Molecular and Genetic analysis of *Desulfovibrio*

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature:…….

ABSTRACT

The current population explosion has resulted in an ever-increasing demand for petroleum-based fuels, consequently global fossil fuel reserves are diminishing at record pace. To provide a sustainable future for the next generation, renewable alternatives to current fuels products are required. *Desulfovibrio* spp. has been reported to microbially synthese hydrocarbons of similar structure to that found in petroleum-based fuel products. Exploration of the hydrocarbon synthesis pathway through transcriptomic analysis highlights the genes and proteins involved. Comparative RNA-seq analysis between two homologous strains of *Desulfovibrio; Desulfovibrio desulfuricans* 8326 and *Desulfovibrio salexigens* 2638 provided growth characterisation and the development of a reliable RNA extraction method when cultivated in Postage medium B. Bioinformatic analyses are currently pending to identify components accountable for hydrocarbon synthesis. Complementary C_{18} alkane and 16S genetic analysis confirmed *D.desulfuricans* hydrocarbon synthesis but highlighted contamination of *D.salexigens* cultures resulting in false-positive alkane production. Additional transformation investigations of *D.desulfuricans* confirmed natural resistance markers. Supplementary work to generate a highly transformable strain lacking the *hsdR* gene examined two methods of gene deletion; TargeTron and Cre-lox. Neither methodology provided viable transformants. Future work in developing a 'tool box' for genetic manipulation using a highly transformable strain of *D.desulfuricans* would allow control of the hydrocarbon synthetic pathway through regulation of genes discovered in the RNA-seq analysis. This new insight would improve our knowledge and enhance the future viability of renewable microbial-derived hydrocarbons as a replacement for the current non-renewable petroleum-based fuels.

Keywords: *Desulfovibrio desulfuricans* 8326*, Desulfovibrio salexigens* 2638*,* comparative transcriptomics, C_{18} alkanes, Postgate medium B, 16S, RNA, transformation, TargeTron, Cre-lox, *hsdR*.

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ABBREVIATIONS

GHG	Greenhouse gas
PGB	Postgate Medium B
MT	Metal Toxicity
D.desulfuricans	*Desulfovibrio desulfuricans* 8326
D.salexigens	*Desulfovibrio salexigens* 2638
D.desulfuricans G20	*Desulfovibrio desulfuricans* G20
Abs_{600}	Absorbance at 600 nm
GC/MS	Gas chromatography- mass spectrometry
GC/GC	Two dimensional gas chromatography
H^2	Deuterium
D_2O	Deuterium oxide (heavy water)
m/z	Mass to charge ratio

CHAPTER 1

INTRODUCTION

1.1: Economic and Political Drivers behind improving Biofuel Production

Global industrialisation and affluence have increased the demand for petroleum based fuels (Agrawal, 2007). The production of a renewable and sustainable fuel is critical for future economic security and in mitigating carbon dioxide emissions and global climate change (Solomon, 2010). The use of biomass as a renewable and sustainable source of fuel is a viable alternative for fuels derived from petroleum (Cook *et al.*, 1991; Singh and Nigam, 2011).

Eighty percent of the primary energy consumed worldwide is derived from fossil fuels, with 58% being used by the transport sector (Escobar *et al.*, 2009). Global production of bioethanol is lead by Brazil and USA, with Europe heading the field of biodiesel producers (Balat and Balat, 2009; Demirbas, 2009). Governments are providing billions of dollars for biofuel initiatives yet this is severely overshadowed by funding for fossil fuel programmes (Robbins, 2011). A major outcome of initiatives is the formulation of targets for increased biofuel production and improved blending levels (Demirbas, 2009). Despite the foreseeable energy crisis, the development of renewable energy at industrial scale is a plausible route for economic growth. Brazil's current rise as global force is a prime example. One of the major drivers behind Brazil's success it's innovation in biofuels (Moraes, 2011). Throughout the world lessons are being learnt from the past mistakes and current experiences of Brazil, whose biofuel industry dates back to the 1970's (Balat and Balat, 2009). Brazil has vast amounts (~335 10^6 ha) of arable land and a climate that is suited to growing energy dense sugar cane (*Saccharum* spp.) (Goettemoeller and Goettemoeller, 2007). Brazil's current position, as a leading producer of bioethanol, is due to the then military government's aim to become energy independent as a result of the 1970's oil shocks (Robbins, 2011). The Brazilian model gave rise to many challenges and has sparked many debates into how a global biofuel industry should be implemented and developed into the size required to meet intensifying demands (Moraes, 2011).

Rapid population expansion in countries such as China and India has severely increased the demand for fossil energy and fuel. As a result initiatives for biofuel production in these countries have resulted in ambitious blending level targets (Robbins, 2011). For example, India intends to have 20% ethanol blending mandate by 2017, with current levels at 5%

(Biofuel digest, 2011). For such ambitious targets to be met stimulation of bio-energy crop cultivation has to occur. Land use change, switching from food crops to bio-energy crops, will be significant, putting a huge strain on the amount of food produced (Reinhardt and von Falkenstein, 2011). A major concern is that the diversion in cultivation of food crop to fuel crop could lead to hunger within the expanding population (Solomon, 2010; Singh and Nigam, 2011). Development of internationally binding regulations or policies is required for an ethically sustainable biofuel industry to be created (Rajagopal and Zilberman, 2007).

The increased public interest in biofuels is a direct result of political and economical pressures. Politicians and media portray biofuels to be a 'green', non-polluting industry, but that image is often far from the truth (Rajagopal and Zilberman, 2007; Robbins, 2011; Fairley, 2011). It is important that potential production methods are analysed in full to understand the exact ecological and environmental implications of increased commercial production.

1.2: Biofuels Overview

A biofuel is defined as a fuel generated from renewable sources, they include fuels such as wood, alcohols (ethanol/methanol), biodiesels, Fisher-Tropsch liquids, hydrogen and methane and are broadly classified as primary and secondary biofuels (Singh and Nigam, 2011).

Primary biofuels generate energy from un-modified chemical energy found in natural, unprocessed biomass such as firewood, wood chips, crop residues *etc*. These are often directly combusted to supply energy for cooking, heating and electricity production.

Secondary biofuels are primary biofuels that have been processed prior to combustion; thus, manipulated to produce an array of multiple state fuels. Applications include fuel for both transportation and industrial processes. Further classification of secondary biofuels into first-, second- generation and advanced biofuels is based on the raw material and technology used in production (Singh and Nigam, 2011).

1.3: First Generation Biofuels

First-generation biofuels derive from sugars, grains or seeds and require simple processes to produce a refined fuel, many of which are currently in commercial production. The major examples of first-generation biofuels are ethanol and butanol produced from crops and biodiesel from vegetable oils (Singh and Nigam, 2011).

Ethanol or butanol is generated by extracting sugars (mainly glucose) from sugarcane via hydrolysis. The sugar is then fermented in the presence of yeast or bacteria that convert six-carbon sugars into ethanol or butanol (Balat and Balat, 2009). Further distillation and dehydration processes generate the final product at desired concentrations. Commonly used carbon sources include sugars from *Saccharum* spp (sugar cane and sugar beet) and starch from *Triticum* spp (wheat), *Zea mays* (maize) and *Manihot esculenta* (cassava) (Zhao *et al.,* 2009). This bioethanol is then combined and blended with petrol at industrial level, these levels range from 10% ethanol (E10) up to 85% ethanol (E85) (Singh and Nigam, 2011).

Biodiesel is a mono-alkyl ester of fatty acids derived from vegetable oils and is a replacement for diesel. Biodiesel feedstock include oils of *Elaeis guineensis* (palm), *Glycine max* (soybean) *Helianthus annuus* (sunflower), *Cocus nucifera* (coconut), *Brassica napus* (rapeseed), and *Vernicia fordii* (tung) (Shahid and Jahmal, 2007). Palm, soybean and rapeseed oil are the most commonly used substrates for biodiesel production. Most of the vegetable oils highlighted showed promise at the manufacturing level, but multiple issues occurred during engine testing. Studies show that extensive use of biodiesel caused erosion and carbon build up within un-modified combustion engines (Bajai and Tyagi, 2006). High viscosity, low volatility and reactivity of the unsaturated hydrocarbon chains, differing amounts of free fatty acids (FFAs) and triglycerides (TAGs) further contribute to make vegetable oils unsuitable as a direct fuel source (Ma and Hanna, 1999). Attempts to improve the viscosity of the product included microemulsion, pyrolysis, catatlytic cracking and transesterfication. Of these the most promising method is transesterfication yielding a biodiesel that consists predominantly of fatty acid methyl esters (FAME). Analysis show its paraffinic hydrocarbon structures allow clean combustion with minimal nitrous oxides (NO_x) and sulphur oxides (SO_x) emissions.

Although bioethanol and biodiesel derived from energy crops are commercially available worldwide poor energy conversion efficiencies deem such practices economically unfavourable. For success, future industries must overcome low efficiencies and the associated impacts on food production (Singh *et al.*, 2011; Singh and Nigam, 2011).

1.4: Second Generation Biofuels

Lignocellulosic biomass from agricultural crops, wood, grasses and the non-edible residues of food crops are used as feedstocks for second-generation biofuel production (Singh and Nigam, 2011). Common feedstocks include corn stover, *Panicum virgatum* (switchgrass) and *Miscanthus giganteus* (miscanthus). Through use of non-edible substrates, second

generation biofuel production eliminates direct conflict between fuel and food production (Barron *et al.,* 1996). Second generation biofuels are derived from lignocelluloses biomass, an array of polysaccharides that can be converted into glucose. Such polysaccharides include cellulose, hemicellulose and lignin (Balat and Balat, 2009).

Cellulose is the most abundant polysaccharide but has a complex structure of poly-anhydroglucose (C_6) molecules (Balat and Balat, 2009) making it difficult to convert into glucose (Singh *et al.*, 2011). Once formed glucose is easily fermented to ethanol. Another polysaccharide component of cellulose is hemicelluloses this consists mainly of sugars such as pentose and xylose. These sugars are easily broken down into simple (C_5) sugars, but conversion of the products to ethanol is more complex (Sheoran *et al.,*1998). Biological conversion of pentose and xylose to ethanol has proved inefficient with biochemical methods proven to have superior yields (Balat and Balat, 2009). Vast improvements in bioethanol production technologies have aided promotion within the transport sector. Challenges, such as engine erosion and low blending levels for un-modified ignition engines, are preventing successful development of an economic ethanol production system.

Butanol is another lignocellulosic fuel product, it is a four carbon alcohol that can be easily blended with gasoline as high concentration levels (85%) without detrimental effects on un-modified engine performance (Brekke, 2007). Higher harvestable energy, improved safety aspects and decreased corrosiveness allows butanol to be used within the current distribution infrastructure. (Wu et al., 2007.) Butanol technologies exhibit many desired characteristics but commercial expansion has been hindered by low yields and high production costs (Ramey, 2004).

Biomass conversions via thermochemical processes involve extreme temperatures and pressures that allow for flexibility of the initial feedstocks and produce a diverse range fuel products (Farias *et al.,* 2007). These include methanol, Fisher-Tropsch liquid (FTL) and dimethyl ester (DME) (Singh and Nigam, 2011). Production begins with either gasification or pyrolysis. FTL production converts the feedstock into carbon monoxide and hydrogen via gasification, these are then catalytically converted into FTL. FTL is a mixture of mainly straight-chained hydrocarbon compounds, similar to semi-refined crude oil, allowing it to be directly refined to produce different fractions that include ‘green diesel’ and jet fuel (Farias *et al.,* 2007). Biomass-derived dimethyl esters (DME) are further products of gasification. Subtle differences in DME and FTL production methods (eg catalysts) determine the different end products (Singh and Nigam, 2011). FTL’s and DME act as ‘clean fuels’ producing minimal

carbon monoxide, sulphurs, aromatics and nitrous compounds. This has encouraged an expansion in the global FTL and DME market (Farias *et al.,* 2007).

Improved land-use efficiencies of second generation fuels minimize the impact on edible crop cultivation, a huge benefit that will promote commercial production of these biofuels.

1.5: Advanced biofuels

A new focus of biofuel production is the manipulation of micro-organisms' natural metabolic process to produce a direct replacement fuel product (Singh *et al.*, 2011). Research has intensified on identifying specific strains of yeasts, fungi and microalgae that synthesise natural oil products such as, lipid, hydrocarbons and complex oils at high yields (Singh and Nigam, 2011). Using micro-organisms for biofuel production is beneficial compared with previous technologies as they present decreased environmental impacts such as reducing greenhouse gas (GHG) emissions, lowering water and energy requirements and potentially sequestering carbon dioxide (CO_2) (Brennan and Owende, 2010). Furthermore, the elimination of the conflict with current food production is a major benefit. However, concerns have been raised about the upstream impacts include access to large quantities of CO_2 and fertilizers (Brennan and Owende, 2010).

Trichosporon fermentans was the first major micro-organism to be studied as a potential source of microbial oil production (Huang *et al.,* 2009). It has played an important role in developing novel methods such as multiple pre-treatments to improved fermentability, metabolising different feedstocks and optimising C/N ratios all leading to improve microbial oil yields. These developments resulted in scientists exploring other organisms as potential microbial oil producers.

Algae are microscopic photosynthetic organisms that utilise the Sun's energy and sequester atmospheric CO_2 to yield lipids, proteins and carbohydrates (Singh *et al.*, 2011). Fast growth rates allow for high yields of desired products but current algal cultivation and extraction methods need to be improved for the industry to become commercially viable (Chisti, 2007). Primary research centres on isolating environmental strains with high growth rates and lipid yields. Further growth optimisation and genetic engineering can develop high yielding strains allowing for production of superior microbial oil (Schenk *et al.*, 2008). Pilot-scale plants have highlighted numerous problems associated with algal growth and its scale-up potential (Savage, 2011). Current industrial platforms use two major methods for algal production; enclosed bioreactors and open ponds. Open methods have faltered due to contamination problems, where large scale bioreactor systems have proved too costly, preventing

microalgal biodiesel from becoming commercially competitive (Benemann and Oswald, 1996). New focus has turned to metabolic engineering to allow for direct control over cellular metabolism, with the main intention of optimising lipid yields (Singh *et al.*, 2011). Whilst there are ongoing problems, the potential of advanced biofuels to deliver huge benefits in producing renewable oil should not be overlooked.

1.6: Metabolic Engineering

Recent technological advances in genomic research have led to a renaissance of microorganism engineering (Berry, 2010). Due to the urgent need to develop alternative sources of fuel, much emphasis has been placed on using synthetic biological approaches (Wackett, 2011). In recent years, biomass-derived fuel used a simple method of cultivating a feedstock to yield sugar that is subsequently converted into a fuel or a product for processing into the desired product. These traditional processes have low efficiencies and involve large costs (Fischer *et al.*, 2008). Pioneering approaches are centered on the idea of efficient bioconversion of sugars into hydrocarbons (Berry, 2010). Through utilising cutting-edge biological engineering methods, research is designed to regulate and optimise biological pathways to gain maximum production efficiency whilst minimising the need for post-processing steps. Simplifying the production of a desired (biofuel) molecule to a single step has significant benefit for commercial viability (Berry, 2010). With finished products having high diversity, improved net energy gain, carbon neutrality and direct use in current infrastructure, these strategies are considered as a big advance towards a usable, economically viable resource (Peralta-Yahya and Keasling, 2010). Start-up biotechnology companies such as LS9 and Joule Unlimited have further developed these technologies with expectations of reaching commercial production levels within the next couple of years (Berry, 2010). Since then, LS9 has fallen behind their initial plan, recently opening a demonstration plant in USA. They have now set a new target to have an operational commercial-scale plant by 2014 or 2015, emphasising the ongoing struggles against scaling up production (LS9, 2012) The genetically tractable organisms *Escherichia coli* and *Saccahromyces cerevisiae* have been the focal point in studies for exploitation of metabolic pathways to produce both known and novel advanced biofuels. The range of pathways are shown in figure 1.1 (Peralta-Yahya and Keasling, 2010)

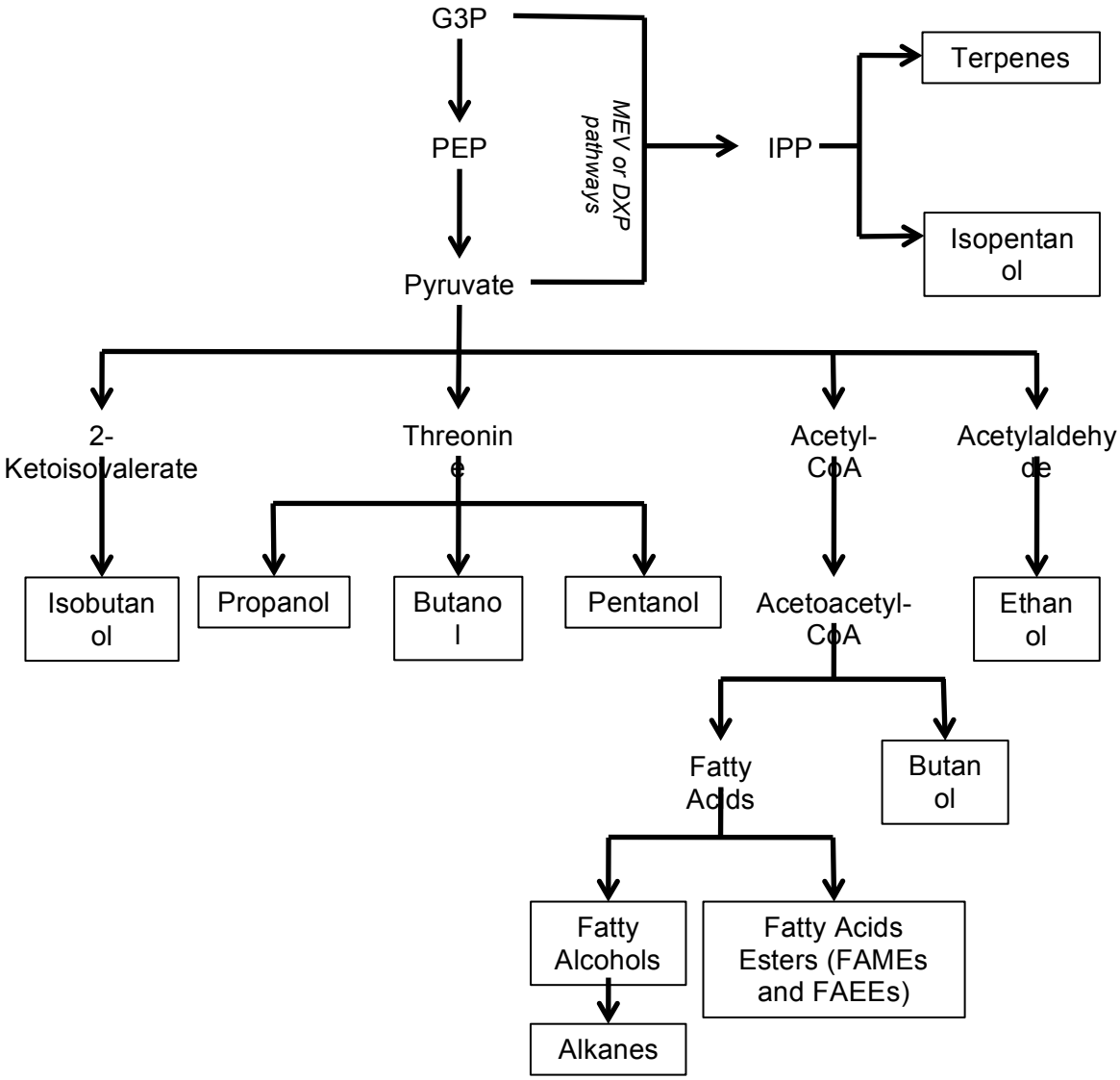


Figure 1.1: Schematic overview of metabolic routes to advanced biofuel production.

Diagram adapted from Peralta-Yahya and Keasling, 2010. Compounds within box indicate suitable fuel product.

The foremost areas of research include; heterologous expression of the *Clostridium* C_3-C_4 biosynthetic pathway for the production of isopropanol and butanol (Hania *et al.*, 2007), re-direction of amino acid biosynthesis to produce higher alcohol (Atsumi *et al.,* 2008) and manipulation of the isopreniod biosynthetic pathway to produce an isoprenoid-based fuel (Withers *et al.,* 2007). A further field is the metabolic engineering of the fatty acid biosynthesis pathway for the production of fatty acid based biofuels (Michinaka *et al.,* 2003; Kalscheuer *et al.,* 2006; Lu *et al.,* 2008; Steen *et al.,* 2010). These numerous research

avenues have led to heightened development of innovative methodologies and approaches to improve yield and productivities required for commercial scale operations.

One widely used approach was to utilise species that naturally produces the desired product, for example, various species of *Clostridium* produce isoproanol and 1-butanol from acetyl-CoA (Yan and Liao, 2009). Isopropanol yields of 4.9 g l^{-1} were achived by heterologous expressing the *Clostridium* C_3-C_4 biosynthetic pathway in *E.coli* (Hania *et al.*, 2007). Improved yield was achieved via optimisation of differing combinations of up/down regulation of genes present within the pathway. Further to this work, Jojima *et al.* reconstructed the isopropanol pathway within *E.coli* by expressing the pathway via a dedicated promoter within a single vector, in contrast to the two vector approach employed by Hania *et al.* This design led to significantly increased yields of 13.5 g l^{-1} emphasising the need for and importance of experimental methodology. A similar approach has been applied to improve yields of universal precursors, isoprenyl phosphate (IPP), and dimethylallyl pyrophosphate (DMAPP) for isoprenoid production (Yan and Liao, 2009) by overexpressing both the deoxyxylose (DXP) and mevalonate (MEV) biosynthetic pathway (figure 1.1). Isoprenoid derived fuels provide precursor molecules for generation of synthetic fuel molecules to replace diesel and jet fuels (Peralta-Yahya and Keasling, 2010).

An alternative, highly successful approach was to produce numerous higher alcohols by re-directing amino acid biosynthesis (Atsumi *et al.,* 2008). But drawbacks, such as regulation via feedback inhibition and irregularity of enzymes, have resulted in decreased yields far from those required. Use of enzyme engineering may overcome such difficulties and alter metabolic pathways to produce novel fuels molecules (Peralta-Yahya and Keasling, 2010).

A final and very exciting field of metabolic engineering is through manipulation of the fatty acid biosynthetic pathway to generate potential alcohol, esters and alkane molecules that resemble diesel and jet fuel (Steen *et al.,* 2010). Fatty acids are derived from monomers of acetyl-CoA and malonyl-CoA that are sequentially condensed into a growing fatty acyl-chain (figure 1.2). In *E.coli,* elongation is carried out by a monofunctioning enzyme, type 2 fatty acid synthase (Peralta-Yahya and Keasling, 2010).

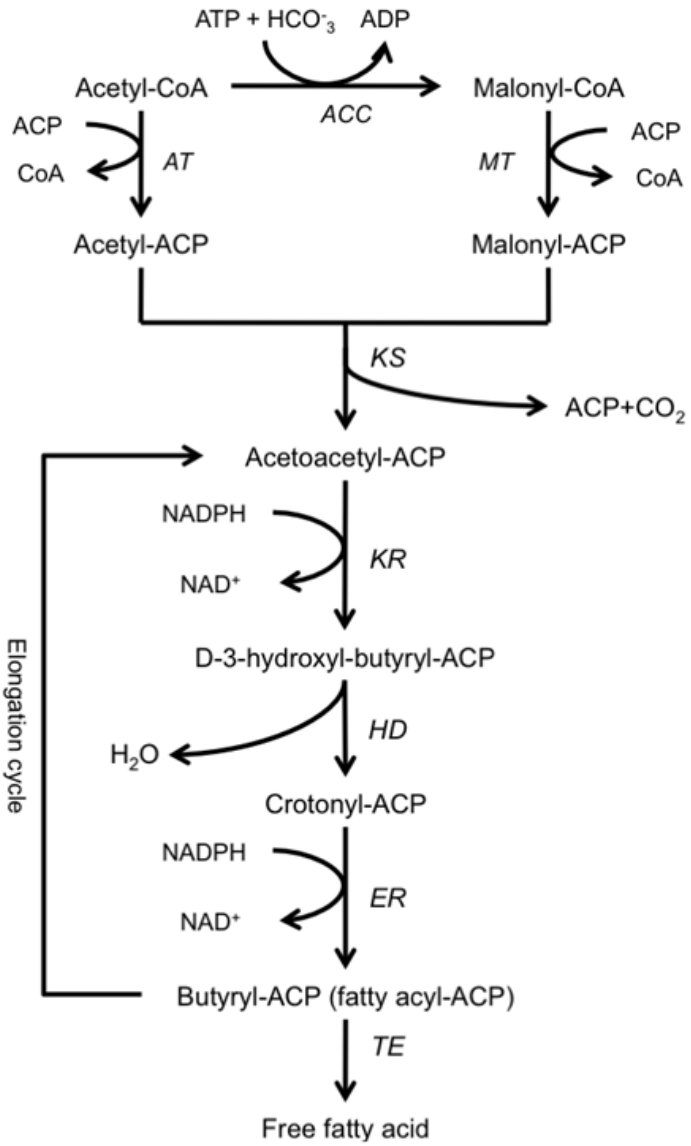


Figure 1.2: Schematic of the Fatty acid biosynthetic pathway.

Symbols and the enzymes they encode (*enzymes in italics*): ACP, acyl carrier protein; *ACC*, acetyl-CoA carboxylase; *AT*, acetyl transacylase; *MT*, malonyl transacylase; *KS*, ß-ketoacyl-ACP synthase; *KR,* ß-ketoacyl-ACP reductase; *HD,* ß-hydroxyacyl-ACP dehydratase; *ER,* enoyl-ACP reductase; *TE*, thioesterase.

The first aim of manipulating fatty acid biosynthesis was to overproduce the amount of free fatty acids (FFA). Michinaka *et al.* used a mutagenesis and screening approach with *S.cerevisiae*. This obtained mutant strains of increased free fatty acid, further complementation assays isolated fatty acyl-CoA sythase (*FAA1*) to be involved in fatty acid degradation. Additional gene deletions of all four *FFA* genes did not improve fatty acid secretion as free fatty acids were utilised for growth.

An alternative approach diverted the actyl-CoA pool to favour fatty acid biosynthesis. By over-expressing enzymes from different species fatty acid production was increased, for example a study on *E.coli* over-expressed acyl-ACP thioesterase from *Cinnamonum camphorum*. This doubled fatty acid production by deregulating fatty acid biosynthesis (Lu *et al.,* 2008; Peralta-Yahya and Keasling, 2010). Thioesterase genes are significant in determining the chain length of the fatty acyl-ACP (Dehesh *et al.,* 1996; Steen *et al.,* 2010). Tailoring chain lengths is possible through use of different thioesterases from other species such as *Cuphea hookeriana* and has resulted in product optimisation (Dehesh *et al.,* 1996; Peralta-Yahya and Keasling, 2010).

Improved production of fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) via metabolic engineering is also being explored. FAMEs/FAEEs can be produced via esterification of fatty acids with ethanol (Peralta-Yahya and Keasling, 2010). By inserting the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) genes from *Zymomonas mobilis* into *E.coli,* Kalscheur *et al.* engineered the bacteria to produce ethanol. Supplementary over-expression of a gene encoding for wax ester synthase/acyl-coenzyme A (diacylglycerol acyltransferase from *Acinetobacter baylyi* (*ws/dgat*)) allowed for the fatty acids and ethanol to be transesterified into FAEEs. A later study highlighted the inefficiencies of using fatty acids as the feedstock (Steen *et al.,* 2010). An *E.coli* strain was engineered to produce FAEEs directly from glucose and ethanol. Analysis found native *fadD* activity to be the limiting factor but over-expression of acyl-CoA ligase (*FAA2)* from *S.cerevisiae* improved yields 2.5 fold over native *fadD* function.

Technological advances within the last few years have allowed for significant progress in metabolic engineering practices; this has proved invaluable for microbial fuel research. Through manipulation of metabolic pathways, scientists are getting ever closer to a strategy that supports yields on a scale required for commercial production. Particular interest surrounding fatty acid bioengineering is emphasised by prevalent patent literature suggestive of the technologies viability.

1.7: *Desulfovibrio* species

Desulfovibrio are a diverse group of gram-negative, sulphate-reducing, obligate anaerobic bacteria that were first isolated in 1895 (Postgate, 1984 and since have been the object of substantial research interest (Voordouw, 1995). The *Desulfovibrio* are of economic importance due to the problems caused such as corrosion of metals, pollution of water, sand and soil, as well as major problems in oil technology such as oil souring (Postgate, 1984;

Little *et al.*, 1994). As a result of their anaerobic growth *Desulfovibrio's* defining electron acceptor is sulphate. Sulphate is reduced to sulphide by a series of cytoplasmic enzymes, resulting in the following reaction (Voordouw, 1995):

$$SO_4^- + ATP + 8H^+_{in} + 8e^- \rightarrow HS^- + AMP + 2P_i$$

However, *Desulfovibrio* have other compound such as formate, lactate, pyruvate, hydrogen, ethanol and organic compounds, for example, crude oil components that can serve as electron donors in sulphate reduction (Postgate, 1984; Bagaeva and Belyeva, 2000). Much is now known about the metabolic capabilities of *Desulfovibrio;* a significant observation was that of high hydrocarbon production (Jankowski and ZoBell, 1944; Davis, 1964; Oppenheimer, 1965; Bagaeva and Chernova, 1994; Bagaeva and Zolotukhina, 1994; Bagaeva, 1997; Bagaeva and Zinurova, 2004).

Desulfovibrio desulfuricans has received increased attention as a result of hydrocarbon production (Jankowski and ZoBell, 1944). Both intracellular and extracellular hydrocarbons of even and odd carbon chain lengths are produced. Intracellular hydrocarbons range between C_{11}-C_{35} and extracellular hydrocarbons between C_{11}-C_{24} (Davis, 1964; Oppenheimer, 1965; Bagaeva and Chernova, 1994; Bagaeva and Zinurova, 2004). However, a major concern is that the type of alkanes reported (C_{18}-C_{30}, *n*-alkanes) is typical of "white oil", a commonly used lubricant present on most manufactured items (Lee, 2011; Internal Shell report). This is confirmed by the GC spectrum found in Davis (1964) who presents a series of alkane peaks between C_{23}-C_{35}, consistent with white oil. Nevertheless, the same spectrum also reveals a major peak at $C_{18,}$ as well as smaller peaks at C_{17}, C_{19} and C_{20}. Further to these observations work was carried out using isotope-labelled water to verify C_{15}-C_{20} alkanes are metabolically derived (Ladygina *et al.,* 2006; Lee, 2011; Internal Shell report). Understanding of the synthetic pathway is necessary to utilise the beneficial *D.desulfuricans* hydrocarbon products. With the exception of one report (Bagaeva, 1998 reviewed in Ladygina *et al.,* 2006) little literature is available this subject. Bagaeva designed a series of isotope labelled experiments to explore the conversion of lactate to hydrocarbons (figure 1.3). The major conclusions were: formate is produced directly from the reduction of CO_2 catalysed by formate dehydrogenase (FDH) (step 1), formate is involved in acetate synthesis (step 2), a decarboxylation reaction is involved in the production of hydrocarbons (step 3) (Ladygina *et al.,* 2006).

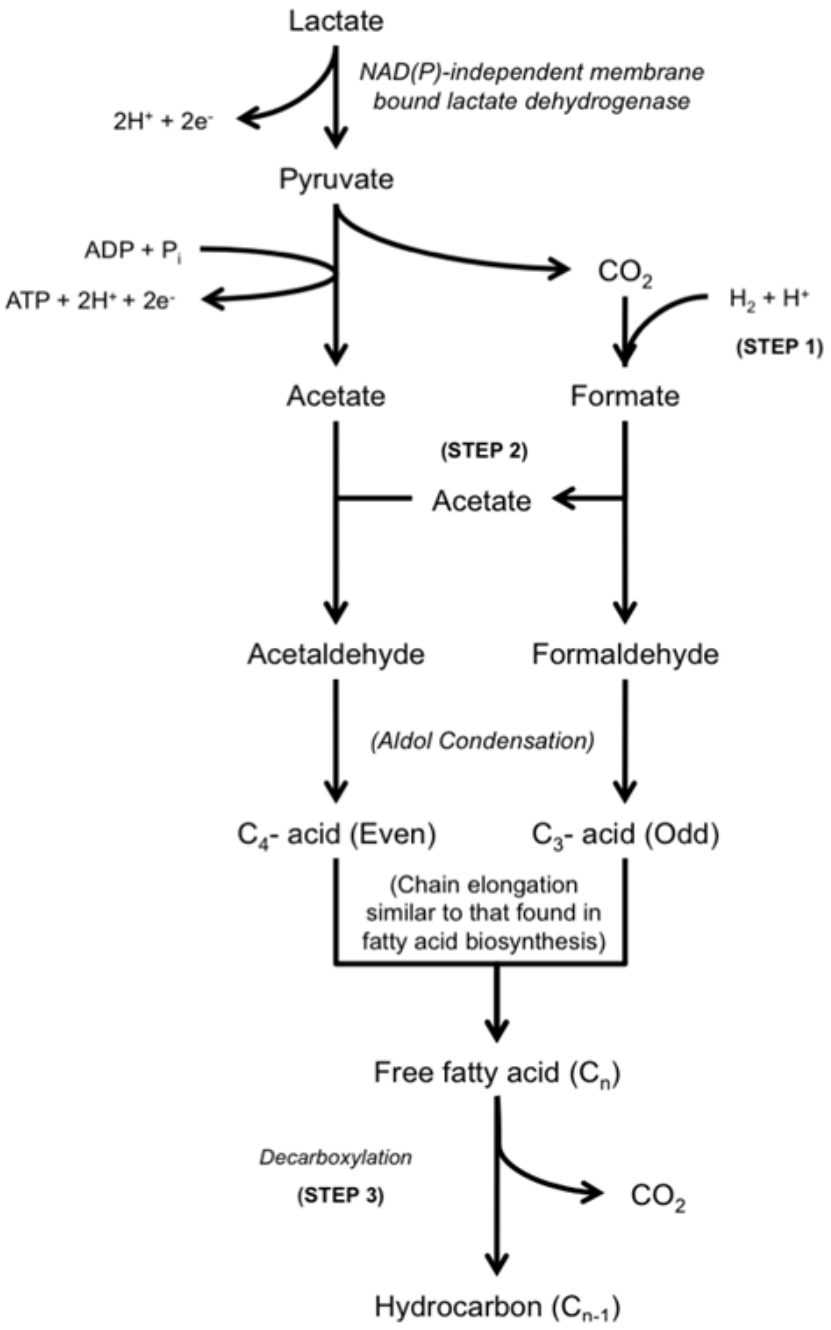


Figure 1.3: Schematic diagram of proposed biosynthetic pathway of hydrocarbons in *D.desulfuricans* (reviewed by Ladygina *et al.,* 2006).

The diagram highlights three major conclusions of the Bagaeva study: formate production via reduction of CO_2 (step 1), formate involvement in acetate synthesis (step 2), decarboxylation reaction to produce hydrocarbons (step 3).

1.8: Hypothesis and scope of project

An internal investigation at Shell provided and alternative and intriguing hypotheses. Metabolic hydrocarbon production in *D.desulfuricans* was verified proving the synthesis of *n*-alkanes; C_{14}, C_{16}, C_{18} and C_{20} and *i*-alkane of C_{15}, C_{17} and C_{19} chain length (Lee, 2011; Internal Shell report). Substantial *i*-alkane production suggests alkanes derive from hydrogen and/or methyl additions to an even carbon number fatty chain, suggesting the pathway to alkanes is via a reductive hydrogenation route, potentially, with a fatty alcohol intermediate (figure 1.4) (Lee, 2011; Internal Shell report). This is contradictory to Bagaeva's study that states hydrocarbon production is via a decarboxylation or decarbonylation route involving an activated aldehyde (Ladygina *et al.,* 2006). Furthermore, Lee eliminates the presence of decarboxylation or decarbonylation reactions by showing no aldehydes are produced, thus, a reductive hydrogenation route. Lee's proposes route results in no carbon loss, presenting a pathway to alkanes with improved carbon efficiency.

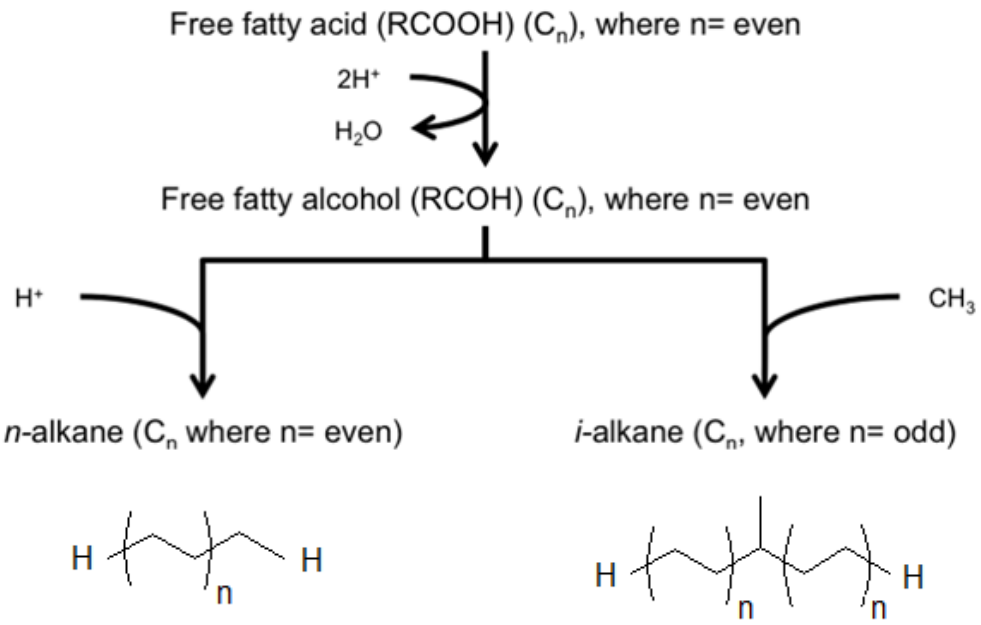


Figure 1.4: Schematic diagram of proposed reductive hydrogenation route via fatty alcohol intermediate (Lee, 2011; Internal Shell report).

By determining the genes and proteins involved within the pathway, particularly the alcohol to alkane step, will further our understanding of carbon flux and provide a novel method of hydrocarbon synthesis without the loss of a carbon.

1.9: Experimental Plan

One method to improve understanding of the pathway is to use transcriptomic methodologies. By discovering which genes are expressed and transcribed at the time of the hydrocarbon production, analysis of the metabolic enzymes and proteins present is possible. This report will analyse the transcriptome expression profiles of two *Desulfovibrio* strains; *Desulfovibrio desulfuricans* NCIB 8326 (hydrocarbon producing) and *Desulfovibrio salexigens* DSM 2638 (non-hydrocarbon producing). Reports show that *D.salexigens* has a 74% protein homology to *D.desulfuricans*, the closest reported (Lee, 2011; Internal Shell report). Using *D.salexigens* as a control allows for comparison of the two transcriptomes, highlighting proteomic differences, thus shedding light on the enzymes and genes used in alkane production. The transcriptomes will be sequenced using RNA-seq application of the Illumina high-throughput HiSeq 2000 sequencing system at the University of Exeter.

Further to the comparative transcriptome study, an investigation to develop a reproducible method of transformation within *D.desulfuricans*. During the development of this protocol, the study will aim to produce a *D.desulfuricans* strain of high transformation efficiency via the deletion of the *hsdR* gene (Keller *et al.,* 2009). The eventual aim is to up/down regulate genes discovered from the transcriptome study allowing metabolic control of hydrocarbon production in *D.desulfuricans.*

CHAPTER 2

MATERIALS AND METHODS

2.1: Strains, Media and Culturing Methods

The following *Desulfovibrio* strains were used; *Desulfovibrio desulfuricans* 8326 (NCIMB Ltd., Aberdeen, Scotland), *Desulfovibrio salexigens* 2638 (DSMZ, Braunschweig, Germany), *Desulfovibrio desulfuricians* G20 (obtained from Prof. Judy Wall, University of Missouri), *Desulfovibrio magneticus* 13731 (DMSZ), *Desulfovibrio piger* 749 (DMSZ) and *Desulfovibrio vulgaris* Hildenborough 8303 (NCIMB).

All chemicals used in media preparation, were supplied by Sigma-Aldrich (St Louis, USA), unless stated.

All bacteria were grown in a modified Postgate medium B (PGB); 0.5 g l^{-1}, KH_2PO_4,; 1.0 g l^{-1}, NH_4Cl; 2.0 g l^{-1}, $MgSO_4 \cdot 7H_2O$; 0.5 g l^{-1}, $FeSO_4 \cdot 7H_2O$; 1.0 g l^{-1}, $CaSO_4$,; 3.5 g l^{-1}, sodium-L-lactate (Alfa aesar, Ward Hill, USA); 0.5 g l^{-1}, yeast extract; 0.1 g l^{-1}, ascorbic acid; 1 vitamin pill (A-Z complete, Santogen; ground, added to 20 ml water, sonicated for 5 min and pellet removed after centrifugation); 0.6 mg l^{-1}, Na_2MoO_4; 0.25 mg l^{-1}, H_3BO_3; 0.2 ml, 60% ammonium thioglycolate in water; at pH 7.2-7.4 (Postgate, 1983). The only alteration made to the media was the addition of sea salts at 10 g l^{-1} and 20 g $l^{-1}$for *D.desulfuricans* and *D.salexigens* respectively.

D.desulfuricans and *D. salexigens* were also grown in metal toxicity medium (MT); 5.1 g l^{-1}, sodium-L-lactate; 2.1 g l^{-1}, Na_2SO_4; 1.0 g l^{-1}, NH_4Cl; 0.06 g l^{-1}, $CaCl_2$ (anhydrous); 1.0 g l^{-1}, $MgSO_4$; 0.05 g l^{-1}, yeast extract; 0.5 g l^{-1}, tryptone; 9.07 g l^{-1}, PIPES; at pH 7.2-7.4.

For experiments requiring solidified media, 15 g l^{-1} bacteriological agar was added prior to autoclaving.

Bacterial samples required for alkane synthesis analyses were grown in media containing 10 % (vol) deuterium oxide (D_2O; Fluka Analytical, Sigma-Aldrich) added prior to pH adjustment.

All media was autoclaved at 121 °C and 15 psi for 15 min (AVS347G, Astell). Prior to inoculation all media were cooled, then degassed by bubbling with argon gas (30 s for 500 ml or 1 l samples, 15 s for 100 ml samples). This included agar-containing media.

All inoculations were performed at a 10 % volume within an anaerobic cabinet (Electrotek AW300SG Cabinet, Shipley, UK) at 37 °C under a 10 % CO_2, 10 % H_2 and 80 % N_2 atmosphere.

2.2 Growth Analysis

D.desulfuricans and *D.salexigens* were grown in 500 ml Duran flasks (DURAN, Mainz, Germany) in both PGB and MT media to determine the effect on growth rates.

Prior to inoculation the mass of oven-dried 50 ml centrifuge tubes were recorded (BD, Franklin Lakes, USA). 40 ml samples were removed from the anaerobic cabinet, centrifuged at 13,000 x *g* for 10 min, washed with 20 ml deionised water twice before freezedrying for 24 hours (Scanvac, CoolSafe in line with Edwards xDS5 pump). Tubes were re-weighed and dry cell mass determined.

A bioinchoninic acid protein (BCA) assay was used to determine the total protein concentration of samples (Pierce, Thermo Scientific, Waltham, USA). Bacterial samples were diluted 20-fold, 50 µl of sample was mixed with 1 ml of working reagent (supplied in kit) and incubated at 37 °C for 30 min. The absorbance at 562 nm was recorded; this value was then analysed against a standard curve to calculate the total protein concentration (Thermo Scientific, Geneysys 10S UV-Vis).

2.3 Comparative Transcriptome Analysis

2.3.1: RNA extraction and purification

D.desulfuricans and *D.salexigens* were grown in Postgate medium B. 250 ml samples were harvested at mid-exponential phase, 40 h and 80 h for *D.salexigens* and *D.desulfuricans* respectively, all were performed in triplicate. Samples were centrifuged at 13,000 x *g* for 10 min to pellet cells, re-suspended in 25 ml of RNAprotect Bacterial reagent (QIAGEN, Hilden, Germany) and incubated at room temperature (15-25 °C) for 5 min. Further centrifugation at 13,000 x *g* for 10 min at 4 °C was required to pellet the bacterium and remove RNAprotect Bacterial reagent.

All samples were digested in an enzymatic buffer; 15 mg ml^{-1} Lysozme (Sigma-Aldrich), 2 mM EDTA [pH 8.0], 0.4 mg $µl^{-1}$ Proteinase K (QIAGEN) and incubated at room temperature for 10 min whilst on a shaker-incubator (KS 130, IKA, Staufen, Germany).

RNA was purified using RNeasy Plus mini kit (QIAGEN). Extracted RNA samples were spun through a gDNA column removing all genomic DNA, subsequent on-column DNase digestion (RNase-free DNase I kit; QIAGEN) removed any contaminating gDNA, however, this was not assessed by PCR. Purified RNA was re-suspended in 60 µl of RNase-free water. A further purification step was performed using RNeasy mini kit (QIAGEN). Concentrated RNA samples were eluted in 40 µl of RNase-free water, a 5 µl aliquot was required for analysis, the remaining volume was immediately stored at -80 °C.

2.3.2: RNA Analysis

RNA concentration was determined using Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Carlsbard, USA). RNA quality and integrity was analysed using Aglient 2100 Bioanalyser on either a Nano or Pico chip (Agilent Technologies, Santa Clara, USA).

2.3.3: RNA-seq method

Total RNA was sent to the University of Exeter. RNA samples were depleted and prepared for sequencing using Illumina TruSeq RNA sample preparation kit (Illumina Inc., San Diego, USA). Sequencing was performed on the Illumina HiSeq 2000, using a fluorescent based method (Illumina; Run ID: 121012_SN982_0177_Bc0jjfacxx)

2.3.4: RNA transcriptomic analysis

RNA-seq data generated was analysed by Dr Thomas Lux, Exeter Microbial Biofuels Group. Transcripts were mapped onto the reference genome and the number of transcripts quantified.

2.4: Alkane Analysis

2.4.1: Sample Preparation

40 ml bacterial suspensions were centrifuged at 13,000 x *g* to pellet cells. Pellets were washed with 20 ml deionised water twice, prior to freezedrying for 24 hours. Weighed samples had 500 µl or 1000 µl dichloromethane (DCM; Sigma-Aldrich) added dependant on initial mass. A 30 min sonication in an ice bath was followed by centrifugation at 13,000 x *g* for 5 min. The supernatants were aliquoted and stored at -20 °C.

2.4.2: GC/MS

Samples extracted in DCM were analysed at the analytical department, Shell Global Solutions Ltd.. 1 µl samples were loaded onto Phenomenex ZB5-HT (30 m x 0.25 mm ID x

0.25 µm film) GC column and ramped from 50 °C to 250 °C at 10 °C min^{-1}, then held at 250 °C for 2 min (Phenomenenex, Macclesfield, UK). Samples were then analysed using Thermofisher DSQ II mass spectrometer (Thermo Scientific) within the mass range of 50-550 Daltons. Peak identification (eg. the presence of deuterated alcohol and aldehydes) was through comparison with known standards, retention time and mass spectral comparisons with the NIST database (NIST, 2011).

2.4.3: GC/GC

Samples extracted in DCM were analysed at the analytical department, Shell Global Solutions Ltd.. The GC/GC analysis was performed on a LECO Pegasus III GCxGC-TOF-MS equipped with a HP-5MS (30 m x 0.25 mm x 0.25 mm) as primary column and DB-17 (2 m x 0.10 mm x 0.10 mm) as a secondary column (LECO, St Josephs, USA). After splitless injection the primary oven was maintained at 40 °C for 10 min, heated to 300 °C at a rate of 5 °C per min, and held at 300 °C for 10 min. The secondary oven was kept at 40 °C for 10 min and heated at a rate of 5 °C per min till the temperature reached 335 °C and was held for 10 min. The mass spectrometer range was set to 35-650 m/z and rate set to 100 scans s^{-1} and 4 s modulation with 400 ms hot pulse. The presence of deuterated alkanes, alcohols, and aldehyde positions were confirmed through the use of standards and analysis against a known library.

2.4.4: 16S gene Sequencing

D.desulfuricans and *D.salexigens* were grown in PGB and MT media containing an additional 10 % (vol) D_2O. Once genomic DNA of each sample was extracted the 16S gene was amplified via the polymerase chain reaction using AccuPrime *Pfx* DNA polymerase (Invitrogen) and the primers 343 F (TAGGGRAGGCAGCAG) and 1047 R (GACGGGCGGTGTGTRC; Integrated DNA Technologies, Coralville, USA). Two of each of the desired PCR fragment (bands) were purifed and sequenced by Genevision (Newcastle Upon Tyne, UK). Analyses were performed using 3730xl DNA Analyser (Applied Bioscience).

2.5: Transformation

2.5.1: Antibiotic resistance test

An antibiotic resistance test was performed on *D.desulfuricans* to determine levels of natural antibiotic resistance. The antibiotics tested were: kanamycin, spectinomycin, chloramphenicol and geneticin (G418). Antibiotic sensitivity was analysed by using antimicrobial discs placed on agar plates.

Solutions of kanamycin, spectinomycin, chloramphenicol and geneticin (G418) were prepared to the following concentrations (μg ml^{-1}): 0, 10, 20, 50, 100, 200, 500, 750 and 1000. Keller *et al.*, 2011 state that *Desulfovibrio* spp have varying susceptibilities to different antibiotics, therefore the range of concentrations for each antibiotic varied. *D.desulfuricans* was exposed to kanamycin and spectinomycin concentrations of up to 1000 μg ml^{-1} and the maximum concentrations of chloramphenicol and geneticin (G418) were 100 μg ml^{-1} and 500 μg ml^{-1} respectively.

100 μl of 10x dilute *D.desulfuricans* was spread per agar plate. Once the bacteria had dried antibiotic-soaked discs were placed onto the agar. Each antibiotic concentration was performed in triplicate. All plates were incubated in the defined environment, daily observations monitored bacterial growth.

2.5.2: TargeTron Plasmid construction

A gene deletion plasmid was constructed using the TargeTron gene-knock out system according to the manufacturer's protocol (Sigma Aldrich). Analysis of *D.desulfuricans* genome using BLAST (basic local alignment search tool; megablast program; http://blast.ncbi.nlm.nih.gov/Blast.cgi) located the nucleotide sequence encoding the hsdR protein, a type 1 site-specific deoxyribonuclease (appendix I). This sequence was input into the TargeTron design website (http://www.sigma-genosys.com/targetron/) to design specific primers required for the TargeTron protocol. Polymerase chain reaction (PCR) amplified designed primers and 4% agarose gel electrophoresis verified DNA fragment sizes. Selected bands were purified using QIAGEN gel purification kit (QIAGEN) for insertion into pACD4K-C vector. Prior to insertion, a double digestion with *Hin*d III and *Bsr*G I linearised pACD4K-C. Ligation using Quick-Link T4 DNA ligation kit (Sigma-Aldrich) inserted the primer sequence into pACD4K-C. A further purification using QIAGEN plasmid purification kit (QIAGEN) followed. The concentration of the final plasmid, pACD4K-C-ΔhsdR, was determined using Quant-iT PicoGreen dsDNA kit (Invitrogen).

2.5.3: Cre-lox ΔhsdR DNA preparation

A gene deletion cassette was designed using Cre-lox recombination technology according to the sequence shown in appendix II. DNA was synthesised by DNA 2.0 (Menlo Park, USA) into a bacterial expression vector containing a kanamycin selection marker. *Esherichia coli* (TOP10; Invitrogen) was transformed via heat shock to allow for amplification and stock of plasmid DNA. Plasmids were extracted using GeneJET plasmid miniprep kit (Thermo Scientific) and digested for 2 hours at room temperature using EcoR1 and PstI (New England

Biolabs, Ipswich, USA). Digested plasmids were analysed via 1% gel electrophoresis, desired bands were extracted using QIAquick gel extraction kit (QIAGEN). Amplified DNA fragments were quantified using Quant-iT PicoGreen dsDNA kit (Invitrogen) and used directly for electroporation.

2.5.3: Electroporation

D.desulfuricans were grown to exponential phase and washed twice in 50 ml chilled electroporation buffer (30 mM Tris-HCl, pH 7.2) by re-suspension and centrifugation at 10,000 x *g* for 10 min at 4 °C.

For the TargeTron protocol, pACD4K-C-ΔhsdR was diluted to 0.2 μg μl^{-1} and pAR1219 (Sigma-Aldrich) to 1 μg μl^{-1}. pAR1219 is required as it carries the T7 RNA polymerase needed for induction of TargeTron intron-mediated gene disruption.

The Cre-lox protocol required the DNA fragments to be diluted to final concentrations of 1.06 ng μl^{-1}, 2.12 ng μl^{-1} and 4.26 ng μl^{-1}.

The electroporation procedure was performed using an Eppendorf Eporator according to the manufacturer's protocol (Eppendorf, Hamburg, Germany). Chilled, gap width 1 mm, aluminium electrodes, sterile electroporation cuvettes were used. 50 μl of bacterial culture was mixed with 50 μl of diluted plasmid in a chilled electroporation cuvette. This was then inserted into the Eporator where samples were electroporated at specific voltages. Samples were immediately placed in the controlled atmosphere and were replenished with in 1 ml fresh medium for 2 hours. Cultures were then plated using 10 μg ml^{-1} chloramphenicol for both TargeTron and Cre-lox methods. Plates were incubated in controlled atmosphere and checked daily for colonies. The voltages tested were; 2500 V, 2000 V, 1500 V and 1000 V. Each experiment was performed in triplicate.

CHAPTER 3:

D.DESULFURICANS AND *D.SALEXIGENS* GROWTH CHARACTERISATION AND OPTIMISATION.

3.1: Optimisation of growth analysis

D.desulfuricans and *D. salexigens* were cultured in Postgate medium B (PGB) and growth analysis was performed. Dry biomass was measure by harvesting 40 ml samples into pre-weighed 50 ml centrifuge tubes. The cell debris was then pelleted and freeze-dried for 24 hrs prior to dry biomass quantification. Optical density was analysed by measuring the absorbance at 600 nm of a diluted culture sample. Total protein concentration (mg ml^{-1}) was determined using the bioinchonic acid protein assay. Growth patterns according to each type of analysis are shown in figures 3.1 – 3.3.

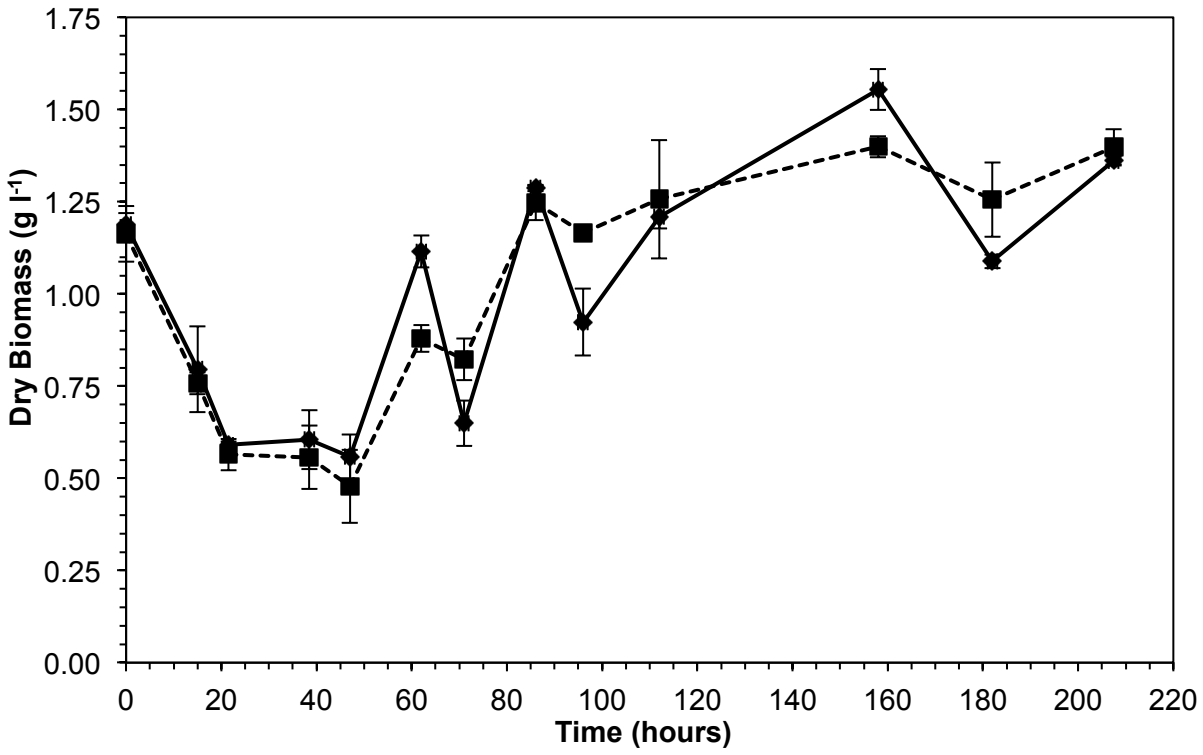


Figure 3.1: Growth of *Desulfovibrio* according to dry biomass (g l^{-1}).

D.desulfuricans (♦; solid line) and *D.salexigens* (■; dashed line) were grown in PGB under anaerobic conditions for 207 hours. Each data points represents the mean of three independent replicates, with the standard deviation of each point shown by error bars. Where, error bars appear missing they are masked by the symbol.

The dry biomass consists of all the insoluble material collected via centrifugation and freeze drying. Figures 3.1 shows an initial decrease followed by a minimal increase in dry biomass over a period of 207 hours. Although standard deviations represented by error bars are small, limited information can be gained from the pattern of both graphs. This is also supported by significant fluctuations in dry biomass values. Furthermore, the total range of 1 g of dry biomass questions the accuracy, thus, alternative methods of analyses were pursued.

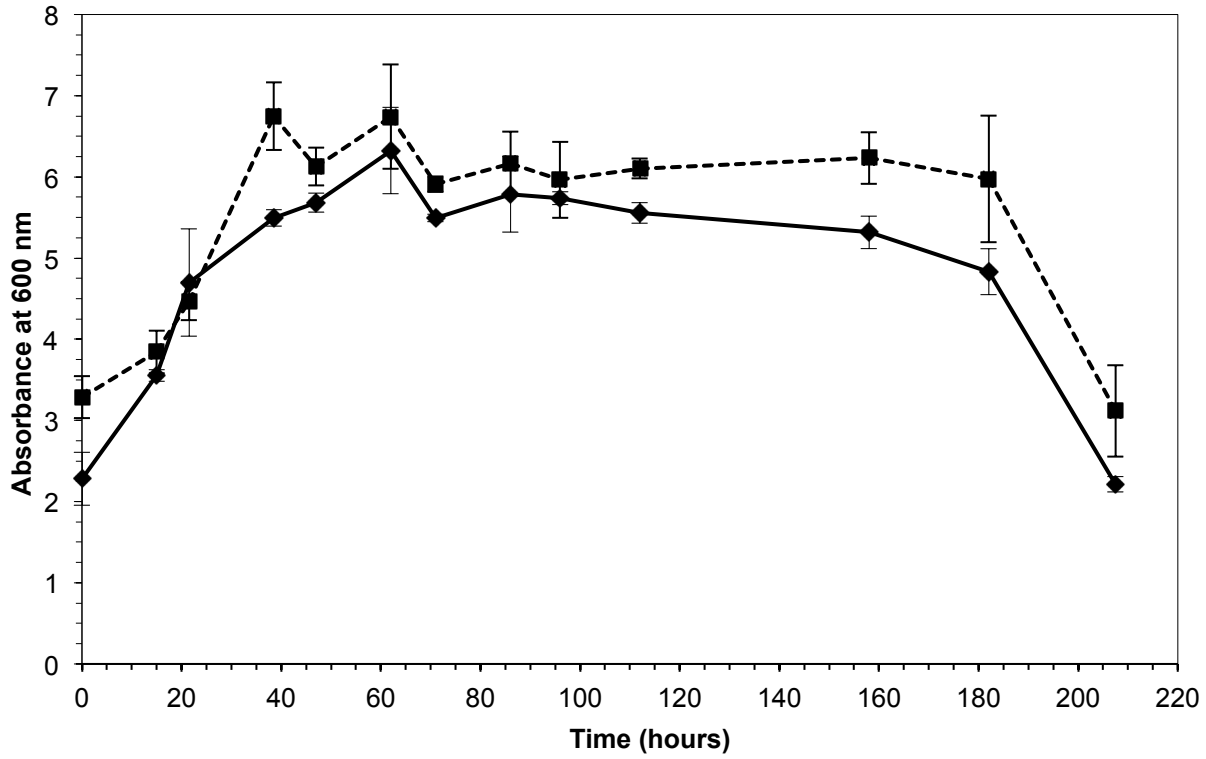


Figure 3.2: *Desulfovibrio* growth according to the optical density at 600 nm.

D.desulfuricans (♦; solid line) and *D.salexigens* (■; dashed line) were grown in PGB under anaerobic conditions for 207 hours. Each data points represents the mean of three independent replicates, with the standard deviation of each point shown by error bars. Where error bars appear missing they have been masked by the symbol.

Figure 3.2 shows the Abs_{600} of both *D.desulfuricans* and *D.salexigens* grown in PGB. Both graphs show similar pattern of increased absorbance after 20 hours that plateaus until 180 hours where both subsequently decrease. These pattern suggests lag, exponential, stationary phases typical to that of bacterial growth. *D.salexigens* data in figure 3.2 contains large error bars throughout growth questioning the level of confidence of using optical density quantifying bacterial growth.

Growth of *Desulfovibrio* is not typical of other bacteria such as *Escherichia coli,* as *Desulfovibrio* produce a black precipitate, iron sulphate (FeS) as a by-product of growth. During experimental procedures this precipitate was found to form large clumps that quickly settled, thus greatly affecting optical density readings. Therefore, this method does not offer confidence in providing a direct reflection of cell number and alternative methods of growth quantification were tested.

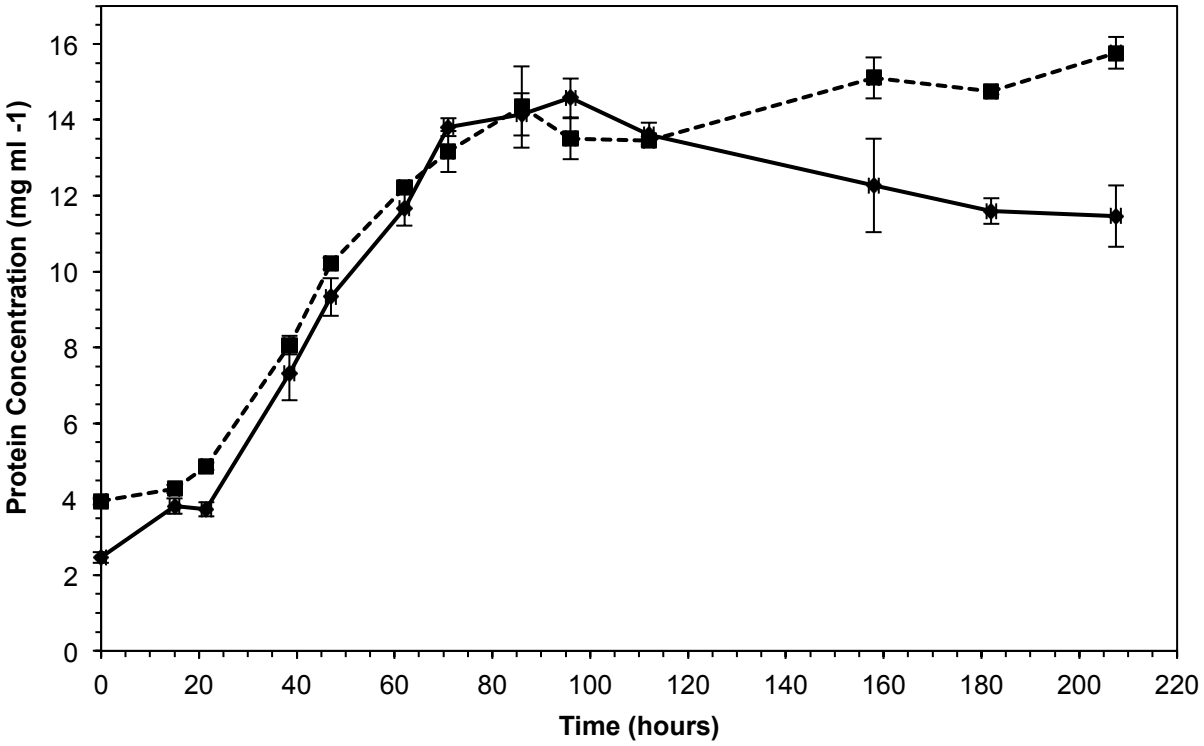


Figure 3.3: *Desulfovibrio* growth according to the protein concentration (mg ml $^{-1}$)

D.desulfuricans (♦; solid line) and *D.salexigens* (■; dashed line) were grown in PGB under anaerobic conditions for 207 hours. Each data points represents the mean of three independent replicates, with the standard deviation of each point shown by error bars. Where error bars appear missing they have been masked by the symbol.

The total protein concentration (mg ml $^{-1}$) of both *Desulfovibrio* strains was quantified using bioinchonic acid protein assay. Figure 3.3 show clear growth phases common to bacterial growth. *D.desulfuricans* is shown to have a lag phase of at least 25 hours with *D.salexigens* showing evident increase after 15 hours. Exponential phases for both strains last for approximately 50 hours, where protein concentrations then level out.

Identification of typical to bacterial growth suggests protein quantification is a good proxy for growth analyses in *Desulfovibrio*. Therefore this method was applied for the characterisation of *D.desulfuricans* and *D.salexigens* growth.

3.2: Characterisation of *D.desulfuricans* and *D.salexigens* growth

As consequence to previous method analysis, total protein concentration was used for in-depth growth characterisation of *D.desulfuricans* and *D.salexigens* grown in PGB. Six repeat analyses, each completed in triplicate, provided detailed protein data, see figure 3.4. All data was normalised by calculating the increase in protein concentration (mg ml^{-1}) for each individual growth prior to data being collated.

Both *D.desulfuricans* and *D.salexigens* show relative slow growth cycles reaching early stationary phase at 90 and 60 hours respectively (figure 3.4). The strains exhibit common bacterial growth phases; lag, exponential and stationary, although, comparative analysis suggests different growth phase lengths. However, once in stationary phase both strains reach similar total protein concentrations of 7500- 8500 mg ml^{-1}.

D.desulfuricans shows a lag phase of approximately 55 hours leading to a 35 hour exponential phase till 90 hours (figure 3.4A). In comparison, *D.salexigens* lag phase is shorter, only lasting approximately 30 hours (figure 3.4B). This is followed by a 30 hour exponential phase.

Growth rates of both *D.desulfuricans* and *D.salexigens* were calculated using the following equation (values were taken during the exponential phase):

$$\text{Growth rate } (\mu) = \frac{\ln IP_{t2} - \ln IP_{t1}}{t_2 - t_1}$$

Where, $\ln IP_{t2}$ is the natural logarithm of the increase in protein concentration at t_2, $\ln IP_{t1}$ is the natural logarithm of increase in protein concentration at t_1. The units of this proxy growth rate are in milligrams per millilitre per hour (mg ml^{-1} h^{-1}).

D.desulfuricans had a growth rate of 45.65 mg ml^{-1} h^{-1} and *D.salexigens* of 259.54 mg ml^{-1} h^{-1}.

These are important observations for future experiments such as comparative transcriptomic analysis that requires RNA extraction at mid-exponential phase.

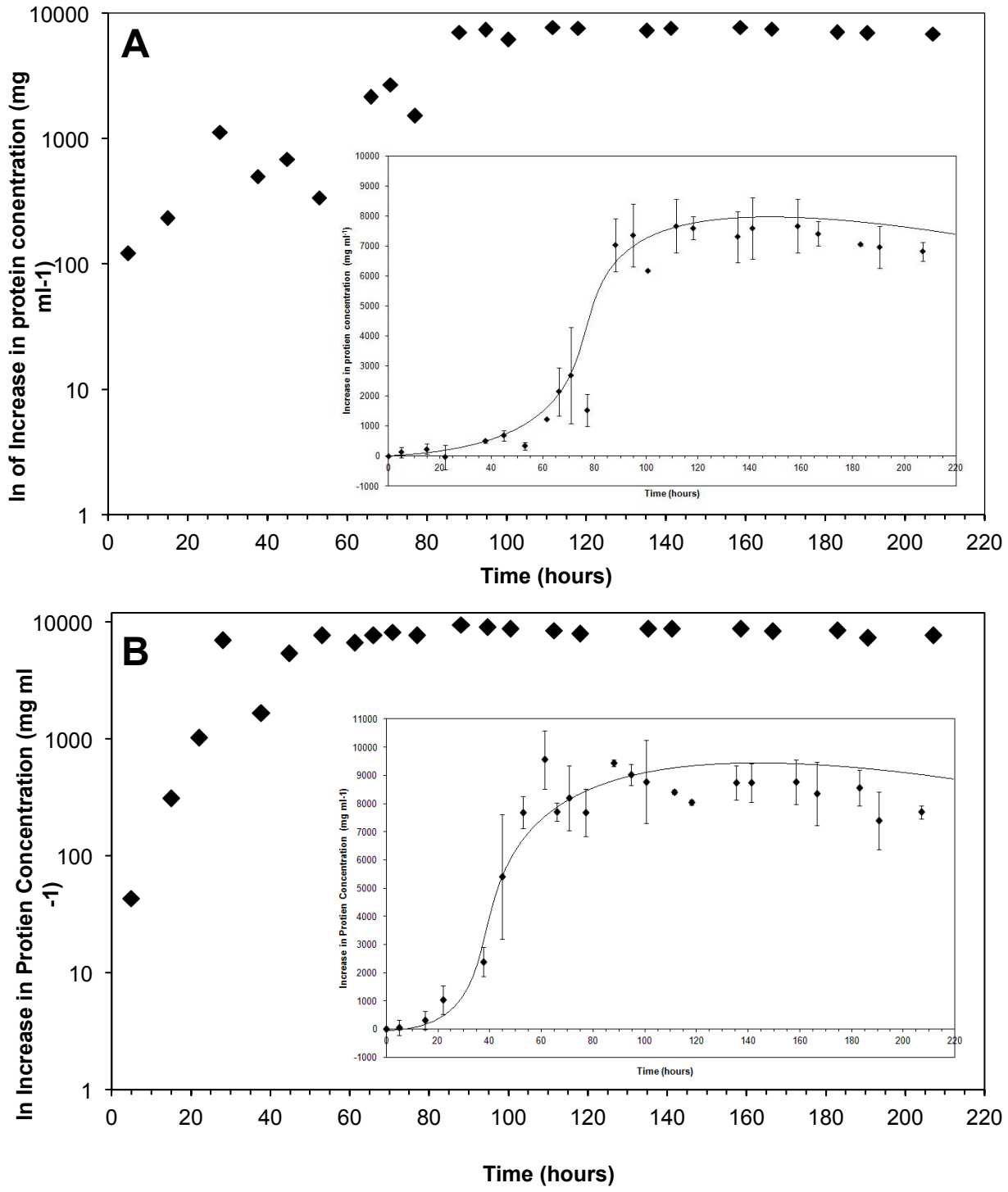


Figure 3.4: *Desulfovibrio* growth represented by the natural logarithium (ln) increase in protein concentration (mg ml $^{-1}$); main graph. Insets show *Desulfovibrio* growth measured by the actual increase in protein concentration (mg ml $^{-1}$)

D.desulfuricans (A) and *D.salexigens* (B) were grown in PGB under anaerobic conditions for 207 hours. Each data points represents the mean of six independent replicates, with the standard deviation of each point shown by error bars. Where error bars appear missing they have been masked by the symbol.

CHAPTER 4

EFFECTS OF MEDIA ON C18 ALKANE SYNTHESIS IN DESULFOVIBRIO

4.1: *Desulfovibrio* Genus Screen:

A genus wide alkane screen was performed to confirm the production of microbial derived hydrocarbons (C_{18} chain length). Metal toxicity medium (MT), a non precipitating media, and Postgate medium B (PGB) were used in the genus wide alkane synthesis (Sani *et al.,* 2001). The bacteria used in the screen were; *Desulfovibrio desulfuricans* 8326, *Desulfovibrio salexigens* 2638, *Desulfovibrio desulfuricians* G20, *Desulfovibrio magneticus* 13731, *Desulfovibrio piger* 749 and *Desulfovibrio vulgaris* Hildenborough 8303. All strains were grown in both media containing 10% (vol) D_2O. Once all cultures reached stationary phase alkanes were extracted using DCM and sent for Gas Chromatography – Mass spectrometry (GC/MS). This determined if any microbial C_{18} alkanes are produced. Blank media samples provided baseline results to determine any media contamination or naturally occurring deuterated C_{18} hydrocarbons.

Samples of *D.desulfuricans* grown in PGB showed microbial C_{18} alkane synthesis, confirming the findings of Lee's 2011 report. Furthermore, both *D.desulfuricans* and *D.salexigens* were found to microbially synthesis C_{18} alkane when grown in MT. All other samples showed no evidence of C_{18} alkane synthesis when grown in either media.

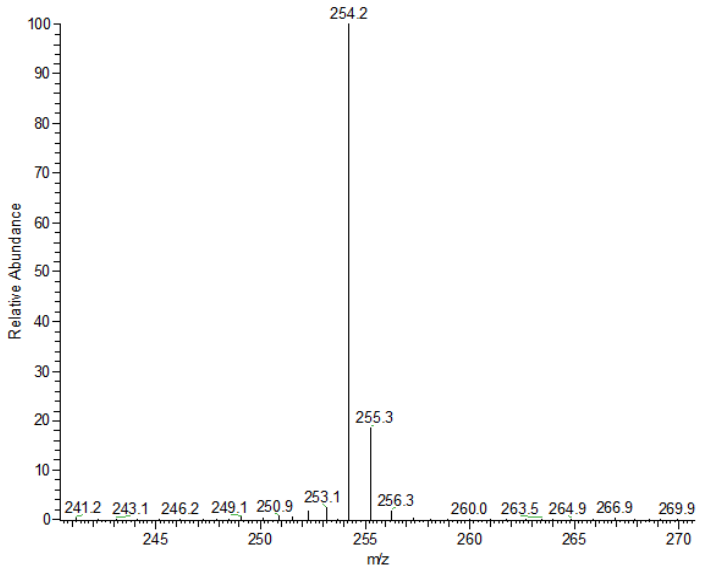


Figure 4.1: Mass spectrum of non-deuterated C_{18} alkane standard, octadecane.

This was used as a negative control when analysing microbiologically derived C_{18} alkanes in strains of *Desulfovibrio.*

Figure 4.1 shows the GC/MS spectrum of a non-deuterated C_{18} alkane, octadecane. The most abundant peak present at m/z 254.2 is representative of the non-deuterated alkane, $C_{18}H_{38}$. This provided a negative control for analysis of synthesised alkanes in *Desulfovibrio* strains. All work completed in the study was to analysis the presence of deuteration of the C_{18} alkane, therefore conclusion are made regarding the relative abundance as opposed to actual amounts. Unfortunately, quantitative analysis of hydrocarbons was not performed in this study.

GC/MS was used as the main method of alkane analysis but where available GC/GC, although, for these analyses the data is not shown. For each GC/MS spectra shown octadecane was used as the standard.

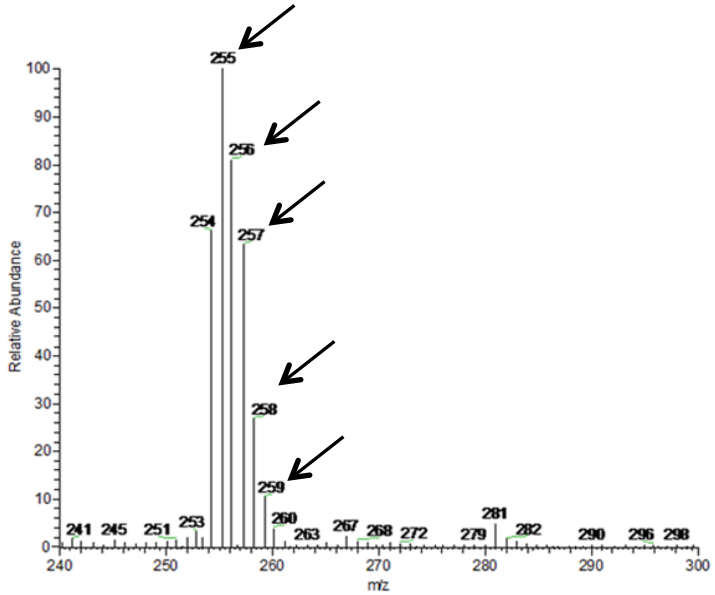


Figure 4.2: Mass spectrum of hydrocarbons extracted from *D.desulfuricans* grown in PGB.

Spectrum show significant m/z peaks of 254, 255, 256, 257, 258 and 259. M/z peaks of 255, 256, 257, 258 and 259, confirming the addition of one, two, three, four or five ^{2}H to the hydrocarbon chain. Arrows identify deuterated peaks.

Figure 4.2 is the mass spectrum of extracted hydrocarbons from *D.desulfuricans* grown in PGB. Deuteration is proved by peaks at m/z 255, 256, 257, 258 and 259, suggesting the addition of one, two, three, four or five ^{2}H respectively. Confirming *D.desulfuricans* ability to synthesis C_{18} alkane through the addition of ^{2}H from the D_2O present in PGB. Similar spectra can be found for extracted hydrocarbons of *D.desulfuricans* and *D.salexigens* grown in MT where abundant m/z 255, 257 and 259 peaks are present (figure 4.3). The *D.desulfuricans* spectrum is shown in appendix III; section I. These spectra confirm both strains ability to microbially synthese C_{18} alkanes.

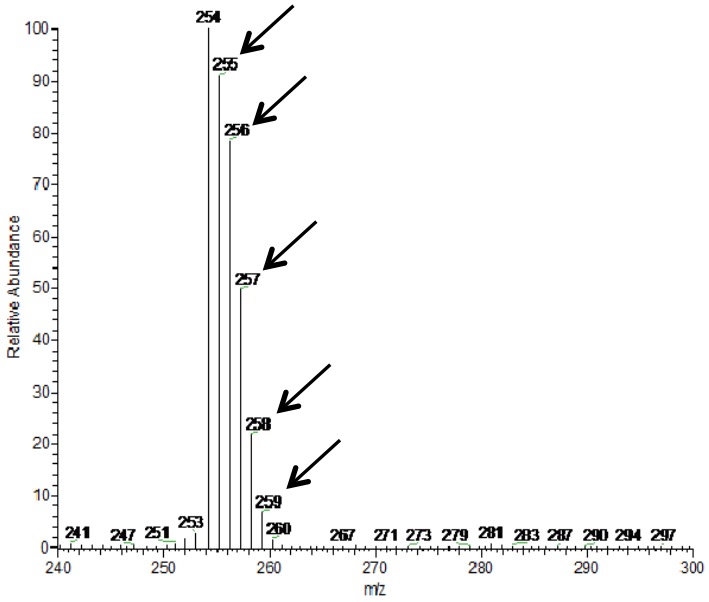


Figure 4.3: Mass spectrum of hydrocarbons extracted from *Desulfovibrio salexigens* grown in MT.

Spectrum show significant m/z peaks of 254, 255, 256, 257, 258 and 259. M/z peaks of 255, 256, 257, 258 and 259, confirming the addition of one, two, three, four or five ^{2}H to the hydrocarbon chain. Arrows identify deuterated peaks

Further confirmation of microbial derived C_{18} alkanes can be found through comparison with the hydrocarbons extracted from *D.salexigens* grown in PGB (figure 4.4).

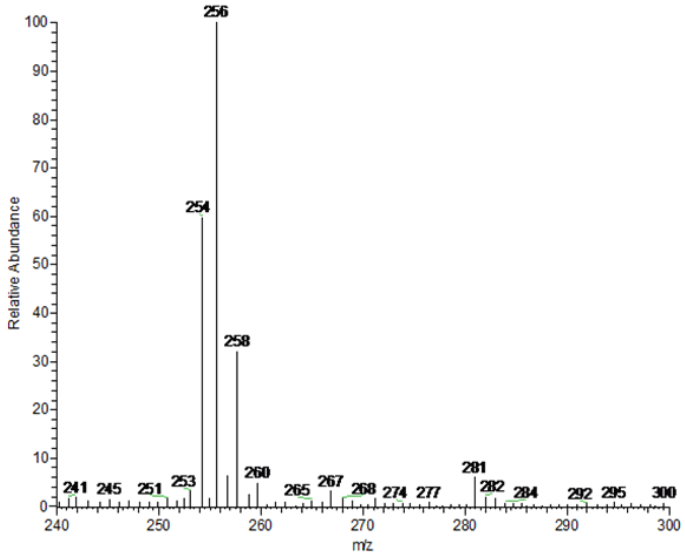


Figure 4.4: Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in PGB.

Spectrum show significants m/z peaks of 254, 256 and 258.

The *D.salexigens* spectrum shown in figure 4.4 is ambiguous, the absence of a peak at 255 suggests no addition of one ^{2}H to the hydrocarbon chain suggesting no deuteration. However, peaks as 256 and 258 could suggest addition of two and four ^{2}H. A concern is that this spectrum could represent, a mix of two or more co-eluting compounds. GC/GC analysis allows the de-convolution of these peaks to determine the exact compound, previous work by Lee showed confirmed these peaks not to be deuteration (Lee, 2011; Internal Shell report). Unfortunately, in the duration of the majority of this study GC/GC analysis was unavailable. Furthermore, if shown a single peak if the spectrum was to show an individual peak

This experiment confirms Lee's conclusions that only *D.desulfuricans* can microbially derive C_{18} hydrocarbons when grown in PGB. Results also shows the strain to synthesise C_{18} hydrocarbons when grown in MT. *D.salexigens* synthesises C_{18} hydrocarbons when grown in MT but not whilst in PGB.

4.2: *D.salexigens* metal toxicity medium analysis

Further experiments were performed to determine if a constituents or mix of constituents resulted in the ability of *D.salexigens* to synthese microbially derived C_{18} hydrocarbons when grown in MT but not in PGB.

Cultures of *D.salexigens* were grown in a modified MT to contain different combinations of substituent's from PGB. All additional ingredients were added at equivalent amounts (g l^{-1}) to that of the PGB recipe. The following combinations were assessed; additional $CaSO_4$, additional $FeSO_4$, additional $MgSO_4$, additional Yeast, additional Ammonium Thioglycate, additional $CaSO_4$, $FeSO_4$, $MgSO_4$, Yeast and Ammonium Thioglycate, additional $FeSO_4$ and $CaSO_4$, additional $FeSO_4$ and $MgSO_4$, additional $FeSO_4$ and Yeast and additional $FeSO_4$ and Ammonium Thioglycate. All cultures were grown in the defined anaerobic atmosphere until they reached stationary phase (7 days) when alkanes were extracted and sent for GC/MS analysis.

All samples analysed showed deuteration of C_{18} hydrocarbons (see appendix III; section II), thus, none of the combinations resulted an inabilty to synthese C_{18} hydrocarbons.

The next step was to analyse the effect of PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)), a major component of MT, on the microbial C_{18} alkane synthesis capabilities of *D.salexigens*. This was completed by growing *D.salexigens* in MT containing no PIPES with other combinations of components from PGB and another MT medium of increasing PIPES concentration. The combination of a modified MT containing no PIPES included; additional $CaSO_4$, additional $FeSO_4$, additional $MgSO_4$, additional Yeast, additional Ammonium Thioglycate, additional $CaSO_4$, $FeSO_4$, $MgSO_4$, Yeast and Ammonium Thioglycate, additional $FeSO_4$ and $CaSO_4$, additional $FeSO_4$ and $MgSO_4$, additional $FeSO_4$ and Yeast and additional $FeSO_4$ and Ammonium Thioglycate. All PGB components added at equivalent amounts (g l^{-1}). The amount of PIPES added was relative to amounts present in the defined media recipe. Amounts included; 0%, 25%, 50%, 75% and 100%. All cultures were grown in the defined atmosphere until they reached stationary phase (7 days) when alkanes were extracted and sent for GC/MS analysis.

Again, all extracted hydrocarbons showed deuteration (appendix III; section III), therefore, neither the amount of PIPES or lack of PIPES plus PGB components resulted in the inhibition of C_{18} alkane synthesis.

Next, *D.salexigens* was grown in modified PGB and MT.

The PGB medium had either, an alternative source of sulphate, or, additional tryptone, or, both added to the media. This experiment was performed to see if either a different type of sulphate or presence of tryptone would cause alkane synthesis. The alternative sulphate was created by removing all of the sulphate sources found in PGB ($MgSO_4 \cdot 7H_2O$; 0.5 g l^{-1},

$FeSO_4 \cdot 7H_2O$; 1.0 g l^{-1}, $CaSO_4$,; 3.5 g l^{-1}) and supplementing this with the type and quantity found in MT (Na_2SO_4; 1.0 g l^{-1}).

MT was altered by having different quantities of tryptone or by having an alternative sulphate source. The amount of tryptone is relative to that found in the defined MT, the quantities analysed were; 0%, 25%, 50% and 100%. The sulphate switch was created by removing the Na_2SO_4 from the medium, supplementing it with the sulphate combination found in PGB ($MgSO_4 \cdot 7H_2O$; 0.5 g l^{-1}, $FeSO_4 \cdot 7H_2O$; 1.0 g l^{-1}, $CaSO_4$,; 3.5 g l^{-}). All cultures were grown in the defined atmosphere until they reached stationary phase (7 days) when alkanes were extracted and sent for GC/MS analysis.

Hydrocarbons extracted from modified PGB cultures showed no evidence of deuteration, suggesting, neither tryptone or an alternative sulphate source acts as a 'switch' for microbial derived C_{18} hydrocarbon production. All extracted hydrocarbons from the modified MT showed evidence of deuteration, therefore, tryptone nor alternative sulphate source resulted in the inhibition of microbial C_{18} alkane synthesis.

4.3: Affect of KH_2PO_4 and Ascorbic acid on alkane synthesis in *D.desulfuricans* and *D.salexigens*

A final media manipulation experiment was performed to test the effect of the remaining untested components; potassium, ascorbic acid and yeast extract. Both *D.desulfuricans* and *D.salexigens* were grown in PGB and MT with varying amounts of potassium, ascorbic acid and yeast extract.

The PGB either lacked its normal amount of potassium and ascorbic acid (–KH_2PO_4 – Ascorbic Acid) or contained the equivalent amount of yeast extract to that in the MT medium.

The MT medium was supplemented with either potassium or ascorbic acid or both at the same concentration as found in PGB. All cultures were grown in the defined atmosphere until they reached stationary phase (7 days) when alkanes were extracted and sent for GC/GC analysis.

The hydrocarbons extracted from all cultures of *D.desulfuricans* confirm its ability to microbially synthese C_{18} alkane, whilst proving none of the component combinations resulted in alkane synthesis inhibition. Hydrocarbons extracted from *D.salexigens* grown in all MT media combinations showed deuteration. In contrast, the hydrocarbons extracted from the same strain grown in the PGB mixtures were undeuterated. Showing that neither

combinations of potassium and ascorbic acid nor minimal amounts of yeast resulted in unexpected hydrocarbon profiles.

Results from this chapter show that extracted hydrocarbons from *Desulfovibrio* grown in PGB support the finding of Lee's 2011 report. The data is unable to explain the ability of *D.salexigens* syntheses C_{18} hydrocarbons when grown in MT but not in PGB. However, the data shows that none of the media components analysed result in the activation or inhibition of the C_{18} alkane pathway.

4.4: Analysis of bacterial culture integrity

16S gene sequence analysis of both *D.desulfuricans* and *D.salexigens* grown PGB and MT was performed to determine the integrity of the cultures. BLAST analysis and alignment genetically confirmed *D.desulfuricans* cultures. However, analysis of the *D.salexigens* grown in MT show contamination with *D.desulfuricans*, thus explaining microbially C_{18} alkane production. All analysis and alignment results can be found in appendix IV.

Further analyses to assess the genetic integrity of the *D.salexigens* culture were performed. A freeze-dried stock of *D.salexigens* received directly from the NCIMB library was replenished in liquid PGB. This was then streaked onto both PGB and MT agar plates. Individual colonies were selected and re-grown in both PGB and MT. 16S gene sequence analysis of *D.salexigens* grown PGB and MT verified the PGB culture contained a bacterium of a single taxon, whereas, multiple bacteria were present in the MT culture. C_{18} hydrocarbon analysis was performed on sequenced samples to verify hydrocarbon production. All analysis and alignment results can be found in appendix V.

CHAPTER 5

RNA EXTRACTION AND COMPARATIVE TRANSCRIPTOME ANALYSIS

5.1: RNA Extraction Optimisation

The aim of these experiments was to develop a reliable and accurate method for extracting and purifying high quality RNA from both strains of *Desulfovibrio*. This high quality RNA was required for sequencing via the RNA-seq application on the Illumina high-throughput HiSeq 2000 sequencing system. A major problem thought to make extractions more complex is the formation of and FeS precipitate, a by-product of growth.

A series of experiments were performed to optimise conditions of the extraction and purification methods. Final yield and integrity improvements were achieved by incubating cells in RNAprotect bacterial reagent for 5 mins and using an optimised digestion buffer (15 mg ml^{-1} Lysozme, 2 mM EDTA [pH 8.0], 0.4 mg μl^{-1} Proteinase K). Additional filtration through a genomic DNA removal column and on-column DNase digestion with RNase-free DNase removed all traces of genomic DNA. An extra purification step using an RNeasy mini kit was found to further improve both yield and integrity of total RNA. Thus, a optimised RNA extraction method was developed and is described in chapter 2 section 2.3.1.

5.2: Comparative Transcriptome Analysis

Total RNA was extracted from *D.desulfuricans* and *D. salexigens* grown in Postgate medium B (PGB) and *D.salexigens* grown in metal toxicity medium using the optimised RNA extraction and purification method. Samples were harvested a mid-exponential phase, 40 and 80 hours for *D.desulfuricans* and *D.salexigens* respectively. Total RNA was sent to the University of Exeter where they were prepared for sequencing using Illumina TruSeq RNA sample preparation kit. Sequencing was performed on the Illumina HiSeq 2000. RNA-seq data generated was analysed by Thomas Lux and transcripts mapped onto the reference genomes using Bowtie software.

The first set transcriptome data initially showed contamination of *D.desulfuricans* by *Bacillus subtilis* this data is presented in appendix VI. However after further analysis and on correspondence with the bioinformatican it has been concluded that the ‘contamination’ was un-depleted *Desulfovibrio*. Currently the second round sequence data is being generated and analysed. Unfortunately, due to time constraints no data can currently be released.

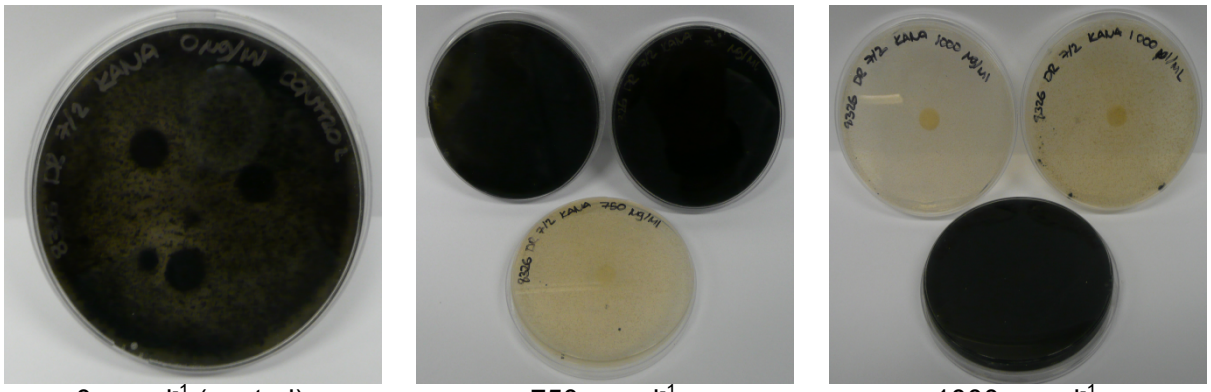
CHAPTER 6

GENERATING A D.DESULFURICANS STRAIN WITH HIGH TRANSFORMATION EFFICIENCY

These series of experiments was designed to produce a strain of *D.desulfuricans* with high transformation efficiency by deleting the gene encoding the hsdR protein. The hsdR protein is a type 1 site-specific deoxyribonuclease present to protect the bacterium from foreign DNA, thus preventing replication of transformed genetic information (Promega website). Therefore a strain lacking the hsdR protein would have improved transformation efficiency. This was attempted using the TargeTron gene-knock out system (Sigma-Aldrich) or using a plasmid containing a hsdR deletion cassette synthesised by DNA 2.0.

6.1: Antibiotic Sensitivity Test

A preliminary investigation to isolate any antibiotic resistance in *D.desulfuricans*. underwent an antibiotic screen to isolate any natural antibiotic resistance. It is known that that *Desulfovibrio* spp have varying susceptibilities to different antibiotics (Keller *et al.,* 2011). Antibiotics are required as resistant markers in the selection of genetically modified bacteria. The anitbiotic tested were kanamycin, spectinomycin, chloramphenicol and geneticin (G418). By growing *D.desulfuricans* on Postgate medium B (PGB) plates containing antimicrobial discs provided visual evidence of growth inhibition (figure 6.1- 6.4). *D.desulfuricans* was exposed to kanamycin and spectinomycin concentrations of up to 1000 mg ml^{-1}. 100 mg ml^{-1} and 500 mg ml^{-1} were the maximum concentrations of chloramphenicol and geneticin (G418) respectively. Each concentration was performed in triplicate.

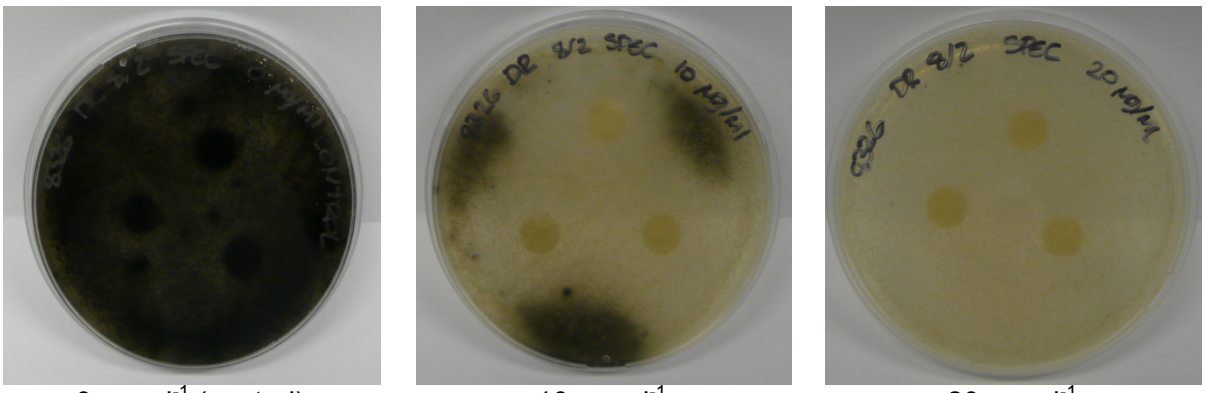


0 µg ml^{-1} (control) 750 µg ml^{-1} 1000 µg ml^{-1}

Figure 6.1: *D.desulfuricans* grown on PGB agar containing kanamycin.

Three concentrations of kanamycin are shown; 0 µg ml^{-1}, 750 µg ml^{-1} and 1000 µg ml^{-1}. Confluent bacterial lawns (complete bacterial coverage) are shown at all 3 antibiotic concentrations. Concentration between 0 µg ml^{-1} and 750 µg ml^{-1} are not represented as all show confluent bacterial lawns.

Figure 6.1 shows natural resistance of *D.desulfuricans* to high levels of kanamycin,indicated by growth at concentrations up to 1000 µg ml^{-1}. Although, two plates at 1000 µg ml^{-1} show no growth suggesting that concentrations of 1000 µg ml^{-1} kanamycin would be sufficient for use as a selection marker. It also provides a method of selecting *D.desulfuricans* through natural resistance to high quanities of kanamycin.

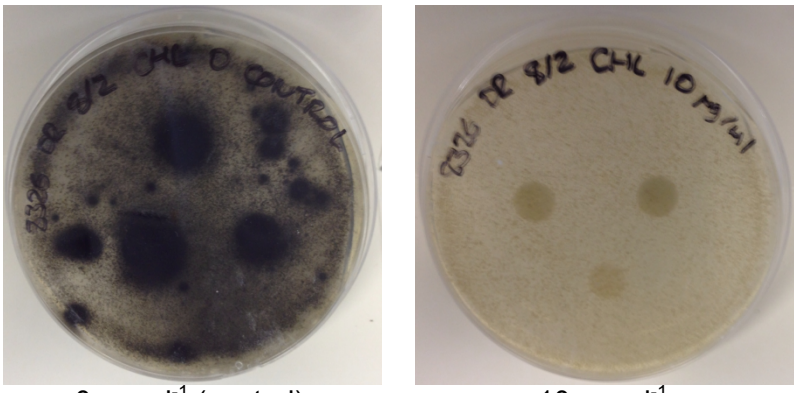


0 µg ml^{-1} (control) 10 µg ml^{-1} 20 µg ml^{-1}

Figure 6.2: *D.desulfuricans* grown on PGB agar containing spectinomycin.

Three concentrations of spectinomycin are shown; 0 µg ml^{-1}, 10 µg ml^{-1} and 20 µg ml^{-1}. Confluent bacterial lawns is present in the 0 µg ml^{-1} (control). Some growth is evident on 10 µg ml^{-1} plate shown by the halo around each disc. No evident growth at 20 µg ml^{-1} concentration.

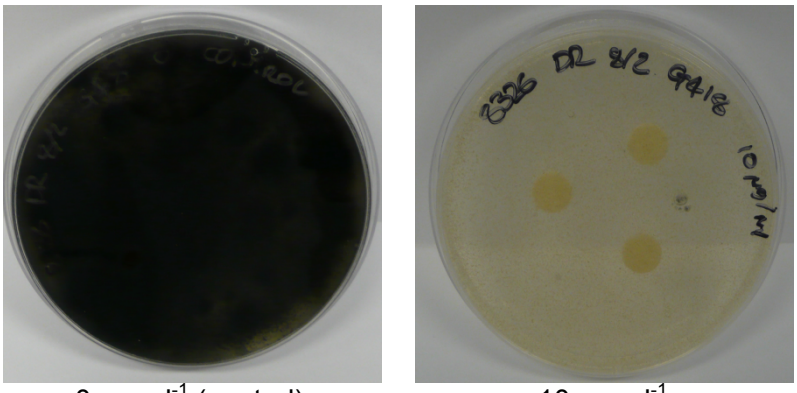
Minimal resistance to spectinomycin is observed on plates containing 10 µg ml^{-1} where a halo is present prior to bacterial growth (figure 6.2). No growth was present on 20 µg ml^{-1} plates suggesting the minimal inhibitory concentration (MIC) of spectinomycin is 20 µg ml^{-1}.



0 µg ml^{-1} (control) 10 µg ml^{-1}

Figure 6.3: *D.desulfuricans* grown on PGB agar containing chloramphenicol.

Two concentrations of chloramphenicol are shown; 0 µg ml^{-1} and 10 µg ml^{-1}. Significant bacterial growth is present in the 0 µg ml^{-1} (control) and no growth is present at 10 µg ml^{-1}.



0 µg ml^{-1} (control) 10 µg ml^{-1}

Figure 6.4: *D.desulfuricans* grown on PGB agar containing genticin (G418).

Two concentrations of genticin (G418)are shown; 0 µg ml^{-1} and 10 µg ml^{-1}. A confluent bacterial lawn is present in the 0 µg ml^{-1} (control) and no growth is present at 10 µg ml^{-1}.

Results show the minimal inhibitory concentrations (MIC) of both chloramphenicol and genticin (G418) are identical (figures 6.3 and 6.4). Both show expected growth on 0 µg ml^{-1}

(control) whilst a lack on plates containing 10 μg ml^{-1} antibiotic. At the time of growth it was noted that *D.desulfuricans* grown under chloroamphenicol took longer than that of the other antibiotic treatments, this may explain the slight discrepancy seen in the nature of the control plate.

The antibiotic screen confirms Keller *et al.* suggestion of different *Desulfovibrio* species have varying natural resistant to numerous antibiotics. This screen shows kanamycin, spectinomycin, chloramphenicol and geneticin (G418) to have different minimal inhibitory concentrations (MIC). Thus, when using these as antibiotic markers in transformation experiments the following concentrations are required; 1000 μg ml^{-1} kanamycin, 20 μg ml^{-1} spectinomycin, 10 μg ml^{-1} chloramphenicol and 10 μg ml^{-1} geneticin (G418).

6.2: *hsdR* Nucleotide sequence

A BLAST search (megablast program) was performed to identify the nucleotide sequence encoding the hsdR protein on the *D.desulfuricans* genomic contigs. The sequence was required for plasmid design for use with TargeTron gene knockout system. The *hsdR* nucleotide sequence was located on node 4 of the *D.desulfuricans* at a length of 2948 bp. A full sequence can be found in appendix I.

6.3: TargeTron Transformation

Plasmid constructed according to the TargeTron protocol were electroporated into *D.desulfuricans* according to the methods described. Different electroporation parameters such as set voltage, volume of plasmids were analysed to determine the most effective method of electroporation. None of the selection plates showed any growth of colonies, proving the protocol was unsuccessful.

6.4: Cre-lox Transformation

A plasmid containing a deletion cassette for the hsdR gene (ΔhsdR) was synthesised by DNA 2.0 and transformed into *E.coli* (TOP10) (appendix II). The plasmid was extracted and digested with EcoR1 and PstI. Gel electrophoresis confirmed the correct size of insert (1049 bp), see appendix II. Each fragment was extracted from the gel, purified and quantified. This was directly electroporated into *D.desufuricans*. Different parameters were tested in an attempt to optimise the electroporation method. Unfortunately, no colonies grew on any of the plates.

CHAPTER 7

DISCUSSION

7.1: Growth Analysis and Characterisation of *D.desulfuricans* and *D.salexigens*

To date, analysis of growth in *Desulfovibrio* has been quantified by optical density (Bryant *et al.,* 1977; Keller *et al.*, 2011). In this project, both optical density and biomass resulted in an inaccurate measurement of growth, due to two different factors. Unpredictable optical density measurements were attributable to formation of a dense black precipitate, FeS. This was noted to quickly settle in the spectrophotometry cuvettes resulting in false readings. Moreover, dry biomass measurements were recorded within a range of 1 g l^{-1}, drawing attention to potential errors in the sampling procedure, decreasing confidence for the future collection of accurate and reliable data. From the analysis methods examined, quantification of total protein concentration proved the most reproducible. Analysis of figure 3.3 highlights consistent and common growth patterns analogous to that of bacterial growth. Thus, indicating protein quantification as a good proxy for direct growth analysis of *Desulfovibrio.*

Further analysis of growth for both *D.desulfuricans* and *D.salexigens* using protein concentration as the method of quantification was performed. Six replicate cultures, each performed in triplicate, demonstrated the growth phases; lag, exponential and stationary all typical of bacterial growth. However, they present significant differences in growth profiles between strains. *D.salexigens* is shown to have a much shorter lag phase (~20 hrs) compared with *D.desulfuricans* (~50 hrs), see figures 3.4A and 3.4B. Thus, *D.salexigens* reached stationary ~40 hours earlier than *D.desulfuricans*. This was emphasised through the differences in growth rates, *D.salexigens*, 259.54 mg ml^{-1} h^{-1} and *D.desulfuricans*, 45.65 mg ml^{-1} h^{-1}.

A major result of the growth characterisation was the accurate determination of a mid-exponential time point, significant for the comparative transcriptomic analysis. mRNA extracted at mid-exponential phase would provide an accurate representation of the transcription of the proteins required for growth and the identification of potential proteins involved in C_{18} alkane synthesis within *D.desulfuricans.*

7.2: C_{18} Alkane Synthesis in *Desulfovbrio*

A genus wide *Desulfovibrio* C_{18} alkane synthesis screen was performed to verify findings of previous alkane synthesis studies (Jankowski and ZoBell, 1944; Davis, 1964; Oppenheimer, 1965; Bagaeva and Chernova, 1994; Bagaeva and Zolotukina, 1994; Bagaeva, 1997; Bagaeva and Zinourova, 2004; Lee, 2011; internal shell report). Results confirm alkane synthesis in *D.desulfuricans* cultivated in PGB and MT. Previously, *D.salexigens* had never been shown to produce alkanes (Lee, 2011), when cultivated in PGB, and was therefore considered incapable of alkane production. In this study, we demonstrated that *D.salexigens* cultivated in MT synthesises alkanes. We therefore predicted that some component(s) in the media may be the cause of the opposing alkane synthesis profiles in *D.salexigens* (figures 4.1 - 4.4). If isolated, this would aid the identification of proteins involved in the alkane synthesis pathway.

However, after a series of media analyses, we were unable to identify a particular, or mixture of components that resulted in alkane synthesis. Additional genetic analysis was therefore performed. This sequencing of the 16S gene of *D.desulfuricans* and *D.salexigens* when cultivated in both media confirmed that the cultures of *D.desulfuricans* and *D.salexigens* in PGB were monophyletic. Conversely, 16S rDNA analysis showed a mixed culture of *D.salexigens* when grown in MT. Alignments of sequences suggest existence of *D.desulfuricans* within this mixed culture, thus accounting for the production of microbially derived C_{18} alkanes (appendix III). Furthermore, to eliminate the possibility of contamination/ human error, *D.salexigens* from NCIMB library stocks were replenished in PGB and MT. Identical genetic analysis again confirmed the integrity of the *D.salexigens* culture in PGB and further verified the mixed culture in MT (appendix IV). This suggests a potential contamination within the NCIMB library stock of *D.salexigens,* identifying *D.desulfuricans* as a potential contaminant. Proving that when cultivated in MT *D.desulfuricans* thrives, outcompeting the *D.salexigens* resulting in microbial C_{18} alkane synthesis. However, other considerations such as in-house contamination of MT media could be another source of the discussed contamination. Moreover the contamination of *D.salexigens* with *D.desulfuricans* could explain peaks 256 and 258 peaks in figure 4.4. These maybe the result of contamination and co-elution with C_{18} alkanes produced by *D.desulfuricans*. Further work would be completed to isolate and characterise the strains present within the mixed culture resulting in additional analysis of the strains, determining the source of any contamination.

7.3: Comparative Transcriptome Analysis

For transcriptomes of *D.desulfuricans* and *D.salexigens* to be sequenced using the Illumina method and compared, high quality total RNA was required. From the outset, extraction of RNA from *Desulfovibrio* proved problematic. Low yields and poor quality RNA was generated from extractions using methods designed for other gram-negative bacteria such as *Escherichia coli*. Significant method development was required to produce RNA of a quality and yield necessary for sequencing. The major problem posed was natural formation of iron sulphide, a natural by-product of growth in *Desulfovibrio* (Postgate, 1984). The tertiary structure of RNA is sensitive to different types and concentrations of cations present at time of extraction (Draper, 2004). It is postulated that the negatively charged RNA molecules electrostatically bind to the positive Fe^{2+} ion in the iron sulphide (personal correspondence Mike Goldsworthy). Thus, as soon as the RNA is extracted it immediately attracts toward the iron resulting in significant degradation. Further degradation and loss in yield will occur during the attempts to separate the iron-RNA complex and throughout the general purification process. Initially, an alternative non-precipitating, MT medium was tested. This provided required yield and RNA quality, however, supplementary analysis proved contaminated library stocks resulting in a mixed bacterial culture. Therefore, rending it unsuitable for this comparative experiment.

The focus consequently turned to producing an optimised method of RNA extraction for *Desulfovibrio* cultivated in PGB. After much adjustment, a reliable method of RNA extraction was developed; this is described in chapter 3, section 2.3.1. This procedure was used to produce the total RNA required for depletion to mRNA then transcriptomic sequencing using the Illumina HiSeq 2000 platform.

To date, sequencing data has highlighted contamination in first round of *D.desulfuricans*, hence, preventing comparative analysis. However, a second sequencing run is currently being completed. Unfortunately, analysis and annotations are due to be completed within the next three months. As a result of time constraints and the complex nature of the comparative analysis between reads of different strains no conclusions can be devised regarding any potential gene candidates involved in alkane synthesis. However, once completed the sequence reads will be aligned against their reference genome using BOWTIE software (Langmead, 2010). Expression profiles would be quantified using a perl script called sam2Refcount. Next, intermediate gene ontology (GO) annotation would be completed using a local version of RAST (Exeter Microbial Biofuels Group) and by BLAST comparison with

public GO annotated sequences. Further functional analysis would be obtained by manual inspection of the intermediate GO annotations and BLAST results (Felliti *et al.,* 2006). Sequences would then be compared to select potential proteins involved in the fatty acid and alkane biosynthetic pathways. Thus, allowing comparisons and the identification of novel proteins within *D.desulfuricans* that may cause the synthesis of *n* and *i*-alkanes from free fatty acids (figure 1.4).

Future work would involve improvement to the current reference genome of *D.desulfuricans* allowing for superior understanding of the organism, consequently resulting in enhanced accuracy of transcriptome read data. An alternative approach would be to further explore the media analysis completed on *D.salexigens* but with *D.desulfuricans*. If *D.desulfuricans* is able to be cultivated in a media where it lacks the ability to synthesis alkanes it would allow for a simpler, more accurate method of comparative analysis to be performed. The potential application of generating superior data would be significant in providing novel information on microbial alkane synthesis.

7.4: Generating a *D.desulfuricans* strain with high transformation efficiency

To generate a highly transformable strain of *D.desulfuricans* a gene knock-out was performed by deleting the gene encoding for a type 1 site-specific deoxyribonuclease, hsdR (Keller *et al.*, 2009). Two alternative gene knock-out methods were tested. The TargeTron method used a protocol designed and developed by Sigma Aldrich that incorporates intron-mediated gene deletion (Karberg, 2001). Conversely, the Cre-Lox method used a deletion cassette to exchanged the hsdR coding sequence with a resistance marker by homologous recombination (Craig, 1988). This method then allows the excision of the resistant marker producing a strain cultivatable without a selection pressure yet lacks the gene of interest. DNA was electroporated using a method developed by Keller *et al.,* 2011. Unfortunately, neither method produced transformants.

The TargeTron method required coupled electroporation of two separate vectors, the designed pACD4K-C-Δ*hsdR* vector and pAR1219 (chapter 2). The pAR1219 vector contains T7 RNA polymerase, absent in *D.desulfuricans* and is required for intron-mediated gene deletion. Although, numerous electroporation attempts were performed no transformants were produced. A concern was that co-transformation of plasmids introduced too much of a ‘burden’ on the cells. Consequently, the Cre-Lox method was developed.

To overcome this 'burden' the Cre-Lox method employed electroporation of a small section of linear DNA. However, this method proved unsuccessful with no viable transformants. Due to time constraints further experimental analyses was unable to be performed. Had this not been the case experimental work would have focused on developing a reliable electroporation method enhancing that developed by Keller *et al.* Such modifications would include the optimisation of initial DNA forms, purities and quantities, electroporation voltages and adjustments to recovery times. Alternative approaches such as the isolation of *D.desulfuricans* grown in MT medium would allow for manipulation of a *Desulfovibrio* strain grown in non-precipitating media. Thus, eliminating any affect caused by iron sulphide formation in PGB. Successful electroporation would therefore allow the completion of Cre-Lox transformation.

The Cre-Lox method requires a further transformation of a plasmids containing Cre recombinase resulting in the excision of the newly added resistance marker. The cre recombinase enzyme recognises the LoxP sites causing the removal. Thus, resulting in a new strain of *D.desulfuricans* lacking the hsdR gene and able to be cultivated without any selection pressure.

If successful, a comparative analysis between the newly transformed (Δ*hsdR*) strain and the original *D.desulfuricans* would be performed the transformation efficiency as number of transformants per ng DNA. The results would show if deletion of the hsdR function has the desired effect. Colony PCR using specific primers flanking the 'deletion' site would provide DNA fragments for sequencing further confirming the deletion.

Developing a reliable method of electroporation and a highly transformable strain (Δ*hsdR*) of *D.desulfuricans* would provide a genetic 'tool box' for future reproducible manipulations. This could be used to engineer *D.desulfuricans* allowing for complete understanding and enhancement of its novel alkane synthetic pathway.

CHAPTER 8

CONCLUSION

This study has improved our understanding of *D.desulfuricans* and *D.salexigens* by characterisation and quantification of growth trends when grown in PGB. C_{18} alkane synthesis analysis confirmed contamination of the NCIMB library stocks of *D.salexigens*, isolating *D.desulfuricans* as a potential contaminant. However, this work uncovered the cultivation in MT medium providing a non-precipitating media. Future applications of molecular techniques using *Desulfovibrio* grown in this medium could be explored allowing for less problematic experimental work. Further work developed a reliable method of RNA extraction of *Desulfovibrio* cultivated in PGB allowing for the generation of RNA-seq data for comparative transcriptome analysis. However, due to time constraints analysis of the data was unable to be completed. Preliminary transformation analysis tested both an intron-mediated gene deletion method (TargeTron; Sigma-Aldrich) and a Cre-lox method, but provided no viable transformants. Thus, emphasising the requirement for the generation of a reliable method of transformation within *Desulfovibrio*.

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APPENDICES

APPENDIX I

***hsdR* Nucleotide sequence:**

Location: Node 4, 88146- 91093 base pairs.

Nucleotide Sequence (2948 bp):

TACTGCCGAATATACCAGAAAGCCTTATCAAATAAATCCTGATCCTTGATCTTGTACTTCACATAAAC
GACCTTGCGCAGAGCCTTCTGGACTTCGCGCTCTCCGGCCTTGGTGTTTTGCCAGCCGGGGAACCGGA
CCAGGCGCACAATCTCGTCAATGTCGGTCACGATCCGCTCGACAACCATCGGCGTCTTGCCGTTTTTC
ACCTCGGCGAACAGTTCGGTCAGGGCCGCCTTAGCCTTGGCCTGTTCATCAATCGGGTCCACCCGCTT
CTCCGCCTGGACGACCTCCCTGGCCAGGGTCAGCAACTCCTTGAGGAAATCGAGGCTGTGAAGCAGGC
CCTGTTCGTGCCGCTCCTTGAGCCTTTCGAGGCGTTCGCCCAGGGCTACAAACTTCGGATTGTCCTTG
TGCTTGCGCAGGCGGGCGATAAGCTTGATCTCGATTTCCTTGGATTTCTTGTCCGGGTCCTTGGCATC
GAGCAGCCCTTCGAGGACCTCGGCGTCCATCACCAGGGTGTCCAGATCGTCGCGCACCGTCTCCAGAT
GCACGTTCTCATGCACCAGCTCGATGGTCTTGGCCCCCAGGGCGTGCCATAGCAGCTTGCCATTGCCG
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GGGATCGGGAGACAGCGCCTCCCACAGCCGGGAGAGCACCGAGTATTCCGCCGCGAATTTGTCCCGAG
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GGTCACCACCTGGGCCTTGAAGCCGTTCGGCTCCACCTTCTCCTGGAAATGCTTGACGATGTGCTGGC
AGATGGCGCTCACCCGTGCCGGGGCCTTGATCAGCACCGCCATTTTGGCGGCCCGCTTGGCCAGGTCG
TCACGATCCAGCTCGCTCAGCTCATCGGTCATCTGAGAGTAGGCTTCGTCGATGGCGTCCTTGTTGAT
GTGCAGCTTCACGTCCACCGCCTCGAAGTGCAGCGGCAGCGTCGCCTTGTCCCTGATCGAGTCCTGGA
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CCCCAAGTTGCCTTCCTGGGTGCGGTGCGCCTCGTCCACCATCACGATGATGTTCGAGCGCTCGTTCA
GGCGGCCGTCCGCCTCGCCGAACTTGTGAATGGTGGTGATGATGATCTTGCGGGTATCGGCCGCCAGC
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GGTGATCTGGGTGTCCAGGTCAATACGGTCCACCACGATCATCACCGTGGGGTTGCCGAGCTTGCGGT
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CCACTGGTTGGTGACCACGTAATCGTTGTTGCTGAGATTCTCGAAGTCGATCAGGCGCACCGGCGTGT
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CGCACGAGGCCTTCGCTCTGCACCGACAGCGGAATGGTCCGCAGGCGGTAGAGCACTTCGTCGGCGCG
GTCGGGCTGGGCCTTTATTTCCGGGTTCAGGCGGATGAGAGCGTCGCGCACCATCGACTCCACCAGCA
TGTCGGAGTGCTGACGGGGCAGCTCCTCGGCGGACACGTAGCGCCAGCCTTTGATCTCGCCGCCATAG
CTGGCGAGTTCTTCGGCAACCATGTTTGAAGTCACGCTGCCGCAGAGCGTGTCGAGAACCATCTGTTC
GACAGTATTTTCTTCGTTAAACATATCGATCGTCGATAGTCGAGTCAGTCGA

APPENDIX II

Cre-lox Recombination DNA design

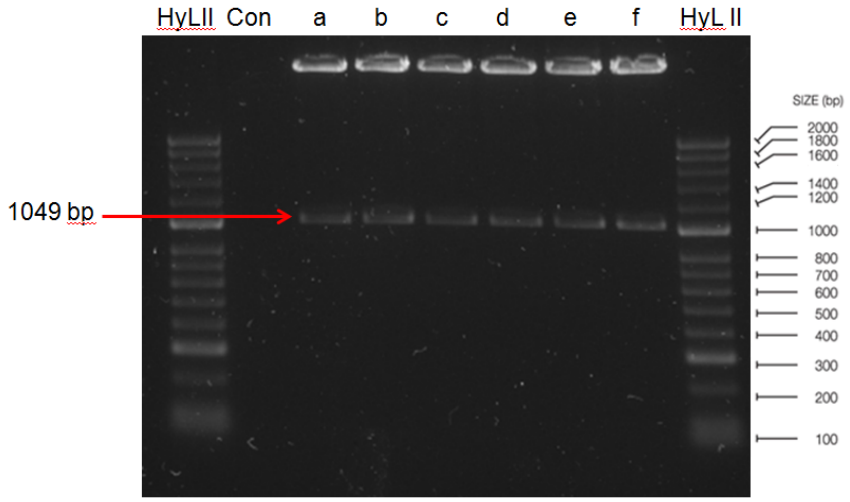
GAATTCTACTGCCGAATATACCAGAAAGCCTTATCAAATAAATCCTATAACTTCGTATAGCATACATT
ATACGAAGTTATTCGACTTTAAAAGCGAACGAAAAACAATTGCAAAAGCAGATTGATTTTTGGCAACG
TGAATTTAGGTTTTGGAAGTAAAATAAGTTTTATTTGATAAAAATTGCTAATTCAGTATAATTAATAT
TTACGAGGTGACATAACGTATGAAAAAATCAGAGGATTATTCCTCCTAAATATAAAAATTTAAAATTT
AGGAGGAAGTTATATATGACTTTTAATATTATTGAATTAGAAAATTGGGATAGAAAAGAATATTTTGA
ACACTATTTTAATCAGCAAACTACTTATAGCATTACTAAAGAAATTGATATTACTTTGTTTAAAGATA
TGATAAAAAAGAAAGGATATGAAATTTATCCCTCTTTAATTTATGCAATTATGGAAGTTGTAAATAAA
AATAAAGTGTTTAGAACAGGAATTAATAGTGAGAATAAATTAGGTTATTGGGATAAGTTAAATCCTTT
GTATACAGTTTTTAATAAGCAAACTGAAAAATTTACTAACATTTGGACTGAATCTGATAAAAACTTCA
TTTCTTTTTATAATAATTATAAAAATGACTTGCTTGAATATAAAGATAAAGAAGAAATGTTTCCTAAA
AAACCGATACCTGAAAACACCATACCGATTTCAATGATTCCTTGGATTGATTTTAGTTCATTTAATTT
AAATATTGGTAACAATAGCAGCTTTTTATTGCCTATTATTACGATAGGTAAATTTTATAGTGAGAATA
ATAAAATTTATATACCAGTTGCTCTGCAACTTCATCATTCTGTATGTGATGGTTACCATGCTTCACTA
TTTATGAATGAATTTCAAGATATAATTCATAGGGTAGATGATTGGATTTAGTTTTTAGATTTTGAAAG
TGAATTTAATTTTATACACGTAAGTGATCATAACTTCGTATAGCATACATTATACGAAGTTATCGAGA
ACCATCTGTTCGACAGTATTTTCTTCGTTAAACATCTGCAG

Key:

EcoR1
Upstream Homologous region
LoxP
Chloramphenicol resistance
LoxP
Downstream Homologous region
PstI

Total size: 1061 bp (1049 bp minus restriction sites)

Cre-lox DNA Fragment gel electrophoresis verification



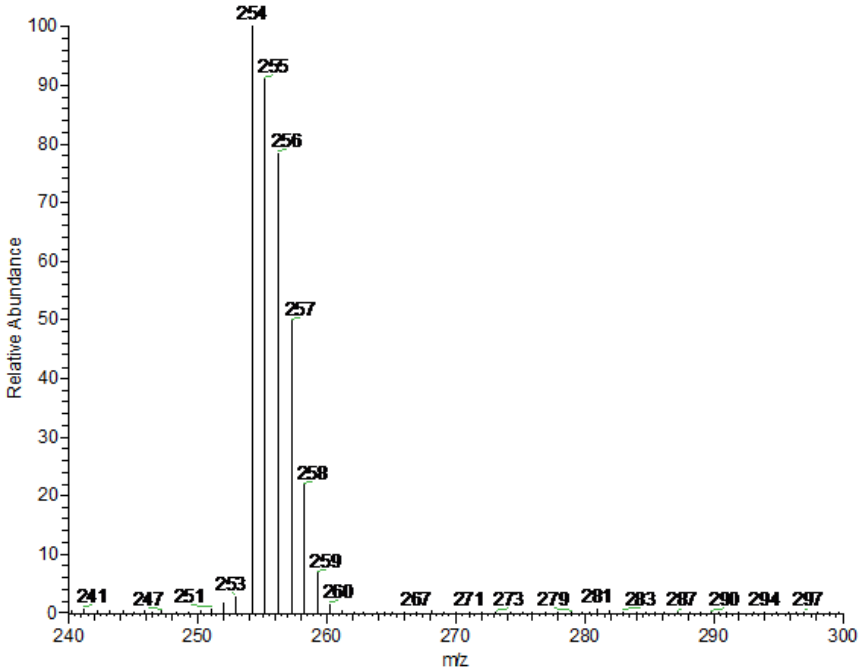
1.5% Agarose gel electrophoresis of digested ΔhsdR plasmid fragments.

1.5% agarose gel showing digested ΔhsdR plasmid fragments Lane design: HyLII = Hyperladder II, 2000-100 bp (Bioline); Con = control; a-f = repeats Key on the right of the gel corresponses to Hyperladder II molecular markers. Samples a-f all show the same size band a 1049 bp.

APPENDIX III

Effects of Media on *Desulfovibrio* C_{18} Alkane Synthesis

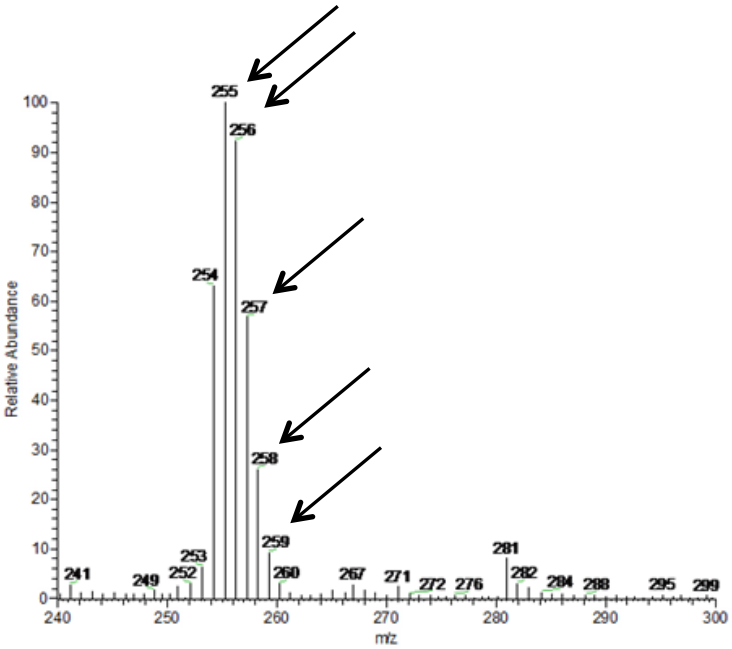
Section I: *Desulfovibrio* Genus Screen



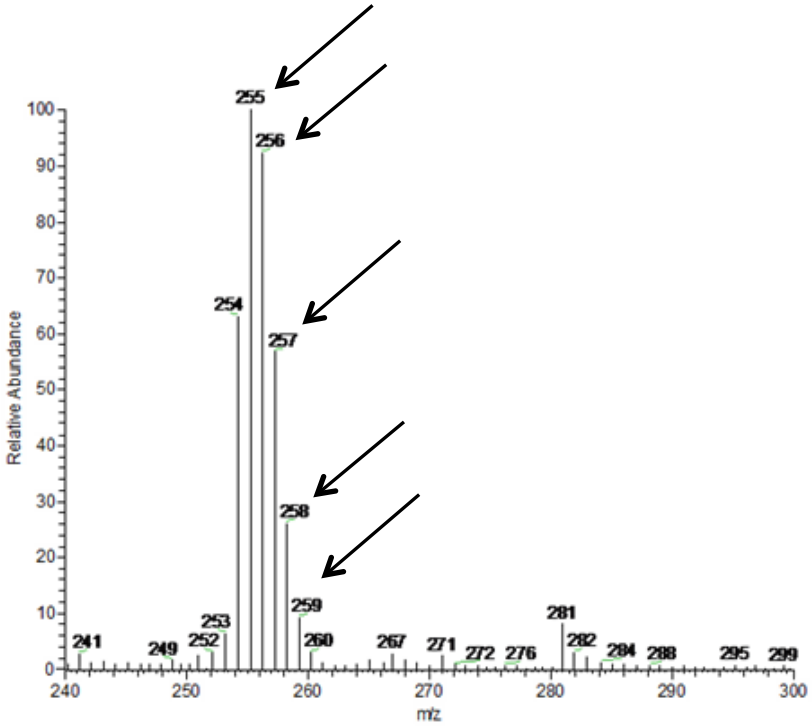
Mass spectrum of hydrocarbons extracted from *D.desulfuricans* grown in Metal Toxicity medium.

Spectrum show significant m/z peaks of 254, 255, 256, 257, 258 and 259. M/z peaks of 255, 256, 257, 258 and 259, confirming addition of one, two, three, four or five ^{2}H to the hydrocarbon chain.

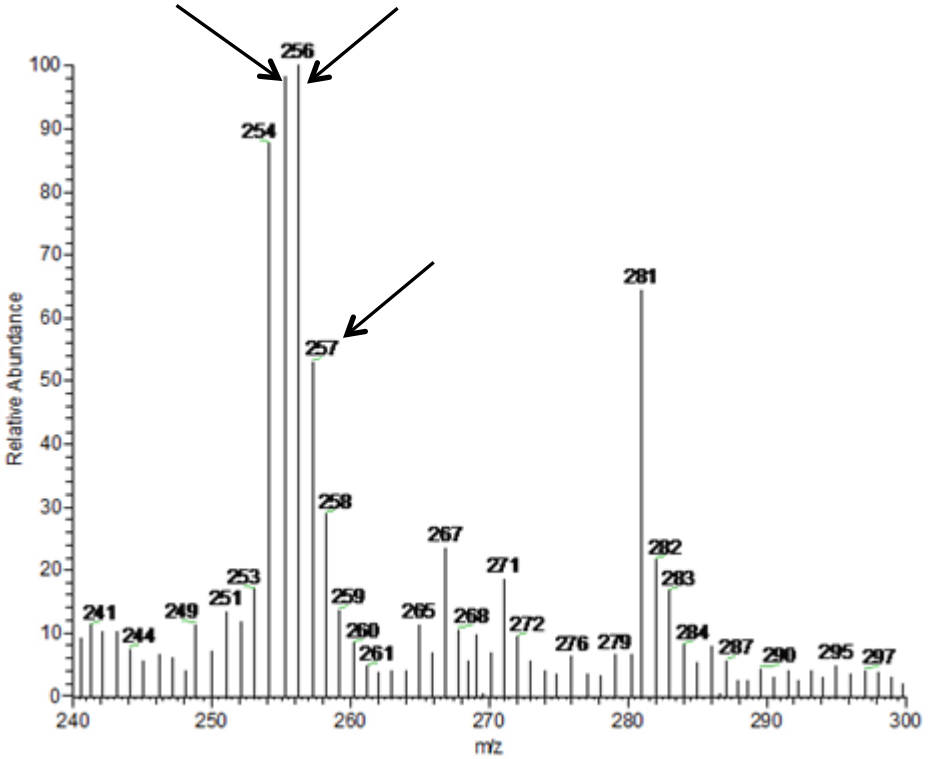
Section II: *D. salexigens* metal toxicity medium analysis



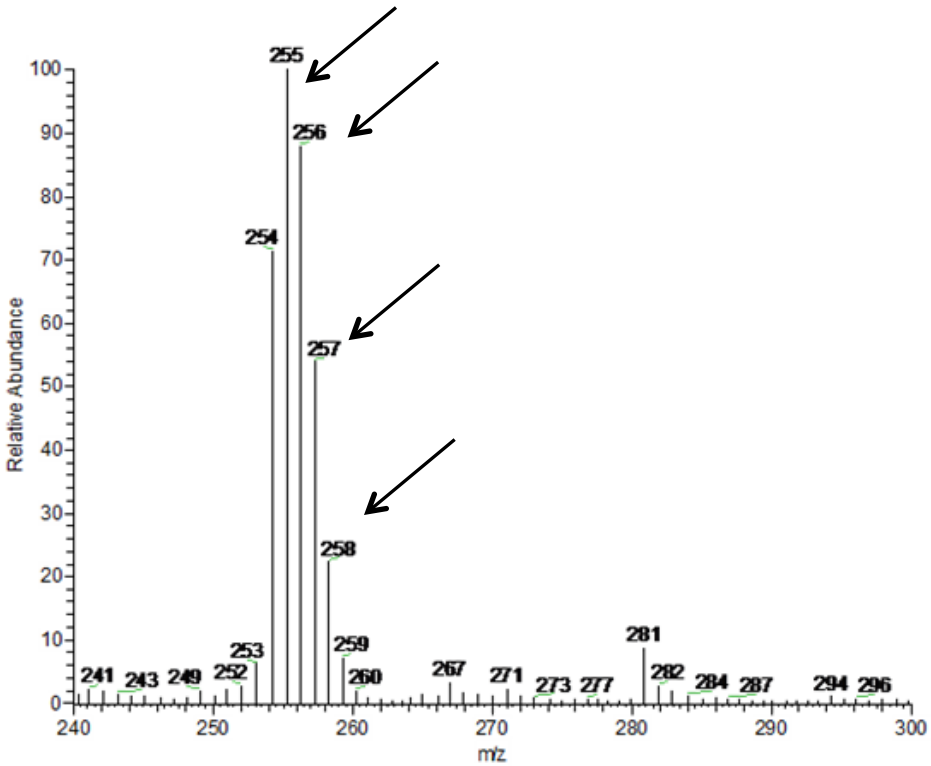
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $CaSO_4$; arrows represent deuteration.



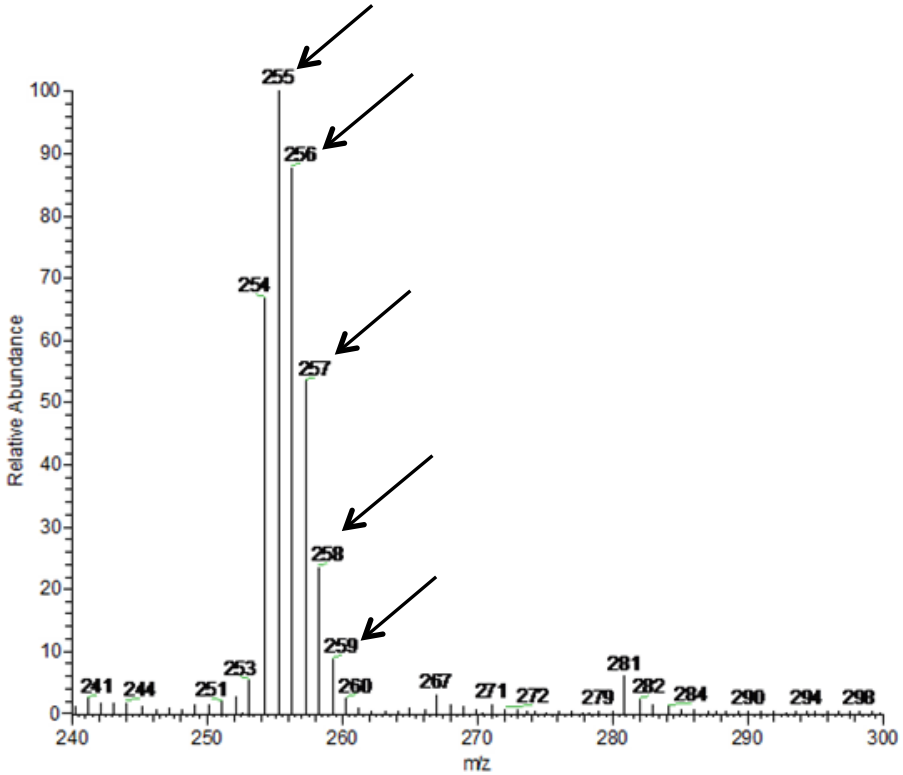
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $FeSO_4$; arrows represent deuteration



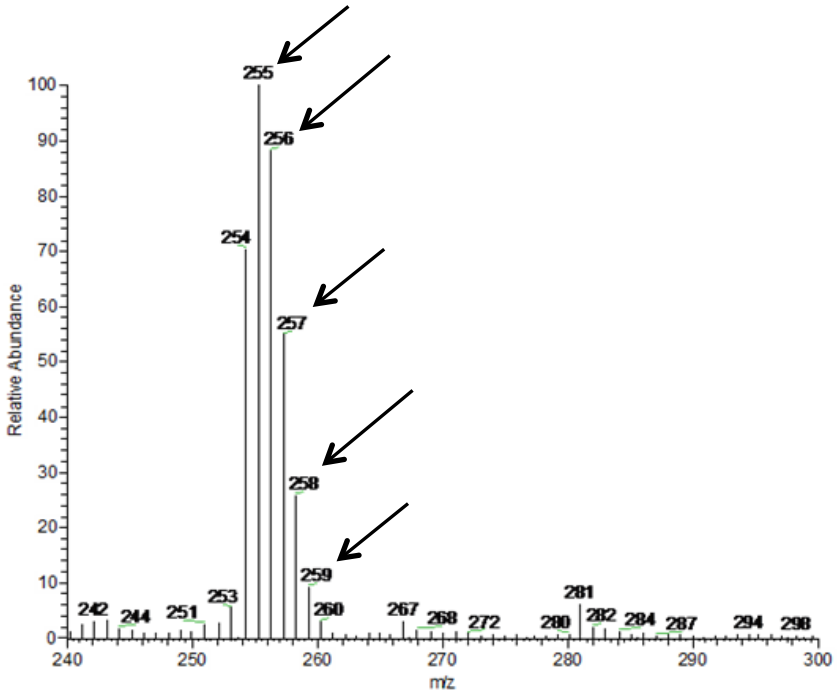
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $MgSO_4$; arrows represent deuteration



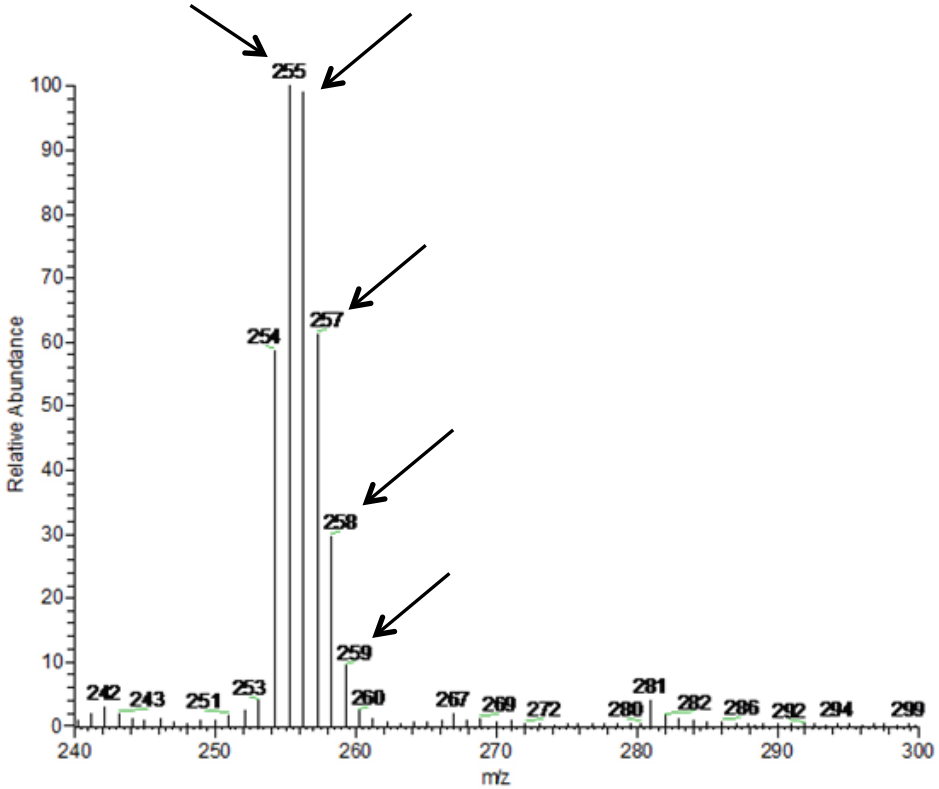
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional Yeast Extract; arrows represent deuteration



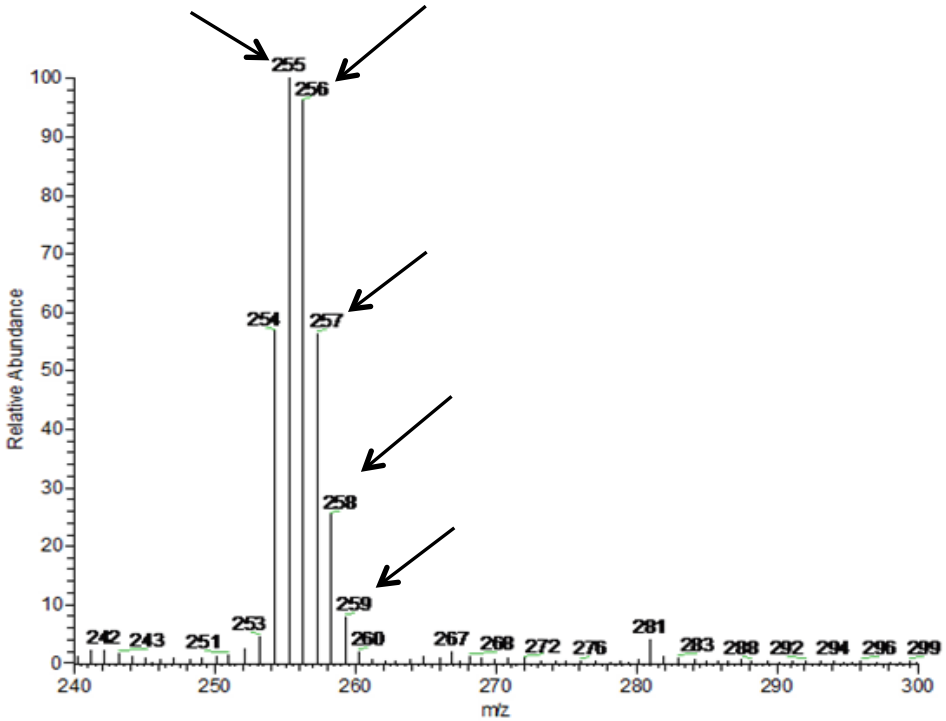
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional Ammonium Thioglycate; arrows represent deuteration.



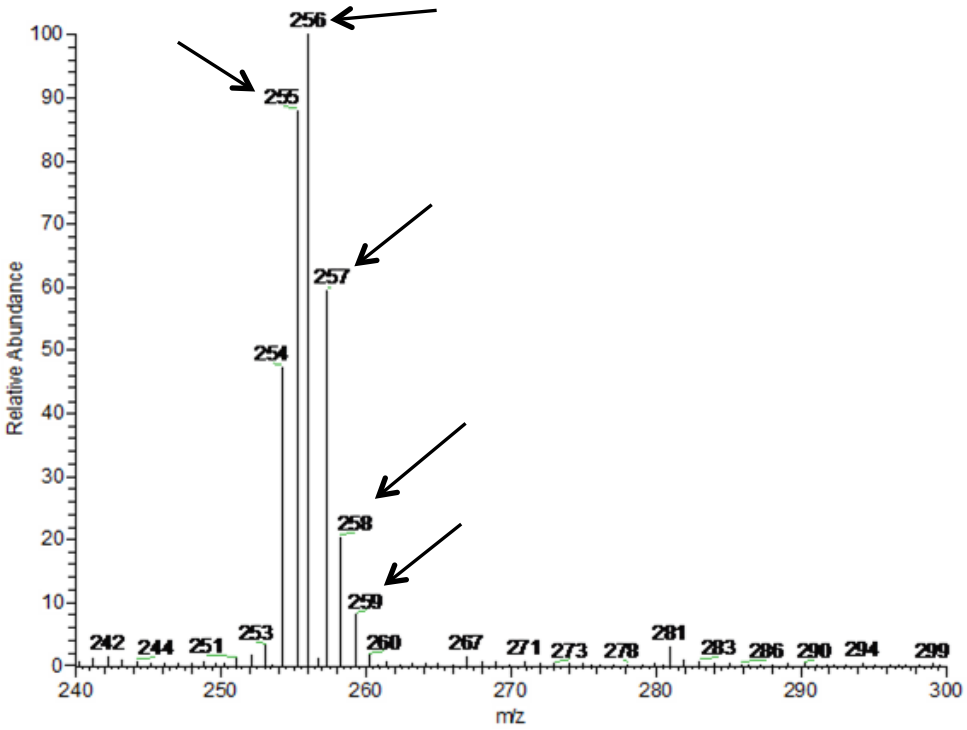
Mass spectrum of hydrocarbons extracted from *D.salexigens* . grown in Metal Toxicity medium plus additional $CaSO_4$, $FeSO_4$, $MgSO_4$, Yeast and Ammonium Thioglycate; arrows represent deuteration



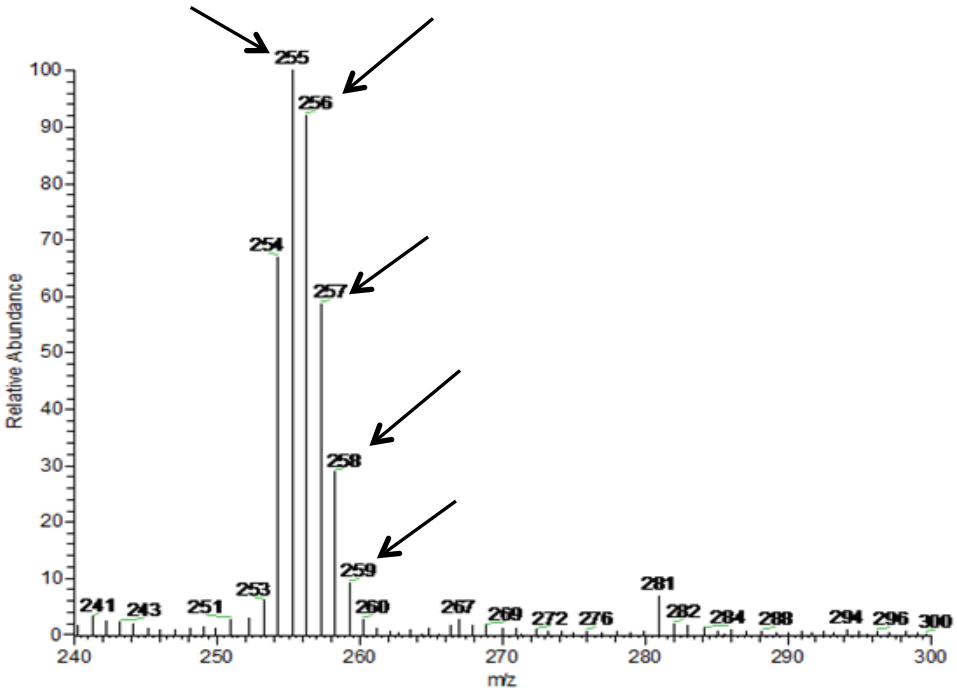
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $FeSO_4$ and $CaSO_4$; arrows represent deuteration.



Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $FeSO_4$ and $MgSO_4$; arrows represent deuteration.

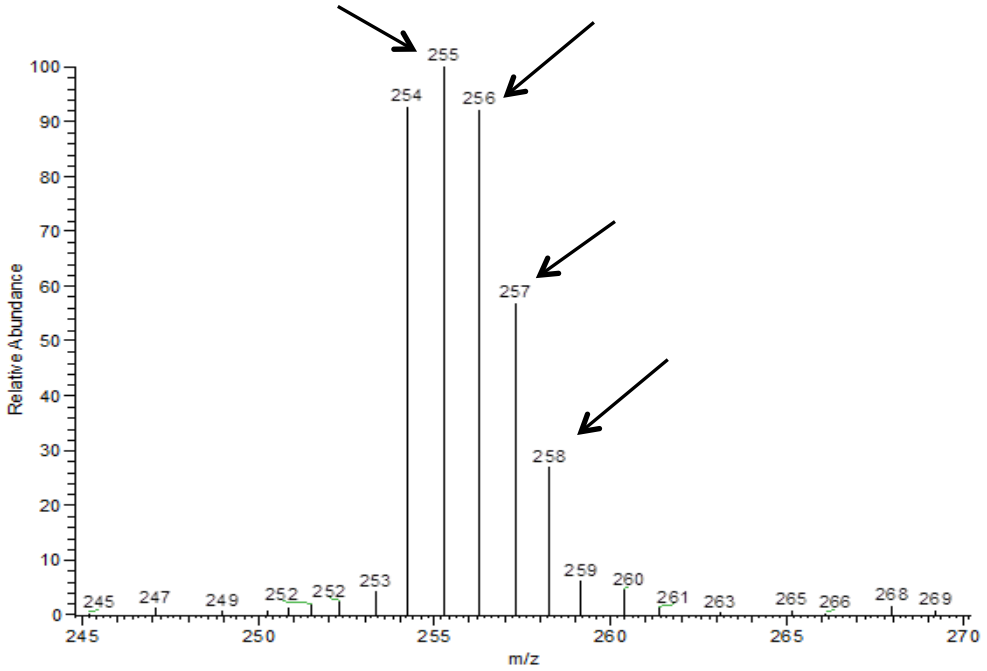


Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $FeSO_4$ and Yeast extract; arrows represent deuteration.

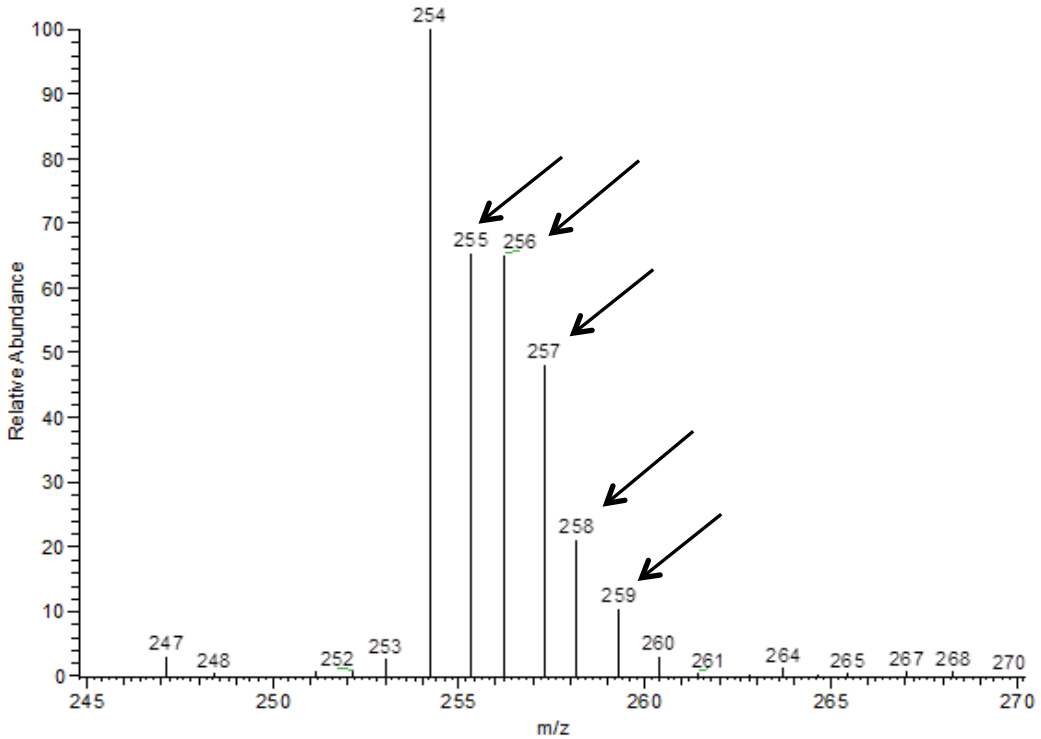


Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium plus additional $FeSO_4$ and Ammonium Thioglycate; arrows represent deuteration

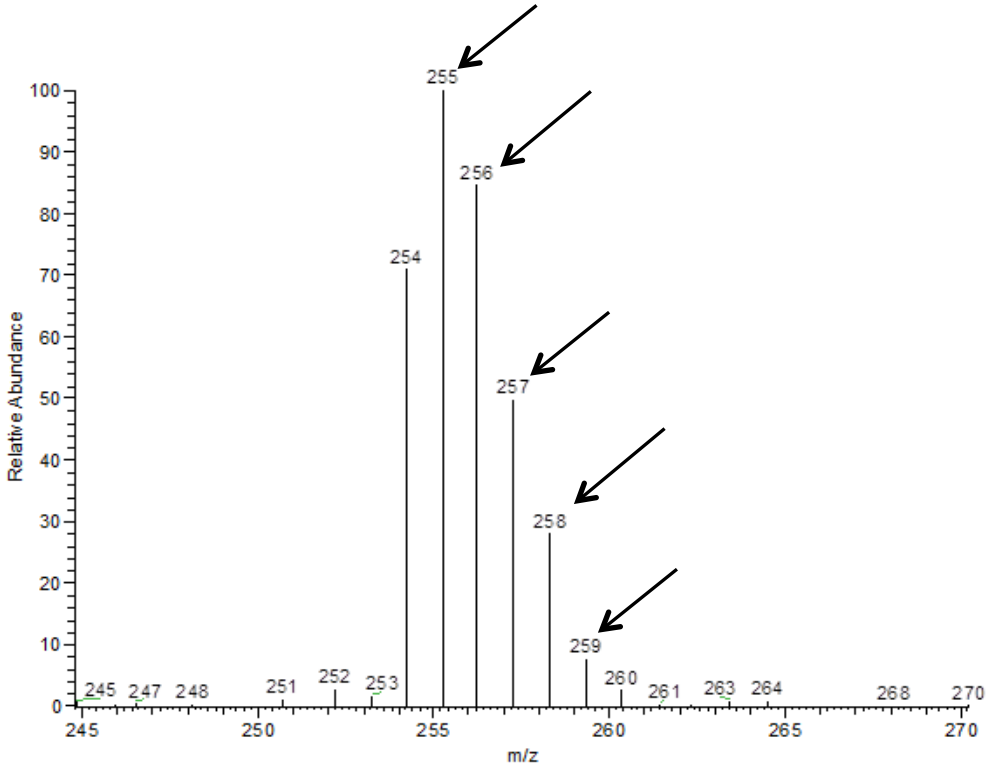
Section III: *D.salexigens* metal toxicity media analysis



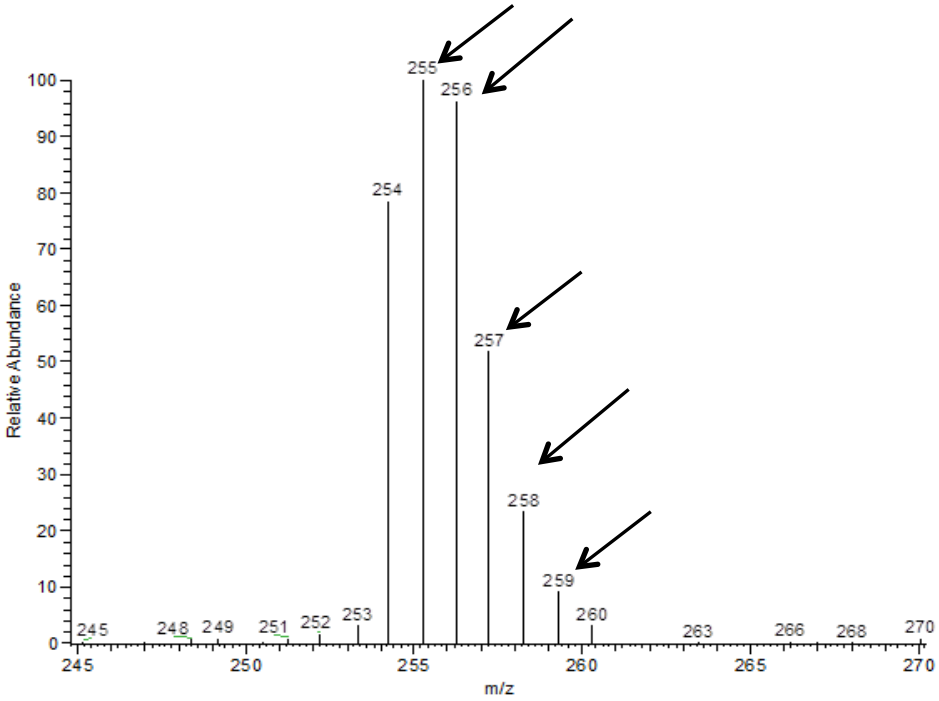
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $CaSO_4$; arrows represent deuteration.



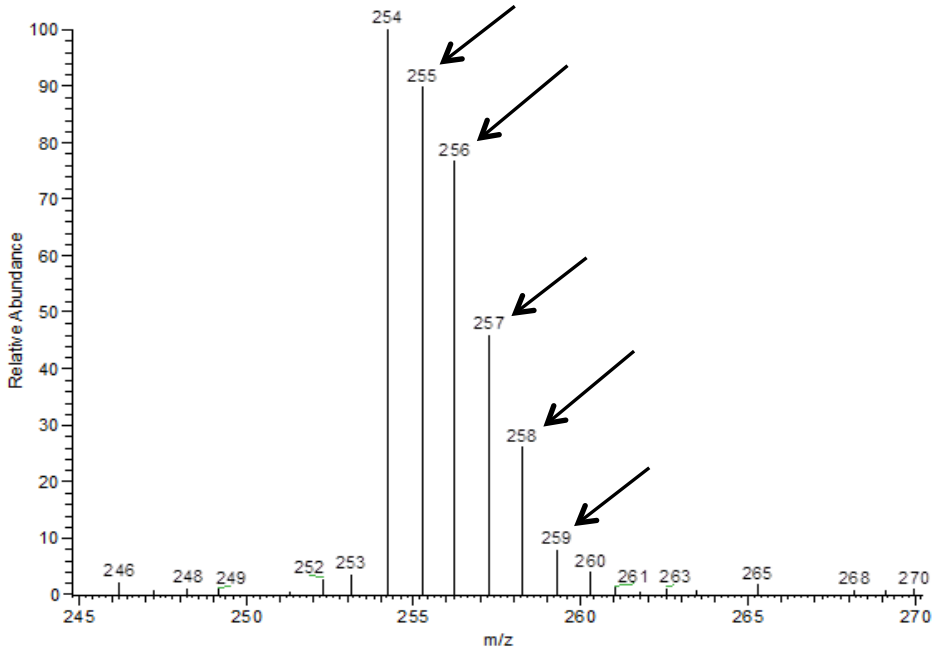
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $FeSO_4$; arrows represent deuteration



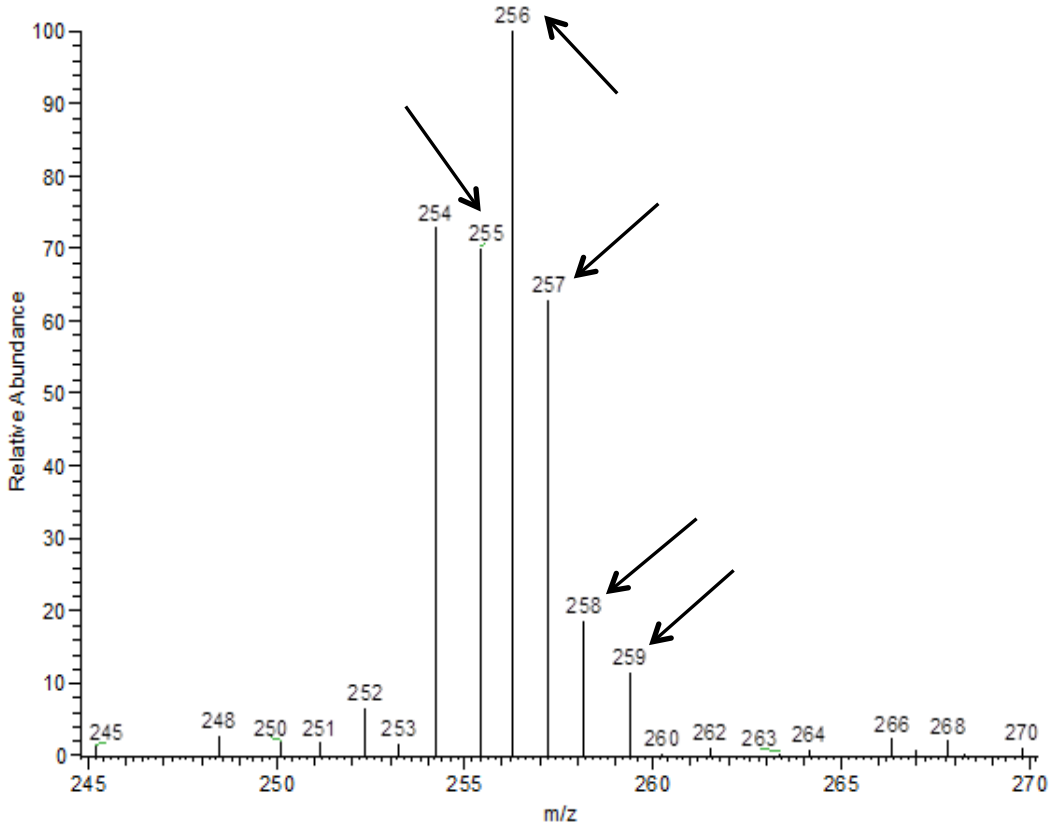
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $MgSO_4$; arrows represent deuteration



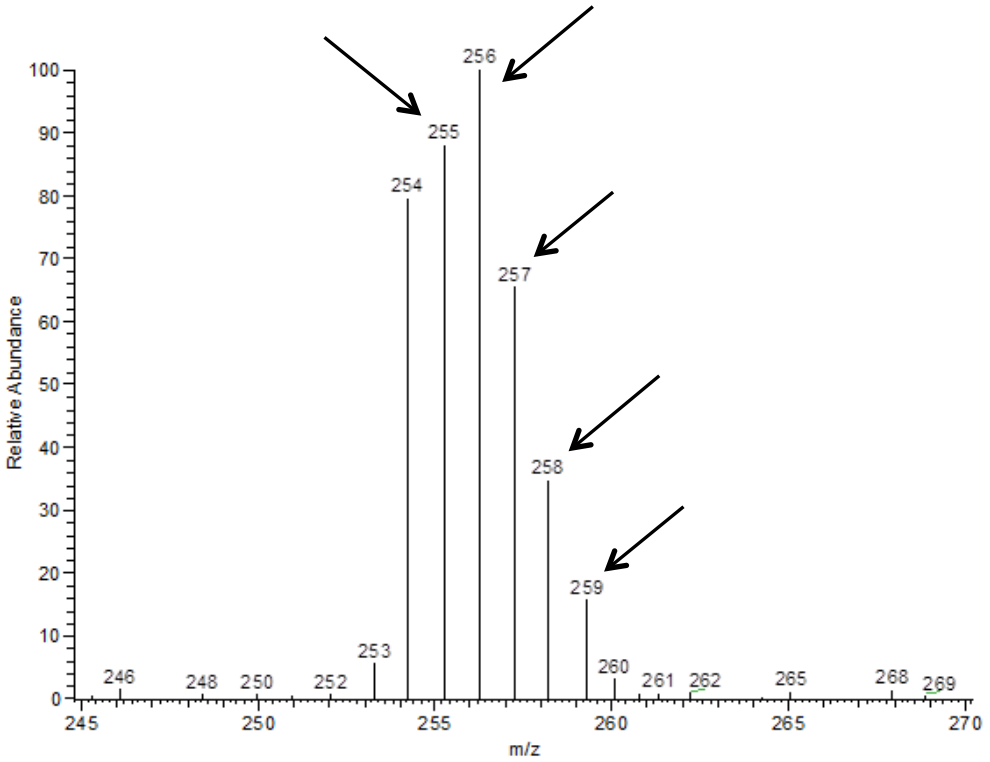
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional Yeast Extract; arrows represent deuteration.



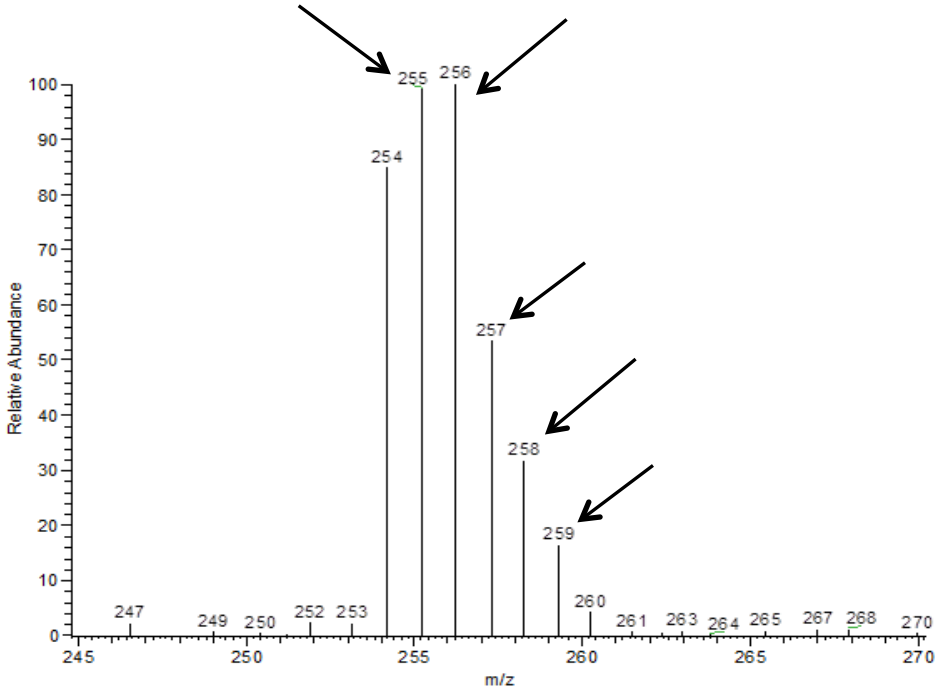
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional Ammonium Thioglycate; arrows represent deuteration.



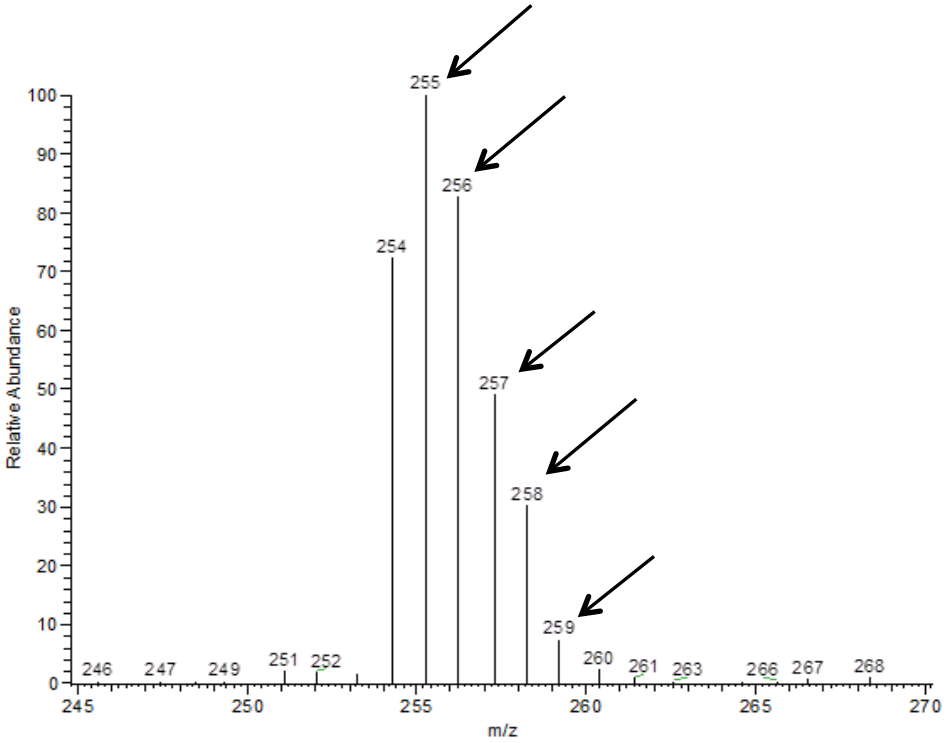
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $CaSO_4$, $FeSO_4$, $MgSO_4$, Yeast and Ammonium Thioglycate; arrows represent deuteration



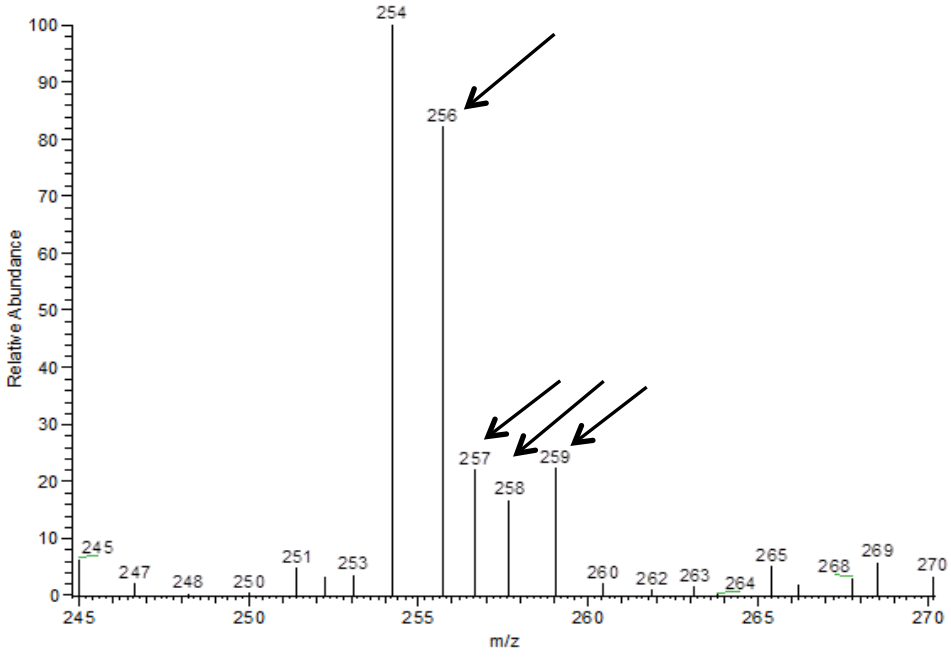
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $FeSO_4$ and $CaSO_4$; arrows represent deuteration



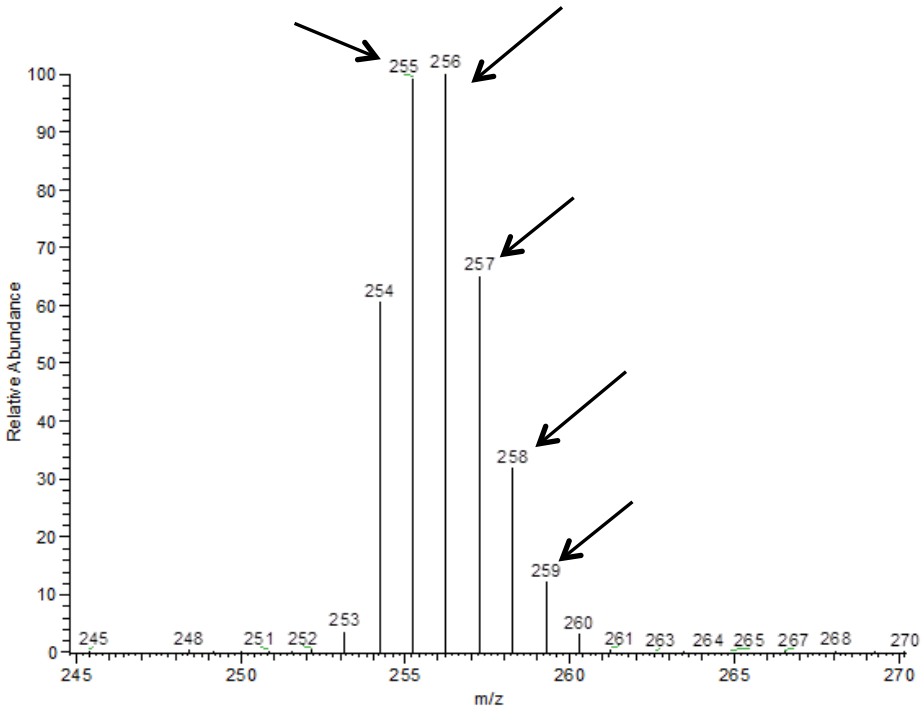
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $FeSO_4$ and $MgSO_4$; arrows represent deuteration



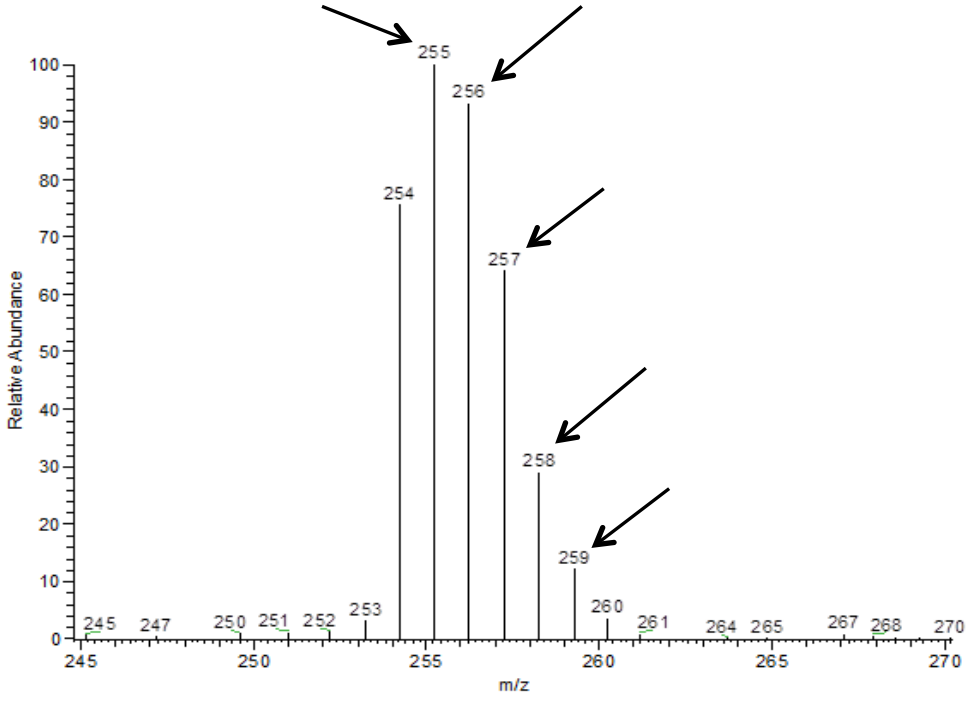
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $FeSO_4$ and Yeast Extract; arrows represent deuteration



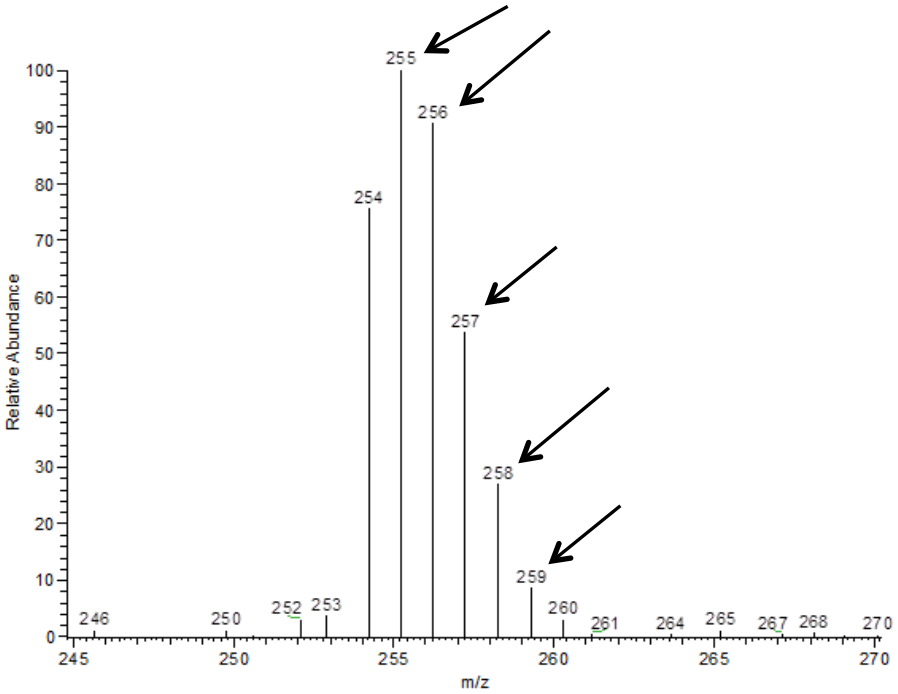
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium plus additional $FeSO_4$ and Ammonium Thioglycate; arrows represent deuteration



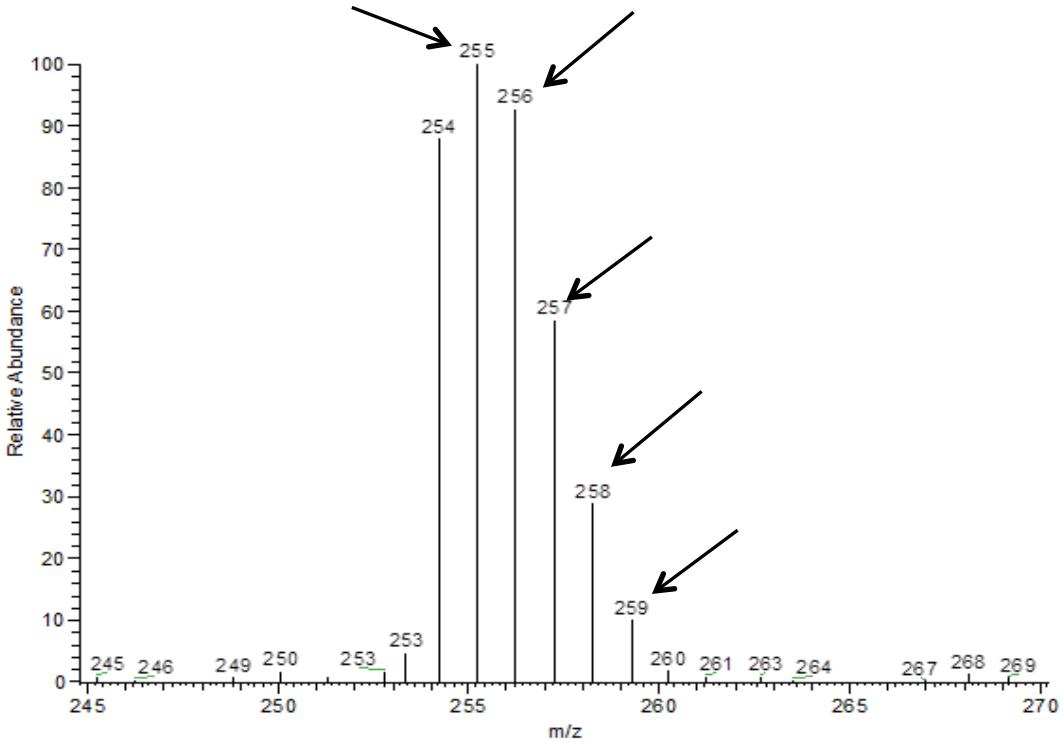
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium containing 0% PIPES; arrows represent deuteration



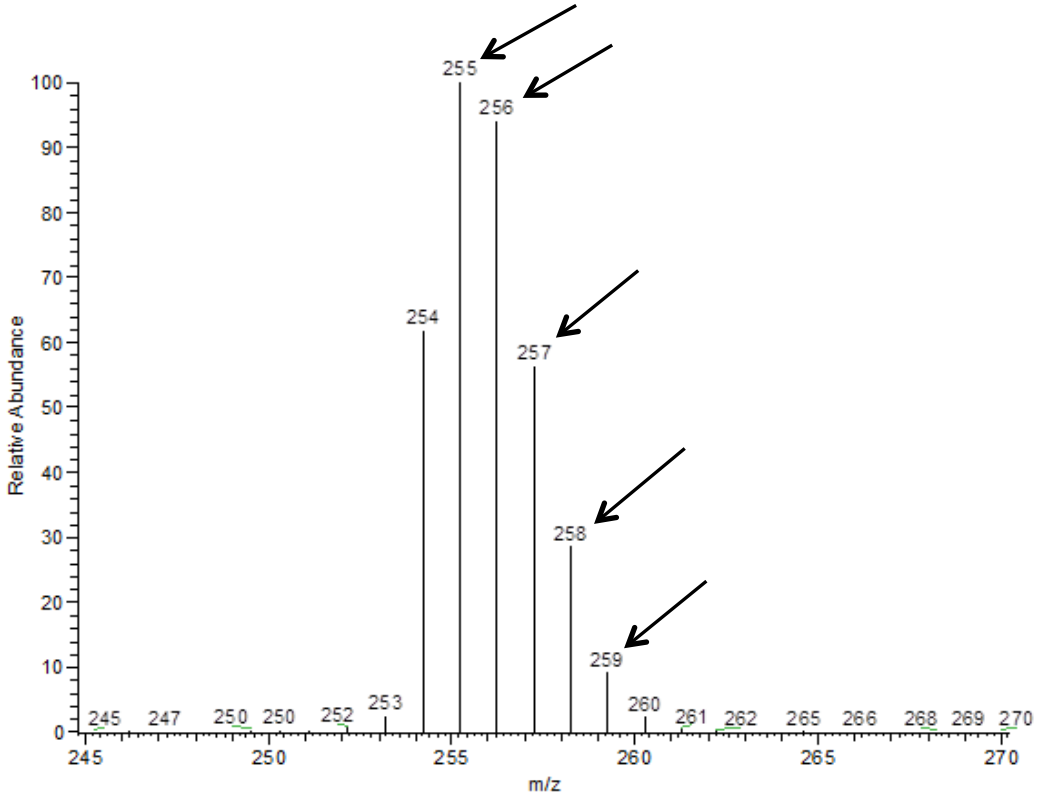
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium containing 25% PIPES; arrows represent deuteration



Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium containing 50% PIPES; arrows represent deuteration



Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium containing 75% PIPES; arrows represent deuteration



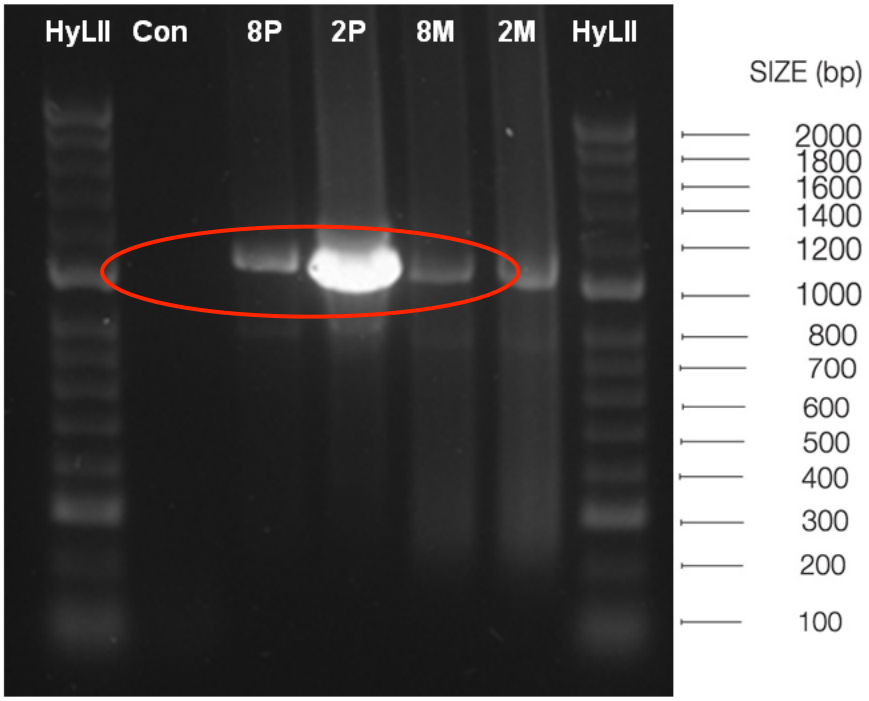
Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Modified Metal Toxicity medium as a control, thus containing 100% PIPES; arrows represent deuteration

APPENDIX IV

D. desulfuricans and *D.salexigens* 16S Sequencing:

Genomic DNA extraction analysis

D.desulfuricans and *D.salexigens* replenshied from glycerol stocks were grown in Postgate medium B and metal toxicity medium, all cultures had there genomic DNA extracted. This DNA was then amplified via PCR using AccuPrime *Pfx* DNA polymerase and primers 343 F (TAGGGRAGGCAGCAG) and 1047 R (GACGGGCGGTGTGTRC) Below shows the gel electrophoresis of expected fragments (1064 bp) using Hyperladder II (Bioline).



Agarose gel electrophoresis of amplified genomic DNA fragments.

1% agarose gel showing amplified genomic DNA fragments. Lane design: HyLII = Hyperladder II, 2000-100 bp (Bioline); Con = control; 8P = *D.desulfuricans* grown in Postgate medium B; 2P = *D.salexigens* grown in Postgate medium B: 8M = *D.desulfuricans* grown in metal toxicity medium and 2M = *D.salexigens* grown in metal toxicity medium. Key on the right of the gel corresponses to Hyperladder II molecular markers. The red circle represents the desried band, 1064 bp.

All samples show amplified DNA fragment represent by the band at 1064 base pairs. The brighter the band the greater the quantity of DNA. A positive negative control is confirmed by the lack of DNA in the control lane. Each sample was quantified using Quant-iT PicoGreen dsDNA Assay. The concentration of genomic DNA of *D.desulfuricans* was; 20.02 ng μl^{-1} and

34.98 ng μl^{-1} and *D.salexigens*; 52.17 ng μl^{-1} and 47.20 ng μl^{-1} when grown in Postgate medium B and metal toxicity medium respectively.

Once the samples had been analysed to meet specific requirements the requirements the samples were sent to Genevision (Newcastle Upon Tyne, UK) for Sanger sequencing.

16S Nucleotide Sequences from Genevision for identification of *Desulfovibrio* strain

D.desulfuricans

Media: Postgate Medium B **Orientation**: Reverse **Size**: 519 bp

TTTAACGCGTTAGCTCCGGCACCGAGGGTCAAGCGCCCGACACCTAACGTCCATCGTTTACAGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTACCTCAGCGTCAGTATCATTCCAGGT
AGCCGCCTTCGCCACTGGTGTTCCTCCCGATATCTACGGATTTCACTCCTACACCGGGAATTCCGCTA
CCCTCTCCTGAACTCAAGCTACGCAGTTTCAAGCGCAATTCCTCGGTTGAGCCGAGGGCTTTCACGCC
TGACTTGCATAGCCGCCTACGCACGCTTTACGCCCAGTGATTCCGATTAACGCTTGCACCCTCCGTAT
TACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTCTGGAGGTACCGTCAGCCGAAGACACTATT
CGCATCCTCGGGGTTCTTCCCTCCTGACAGAGGTTTACGACCCGAAGGCCTTCTTCCCTCACGCGGCG
TCGCTGCGTCAGACTTTCGTCCATTGCGCAATATTCCCCACTG

D.desulfuricans

Media: Metal Toxicity **Orientation**: Forward **Size**: 492 bp

GAAATCCGTAGATATCGGGAGGAACACCAGTGGCGAAGGCGGCTACCTGGACTGATACTGACGCTGAG
GTACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGGACGTTA
GGTGTCGGGGGCTTGACCCTCGGTGCCGGAGCTAACGCGTTAAACGTCCCGCCTGGGGAGTACGGTCG
CAAGGCTGAAACTCAAAGAAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATG
CAACGCGAAGAACCTTACCTGGGCTTGACATCCCGCGTACCCTCCCGAAACGGAGGGGTGCCCTTCGG
GGAGCGCGGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCCTATTCTTAGTTGCCAGCAAGTAATGTTGGGCACTCTAAGGAGACTGTCTCGGT
CAACGGGGAGGAAGGT

D.desulfuricans

Media: Metal Toxicity **Orientation**: Reverse **Size**: 520 bp

AGTTTACGCGTTAGCTCCGGCACCGAGGGTCAAGCCCCCGACACCTAACGTCCATCGTTTACAGCGTG
GACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTACCTCAGCGTCAGTATCAGTCCAGG
TAGCCGCCTTCGCCACTGGTGTTCCTCCCGATATCTACGGATTTCACTCCTACACCGGGAATTCCGCT
ACCCTCTCCTGAACTCAAGCTACGCAGTTTCAAGCGCAATTCCTCGGTTGAGCCGAGGGCTTTCACGC

CTGACTTGCATAGCCGCCTACGCACGCTTTACGCCCAGTGATTCCGATTAACGCTTGCACCCTCCGTA
TTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTCTGGAGGTACCGTCAGCCGAAGACACTAT
TCGCATCCTCGGGGTTCTTCCCTCCTGACAGAGGTTTACGACCCGAAGGCCTTCTTCCCTCACGCGGC
GTCGCTGCGTCAGACTTTCGTCCATTGCGCAATATTCCCCACTG

D.salexigens

Media: Metal Toxicity **Orientation**: Forward **Size**: 343 bp

GGCGTAAGCGTGCGTAGGCGGCTATGCAAGTCAGGCGTGGAAGCCCTCGGCTCACCGAGGAATTGGGC
TTGAAACTGCGTTATCTGCAAAAAGGAGTTAATATACCTCTATACTTTAACGTCAGGGAGAAAAAACC
CCGGATCTAATGGAAGGGTCCGGTGAACAACCAAAAAGTGGGGGTCCAACCTCCTCTGAACAAATCAT
GAAGACTGGTGCCTTGTTGCTTCAAGGTTTCATCCAGGACGGAGCTGGTAGAATGGGTGGTGAAGCTC
CTGAATTGGCCTTGGACCCAGTTCCACAAGACGCTTCCACCAAGAAATTGTCTGAATGTTTGAAAAAA
ATC

D.salexigens

Media: Metal Toxicity **Orientation**: Reverse **Size**: 516 bp

TTACGCGTTACTCCGGCACCGAGGGTCAAGCCCCCGACACCTAACGTCCATCGTTTACAGCGTGGACT
ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTACCTCAGCGTCAGTATCAGTCCAGGTAGC
CGCCTTCGCCACTGGTGTTCCTCCCGATATCTACGGATTTCACTCCTACACCGGGAATTCCGCTACCC
TCTCCTGAACTCAAGCTACGCAGTTTCAAGCGCAATTCCTCGGTTGAACCGAGGGCTTTCACGCCTGA
CTTGCATAGCCGCCTACGCACGCTTTACGCCCAGTGATTCCGATTAACGCTTGCACCCTCCGTATTAC
CGCGGCTGCTGGCACGGAATTAGCCGGTGCTTCCTCTGGAGGTACCGTCAGCCGAAGACACTATTCGC
ATCCTCGGGGTTCTTCCCTCCTGACAGAGGTTTACGACCCGAAGGCCTTCTTCCCTCACGCGGCGTCG
CTGCGTCAGACTTTCGTCCATTGCGCAATATTCCCCACTG

16S sequenced analysis and alignment:

BLAST Analysis: Megablast program, blastn against *D. desulfuricans* contigs as reference genome.
Query:
Strain: *D. desulfuricans*
Orientation: Forward
Media: Postgate medium B
Location on reference genome:
Node 20 between 4299-4817 base pairs
Score:
Score = 948 bits (513), Expect = 0.0, Identities = 517/519 (99%), Gaps = 0/519 (0%)

Query:

Strain: *D. desulfuricans*
Orientation: Reverse
Media: Metal Toxicity
Location on reference genome:
Node 20 between 4298-4817 base pairs
Score:
Score = 953 bits (516), Expect = 0.0, Identities = 519/520 (99%), Gaps = 1/520 (0%).

Query:
Strain: *D. desulfuricans*
Orientation: Forward
Media: Metal Toxicity
Location on reference genome:
Node 20 between 3887-4478 base pairs
Score:
Score = 904 bits (489), Expect = 0.0, Identities = 491/492 (99%), Gaps = 0/492 (0%)

BLAST Analysis: Megablast program, blastn against *Desulfovibrio salexigens* 16S ribosomal rRNA as the reference sequence (gb|M34401.1|DVURR162)
Query:
Strain: *D.salexigens*
Orientation: Forward
Media: Metal Toxicity
Score:
Score = 588 bits (318), Expect = 5e-172, Identities = 456/526 (87%), Gaps = 13/525 (2%)
Query:
Strain: *D.salexigens*
Orientation: Reverse
Media: Metal Toxicity
Score:
Score = 575 bits (311), Expect = 4e-168, Identities = 456/528 (86%), Gaps = 19/528 (4%)

Alignment analysis:
Query:

Strain: *D. desulfuricans*		Strain: *D. desulfuricans*
Orientation: Reverse	against	Orientation: Reverse
Media: Postgate medium B		Media: Metal Toxicity

Score:
Score = 941 bits (509), Expect = 0.0, Identities = 516/519 (99%), Gaps = 1/519 (0%)

Query:

Strain: *D.salexigens*		Strain: *D. desulfuricans*
Orientation: Reverse	against	Orientation: Forward
Media: Metal toxicity		Media: Metal Toxicity

Score:
Score = 318 bits (172), Expect = 3e-91 Identities = 175/176 (99%), Gaps = 1/176 (1%)

Query:

Strain: *D.salexigens*		Strain: *D. desulfuricans*
Orientation: Reverse	against	Orientation: Reverse
Media: Metal toxicity		Media: Metal Toxicity

Score:
Score = 937 bits (507), Expect = 0.0, Identities = 514/517 (99%), Gaps = 1/517 (0%).

Query:

Strain: *D.salexigens*		Strain: *D. desulfuricans*
Orientation: Reverse	against	Orientation: Reverse
Media: Metal toxicity		Media: Postgate Medium B

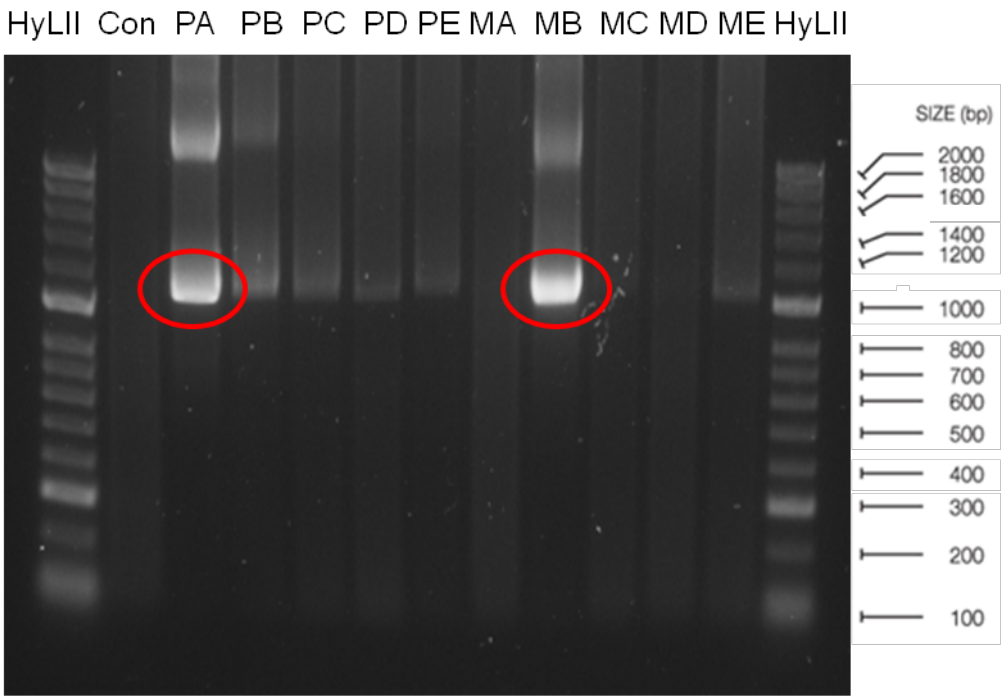
Score:
Score = 922 bits (499), Expect = 0.0, Identities = 510/515 (99%), Gaps = 1/515 (0%)

APPENDIX V

***D.salexigens* 16S Sequencing:**

Genomic DNA extraction analysis:

D.salexigens replenished from NCIMB stocks were cultivated in PGB, spread onto PGB and MT agar plates all cultures. Individual colonies were selected and grown in their corresponding liquid media. Once all cultures reached stationary phase genomic DNA was extracted. This DNA was then amplified via PCR using AccuPrime *Pfx* DNA polymerase and primers 343 F (TAGGGRAGGCAGCAG) and 1047 R (GACGGGCGGTGTGTRC). Below shows the gel electrophoresis of expected fragments (1064 bp) using Hyperladder II (Bioline).



Agarose gel electrophoresis of amplified genomic DNA fragments.

1% agarose gel showing amplified genomic DNA fragments. Lane design: HyLII = Hyperladder II, 2000-100 bp (Bioline); Con = control; PA–PE = *D.salexigens* grown in Postgate medium B; MA-ME = *D.salexigens* grown in metal toxicity medium. Key on the right of the gel corresponses to Hyperladder II molecular markers. Red circle highlight desired fragment, 1069 bp. Samples PA and MB were sent for sequencing.

Both the forward and reverse orientations of *D.salexigens* grown in PGB show a single bacterium present that when analysed via BLAST identifies it as *D.salexigens*. Further GC/MS analysis confirms lack of C_{18} alkane production in *D.salexigens* grown in PGB see

spectrum below. Both sequences extracted from *D.salexigens* grown in MT showed a mix culture although being selected from an individual colony. However, GC/MS analysis of this culture show no microbially derived C_{18} alkanes.

D.salexigens

Media: Postgate Medium B **Orientation**: Reverse **Size**: 522 bp

TATCGCGTTAACTTCGACACCGAACCGGTTAAGGCCCGACATCTAGCATCCATCGTTTACGGCGTGGA
CTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTACTCGTCCAGGTG
GCCGCCTTCGCCACTGGTGTTCCTCCAGATATCTACGGATTTCACTCCTACACCTGGAATTCCGCCAC
CCTCTCCGAGACTCAAGCACGACAGTATCAAGCGCAATTCCCCGGTTGAGCCGAGGGCTTTCACGCCT
GACTTATCGCGCCGCCTACGCGCGCTTTACGCCCAGTGATTCCGATTAACGCTCGCACCCTCCGTATT
ACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTCTGGAGGTACCGTCAGTGAAAGAGGGTATTA
GCCTCAAACAGTTTCTTCCCTCCTGACAGAGGTTTACGACCCGAAAGCCTTCGTCCCTCACATGGCGT
CGCTGCGTCAGGGTTTCCCCCATTGCGCAATATTCCCCACTGCTGC

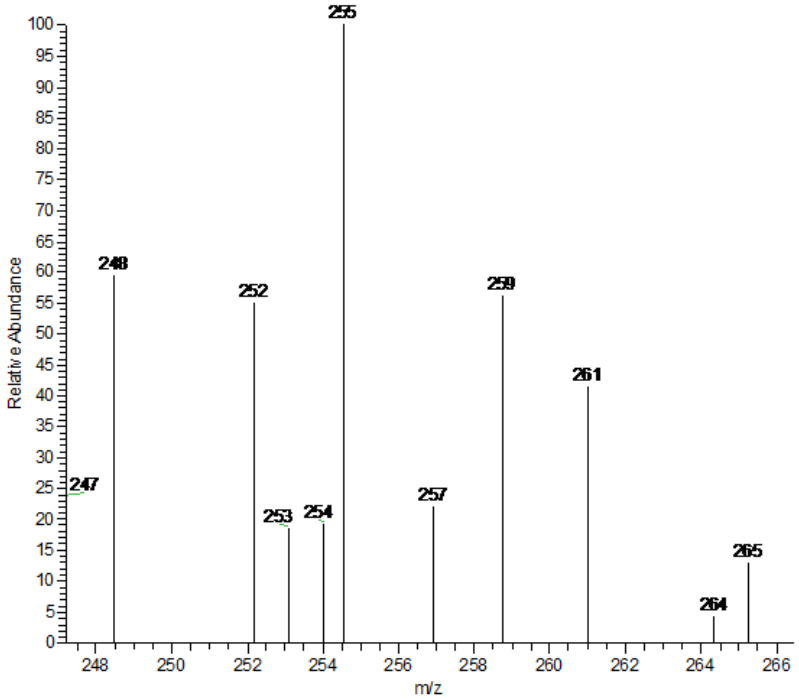
D.salexigens

Media: Postgate Medium B **Orientation**: Forward **Size**: 340 bp

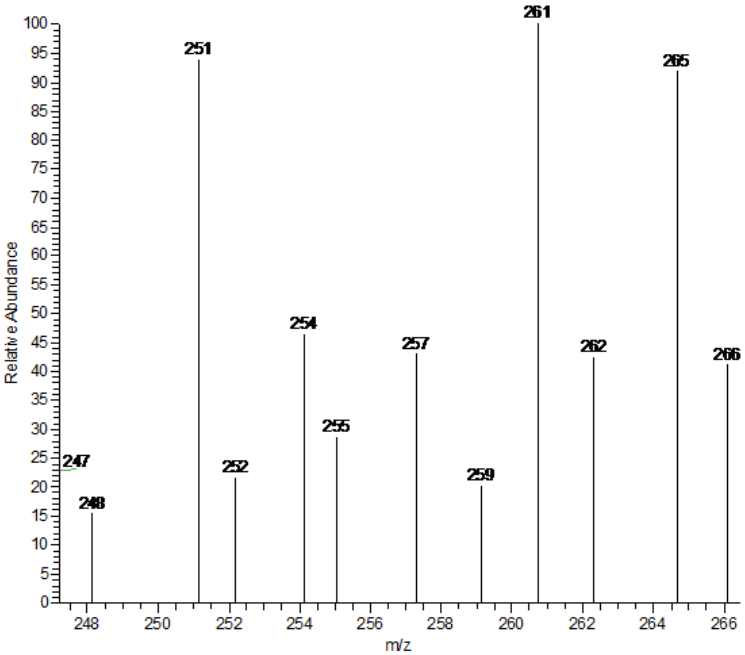
GAAAGCCCTCGGCTCAACCGGGGAATTGCGCTTGATACTGTCGTGCTTGAGTCTCGGAGAGG
GTGGCGGAATTCCAGGTGTAGGAGTGAAATCCGTAGATATCTGGAGGAACACCAGTGGCGAA
GGCGGCCACCTGGACGAGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGGATGCTAGATGTCGGGCCTTAACCGGTTCGGT
GTCGAAGTTAACGCGATAAGCATCCCGCCTGGGGAGTACGGTCGCAAGGCTGAAACTCAAAG
AAATTGACGGGGGCCCGCACAAGCGGTGGA

BLAST Analysis Megablast program, blastn against *D.salexigens* 16S ribosomal rRNA as the reference sequence (gb|M34401.1|DVURR162).
Query:
Strain: *D.salexigens*
Orientation: Forward
Media: Postgate Medium B
Score:
Score = 492 bits (266), Expect = 3e-143, Identities = 271/274 (99%), Gaps = 1/274 (0%)

Query:
Strain: *D.salexigens*
Orientation: Reverse
Media: Postgate Medium B
Score:
Score = 902 bits (488), Expect = 0.0, Identities = 509/524 (97%), Gaps = 2/524 (0%)



Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Postgate medium B peaks suggest no deuteration.



Mass spectrum of hydrocarbons extracted from *D.salexigens* grown in Metal Toxicity medium peaks suggest no deuteration.

APPENDIX VI

Transcriptome RNAseq Bowtie alignment analysis:

Total RNA samples extracted from mid-exponential phase of *D.salixegens* and *D.desulfuricans* were depleted and prepared for sequencing using Illumina TruSeq RNA sample preparation kit. Sequencing was performed on the Illumina HiSeq 2000, reads were aligned using Bowtie software.

Sample	Total Corrected Reads	Aligned Reads	%
D.salexigens A	11733743	6293443	53.64
D.salexigens B	9593995	5363008	55.90
D.salexigens C	11194074	5774239	51.58
D.desulfuricans A	9479032	2921	0.03
D.desulfuricans B	11194074	2699	0.03
D.desulfuricans C	10443901	3550	0.03

Table shows the Bowtie alignment of RNAseq reads against the *D.salexigens* genome. A-C represents repeat samples.

This data confirms *D.salexigens* integrity, whilst confirming *D.desulfuricans* is not *D.salexigens*.

Sample	Total Corrected Reads	Aligned Reads	%
D.salexigens A	11733743	510307	4.35
D.salexigens B	9593995	721291	4.66
D.salexigens C	11194074	427525	3.82
D.desulfuricans A	9479032	721291	7.61
D.desulfuricans B	11194074	721592	7.20
D.desulfuricans C	10443901	602515	5.77

Table the Bowtie alignment of RNAseq reads against the *D.desulfuricans* genome.

This data shows that *D.desulfuricans* is contaminated as the average percentage of reads is 6.86%. Therefore suggesting contamination in the sample. Furthermore, the data further confirms the integrity of *D.salexigens*.

Further analysis of the reads show *D.desulfuricans* is contaminated with high levels of *B.subtilis.* Further alignment via bowtie confirms this level of contamination. This ‘contamination’ was later determined to be undepleted *Desulfovibrio.*

Sample	Total Corrected Reads	Aligned Reads	%
D.salexigens A	11733743	3482	0.030
D.salexigens B	9593995	5579	0.058
D.salexigens C	11194074	3699	0.033
D.desulfuricans A	9479032	1842550	19.438
D.desulfuricans B	11194074	2148913	21.454
D.desulfuricans C	10443901	2359219	22.589

Table shows the Bowtie alignment of RNAseq reads against the *B.subtilis* genome.