutants that were able to degrade increasingly complex compounds.

Chemostats have also been utilized to change the sensitivity that organisms can have to toxic or inhibitory compounds. *Gordonia alkanivorans* exhibited a high sensitivity to 2-HBP in normal culture growth, but, when exposed to this potentially toxic compound in chemostat culture, a higher resistance to 2-HBP was observed (Alves and Paixão, 2011).

Self-cycling fermentation (SCF) is another method of semi-continuous cultures that could be utilized for process-based directed evolution. In SCF, the culture is grown until the onset of stationary phase, at that point half the culture is removed and replaced with fresh media and the next cycle continues (Sauvageau et al., 2010). The cycling process is based on a feedback control loop that measures dissolved oxygen (Sarkis and Cooper, 1994) or carbon dioxide evolution rate (CER) (Sauvageau et al., 2010) to determine the end of exponential growth. Each cycle corresponds to one generation (Sheppard and Cooper, 1990). Sarkis and Cooper (1994) demonstrated that the SCF could be used to adapt microorganisms for more efficient growth on inhibitory compounds, which would be advantageous for directed evolution strategies. Brown (2001) addressed some of the many advantages that an SCF could bring to environmental remediation. Of great interest was the fact that degradation rates have been shown to increase as the number of cycles increased. This would indicate that faster growing organisms are being selected for in the cycling process. In addition, forcing early cycling in SCF should also allow for selection of faster growing organisms. The use of SCF for process-based directed evolution strategies has not been implemented but shows great potential.

Directed evolution techniques have been utilized to improve the rate and extent of desulfurization of DBT and its derivatives in the 4S biodesulfurization pathway of *R. rhodochrous* IGTS8 (Arensdorf et al., 2002). A combination of directed evolution techniques like chemical mutagenesis and process-based directed evolution techniques would result in more mutants existing in the inoculum and allow for faster selection processes.

2.5 Process challenges for the implementation of biodesulfurization

Improvements to the biocatalyst are necessary to make the biodesulfurization process industrially viable. However there are challenges on the process side that also need to be addressed before implementing biodesulfurization. Shennan (1996) discussed some of those challenges: (i) a carbon substrate such as acetate, glucose or yeast extract would be required for cell growth and could be a costly addition for the biodesulfurization of fuel products; (ii) aerobic processes will have significant power requirements to supply substantial aeration and agitation for cell growth; (iii) costly disposal of bulk biomass and the treatment of high oxygen downstream aqueous waste streams must be taken into account; (iv) the rate of transportation of sulfur compounds from the oil phase to the water phase will be a limiting factor for the rate of biodesulfurization; and (v) the effects of chemical surfactants used to create oil/water emulsions will have to be tested on the organisms.

Additionally, Schilling et al. (2002) utilized a CSTR with and without recycled biocatalyst. They found that the recycled biocatalyst had desulfurization activity one order of magnitude lower than fresh biocatalyst. This represents another challenge to the implementation of industrial biodesulfurization as the biocatalyst will need to be regenerated if it is to be reused.

3. CHAPTER 3 – MATERIALS AND METHODS FOR DESULFURIZATION OF OXIDIZED BITUMEN USING MICROORGANISMS THROUGH PROCESS-BASED DIRECTED EVOLUTION

3.1 Introduction

Dibenzothiophene (DBT), a predominant compound in bitumen, is, like many aromatic thiophenes, recalcitrant to hydrodesulfurization. Biodesulfurization is being investigated as an alternative strategy to remove DBT from fuel (**Chapter 2.)** Many *Rhodococcus* species have been shown to desulfurize dibenzothiophene (DBT) by the 4S pathway to produce 2-hydroxybiphenyl (2-HBP) and sulfite. *Rhodococcus rhodochrous* IGTS8, in particular, has shown good potential and has been the focus of many genetic improvement studies (**Section 2.4.2**). *Rhodococcus* K1bD shows selective preference to degrading DBT over simpler heterocyclic compounds making it advantageous for the commercialization of the process (Kirkwood et al., 2005). *Rhodococcus erythropolis EPWF* has been shown to oxidize aliphatic sulfides but not DBT, making it an interesting candidate for gain of function evolution strategies (Kirkwood et al., 2005). The first steps in the 4S pathway involve the oxidation of DBT to dibenzothiophene sulfone ($DBTO_2$) (**Section 2.3.1**). Some studies have found faster growth rates and increased desulfurization activity when using $DBTO_2$ instead of DBT (Setti et al., 1994; Wang and Krawiec, 1996) while some studies have found growth on DBT to exhibit higher desulfurization activity (Ohshiro et al.,1996). Other studies have found that growth with DBT and $DBTO_2$ were approximately the same (Gallagher et al., 1993).

In order to make biodesulfurization a feasible process, desulfurization activity must be improved. Many studies have used genetic modifications and directed evolution to generate point mutations or single enzyme modification in the cells (**Section 2.4.2**). The main limitation with these strategies is that the modification is targeted and discrete. Additionally, the point mutation may not function or be expressed at optimal process conditions. Process-based directed

evolution strategies, such as through chemostats, apply a selective pressure on the cells that allows mutant cells with advantageous traits (e.g. faster growth rates) to take over populations. It is not necessary to have an understanding of the mechanism of the cells to utilize process-based directed evolution; only the desired activity must be understood so that appropriate selective pressure can be applied. Advantageous mutations improve cell activity as a whole. Process-based directed evolution technologies can also be run under ideal process conditions, ensuring that the mutant cells exhibit optimal functionality at those conditions. For example, beyond achieving faster desulfurization, successful desulfurization mutants must account for the presence of sulfate in the cell media, which has been found to significantly inhibit desulfurization (**Section 2.3.5**). Overcoming the inhibition of sulfate is thus another important factor for increasing the desulfurization activity of the cells.

It is hypothesized that desulfurizing $DBTO_2$ directly would be advantageous to the cells as it requires less enzymes and cofactors, and its solubility is greater to that of DBT. Utilizing process-based directed evolution strategies with $DBTO_2$ as a sole source of sulfur is expected to generate increased desulfurization activity and growth rate. Process-based directed evolution was utilized on *R. rhodochrous* IGTS8 through two strategies; (i) sequential transfers and (ii) self-cycling fermentation (SCF). Sequential transfers involved growing the organisms to stationary phase (~48 hours) then transferring 0.08-0.4% v/v of the culture to fresh media to continue cell growth. Sequential transfers were also conducted with *R. erythropolis* EPWF and *R.* K1bD. Sequential transfers were conducted in sulfate-free mineral media (SFMM) and minimal salt media (MSM) both with 1.0mM $DBTO_2$. MSM media contains sulfate well above the inhibitory concentrations discussed in **Section 2.3.5**. In order for the cells to desulfurize $DBTO_2$ in MSM media they will need to overcome sulfate inhibition therefore adding an additional selective pressure to the media. Cultures grown in the SCF were grown to stationary phase (~12 hours) at which point half the volume of cells and media were removed from the system and replaced with fresh media to continue cell growth. Each cycle was one generation time, except for the first

cycle which resembled batch growth and took ~48 hours. Growth in the SCF applies a stronger selective pressure then the sequential transfers requiring less generations for a mutant to take over the population. The desulfurization yield was determined by measuring the accumulation of 2-HBP in the culture media after 48 hours of growth. The specific desulfurization activity was measured as the desulfurization yield (at 48 hours), divided by the optical density value of the culture at 48 hours, divided by 48 hours (μM/OD/hr). The desulfurization yield of the cells, and therefore the presence of 2-HBP in the media was confirmed by gas chromatography-mass spectrometry analysis. The 2-HBP concentration was determined by gas chromatography analysis of solvent extracted media.

3.2 Chemicals

The manufacturer and purity of purchased chemicals are given in **Table 1.**

Table 1 List of chemicals used along with the purity and manufacturer

Chemical Name	Purity	Manufacturer
2-Phenylphenol (2-HBP)	>99%	Acros Organics
Agar Technical		BD
Ammonium chloride	>99.5%	Fisher Scientific
Ammonium nitrate	>98%	Sigma-Aldrich
Barium chloride dehydrate	>99%	Fisher Scientific
Biotin	99%	Sigma-Aldrich
Boric acid	>99.5%	Sigma-Aldrich
Calcium chloride dehydrate	>99%	Fisher Scientific
Chloroform	>99.8%	Acros Organics
Cobalt chloride hexahyrdate	>98%	Sigma-Aldrich
Copper (II) chloride dihydrate	>95%	Sigma-Aldrich
Dextrose (D-glucose) anhydrous	ACS	Fisher Scientific
Dibenzothiophene (DBT)	98%	Sigma-Aldrich
Dibenzothiophene sulfone ($DBTO_2$)	97%	Sigma-Aldrich
Drieriter 8 mesh (with indicator)		Acros Organics
Ethanol	95%	Fisher Scientific
Glycerol	99.5%	Fisher Scientific
Hydrochloric acid 1N	99.5%	Fisher Scientific
Iron (III) chloride	97%	Sigma-Aldrich
Iron sulfate heptahydrate	99.5%	Acros Organics
Magnesium chloride hexahydrate	>99%	Sigma
Magnesium sulfate heptahydrate	>98%	Fisher Scientific
Manganese chloride tetrahydrate	>99%	Sigma-Aldrich
N,N-Dimethylformamide	>99.8%	Fisher Scientific
Nutrient Broth (NB)		BD
Octadecane (C_{18})	99%	Acros Organics
Potassium phosphate dibasic	>98%	Sigma-Aldrich
Potassium phosphate monobasic	>99%	Fisher Scientific
Sodium acetate trihyrdate	99%	Fisher Scientific
Sodium chloride	>99%	Fisher Scientific
Sodium molybate dehydrate	>99.5%	Sigma-Aldrich
Sodium phosphate dibasic	>99%	Fisher Scientific
Tetrasodium EDTA	99%	Fisher Scientific
Thiamine Hydrochloride	>98%	Fisher Scientific
Tryptic soy broth (TSB)		BD
Vitamin B-12	98.5	Sigma-Aldrich
Yeast Extract		BD
Zinc chloride	>98%	Sigma-Aldrich
ρ-aminobenzoic acid	>99%	MP Biomedicals

3.3 Growth conditions and bioreactors

3.3.1 Microorganisms and cryopreservation

Rhodococcus rhodochrous ATCC 53968 (*R. rhodochrous* IGTS8) was purchased from the American Type Culture Collection (ATCC). This strain was formerly known as *R. erythropolis* IGTS8. *Rhodococcus erythropolis* EPWF and *Rhodococcus* K1bD were graciously provided by Dr. Julia Foght (University of Alberta, Department of Biological Sciences). Initial cell banks were prepared and preserved at -80^{o}C prior to starting experiments. 1-mL aliquots of cell cultures were suspended in tryptic soy broth (TSB) (BD, Edmonton, AB) with 20% v/v glycerol (Fisher Scientific, Edmonton, AB). Cell banks were also periodically prepared for sequentially transferred cultures and cultures undergoing self-cycling fermentation to preserve any evolutionary changes.

3.3.2 Growth media and stock solutions

TSB and nutrient broth (NB) (BD, Edmonton, AB) were prepared with distilled water according to the recipes provided by the manufacturer. Minimal salt medium (MSM), M9 medium, sulfate-free mineral medium (SFMM), and phosphate buffer were all prepared using distilled water. The recipes for the media and stock solutions are shown in **Table 2**. The sulfate-free mineral medium recipe was taken from Kirkwood (2006).

Table 2. Media and stock solution recipes

Sulfate-Free Mineral Medium (SFMM)
0.5g K_2HPO_4
1.5g NH_4Cl
1.0g NaCl
0.1g $MgCl_2 \cdot 6H_2O$
4.0g $CH_3COONa \cdot 3H_2O$
1mL Trace Metal Solution*
1ml Pfennig's Vitamin Solution**
1L Distilled water

Trace Metal Solution (not sterilized)
3.7g $CaCl_2 \cdot 2H_2O$
2.5g H_3BO_3
1.3682g $MnCl_2 \cdot 4H_2O$
0.65g $FeCl_3$
0.44g $ZnCl_2$
0.29g $Na_2MoO_4 \cdot 2H_2O$
0.0183g $CoCl_2 \cdot 6H_2O$
0.000131g $CuCl_2 \cdot H_2O$
1L Distilled water

Minimal Salt Medium (MSM)
6g Na_2HPO_4
4g NH_4NO_3
4g KH_2PO_4
0.2g $MgSO_4 \cdot 7H_2O$
0.014g disodium EDTA
0.01g $FeSO_4 \cdot 7H_2O$
0.01g $CaCl_2 \cdot 2H_2O$
1g Yeast Extract
900ml of Distilled water
100ml of 20g/L Glucose **

Phosphate Buffer
0.6g KH_2PO
0.4g K_2HPO_4
1L Distilled Water

Pfennig's Vitamins (Filter Sterilized)
50mg ρ-aminobenzoic acid
50mg vitamin B-12
10mg biotin
100mg thiamine
1L Distilled water

M9 Medium recipe (1L)
200ml 5X M9 salts
0.240 g $MgSO_4$
0.011g $CaCl_2$
1g Yeast Extract
900ml of Distilled water
100ml of 20g/L Glucose **

5x M9 Salts (1L)
64g $Na_2HPO_4 \cdot 7H_2O$
15g KH_2PO_4
2.5g NaCl
5g NH_4Cl
1L Distilled Water

*(added before autoclaving)

**(added after autoclaving and cooling)

3.3.3 Growth conditions

Cell cultures were incubated in incubator shakers (New Brunswick Scientific CO. Inc (G25) and Infors HT Ecotron) at 30ºC and 150rpm.

3.3.4 Agar plate preparation and culture streaking

Agar plates were prepared with 3% TSB and 1.5% agar technical (Fisher Scientific, Edmonton, AB). Globe inoculation loops (1uL) were utilized to streak cultures onto the agar plate using the T-streak method. Agar plates were incubated at either room temperature or 30ºC.

3.4 Measurements

3.4.1 Optical density measurements and cell number determination

Cell concentration was determined by optical density, microscopy and cell dry weight.

Optical density of the cultures was measured at a wavelength of 600nm using a spectrophotometer (biochrom Ultrospec 50 UV-Vis). Initial growth curves were compiled using a Shimudzu (UV-160) UV-spectrophotometer at 600nm. In all cases, distilled water was used as a reference due to solubility issues with $DBTO_2$ in the media.

Cell number was determined using a Leica DMRAX2 microscope with a Qimaging (regtiga exi) camera. 10µl of culture was placed on a hemocytometer (Neubauer ruling). If cell dilution was required for the cell count, cells were diluted in phosphate buffer. Cell counts were performed using the software imageJ (from NIH) and were converted to cells/ml.

3.4.2 Cell dry weight

For cell dry weight experiments, 30-35ml samples of cells were centrifuged in a Sorvall (RC 6 Plus) centrifuge with a Thermo Scientific (S-34) rotor at 20,000 rcf and 25ºC for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20ml-25ml of phosphate buffer. The re-suspended cell pellet was centrifuged again under the same conditions. The supernatant was once again discarded and the pellet was rinsed out of the centrifuge tube with a minimal amount of distilled water into a previously weighed aluminum weigh dish. The weigh dish was dried in an oven at 60ºC for a minimum of 10h. Once the sample was dried, the aluminum weigh dish was weighed to determine the dry weight biomass concentration (mg/ml).

3.4.3 Measure of desulfurization yield and specific desulfurization activity

Desulfurization yield was defined as the accumulation of 2-HBP (mM) after 48 hours of incubation. This measure was determined using gas chorography following the method outlined in **Section 3.6.1** The desulfurization yield was used to compare the desulfurization capacity of the three *Rhodococcus* species. Specific desulfurization activity was determined by taking the desulfurization yield after 48 hours, and dividing it by the optical density value of the culture at 48 hours, then dividing by 48 hours (µM/OD/hr). Specific desulfurization activity was used when comparing activity of a single *Rhodococcus* species. This measure of specific desulfurization activity would not be an accurate measure of comparison for comparing the three *Rhodococcus* species. This is due to the fact that the same optical density values do not correspond to the same cell concentration for each species.

3.4.4 Standard Deviation Measurements

Standard deviation measurements were calculated on samples using the n-1 method. One standard deviation was reported with the mean of the samples. Three samples were used to determine the mean and standard deviation unless otherwise stated.

3.5 Process-based directed evolution methods

3.5.1 Sequential batches of transferred cells

R. rhodochrous IGTS8, *R. erythropolis* EPWF, and *R.* K1bD were individually grown in 125-mL shake flasks containing 25mL of medium in incubated shakers at 30ºC and 150 rpm. These conditions were chosen as they were recommended by ATCC for *R. rhodochrous*.

The media used for this section of the study were SFMM or MSM, each containing 1.0mM dibenzothiophene sulfone ($DBTO_2$). The cultures were transferred once they had reached stationary phase, every two or three days. When cultures were to be transferred after two days, 100µl of culture were used to inoculate a new shake flask containing 25ml of fresh medium. When cultures were to be transferred after three days, 20µl of culture were transferred to a new shake flask containing 25ml of fresh medium. Each inoculation of a new culture was considered one transfer. The optical density of the transferred cells was also recorded at the time of the transfer. Cell banks were prepared as described above to preserve any changes in the cells. Cell plating was also done periodically on TSB plates to test for contamination. If contamination was detected the culture was restarted from the previous uncontaminated plate or from the most recent cell bank.

For the growth and specific desulfurization activity comparison of sequentially transferred cells, transferred in SFMM with 1.0mM $DBTO_2$, the cultures were grown in SFMM with 0.25mM $DBTO_2$ and 14mM

dimethylformamide (DMFA). The cultures were started with approximately $2.5x10^5$ cells/ml.

Comparison of MSM sequentially transferred cells of *R. rhodochrous* were grown in MSM with 0.25mM DBTO2 and 14mM DMFA. The cultures were started with approximately $5x10^5$ cells/ml.

3.5.2 Self-cycling fermentation (SCF)

R. rhodochrous IGTS8 was grown in a self-cycling fermenter (SCF) for 69 cycles. **Figure 3-1** shows a schematic of the SCF set-up utilized in this experiment. The SCF set-up used was based on a previously described SCF set-up and control system (Sauvageau et al., 2010). LabView 8.0 (National Instruments) was utilized as the control system.

A 1L glass bottle was used as the reactor vessel with a rubber stopper. The working volume inside the glass reactor was 500ml. The operating conditions for the 500mL working volume were ~150rpm at room temperature with an aeration rate of 492.5ml/min. A magnetic stirrer and stir bar was used to generate mixing in the system. In each cycle, the cells were grown for 12 hours to stationary phase, except the first cycle which resembled batch growth and took ~48 hours. At 12 hours the feedback control system opened solenoid valve 2 and turned on peristaltic pump 2 to remove half the volume of cells and media (250ml). Upon completion solenoid valve 1 and peristaltic pump 1 were activated to pump fresh media into the system and fill the working volume back up to 500ml. The time required to remove and fill up half of the working volume in the glass reactor were utilized in the feedback control system to maintain accurate volumes. Liquid isolators were used to prevent contact of the reactor working volume with the feed media and waste carboys. Oxygen and carbon dioxide output were also monitored for the system using a CO_2 gas sensor (CO2-BTA, Vernier) and an O_2 gas sensor (O2-BTA, Vernier). The sensors were located after a Liebig water cooled condenser and a desiccator containing Drierite (indicator 8 mesh, Acros Organics, Edmonton AB) to ensure no moisture reached the sensors. The CO_2 and O_2 data

was recorded by the control system. A sample port was connected to the glass reactor so that samples could be taken for optical density measurements.

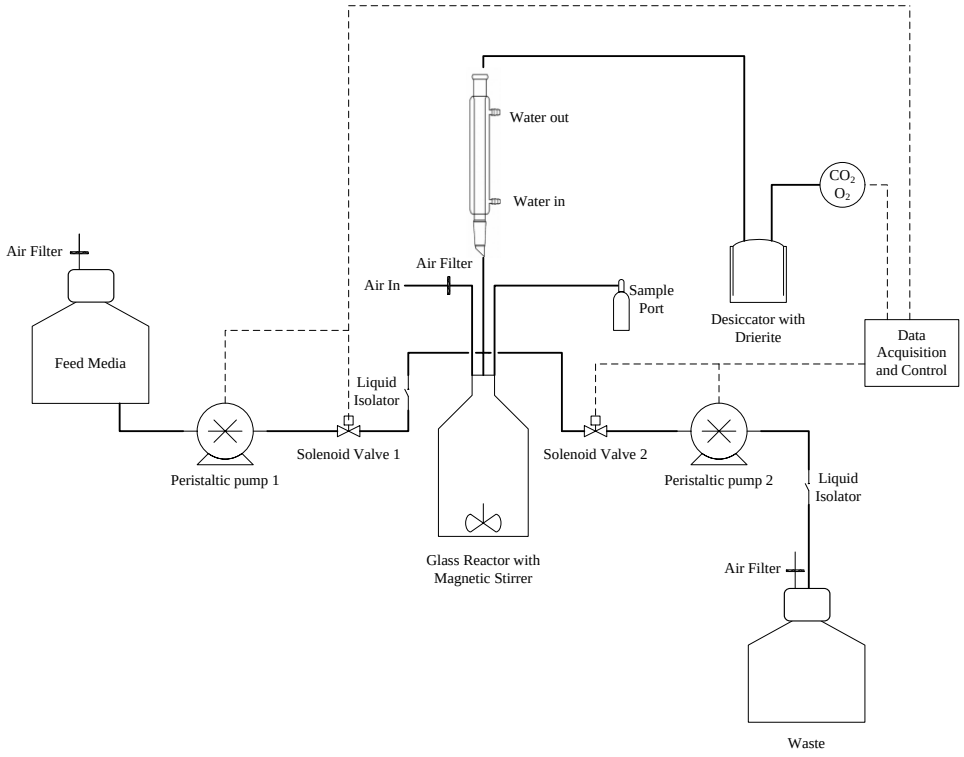

Figure 3-1 Schematic of the Self-Cycling Fermentation (SCF) system

3.6 Analyses

3.6.1 Quantification of dibenzothiophene sulfone and 2-hydroxybiphenyl using gas chromatography

The quantification of $DBTO_2$ and 2-HBP in the media was achieved using organic chloroform extraction followed by gas chromatography. The identification and accumulation of 2-HBP was confirmed using gas chromatography-mass spectrometry; the method is outlined in **Section 3.6.2**.

A solution of chloroform, containing 2mM octadecane (C_{18}) as internal standard, was added at a ratio of 1:2 to the desired sample (cell culture and media) in a separatory funnel. After mixing and leaving time for the hydrophilic and

hydrophobic phases to separate, the latter was recovered and stored at 4°C prior to analysis.

An Aglient 7890A gas chromatograph with a flame ionization detector (FID) and a J&W 122-5532 column (30m x 250um x 0.25um) were used to analyze the samples. 10µl of sample was injected into the column. Helium was used as carrier gas at a flow rate of 2ml/min. The operating conditions for the GC can be found in **Table 3**.

Table 3 Gas Chromatograph operating conditions for the analysis of dibenzothiophene sulfone and 2-hydroxybiphenyl

Operating Condition	Value
Injection Temperature	250^{o}C
Initial Column Temperature	75^{o}C for 0.5min
Temperature Ramp	20^{o}C/min
Final Column Temperature	325^{o}C hold for 5 min
Detector Temperature	300^{o}C

3.6.2 Identification of 2-hydroxybiphenyl using gas chromatography-mass spectrometry

The accumulation of 2-HBP in the SFMM samples was confirmed using gas chromatography coupled with mass spectrometry. Samples were analyzed at the Mass Spectrometry Facility in the department of Chemistry at the University of Alberta.

The samples were prepared using the chloroform extraction method outlined in **Section 2.3.1**.

An Agilent Technologies 7890 GC with 5975C mass spectrometric detector (MSD) was used to analyze the samples. 1µL of sample was injected into a Zebron ZB-5MS column (30 m x 250 µm x 0.25 µm). The operating conditions for the gas chromatograph-mass spectrometer can be found in **Table 4.**

Table 4 Gas chromatograph coupled with a mass spectrometer operating conditions for the identification of 2-hydroxybiphenyl

Operating Condition	Value
Injection Temperature	290ºC
Initial Column Temperature	75ºC
Temperature Ramp	20ºC/min
Final Column Temperature	325ºC

3.6.3 Elemental analysis for the determination of sulfur concentration in *Rhodococcus rhodochrous* IGTS8 cells

The elemental analysis of *R. rhodochrous* IGTS8 was conducted using a Carlo Erba Elemental Analyzer 1108 in the Analytical and Instrumentation Lab in the Chemistry Department at the University of Alberta. The sample was transferred to a tin (Sn) cup for analysis by the elemental analyzer. The autosampler, (which was continuously swept with helium gas) dropped the tin (Sn) cup containing the sample into the combustion reactor (maintained at 1000ºC). The combustion reactor contained an oxidation catalyst (tungstic oxide (WO_3) on alumina) and pure reduced copper wires. When the sample entered the combustion reactor, a small volume of pure oxygen was added to the helium carrier gas creating an oxidizing atmosphere that completely burned the sample and tin cup container. This resulted in the conversion of the carbon, hydrogen, nitrogen, and sulfur present in the samples to CO_2, H_2O, NO_x, and SO_2 respectively. The combustion products were then swept onto the chromatographic column (Porapak QS, 4 mm ID, 2 m long), where the gases were separated. A thermal-conductivity detector was used to detect the individual gases. Eager Xperience software converted the output signal from the thermal conductivity detector into a chromatogram and generated the area count data used to calculate the percentage of each compound in the sample. The analytical run lasted for 12 minutes.

Rhodococcus rhodochrous IGTS8 cells from different transfers of the sequential batch experiments (original inoculum and T.50) were grown in triplicates in 200ml of SFMM containing 0.25mM $DBTO_2$ and 14mM DMFA. Cells were sampled once the cultures had reached stationary phase, which took approximately 48 hours.

The cultures were diluted to an optical density of 1.5 for the original inoculum samples and 1.7 for T.50 samples. Cell counts were performed on the samples according to the method outlined in **Section 3.4.1.**

Cell dry weight samples were also prepared using the method outlined in **Section 3.4.2**. Once cell dry weight was determined, 6-9 mg of biomass was scraped off of the aluminum weigh dish into small sample vials for elemental analysis. 5mg of sample was required for duplicate runs in the elemental analyzer.

3.6.4 Sulfate determination by absorbance at 420nm

Sulfate concentration in SFMM after 48 hours of cell growth was determined using the method outlined by Kolmert et al. (2000). 1ml of sample was taken from the culture and centrifuged in a microcentrifuge (5424 R, Eppendorf). The supernatant was removed and mixed with 1ml of conditioning reagent (**Table 5**) and ~60mg of Barium Chloride (Fisher Scientific, Edmonton, AB); the cell pellet was discarded. The 2ml mixture was vortexd for 30 seconds. The mixture was then poured into a cuvette and the absorbance was analyzed in a spectrophotometer (biochrom Ultrospec 50 UV-Vis) at 420nm.

Table 5 Conditioning reagent recipe for sulfate determination by absorbance (1L)

Chemical	Amount
Sodium Chloride	150g
Glycerol	100ml
Ethanol (95%)	200ml
Hydrochloride Acid 1N	180ml
Distilled water	520ml

3.7 Calibrations

3.7.1 Gas chromatography calibration

Figure 3-2 is a gas chromatography calibration curve developed for 2-HBP. The concentration of 2-HBP was determined by correlating the ratio of the area under the 2-HBP peak to the area under the octadecane (C_{18}) peak, to a known concentration of 2-HBP.

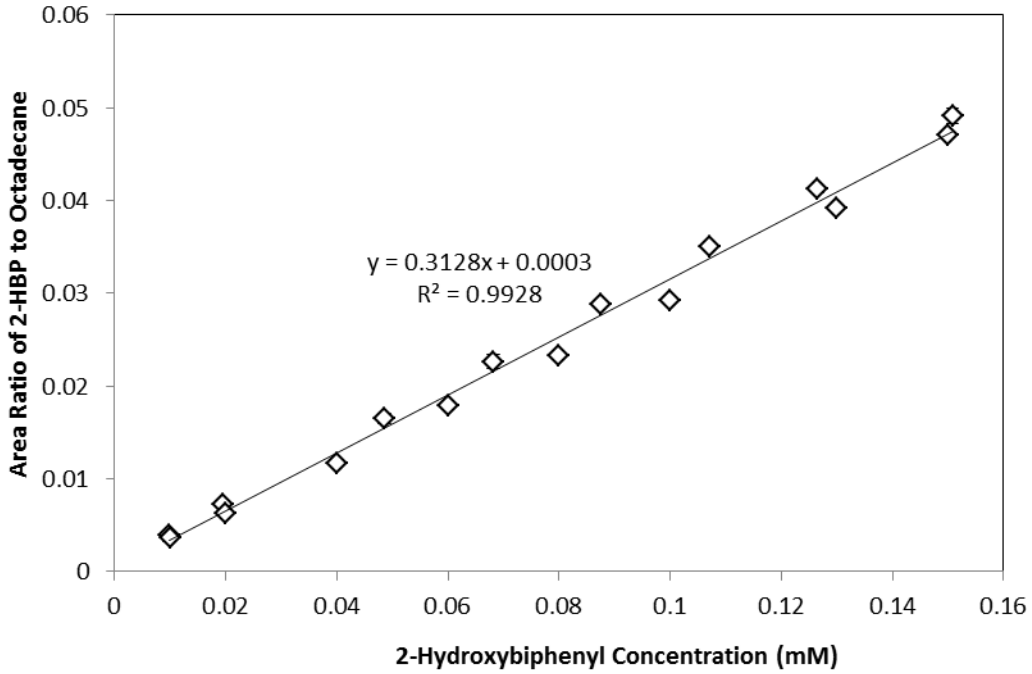

Figure 3-2 Gas chromatography calibration curve for 2-HBP with octadecane as an internal standard

A gas chromatography calibration curve was also obtained for $DBTO_2$ and is shown in **Figure 3-3**. Solubility issues with $DBTO_2$ in the sulfate-free mineral media (SFMM) lead to discrepancies between the actual and experimentally determined concentrations. Therefore the appearance and accumulation of 2-HBP was used as an indicator of desulfurization of $DBTO_2$.

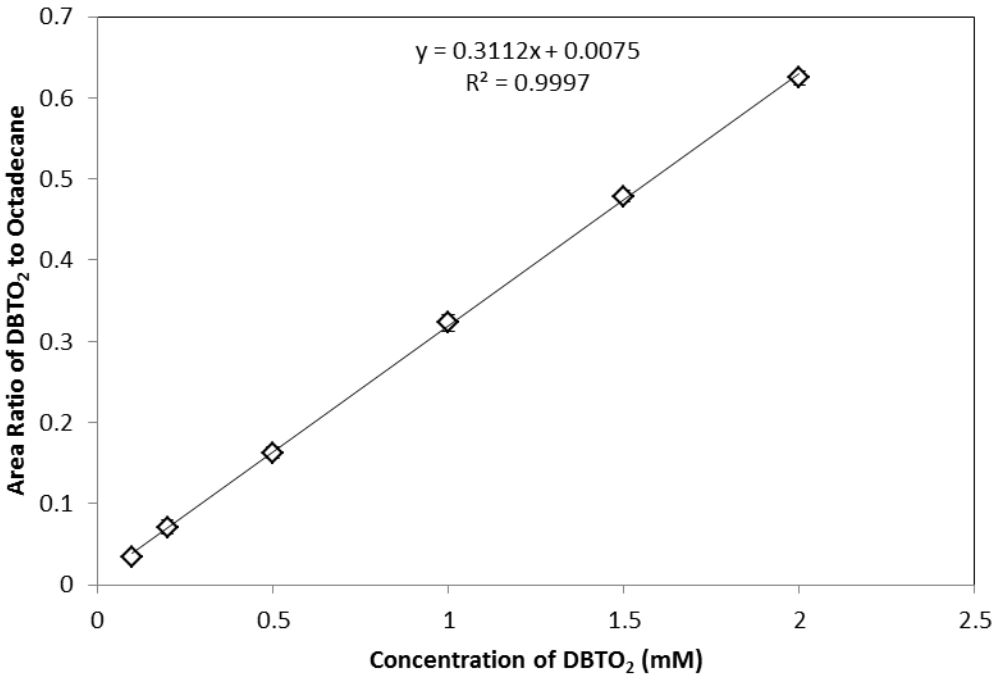

Figure 3-3 Gas chromatography calibration curve for $DBTO_2$ with octadecane as an internal standard

3.7.2 Sulfate absorbance calibration

A calibration curve was obtained for sulfate absorbance at 420nm using the method outlined in **Section 3.6.4.** Magnesium sulfate heptahydrate (Fisher Scienetific, Edmonton AB) was used in varying concentrations to make the calibration curve.

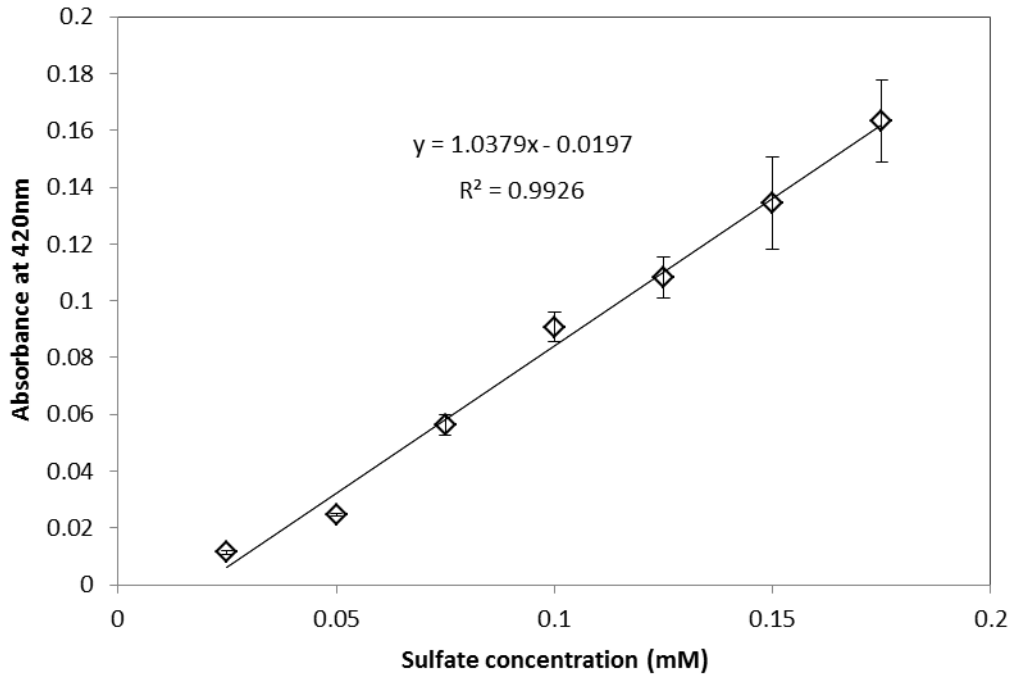

Figure 3-4 Calibration curve for absorbance at 420nm for varying concentrations of sulfate

4. CHAPTER 4 – RESULTS FOR DESULFURIZATION OF OXIDIZED BITUMEN USING MICROORGANISMS THROUGH PROCESS-BASED DIRECTED EVOLUTION

4.1 Cell growth and conditions

In order to determine optimal growth and media conditions for the microorganisms used in the present study, a number of conditions were tested. The microorganisms – *Rhodococcus rhodochrous* IGTS8 (ATCC 53968), *Rhodococcus* K1bD and *Rhodococcus erythropolis* EPWF – were grown in five different media; tryptic soy broth (TSB) (Fisher Scientific, Edmonton, AB), nutrient broth (NB) (Fisher Scientific, Edmonton, AB), M9 medium, minimal salt medium (MSM), and sulfate-free mineral medium (SFMM). The effect of varying the concentration of dibenzothiophene sulfone ($DBTO_2$) (Sigma-Aldrich, Oakville, ON) in the medium was tested in TSB and SFMM. The effect of varying the concentration of the carbon source (sodium acetate – Fisher Scientific, Edmonton, AB) was tested in SFMM.

Figure 4-1 shows a growth curve of *R.* K1bD grown in TSB, NB, and M9 media. The purpose of this experiment was to determine which media resulted in the greatest cell yield. Growth in TSB resulted in the greatest optical density value (5.6) after 24 hours of growth. NB and M9 media resulted in optical density values of 3.0 and 2.5 respectively at 24 hours.

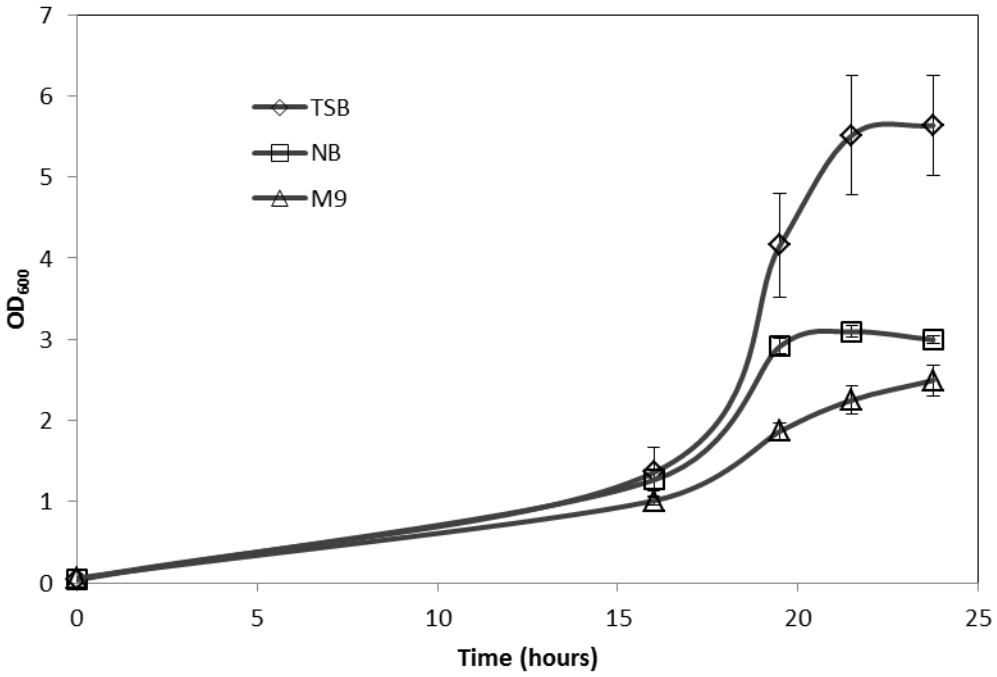

Figure 4-1 Growth curve of *R*.K1bD in TSB(◇), NB (□) and M9 (△) media

Figure 4-2 shows the growth of *R.* K1bD with varying concentrations of $DBTO_2$ from 0mM to 2.5mM in TSB. All cultures showed a similar exponential growth rate of $0.27 \pm 0.02 h^{-1}$ and reached final optical densities ranging from 11.2 to 14.2 with an average of 12.7 ± 1.1. Stationary phase was reached at approximately 40 hours for all cultures. No significant difference was observed with varying $DBTO_2$ concentrations from 0 to 2.5mM.

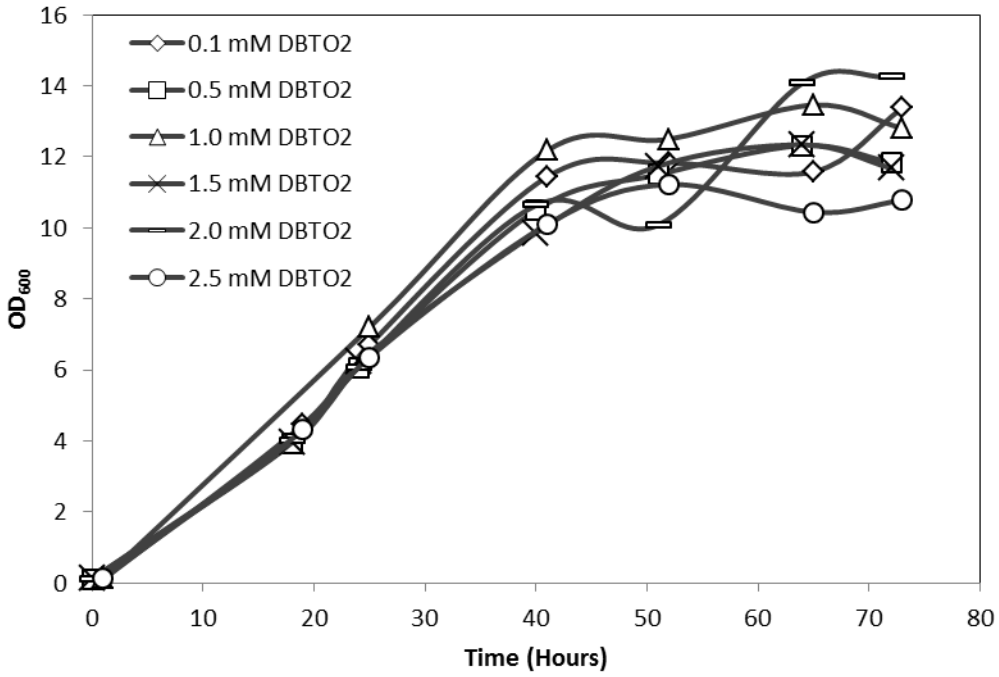

Figure 4-2 Optical density measurements for cultures of *R.* K1bD growing in TSB with varying concentrations of $DBTO_2$ (◇ 0mM, △ 0.1mM, x 0.5mM, ∗1.0mM, o 1.5mM, □ 2.0mM, and − 2.5mM)

Figure 4-3 shows the growth of *R. erythropolis* EPWF with varying concentrations of $DBTO_2$ from 0mM to 2.5mM in TSB. All cultures containing $DBTO_2$ showed a similar exponential growth rate of $0.29 \pm 0.04 h^{-1}$ and reached final optical densities ranging from 13.7 to 15.7 with an average of 14.6 ±0.7. Stationary phase was reached at approximately 50 hours for all cultures containing $DBTO_2$. Similarly to the case with *R.* K1bD, no significant differences were noted. A longer lag phase was observed in the case of *R. erythropolis* EPWF grown without $DBTO_2$; this can be explained by the fact that the culture was inoculated from colonies growing on agar plates rather than from planktonic cultures. Older colonies growing on a solid surface will often lead to longer lag phases.

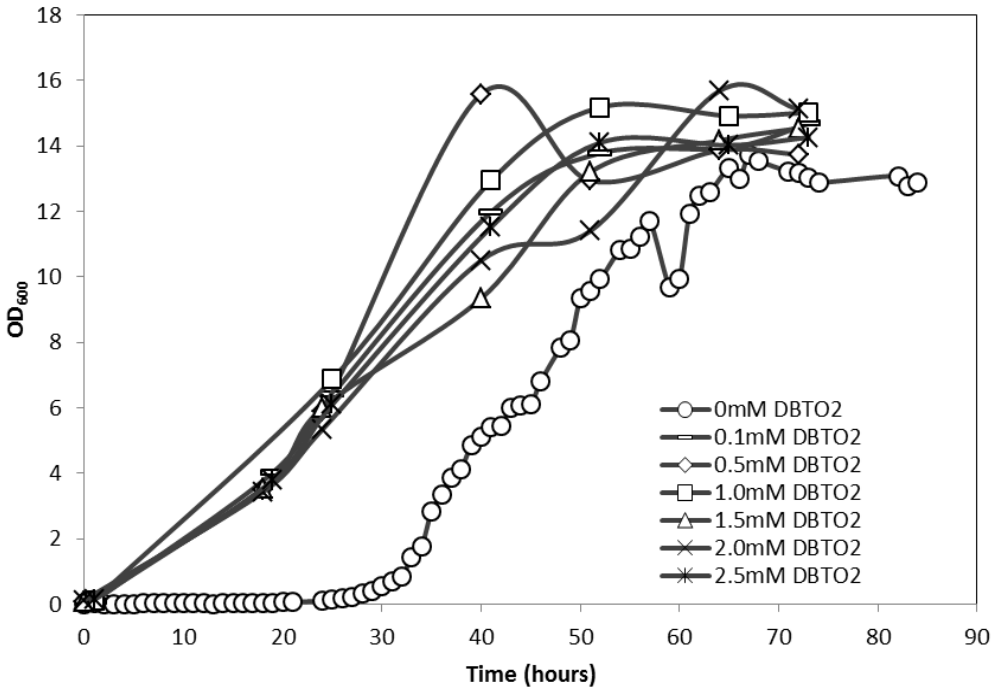

Figure 4-3 Optical density measurements for cultures of *R. erythropolis* EPWF growing in TSB with varying concentrations of $DBTO_2$ (O 0mM,− 0.1mM, ◇ 0.5mM, □ 1.0mM, △1.5mM, x 2.0mM, and∗ 2.5mM)

Figure 4-4 shows a growth comparison of *R.* K1bD in MSM with and without $DBTO_2$ (1.0mM). Similar growth rates of 0.152 ± 0.003 h^{-1} were observed with final optical densities of approximately 4. Stationary phase was reached after approximately 35 hours of growth. The addition of 1.0mM $DBTO_2$ was shown to have no effect on the growth of *R.* K1bD in MSM.

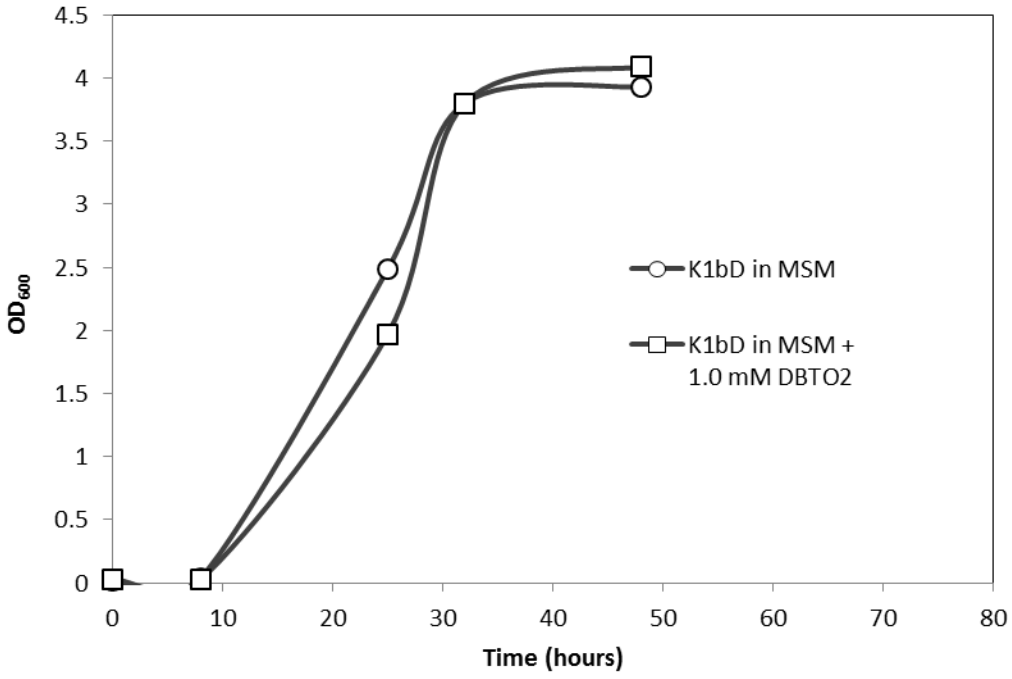

Figure 4-4 Optical density measurements for cultures of *R.* K1bD grown in MSM (O**)** and MSM with 1.0mM (□)

Figure 4-5 shows a growth comparison of *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF in SFMM with 1.0mM of $DBTO_2$. It can be seen that *R. erythropolis* EPWF grew significantly slower than *R. rhodochrous* IGTS8 and *R.* K1bD. The growth rate for *R. rhodochrous* IGTS8 was $0.059h^{-1}$ compared to $0.068h^{-1}$ and $0.032h^{-1}$ for *R.* K1bD and *R. erythropolis* EPWF, respectively. *R. rhodochrous* IGTS8 reached the greatest final optical density value (1.8, compared to 1.7 and 1.4 for *R.* K1bD and *R. erythropolis* EPWF, respectively). *R. rhodochrous* IGTS8 and *R.* K1bD reached stationary phase after approximately 35 hours of growth, whereas *R. erythropolis* EPWF reached stationary phase after 50 hours.

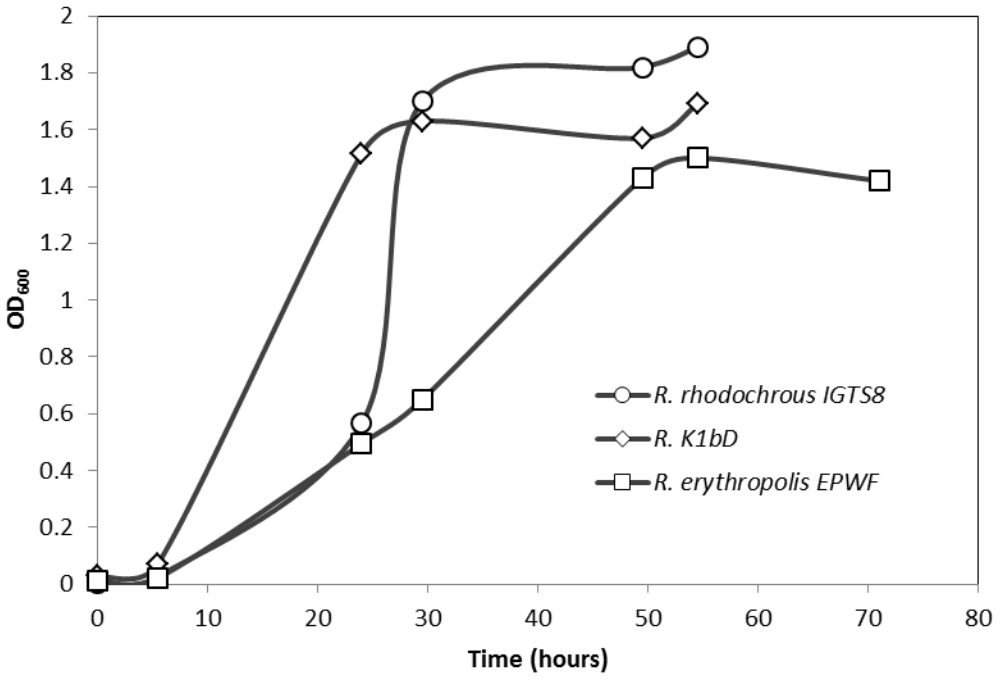

Figure 4-5 Optical density measurements of *R. rhodochrous* IGTS8 (O), *R.* K1bD (◇), and *R. erythropolis* EPWF (□) in SFMM with 1.0mM $DBTO_2$

4.2 Desulfurization

The presence of organic compounds associated with the desulfurization of $DBTO_2$ was investigated by chloroform extraction of the growth media. The chromatograms for *R. rhodochrous* IGTS8 at time zero and after 48 hours of incubation are shown in **Figure 4-6**. After 48 hours of incubation, a new peak appeared at a retention time of 7.45 minutes. This indicated the accumulation a new compound during cell growth. GC-MS analysis and information from literature led to the identification of this compound as 2-hydroxybiphenyl (2-HBP). $DBTO_2$ had a retention time of 11.18 minutes and the internal standard octadecane (C_{18}) had a retention time of 9.13 minutes.

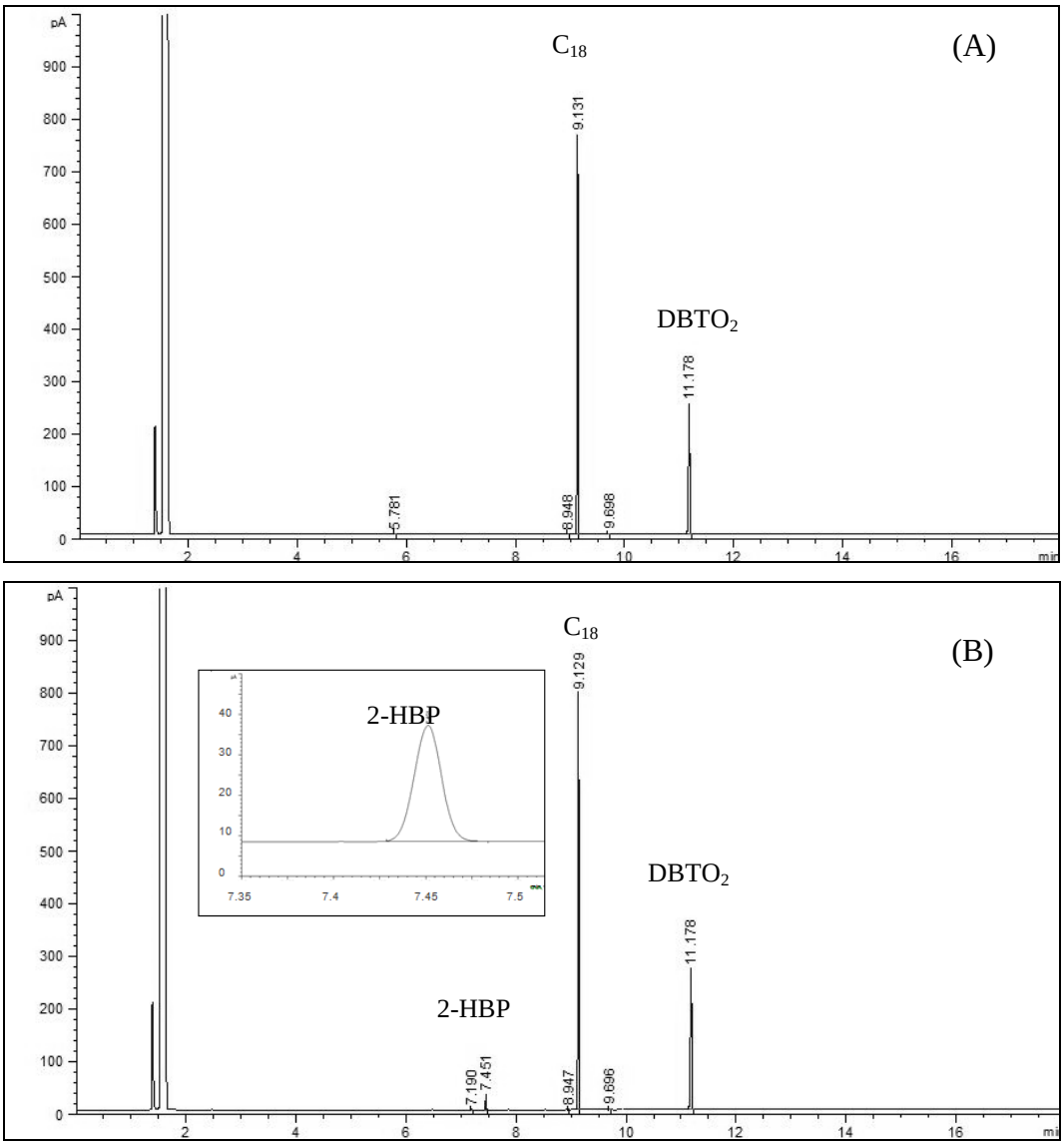

Figure 4-6 Comparison of Gas Chromatograms for *R. rhodochrous* IGTS8 grown in SFMM with 1.0mM $DBTO_2$. Samples were taken at time zero **(A)** and after 48 hours **(B)** of incubation

GC-MS analysis was used to confirm the identity of 2-HBP that was found to accumulate in cultures of *R. rhodochrous* IGTS8. **Figure 4-7A)** shows the chromatogram for *R. rhodochrous* IGTS8 in SFMM with 1.0mM $DBTO_2$ after 48 hours of growth. The identification of the peaks at 7.74 minutes and 9.46 minutes were confirmed by mass spectrometry to be, as expected, the internal standard C_{18}

and $DBTO_2$, respectively. The spectrogram of the new compound with a residence time of 6.17 minutes, was consistent with the expected signature of 2-hydroxybiphenyl (2-HBP), an accumulating metabolite of desulfurization in the 4S pathway (**Figure 4-7B)**).

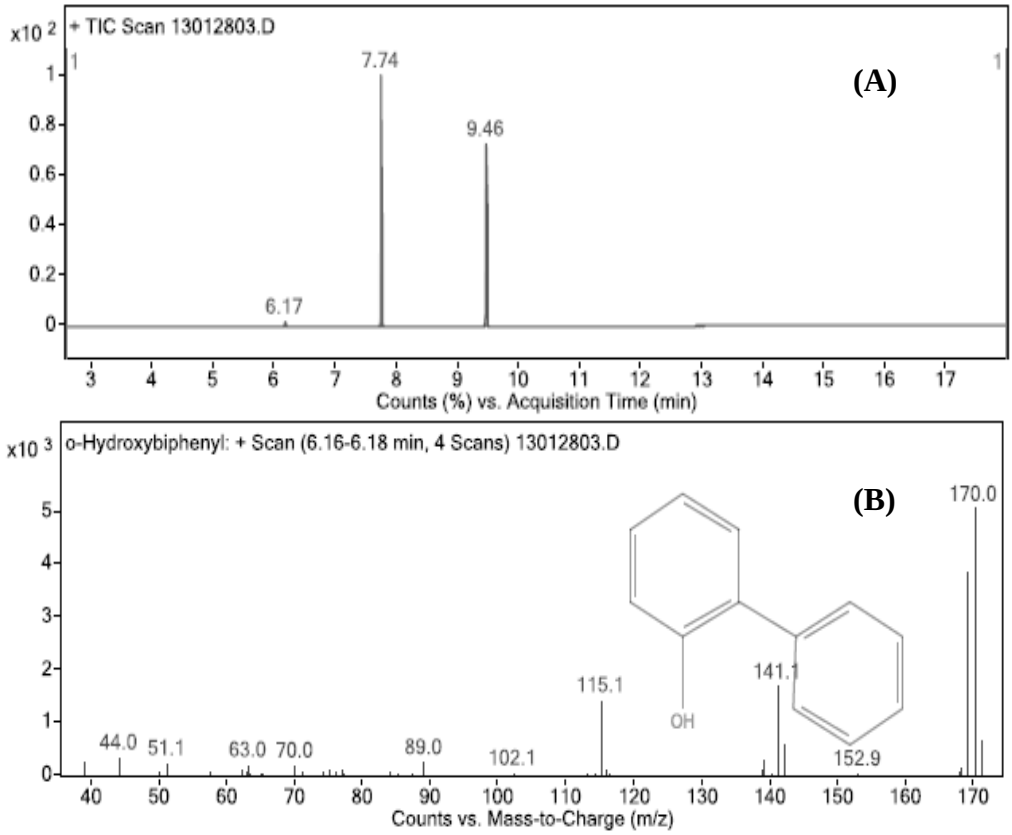

Figure 4-7 (A) Chromatogram from the gas chromatography-mass spectrometry analysis and **(B)** identification of 2-HBP by gas chromatography -mass spectrometry for *R. rhodochrous* IGTS8 in SFMM with 1.0mM $DBTO_2$ at 48 hours

Figure 4-8A) shows the growth of *R. rhodochrous* IGTS8 in SFMM with 0.5mM $DBTO_2$. *R. rhodochrous* IGTS8 grew at a growth rate of $0.077h^{-1}$, reaching a final optical density of approximately 1.7 after 40 hours. **Figure 4-8B)** shows the change in $DBTO_2$ concentration as well as the accumulation of 2-HBP. No discernible trend was observed in $DBTO_2$ concentration during the

experiment. However, a significant increase was observed for 2-HBP which increased from 0 to 0.042mM over 40 hours. As the cells reached stationary phase at 40 hours of growth, the accumulation of 2-HBP reached a plateau and no further increase was observed after 40 hours.

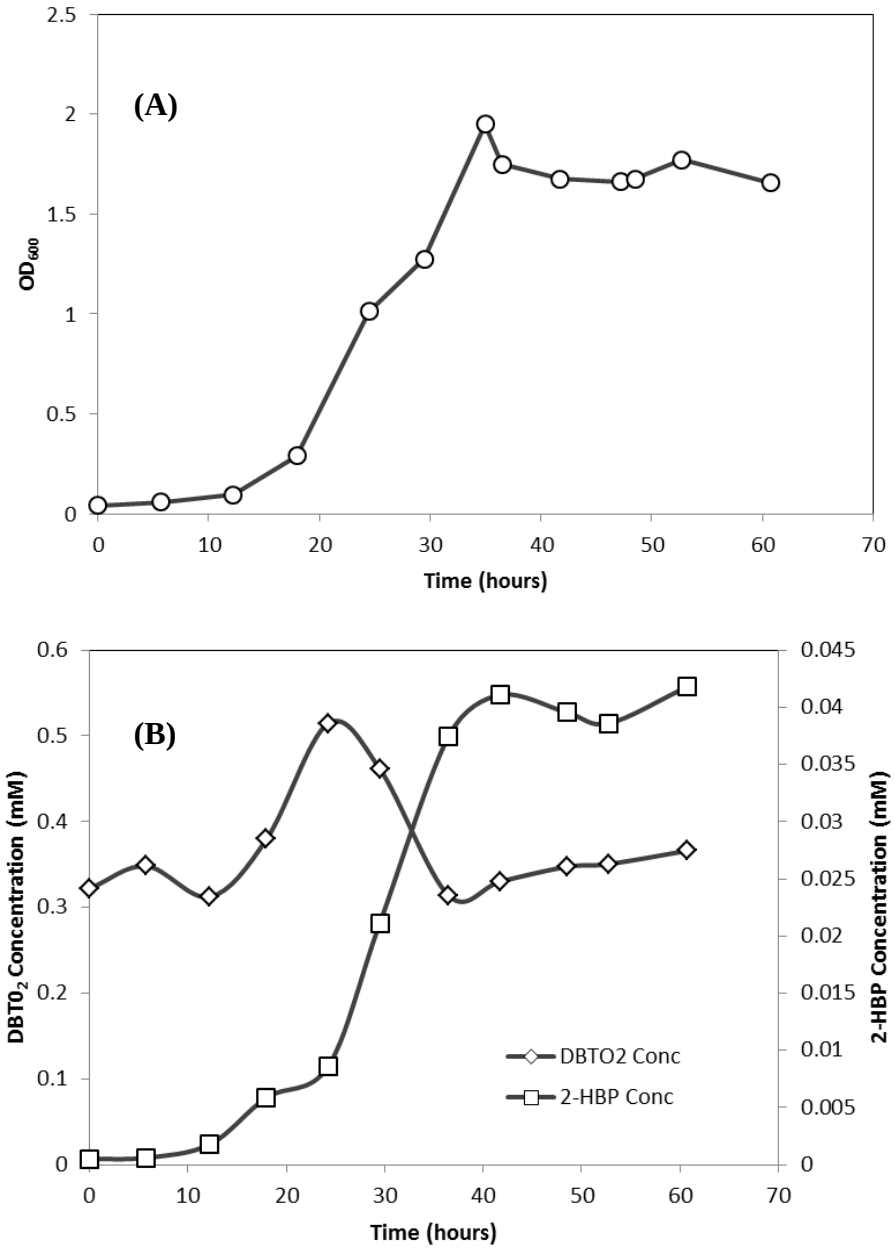

Figure 4-8 (A) Optical density values and **(B)** concentration of $DBTO_2$ (◇) and 2-HBP in cultures of *R. rhodochrous* IGTS8 grown in SFMM with 0.5mM $DBTO_2$

In a further sets of experiments, dimethylformamide (DMFA) was added to media containing $DBTO_2$ to increase its solubility. Indeed, the addition of DMFA improved the extraction efficiency of $DBTO_2$. Furthermore, experiments showed that the presence of DMFA in concentrations varying from 5mM to 28mM had no impact on cell growth (data not shown). As can be observed in **Figure 4-9**, the concentration of $DBTO_2$ decreased in cultures of *R. rhodochrous* IGTS8 growing in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. Fluctuations in the $DBTO_2$ concentrations, especially early on in the incubation, are likely due to the poor solubility of $DBTO_2$, even in the presence of DMFA. This rendered $DBTO_2$ an unreliable indicator of desulfurization. In contrast, the accumulation of 2-HBP, one of the end products of the desulfurization of $DBTO_2$, was found to be a reliable indicator of desulfurization. An example of such a trend is shown in **Figure 4-9** where 2-HBP accumulated throughout the incubation of *R. rhodochrous*. **Figure 4-9** also shows the growth of the culture, through optical density measurements. An exponential growth rate of $0.76h^{-1}$ was observed, with a final optical density of 1.54.

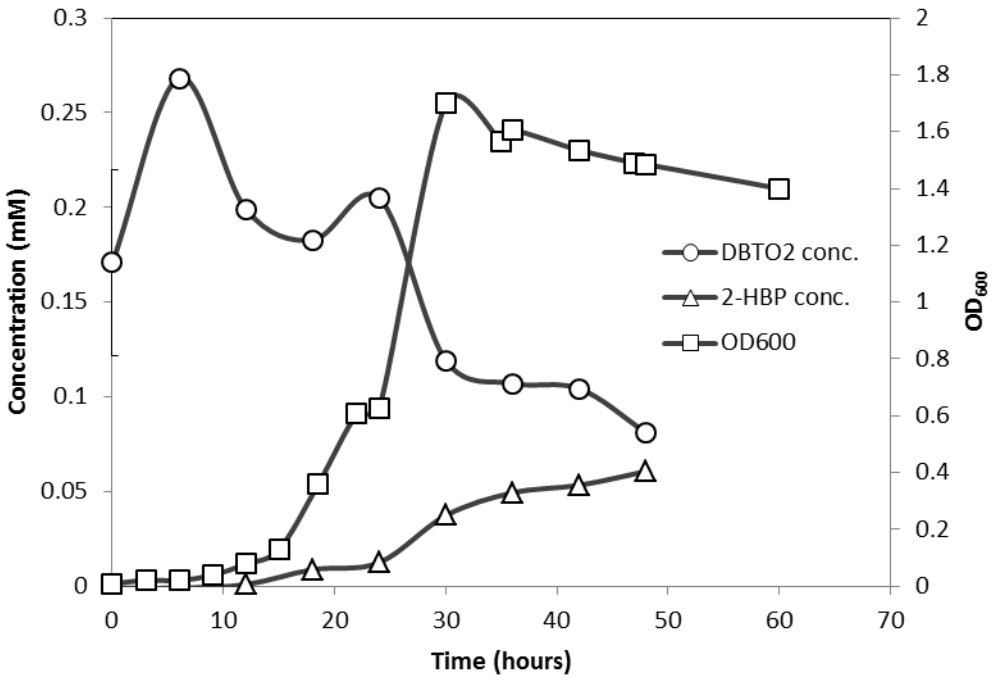

Figure 4-9 $DBTO_2$ concentration (O), 2-HBP concentration (△), and optical density values at 600nm (□) for *R. rhodochrous* IGTS8 grown in SFMM with 0.25mM DBTO2 and 14mM DMFA

The growth of *R. rhodochrous* IGTS8 in SFMM with varying concentrations of $DBTO_2$ is compared in **Figure 4-10**. $DBTO_2$ concentrations of 0.1, 0.25, 0.5 and 1.0mM were used to compare growth (**Figure 4-10A)**) and specific desulfurization activity (**Figure 4-10B)**). Growth rates of 0.072, 0.095, 0.082, and 0.077 h^{-1}, respectively, were determined for each concentration tested. The final optical density values and desulfurization yields were determined to be 1.6, 1.6, 1.6 and 1.9 and 0.028, 0.059, 0.051, and 0.063mM 2-HBP for growth with 0.1, 0.25, 0.5 and 1.0mM $DBTO_2$ after 48 hours respectively. The resulting specific desulfurization activity is shown in **Figure 4-10B)**. The specific desulfurization activity was found to be 0.037, 0.075, 0.069 and 0.067µM/OD/hr for the respective concentrations tested. No significant difference in growth, or specific desulfurization activity was observed for *R. rhodochrous* IGTS8 grown in SFMM with 0.25, 0.5 and 1.0mM $DBTO_2$. However, a lower specific desulfurization activity was observed for growth with 0.1mM $DBTO_2$.

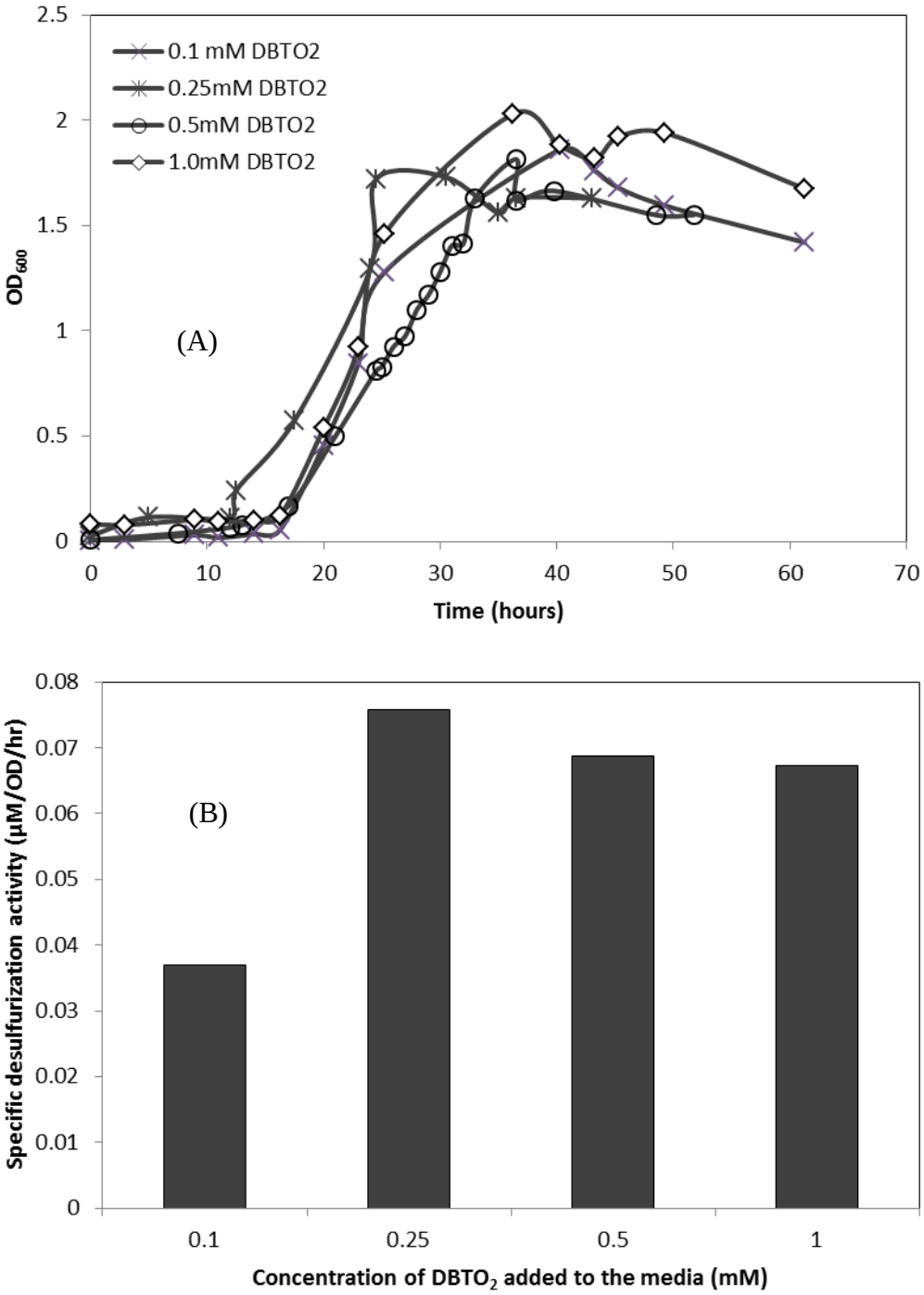

Figure 4-10 Comparison of growth **(A)** and specific desulfurization activity **(B)** for *R. rhodochrous* IGTS8 in SFMM with varying concentrations of $DBTO_2$ (0.1mM (×), 0.25mM (∗), 0.5mM (O) and1.0mM (◇))

The growth of *R. rhodochrous* IGTS8 was also tested with varying concentrations of carbon source (sodium acetate) in SFMM with 1.0mM $DBTO_2$ and is shown in **Figure 4-11**. Growth rates of 0.077, 0.086, and 0.088 h^{-1} were determined for growth with 1.0mM $DBTO_2$ and 2, 4, and 6g/L sodium acetate respectively (**Figure 4-11A)**). The final optical density values were determined to be 1.0, 1.9, and 2.6, with a desulfurization yield of 0.037, 0.063, and 0.070mM 2-HBP at 48 hours for the respective concentrations tested. The specific desulfurization activity of *R. rhodochrous* IGTS8 was also determined and is shown in **Figure 4-11B)**. The desulfurization activity was found to be 0.077, 0.067 and 0.057µM/OD/hr for 2, 4 and 6g/L sodium acetate respectively. A decrease in growth rate and desulfurization yield was observed for growth with 2g/L sodium acetate, and an increase in growth rate and desulfurization yield is observed for 6g/L sodium acetate when compared to growth at 4g/L. The specific desulfurization activity was found to be the highest for growth with 2g/L sodium acetate and the lowest for growth with 6g/L sodium acetate.

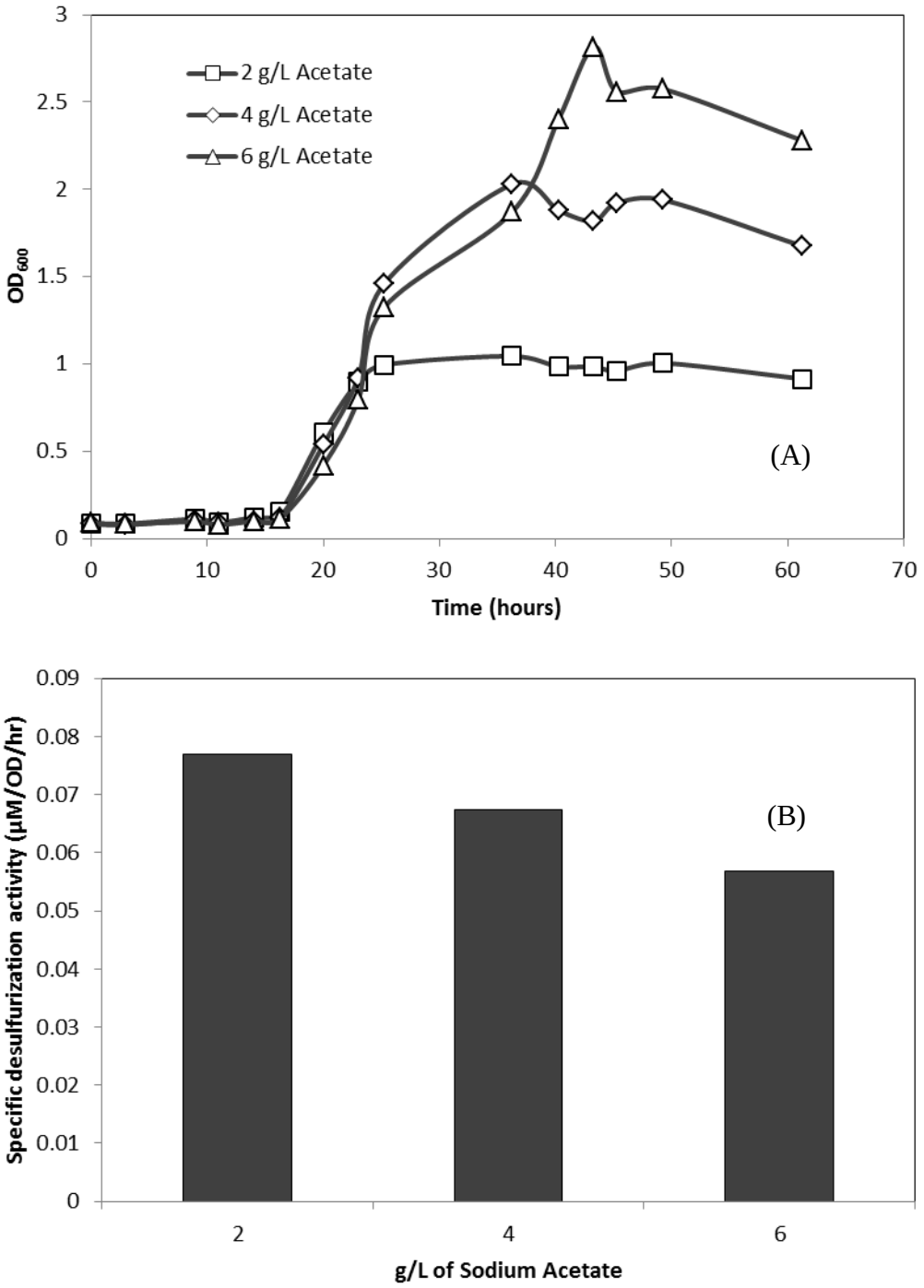

Figure 4-11 Comparison of growth **(A)** and specific desulfurization activity **(B)** for *R. rhodochrous* IGTS8 in SFMM with varying concentrations of sodium acetate (2g/L (□), 4g/L (◇), and 6g/L (△)) with 1.0mM $DBTO_2$

The desulfurization yield of 2-HBP after 48 hours of incubation with *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF cultures growing in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA is compared in **Figure 4-12**. No 2-HBP was detected for the abiotic control and the *R. erythropolis* EPWF culture. 0.052mM and 0.056mM 2-HBP accumulated in the cases of *R. rhodochrous* IGTS8 and *R.* K1bD after 48 hours, respectively.

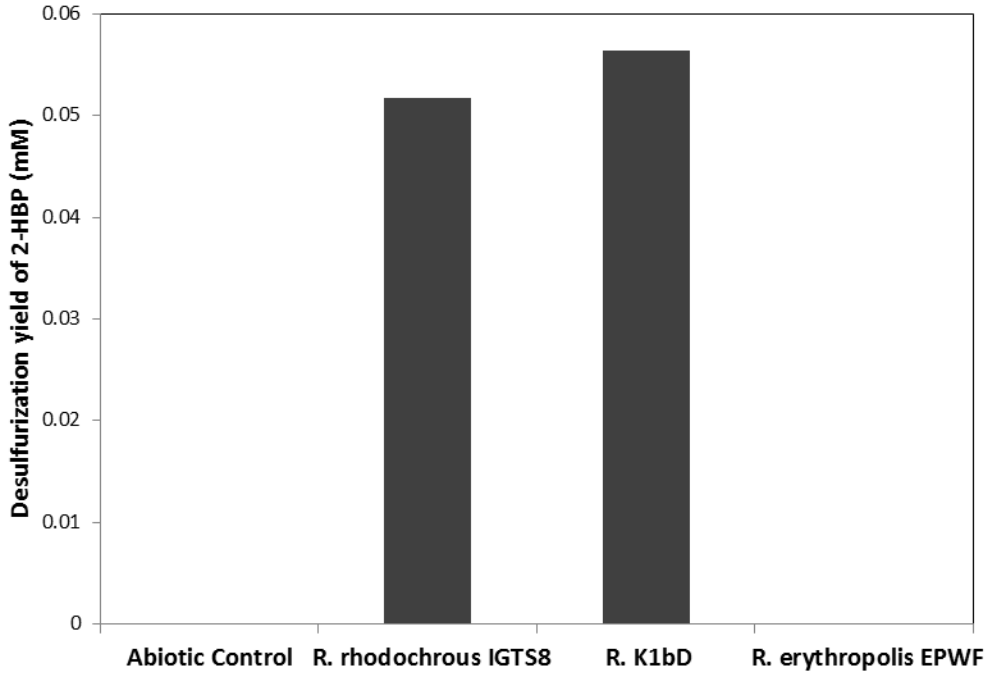

Figure 4-12 Desulfurization yield of 2-HBP after 48 hours in cultures of *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. An abiotic control is included for comparison

4.3 Selection through sequential batches

The cell concentration at the end of sequential batch growth, as measured by optical density, is shown in **Figure 4-13** for cultures of *R.* K1bD, *R. erythropolis* EPWF, and *R. rhodochrous* IGTS8 grown in either SFMM or MSM with 1.0 mM $DBTO_2$. The transfers were performed to accustom the cells to the

medium and, in the case of SFMM, apply a selective pressure for desulfurization, as $DBTO_2$ was the only source of sulfur. For *R. rhodochrous* IGTS8, a decrease in final optical density of 0.40 units was observed after the 107 and 100 transfers performed in MSM and SFMM, respectively. This corresponded to 12% and 25% reductions in cell production. For *R.* K1bD, decreases in final optical density of 1.67 (37% reduction) and 0.02 (1.3% reduction) were observed over the 107 and 100 transfers, respectively. In the case of *R. erythropolis* EPWF, the decrease in MSM was 2.09 (65% reduction) after 107 transfers. Interestingly, for *R. erythropolis* EPWF grown in SFMM there was an increase in final cell concentration of 1.54 after 100 transfers. This corresponded to an increase of 375% in biomass yield.

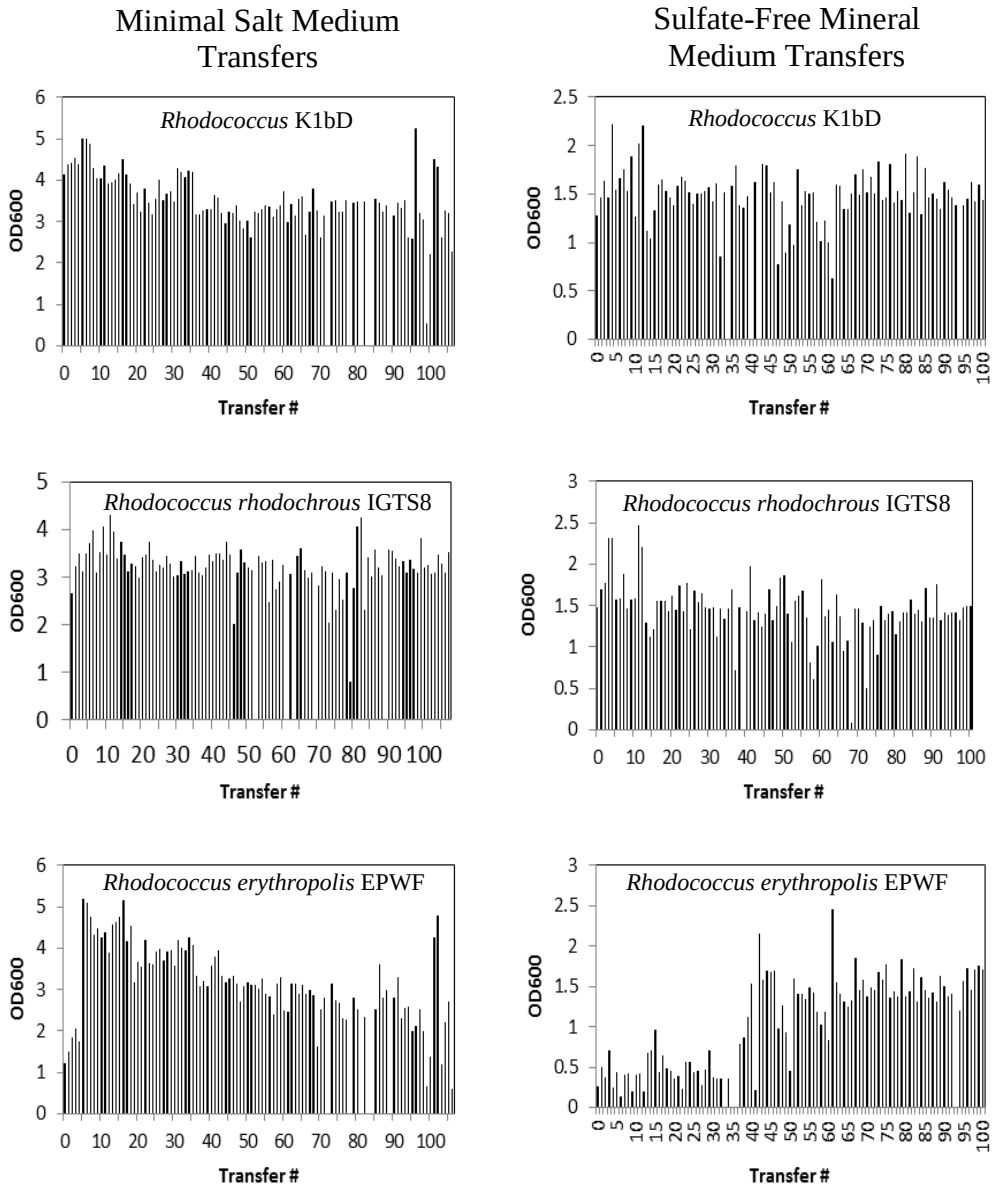

Figure 4-13 Sequential transfers of *R.* K1bD, *R. rhodochrous* IGTS8, and *R. erythropolis* EPWF in SFMM and MSM with 1.0mM $DBTO_2$

4.3.1 Specific desulfurization activity of sequential batches in SFMM

Experiments were performed on cultures taken from different transfers to assess their growth and desulfurization potential. **Figure 4-14** shows the specific desulfurization activity obtained from transferred cultures of *R. rhodochrous* IGTS8 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA for 48 hours of

incubation. Extractions were performed at 48h, once the 2-HBP concentration had stabilized. The cultures used for comparison were taken from the initial inoculum and the transfer numbers 25, 33, 50, 68, 80 and 100. Results show that after 25 transfers, the specific desulfurization activity (0.057µM/OD/hr) actually decreased by 34% in comparison to the initial inoculum (0.085µM/OD/hr). As more transfers were performed, the specific desulfurization activity increased until it reached a peak at approximately 0.101µM/OD/hr at transfer number 68. The specific desulfurization activity then decreased by 21% to 0.079 µM/OD/hr for transfer number 80, and by a further 9% to 0.0722 µM/OD/hr for transfer number 100. Specific desulfurization activity results for the initial inoculum were found to be statistically equivalent (using a t-test with 95% confidence) to those of transfer numbers 68. The specific desulfurization activity results of transfer number 33 were found to be statistically equivalent to those of transfers 50 and 100. Additionally, the specific desulfurization activity results of transfers 50, 80, and 100 were all found to be statistically equivalent.

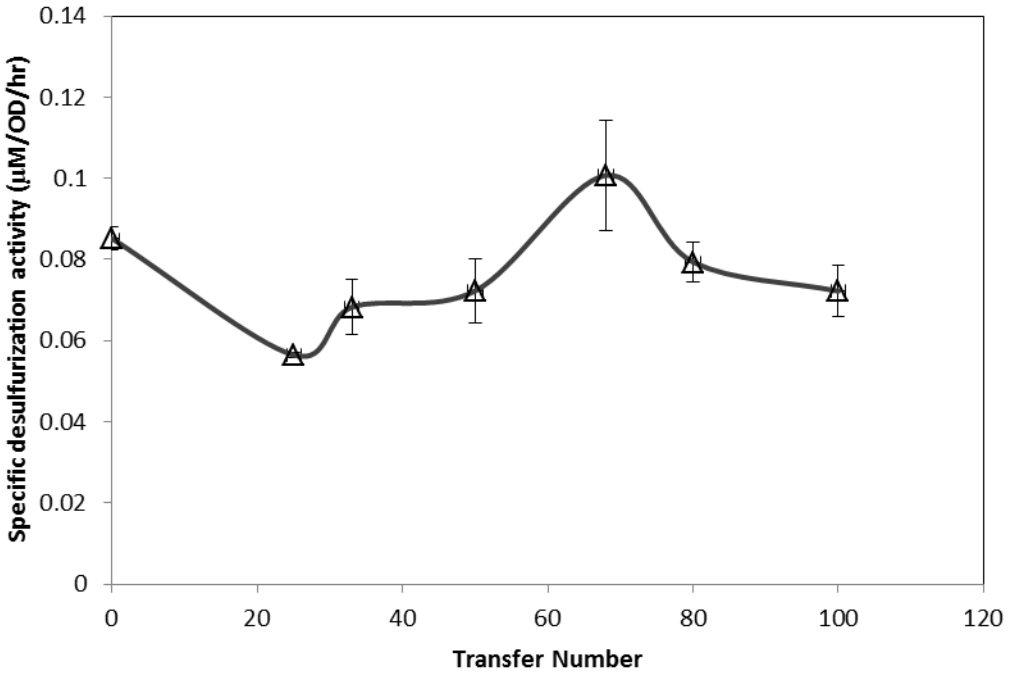

Figure 4-14 Comparison of specific desulfurization activity for transferred cells of *R. rhodochrous* IGTS8 growing in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA

4.3.2 Cell utilization of 2-hydroxybiphenyl

Experiments were conducted to determine if cultures of *R. rhodochrous* IGST8 transferred in SFMM with 1.0mM $DBTO_2$ were utilizing, rather than accumulating, 2-HBP. Growth experiments were performed with 2-HBP present as a co-substrate. **Figure 4-15** shows the values of 2-HBP concentrations, normalized with respect to the initial concentration, throughout cell growth in SFMM and MSM for the initial inoculum of *R. rhodochrous* IGTS8, transfers number 25 and 50. The figure shows no obvious change in the amounts of 2-HBP. An analysis of the residuals of the normalized 2-HBP results for SFMM with 2-HBP and MSM with 2-HBP can be found in **Figure 4-16**. No significant trends were observed in the residuals to indicate any significant change in 2-HBP concentration in the media. This confirms that *R. rhodochrous* IGTS8 is not readily utilizing 2-HBP as a carbon source even after 50 transfers. The residuals

for the original inoculum of *R. rhodochrous* IGTS8 grown in SFMM with 2-HBP does show a slight upward trend. No $DBTO_2$ was added to the media so that the accumulation of 2-HBP could be avoided. Therefore this upward trend can be attributed to error in measurements of the initial concentration at time zero.

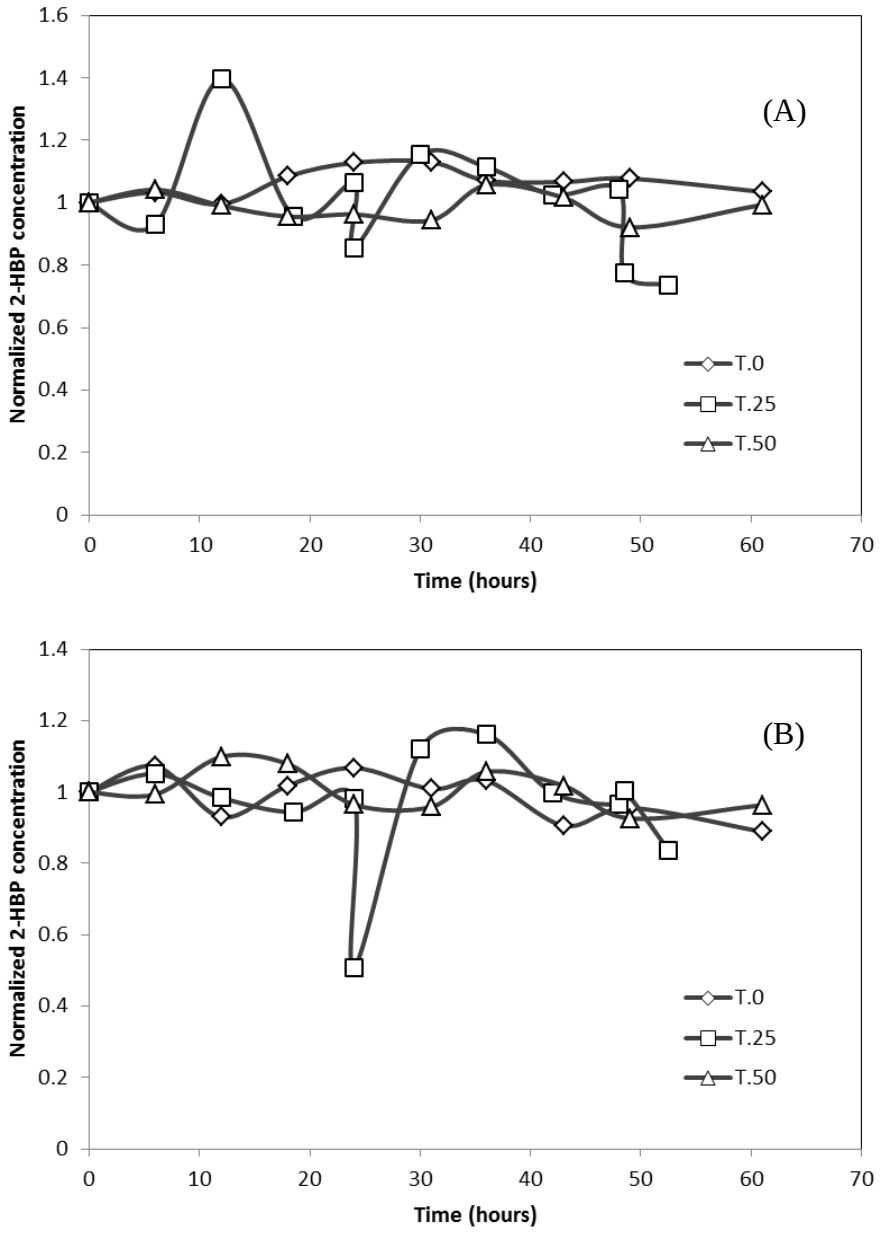

Figure 4-15 Normalized values of 2-HBP concentration for *R. rhodochrous* IGTS8 original inoculum (◊), T.25 (□) and T.50 (△) (transferred in SFMM + 1.0mM $DBTO_2$) grown in SFMM with 2-HBP **(A)** in MSM with 2-HBP **(B)**

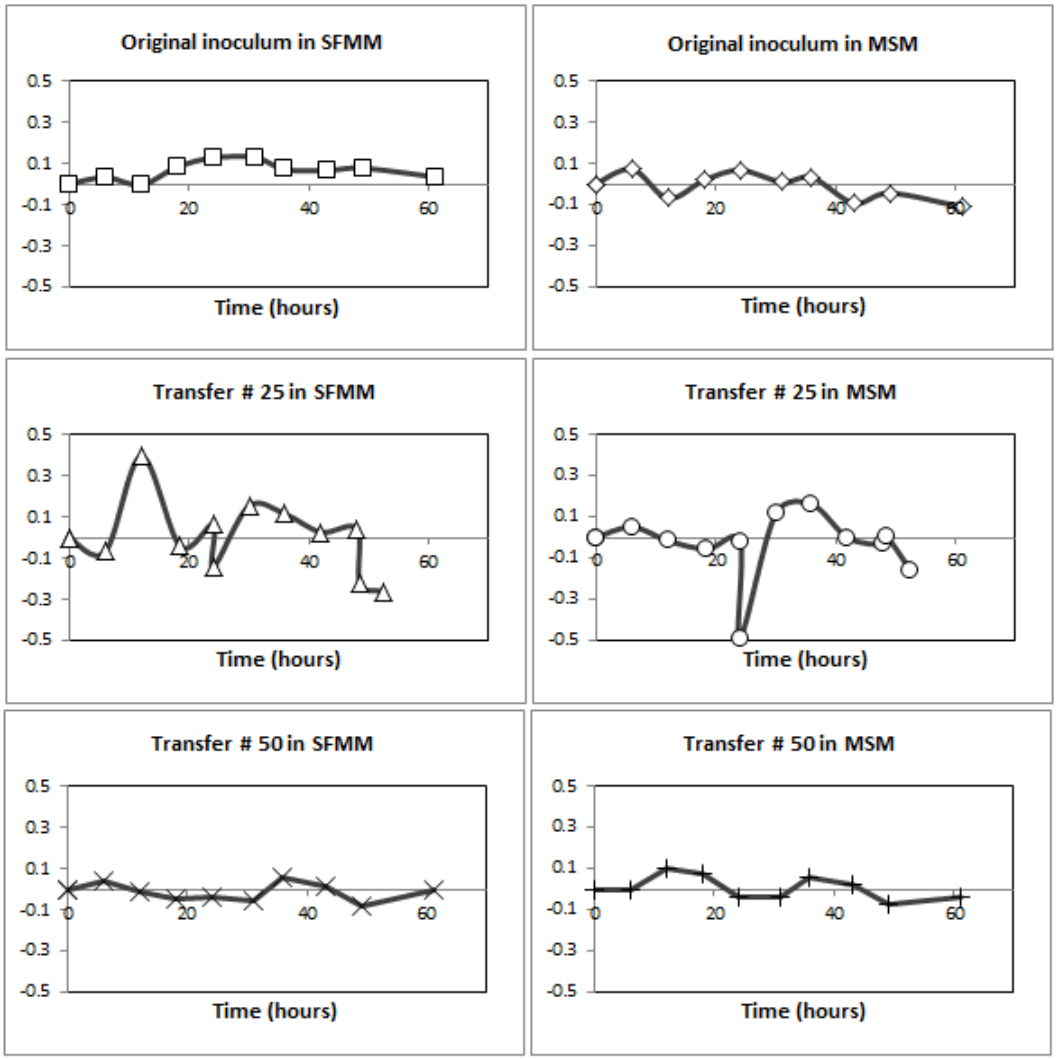

Figure 4-16 Analysis of residual of 2-HBP concentration for *R. rhodochrous* IGTS8. Results from the original inoculum (□,◇), transfer 25 (△,O) and transfer 50 (×,+) (transferred in SFMM with 1.0mM $DBTO_2$) grown in SFMM or MSM with added 2-HBP

4.3.3 Comparison of sulfur content in the cell biomass

Microscopy and cell dry weight measurements were conducted at the end of a batch experiment to assess the differences in growth between the original inoculum of *R. rhodochrous* IGTS8 and transfer number 50. The difference in cell concentrations is shown in **Figure 4-17**. The values were found to be 6.14E+07

cells/ml for the original inoculum and 5.77E+07 cells/ml transfer number 50. A t-test (with 95% confidence) confirmed that there these values were equivalent.

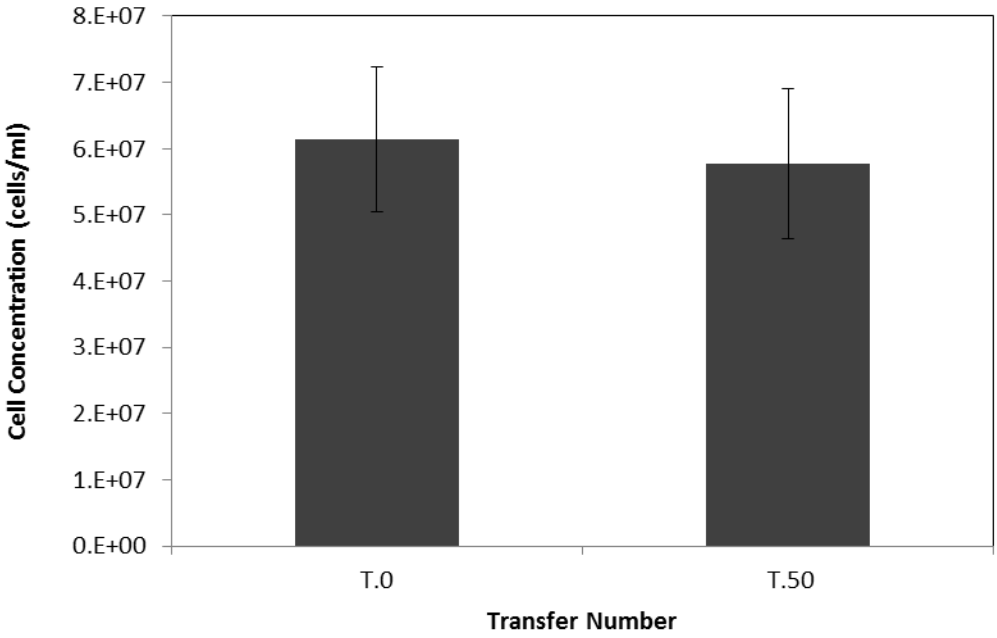

Figure 4-17 Cell concentration as determined by microscopy of the original inoculum of *R. rhodochrous* IGTS8 and transfer number 50 in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA

These results allowed the assessment of the yields of biomass and cells in the medium. **Figure 4-18** shows the results for the final biomass dry weight. The concentrations were found to be 0.53mg/ml and 0.52mg/ml, respectively. A t-test (with 95% confidence) confirmed that these results were equivalent.

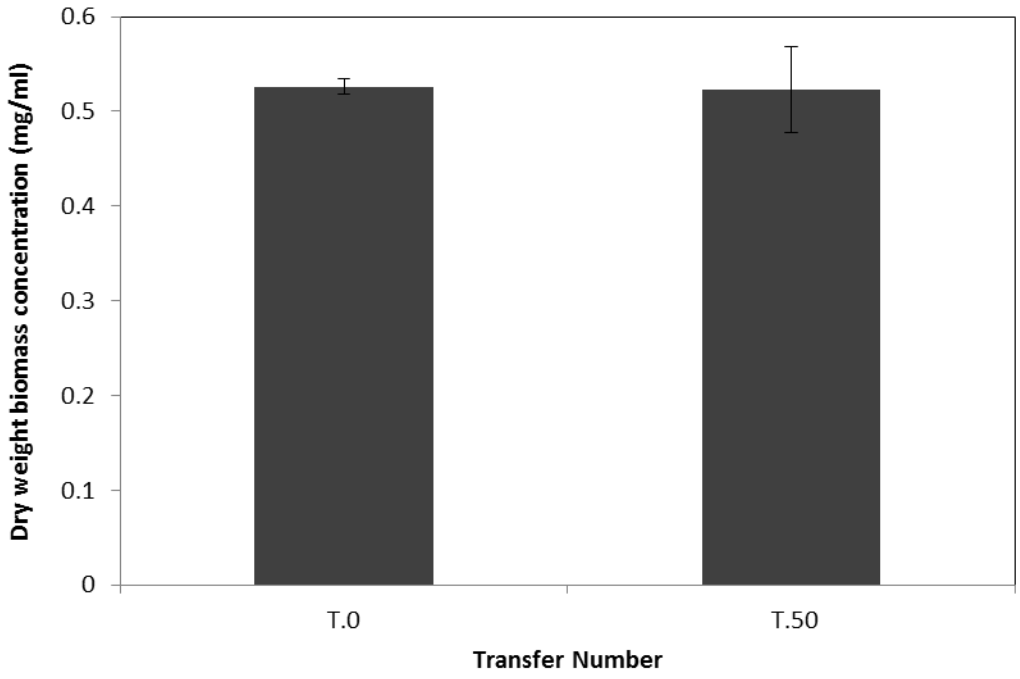

Figure 4-18 Dry weight biomass concentration for transferred cells of *R. rhodochrous* in sulfate-free mineral media with 0.25mM DBTO2 and 14mM DMFA

Elemental analyses were conducted on cells from the initial inoculum and from transfer number 50 of *R. rhodochrous* IGTS8 growing in SFMM with 0.25mM $DBTO_2$. **Figure 4-19** shows the sulfur concentration in weight % of the biomass. The standard deviation was obtained from two different cultures for the initial inoculum and three different cultures for transfer number 50. The average sulfur concentration was found to be 0.42% and 0.40%, respectively. A t-test (with 95% confidence) conducted on these results confirmed that there was no significant difference between the sulfur concentration in the original culture and the culture obtained after 50 transfers.

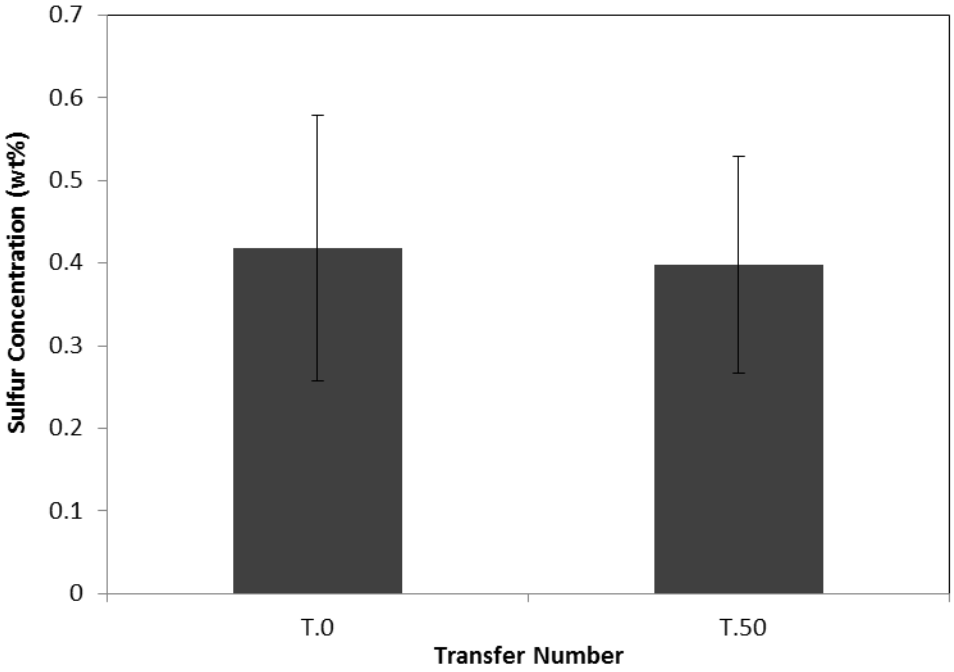

Figure 4-19 Sulfur elemental analysis results for the original inoculum of *R. rhodochrous* IGTS8 and transfer number 50 in SFMM with 0.25mM DBTO2 and 14mM DMFA

4.3.4 Production of sulfate and 2-hydroxybiphenyl

Figure 4-20A) compares the sulfate concentration in the media for the original inoculum and transfer number 50 and 100 of *R. rhodochrous* IGTS8 in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. The sulfate concentrations (mM) were determined to be 0.068 ± 0.005, 0.080 ± 0.001, and 0.067 ± 0.003 for the original inoculum and transfers number 50 and 100 of *R. rhodochrous* IGTS8 respectively. A t-test (with 95% confidence) conducted on these results determined that the sulfate concentration of the original inoculum was equivalent to that of transfer number 100.

Figure 4-20B) presents a comparison of the normalized amount of sulfate produced in mM per mM of 2-HBP produced in desulfurization experiments conducted with the original inoculum and transfers number 27, 50 and 100 of *R. rhodochrous* IGTS8 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA.

The ratio of sulfate to 2-HBP for the original inoculum and transfers number 50 and 100 of *R. rhodochrous* IGTS8 was determined to be 1.11, 1.49, and 1.12 respectively. A t-test (with 95% confidence) was conducted on these results. The original inoculum of *R. rhodochrous* IGTS8 was found to be statistically equal to that of transfer number 100. Transfer number 50 was found to be statistically different than the original inoculum and transfer number 100.

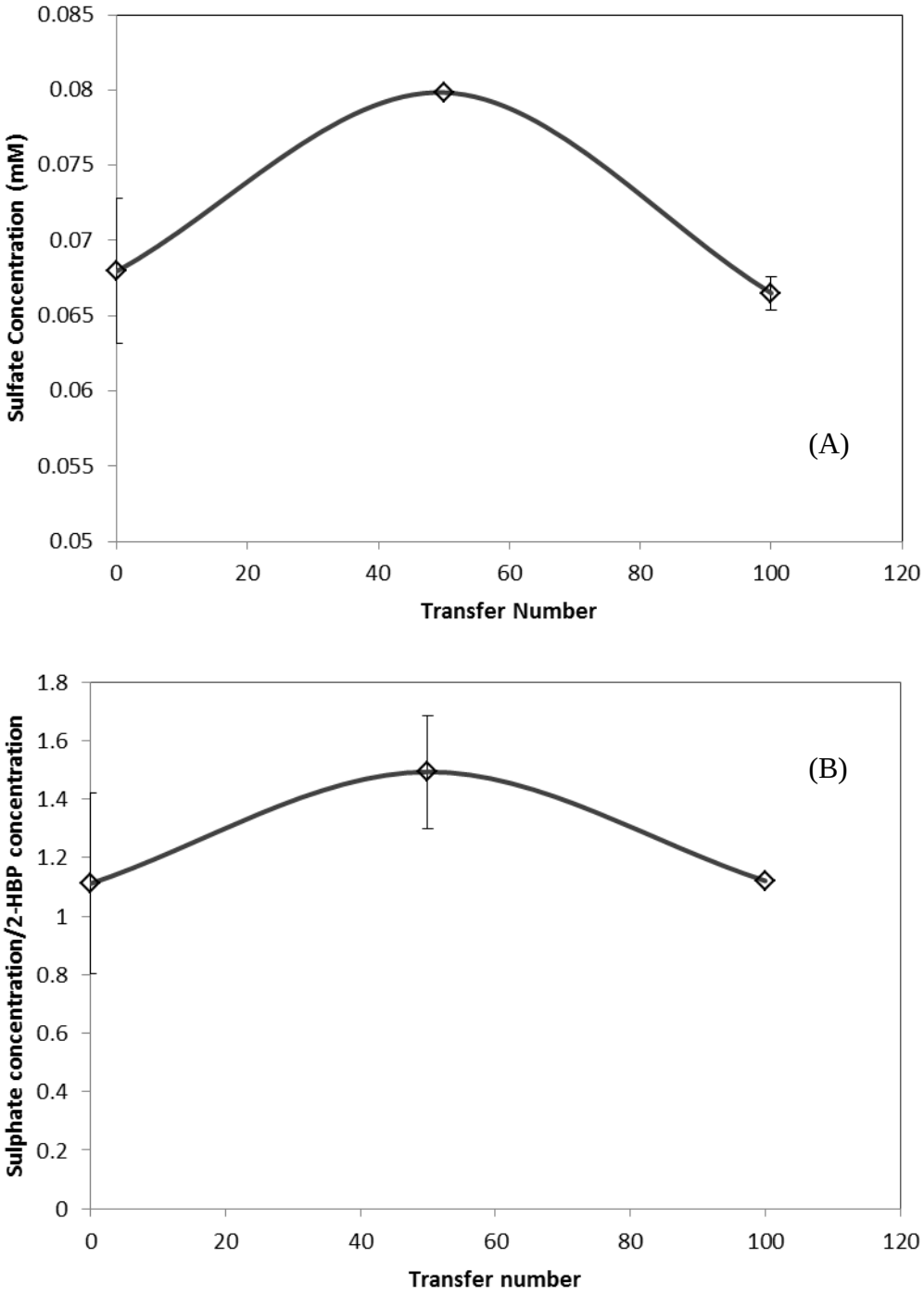

Figure 4-20 (A) sulfate concentration in the media for sequentially transferred cells of *R. rhodochrous* IGTS8 after 48 hours of growth **(B)** Comparison of sulfate produced (mM) to the yield of 2-HBP (mM)

4.3.5 Desulfurization activity of sequential transfers of *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF in MSM and *R.* K1bD and *R. erythropolis* EPWF in SFMM

Figure 4-21 shows the desulfurization yield of *R.* K1bD transfer number 51 and *R. erythropolis* EPWF transfer number 51 from sequential batches in SFMM with 1.0mM $DBTO_2$ after 48 hours. In these experiments, the cells were grown in SFMM with 0.25mM $DBTO_2$ and 14 mM DMFA. **Figure 4-21** also shows the desulfurization yield after 48 hours of growth for *R.* K1bD transfer number 95, *R. erythropolis* EPWF transfer number 95 and *R. rhodochrous* IGTS8 transfer number 74 from sequential batches in MSM with 1.0mM $DBTO_2$ and grown in MSM with 0.5mM $DBTO_2$ and 28mM DMFA for the comparison. The desulfurization yield of *R.* K1bD and *R. erythropolis* EPWF transfers 51 in SFMM were determined to be 0.058mM and 0.055mM respectively after 48 hours. No desulfurization was observed for *R.* K1bD and *R. erythropolis* EPWF transfers 95 in MSM. However the desulfurization yield of *R. rhodochrous* IGTS8 transfer number 74 in MSM was found to be 0.004mM after 48 hours.

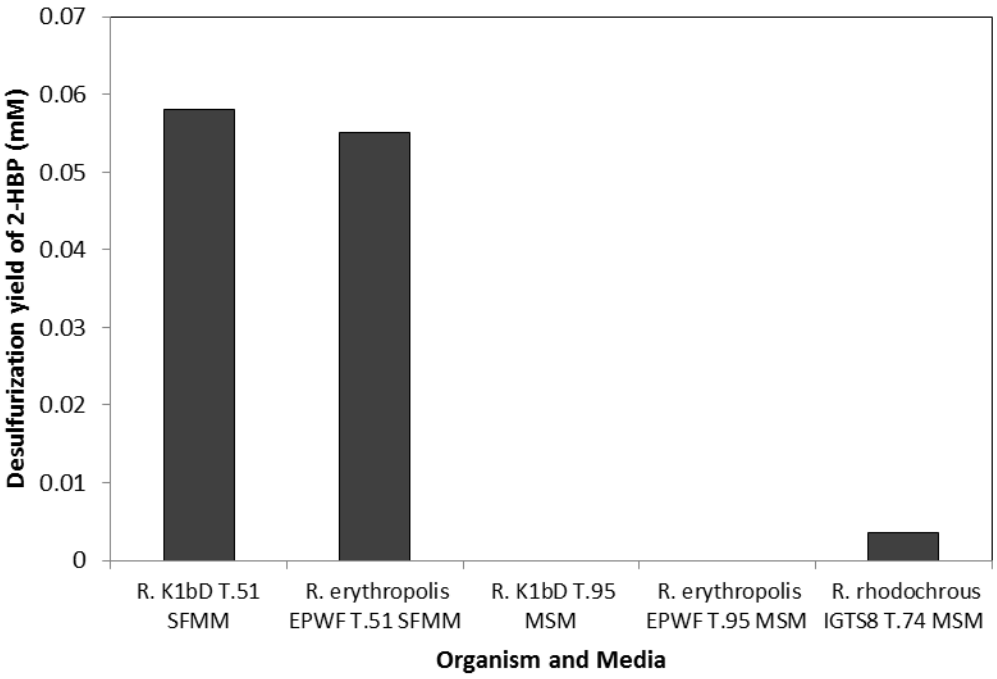

Figure 4-21 Desulfurization yield of *R*. K1bD T.51 and *R. erythropolis* EPWF T.51 in SFMM with 0.25mM $DBTO_2$and 14mM DMFA and *R*. K1bD T.95, *R. erythropolis* EPWF T. 95, and *R. rhodochrous* IGTS8 T.74 in MSM with 0.5mM $DBTO_2$ and 28mM DMFA

The specific desulfurization activity was compared for the initial inoculum and transfers number 36, 74, 92 and 100 of *R. rhodochrous* IGTS8 transferred in MSM with 1.0mM $DBTO_2$ and grown in MSM with 0.5mM $DBTO_2$ with 28mM DMFA for the comparison. The results can be found in **Figure 4-22**. A t-test (with 95% confidence) conducted on these results found the specific desulfurization activity of transfer number 36 was equivalent to that of transfers 74 and 92. The specific desulfurization activity of the original inoculum of *R. rhodochrous* IGTS8 was found to be statistically equivalent to that of transfer number 107.

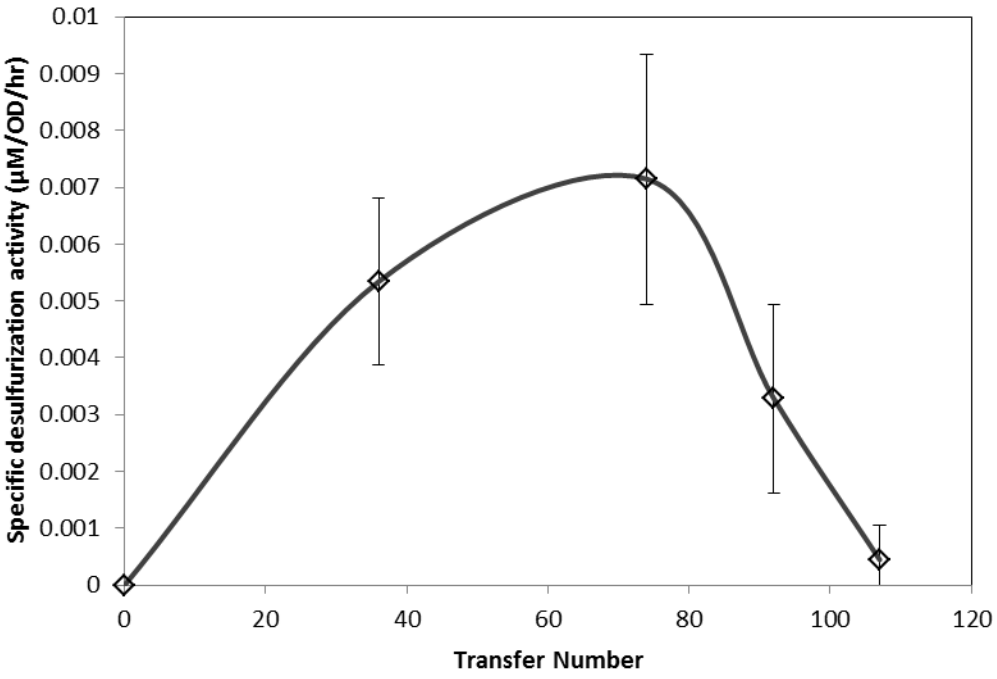

Figure 4-22 Comparison of the specific desulfurization activity for transferred cells of *R. rhodochrous* IGTS8 in MSM with 0.5mM $DBTO_2$ and 28mM DMFA

4.3.6 Comparison for the utilization of dibenzothiophene (DBT)

The growth and specific desulfurization activity of the original inoculum of *R. rhodochrous* IGTS8 and transfers number 25 and 50 were compared for growth in SFMM with 0.25mM dibenzothiophene (DBT) and 14mM DMFA as shown in **Figure 4-23**. **Figure 4-23A)** compares the growth of the original inoculum and transfers number 25 and 50. The growth rates were found to be 0.068, 0.074, and 0.078h^{-1} with final optical densities of 1.2, 1.2, and 1.4, respectively. The growth between the original inoculum and transfer number 25 are similar and reach the same final optical density. However, the growth of transfer 50 had a longer lag phase and the optical density increased rapidly to reach 1.8 at 36 hours. The optical density eventually decreased to 1.4 at 51 hours. The specific desulfurization activity of the original inoculum and transfers number 25 and 50 is shown in **Figure 4-23B).** The specific desulfurization activity was determined to be 0.067, 0.062, and 0.059µM/OD/hr for the original

inoculum and transfers number 25 and 50, respectively. A t-test (with 95% confidence) conducted on these results and they were found to be statistically equal to one another.

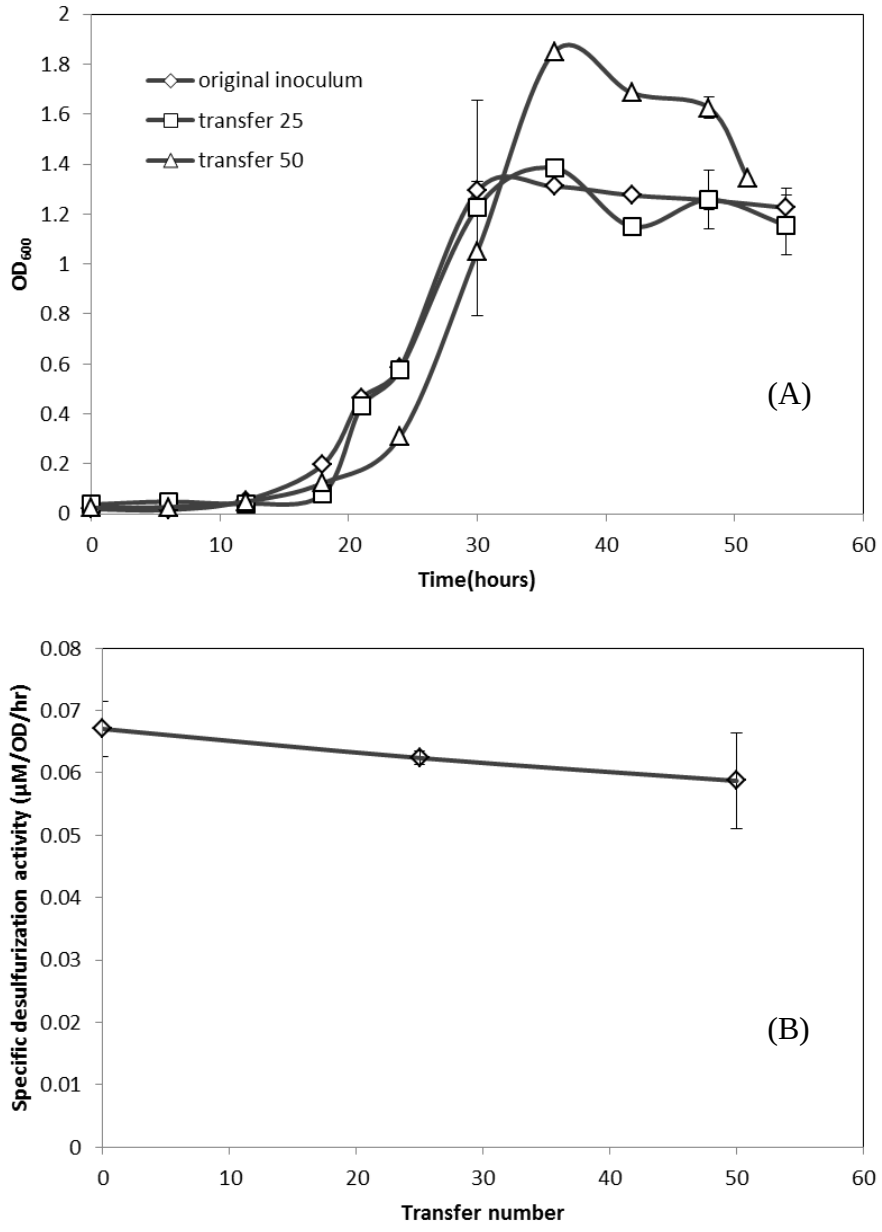

Figure 4-23 Comparison of growth (original inoculum (◇), transfer 25 (□), and transfer 50 (△)) **(A)** and specific desulfurization activity **(B)** from transferred cells of *R. rhodochrous* IGTS8 growing in SFMM with 0.25mM DBT and 14mM DMFA

4.4 Self-cycling fermentation (SCF) of *R. rhodochrous* IGTS8

Cultures of *R. rhodochrous* IGTS8 were grown by self-cycling fermentation (SCF) in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA to accustom the cells to the medium and apply a stronger selective pressure for desulfurization of $DBTO_2$. In each cycle the cultures were grown to stationary phase, which took approximately 12 hours. It should be noted that the initial cycle was equivalent to a batch and took approximately 48 hours. Values of optical density, taken before and at the end of the cycles, are shown in **Figure 4-24**. It should be noted that three subsequent sets of cycles were conducted to perform maintenance on the reactor. New sets of cycles were initiated at 220 hours and 580 hours. In theory, the optical density values at the start of a cycle should be half of the optical density at the end of the previous cycle. This oscillating pattern can be seen in **Figure 4-24.** The end of cycle optical density value of cycle 1 was 1.24. A decrease in the end of cycle optical density values of 11% occurred over cycles 1-15 (time zero to 235 hours). This is significantly lower than the decrease in end of cycle optical density values of cycles 16-42 (235 hours to 594 hours) which was found to be 30%. Optical density values weren't taken as frequently during cycles 43-69 (594 hours to 956 hours) making the trend in optical density values less obvious, however a decrease in end of cycle optical density values of 19% was observed.

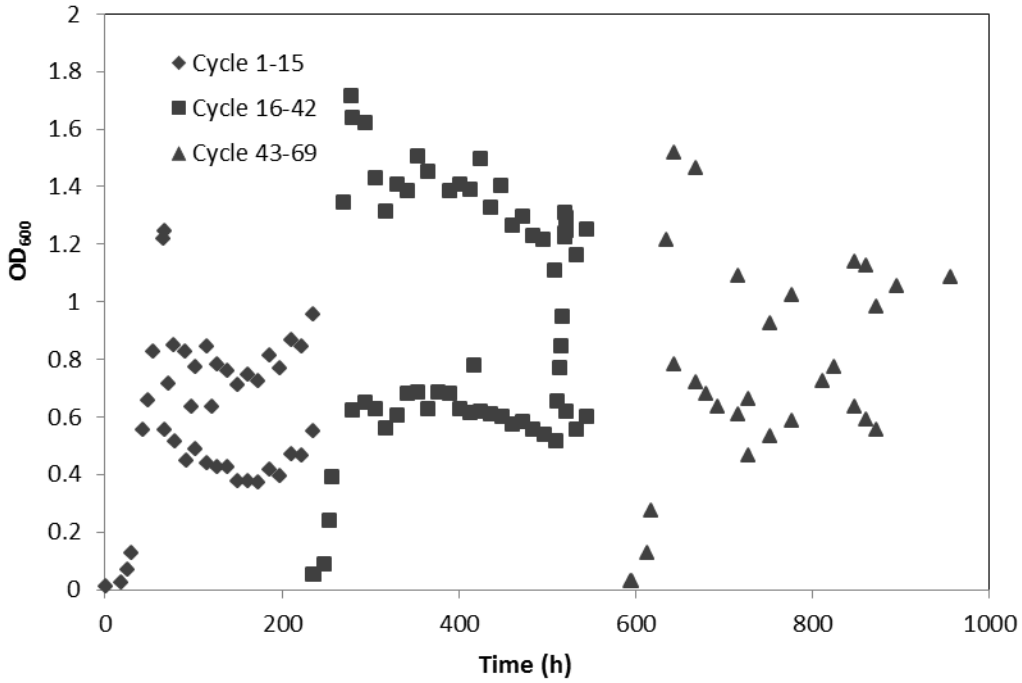

Figure 4-24 Optical density measurements during SCF operation of *R. rhodochrous* IGTS8 for 69 cycles in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA

Figure 4-25 shows the decrease in $DBTO_2$ and the desulfurization yield of 2-HBP (**Figure 4-25A)**) along with the optical density values (**Figure 4-25B)**) for cycle 36 of *R. rhodochrous* IGTS8 in the SCF with SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. It should be noted that 0mM of $DBTO_2$ and 0.032mM 2-HBP were present in the medium as residuals at the end of cycle 35. Thus, at the onset of cycle 36, after cycling, the concentration of $DBTO_2$ and 2-HBP were 0.040 and 0.013mM, respectively. The $DBTO_2$ depleted completely between 2 and 4 hours of growth in the fermenter. However the desulfurization yield of 2-HBP continued to increase over the 12 hours cycle up to 0.036mM. The yield of 2-HBP at the end of cycle 36 was 13% higher than the 2-HBP yield at the end of cycle 35. **Figure 4-25B)** shows that the optical density at the end of cycle 35 was 1.11 with an after cycle optical density value of 0.52 for the onset of cycle 36. The optical density values of cycle 36 increased until 10 hours, then remained stable around 1.24. The

optical density values at the end of cycle 36 are 12% greater than the end of cycle optical density value of cycle 35.

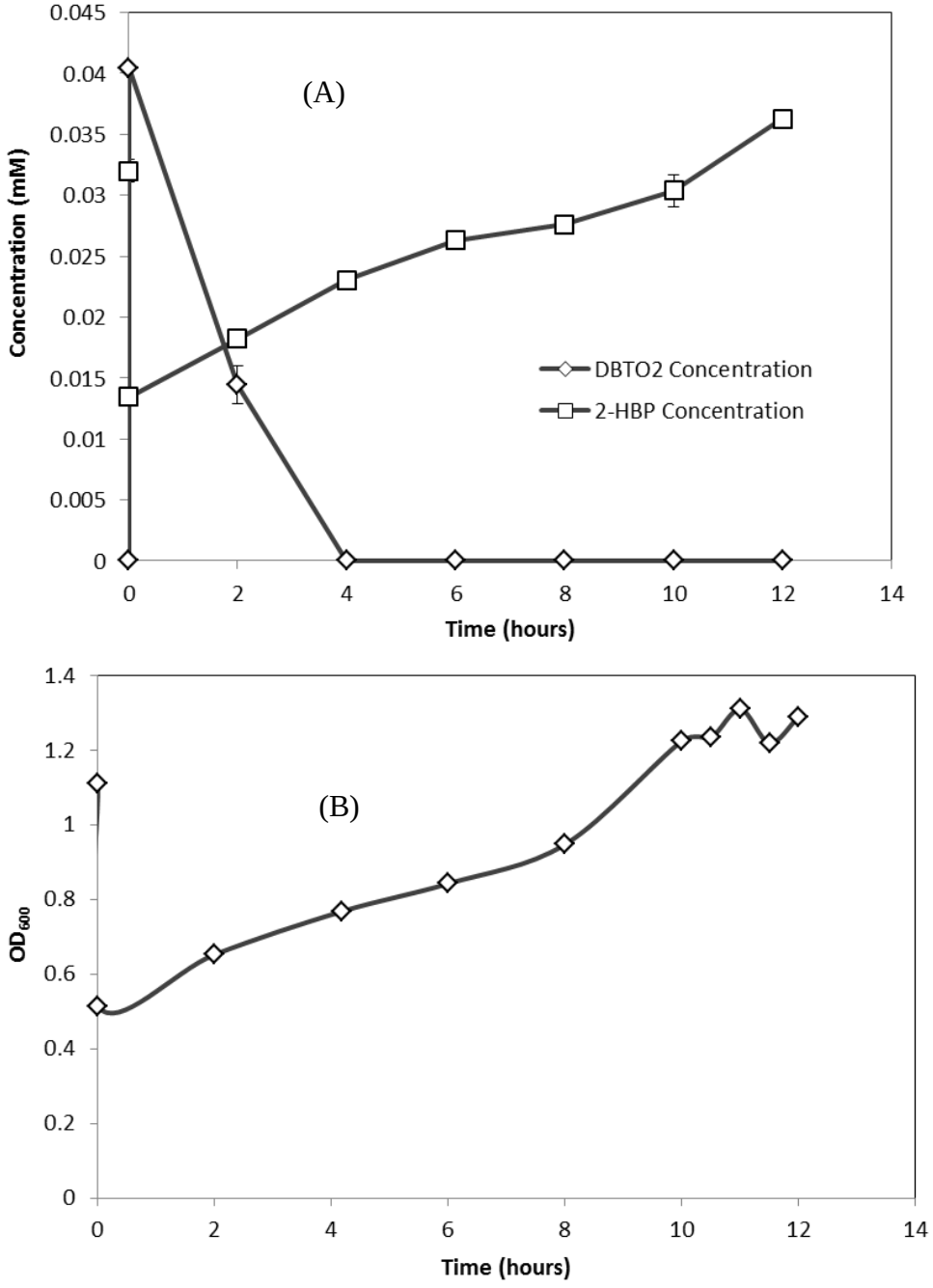

Figure 4-25 2-HBP accumulation (□) and $DBTO_2$ depletion (◇) **(A)** and optical density values **(B)** for cycle 36 of *R. rhodochrous* IGTS8 grown in the SCF with 0.25mM $DBTO_2$ and 14mM DMFA

Experiments were performed on cultures taken from different cycles to assess their growth and desulfurization potential. **Figure 4-26** shows the specific desulfurization activity obtained from cycled cultures (cycles 1, 15, 42, and 54) of *R. rhodochrous* IGTS8 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. Extractions were taken after 48 hours of incubation, once the 2-HBP concentration had stabilized. The cycled cells appear to oscillate in their desulfurization activity. The resulting specific desulfurization activities and standard deviation from the compared cycles were evaluated using a t-test with 95% confidence. No significant difference in specific desulfurization activity was observed for the cycled cells.

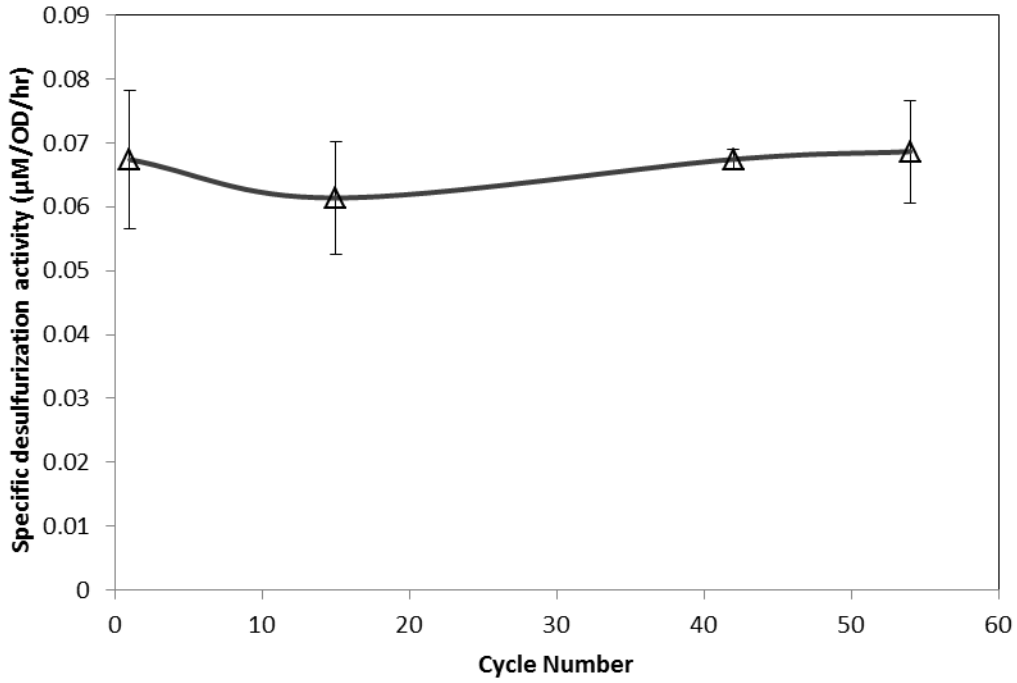

Figure 4-26 Comparison of specific desulfurization activity from cycled cells of *R. rhodochrous* IGTS8 in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA

5. CHAPTER 5: DISCUSSION FOR DESULFURIZATION OF OXIDIZED BITUMEN USING MICROORGANISMS THROUGH PROCESS-BASED DIRECTED EVOLUTION

5.1 Toxic and inhibitory compounds

5.1.1 Dibenzothiophene sulfone ($DBTO_2$)

The cell yield of *Rhodococcus* K1bD was tested in tryptic soy broth (TSB), M9 medium, and nutrient broth (NB) (**Figure 4-1**). The cultures reached the highest optical density values in TSB, so it was used to determine if dibenzothiophene sulfone ($DBTO_2$) had any toxic effects on the cultures. Seymour et al. (1997) was unable to determine a toxicity for $DBTO_2$ using a microtox assay as it was above the aqueous solubility limit of the compound (0.027mM). However, Gou et al. (2003) found that $DBTO_2$ concentrations of 0.4mM completely inhibited the growth of *Rhodococcus erythropolis* LSSE8-1. $DBTO_2$ ranging from 0mM to 2.5mM in TSB was used to test the growth of *R.* K1bD and *Rhodococcus erythropolis* EPWF. No significant differences in growth rate or biomass yield (**Figures 3-2** and **3-3**) were observed for either culture. Similarly, no differences were observed when *R.* K1bD was grown in minimal salt medium (MSM) with and without 1.0mM $DBTO_2$ (**Figure 4-4)**. It was then inferred that, under the range of concentrations tested (0mM to 2.5mM) that $DBTO_2$ did not display toxicity for *R.* K1bD and *R. erythropolis* EPWF.

In all cases studied, the addition of $DBTO_2$ also did not improve the growth of cells (**Figures 3-2, 3-3** and **3-4**). This had two implications: firstly, that $DBTO_2$ and its metabolites were not used as carbon sources; and secondly, that the sulfur contained in TSB and MSM was not limiting to the growth of the organisms.

5.1.2 Sulfate

Ohshiro et al. (1996) found that concentrations of dibenzothiophene (DBT) higher than 3mM repressed the desulfurization activity. However they suggested that this was not caused by the DBT itself, but by the enzymatic release of sulfate caused by the desulfurization of DBT. They determined that, in cultures of microorganisms degrading 0.3mM DBT, the presence of sodium sulfate at concentrations as low as 0.1mM in the cell medium resulted in a reduction of specific desulfurization activity of 72% when compared to cases in which only DBT was present. The addition of 0.5mM sodium sulfate to the media containing 0.3mM DBT was found to completely repress the activity. Additionally, they found that the presence of sulfate added to cell free extracts did not inhibit activity even up to sulfate concentrations of 1mM. These results were also confirmed by Wang and Krawiec (1996) who found that sulfate represses the expression of the phenotype but does not inhibit the desulfurization activity of the enzymes. Ma et al. (2006) demonstrated that sulfate concentrations of 0.04mM did not have a noticeable effect on cell growth; however they also found that the presence of sulfate could alleviate some of the inhibition to cell growth caused by 2-hydroxybiphenyl (2-HBP) accumulation.

In the present study, the concentration of sulfate in MSM medium was 0.85mM. This is well above the complete repression limit for the 4S pathway of 0.5mM found by Ohshiro et al. (1996). Gas chromatography analysis of extractions conducted on *R.* K1bD cultures grown in TSB and MSM showed no formation of 2-HBP, the end product of the 4S desulfurization pathway. As *R.* K1bD was shown to desulfurize $DBTO_2$ in sulfate free mineral medium (SFMM) and produce 2-HBP (**Figure 4-12**), the lack of desulfurization in TSB and MSM was consistent the presence of sulfate inhibiting the 4S biodesulfurization pathway.

5.1.3 2-Hydroxybiphenyl

2-HBP has been shown to exhibit toxicity to microbial cells. Alves and Paixão (2011) found that 0.52mM 2-HBP resulted in a 50% inhibition of the bacterial baseline respiration rate after 3 hours. Ohshiro et al. (1996) showed that the inhibitory effects of 2-HBP were greater in media containing DBT than in media containing sodium sulfate. This suggests that the accumulation of 2-HBP has a direct impact on the 4S desulfurization pathway and not on the growth of the cell. The effects of biphenyl were also tested by Ohshiro et al. (1996) and no inhibitory effects were observed; this indicates that the hydroxyl group on 2-HBP plays an important role in the inhibition activity. In the present study, the maximum 2-HBP concentration obtained was 0.063mM for *R. rhodochrous* IGTS8 grown in SFMM with 1.0mM $DBTO_2$ (**Figure 4-10**). Therefore, the toxic effects of 2-HBP were not a concern for this study. However, increased desulfurization activity is desired and the toxicity of 2-HBP is an issue that will need to be overcome. Alves and Paixão (2011) showed that the toxic effects of 2-HBP had more of an impact in the late growth phase. They hypothesized that if a microorganism were evolved to exhibit faster generation times they may be more resistant to the toxic 2-HBP accumulation. A comparison on the toxicity of 2-HBP on the evolved organisms was not conducted in this study.

5.1.4 Dimethylformamide

Dimethylformamide (DMFA) was utilized to increase the solubility of $DBTO_2$ in the media. Alves and Paixão (2011) showed that DMFA concentrations of 9.91% v/v resulted in an inhibition of 50% of the bacterial respiration rate after 3 hours. The effect of DMFA addition on the growth of *Rhodococcus rhodochrous* IGTS8 in SFMM was tested in the present study using concentrations of 0.04%, 0.12%, and 0.215% v/v. No change in final cell yield was observed (data not shown). The DMFA concentration utilized in this study

was 0.108% v/v (14mM) which is well below the inhibitory concentration observed by Alves and Paixão (2011).

5.2 Comparison of growth rates and final optical density values

5.2.1 *Rhodococcus* K1bD

A comparison of the cell yield of *R.* K1bD grown in TSB, NB, and M9 media was performed to determine which media would result in the greatest cell yield (**Figure 4-1**). TSB showing the greatest cell yield was then utilized to determine the effect of varying $DBTO_2$ concentrations. The higher cell yields observed for TSB and NB were expected since these media contain excess nutrients whereas M9 is a minimal synthetic medium. Minimal media were preferred in process based directed evolution experiments with $DBTO_2$ as they ensured that the only selective pressure on the cultures was the presence of the sulfur compound and not of other nutrients in the medium. These results were similar to those observed for the growth of *R.* K1bD in **Figure 4-2** and **Figure 4-4**. *R.* K1bD grew faster ($0.27h^{-1}$ compared to $0.15h^{-1}$) and reached higher optical density values (12.6 compared to 4.0) in TSB compared to MSM, a minimal salt media.

The addition of $DBTO_2$ in concentrations ranging from 0 to 2.5mM in TSB media showed no effect on the growth rate ($0.27\pm0.02\ h^{-1}$) and final optical density values (12.7±1.1) of *R.* K1bD (**Figure 4-2**). Stationary phase was reached for all cultures at approximately 40 hours**.** This further confirms that *R.* K1bD was not utilizing $DBTO_2$ as a sulfur source in TSB. Although there was a range in final optical density values between 10.8 and 14.2, there was no trend showing that the addition of $DBTO_2$ affected the final cell yields.

Similar results were found for the addition of $DBTO_2$ (1.0mM) in MSM (**Figure 4-4)**: no difference was observed in growth rate ($0.152 \pm 0.004\ h^{-1}$) or final optical density values (4.0 ± 0.1) for cultures grown with and without $DBTO_2$. The stationary phase occurred around 35 hours. This result can be

explained by the fact that the presence of sulfate in the medium inhibited the 4S pathway and prevented the utilization of $DBTO_2$ as a sulfur source as discussed in **Section 5.1.**

The growth rate of *R.* K1bD in SFMM with 1.0mM $DBTO_2$ as the only sulfur source was 0.078 h^{-1} with a final optical density value of 1.7. Stationary phase was reached around 35 hours. The growth rate and final cell yield of *R.* K1bD grown in SFMM with 1.0mM $DBTO_2$ were significantly lower than the growth in other media. This is consistent with the fact that $DBTO_2$ was a limiting nutrient in SFMM.

5.2.2 *Rhodococcus erythropolis* EPWF

Similar results were observed for *R. erythropolis* EPWF grown in TSB with $DBTO_2$ (**Figure 4-3**). The addition of $DBTO_2$ at concentrations ranging from 0 to 2.5mM in TSB medium showed no change in growth rate (0.29 ± 0.04 h^{-1}) or final optical density values (14.6 ± 0.7). Stationary phase was reached after approximately 50 hours. Therefore *R. erythropolis* EPWF was unable to utilize $DBTO_2$ as a carbon source for growth (**Figure 4-5**). *R. erythropolis* EPWF has been shown to oxidize aliphatic sulfides but not DBT, in fact it does not utilize the 4S pathway (Kirkwood et al., 2005). Therefore, *R. erythropolis* EPWF was not expected to readily grow in SFMM with $DBTO_2$ over many generations. However, the organism grew at a growth rate of 0.03 h^{-1} and reached a final optical density of 1.4 after 55 hours (**Figure 4-5**). This final optical density value was not replicated in the first 35 sequential transfers of *R. erythropolis* EPWF in SFMM with 1.0mM $DBTO_2$ (**Figure 4-13)**. The culture grown in **Figure 4-5** was initially grown in MSM with 1.0mM and then transferred to SFMM with 1.0mM $DBTO_2$ to continue growth. It is assumed that minimal amounts of sulfate present in the transfer volume supported the cell growth observed in **Figure 4-5**.

5.2.3 *Rhodococcus rhodochrous* IGTS8 (ATCC 53968)

The growth of *R. rhodochrous* IGTS8 in SFMM with 1.0mM $DBTO_2$ was compared to the growth of *R.* K1bD and *R. erythropolis* EPWF (**Figure 4-5**). *R. rhodochrous* IGTS8 grew at a growth rate of $0.059h^{-1}$.

R. rhodochrous IGTS8 was used to test the kinetics of varying the concentrations of the carbon source and the sulfur source in SFMM with $DBTO_2$. Again, as with the two other strains tested, the results showed that $DBTO_2$ had no effect on cell growth or final cell yield (**Figure 4-10A)**).

Varying the concentration of the carbon source, sodium acetate, for 2 g/L to 6g/L while keeping the concentration of $DBTO_2$ constant in SFMM with 1.0mM $DBTO_2$ led to increasing yields of biomass (**Figure 4-11A)**) – suggesting $DBTO_2$ was not limiting. Although it was important to have strong growth cultures, it was also important to select growth conditions that resulted in the high desulfurization activity. Specific desulfurization activity of *R. rhodochrous* IGTS8 in varying $DBTO_2$ and sodium acetate concentrations was compared in **Figure 4-10B)** and **Figure 4-11A)** and is further discussed in **Section 5.3.3**.

5.2.4 Comparison of *Rhodococcus* species

Comparing the growth of *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF in SFMM with 1.0mM $DBTO_2$ (**Figure 4-5**) showed that *R. rhodochrous* IGTS8 and *R.* K1bD grew fairly similarly, with growth rates of $0.06h^{-1}$ and $0.078h^{-1}$, respectively. *R. erythropolis* EPWF, however, grew much slower at a rate of $0.03h^{-1}$. *R. rhodochrous* IGTS8 grew to the highest optical density of 1.8. Based on its strong growth and relatively fast growth rate, *R. rhodochrous* IGTS8 was chosen to further investigate some aspects of the present study.

5.3 Desulfurization and 2-HBP accumulation

5.3.1 Gas chromatography and gas chromatography-mass spectrometry analysis

A comparison of the gas chromatography chromatograms obtained for *R. rhodochrous* IGTS8 growing in SFMM with 1.0mM $DBTO_2$ is shown in **Figure 4-6**. Both chromatograms show a similar amount of octadecane (C_{18}) and $DBTO_2$. However the appearance of a new peak is observed in **Figure 4-6B)** after 48 hours of incubation.

Gas chromatography-mass spectrometry of the sample in **Figure 4-6** identified the new peak as 2-HBP (**Figure 4-7B)**). The retention times were shorter in the gas chromatography-mass spectrometry run due to slight differences in the column type and in the method. However, the peaks were determined to be (in order of appearance) 2-HBP, the internal standard octadecane (C_{18}), and $DBTO_2$ **Figure 4-7**. It is known that 2-HBP is the end product of the 4S biodesulfurization pathway for DBT (Soleimani et al., 2007). *R. rhodochrous* IGTS8 has been shown to oxidize DBT to $DBTO_2$ and ultimately produce 2-HBP through the use of the 4S pathway (Soleimani et al., 2007). The results found in **Figure 4-7** confirms that *R. rhodochrous* IGTS8 uses $DBTO_2$ through the 4S pathway to produce 2-HBP. The impurities that appear in the gas chromatography samples, such as the peak at a retention time of 9.69 minutes as shown in **Figure 4-6**, did not appear in the gas chromatography-mass spectrometry results of the sample shown in **Figure 4-7** therefore confirming their insignificance.

A sample of *R.* K1bD grown SFMM with 1.0mM $DBTO_2$ was also ran through the gas chromatograph-mass spectrometer. The same peaks were identified, showing that *R.* K1bD also utilizes $DBTO_2$ through the 4S pathway to produce 2-HBP. This is not a surprise as *R.* K1bD has also been shown to utilize DBT through the 4S pathway and produce 2-HBP (Kirkwood et al., 2005). The use of $DBTO_2$, and therefore the desulfurization yield of 2-HBP, via the 4S pathway for Rhodococcus species was also observed by Ohshiro et al. (1996).

Extractions and gas chromatography analysis was also conducted on cultures of *R.* K1bD and *R. erythropolis* EPWF grown in TSB and MSM with 1.0mM $DBTO_2$ and no desulfurization activity was observed. Additionally, no desulfurization activity was observed for cultures of *R. rhodochrous* IGTS8 grown in MSM with 1.0mM $DBTO_2$.

5.3.2 Measures of desulfurization

It is expected that a decrease in $DBTO_2$ concentration would occur as the microorganisms utilize the sulfur and form 2-HBP. However the aqueous solubility of $DBTO_2$ is 0.027mM (Seymour et al., 1997), therefore issues with the solubility of $DBTO_2$ are accentuated when in larger concentrations such as 1.0mM. This leads to inaccurate $DBTO_2$ concentration results from the extractions and gas chromatography analysis. A steady increase in 2-HBP concentration over 48 hours of growth for *R. rhodochrous* IGTS8 in SFMM with 0.5mM $DBTO_2$ is observed in **Figure 4-8B)**. However, no discernible trend was observed for the concentrations of $DBTO_2$ over 48 hours giving no quantitative indication that the accumulation of 2-HBP was in fact due to the desulfurization of $DBTO_2$. There appears to be an initial increase in $DBTO_2$ concentration in **Figure 4-8B)**, which is contradictory to the increase in accumulation of 2-HBP. As the change in $DBTO_2$ concentration has been shown to be an unreliable indicator of desulfurization, the accumulation of 2-HBP was used, as in other studies (Ma et al., 2006; Ohshiro et al., 1996) as monitor of desulfurization.

Several studies have dissolved DBT in ethanol or methanol before adding it to the media (Duarte et al., 2001; Kirkwood et al., 2005; Wang and Krawiec, 1996). However, ethanol was unsuccessful at dissolving $DBTO_2$ in in the same concentrations used for DBT. DMFA has also been utilized to dissolve DBT along with $DBTO_2$ (Alves and Paixão, 2011). In the present study, *R. rhodochrous* IGTS8 was grown in SFMM with 0.25mM $DBTO_2$ and 14mM (0.108% v/v) DMFA (**Figure 4-9)**.

Based on these conditions, a downward trend in $DBTO_2$ over 54 hours of cell growth was observed. This indicated that the addition of DMFA improved the solubility and extraction of $DBTO_2$. However, the concentration results still varied quite significantly in the early stages of growth (**Figure 4-9)** confirming that change in $DBTO_2$ concentration was still unreliable to determine desulfurization activity. The change in solubility of $DBTO_2$ with the addition of DMFA was not determined; however an improvement in solubility would make $DBTO_2$ more bioavailable to the microorganisms and would improve desulfurization activity. A similar increase in 2-HBP concentration for *R. rhodochrous* IGTS8 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA in **Figure 4-9** was observed in **Figure 4-8**. This shows that the addition of DMFA did not affect the concentration of 2-HBP and that the accumulation of 2-HBP was still the best indicator of desulfurization activity.

Wang and Krawiec (1996) found that the appearance of 2-HBP lagged behind the disappearance of $DBTO_2$. In the present study, the accumulation of 2-HBP was found to become stable around 48 hours, even though the optical density results indicate stationary phase was reached around 30 hours (**Figure 4-9**). Therefore, a 48-hour time period was utilized for experiments comparing the desulfurization yields and the specific desulfurization activities of the cultures.

5.3.3 Effect of carbon source and sulfur source concentrations on the specific desulfurization activity

A comparison of the growth curves obtained for *R. rhodochrous* IGTS8 grown in varying concentrations of $DBTO_2$ from 0.1 to 1.0mM in SFMM showed similar growth rates – 0.072, 0.095, 0.082 and $0.077h^{-1}$ (**Figure 4-10A)**). Similarly, the final optical density values were not significantly affected by the different concentrations – ranging from 1.6 to 1.9. The specific desulfurization activity of *R. rhodochrous* IGTS8 was also determined (**Figure 4-10B)**) from these conditions. The specific desulfurization activities of *R. rhodochrous* IGTS8 grown in SFMM with 0.1, 0.25, 0.5, and 1.0mM $DBTO_2$ were found to be 0.037,

0.075, 0.069 and 0.067µM/OD/hr respectively. No trends were observed for the growth rate and final optical density values. The specific desulfurization activity was found to be similar for growth with 0.5 and 1.0mM $DBTO_2$. The specific desulfurization activity was found to be the highest in SFMM with 0.25mM $DBTO_2$ even though the growth rates and optical density values were similar. This indicates that higher concentrations of $DBTO_2$, such as 0.5 and 1.0mM, may have an impact on enzymatic activity. Additionally the lower specific desulfurization activity observed for growth with 0.1mM could be due to the fact that as desulfurization occurred there reached a point where there was not enough $DBTO_2$ present to further induce the desulfurization activity. It has been suggested that DBT is required to induce the 4S desulfurization pathway (Ma et al., 2006). It can then be assumed that desulfurization studies starting with $DBTO_2$ would also require $DBTO_2$ present to induce the desulfurization pathway. The present results confirm that $DBTO_2$ is capable of inducing desulfurization via the 4S pathway. Growth with $DBTO_2$ concentrations of 0.25mM were utilized in the majority of experiments, as it was the lowest concentration tested that resulted in the highest specific desulfurization activity.

The growth of *R. rhodochrous* IGTS8 was also tested with varying concentrations of carbon source (sodium acetate) in SFMM with 1.0mM $DBTO_2$ to determine if sulfur, rather than carbon was limiting under the conditions tested (**Figure 4-11**). A comparison of the growth curves obtained for *R. rhodochrous* IGTS8 grown in varying sodium acetate concentrations – from 2 to 6 g/L – with 1.0mM $DBTO_2$ shows similar growth rates and increasing final cell yield (**Figure 4-11A)**). The specific desulfurization activity of *R. rhodochrous* IGTS8 was shown to decrease with increasing concentrations of the carbon source – from 0.077 to 0.057µM/OD/hr (**Figure 4-11B)**). The specific desulfurization activity was the highest for medium containing 2g/L sodium acetate. However, the final cell concentration for growth in 2g/L sodium acetate was significantly lower than the growth with 4g/L and 6g/L (**Figure 4-11A)**). This indicated that the decrease in carbon substrate was substantially limiting cell growth which was undesirable. The final cell yield increased significantly for medium containing 6 g/L sodium

acetate; however, the specific desulfurization activity was lower. This was brought about by a lower yield of 2-HBP, indicating that the increase in 2-HBP was not proportional to the increase in cell concentration. Since increasing the sodium acetate concentration from 4 to 6g/L did not have a beneficial impact on the desulfurization activity of the cells, the recommended SFMM recipe with 4g/L sodium acetate was utilized.

5.3.4 Comparison of desulfurization yield for *Rhodococcus* species

A quantitative comparison of the desulfurization yield of *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA after 48 hours was performed (**Figure 4-12**). Similar desulfurization was observed for *R. rhodochrous* IGTS8 and *R.* K1bD (0.052mM and 0.056mM 2-HBP were produced over 48 hours, respectively). No accumulation of 2-HBP was observed for *R. erythropolis*. This was expected as *R. erythropolis* EPWF is unable to oxidize DBT and utilize the 4S pathway to produce 2-HBP (Kirkwood et al., 2005). The fact that no formation of 2-HBP was observed in the abiotic control confirms that the microorganisms were solely responsible for the production of the 2-HBP.

5.4 Sequentially transferred cells

5.4.1 Change in cell yields over the sequential transfers

Investigating the impact of sequential transfers on cultures of *R. rhodochrous* IGTS8, *R.* K1bD and *R. erythropolis* EPWF in MSM with 1.0mM $DBTO_2$, and SFMM with 1.0mM $DBTO_2$ led to interesting results (**Figure 4-13**). 107 transfers were completed for *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF in MSM with 1.0mM $DBTO_2$. 100 transfers were completed for *R. rhodochrous* IGTS8, *R.* K1bD and *R. erythropolis* EPWF in SFMM with 1.0mM $DBTO_2$. Each transfer represents approximately 8 generations. Some of the fluctuations that appear in the optical density values can be attributed to

variations in the growth time for the cultures. The cultures were usually transferred every 45-55 hours, however occasionally they were not transferred until 72 hours. Additionally slight variations in medium composition lead to more variations. However, neglecting the minimal impact of these slight variations, overall trends in final cell yields can be observed. For *R. rhodochrous* IGTS8 grown in MSM and SFMM with 1.0mM $DBTO_2$, a slight decrease in final optical density of 12 and 25% was observed over the 107 and 100 transfers, respectively. For *R.* K1bD, a significant decrease (37%) was only observed when grown in MSM with 1.0mM $DBTO_2$. Finally, in the case of *R. erythropolis* EPWF, a significant decrease (65%) was observed when growing in MSM with 1.0mM $DBTO_2$, but, more interestingly, an increase of 375% in final cell yield was observed after 100 transfers in SFMM with 1.0mM $DBTO_2$. The decreases can be attributed to growth in synthetic media which have low concentrations of rare or trace nutrients. The significant increase in yield may be the result of an adaptation of *R erythropolis* EPWF to the desulfurization of $DBTO_2$.

R. rhodochrous IGTS8 had the smallest decrease in final optical density for growth in MSM with 1.0mM $DBTO_2$ when compared to *R.* K1bD and *R. erythropolis* EPWF. Its decrease in final optical density values of 12% was relatively small. This shows that *R. rhodochrous* IGTS8 was able to maintain a relatively stable cell yield over 107 transfers in MSM with 1.0mM $DBTO_2$. This decrease can be attributed to *R. rhodochrous* IGTS8 shifting its sulfur utilization from sulfate to $DBTO_2$ as indicated in **Figure 4-21** and shown in **Figure 4-22**; this change is discussed further in **Section 5.6.** The decrease in final optical density in SFMM with 1.0mM $DBTO_2$ was more significant (approximately 25%). This may also correspond to a change in desulfurization activity throughout the sequential transfers which is further discussed in **Section 5.4.2.**

The decrease in final optical density values for *R.* K1bD transferred in MSM with 1.0mM $DBTO_2$ was also quite significant, at 37%. **Figure 4-21** indicates that this decrease was not due to a shift in sulfur utilization from sulfate to $DBTO_2$. *R.* K1bD had an insignificant decrease (1.3%) in final optical density

values after the 100 transfers in SFMM. This indicates that the final optical density values of *R.* K1bD remained stable over the 100 transfers.

The greatest decrease in final optical density after 107 transfers in MSM with 1.0 mM $DBTO_2$ was observed with *R. erythropolis* EPWF (63%). It was also confirmed not to be the result of utilization of $DBTO_2$ as shown in **Figure 4-12**. As this decreasing trend was observed for both *R.* K1bD and *R. erythropolis* EPWF it indicates that something else is being changed in the cell to result in the decreased final optical density values, likely an absence or limitation of a trace nutrient.

Most surprisingly, a 375% increase in final optical density was observed after 100 transfers in *R. erythropolis* EPWF transferred in SFMM with 1.0mM $DBTO_2$. **Figure 4-21** shows that the 51st transfer, *R. erythropolis* EPWF is able to desulfurize $DBTO_2$ and produce 2-HBP. The adaptation to $DBTO_2$ as a source of sulfur would explain the significant increase in cell yield, as this would render sulfite available to the cells through desulfurization. While it is still not possible to completely discard at the moment, preliminary tests have shown that contamination was not a factor.

5.4.2 Comparison of the desulfurization activity of sequentially transferred cells of *R. rhodochrous* IGTS8

The impact of transfers on the specific desulfurization activity of *R. rhodochrous* IGTS8 was investigated. *R. rhodochrous* IGTS8 cells from different sequential batches in SFMM with 1.0 mM $DBTO_2$ were compared by growing the cells in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA and determining the specific desulfurization activity. The cultures used for comparison were taken from the initial inoculum and transfers number 25, 33, 50, 68, and 80. The desulfurization activity was observed to decrease between the original inoculum and transfer number 25 and then increase to a peak at transfer number 68 then decrease to a plateau that remained constant for transfers 80 to 100 (**Figure 4-14**). Each transfer was approximately 8 generations which means that approximately

850 generations occurred over the sequential transfers. Errors in DNA replication can occur at frequencies between 10^{-6} and 10^{-7} errors per kilobase pair during one round of replication (Brock, 2012). *Rhodococcus rhodochrous* has been shown to have up to 6870 kilobase pairs in its genome (National Center for Biotechnology Information (NCBI) A)). Therefore each transfer would results in 4.2E+05 mutations per ml. As only 100ul of each culture was transferred to the next sequential batch, only 4.2E+04 mutations would be carried onward. However it is important to note that not all of these mutations will be beneficial to the cell or result in a change to the desired activity. Further sequential batches result in a progressive accumulation of mutations which increases the probability that a beneficial mutation will occur and begin to overtake the population. Therefore, it is not a surprise that by 25 transfers we observe a significant change in specific desulfurization activity (**Figure 4-14**). It was thought that the selective pressure from $DBTO_2$ would generate a mutant that exhibited increased desulfurization activity; however the opposite effect was observed. This indicated that the cells needed to undergo other mutations first before they could improve their specific desulfurization activity. It was hypothesized that changes needed to be made to the way that the cells manage their sulfur intake and the effect that sulfate had on the cells before the specific desulfurization activity could be improved.

5.5 Investigation to explain the change in desulfurization activity observed in sequentially transferred cells of *R. rhodochrous* IGTS8

5.5.1 2-HBP utilization as a carbon source

Before trying to explain the changes observed in 2-HBP concentration, it was necessary to confirm that the decrease in 2-HBP observed after 25 transfers was not due to the microorganisms utilizing 2-HBP as a carbon source. **Figure 4-15A)** and **B)** are comparisons of the normalized 2-HBP concentrations after desulfurization by the initial inoculum and transfers number 25 and 50 for *R. rhodochrous* IGTS8 grown in SFMM and MSM, respectively, with 2-HBP

(~0.04mM). Both media were utilized to compare the differences between growth with and without sulfate present. As mentioned, sulfate is a byproduct of the 4S desulfurization pathway, and its presence could allow the cells to utilize 2-HBP. No $DBTO_2$ was added to either culture to ensure that any observed fluctuations in the 2-HBP concentrations were not due to the cells producing it through desulfurization of $DBTO_2$. 2-HBP has previously been shown to result in a 50% inhibition of cell growth at a concentration of 0.52mM (Alves and Paixão, 2011). In order to ensure that toxicity of the 2-HBP would not be an issue, the desired concentration in the medium was 0.04mM – a level on the order of the minimum concentrations produced by the cells in other experiments (*e.g.* **Figure 4-9**). The initial concentrations of 2-HBP in the culture varied due to difficulties in measuring out small amounts of 2-HBP (variations were from 0.015 to 0.045mM). Therefore the values were normalized in **Figure 4-15** to allow a comparison. No significant variations were observed (**Figure 4-15A)** or **B)**), which indicated that the organisms were not utilizing the 2-HBP as carbon source. This was further confirmed by an analysis of the residuals (**Figure 4-16**). Wang and Krawiec (1996) also determined that the *Rhodococcus* cultures used in their study were not capable of utilizing 2-HBP as a carbon source.

5.5.2 Comparative sulfur content analysis

As the specific desulfurization activity was shown to change as sequential batches were performed on *R. rhodochrous* IGTS8 (**Figure 4-14)**, and since these changes were shown not to be attributed to cells utilizing 2-HBP as a carbon source (**Figure 4-15**), it was hypothesized that the cells may have adapted to incorporate less sulfur. Sulfur makes up between 0.5% and 1% of bacterial cell dry weight (Soleimani et al., 2007). Elemental analysis was conducted on the original innoculum of *R. rhodochrous* IGTS8 and transfer number 50 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. Cultures from these transfers exhibited high levels of specific desulfurization activity (0.085 and 0.0723µM/OD/hr respectively). However, considering that cultures taken from

transfer 25 showed significantly lower specific desulfurization activity and that a potential mutation in this transfer would be carried over to further generations, should such an adaption leading to lower sulfur contents in the biomass have occurred, transfer 50 should have exhibited a lower sulfur percentage in the elemental analysis results.

Significant sample preparation was involved to accurately compare the original inoculum of *R. rhodochrous* IGTS8 and transfer number 50. It was important that the same concentration of cells were sent for analysis. The samples were diluted and the concentration of the cells was compared using microscopy as shown in **Figure 4-17**. The values of cell concentration were found to be statically equivalent.

Additionally, the weight of the dried biomass obtained for elemental analysis was also compared (**Figure 4-18**). As the same concentration of cells was prepared in all cases (**Figure 4-17**), it was also expected that the biomass yields would be the same. The dry weight concentrations (mg/ml) were found to be statistically equivalent for the original inoculum and transfer number 50 (**Figure 4-18).** These results give confidence to the elemental analysis results in **Figure 4-19** that a valid comparison was conducted.

The elemental analysis results for the sulfur content in weight % of the cells of the original inoculum and transfer number 50 of *R. rhodochrous* IGTS8 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA are shown in **Figure 4-19**. The average sulfur concentration in weight % was found to be 0.42% and 0.40% for the original inoculum and transfer number 50, respectively. There was no statistical difference between the sulfur concentration in the original culture and the culture obtained after 50 transfers. This indicated that the sulfur uptake into the cell biomass had not been changed by the cells. One factor impacting these results and possibly masking differences is the significant error in the measurements, it is important to take this into consideration when evaluating the significance of these results.

It was interesting to note that the sulfur concentration in the cells determined by elemental analysis was lower than that indicated by the literature

(0.5 -1% (Soleimani et al., 2007)). This indicated that the cells should be capable of increased desulfurization to incorporate more sulfur into the biomass, but that something is inhibiting their ablilty.

5.5.3 Comparison of the sulfate accumulation in the media

Aggarwal et al., (2012) proposed that sulfate plays an important role in the regulation of the 4S desulfurization pathway. They propose two enzymes, a sulfite reductase (SR) and a sulfite oxidoreductase (SOR), are responsible for converting the sulfite produced from the 4S pathway into sulfide and sulfate, respectively (**Figure 2-3)**. They proposed two hypotheses for the potential fate of sulfur: (i) any sulfite, which is toxic to the cell, that cannot be converted into sulfide for biomass will be converted to sulfate via SOR to avoid accumulation; (ii) a second possibility is that the cells are unable to directly convert sulfite into sulfide through SR, and therefore must first use SOR to produce sulfate that can then be converted by SR into sulfide for biomass. Gallagher et al. (1993) observed a small increase in sulfate concentration at 2 and 4 hours of cell growth for *R. rhodochrous* IGTS8 grown with DBT, followed by a decrease in sulfate to the detection limit; no sulfite was detected. This result could confirm the second hypothesis by Aggarwal et al. (2012), where sulfate first has to be formed before cells produce sulfide for biomass through SR. Aggarwal et al. (2012) concluded that the activities of SR and SOR enzymes may be critical to the improvement of desulfurization activity. In particular they suggested that an increase in the activity of SOR coinciding with a decrease in SR activity would starve the cells of sulfur and result in increased desulfurization activity. It is important to note that the study by Aggarwal et al. was based on modelled flux analyses and that, until the present study, no experimental results were available to corroborate these hypotheses.

Based on the results observed in sequential batches, it is hypothesized that a change in SR or SOR was responsible for the change in specific desulfurization activity observed in **Figure 4-14**. It is hypothesized that an up-regulation or a

positive mutation of the SOR enzyme occurred between the original inoculum and transfer number 50 of *R. rhodochrous* IGTS8 transferred in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. This would explain the increased amount of sulfate produced for transfer number 50 (0.080mM ± 0.001) compared to the sulfate concentrations of 0.068mM ± 0.005 produced by the original inoculum. Ma et al. (2006) found that sulfate concentrations of 0.04mM did not have a noticeable effect on cell growth, but Ohshiro et al. (1996) determined that the presence of sulfate at 0.1mM resulted in a reduction in desulfurization activity of 72%. All sulfate concentrations found in the media of the cultures after 48 hours of growth fall between the range of 0.04mM and 0.1mM. It is assumed that the higher sulfate concentrations (transfer number 50) shown in **Figure 4-20A)** would have a greater inhibitory effect than the lower sulfate concentrations (original inoculum and transfer number 100). Therefore, this could explain why the specific desulfurization activity of transfer number 50 is lower than that of the original inoculum (**Figure 4-14**) despite the fact that it shows a higher sulfate concentration**.** However, the decrease in sulfate concentration between transfer number 50 and transfer number 100 of *R. rhodochrous* IGTS8 (**Figure 4-20A)**) does not coincides with an increase in desulfurization activity between the transfers (**Figure 4-14**). The change in sulfate concentration with no change in specific desulfurization activity of transfer number 100 of *R. rhodochrous* IGTS8 after 48 hours of incubation could thus be caused by one of two hypotheses: (i) a down-regulation or negative but non deleterious mutation of SOR may have a occurred leaving more sulfite in the media instead of converting it to sulfate, (ii) an up-regulation or positive mutation of SR may be converting more sulfate/sulfite into sulfide for biomass. The biomass sulfur concentration of transfer number 100 of *R. rhodochrous* IGTS8 was not determined in this study. Both of these hypotheses suggest that the transferred cultures of *R. rhodochrous* IGTS8 in SFMM adapted to the selective pressure ($DBTO_2$ as sole sulfur source) and evolved to improve the regulation of sulfur in the cells and media and not necessarily the direct desulfurization steps.

Comparisons of the amount of sulfate produced per 2-HBP produced for the original inoculum and transfers number 50 and 100 of *R. rhodochrous* IGTS8 were also conducted (**Figure 4-20B**). The ratios of sulfate to 2-HBP ranged from 1.11 to 1.49. All the values measured were above 1.0, which indicates some measurement error as the concentration of sulfate should be less than that of 2-HBP as at least some of the sulfur released from $DBTO_2$ went to biomass. One possible explanation for ratio measurements being above 1.0 is that at least some of the 2-HBP produced was not extracted from solution and, therefore, not accounted for in GC analysis. The measurements of desulfurization activity reported would thus be a pessimistic estimation of the true desulfurization potential of the organism. Despite these limitations, comparative analysis still demonstrated through t-test (with 95% confidence) that none of the results were equivalent to that of transfer number 50 further confirming a change in sulfur management in the cell.

5.6 Comparison of the desulfurization yield of sequential transfers of *R. rhodochrous* IGTS8, *R.* K1bD, and *R. erythropolis* EPWF in MSM and *R.* K1bD and *R. erythropolis* EPWF in SFMM

The desulfurization yield of 2-HBP for transfers number 51 of *R.* K1bD and *R. erythropolis* EPWF transferred in SFMM, transfers number 95 of *R.* K1bD and *R. erythropolis* EPWF transferred in MSM and transfer number 74 of *R. rhodochrous* IGTS8 transferred in MSM was compared after 48 hours of incubation (**Figure 4-21**)**.** The desulfurization yield of *R.* K1bD transfer number 51 transferred in SFMM was found to be similar to the desulfurization yield of the original inoculum (0.056mM compared to 0.058mM 2-HBP – **Figure 4-12**). No desulfurization yield was observed for early transfers of *R. erythropolis* EPWF grown in SFMM (**Figure 4-12**), which was expected since this organism does not contain the 4S pathway. However, in the case of transfer number 51, *R. erythropolis* EPWF significant desulfurization yield was observed (0.055mM 2-HBP). This apparition of desulfurization activity corresponds to an increase in

final optical density value from transfer number 35 onwards (**Figure 4-13**) – approximately 280 generations. This change in final optical density and the accumulation of 2-HBP indicate that *R. erythropolis* EPWF was now capable of utilizing $DBTO_2$ as a sulfur source, therefore providing more sulfide to the biomass and increasing the cell yield. *Rhodococcus erythropolis* sp. have been shown to have a similar genome size to that of *Rhodococcus rhodochrous* sp. (National Center for Biotechnology Information (NCBI) B)). Therefore the previously mentioned mutation calculations for *R. rhodochrous* sp. would also be applicable for *R. erythropolis* sp. Therefore, at transfer number 35 of *R. erythropolis* EPWF in SFMM with 1.0mM $DBTO_2$ the number of mutations that occurred in the cultures would have reached approximately 1.47E+07. This should be enough mutations to for a mutant population with desulfurizing activity to take over the population. The mutation that would explain this change in behavior was not identified in this study. Plating and microscope evaluation do not show significant contamination in the culture; however until genetic analysis is used to corroborate the results, this possibility cannot be completely discarded.

As discussed in **Section 5.3.1**, no 2-HBP was detected with the original cultures of *R.* K1bD and *R. erythropolis* EPWF grown in MSM with 1.0mM $DBTO_2$. After 95 transfers, experiments were conducted with these strains to determine if any 2-HBP could be detected. Again, the results showed no yield of 2-HBP (**Figure 4-21**). This indicated that, in the absence of a selective pressure on sulfur (sulfate is readily available in MSM), the organisms were not able to overcome sulfate inhibition.

However, an accumulation of 2-HBP was observed for transfer number 74 of *R. rhodochrous* IGTS8 in MSM (**Figure 4-21**). The desulfurization yield of 2-HBP was determined to be 0.004mM which is significantly smaller than the 2-HBP accumulation found for transfers of *R. rhodochrous* IGTS8 in SFMM which ranges from 0.039 – 0.063mM. The specific desulfurization activity for *R. rhodochrous* IGTS8 transferred in MSM was compared for the original inoculum and transfers number 36, 72, 94, and 107 (**Figure 4-22**). No specific desulfurization activity was detected in the original culture of *R. rhodochrous*

IGTS8 transferred in MSM with 1.0mM $DBTO_2$ as previously observed. However transfers number 36, 74, 92, and 107 all showed specific desulfurization activities (0.0053, 0.0071, 0.0033, and 0.0005µM/OD/hr, respectively). The specific desulfurization activity of transfer number 36 was found to be statistically equivalent to the activities of transfer number 72 and 92. However the desulfurization activity of transfer number 72 was not found to be equivalent to that of transfer number 92 therefore confirming the significance of the decrease in specific desulfurization activity for transfers number 92 and 107. The fact that 2-HBP accumulation is observed after 36 transfers, when it was initially not present, suggests that a mutation that enables desulfurization of $DBTO_2$ despite elevated sulfate contents is present. This result is of great importance as sulfate inhibition is one of the main limitations on reaching improved desulfurization activity and making the biodesulfurization process industrially appealing. The observed decrease in specific desulfurization activity could be similar to the observed decrease in transfer number 25 of *R. rhodochrous* IGTS8 transferred in SFMM, and may indicate that *R. rhodochrous* IGTS8 transferred in MSM is also adapting to change sulfur management within the cell.

5.7 Desulfurization with DBT vs. $DBTO_2$

Figure 4-23A) compares the growth of the original inoculum and transfers number 25 and 50 of *R. rhodochrous* IGTS8 (transferred in SFMM with 1.0mM $DBTO_2$) grown in SFMM with 0.25mM DBT and 14mM DMFA. Growth rates and final optical density values at 48 hours were similar for all three cases. These growth rates and final optical densities were found to be similar to those of the original inoculum and transfers number 25 and 50 grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA (data not shown). However, a lower specific desulfurization activity was observed for the cultures grown with DBT (**Figure 4-23B)**) than those grown with $DBTO_2$ (**Figure 4-14**). The specific desulfurization activity of the original inoculum and transfers number 25 and 50 were found to be 0.067, 0.062, 0.059 µM/OD/hr, respectively (**Figure 4-23B)**).

All values were found to be statistically equivalent. The specific desulfurization activity of *R. rhodochrous* IGTS8 grown with DBT (**Figure 4-23B)**) was found to be 21 and 19% lower than the specific desulfurization activities found for the original inoculum and transfer number 50 grown with $DBTO_2$, respectively (**Figure 4-14**). However the specific desulfurization activity of transfer number 25 grown with DBT (**Figure 4-23B)**) was found to slightly higher (9%) then the specific desulfurization activity for transfer number 25 grown with $DBTO_2$ (**Figure 4-14**). Regardless, these results demonstrate the relevance of using preliminary oxidizing steps for the desulfurization of thiophenes. $DBTO_2$ is more bioavailable to cells – it has a higher solubility (5.8mg/L) than that of DBT (1.0mg/L) at 25ºC (Seymour et al., 1997) – and is further down the 4S desulfurization pathway than DBT – requiring fewer enzymes and cofactors for its desulfurization making it easier for the bacteria to utilize.

Experimental studies have observed varying results for growth with $DBTO_2$ versus DBT. Ohshiro et al. (1996) grew *R. erythropolis D-1* in cultures with DBT and with $DBTO_2$. They found that the cultures in contact with DBT grew to a higher specific activity than those growing in the presence of $DBTO_2$. However the cultures containing $DBTO_2$ reached a higher optical density value than the cultures containing DBT. Gallagher et al. (1993) found that growth with DBT and $DBTO_2$ were approximately the same. Setti et al. (1994) and Wang and Krawiec (1996) all observed increased growth rates and increased desulfurization activity in *R. erythropolis* N1-36 and *Pseudomonas* sp., respectively, when grown in $DBTO_2$ versus DBT. They also observed that production of 2-HBP occurred quicker and in a greater extent for growth with $DBTO_2$. These results could partially explain the decreased specific desulfurization activities for growth in DBT (**Figure 4-23B)**) compared to growth in $DBTO_2$ (**Figure 4-14**). It also indicates that if the experiment had continued longer (i.e. 60 hours) the desulfurization yield of 2-HBP may have increased further. However, since it is desired to have faster growing cultures with increased specific desulfurization activities, utilizing $DBTO_2$ as a desulfurization substrate is preferred over utilizing DBT.

5.8 Cycled cells obtained through self-cycling fermentation (SCF)

Self-cycling fermentation (SCF) was utilized to apply a stronger selective pressure on *R. rhodochrous* IGTS8 to increase desulfurizing activity of $DBTO_2$ in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. 69 cycles of *R. rhodochrous* IGTS8 were conducted in three separate sets.

A decrease in the end of cycle optical density values of 0.14 occurred over cycles 1-15 (0 to 235 hours) (**Figure 4-24)**. The end of cycle optical density value of cycle 1 was 1.24. This is significantly lower than the end of cycle optical density values observed for cycles 16-69. This may be attributed to the fact that a foam stopper, which reduced the oxygen supply in the reactor was used in the first set of cycles, whereas a rubber stopper was used for cycles 16-69. Schilling et al. (2002) found that the rate of desulfurization in a continuous stirred tank reactor (CSTR) is partially limited by the transfer rate of the substrates DBT and oxygen. The decrease in final optical density values in cycles 1-15 (0 to 235 hours) of 0.14 is significantly lower than the decrease in end of cycle optical density values of cycles 16-42 (235 to 594 hours) which was found to be 0.50. The end of cycle optical density value of cycle 16 was 1.64, so a decrease to 1.14 occurred over the 26 cycles. A decrease in final optical density was also observed in the third set of experiments (cycles 43 to 69 – 594 hours to 956 hours) this decrease in final optical density values – observed over each set of cycles – can be explained by observed biomass accumulation on the sides of the reactor. The formation of a bacterial mat by *Rhodococcus* strains in the presence of hydrophobic compounds is well known and is due to the presence of mycolic acids extending from the cell membrane (Ma, 2010). Schilling et al., (2002) preformed continuous desulfurization of DBT experiments with *R. rhodochrous* IGTS8 in a 2-L continuous stirred tank reactor (CSTR). They observed a high mycolic acid content in the cell culture resulting in a high degree of hydrophobicity. This resulted in cell mass accumulation on the reactor wall as well as the presence of foam. Similar observations were made during SCF operation. As the biomass accumulated on the reactor wall, a decrease in final optical density value was

observed. Additionally, 2-HBP is a hydrophobic compound that may accumulate along the cell wall and in the foam produced in the reactor (Schilling et al., 2002). The same accumulation may have occurred for other toxic compounds (e.g. elevated concentration of $DBTO_2$). This accumulation may have resulted in bacteria being exposed to toxic levels of these compounds further decreasing the optical density values. The accumulation of biomass and desulfurization products on the reactor wall is also why a maximum of 26 cycles were ran in a set. The reactor was cleaned and autoclaved before a new set of cycles was initiated.

Figure 4-25 shows the decrease in $DBTO_2$ and the accumulation of 2-HBP along with optical density values for the 12-hour cycle 36 of *R. rhodochrous* IGTS8 in the SCF with SFMM with 0.25mM $DBTO_2$ and 14mM DMFA**).** The concentration of $DBTO_2$ in the media after cycling was 0.04mM (**Figure 4-25A)**) and its desired concentration in the feed media added to the system was 0.25mM. This would result in a $DBTO_2$ concentration of 0.125mM in the SCF at the start of cycle 36 as the end of cycle 35 indicated that no $DBTO_2$ was remaining in the system. This is an issue that can be associated with its solubility. In fact, $DBTO_2$ can accumulate along the walls of the feed carboy and in the tubing from the carboy to the SCF, leading to variation of the actual concentration of $DBTO_2$ present in the feed medium to the reactor. This is a significant challenge that needs to be overcome for process-based directed evolution strategies involving hydrophobic compounds, as the selective pressure needs to be consistent on the cells. Since the $DBTO_2$ concentration is significantly lower than expected at the start of cycle 36, it indicates that other cycles would have significantly more $DBTO_2$ present in the media to act as a selective pressure on the cells.

The $DBTO_2$ concentration was shown to be depleted completely between 2 and 4 hours of growth in the fermenter (**Figure 4-25A)**). However, the 2-HBP concentration continued to increase over the 12 hours cycle up to 0.036mM. Again, this can be linked to the solubility and bioavailability of the sulfur source. The concentration of 2-HBP at the end of cycle 36 is 13% higher than the 2-HBP concentration at the end of cycle 35. This may indicate that the increased initial concentration of $DBTO_2$ in cycle 36 may have resulted in increased

desulfurization activity throughout the cycle. However no conclusions can be drawn as the initial concentration of $DBTO_2$ in cycle 35 was not measured.

The end of cycle optical density value for cycle 35 was determined to be 1.11 with an after cycling optical density value of 0.52, which is approximately half, as expected (**Figure 4-25B)**). The optical density values of cycle 36 increased until 10 hours were the optical density values remained stable around 1.24. The optical density values at the end of cycle 36 were 12% greater than the end of cycle optical density value of cycle 35. This increase in optical density value may also be related to an increase in desulfurization activity between the two cycles though no definite conclusions can be drawn.

Figure 4-26 compares the specific desulfurization activity of cultures taken from cycles 1, 15, 42, and 54 of *R. rhodochrous* IGTS8 cycled in the SCF, and grown in SFMM with 0.25mM $DBTO_2$ and 14mM DMFA. The cycled cells did not show change in specific desulfurization activity.

Arensdorf et al. (2002) were successful in utilizing process-based directed evolution strategies (chemostat) to develop gain of function mutants that exhibited an expanded substrate range for the enzymes involved in the 4S desulfurization pathway. They found that it took 104 days (54 generations) in the chemostat to achieve a gain of function mutant. This confirms the advantage of utilizing process-based directed evolution strategies as they apply a stronger selective pressure on the culture and are able to gain a mutation in less time (i.e. 54 generations). For instance, the SCF carries forward more mutations in each cycle (2.6E+06) than then in the sequential transfers (4.2E+04). This further supports the use of the SCF as a preferred method for process-based directed evolution. The fact that Arensdorf et al., (2002) only saw a mutation after 54 generations indicates that SCF was likely not operated long enough to allow a mutant to take over the population – recalling that each cycle corresponds to 1 generation, and that solubility/bioavailability issues would need to be resolved. Additionally, the SCF would be able to complete 54 generations in approximately 30 days (depending on how frequently the system need to be cleaned) compared to the 104 days it took for Arensdorf et al. (2002), further supporting its preferred use.

Arensdorf et al. (2002) also found that changing the selective pressure in the chemostat resulted in the accumulation of different evolved phenotypes in a single strain. This further confirms the advantage of process-based directed evolution strategies to create an overall mutation, rather than single enzyme targets, in the cell. This strategy could also be utilized in the SCF and result in more evolved phenotypes in a shorter period of compared to the chemostat.

6. CHAPTER 6: CONCLUSIONS

Rhodococcus rhodochrous IGTS8 has exhibited changes in its desulfurization activity over 100 sequential transfers in sulfate-free mineral medium (SFMM) with 1.0mM $DBTO_2$ added as a source of sulfur (selective pressure). These changes appear to involve sulfur management by the culture and suggest a reduction in sulfate inhibition which improved desulfurization activity. Future work will need to be conducted to determine if these changes correspond to modifications in the enzymes involved or changes in their regulations.

Sequential transfers were also conducted with *R. rhodochrous* IGTS8 in minimal salt medium (MSM) with 1.0mM $DBTO_2$ for 107 transfers. *R. rhodochrous* IGTS8 has been shown to overcome the inhibitory concentration of sulfate in MSM and exhibit desulfurization of $DBTO_2$. Desulfurization activity was quite low in MSM compared to the activity observed in SFMM. Identifying and understanding the changes that occurred in the *R. rhodochrous* IGTS8 cells transferred in MSM will be important to further increase the desulfurization activity.

R. rhodochrous IGTS8 was grown under SCF for 69 cycles. No noticeable change in desulfurization activity was observed over 54 cycles (54 generations). It can be concluded that not enough cycles were conducted in the SCF to allow a mutation to take over the population. Future work will need to overcome some of the challenges faced in running the SCF process to be able to conduct more cycles.

Sequential transfers were also conducted with *Rhodococcus* K1bD in SFMM with 1.0m $DBTO_2$ and MSM with 1.0mM $DBTO_2$. *R.* K1bD was shown to be able to desulfurize $DBTO_2$ in SFMM but not MSM. No significant changes were noticed over 100 transfers of *R.* K1bD in SFMM with 1.0mM $DBTO_2$. However, the desulfurization activity of *R.* K1bD in SFMM was confirmed to still be present in the cells half way through the 100 sequential transfers. No desulfurization activity was observed after 95 sequential transfers in MSM, though 107 sequential transfers were conducted.

Sequential transfers were also conducted with *Rhodococcus erythropolis* EPWF in SFMM with 1.0mM $DBTO_2$ and MSM with 1.0mM $DBTO_2$. Initially, *R. erythropolis* EPWF could not desulfurize $DBTO_2$ in SFMM or MSM. However a significant increase in cell yield around 35 sequential transfers, and desulfurization activity at transfer number 51 in SFMM with 1.0mM $DBTO_2$ were observed. No desulfurization activity was observed for *R. erythropolis* EPWF sequentially transferred in MSM with 1.0mM $DBTO_2$ after 95 sequential.

Future work to identify the sources of the changes that occurred in the cells and how they relate to the observed desulfurization activities will be important to develop better biodesulfurization processes.

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