Universidade do Minho
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Ana Rita Castro Carvalho

Hydrocarbonoclastic bacteria: from bioremediation to bioenergy feedstock

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Trabalho efetuado sob a orientação da
Doutora Maria Alcina Alpoim de Sousa Pereira
e
Doutora Isabel Cristina de Almeida Pereira da Rocha

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Hydrocarbonoclastic bacteria: from bioremediation to bioenergy feedstock

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Universidade do Minho, 28 / 09 / 2015

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"Life is not about waiting for the storm to pass, is about learning how to dance in the rain."

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Abstract

Bacterial storage lipids are being considered as viable alternative feedstocks for industrial and biotechnological applications, compared to conventional ones. The production of these bacterial compounds can be obtained from different carbon sources, including inexpensive and recalcitrant wastes. This thesis explores the potential of using hydrocarbonoclastic bacteria to obtain lipid reserve substances from hydrocarbon-based wastes, promoting a more economic and environmentally sustainable biotechnological process combining pollution reduction and production of added value compounds.

The production of storage lipids by a hydrocarbon degrading bacterium, *Rhodococcus opacus* B4, from glucose, acetate and hexadecane, was reported for the first time and compared to *Rhodococcus opacus* PD630, the best known triacylglycerols (TAG) accumulating bacterium. TAG was the main storage compound produced by both strains. *R. opacus* B4 presented 2 to 3 fold higher TAG volumetric productions than *R. opacus* PD630 when cultivated in hexadecane.

Subsequently, the ability of *R. opacus* B4 to decontaminate hexadecane-contaminated cork sorbents while producing lipid storage compounds was shown. Two types of cork sorbents, natural and thermally treated cork, were used. *R. opacus* B4 was able to degrade practically all hexadecane impregnated in both cork sorbents and TAG was the main neutral lipid compound produced. The potential of the obtained lipid-rich biomass for biomethane production was assessed and an efficent conversion to methane was observed.

An indigenous microbial community enriched in carbon storage producing hydrocarbon degrading bacteria was developed from a hydrocarbon-based wastewater (containing lubricant and engine oil waste), using feast and famine conditions in the form of alternating periods of presence of the carbon substrate followed by its absence. The most dominant bacteria identified belongs to hydrocarbon degrading *Rhodococcus*, *Acinetobacter* and *Pseudomonas* genera, known for producing mainly triacylglycerols (TAG), wax esters (WE) and polyhydroxyalkanoates (PHA), respectively. The enriched community was able to accumulate small amounts of TAG and PHA, and higher levels of a non-identified lipid as well as to efficiently degrade the wastewater hydrocarbons. The effect of different cultivation parameters on storage compound production by the developed enriched community was evaluated, using central composite circumscribed design based on surface response methodology. The interaction between carbon and nitrogen concentrations positively influenced PHA accumulation whereas interaction between carbon and nitrogen concentrations with cultivation time affected PHA in a negative way. Regarding neutral lipids production, nitrogen concentration and the interaction between carbon and nitrogen concentrations were the significant parameters. Neutral lipids produced were essentially TAG, presenting a highly diversity of chemical structures composed by a narrow range of fatty acids.

Finally, a genome-based comparative analysis of genes and metabolic reactions responsible for TAG, WE and polyhydroxybutyrate (PHB) biosynthesis in members of *Rhodococcus*, *Acinetobacter*, *Alcanivorax* and *Pseudomonas* genera was performed to get deeper knowledge on carbon storage compounds metabolism in hydrocarbonoclastic bacteria. The presence of genes coding for complete metabolic pathways for TAG and PHB biosynthesis was detected in all species. *Rhodococcus* strains are highly enriched in genes involved in TAG and PHB metabolism, whereas *Alcanivorax*, *Acinetobacter* and *Pseudomonas* have a high number of genes coding for enzymes involved in PHB production.

Resumo

Os lípidos de reserva bacterianos têm sido considerados como matéria-prima alternativa aos lípidos convencionais para aplicações biotecnológicas e industriais. A produção destes compostos pode ser obtida a partir de diferentes fontes de carbono, incluindo resíduos recalcitrantes e economicamente acessíveis. Esta tese teve como principal objetivo explorar o potencial de bactérias hidrocarbonoclásticas para a produção de lípidos de reserva a partir de resíduos contendo hidrocarbonetos. Esta capacidade pode contribuir para o desenvolvimento de um processo biotecnológico enconomicamente e ambientalmente sustentável, envolvendo a descontaminação biológica de resíduos e a produção de compostos de valor acrescentado.

A produção de lipidos de reserva em *Rhodococcus opacus* B4, uma bactéria degradadora de hidrocarbonetos, a partir de glucose, acetato e hexadecano, foi descrita pela primeira vez e comparada com a bactéria *Rhodococcus opacus* PD630, considerada como a melhor produtora de triacilgliceróis (TAG). Os triacilgliceróis foram os principais compostos produzidos por ambas as estirpes. *R. opacus* B4 apresentou uma produção volumétrica de TAG duas a três vezes superior à de *R. opacus* PD630 quando cultivadas em hexadecano.

A capacidade de *R. opacus* B4 para degradar hexadecano adorvido a cortiça e produzir lípidos de reserva foi avaliada. Foram utilizados dois tipos de adsorventes de cortiça, cortiça natural e cortiça termicamente tratada. *R. opacus* B4 degradou eficazmente o hexadecano impregnado em ambas as cortiças e os triacilgliceróis foram os principais lípidos de reserva produzidos. O potencial de produção de metano a partir da biomassa rica em lípidos obtida foi analisado, tendo-se observado uma eficiente conversão a metano.

O desenvolvimento de uma comunidade microbiana enriquecida em bactérias hidrocarbonoclásticas produtoras de compostos de reserva foi obtido a partir de águas residuais contendo lubrificantes e óleo de motor, aplicando períodos alternados de presença e ausência de fonte de carbono. As bactérias mais dominantes identificadas pertencem a géneros hidrocarbonoclásticos, nomeadamente *Rhodococcus*, *Acinetobacter* e *Pseudomonas*, conhecidas como produtoras de TAG, ésteres de cera (WE) e polihidroxialcanoatos (PHA), respetivamente. A comunidade enriquecida acumulou baixas quantidades de TAG e PHA e quantidades superiores de um lípido desconhecido, tendo degradado eficientemente os hidrocarbonetos presentes na água residual. O efeito de diferentes parâmetros de cultivo na produção de compostos de reserva pela comunidade enriquecida foi avaliado através de um desenho fatorial de experiências baseado na metodologia de superfície de resposta (RSM). A interação entre as concentrações de carbono e azoto influenciou positivamente a acumulação de PHA enquanto que as interações entre o período de cultivo e as concentrações de carbono e azoto e o tempo de cultivo afetaram negativamente a produção de PHA. A produção de lípidos neutros foi significativamente influenciada pela concentração de azoto e pela interação entre as concentrações de carbono e azoto. Os lípidos neutros produzidos foram essencialmente TAG, apresentando uma elevada diversidade de estruturas químicas compostas por uma ampla gama de ácidos gordos.

Foi efetuada uma análise comparativa de genes e reações metabólicas responsáveis pela síntese de TAG, WE e polihidroxibutiratos (PHB) em espécies pertencentes aos géneros *Rhodococcus*, *Acinetobacter*, *Alcanivorax* e *Pseudomonas*. A presença de genes que codificam para enzimas pertencentes a todas as vias metabólicas relativas à produção de TAG e PHB foi detetada em todas as espécies estudadas. As estirpes de *Rhodococcus opacus* apresentam um número elevado de genes envolvidos no metabolismo de TAG e PHB, enquanto que *Alcanivorax borkumensis* SK2, *Acinetobacter baylyi* ADP1 e *Pseudomonas putida* KT2440 possuem uma grande diversidade de genes que codificam para enzimas envolvidas na produção de PHB.

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3-HB	3-hydroxybutyrate
3-HP	3-hydroxypropionate
3-HV	3-hydroxyvalerate
3HHp	3- hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3-HTD	3-hydroxytetradecanoate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BAF	biological aerated filter
BLAST	Basic Local Alignment Search
BLASTP	Protein Basic Local Alignment Search
Bp	base pair
BMP	biomethane production
BN	2-bromonaphthalene
C16_NC	hexadecane contaminated natural cork
C16-TTC	hexadecane contaminated thermally treated cork
CDP	cytdine diphosphate
Cdw	cellular dry weight
CH_4	methane
C/N	carbon/nitrogen
CO_2	carbon dioxide
CoA	coenzyme A
COD	chemical oxygen demand
C.O.D	chlorooctadecane
CMD	cytdine monophosphate
CTD	cytdine triphosphate
DAG	diacylglycerols
DGGE	denaturant gradient gel electrophoresis

DNA	deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
EC	Enzyme Commission
ESI-Q-TOF	electrospray ionization quadrupole time-of-flight
FA	fatty acids
FAEE	fatty acid ethyl esters
FAME	fatty acid methyl esters
FID	flame ionization detector
FBR	fed-batch reactors
GC-MS	gas chromatography-mass spectrometry
G3P	glycerol-3-phosphate
GC	gas chromatography
H_2	hydrogen
H_2O	water
HPLC	high-performance liquid chromatography
IBAF	immobilized biological aerated filters
LCFA	long chain fatty acids
lcl-PHA	long chain length-PHA
MAH	monocyclic aromatic hydrocarbons
MAG	monoacylglycerols
MB	membrane bioreactors
mcl-PHA	medium chain length PHA
MS	mineral salts
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NBRC	National Biological Resource Centre
NC	natural cork
NCBI	National Centre for Biotechnology Information
NH_4^+	amonium
OO	oleyl oleate
OTP	ortho-terphenyl
PAH	polycyclic aromatic hydrocarbons

PCR	polymerase chain reaction
PDAT	phospholipid:diacylglycerol acyltransferase
PE	polyethylene
PET	polyethylene terephthalate
PHA	polyhydroxyalkanoates
PHB	polyhydroxybutyrate
PHV	polyhydroxyvalerate
PO_4^{3-}	phosphate
PP	polypropylene
PS	polystyrene
PVC	polyvinylchloride
RBC	rotating biological contactor
RF	retention factor
RI	refraction Index
RNA	ribonucleic acid
Rpm	revolutions per minute
Rrna	ribosomal ribonucleic acid
SBR	sequencing batch reactors
Scl-PHA	short chain length PHA
SPE	solid phase extraction
STP	standard temperature pressure
TAG	triacylglycerols
TAN	total ammonia nitrogen
TCA	triacarboxylic acid
TH	total hydrocarbons
TLC	thin layer chromatography
TN	total nitrogen
TPH	total petroleum hydrocarbons
TTC	thermally treated cork
UASB	upflow anaerobic sludge blanket
UCM	unresolved complex mixture
UV	ultraviolet

v/v	vol/vol
w/w	weight/weight
WE	wax esters

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Chapter 1

Context, Aim and Thesis Outline

1.1 Perspective and motivation

1.1.1 Fossil fuels depletion and contribution to greenhouse effect

Fossil fuels are non-renewable resources since its formation is considered a quite long process and reserve consumption rates are much faster than the reserve production ones. Worldwide, the burning of fossil fuels generated 31.7 billion tons of carbon dioxide (CO_2) per year in 2012 (IEA, 2014). CO_2 is one of the greenhouse gases that contribute to global warming, causing the average surface temperature of the Earth to rise in response, causing several adverse effects. In the United States, more than 90 % of greenhouse gas emissions come from the combustion of fossil fuels, contributing to a total of 6673 million metric tons of carbon dioxide equivalents (USEPA, 2015b). Petroleum is the main energetic vehicle responsible for the economical sustainable development during the last decades. Currently, the world total petroleum production is about 95.66 million barrels per day, while the world total petroleum consumption is 93.62 million barrels per day in 2015, indicating that in a near future the increasing petroleum demand cannot meet petroleum production levels (USEPA, 2015a). Comparing the forecasted energy demand and accessible resources of crude oil, it is obvious that the future energy demand cannot solely be met by fossil fuels. In a recent evaluation of the global oil depletion, it was concluded that a peak of conventional oil production will be reached between 2020 and 2030 due to the exhaustion of accessible sources (Al-Husseini, 2009). Without appropriate alternatives to crude oil, the global economy will suffer a collapse due to the dramatic increase in oil prices as well as in the demand for oil production. Consequently, it is mandatory to overcome the dependence on crude oil and its detrimental impact on environment by developing a sustainable and competitive alternative based on renewable and abundant feedstock like biomass (Narasimharao *et al.*, 2007). In fact, the continuous increase of crude oil prices and the increasing environmental concern are driving the development towards new bio-based, renewable and therefore environmental-friendly substitutes for petroleum, like bioplastics or biofuels (Uthoff *et al.*, 2009).

1.1.2 Global anthropogenic pollution and biological removal strategies

Other prominent worldwide concern is the global anthropogenic pollution generated by crude oil extraction and refinery processes and also by oil-based industries. The development of the petroleum industry, the spillages that occur during routine and transport operations, have called for

more studies into oil pollution problems, which has been recognized as the most significant contamination problem worldwide (Lefebvre & Moletta, 2006; Das & Chandran, 2011). Even small releases of petroleum hydrocarbons into aquifers can lead to concentrations of dissolved hydrocarbons far in excess of regulatory limits (Spence *et al.*, 2005). The resulting hydrocarbon-rich wastewaters have led to the accumulation of a wide variety of organic xenobiotic pollutants causing detrimental effects on human health and on both the biotic and abiotic components of several types of ecosystems (Head *et al.*, 2006). The processes leading to the removal of hydrocarbon pollutants from the environment has been extensively reported and involves different physical, chemical and biological alternatives. The currently accepted disposal methods of incineration or burial in secure landfills (Wang *et al.*, 2004) can become incredibly expensive when there are high amounts of contaminants to be treated. The biodegradation of oil pollutants is not a new technique but it is relevant as an increasingly effective and potentially inexpensive cleanup technology. Bioremediation, which uses the biodegradative metabolic machinery of organisms, is an effective technology that can be used to reduce organopollutant levels and is useful in the recovery of sites contaminated with oil and hazardous wastes. Besides, bioremediation technology is non-invasive, relatively cost effective and probably the most reliable option for exploitation in solving some chemical pollution problems (Koul, 2013). Alkanes, as main components in fuels and oils, are relative inert compounds, originating dramatic ecological problems upon their release to the environment (Singh *et al.*, 2012). However, microorganisms have established effective strategies involving specialized enzyme systems and metabolic pathways to access n-alkanes as a carbon and energy source. Such microorganisms are capable of degrading alkanes and converting them to easily degradable substrates (Wentzel *et al.*, 2007). In fact, microbial communities are known to colonize polluted environments and have the ability to metabolize these recalcitrant xenobiotic compounds. Currently, several microbial biodegradation strategies have been developed as an indispensable, ecofriendly and cost-effective solution for restoring polluted ecosystems (Desai *et al.*, 2010) and biodegradation of the hydrocarbons by natural populations of microorganisms has been reported to be the main process acting in the removal of hydrocarbon-polluted environments (Hassanshahian *et al.*, 2014; Bargiela *et al.*, 2015).

1.1.3 Biofuels and bioplastics industry

One of the most prominent renewable energy resources is biodiesel, which is produced from renewable biomasss by transesterification of triacylglycerols, yielding monoalkyl esters of long-chain

fatty acids with short-chain alcohols, for example, fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) (Meng *et al.*, 2009). This fuel has been reported as a possible substitute or extender for conventional petrol-based diesel. Biodiesel is rapidly moving towards the mainstream as an alternative source of energy and has become more attractive recently because of its environmental benefits. This biofuel offers several interesting and attractive properties, e.g., biodegradability and non-toxicity, compared to petroleum-based diesel. The most important advantage of biodiesel, as a renewable material, is the maintenance of balanced carbon dioxide cycle. Additionally, biodiesel combustion results in reduced emission of carbon monoxide, sulfur, and aromatic hydrocarbons (Adamczak *et al.*, 2009). The global biodiesel industry has grown significantly over the past decade. In 2012, the overall biodiesel production was 5.94 billion gallons (Babu *et al.*, 2013b) and the global biodiesel market is estimated to reach 37 billion gallons by 2016, growing at an average annual rate of 42 % (Fan, 2010). Among the conventional chemical compounds, plastics assume a prominent role in different areas of modern society (Andrady & Neal, 2009). In 2012, the global plastic production and consumption was 288 million tones, possibly reaching 297.5 million tons in 2015 (PlasticsEurope, 2013). The plastic demand in Europe was 50 million tons in 2012, representing 20.4 % of the global plastic production of which 61 % was composed by mainly five polymer classes - polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET). However, these plastics are produced based on petroleum feedstocks, presenting toxic properties, being resistant to biodegradation, causing severe environmental consequences and an increase of carbon dioxide emissions to the atmosphere (Pei, 2011). Due to these drawbacks, there is currently wide interest for bioplastics production. Nowadays, the worldwide production of bioplastics is around 1.6 million tonnes, representing approximately 1 % of total plastic-based industry. Based on increasing development in bio-based plastics technologies, it is estimated that bioplastics production will reach a total of 6.7 million tonnes by 2018 (Bioplastics, 2014).

1.1.4 Feedstocks for biofuels industry

Currently, different types of animal fats, lignocellulosic biomass, vegetable oils such as soybean oils, rapeseed oils, palm oils, and waste cooking oils and agricultural products are usually adopted as feedstocks for biofuels production (Felizardo *et al.*, 2006; Yang, 2007; Moser, 2009; Karmakar *et al.*, 2010; Gnanaprakasam, 2013). However, all these plant oil materials need energy for sufficient production of oilseed crops and animal fat oils require more cattle breeding. In fact, these

edible feedstocks directly compete with food chain supply and cause environmental issues namely destruction of soil resources, intensive use of large areas for cultivation and high levels of deforestation (Atabani *et al.*, 2012; Arikan, 2015). Particularly, one of the most important drawbacks is the dramatic increase in vegetable oils prices, committing biofuels production market (Balat, 2011; Mekonnen *et al.*, 2013). To assure the needs for these bio-based compounds production, other oil sources, especially nonedible oils need to be explored. Therefore, exploring ways to mitigate biofuels disadvantages is of much interest in recent research, especially for those technologies allowing the reduction of raw material price. Microorganisms have often been considered for the production of oils and chemicals as an alternative to agricultural and animal sources (Yu & Stahl, 2008; Meng *et al.*, 2009). Compared to plant and animal oils, microbial oils derived from fungi, yeast and microalgae are being considered as potential feedstocks for biofuels and bioplastics production since they present several advantages, such as short life cycle, lower manpower required, relative independence on climacteric and seasonal conditions, easier scaling up, no arable land requirements, a wide range of available raw materials, lower water consumption and mitigation of atmospheric CO_2 (Li *et al.*, 2008; Yu & Stahl, 2008; Hempel, 2011; Markou & Nerantzis, 2013; McCurdy*, et al.*, 2014; Medipally, 2015). Moreover, oleaginous microorganisms present promising oil productivity, since some members can accumulate lipids ranging between 20 % and 80 % of their cellular dry weight. They can be cultivated in more controlled conditions, namely in terms of pH, nutrient amounts, temperature and using a wide range of carbon sources, which ultimately determine the fatty acids composition of lipids. Finally, microorganisms can be grown on different systems, such as open ponds, raceways, photo-bioreactors or fermenters, reducing space requirements (McCurdy *et al.*, 2014). Microalgae are one of the most significant microbial oil producers. Microalgal cells can accumulate lipids between 1 % and 90 % of their cellular dry weight (Metting, 1996; Gouveia & Oliveira, 2009; Mandal & Mallick, 2009; Yoo*, et al.*, 2010), being the most common a lipid content between 20 % and 50 %. In some cases, microalgae have the potential to yield higher oil levels when compared to the most productive oil crops (Chisti, 2007; Schenk *et al.*, 2008; Atabani *et al.*, 2012). Despite the above mentioned advantages of using microalgae as feedstocks for biofuels and bioplastics production, there are several limitations hindering the use of these microorganisms at industrial scale. Some of the most significant drawbacks are the dependence of sunlight for the cultivation process, which is daily and seasonal variable (Liang & Jiang, 2013), requirement of additional organic carbon sources (Li *et*

al., 2008), the high cost of harvesting and dewatering methods (Atabani *et al.*, 2012; Wahlen *et al.*, 2013) and the requirement of high levels of CO_2 (Ratledge, 2013).

1.1.5 Bacterial oils as feedstocks for bio-based compounds production

In the last decades, bacteria have gained increasing importance over microalgae in oil production since they do not require acreage for cultivation, presents shorter fermentation periods and higher growth rates, are easier to cultivate, require a reduced source of carbon to meet their energy demands, becoming emerging candidates for biofuels and bio-based compounds production (Li *et al.*, 2008; Lennen & Pfleger, 2013; Thevenieau & Nicaud, 2013).

Generally, bacteria produce polyhydroxyalkanoates (PHA) as the main carbon and energy reserve compounds, being used as feedstock for bioplastic and bio-based chemical compounds production (Chen, 2010; Babu *et al.*, 2013a; Singh *et al.*, 2014). However, bacteria belonging to the actinomycetes group such as species of *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia* and *Gordonia* (Leman, 1997; Alvarez & Steinbuchel, 2002; Li *et al.*, 2008) and some gram-negative bacteria belong to *Alcanivorax*, *Marinobacter* and *Acinetobacter* genera (Kalscheuer & Steinbuchel, 2003; Kalscheuer *et al.*, 2007; Manilla-Pérez*, et al.*, 2011) are able to synthesize intracellular lipid storage compounds in the form of triacylglycerols (TAG) and wax esters (WE). The content of fat in these oleaginous bacteria can reach up to 80 % of the cellular dry weight (Alvarez *et al.*, 1996). Moreover, a wide range of substrates are utilized by oleaginous bacteria for oil synthesis, e.g., sugars, organic acids, alcohols, oils, and different waste products, such as whey and agro-industrial wastes (plant oils, sugar-cane molasses, wheat bran, vegetable and fruit waste), dairy wastewaters, light oil derived from pyrolysis and lignin derived from pulp and paper industry (Alvarez *et al.*, 1996; Alvarez *et al.*, 1997a; Alvarez, 2003; Gouda *et al.*, 2008; Hori *et al.*, 2009; Kurosawa *et al.*, 2010; Manilla-Pérez *et al.*, 2011; Kumar *et al.*, 2015; Wei *et al.*, 2015b; Wei *et al.*, 2015a), making biofuels and bio-based chemicals production processes more attractive and more economically feasible.

In last years, with the concomitant increasing development of Systems Biology and Bioinformatics tools and an expanding knowledge on physiology and metabolic pathways of oil accumulation process in bacteria, efforts have been focused on biological engineering technology, genetic and metabolic engineering to alter bacteria performance to improve oil accumulation (Kosa & Ragauskas, 2011). Recently, it was described the ability of a metabolically engineered *Escherichia coli* to produce fatty acid ethyl and butyl esters (FAEE/FABE)-based biodiesel, generally designated

as microdiesel and also jojoba oil-like wax esters at the expense of renewable carbon sources (Kalscheuer *et al.*, 2006a; Kalscheuer *et al.*, 2006b; Steen *et al.*, 2010; Zhang *et al.*, 2012). Very recently, a particular focus has been attributed to the use of lignocellulosic biomass as raw material for bacterial oil production, reducing the dependence on crop cultures. In this context, *R. opacus* PD630, a model bacterium for TAG synthesis, has been subjected to several genetic and metabolic engineering modifications with the aim of converting lignocellulosic components into renewable fuels (Kurosawa, 2013; Kurosawa, 2014; Kurosawa *et al.*, 2015b; Kurosawa, 2015c; Kurosawa, 2015d). Furthermore, *Acinetobacter baylyi* ADP1, a known bacterial WE producer, has been manipulated to increase TAG and WE levels for biofuels and bio-based chemicals production (Santala *et al.*, 2011b; Santala *et al.*, 2014).

1.2 Research Aim

The aim of the research presented in this thesis was to evaluate the potential of hydrocarbnoclastic bacteria to produce lipid storage compounds using hydrocarbon-based wastes/wastewaters as a cheap raw material. The possibility of combining bacterial lipid production with the bioremediation of these hazardous wastes is a powerful strategy to achieve environmental remediation while producing added value compounds. For this purpose, an integrated experimental strategy was applied combining the use of defined hydrocarbonoclastic bacterial and enriched mixed cultures to obtain bacterial lipids. Furthermore, the application of a bioinformatics-based approach was used to unravel the genomic basis of metabolic pathways involved in carbon storage biosynthesis in several hydrocarbonoclastic bacterial species with different lipid storage compounds abilities.

1.3 Thesis Outline

In this chapter, a general contextualization and motivation for the research on bacterial storage lipids production for application in biofuels and bio-based chemicals industries using hydrocarbon-based substrates is presented, giving emphasis to the current scenario on the generation of hydrocarbon-based wastes derived from oil-based industries and the need for further biological decontamination and also on the development of biofuels and bioplastics industries and the demand to explore alternative bacterial feedstocks for bio-based compounds production. A global overview of the current knowledge on existing hydrocarbon remediation technologies, with

particular focus on biological ones, on the diversity of hydrocarbonoclastic bacterial species and its role in pollution reduction and bioenergy feedstock production as well as in the physiology and pathways underlying bacterial lipid storage metabolism is given in Chapter 2. In the following two chapters *Rhodococcus opacus* B4 ability to produce TAG using hydrocarbon-based compounds was assessed. In Chapter 3 TAG synthesis by *R. opacus* B4 using sugars, organic acids and hydrocarbons as carbon and energy sources was demonstrated. This capability was compared to *R. opacus* PD630, the best known bacterial TAG producer, revealing higher TAG yield when cultivated in hexadecane. Based on the results obtained in Chapter 3, *R. opacus* B4 capacity to produce TAG from hydrocarbon-rich solid residues, using hexadecane contaminated cork, a commercial product used as sorbent in marine and terrestrial oil spills and leaks, was investigated. The potential use of the obtained lipid-rich biomass for biomethane production was also evaluated (Chapter 4). In Chapter 5 a stable microbial community enriched in lipid-accumulating and hydrocarbon-degrading bacteria was developed using hydrocarbon-adapted sludge as source of autochthonous microorganisms and hydrocarbon-rich wastewater as carbon and energy sources. The microbial community was characterized in terms of storage compounds production and hydrocarbon degradation and some of the most dominant bacterial species were detected and identified using 16S rRNA gene cloning and sequencing techniques. The effect of cultivation conditions on storage compounds production by the obtained microbial community was analysed in Chapter 6 using an experimental design methodology. Neutral lipid species were identified and characterized by GC-MS. In Chapter 7 a genome-based comparative analysis of genes and metabolic reactions responsible for TAG, WE and PHB biosynthesis of four hydrocarbonoclastic bacterial genera with recognized capabilities in lipid storage compounds production was performed and genetic differences between bacterial species were detected and identified. Finally, in Chapter 8 the general conclusions of this thesis are resumed and perspectives for future scientific research in this field are discussed.

Chapter 2

Literature review

2.1 The problematic of oil-based industries

In the last decades, an increasing request for petroleum-derived products has led to a rapid development of several types of oil-based industries. As a consequence, considerable amounts of different hydrocarbon-contaminated wastes are produced, being the most significant oil sludge and oil-based wastewaters. Oil sludge is a mixture of oil, solids and water, and is mainly generated during crude oil routine exploration processes, being accumulated in the bottom of storage and transportation tanks and also in wastewater treatment plants located in crude oil refineries (Mrayyan & Battikhi, 2005; Ramaswamy *et al.*, 2007; Xu *et al.*, 2009; Wang *et al.*, 2010). According to several studies it is assumed that around 60 million tons of oily sludge is produced every year and more than 1 billion tons is accumulated around the globe (Tahhan *et al.*, 2011; da Silva *et al.*, 2012). Oil-based wastewaters are produced from several industries, namely crude oil production and refinement; lubricants and petrochemical manufacturing; automotive repair stations and industrial equipment maintenance units (Diya'uddeen *et al.*, 2011). These activities were reported to produce large amounts of wastewaters, representing a production of 33.6 million barrels per day of effluents worldwide (Doggett and Rascoe, 2009).

The petroleum industry also contributes to an increase in the number of oil spills throughout the world. Accidental and deliberate releases of petroleum occur during routine operations of extraction, production, transportation, refining and storage processes in land and marine environments and also during illegal disposal practices such as direct discharge from effluent (Atlas, 1995; Essaid *et al.*, 1995; Thorn & Aiken, 1998; Kingston, 2002; Das & Chandran, 2011). In particular, around 56 million barrels of petroleum were transported through maritime routes in 2013 (EIA, 2014) and approximately 4000 tonnes of petroleum entered the sea in 2014 (ITOPF, 2014).

Crude oil is composed by a complex and natural mixture of hydrocarbons, being already identified approximately 20 000 chemical components (Marshall & Rodgers, 2003). Additionally, crude oil is a heterogeneous material, exhibiting different physico-chemical characteristics, such as viscosity, solubility and absorption capacity and consequently different levels of bioavailability and toxicity that influence its susceptibility to biodegradation and environmental fate (Head *et al.*, 2006). The composition of crude oil can be divided in four main fractions: 1) Saturates (branched, unbranched and cyclic alkanes); 2) Aromatics (ring conformation hydrocarbon molecules namely monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs)); 3) Resins (polar oil-surface

structures dissolved in saturates and aromatics); 4) Asphalthenes (indeterminated solids colloidally dissipated between saturates and aromatics) (Balba *et al.*, 1998; Speight, 1999; Marshall & Rodgers, 2003; Yemashova *et al.*, 2007) Furthermore, substances with sulfur, oxygen and nitrogen in their chemical composition and vestigial quantities of phosphorous and heavy metals are also present (Van Hamme *et al.*, 2003). Saturated hydrocarbons represent the most predominant fraction of crude oil and therefore are considered the major pollutants. For these reasons the degradation of saturated hydrocarbons is the most significant process in the treatment of crude oil in the environment. The aromatic hydrocarbons with less than three aromatic rings are also efficiently degraded whereas those with more than four aromatic rings are quite toxic and persistent and consequently more resistant to degradation (Harayama, 1999). The asphaltene fraction have compounds characterized by several heavy and viscous complex non-hydrocarbon polar compounds and for that it is difficult to be degraded (Tavassoli *et al.*, 2012), whereas resins are more easily degraded because they are light polar compounds (Spiecker *et al.*, 2003). Oil-rich wastewaters and oil sludge derived from industrial activities contain several toxic compounds, namely linear and aromatic hydrocarbons, phenols, lubricants, cutting fluids, tars, grease and diesel oil. They can also be composed by emulsified oils, cleaning agents, solvents, sulphides and heavy metals (Diallo *et al.*, 2000). Several reports have demonstrated that high amounts of these residues are discharged into the environment every year, causing severe pollution problems particularly in soil and water (Holliger *et al.*, 1997; Wake, 2005). These wastes can persist for years in the ecosystems due to the poor solubility and low volatility of these compounds. Contamination with hydrocarbons originate deleterious impacts on local systems since accumulation of contaminants in animals and plants may cause severe mutations and in an indirect way affect human health (Alvarez & Vogel, 1991; Lefebvre & Moletta, 2006).

2.2 Treatment techniques for hydrocarbon-based wastes

The development of industrial activities led to an increase in the formation of huge amounts of waste products. Several ecosystems have greatly suffered dramatic changes due to insufficient treatment and to the discharge of several hydrocarbon-based waste products. Consequently, these wastes were already classified as priority contaminants and several tight directives were approved to control this type of discharges and to maintain wastes concentrations below levels indicated by the regulations (EPA, 2015). In order to mitigate these problems, during the last decades several researchers have been developing new different treatment technologies to decontaminate land and marine environments

as well as oil-based wastewaters and sludge (Diya'uddeen *et al.*, 2011; Hu *et al.*, 2013; Yu *et al.*, 2013; Akpor, 2014). These treatment technologies include physical, chemical and biological processes.

2.2.1 Physical and chemical processes

In the last decades, several physical and chemical remediation treatments have been developed and used for pollution control of oil-based wastewaters and sludge. The most significant remediation methods for treatment of contaminated wastewater are basically divided in two stages: 1) mechanical and physicochemical treatments; 2) advanced treatment of the pre-treated effluent (Renault *et al.*, 2009). These techniques are flotation, adsorption, membranes application, coagulation and advanced oxidation processes (Moosai & Dawe, 2003; Bansode *et al.*, 2004; Juang *et al.*, 2007; El-Naas *et al.*, 2009; Oller *et al.*, 2011). Regarding treatment of oily sludge, the strategies are also grouped in two types. The first is related to the recovery and restore of fuel which still remains in the oily sludge after crude oil refinement and includes solvent extraction, surfactants application, freeze/thaw treatment, pyrolysis, microwave irradiation, electrokinetic methods, ultrasonic irradiation and froth flotation (Hu *et al.*, 2013). The second type of treatment strategies is related to the disposal of the remaining oil sludge in the case of failure in the application of the first strategy (da Silva *et al.*, 2012). The main techniques are incineration, stabilization/solidification and oxidation treatment (Malviya & Chaudhary, 2006; Ferrarese *et al.*, 2008; Zhou *et al.*, 2009). Nevertheless, the complexity and recalcitrance of oily sludge and wastewaters makes ineffective the use of a single remediation technique, since it cannot fulfill all demands in terms of disposal and reutilization of wastes. In fact, there are not many technologies reducing contaminant levels and satisfying environmental regulations. Furthermore, some of the remediation techniques mentioned above can be potentially effective regarding oil recovery as well as remediation of unrecoverable wastes, but their prohibitive costs makes impracticable their implementation to large-scale fields. Hence, it is imperative to consider and develop more economical, effective and environmentally friend remediation techniques as well as to conjugate them with the ones already implemented.

2.2.1.1 Mechanism of bacterial hydrocarbon degradation

Hydrocarbon-degrading microorganisms possess multiple metabolic pathways and enzymes which catabolize hydrocarbon compounds in a series of sequential reactions. Depending on the type of hydrocarbon, there are differences in the easiness in terms of bacterial degradation: linear alkanes >

branched alkanes > small aromatics > cyclic alkanes > polycyclic aromatic hydrocarbons > polar compounds (Ollivier & Magot, 2005; Ulrici, 2008). Alkanes are initially degraded by the action of oxygenases, activating the molecule and inserting oxygen. The alcohol molecules are converted to aldehydes or carboxylic acids. The carboxylic acids, which are intermediates of the metabolic pathways of central carbon metabolism, are then transformed via β-oxidation, producing acetyl coenzyme A (acetyl-CoA) and other intermediates (Fritsche & Hofrichter, 2008). In contrast, cycloalkanes are more difficult to degrade when compared to *n*-alkanes due to their physical state and conformation. The first reaction in cycloalkane degradation is also performed by an oxidase system, converting it into a cyclic alcohol, which is further converted to a ketone. Then, ketone is used as a substrate by a monooxygenase and hydrolase with subsequent ring cleavage. Bacterial biodegradation of aromatic compounds is highly dependent on the number of rings associated to the molecule. First, the aromatic compound is transformed in a dihydrodiol by the action of a dioxygenase and then the diol group is broken with subsequent formation of a carboxylic acid by a dehydrogenase (Wang *et al.*, 1998). Asphaltenes are even more resistant to biodegradation than polycyclic compounds since their concentration increases due to the formation of condensation products as a result of biodegradation reactions. Considering crude oil and wastes generated from oil-based industries, hydrocarbons are present as a complex mixture, where the degradation of the different compounds is dependent on their own presence and concentration (Wackett, 1996).

2.2.1.2 Factors affecting bacterial hydrocarbon degradation

The process of bacterial degradation of hydrocarbon-contaminated environments and wastes is complex and its efficiency requires the establishment and maintenance of a set of optimal conditions to achieve the best rates of hydrocarbon bioremediation. Therefore, there are several factors influencing biological decontamination methods (Van Hamme *et al.*, 2003). The type and concentration of hydrocarbons present in oily residues together with its physical and chemical characteristics affect biodegradation processes. Additionally, some environmental parameters such as temperature, pH, oxygen levels, the availability of electron donors and acceptors and the adequate availability of nutrients can be determinant for improving pollutants availability and/or to promote an effective hydrocarbon-degrading bacterial community (Atlas, 1995; Boopathy 2000; Oh *et al.*, 2001; Venosa & Zhu, 2003; Kim *et al.*, 2005; Okoh, 2006; Fountoulakis *et al.*, 2009). In fact, hydrocarbon biodegradation processes are highly dependent on the establishment of an adapted microbial consortium with specific physiological and metabolic properties (Van Hamme *et al.*, 2003; Olajire, 2014).

2.2.2 Biological techniques

Biological pollutant treatment is a decontamination process that can be defined as the use of biological agents namely bacteria, fungi and plants, to degrade and remove environmental contaminants (Caplan, 1993). In particular, the majority of bioremediation studies are performed with bacteria since they possess robust enzymatic machinery and metabolic capabilities to transform or reduce the concentration of toxic substances into less harmful compounds (Pala *et al.*, 2006). In the last decade several efforts have been performed to study biological degradation of hydrocarbons. These techniques can be applied to different systems, namely soil and marine environments and more recently to effluents and sludge resulted from petroleum industry (Medina-Bellver *et al.*, 2005; Head *et al.*, 2006; McGenity *et al.*, 2012; Singh, 2013; Akpor, 2014; Battikhi 2014; Olajire, 2014). In addition, bioremediation technology is noninvasive, environmental friendly and relatively cost-effective since it is easy to operate, can be applied in large areas, and allows in many cases the total pollutant degradation (Leahy & Colwell, 1990; Makkar & Rockne, 2003). Since bacteria are the most important microorganisms in crude oil degradation, presenting an active function in petroleum contaminated environments, several works reported the identification and isolation of many genera of oil-degrading bacteria (Davis, 1967). However, one single bacterial species is not able to fully degrade complex hydrocarbon mixtures frequently found in crude oil and oily wastes. Instead, the synergistic interactions between mixed bacterial consortia will enhance bioremediation by attaining better degradation efficiencies and rates (Olajire, 2014).

There are several biological techniques that can be applied depending on the type of residue/environment to be treated. Therefore, bioremediation strategies can generally be divided in two main categories: *in situ* and *ex situ*. *In situ* technologies are described as the processes responsible for the biological decontamination of organic pollutants in the original place and are mostly applied on hydrocarbon contaminated soils and waters. This type of approach offers some advantages since it is economically favorable, sustainable and does not cause environmental perturbations in the ecosystems. On the other hand, *ex situ* technologies are the ones that involve the treatment of contaminants by removing the polluted material from the site or before the disposal of oil based residues into the environment, being used particularly in contaminated industrial wastewaters and sludge. In this way, biodegradation process is more efficiently monitored and faster. Nevertheless, the transport and removal of contaminated material presents high costs and potential damage to the

surrounding environment (Megharaj *et al.*, 2011; Ubani, 2012). In the last decades, several efforts have been made to develop, improve and increase the number of bioremediation techniques. The most studied techniques are landfarming/landfilling; bioaugmentation/biostimulation; biosparging/bioventing; composting and bioreactors. However, the biological treatments most applied to oil-base wastewaters and sludge are landfarming, composting and bioreactors.

2.2.2.1 Landfarming

Landfarming consists in the application and mixture of oil wastes with the upper part of soil in a given contained site treatment by tilling. The addition of water and nutrients and a proper oxygenation and pH are essential to promote microbial activity and consequently more efficient indigenous microbial hydrocarbons degradation. The main drawback of this technique is the long time required for total oil biodegradation. However, if properly applied, this technique has no negative impact on the surrounding environment, is economically viable (low cost and low energy requirements), easy to operate and can treat large amounts of wastes. In fact, in last years, several oil refining industries adopted this technique, in accordance to standards created for landfarming of oil wastes (Hu *et al.*, 2013). Several papers were published reporting the long-term effectiveness of landfarming in the treatment of different types of oil sludge (Bossert *et al.*, 1984; Mishra *et al.*, 2001; Hejazi *et al.*, 2003; Marin *et al.*, 2005).

2.2.2.2 Composting

Composting is a biological process that consists in the controlled mixture of different organic substrates, where a set of active indigenous microorganisms previously adapted promotes substrate degradation, when an organic waste is added to the compost. In the particular case of oil sludge and to increase biodegradation efficiency, the addition of oxygen, water, nutrients (nitrogen and phosphorous) and bulking agents as well as adequate pH and temperature are of crucial relevance to maintain high microbial metabolic activity (Gibb, 2001; Ball *et al.*, 2012; Hu *et al.*, 2013). This technique has already been considered as an alternative approach to landfarming in the biological treatment of hydrocarbon-rich sludge (De-qing *et al.*, 2007). The main benefits of composting are the low space requirement and waste content reduction, since microbial degradation will convert organic substrates into innocuous inorganic compounds.

Several works have described the application of composting as the most suitable technology to treat oily sludge. Several bulking agents, different levels of moisture, nutrients, and co-substrates were tested and microbial degradation of different types of oil residues (from petrol station and from refineries)

were analysed. The results showed that generally total petroleum hydrocarbons (TPH) were reduced between 50 and 93 % in a time period ranging between 3 months and 1 year (Kirchmann & Ewnetu, 1998; Marín *et al.*, 2006; Liu *et al.*, 2010; Wang *et al.*, 2012; Atagana, 2014), revealing the viability of treating oily sludges by composting.

2.2.2.3 Bioreactors

Bioremediation processes that occur in bioreactors consist in the addition of polluted wastes (liquid or solid) into a confined reservoir containing an adapted indigenous bacterial consortium. For an effective biological treatment, a cocktail of selected nutrients must be added. Inside the reactor a three-phase system is created (gas, liquid and solid phases) to increase the efficiency of biodegradation system, since a mixture of sludge and water is formed, promoting a higher hydrocarbons solubility and availability. The reactor design with the associated devices and operational conditions will allow a better homogenization between hydrocarbons, microbial consortium, nutrients and oxygen, potentiating a faster hydrocarbon conversion by microbial community (Weber & Kim, 2005).

When compared to landfarming and composting, bioreactor-based bioremediation presents some important adavantages. It is characterized by presenting higher levels of pollutant degradation rates, only requires a small working area, avoiding the use of large land areas and is a less time consuming technique since the contaminants are biodegraded in a shorter time period (Castaldi, 2003; Hu *et al.*, 2013; Yu *et al.*, 2013). Notwithstanding the innumerous advantages of bioreactors in the biological treatment of oily wastewaters and sludge, there are some drawbacks. One of the most significant limitations is the elevated treatment expense, mainly derived from the high energy consumption and costs associated to contaminated residues digging and transport operations of contaminated residues (Sorek *et al.*, 2008; Burken *et al.*, 2011).

Different reactor designs are being efficiently used in the biological treatment of hydrocarbon-based wastes, especially wastewaters. Fed-batch reactors (FBR), sequencing batch reactors (SBR), upflow anaerobic sludge blanket (UASB) linked to immobilized biological aerated filters (IBAFs), membrane bioreactors (MB), rotating biological contactor (RBC) and biological aerated filter (BAF) reactors were already used in biodegradation of different types of oil wastes, presenting high total petroleum hydrocarbons removal efficiencies that range between 50 % and 99 % after 3-6 month treatment (Zhao *et al.*, 2006; Alberti *et al.*, 2007; Chavan & Mukherji, 2008; Liu *et al.*, 2013; Shabir *et al.*, 2013).

2.3 Hydrocarbonoclastic bacteria: masters of oil degradation and bioenergy feedstock production

Hydrocarbonoclastic bacteria are a group of bacteria that uses hydrocarbons as energy and carbon source for growth (McKew *et al.*, 2007). The most recent report indicates that approximatelly 80 bacterial generahave been described to be able to degrade hydrocarbon compounds (Prince, 2005). They are widely distributed throughout the world, colonizing a broad range of environments, namely rivers, aquifers, oceans, soils, sediments and surviving in several extreme conditions such as high and low temperatures, high pressure, elevated salinity and extreme pH (Margesin & Schinner, 2001; Obuekwe *et al.*, 2001, Margesin *et al.*, 2003; McGenity *et al.*, 2010; Röling, 2010; Zhuang *et al.*, 2010; Castillo-Carvajal *et al.*, 2014; Fathepure, 2014; Schedler *et al.*, 2014). Although usually in low numbers, their abundance dramatically increases when exposed to hydrocarbon contamination (Kostka *et al.*, 2011; Chronopoulou *et al.*, 2015; Yang *et al.*, 2015). The ability to consume hydrocarbons is directly related to the enzymatic machinery to decompose this type of compounds and there are several reports describing the metabolic pathways responsible for the degradation of aliphatic and aromatic hydrocarbons in different bacteria (Rojo, 2009; Shao & Wang, 2013; Vila *et al.*, 2015) For these reasons, hydrocarbonoclastic bacteria are already being used in environmental biotechnology processes involving bioremediation of oily wastes and contaminated environments (Płaza *et al.*, 2008; Gargouri *et al.*, 2014; He *et al.*, 2014; Nunal *et al.*, 2014). Nevertheless, most of oil-based contaminations are composed by a mixture of complex hydrocarbons and no single bacterium is able to fully degrade all the hydrocarbons compounds present in these mixtures. In order to obtain an effective decontamination, a bacterial consortium composed by several members with affinities for aliphatic and aromatic hydrocarbons is fundamental. Bacterial strains will act in a synergistic mode to promote a more efficient waste decomposition, until total mineralization of substrate is obtained (Vázquez *et al.*, 2009; King *et al.*, 2015). In fact, several hydrocarbonoclastic bacteria genome sequencing projects were already performed (Nelson *et al.*, 2002; Golyshin *et al.*, 2003; Barbe *et al.*, 2004; Di Gennaro *et al.*, 2014) and several other are currently ongoing with the aim to better understand molecular mechanisms beyond hydrocarbon degradation to improve bioremediation processes.

In marine oil-spilled environments the most important genera are *Oleispira* (Yakimov*, et al.*, 2003), *Oleiphilus* (Golyshin *et al.*, 2002), *Thalassolituus* (Yakimov *et al.*, 2004), *Alcanivorax* (Yakimov *et al.*, 1998), *Cycloclasticus* (Dyksterhouse *et al.*, 1995) and *Oleibacter* (Teramoto *et al.*, 2011). These

genera are commonly designated as obligate hydrocarbonoclastic bacteria (OHCB), since they are able only to use hydrocarbons as carbon and energy sources (Yakimov *et al.*, 2007), differing in the type of consumed hydrocarbons. The genera *Cycloclasticus* only consume polycyclic aromatic hydrocarbons (PAH), whereas *Alcanivorax*, *Oleiphilus, Oleispira*, *Thalassolituus and Oleibacter* use exclusively straight and branched chain hydrocarbons (aliphatics).

The most dominant genera degrading aliphatic hydrocarbons, especially in temperate sea water, is *Alcanivorax*. At the present moment, 9 *Alcanivorax* strains were already isolated (Yakimov *et al.*, 1998; Fernández-Martínez *et al.*, 2003; Liu & Shao, 2005; Rivas *et al.*, 2007; Wu *et al.*, 2009; Lai *et al.*, 2011; Lai *et al.*, 2013; Rahul *et al.*, 2014; Kwon *et al.*, 2015) from different marine habitats, namely surface and deep seawater, tidal sediments and saline lakes. The most representative strain is *Alcanivorax borkumensis* SK2, since it is considered the main alkane degrader in these environments (Kasai *et al.*, 2001; Yakimov *et al.*, 2005; Cappello *et al.*, 2007; Sabirova *et al.*, 2008; Gertler *et al.*, 2012). Due to its predominance, *A. borkumensis* SK2 has been extensively studied regarding the ability to metabolize alkanes. Several works demonstrated the presence of an efficient network of genes and enzymes responsible for the degradation of aliphatic hydrocarbons, composed by several alkane hydoxylases and cytochrome P450-dependent alkane monooxygenases (Hara *et al.*, 2004; Van Beilen *et al.*, 2004; Sabirova *et al.*, 2006a; Schneiker *et al.*, 2006; van Beilen & Funhoff, 2007; Reva *et al.*, 2008; Naether *et al.*, 2013). Under this context many works demonstrated the capability of several strains of this genus to degrade hydrocarbons and therefore to be used in bioremediation of different oil-spilled marine environments (Cappello *et al.*, 2007; Genovese *et al.*, 2014; Hassanshahian *et al.*, 2014; Dashti *et al.*, 2015; Gao *et al.*, 2015). Following *Alcanivorax,* the genus *Cycloclasticus* is one of the most predominant genera in marine environments. These bacteria use as carbon source polycyclic aromatic hydrocarbons (PAHs), namely biphenyl, phenantrene, tolyene, xylene and naphtalene, (Kasai *et al.*, 2002) the principal components of crude oil. Generally, it is known that bacterial PAH degradation is initiated by the cleavage of PAH molecule by the action of a composed enzyme called hydroxylating dioxygenase, constituted by reductase, oxygenase and ferredoxin components (Gibson & Parales, 2000; Habe & Omori, 2003). However, knowledge on metabolic pathways responsible for PAH degradation in marine bacteria is still very scarce. Only some works demonstrated the presence of PAH degrading genes in *Cyclolasticus* strains (Geiselbrecht *et al.*, 1998; Kasai *et al.*, 2003; Lozada *et al.*, 2008; Staley, 2010). This genus is typically found in marine environments, especially in temperate seawaters (Kasai *et al.*, 2002; Maruyama *et al.*, 2003; Coulon *et al.*, 2007; Teira *et al.*, 2007). However, strains of *Cycloclasticus* were more recently found in high temperature environments

(Teramoto *et al.*, 2010; Teramoto *et al.*, 2013). There are experimental evidences that show the potential of *Cyclolasticus* to degrade PAHs and to actively participate in bioremediation of crude oil contaminated sites (Niepceron *et al.*, 2010; Teramoto *et al.*, 2010; McGenity *et al.*, 2012; Genovese *et al.*, 2014; Vila *et al.*, 2015).

In soil environments different genera of hydrocarbnoclastic bacteria are present, being able to degrade only a class of hydrocarbons (aliphatic or polycycyclic) or a mixture of both. These groups are globally spread through different types of soils and environmental conditions, particularly in oil contaminated environments (Margesin *et al.*, 2003; Zhang *et al.*, 2010; Etuk *et al.*, 2012; Mahjoubi *et al.*, 2013; Mansur *et al.*, 2014; Al-Mailem *et al.*, 2015; Yang *et al.*, 2015). Some of the most abundant and significant genera are *Bacillus*, *Sphingomonas*, *Alcanigenes*, *Pseudomonas*, *Acinetobacter* and *Rhodococcus* (Leys *et al.*, 2005; Das & Mukherjee, 2007; Song *et al.*, 2011; Rocha *et al.*, 2013; Ismail & Dadrasnia, 2015; Táncsics *et al.*, 2015).

The genus *Pseudomonas* has been reported as containing species able to degrade aliphatic and polycyclic aromatic hydrocarbons and currently, *P. aeruginosa* (Zhang *et al.*, 2011); *P. oleovorans* (van Beilen *et al.*, 1994); *P. stutzeri* (Shimada *et al.*, 2012); *P. cepacia* (Silva *et al.*, 2014) and *P. putida* (Doong & Lei, 2003) species are the ones described as possessing this metabolic feature. The genetic basis of alkane assimilation is very well described in *P. putida* and alkane hydroxylase (*alk*B) genes are currently being used as a functional marker to assess hycrodacrbonoclastic activity in contaminated environments (van Beilen *et al.*, 1994; Rojo, 2009; Paisse *et al.*, 2011; Shao & Wang, 2013). In fact, the biodegradation potential of these species is being studied in order to improve bacterial hydrocarbon bioremediation processes of oil-based wastes (Raghavan & Vivekanandan, 1999; Obayori *et al.*, 2009; Di Martino *et al.*, 2012; Isaac *et al.*, 2015). *Pseudomonas* species are widely recognized as efficient PAH degraders, since they are able to degrade the majority of aromatic compounds present in gasoline and crude oil (Obayori *et al.*, 2009; Striebich *et al.*, 2014). *P. putida* has the best characterized degradation pathway for naphthalene, flourene, and phenanthrene (Yang *et al.*, 1994; Resnick *et al.*, 1996; Takizawa *et al.*, 1999; Jiménez *et al.*, 2002), composed by a complex network of enzymes catalyzing a series of sequential reactions until complete substrate degradation.

There are several reports describing the presence of genus *Acinetobacter* in regions with extreme climacteric and geographical conditions, contaminated by crude oil (Cormack & Fraile, 1997; Kostka *et al.*, 2011; Mahjoubi *et al.*, 2013; Zhang *et al.*, 2014; Zou *et al.*, 2014). The genus *Acinetobacter* is composed by bacteria specialized in alkanes degradation, from short to long carbon chain (C10 to C44) (Asperger *et al.*, 1981; Sakai *et al.*, 1994; Throne-Holst *et al.*, 2006), being also able to degrade

crude oil components (Lal & Khanna, 1996; Palanisamy, 2014) and alkanes in spent engine oil (Koma *et al.*, 2001). Currently, different alkane degradation pathways are characterized for this genus, according to its structure which is dependent on the carbon chain length. For medium chain alkanes, the alkane hydroxylase involved is a cytochrome P-450 while for long chain alkanes rubredoxin- and rubredoxin reductase-dependent alkane hydroxylase is responsible for the initial attack to the molecule (Asperger *et al.*, 1981; Maeng *et al.*, 1996; Ratajczak *et al.*, 1998; Geißdörfer *et al.*, 1999; Tani *et al.*, 2002; Throne-Holst *et al.*, 2006; Throne-Holst *et al.*, 2007).

The genus *Rhodococcus* is one of the most impressive and versatile genera concerning hydrocarbon degradation. Members of this group are able to catabolize a wide range of hydrocarbons, from simple alkanes to complex polycyclic aromatic compounds, present in complex mixtures such as gasoline, diesel, engine and crude oil (Koma *et al.*, 2003; Koma *et al.*, 2004; Kunihiro *et al.*, 2005; de Carvalho *et al.*, 2007; Auffret *et al.*, 2009; Binazadeh *et al.*, 2009; de Carvalho *et al.*, 2009; Nhi-Cong *et al.*, 2009; Song *et al.*, 2011; Zheng *et al.*, 2012; Auffret *et al.*, 2014). They are found widespread through different types of environments, from soils to sediments and in sites characterized by extreme temperatures and salinities (Jones *et al.*, 1983; Sorkhoh *et al.*, 1990; Bej *et al.*, 2000; Margesin & Schinner, 2001; de Carvalho & da Fonseca, 2005; Alvarez *et al.*, 2007; Mahjoubi *et al.*, 2013). Due to its remarkable catabolic versatility, vast number of studies have been performed to apply *Rhodococcus* strains in oil bioremediation strategies (Liu *et al.*, 2009; Lin *et al.*, 2010; Gargouri *et al.*, 2011; de Carvalho, 2012; Ganesh Kumar *et al.*, 2014; Suja *et al.*, 2014; Chen *et al.*, 2015, Rodrigues *et al.*, 2015).

As in *Acinetobacter, Alcanivorax* and *Pseudomonas* genera, the alkane degradation metabolic pathways in *Rhodococci* are present but poorly characterized. The *alk*b gene cluster was previously described in *Rhodococcus* strains, namely in *Rhodococcus* sp. Q15 (Whyte *et al.*, 2002), *R. opacus B4* (Sameshima *et al.*, 2008), *R. opacus* R7 (Zampolli, 2014) and others (Andreoni *et al.*, 2000; van Beilen & Funhoff, 2007; Takei *et al.*, 2008; Amouric *et al.*, 2010; Likhoshvay *et al.*, 2014; Táncsics *et al.*, 2015). *Rhodococcus* species are also able to catabolize different types of polycyclic aromatic hydrocarbons with lower molecular weight (naphthalene and phenanthrene) and higher molecular weight (pyrene, phenanthrene, fluoranthrene anthracene, chrysene, and benzopyrene) (Walter *et al.*, 1991; Grund *et al.*, 1992). However, there is a lack of studies about total degradation pathways of these compounds in *Rhodococci*. The most, however partially, characterized catabolic pathways are for naphthalene (Kulakov *et al.*, 2005; Di Gennaro, *et al.*, 2010; Tomás-Gallardo *et al.*, 2014), benzene (Luz Paje &

Couperwhite, 1996; Na*, et al.*, 2005), isopropylbenzene, ethylbenzene, xylene and toluene (Kesseler *et al.*, 1996; Kim *et al.*, 2004; Maruyama *et al.*, 2005).

Coupled to hydrocarbon degradation, some hydrocarbonoclastic bacteria have the ability to produce lipid storage compounds, namely triacylglycerols (TAG), wax esters (WE) and polyhydroxyalkanoates (PHA) (Table 2.1).

Biosynthesis and accumulation of TAG seems to be a common feature of bacteria belonging to the actinomycetes group, such as *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia* or *Gordonia* (Alvarez & Steinbuchel, 2002). Members of these genera produce variable amounts of neutral lipids during cultivation on different carbon sources. These microorganisms may be defined as oleaginous bacteria since they accumulate more than 20 % of their biomass as lipids (Alvarez & Steinbuchel, 2002).

Gram-positive, aerobic bacteria belonging to the genus *Rhodococcus* are of considerable interest to biotechnologists due to their ability to catalyze the biotransformation and degradation of different hydrocarbons (Finnerty, 1992; Larkin *et al.*, 2005; Martinkova *et al.*, 2009; Kuyukina & Ivshina, 2010; Yam *et al.*, 2010). During the last decade it has become obvious that various species of this genus also have interesting capabilities regarding the biosynthesis of PHA (Füchtenbusch & Steinbüchel, 1999; Alvarez *et al.*, 2000; Hernandez *et al.*, 2008; Hernandéz & Alvarez, 2010) and TAG from different carbon sources, including hydrocarbons, during cultivation under nitrogen-starvation conditions (Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000; Kalscheuer *et al.*, 2001; Alvarez, 2003, Alvarez*; et al.*, 2008; Silva*, et al.*, 2010). Alvarez and co-workers reported on an oleaginous hydrocarbon-degrading *R. opacus* strain, PD630, which is able to accumulate up to 87 % of cellular dry weight of TAG when grown on different carbohydrate and non-carbohydrate carbon sources under nitrogen-limiting conditions, which is probably the highest TAG content ever detected in bacteria (Alvarez *et al.*, 1996; Alvarez*, et al.*, 2000). However, this strain is unable to synthesize PHA. Several reports considered the potential of these bacteria for *in situ* bioremediation of contaminated environments (Whyte *et al.*, 1998; Kuyukina & Ivshina, 2010) as well as good candidates for biotechnological processes involving TAG production (Kurosawa *et al.*, 2010).

Table 2.1 Synthesis of carbon storage compounds in some of the most significant hydrocarbonoclastic bacteria. (cdw - cellular dry weight, n.r. - not reported)

Bacterium	Storage compound	Carbon source	Content	Reference
Acinetobacter baylyi ADP1	TAG; WE	Gluconate	1.4 % TAG; 6.9 % WEs (cdw)[a]	(Kalscheuer & Steinbuchel, 2003)
	TAG	n-hexadecane	3 – 4 % (cdw) [a]	(Reiser & Somerville, 1997)
	TAG; WE	Glucose	4.0 mg L^{-1} medium (TAG)[b] 5.2 mg L^{-1} medium (WE)[b]	(Santala *et al.*, 2011b) (Santala *et al.*, 2011a)
Acinetobacter sp. strain H01-N	WE	n-hexadecane	17 % (cdw)[a]	(Ishige *et al.*, 2002) (Makula *et al.*, 1975)
	WE	n-hexadecanol	1.9 % (cdw [a]	(Singer *et al.*, 1985)
Acinetobacter sp. strain 211	TAG	Olive oil	25 % (cdw)[a]	(Alvarez *et al.*, 1997b)
Alcanivorax borkumensis SK2	TAG WE	Pyruvate n-hexadecane	23 % (cdw)[b] 9.2 % (cdw)[b]	(Kalscheuer *et al.*, 2007); (Manilla-Pérez *et al.*, 2011)
Alcanivorax jadensis T9	TAG TAG; WE	Pyruvate; n-hexadecane	7 % (cdw)[b] 8.6 % TAG; 13.4 % WE (cdw)[b]	(Bredemeier *et al.*, 2003); (Manilla-Pérez *et al.*, 2011)
Marinobacter hydrocarbonoclasticus SP17	WE	Pyruvate; n-hexadecane	30.6 % (cdw)[b] 2.4 % (cdw)[b]	(Klein *et al.*, 2008); (Manilla-Pérez *et al.*, 2011); (Holtzapple & Schmidt-Dannert, 2007)
Marinobacter sp. strain CAB	WE	Phytol; 6,10,14-tri-methylpentadecan-2-one	n.r.	(Rontani *et al.*, 1999)
Marinobacter squalenivorans	WE	Squalene	n.r.	(Rontani *et al.*, 2003)

[a] Total amounts of cellular fatty acids; [b] Total amounts of storage compound

Table 2.1 (Continued)

Bacterium	Storage compound	Carbon source	Content	Reference
Rhodococcus opacus PD630	TAG	Gluconate	76 % (cdw)[a]	(Alvarez*, et al.*, 1996a)
		Olive oil	87 % (cdw)[a]	
		Frutose	18 % (cdw)[a]	
		Acetate	31 % (cdw)[a]	
		Propionate	40 % (cdw)[a]	
		Pentadecane	39 % (cdw)[a]	
		n-hexadecane	38 % (cdw)[a]	
		Heptadecane	28 % (cdw)[a]	
		Octadecane	39 % (cdw)[a]	
		Citrate	37 % (cdw)[a]	(Alvarez *et al.*, 1997a)
		Succinate	22 % (cdw)[a]	
		Propionate	18 % (cdw)[a]	
		Valerate	38 % (cdw)[a]	
		Phenyldecane	38 % (cdw)[a]	
		Agro-industrial wastes	45.3 g L^{-1} medium[b]	(Gouda *et al.*, 2008)
		Glucose	53.9 % (cdw)[a]	(Kurosawa *et al.*, 2010)
		Lignocellulosic autohydrolysates	24.8 - 28.6 % (cdw)[a]	(Wei *et al.*, 2015a)
		Vanilic acid	14.6 % (cdw)[a]	(Kosa & Ragauskas, 2012)
		Dairy wastewater	2 g L^{-1} medium[a]	(Kumar *et al.*, 2015)
		Light oil	25.8 % (cdw)[a]	(Wei*et al.*, 2015b)
	TAG; WE	Phenyldecane	n.r.	(Alvarez *et al.*, 2002)

[a] Total amounts of cellular fatty acids; [b] Total amounts of storage compound

Table 2.1 (continued)

Bacterium	Storage compound	Carbon source	Content	Reference
Rhodococcus opacus DSM 1069	TAG	Light oil	22 % (cdw)[a]	(Wei *et al.*, 2015b)
		Glucose; vanilic acid; 4-hydroxybutyl acrylate (4-HBA)	17.9 % (glucose); 16.8 % (4-HBA); 6.7 % (vanilic acid) (cdw)[a]	(Kosa & Ragauskas, 2012)
Rhodococcus ruber	TAG	Glucose; valerate; n-hexadecane	19.0 %; 12.2 %; 26.0 % (cdw)[a]	(Alvarez *et al.*, 1997a)
	PHA		21.0 % (glucose); 85.4 % (valerate) (cdw)[a]	
Rhodococcus aetherivorans IAR1	TAG	Acetate; toluene	24 % (acetate; toluene) (cdw)[a]	(Hori *et al.*, 2009)
	PHA		12 % (acetate) (cdw)[a]	
			10 % (toluene) (cdw)[a]	
Rhodococcus sp. 602	TAG	Gluconate; Benzoate	71.2 % (gluconate); 64.9 % (benzoate) (cdw)[a]	(Silva *et al.*, 2010)
	PHA		9.0 % (gluconate); 8.2 % (benzoate) (cdw)[a]	
Rhodococcus jostii RHA1	TAG; PHA	Glucose; gluconate; acetate;	n.r.	(Hernandez *et al.*, 2008)
	WE	Hexadecane; hexadecanol	n.r.	
Rhodococcus sp. A5	TAG	Glucose; n-hexadecane	11.0 % (glucose); 32.1 % (n-hexadecane) (cdw)[a]	(Bequer Urbano *et al.*, 2013)
	PHA		2.9 % (glucose); 1.3 % n-hexadecane) (cdw)[a]	

[a] Total amounts of cellular fatty acids; [b] Total amounts of storage compound

Table 2.1 (continued)

Bacterium	Storage compound	Carbon source	Content	Reference
Rhodococcus corynebacteriodes DSM 20151	TAG	Glucose; n-hexadecane	9.2 % (glucose) 17.9 % (n-hexadecane) (cdw)[a]	(Bequer Urbano *et al.*, 2013)
	PHA		2.9 % (glucose); 1.9 % (n-hexadecane)[a]	
Pseudomonas aeruginosa 44T1	TAG	Olive oil	38 % (cdw)[a]	(de Andrés *et al.*, 1991)
Pseudomonas putida KT2440	PHA	Nonanoic acid	75 % (cdw)[a]	(Sun *et al.*, 2007)
Pseudomonas putida KT2442	PHA	Oleic acid	45 % (cdw)[a]	(Huijberts & Eggink, 1996)
		Glucose; fructose; glycerol	16.9 %; 24.5 %; 22.0 % (cdw)[a]	(Huijberts *et al.*, 1992)
Pseudomonas oleovorans	PHA	Alkanoic acids (C6–C10)	5 % - 28 % (cdw)[a]	(Brandl *et al.*, 1988)
		n-alkanes; n-alkenes (C6- C12)	2.0 % - 24.3 % (n-alkanes); 5.1 % - 13.4 % (n-alkenes) (cdw)[a]	(Lageveen *et al.*, 1988)
Pseudomonas sp. Strain DR2	PHA	Waste vegetable oil	23.5 % (cdw)[a]	(Chen *et al.*, 2014b)
Pseudomonas putida S12	PHA	Styrene	14 % (cdw)[a]	(Tobin & O'Connor, 2005)
Pseudomonas sp. TN301	PHA	Aromatic hydrocarbons	19.2 % (xylene and methylbenzene) (cdw)[a]	(Narancic *et al.*, 2012)
Pseudomonas chlororaphis	PHA	Animal-derived waste	29.4 % (cdw)[a]	(Narancic *et al.*, 2012)

[a] Total amounts of cellular fatty acids; [b] Total amounts of storage compound.

A separated group of gram-negative bacteria, belonging to the genus *Acinetobacter*, has also been shown to accumulate TAG but in minor amounts compared to WE, the major detected storage compounds in this genus (Ishige *et al.*, 2002; Kalscheuer *et al.*, 2003; Santala *et al.*, 2011a).

Accumulation of TAGs corresponding to more than 23 % of the cellular dry weight was found in the hydrocarbonoclastic bacteria *Alcanivorax borkumensis* when utilizing simple organic acids like acetate and pyruvate (Kalscheuer *et al.*, 2007) but this organism also accumulates TAG to a minor extent and majority WE when cultivated in hexadecane (Manilla-Pérez *et al.*, 2010a, Manilla-Pérez *et al.*, 2011). This was the first example of substantial TAG accumulation in a gram-negative bacterium. *A. borkumensis* is one of the most well-known marine hydrocarbonoclastic bacterium with a demonstrated pivotal role in bioremediation of crude oil contaminated environments (Kasai *et al.*, 2001; Harayama *et al.*, 2004; Röling, 2010).

Among hydrocarbonoclatic bacteria, the genus *Pseudomonas* is the main producer of PHA. Several *Pseudomonas* species can produce PHA using a wide range of carbon sources, from simple sugars and fatty acids as glucose and oleic acid to complex wastes such as cosmetic co-products, animal-derived waste, agro-industrial oily wastes, acidogenic effluents, olive oil mill wastewaters, milk whey and dairy wastewater (Fernández *et al.*, 2005; Kourmentza *et al.*, 2009; Simon-Colin *et al.*, 2009; Bosco & Chiampo, 2010; Simon-Colin *et al.*, 2012; Venkateswar *et al.*, 2012; Dalal *et al.*, 2013; Muhr *et al.*, 2013). *Pseudomonas putida*, *Pseudomonas oleovorans* and *Pseudomonas citronellolis* have been associated to PHA production from different types of hydrocarbons, revealing satisfactory product yields (Tobin & O'Connor, 2005; Tan *et al.*, 2015).

2.4 Bacterial lipid storage compounds

2.4.1 Polyhydroxyalkanoates

PHA are biopolyesters composed by hydroxyalkanoic acids synthesized by a broad range of bacteria. These compounds are produced under unbalanced growth conditions, namely in the presence of an excess of carbon followed by a limitation of one or more nutrients (typically phosphourous or nitrogen) and are further accumulated in the cytoplasm as storage granules to be used as carbon and energy sources (Anderson & Dawes, 1990; Lee, 1996a; Rehm, 2010). Depending on monomer composition, PHA can be classified in three main categories: short chain length PHA (scl-PHA), composed by 3 to 5 carbon monomers, namely 3-hydroxypropionate (3-HP), 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerate (3-HV); medium chain length PHA (mcl-PHA), containing 6 to 14 carbon monomers and long chain length PHA (lcl-PHA), composed by more than 14 carbon monomers (Figure 2.1).

Additionally, PHA composed by a mixture of scl-PHA and mcl-PHA can also be produced (Sudesh *et al.*, 2000; Khanna & Srivastava, 2005; Keshavarz & Roy, 2010).

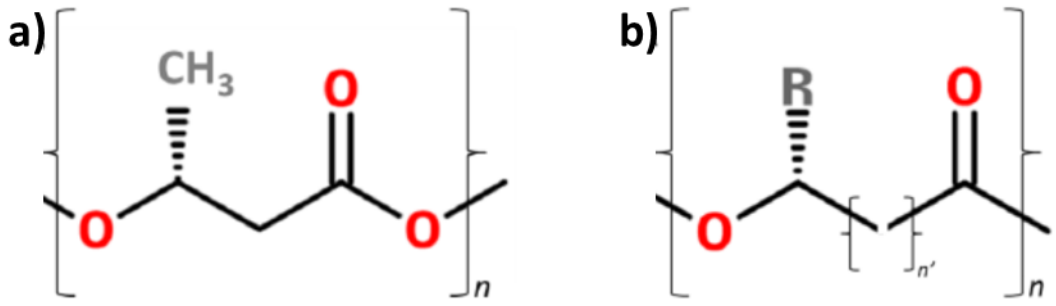


Figure 2.1 Molecular structure of scl-PHA (a) and mcl-PHA (b)

PHA composition is dependent on several factors such as the type of carbon source consumed, culturing conditions and the presence of suitable PHA synthesizing enzymes (Steinbüchel & Valentin, 1995; Rehm, 2003; Verlinden *et al.*, 2007). Currently, a high diversity of different PHA were already identified and it is predictable that an increase in the number of novel bacterial PHA compounds will occur, due to the development of engineered strains with new PHA producing capabilities as well as optimization of the native PHA by physical and chemical alterations (Steinbüchel 2001; Zinn & Hany, 2005; Escapa *et al.*, 2011). The most representative PHA synthesized among prokaryotes is the short chain length polyhydroxybutyrate (PHB). It was the first bacterial PHA compound to be identified and it has been extensively studied and characterized (Anderson & Dawes, 1990; Lee, 1996a; Suriyamongkol *et al.*, 2007).

Due to their particular characteristics, PHA are considered a potential alternative to petrochemical-based polymers, namely polypropylene (PP) and polyethylene (PE) (Lee 1996b; Khardenavis *et al.*, 2007: Pijuan *et al.*, 2009). The main advantageous properties are biodegradability, biocompatibility and non-toxicity (Steinbüchel, 2001; Shah *et al.*, 2008; Akaraonye *et al.*, 2010). Additionally, they present a wide range of structures, with physical and chemical features similar to conventional plastics (Lee, 1996a; Prieto *et al.*, 2007).

Currently, the application of bacterial PHA in industrial and biotechnological activities relies on the cultivation of pure strains on defined growth media composed by simple carbon sources (mainly sugars and organic acids) under sterilized conditions (Suzuki *et al.*, 1986; Zhao & Chen, 2007; Castilho *et al.*, 2009). However, these operational conditions lead to an increase of the process cost when compared to conventional plastics production, limiting its commercialization (Choi & Lee, 1997; Chanprateep,

2010). To overcome this limitation, in the last years intense research has been performed focusing on the replacement of pure cultures by mixed microbial cultures, namely activated sludge, for PHA production (Reis *et al.*, 2003; Serafim*, et al.*, 2004; Johnson *et al.*, 2009, Liu*; et al.*, 2011; Villano *et al.*, 2014). Furthermore, to decrease substrate cost, alternative inexpensive carbon sources, like industrial residues and wastewaters, have been explored as feedstocks for microbial PHA synthesis (Serafim *et al.*, 2008; Beccari *et al.*, 2009; Albuquerque *et al.*, 2010; Sudesh *et al.*, 2011; Queirós *et al.*, 2014).

2.4.2 Triacylglycerols

TAG are neutral reserve lipids composed by glycerol esterified with three fatty acids, with apolar and water insoluble properties (Figure 2.2). They are considered feasible storage compounds due to their low biological toxicity and lack of interference in cellular osmotic balance. When compared to proteins or carbohydrates, TAG present important advantages due to the higher energetic value and density, yielding much more energy upon oxidation (Murphy, 1993; Yen *et al.*, 2008). Like PHA, TAG are produced in the cells under unbalanced growth conditions, namely when an essential nutrient is absent from the culture medium, but still an excess of carbon is available to be consumed for storage lipid synthesis. These compounds are kept in the form of cellular inclusions in the cytoplasm, assuming different forms and sizes which are dependent on the organism and cultivation conditions (Wältermann & Steinbüchel, 2006). TAG are mainly produced by eukaryotes, namely mammals, plants, algae, fungi and yeast (Leman, 1997). However more recently it was found that these storage lipid compounds are also synthesized by prokaryotes, being restricted to some genera, namely *Rhodococcus*, *Acinetobacter*, *Nocardia*, *Mycobacterium*, *Alcanivorax* and *Streptomyces* (Barksdale & Kim, 1977; Olukoshi & Packter, 1994; Alvarez *et al.*, 1997a; Alvarez & Steinbuchel, 2002; Daniel *et al.*, 2004; Kalscheuer *et al.*, 2007; Santala *et al.*, 2011b).

In last decades, different physiological functions have been attributed to bacterial TAG, being the most significant its role as endogenous storage energy and carbon sources. The majority of TAG accumulating bacteria colonizes environments exposed to drastic environmental conditions, such as dryness, wide range of temperatures and limited nitrogen supply, like soils and oil contaminated waters (Juni, 1978; Finnerty, 1992; Alvarez *et al.*, 2004; Luz *et al.*, 2004; Head *et al.*, 2006). Therefore, TAG granules are used as a strategy to cope with starvation periods in these environments, maintaining cellular viability (Alvarez & Steinbuchel, 2002).

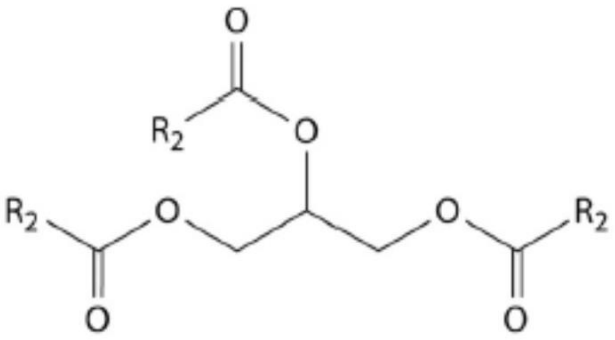


Figure 2.2 Molecular structure of triacylyglycerol. R2 represents the acyl groups attached to glycerol backbone.

The channeling of free fatty acids to TAG production under nitrogen limiting conditions can be a way to prevent fatty acid-based toxicity in the cells. This can occur particularly when bacteria use recalcitrant compounds (for instance complex hydrocarbons) as carbon and energy sources, forming uncommon fatty acids which can damage cellular membranes integrity (Alvarez *et al.*, 2001; Alvarez *et al.*, 2002; Alvarez & Steinbuchel, 2002; Wältermann & Steinbüchel, 2005; Alvarez, 2006; Silva *et al.*, 2010). TAG can also act as source of reducing equivalents, when an excess of acetyl-CoA is produced, particularly in oxygen limiting conditions. In these conditions, the high reductive power can interfere with the activity of enzymes of central metabolism. Consequently, fatty acid production for TAG synthesis will use reduced pyridine nucleotides. In this way, pyridine nucleotides are not accumulated, preventing cellular unbalance and disturbance of central carbon metabolism (Alvarez & Steinbuchel, 2002; Alvarez, 2006).

Bacterial TAG can be used as intermediate for the production of secondary metabolites, namely as fatty acid donor for antibiotic biosynthesis, using acetyl-CoA or malonyl-CoA as precursors (Olukoshi & Packter, 1994; Banchio & Gramajo, 2002).

Generally, the chemical composition, properties and amount of TAG are determined by the type of fatty acids, carbon chain length and the degree of fatty acid saturation and are highly influenced by the carbon source used and cultivation conditions. TAG levels increase substantially when bacterial cells reach stationary phase of growth and/or when bacteria are cultivated in nitrogen limiting conditions. When bacterial cells are actively growing, fatty acids production is channeled to phospholipid synthesis, an essential component of cellular membranes. In stationary phase or under imposed nitrogen limiting conditions, fatty acids flow is redirected leading to TAG biosynthesis (Packter & Olukoshi 1995; Wältermann *et al.*, 2005).

The composition of bacterial TAG mainly depends on the type of carbon source consumed. *R. opacus* PD630 is considered a model organism to study TAG biosynthesis since it can accumulate TAG from a wide range of substrates, reaching 76 to 87 % of the cellular dry weight in fatty acids of acylglycerols when cultivated on gluconate and olive oil, respectively (Alvarez *et al.*, 1996).

The biosynthesis of bacterial TAG from gluconate/glucose/organic acids and hexadecane involves different metabolic pathways. When cells are grown on gluconate and glucose (unrelated carbon sources), the substrate is converted in acetyl-CoA from pyruvate derived from glycolysis, which serves as precursor for fatty acid biosynthesis. On the other hand, during cultivation with hexadecane (related carbon source) the cells oxidize hydrocarbons to originate fatty acids for TAG biosynthesis (Alvarez *et al.*, 1997a; Alvarez, 2003; Manilla-Pérez *et al.*, 2011).

Generally, bacterial TAG have in its composition straight even-numbered fatty acids, ranging between 14 and 18 carbons. However, when *Rhodococcus* cells were grown on glucose and gluconate, a considerable percentage of odd-numbered fatty acids were found, namely heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1). Using organic acids like citrate, succinate, acetate, propionate and valerate, an increase in odd-numbered fatty acids was observed (Alvarez *et al.*, 1996; Alvarez *et al.*, 1997a). This can be related to an effective production of propionyl-CoA, used as precursor for odd-numbered fatty acid synthesis (Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000). On the other hand, when hydrocarbons are used as carbon source, only even-numbered fatty acids are produced, being directly related to hydrocarbon chain length and also to the ß-oxidation metabolic pathway (Alvarez *et al.*, 2002; Alvarez, 2003). The distribution of fatty acids in the glycerol molecule is also influenced by fatty acid chain length and saturation degree of chemical bonds. In *Rhodococcus* strains, short and saturated carbon chain length fatty acids were preferentially incorporated in the sn-2 carbon atom whereas longer unsaturated chain length fatty acids were located at sn-3 position of TAG molecule (Wältermann *et al.*, 2000). In *Mycobacterium* TAG, longer chain length fatty acids were allocated in sn-3 position, 16 carbon fatty acids were present in sn-2 position and C18:0 and C18:1 fatty acids were present at sn-1 position (Purdy *et al.*, 2013), revealing that distribution of fatty acids in the glycerol molecule is a strain-dependent feature.

2.4.3 Wax esters

WE are a class of neutral lipid compounds mainly composed by oxoesters of long chain fatty alcohols and long chain fatty acids (Jetter & Kunst, 2008) (Figure 2.3). They are known for their compactness and solubility in apolar solvents. The physical properties of WE are mainly determined by the carbon

chain length of fatty acyl and fatty alcohol moieties, by the degree of saturation, the level of ramification and the presence of unusual chemical groups (Rontani *et al.*, 1999; Patel *et al.*, 2001; Uthoff *et al.*, 2005). Currently, major source of WE derives from cuticule of jojoba plant (*Simmondsia chinensis* L.) and beeswax and sperm whale. However, there are reports on prokaryiotic WE, being mainly restricted to some bacterial genera. *Moraxella* (Bryn *et al.*, 1977), *Micrococcus* (Russell & Volkman, 1980), *Alcanivorax* (Kalscheuer *et al.*, 2007), *Marinobacter* (Rontani *et al.*, 1999), *Rhodococcus* (Wältermann & Steinbüchel, 2005) and *Acinetobacter* are the main bacterial WE producing genus (Makula *et al.*, 1975).

Bacterial WE are functionally similar to the other bacterial reserve compounds, polyhydroxyalkanoates (PHA) and triacylglycerols (TAG) (Alvarez & Steinbuchel, 2002). Like PHA and TAG, bacterial WE are mainly synthesized under stressful cultivation conditions, such as limited nitrogen concentrations and an excessive presence of carbon source and also under high hydrostatic conditions (Wältermann & Steinbüchel, 2005; Grossi *et al.*, 2010; Rontani, 2010). The majority of WE producing strains uses hydrocarbons as carbon and energy sources (Alvarez *et al.*, 2000; Ishige *et al.*, 2002; Manilla-Pérez *et al.*, 2011). The highest WE content, corresponding to 25 % of the cellular dry weight, was obtained with *Acinetobacter* sp. (Fixter *et al.*, 1986). Additionally, some hydrocarbonoclastic strains can produce specialized wax diesters in the presence of hexadecane (Kalscheuer *et al.*, 2003). In this context, WE producing bacteria can be considered potential candidates to produce WE from hydrocarbon-rich wastewaters. Recent works demonstrated that some members of hydrocarbonoclastic bacteria also produce WE from other types of substrates other than hydrocarbons, namely acetate and sugars (Alvarez, 2010; Kalscheuer, 2010). In order to optimize WE bacterial synthesis it is crucial to understand and identify metabolic pathways leading to polymer production. Currently, research works are being performed to engineer *A. baylyi* ADP1 for improved storage compounds production abilities (Santala *et al.*, 2011b; Santala *et al.*, 2014).

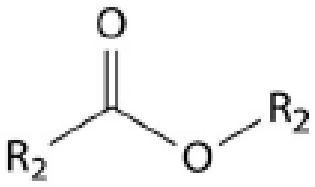


Figure 2.3 Molecular structure of wax esters. R2 represents the fatty acyl and fatty alcohol groups

2.5 Metabolic pathways involved in synthesis of lipid storage compounds

2.5.1 Polyhydroxyalkanoates

Bacterial PHA biosynthesis and accumulation involves the presence of distinct metabolic pathways: formation of PHA precursors deriving from the metabolism of carbon sources (non-PHA related), namely fatty acids; central carbon metabolic pathways such as fatty acid ß-oxidation and *de novo* fatty acid production, originating fatty acid or acetyl-CoA from non-PHA-related intermediates, which are converted into different (R)-3-hydroxyalkanoyl-CoAs, the substrates of PHA synthases; and specific PHA production metabolic pathway, composed by a set of Pha genes. Depending on the PHA chain length, two metabolic pathways for PHA biosynthesis were described. Regarding scl-PHA prodcution, particularly PHB which is the most known scl polymer, three main metabolic reactions and respective enzymes are required (Steinbuchel, 1993; Steinbüchel, 2001; Steinbüchel & Hein, 2001) (Figure 2.4). Two precursor acetyl-CoA molecules are chemically linked by acetyl-CoA C-acetyltransferase enzyme (PhaA), forming acetoacetyl-coA followed by its reduction to (R)-3-hydroxybutyryl-CoA by the action of NADPH-dependent acetoacetyl-CoA reductase (PhaB) (Peoples & Sinskey, 1989). The last reaction is catalyzed by PHA synthase (PhaC), promoting the condensation of (R)-3-hydroxybutyryl-CoA monomers to form PHB polymers (Peoples & Sinskey, 1989; Li *et al.*, 2009).The general metabolic pathway responsible for mcl-PHA uses acyl-CoA as precursors which are sequentially oxidized to enoyl-CoA, (S)-3-hydroxyacyl-CoA and (R)-3-ketoacyl-CoA. These metabolites are then converted in (R)-3-hydroxyacyl-CoA by 3 different enzymes: trans-enoyl-CoA hydratase (PhaJ), an epimerase, and a specific (R)-ketoacyl-CoA reductase (PhaG), respectively. Finally, (R)-3-hydroxyacyl-CoA molecules are condensed by the action of PHA synthase (PhaC) (Rehm & Steinbuchel; 1999, Hisano *et al.*, 2003; Tsuge *et al.*, 2003).

Two main different metabolic pathways are involved in bacterial mcl-PHA production, depending on the carbon source that is consumed.

When cells are cultivated on alkanes, these compounds are sequentially oxidized into the correspondent fatty acids by the alkane oxidation pathway followed by subsequent conversion to the corresponding acyl-CoA thioesters which in turn are oxidized through ß-oxidation pathway to form 3-ketoacyl-CoA. This molecule is broken by the action of ß-ketothiolase into acetyl-CoA and acyl-CoA with two less carbon atoms. The intermediates enoyl-CoA, (S)-3-hydroxyacyl-CoA and (R)-3-ketoacyl-CoA are

used for (R)-3-hydroxyacyl-CoA synthesis, which in turn is polymerized by PHA synthase. If unrelated carbon sources are used for growth (sugars, organic acids and alcohols), they are oxidized to acetyl-CoA which is further used in *de novo* fatty acid biosynthesis pathway. The acetyl-CoA is converted to malonyl-CoA and both are acylated to acyl-carrier protein (ACP). Malonyl- and acyl-ACP molecules are converted to the corresponding (R)-3-hydroxyacyl-ACP through a sequential set of metabolic reactions, which can be later increased by the addition of successive two-carbon units. Acyl-ACP intermediates can then be converted into (R)-3-hydroxyacyl-CoAs, mediated by by the specific transacylase (PhaG) and thus channeled into mcl-PHA synthesis (Sudesh *et al.*, 2000) (Figure 2.4). At the present moment, PHA synthase genes were already detected in different bacterial genera, namely *Burkholderia, Aeromonas, Bacillus*, *Alcanivorax*, *Rhodococcus*, *Ralstonia*, among others (Pieper & Steinbuchel, 1992; Antonio *et al.*, 2000; McCool & Cannon, 2001; Sabirova *et al.*, 2006a; Matsumoto *et al.*, 2009; Lau & Sudesh, 2012). However, *Pseudomonas* is the most dominant genus for PHA biosynthesis and different PHA biosynthetic enzymes were identified in *P. putida*, *P. aeruginosa*, *P. mendocina*, *P. oleovorans* and *P. nitroreducens* (Hoffmann *et al.*, 2000; Hoffmann *et al.*, 2002; Zheng *et al.*, 2005).

2.5.2 Triacylglycerols

Generally, the metabolic pathway that leads to bacterial TAG biosynthesis is known as Kennedy pathway, being extensively studied in plants and yeasts. The precursor glycerol-3-phosphate (G3P), which is a component of glycerophospholipid production pathways and a precursor of glyceraldehyde-3-phosphate from glycolysis, is sequentially acylated in sn-1,2 positions to form diacylglycerol (DAG). The phosphate group is removed before the last acylation reaction, forming triacylglycerol (TAG) (Lehner & Kuksis, 1996) (Figure 2.4). The three acylation steps are mediated by different types of acyltransferases. The substrate specificitiy of acyltransferases highly influences the position of acyl groups on the hydroxyl groups of the glycerol molecule and, consequently, the acyl composition of TAG (Alvarez & Steinbüchel, 2010). The first acylation reaction is catalyzed by glycerol-3-phosphate acyltransferase, using acyl-CoA and fatty acids bound to acyl carrier protein (ACP) derived from fatty acid *de novo* biosynthesis as precursors, resulting in the formation of 1-acylglycerol-3-phosphate (lysophosphatidate) (Wilkinson, 1997). The second acylation step is performed by lysophosphatidate acyltransferase, where lysophosphatidate is converted to phosphatidate/phosphatidic acid (Coleman, 1990). Phosphatidic acid is involved in two metabolic pathways: TAG and phospholipid synthesis. This compound can be transformed in CDP-diacylglycerol by the action of CDP-diacylglycerol synthase, which in turn is used as precursor for phosphatidylinositol, phosphatidylglycerol, phosphatidylcholine

and cardiolipin synthesis, essential components of membrane glycerophospholipids (López-Lara *et al.*, 2003; Martínez-Morales *et al.*, 2003; Zhang & Rock, 2008; Parsons & Rock, 2013). In Kennedy pathway, phosphatidic acid is converted in DAG by a desphoshorilation reaction mediated by phosphatidic acid phosphatase (PAP), being the first enzymatic reaction specific of TAG biosynthesis pathway (Icho & Raetz, 1983). PAP enzymes were already extensively studied in eukaryotes, yeasts, microalgae, and fungi and two main PAP enzymes families were identified: PAP type 1 (PAP1), which are dependent of magnesium and PAP type 2 (PAP2), magnesium independent (Carman & Han, 2006). However, there are few reports describing PAP2 enzymes in bacteria, namely in *E. coli* (Dillon *et al.*, 1996; Touzé *et al.*, 2008), *Streptomyces coelicolor* (Comba *et al.*, 2013) and more recently in *Rhodococcus* strains (Hernández *et al.*, 2015). Like phosphatidic acid, also DAG can be used as precursor for TAG, phosphatidylcholine and phosphatidylethanolamine biosynthesis. Additionally, DAG can also be formed in phospholipid metabolic pathways catalysed by phospholipase C enzyme (Pelech & Vance, 1989).

The final acylation reaction in bacterial TAG biosynthesis converting DAG in TAG is mediated by an acyltrasnferase enzyme known as wax ester/diacylglycerol acyltransferase (WS/DGAT), with unknown similarity to eukaryotes acyltransferase enzymes (Waltermann *et al.*, 2007). This enzyme was firstly identified in *A. baylyi* ADP1, revealing both DGAT and acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS) activities (Kalscheuer & Steinbuchel, 2003). A high number of genes coding for WS/DGAT enzymes were found in actinomycetes group, namely in *Mycobacterium*, *Nocardia* and *Streptomyces* genera (Daniel *et al.*, 2004; Sirakova *et al.*, 2006; Arabolaza *et al.*, 2008) and in several strains of *Rhodococcus* genus (Alvarez *et al.*, 2008; Hernandez *et al.*, 2008; Holder *et al.*, 2011; Hernandéz *et al.*, 2013; Villalba, 2013). Additionally, genes coding for this enzyme are also present in the genomes of some marine proteobacteria such as *Alcanivorax* and *Marinobacter* genera (Holtzapple & Schmidt-Dannert, 2007; Kalscheuer *et al.*, 2007). Although several studies indicate that WS/DGAT enzymes are the key components in neutral lipid biosynthesis in TAG accumulating bacteria, there are experimental evidences indicating possible additional TAG production metabolic routes. Mutant strains of *A. baylyi* ADP1 (Kalscheuer & Steinbuchel, 2003), *A. borkumensis* SK2 (Kalscheuer *et al.*, 2007), *S. coelicolor* (Arabolaza *et al.*, 2008) and *R. opacus* PD630 (Alvarez *et al.*, 2008; Hernandéz *et al.*, 2013) defective in WS/DGAT activity exhibited residual levels of TAG and low but significant DGAT activity, clearly indicate the possible presence of alternative, non-WS/DGAT-type acyltransferases, contributing to low amounts of synthesized TAG. In *S. coelicolor* phospholipid:diacylglycerol acyltransferase (PDAT) enzyme was also identified, using phospholipid as acyl donor (Arabolaza *et al.*, 2008), responsible for

TAG synthesis in yeasts and plants (Dahlqvist *et al.*, 2000), suggesting the presence of this metabolic pathway in other TAG accumulating bacteria.

2.5.3 Wax esters

The metabolic pathway involved in bacterial WE synthesis is mainly composed by three sequential reactions (Figure 2.4). Long chain-acyl-CoA is converted to a long-chain fatty aldehyde by an acyl-CoA reductase Acr1. The gene coding for this enzyme (Acr1) was firstly discovered in *A. baylyi* ADP1 mutants defective in WE production (Reiser & Somerville, 1997) and has no similarity with genes related to lipid synthesis. Furthermore, at the present moment, this gene was not identified in any WE accumulating bacteria. In the second reaction, the fatty aldehyde is reduced to the correspondent fatty alcohol by the action of a NADPH-dependent aldehyde reductase. In recent studies, it was suggested the presence of a NADPH-dependent alcohol dehydrogenase (AlrA) in *Acinetobacter* strains as the main responsible for the conversion of fatty aldehydes into the corresponding fatty alcohols (Tani *et al.*, 2000; Uthoff & Steinbüchel, 2012). Additionally, different alcohol and aldehyde reductase enzymes were found in *Marinobacter aquaeolei* VT8 (Wahlen *et al.*, 2009; Willis *et al.*, 2011; Lenneman *et al.*, 2013). The final and crucial reaction in bacterial WE biosynthesis is promoted by an acyl–CoA:alcohol transferase, catalyzing a condensation reaction between long chain acyl-coA and long chain fatty alcohol. This enzyme was firstly characterized in *A. baylyi* ADP1 as a novel bifunctional wax ester synthase/acyl–CoA:diacylglycerol acyltransferase (Kalscheuer & Steinbuchel, 2003). It was shown that wax ester/diacylglycerol acyltransferase (WS/DGAT) is located at the cytoplasmic membrane and cytoplasm level and also at the surface of neutral lipid granules (Stöveken *et al.*, 2005; Wältermann *et al.*, 2005; Kalscheuer *et al.*, 2006b). This enzyme is characterized by the utilization of a wide range of fatty alcohols (from C2 to C30) and fatty acids (C2 to C20), including unsaturated and branched ones, smaller alchohols such as ethanol and butanol and also in minor amounts, DAG as acyl acceptors, being responsible for low level TAG production in *A. baylyi* ADP1 (Stöveken *et al.*, 2005; Uthoff *et al.*, 2005). In fact, WS activity is much higher when compared to DGAT activity, which corroborates higher WE in comparison to TAG, after cultivation on unrelated carbon sources (Kalscheuer & Steinbuchel, 2003). C16-CoA and C14 to C18 fatty alcohols promoted the highest WS activity and 32 to 36 carbon chain length WE was the most dominant WE type (Rattray, 1984; Fixter *et al.*, 1986; Stöveken *et al.*, 2005). This broad range of substrate affinity has already let to intensive research in the production of fatty esters for use in industrial and biotechnological processes, namely in biofuel and oleochemical industries (Stöveken & Steinbüchel, 2008; Röttig*, et al.*, 2010). In addition to *A. baylyi* ADP1, enzymes

similar to WS/DGAT enzyme were detected in other WE accumulating genera, namely marine hydrocarbonoclastic bacteria such as *Alcanivorax* and *Marinobacter* strains, *Rhodococcus* and *Streptomyces* genera, among others (Röttig & Steinbüchel, 2013b). In *M. hydrocarbonoclasticus* were identified different genes (ws1 to ws4 genes) coding for enzymes involved in WE biosynthesis, with 27 to 45 % similarity to the one present in *A.baylyi* ADP1 (Kalscheuer & Steinbuchel, 2003; Holtzapple & Schmidt-Dannert, 2007). Regarding substrate affinities, WS1 and WS2 enzymes use preferentially long-chain acyl-CoAs to acylate a broad range of fatty alcohols (C10 to C16) or isoprenoid alcohols. Interestingly, WS2 has a wider substrate range, with higher affinity for longer-chain alcohols as well as a higher activity for isoprenoid WE formation, when compared to WS1. WS2 enzyme from *A. baylyi* ADP1 was used in a comparative study of five acyltransferases from different organisms expressed in *S. cerevisiae* in terms of possible applications for biodiesel production, where it revealed the highest activity for all used alcohols (Shi *et al.*, 2012). Recently, comparisons between WS/DGAT enzymes present in *Rhodococcus jostii* RHA1, *Marinobacter aquaeolei* VT8 and *Psychrobacter cryohalolentis* revealed that WS from *M. aquaeolei* was the one with the highest activity, using palmitoyl-CoA and dodecanol or hexadecanol, being dodecanol the preferred alcohol (Barney *et al.*, 2012). In *A. borkumensis* SK2 two WS/DGAT enzymes (AtfA1 and AtfA2) were detected with high levels of similarity to the one identified in *A. baylyi* ADP1 (Kalscheuer *et al.*, 2007). Only AtfA2 have WS activity, using a wide range of substrates particularly medium-chain-length, linear alcohols and even cyclic or phenolic alcohols. Additional studies reveal that WS activity is much higher than DGAT activity in natural cells. However *A. borkumensis* accumulates essentially TAG when cultivated in unrelated carbon sources, such as pyruvate. When cells are grown on hexadecane, both TAG and lower amounts of WE are accumulated (Kalscheuer *et al.*, 2007; Manilla-Pérez *et al.*, 2011). This is possibly related to the presence of an inactive acyl-CoA reductase, being unable to produce fatty aldehydes and alcohols, the main precursors of WE synthesis. A double mutant defective in AtfA1 and AtfA2 activities exhibits a total absence of WS activity, indicating that both enzymes are crucial for WE biosynthesis (Kalscheuer *et al.*, 2007). Other marine oil degrading bacteria, namely *Alcanivorax jadensis* and *Thalassolituus oleivorans* are able to synthesize WE or wax diesters when cultivated in n-alkanes (Bredemeier *et al.*, 2003; Manilla-Pérez *et al.*, 2010b; Manilla-Pérez *et al.*, 2011). However, the respective wax ester synthase(s) was not yet identified in those organisms. *R. opacus PD630*, a model organism used to study TAG metabolism has also the ability to produce WE under very specific conditions. Although no significant progresses have been made in the identification of WS/DGAT enzymes responsible for WE synthesis in

this bacterium, when cultivated in phenyldecanoate, phenyldecyl-phenyldecanoate WE is produced (Alvarez *et al.*, 2002).

Different metabolic routes seem to be involved in bacterial WE biosynthesis, depending on the carbon source used for growth. When cultivated on unrelated carbon sources, the production of fatty acyl-CoA from glycolysis and *de novo* fatty acid biosynthesis is the precursor for the production of fatty aldehydes by the action of fatty acyl CoA reductase enzyme (Reiser & Somerville, 1997). The formed fatty aldehydes are then reduced to the correspondent fatty alcohols by a fatty aldehyde reductase (Wahlen *et al.*, 2009). Afterwards, the fatty alcohol and fatty acyl-CoA moieties are condensed with the release of CoA to form WE through the action of WS/DGAT enzyme (Kalscheuer & Steinbüchel, 2003). In contrast, when bacterial cells are grown in n-alkanes, these compounds are sequentially oxidized, forming aldehydes and alcohols as intermediates, leading to the production of fatty acids. Finally, fatty acid and fatty alcohols formed are condensed by WS/DGAT enzyme, forming WE (Tani *et al.*, 2000; Tani *et al.*, 2002; Ishige *et al.*, 2003)

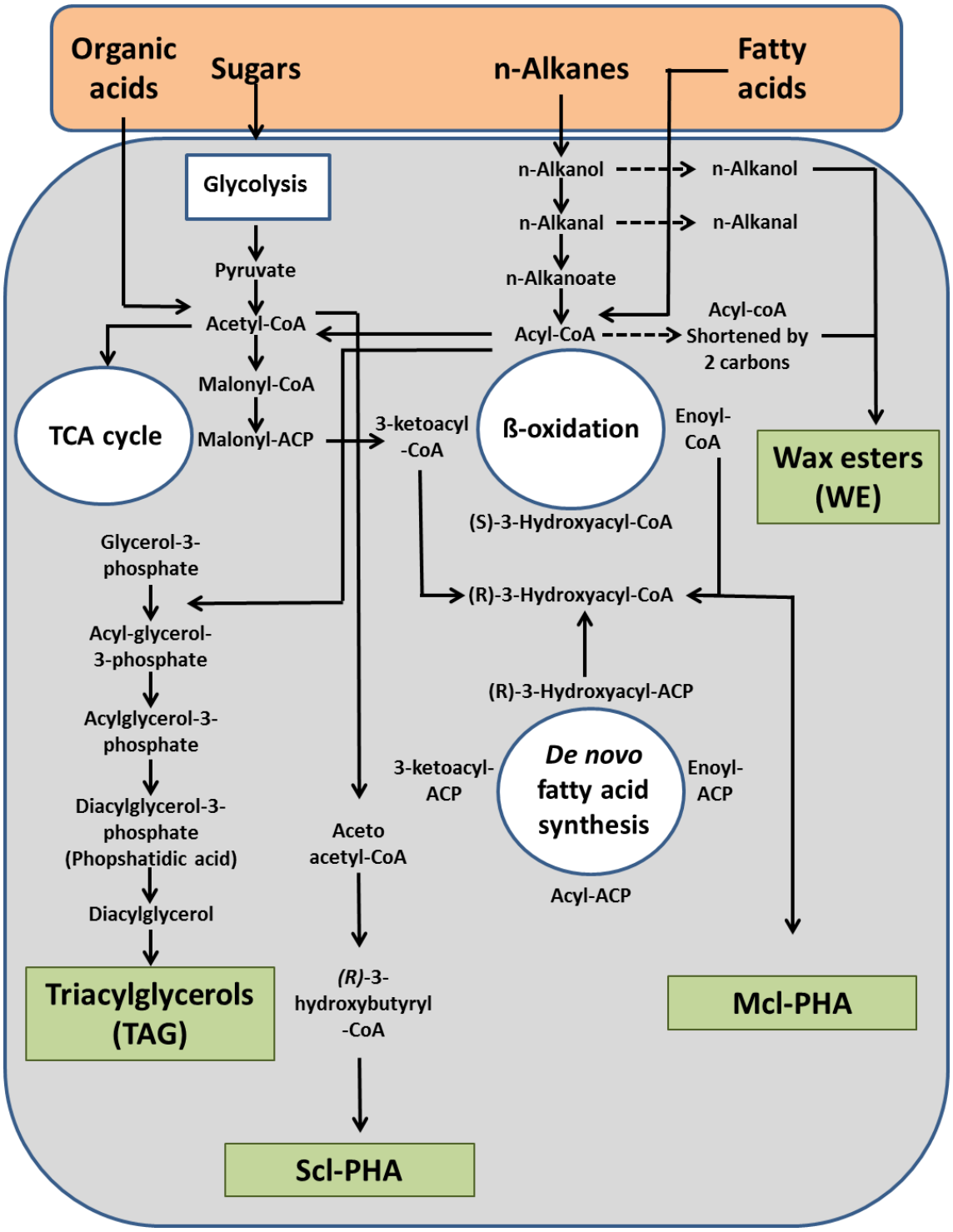


Figure 2.4 Proposed metabolic pathways involved in bacterial synthesis of TAG, WE and PHA from unrelated (sugars and organic acids) and related (fatty acids and n-alkanes) carbon sources.

2.6 Biotechnological and industrial applications of lipid storage compounds

Considering the wide range of properties, bacterial PHA compounds can be used in different types of applications, namely in medical, pharmaceutical, industrial and agricultural areas (Philip *et al.*, 2007; Chen, 2009). The main application is the use of PHA as substitutes of conventional petrochemical plastics. Currently, PHA are being used in packaging and coating materials and films as well as in hygienic products and cosmetic vessels. They are also used in toners, adhesives and in components of electronic material (Madison & Huisman, 1999; Bucci *et al.*, 2005; Verlinden *et al.*, 2007). In the agricultural field, PHA are constituents of herbicides, insecticides, seeds capsules and films for crop maintenance (Reddy *et al.*, 2003; Verlinden *et al.*, 2007). Due to its biocompatibility, PHA are applied on medical devices and implant materials, namely on bones and ligaments regeneration and cardio-vascular valves and cellular tissue treatment (Chen & Wu, 2005; Valappil *et al.*, 2006; Chen, 2009). In pharmaceutical industry, PHA are considered active compounds and their role as drug delivery carrier in antibiotics, and drugs for cancer and HIV treatment are being investigated (Chen, 2009; Rai *et al.*, 2011). PHA are also being developed for the production of stereo regular compounds as the chiral base for synthesis of fine chemical compounds (Gao *et al.*, 2002; Qu *et al.*, 2005) as well as additive in the production of biofuels (Philippe *et al.*, 2006; Philip *et al.*, 2007; Chen, 2009).

Bacterial TAG has been recently recognized as a suitable feedstock for different types of applications. One possible application of bacterial TAG is in the production of edible oil and fats. Currently, major TAG used in nutritional area are from animal and vegetable sources. It was shown that *R. opacus* PD630 cultivated on octadecane produced TAG with a fatty acid profile much similar to those from vegetable sources but not with gluconate (Alvarez*, et al.*, 1996; Alvarez & Steinbuchel, 2002). However engineered strains of *R. opacus* PD630 grown on gluconate were able to produce TAG similar to those present in cocoa butter oil (Wältermann *et al.*, 2000). Bacterial TAG can also be applied in therapeutic and pharmaceutical industries as carriers for drug target, namely in the preparation of vehicles for different types of medicaments such as demulcents, emollients and laxatives (Alvarez & Steinbuchel, 2002).

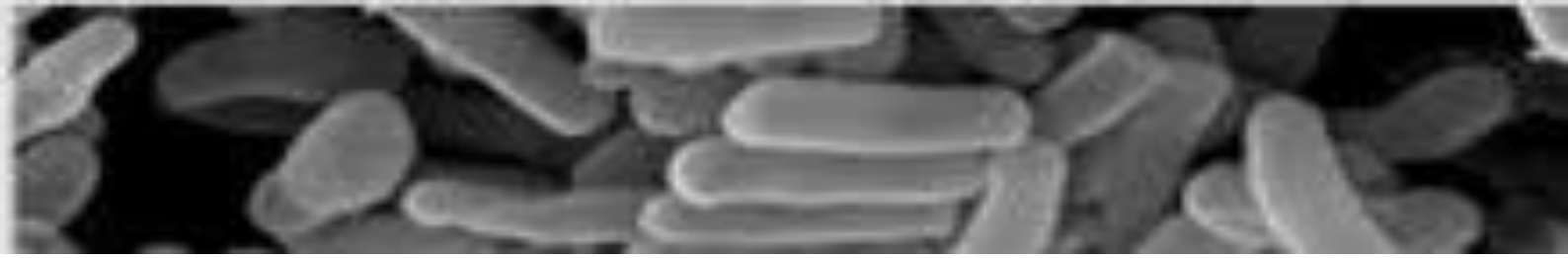
Further studies on bacterial TAG are needed to assess economic feasibility and health security for nutritional purposes. Additionally, bacterial TAG can also be used in oleochemicals and other manufactured products as possible substitutes for petrochemicals and agricultural lipids. They can be used in the production of valuable products such as cleaning and cosmetic products, detergents,

paints, resins, lubricants among others (Wältermann *et al.*, 2000; Alvarez & Steinbuchel, 2002; Alvarez, 2010). Not only bacterial TAG but also TAG synthesizing enzymes can be determinant for industrial purposes. Several hydrocarbonoclastic bacteria have wax ester synthase/diacylglycerol acyltrasnferase (WS/DGAT) (Kalscheuer & Steinbuchel, 2003; Kalscheuer *et al.*, 2007; Alvarez *et al.*, 2008). This is a novel bifunctional enzyme family not found until now in other organisms mediating the last acylation reaction in TAG and WE synthesis. One interesting feature of this enzyme is the low substrate specificity, allowing the use of acyl groups with different compositions, forming TAG with a wide range of fatty acids. Therefore, this ability makes this enzyme a promising alternative for the production of different types of fine chemicals and/or oleochemicals (Stöveken *et al.*, 2009).

The most prominent bacterial TAG application is for biofuels production. Currently, TAG are being used as feedstock for lipid-based biofuels, where the principal TAG sources are plant oils, animal fats or waste cooking oils (Brigham, 2011). However, in last years, bacteria have already being pointed as potential candidates for lipid-based biofuels production. In fact, they present some important advantages over other TAG feedstocks, namely high growth rates, low physical space requirements, easiness of cultivation and are renewable biomass (Li *et al.*, 2008; Meng *et al.*, 2009; Rude & Schirmer, 2009; Kosa & Ragauskas, 2011). Several efforts with the aim to optimize bacterial TAG-based biofuels productions were already achieved by using inexpensive wastes and by-products as carbon sources, by the development of fermentation processes at high scale and by the construction of engineered strains with improved TAG ability biosynthesis. Different agro-industrial wastes were consumed by *Rhodococcus opacus* PD630 and *Gordonia* sp. DG, exhibiting high TAG volumetric productivities (Gouda*, et al.*, 2008). Different *Rhodococcal* strains successfully produced TAG as biodiesel feedstock from light oil from pyrolysis as a sole carbon source (Wei *et al.*, 2015a). A dairy wastewater from food industry was used as carbon source by *R. opacus* PD630 in a batch bioreactor to produce TAG composed by fatty acids suitable for biodiesel production (Kumar *et al.*, 2015). TAG production in *R. opacus* PD630 was already achieved in high cell density fermentation in a 30 and 500 L bioreactors using sugar beet molasses and glucose as substrates (Voss & Steinbuchel, 2001). One promising alternative to biofuels production is the fatty acid methyl or ethyl esters production. Wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) gene from *A. baylyi* ADP1, the first discovered WS/DGAT enzyme and pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis*, was successfully cloned in *Esherichia coli*, producing considerable amounts of fatty acid ethyl esters (FAEE), using glucose and oleic acid as carbon sources. These compounds were considered the first microbial biodiesel, commonly known as microdiesel (Kalscheuer *et al.*, 2006a).

More recently, there has been an increasing interest in lignocellulosic biomass, as an abundant and low-cost renewable substrate for TAG-based biofuels production. However, it is a very complex substrate composed by a mixture of lignin, cellulose and hemicellulose that must be previously treated to obtain the corresponding sugars, accessible to bacterial consumption (Galbe & Zacchi, 2007; Kumar *et al.*, 2009). There are already several reports describing TAG production from natural and engineered *Rhodococcus* strains using glycerol, arabinose, xylose, hydroxybenzoic and vanillic acid for renewable biofuels (Kurosawa *et al.*, 2010; Kosa & Ragauskas, 2012; Kosa & Ragauskas, 2013; Kurosawa, 2014; Kurosawa*, et al.*, 2015b; Kurosawa*, et al.*, 2015d; Wei *et al.*, 2015a). Consequently, recent papers described bacterial TAG accumulation in the presence of inhibitory compounds of lignocellulosic hydrolysates (Wang *et al.*, 2014, Kurosawa *et al.*, 2015a).

Similar to PHA and TAG, bacterial WE can also be applied to different biotechnological and industrial fields. The most significant applications are in the manufacturing of cosmetics and additives for food industry. In oleochemical industry, they are used in the production of candles, lubricants, polishes, surfactants, coatings and ultimately in biofuels industries (Ishige *et al.*, 2003; Alvarez, 2010; Westfall & Gardner, 2011).



Chapter 3

Rhodococcus opacus B4: a promising bacterium for biofuels and bio-based chemicals production

Production of storage lipids is a common phenomenon in members of *Actinomycetes* group. Among them, the genus *Rhodococcus* is one of the most relevant due to the capability to accumulate neutral lipids when cultivated on different substrates, namely sugars, organic acids and hydrocarbons. In this work, production of storage lipids by *Rhodococcus opacus* B4 using glucose, acetate and hexadecane, under nitrogen restricting conditions, is reported for the first time and compared with *Rhodococcus opacus* PD630, a well-known oleaginous bacterium. Both strains accumulated mainly triacylgycerols (TAG) from all carbon sources, being influenced by the carbon source itself and also by the duration of the accumulation period. *R. opacus* B4 produced 0.09 g L^{-1} and 0.14 g L^{-1} at 24 h and 72 h, with hexadecane as carbon source, which was 2 and 3.3 fold higher than the volumetric production obtained by *R. opacus* PD630. Both strains presented similar fatty acids profiles in intact cells while in TAG produced fraction, *R. opacus* B4 revealed a higher variability in fatty acid composition than *R. opacus* PD630, when both strains were cultivated on hexadecane. The obtained results open new perspectives for the use of *R. opacus* B4 to produce TAG, in particular using oily (alkane-contaminated) waste and wastewater as cheap raw-materials. Combining TAG production with hydrocarbons degradation is a powerful strategy to achieve environmental remediation while producing added value compounds.

3.1 Introduction

In the last decades much attention has been paid to microbes as alternative lipid sources for biotechnological and industrial applications. This is due mainly to several advantages in terms of cultivation requirements, namely short life cycles; less physical space to grow; no dependence of climacteric conditions and easiness to scale up production processes (Li 2010; Subramaniam *et al.*, 2010; Ageitos *et al.*, 2011)

Bacterial lipids such as triacylgycerols (TAG - triesters of glycerol and long-chain fatty acids) and wax esters (WE - esters of primary long-chain fatty acids and primary long chain fatty alcohols), have relevant applications in the production of food additives, cosmetics, lubricants, oleochemicals, candles and biofuels (Holder *et al.*, 2011; Röttig *et al.*, 2010) The majority of the bacterial species synthesizes PHA as storage compounds (Alvarez *et al.*, 1997a; Steinbüchel, 2001) whereas the ability to accumulate TAG and WE has been reported for only some genera (Alvarez *et al.*, 2002). The amount, composition and structure of bacterial lipids are highly dependent on several factors, including the species itself, the carbon source used, the time of cultivation and also the amount of carbon and nitrogen present in the culture medium (Packter & Olukoshi, 1995; Alvarez *et al.*, 1997a; Alvarez *et al.*, 1997b; Wältermann *et al.*, 2005). Among several TAG accumulating genera, the genus *Rhodococcus* is one of the most promising, because some strains can accumulate more than 20 % of their biomass as TAG, being considered oleaginous bacteria. Members of this genus can be found in several types of natural environments, from arid and tropical soils in cold ecosystems and also in marine sediments (Whyte *et al.*, 1999; Heald *et al.*, 2001; Luz *et al.*, 2004; Peng *et al.*, 2008) having therefore also a potential for use in environmental biotechnology processes.

Additionally, *Rhodococcus* can produce and accumulate TAG from several types of substrates under nitrogen-limiting conditions, including defined carbons sources like sugars, organic acids or hydrocarbons (Alvarez *et al.*, 199; Alvarez, Kalscheuer*, et al.*, 1997a; Alvarez *et al.*, 2000; Silva *et al.*, 2010) but also complex carbon sources present in industrial wastes (Voss & Steinbuchel, 2001; Gouda *et al.*, 2008), revealing a remarkable versatility in terms of substrate degradation. Within the last decade, reports of new TAG accumulating species of *Rhodococcoccus* have considerably increased, for example *R. ruber*, *R. fascians*, *R. erythropolis*, *R. jostii*, *R. aetherivorans*

IAR1, *R. sp.* 602 and *R. sp.* A5 (Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000; Kalscheuer *et al.*, 2001; Alvarez, 2003; Hernandez *et al.*, 2008; Hori *et al.*, 2009; Silva *et al.*, 2010; Bequer Urbano *et al.*, 2013). *Rhodococcus opacus* PD630 is the best studied bacterium concerning TAG production and accumulation. This bacterium has the ability to accumulate significant amounts of lipids, namely 76 % and 87 % (w/w) of the cellular dry weight, when grown on gluconate and olive oil, respectively (Alvarez *et al.*, 1996; Voss & Steinbuchel, 2001). Additionally, it can also accumulate TAG under cultivation on other carbon sources, such as alkanes, acetate, glucose, propionate, among others (Alvarez *et al.*, 1996; Wältermann *et al.*, 2000; Alvarez & Steinbuchel, 2010). For these reasons, *R. opacus* PD630 is considered a model bacterium. However, in order to develop sustainable lipids producing processes using this species and to increase the knowledge of TAG metabolism and physiology in *Rhodococcus* it is mandatory to go deeper on lipid accumulation studies and also to identify new *Rhodococcus* strains possessing this feature. *R. opacus* B4 was isolated from a gasoline-contaminated soil in Japan. This bacterium is highly tolerant to several organic solvents, can use benzene as a sole source of carbon and energy and metabolizes aromatic and aliphatic hydrocarbons (Na *et al.*, 2005; Yamashita *et al.*, 2007; Sameshima *et al.*, 2008) Moreover, it has the capacity to stabilize water-oil emulsions, which can be important in bioremediation processes (Hamada *et al.*, 2008; Honda *et al.*, 2008). However, the ability to produce and accumulate lipid storage compounds was never reported. In the present work, the ability of *R. opacus* B4 to produce and accumulate lipid storage compounds was investigated, using different carbon sources and accumulation period lengths. *R. opacus* PD630 was used under the same cultivation conditions as a comparative well characterized bacterium.

3.2 Materials and methods

3.2.1 Bacterial strains, media and cultivation conditions

3.2.1.1 Strains and media

The bacterial strains used in this work were *Rhodococcus opacus* PD630 (DSM 44193) and *Rhodococcus opacus* B4 (NBRC 108011) and were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and Biological Resource Centre, NITE (NBRC),

respectively. *R. opacus* PD630 was isolated from a soil sample collected at a gas-works plant in Germany (Alvarez *et al.*, 1996) and *R. opacus* B4 was isolated from an oil sample taken from chemical plants and roadsides in Hiroshima, Japan (Na *et al.*, 2005). The culture media used for maintenance and growth of bacterial strains were 802 medium containing (g L^{-1}) 10.0 polypeptone; 2.0 yeast extract and 1.0 $MgSO_4 \cdot 7H_2O$ and mineral salts (MS) medium according to Schlegel *et al.* (1961). Glucose (40 g L^{-1}), sodium acetate (10 g L^{-1}) or hexadecane (1 g L^{-1}) were used as carbon and energy sources. For solid media, 1.5 % agar was added to the MS and 802 media. The cells were grown at 30 °C and 150 rpm under the conditions described below.

3.2.1.2 Preparation of seed cultures

Cells from a single colony of *R. opacus* B4 and *R. opacus* PD630, grown on 802 medium agar plates at 30 °C for 4 days, were separately inoculated in 50 mL of 802 medium in 250 mL flasks. The seed cultures were incubated on a rotary shaker (150 rpm) at 30°C until the middle of the exponential growth phase was reached (48 hours for *R. opacus* B4 and 24 hours for *R. opacus* PD630). Growth of the seed cultures was determined by measuring optical density at 600 nm wavelength with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan).

3.2.1.3 Growth and lipid accumulation experiments

The experiments were divided in two cultivation stages. In stage I, culture conditions promoted high cell densities (growth conditions) while in stage II neutral lipid accumulation was promoted (storage conditions). MS medium supplemented with glucose (4 %, w/v), sodium acetate (0.6 %, w/v) or hexadecane (0.3 %, w/v) as sole carbon sources was used for cultivation (see Figure 3.1) for a schematic representation of the experimental procedure).

The two-stage experiments were carried out under sterile conditions in duplicate using 250 mL conical flasks containing 50 mL of defined medium and incubated on a rotary shaker (150 rpm) at 30 ◦C.

In stage I, cells of seed cultures were harvested**,** washed with sterile sodium chloride solution (0.9 %, w/v), and suspended in fresh MS medium. Cells were used to inoculate flasks to an optical density (OD) at 600 nm of 0.1. Cultures of both strains were cultivated in MS medium at a molar carbon to nitrogen ratio of 68 for glucose (C/N=68), 8 for acetate (C/N=8) and 4 for hexadecane (C/N=4). Cells were grown until the middle of the exponential growth phase, namely 32, 50 and 150 hours for glucose, acetate and hexadecane, respectively.

For stage II, cells from stage I were collected by centrifugation (4°C, 10 min., 10000 *g*), washed twice with sterile sodium chloride solution (0.9 % w/w) and transferred to fresh MS medium with lower nitrogen concentration, with a C/N ratio of 300 for all carbon sources, in order to promote neutral lipid accumulation. After 24 and 72 hours of cultivation, cells were harvested, washed and kept at -80 °C until further lyophilization.

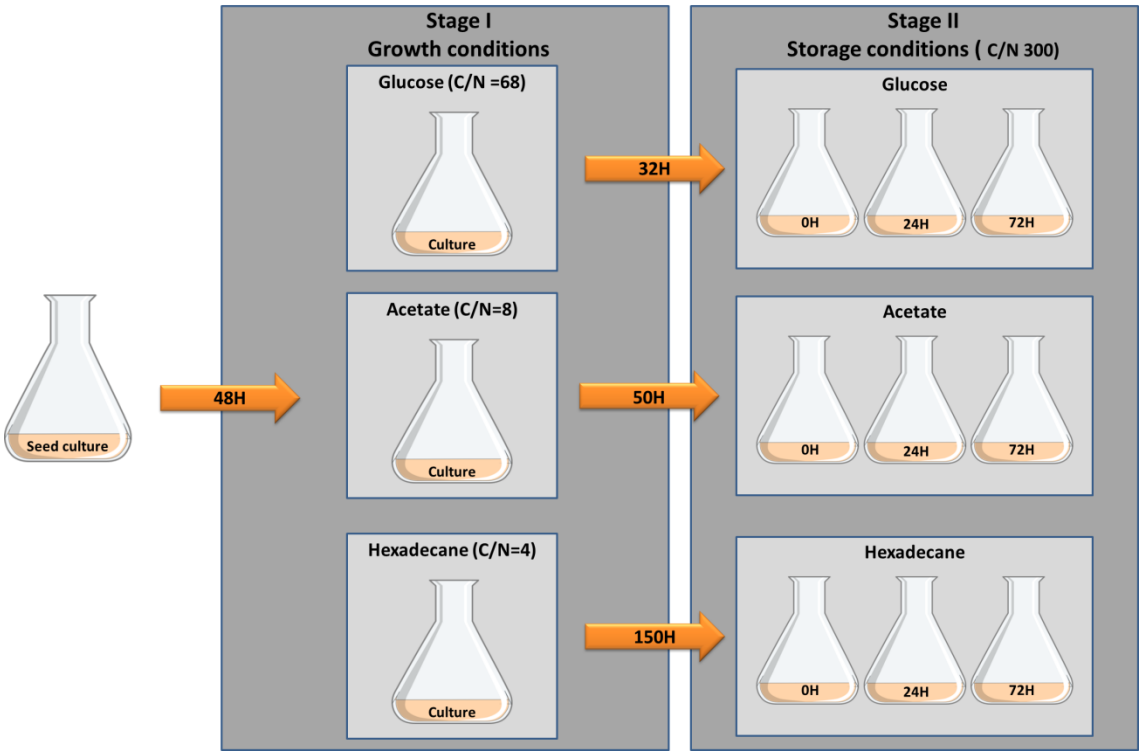


Figure 3.1 Schematic representation of the experimental procedure adopted for accumulation of lipid storage compounds in *R. opacus* B4 and *R. opacus* PD630.

3.2.2 Substrate consumption

3.2.2.1 Glucose and acetate

Samples from the culture medium of both strains were collected in stage II at 0 hours, 24 hours and 72 hours to analyze substrate concentration. Glucose and acetate were measured by high-performance liquid chromatography (HPLC) (Jasco, Japan) using a Chrompack column (6.5 x 30 mm^2). The mobile phase used was sulfuric acid (0.01 N) at a flow rate of 0.6 mL min^{-1}. The column temperature was set at 60 °C. Detection of compounds was performed by using an UV detector at 210 nm for acetate and a Refraction Index (RI) detector for glucose.

3.2.2.2 Hexadecane

For hexadecane analysis, total content of the cultivation flask (50 mL of culture medium) was acidified with hydrochloric acidl8 M to pH 2 and stored at 4 °C (for no longer than 14 days) until extraction. Hexadecane present in the culture medium was extracted using a liquid-liquid extraction procedure. Before extraction, tetradecane (C14) was added as a surrogate to evaluate the efficiency of the extraction process. The extraction was serially performed three times with 15 mL of hexane. Between the serial extractions, the funnels were vigorously shaken for 2 minutes. After 10 to 30 minutes rest (depending on the sample), the organic layer was collected.

The extract was passed through a Sep-Pak Florisil 6cc column (Waters, USA) using a Supelco® vacuum Manifold system. The column was previously preconditioned with 5 mL n-hexane. In the end, an additional 5 mL n-hexane was added to the column to ensure that all hexadecane was eluted. Then, sodium thiosulfate was added to remove residual aqueous residues. The extracts were kept in glass flasks in fume hood until dryness, reconstituted in 1 mL n-hexane and stored at -20°C until analysis. Hexadecane concentration was determined using a gas chromatograph with a flame ionization detector (GC-FID) (GC Varian® star 3400CX, USA). Undecane (C11) was used as internal standard and was added to the samples before GC analysis. The column used was the model VF-1ms (Agilent, USA) 30 m length long x 0.025 mm internal diameter, made from fused silica coated with dimethylpolysiloxane as stationary phase. Helium was used as carrier gas at 1 mL min^{-1}. The temperature of the detector and the injector were set at 300 °C and 285 °C, respectively. The column's temperature was maintained at 60 °C for 1 minute and then programmed up to 290 °C at a rate of 8 °C min^{-1}.

3.2.3 Extraction and analysis of cellular lipids

3.2.3.1 Lipid extraction

Lipids were extracted according to Folch method (Folch *et al.*, 1957), with some modifications. For this, 15 mg of lyophilized cells were extracted with 3 mL of a mixture of chloroform/methanol (2:1, v/v) (Wältermann *et al.*, 2000). The mixture was incubated at room temperature with shaking for two hours. Afterwards, the lipid extracts were obtained after filtration through glass wool and evaporated to dryness.

3.2.3.2 Neutral lipid detection by TLC

Analysis of lipid content in *R. opacus* B4 and *R. opacus* PD630 was performed by thin-layer chromatography (TLC). Lipid extracts were dissolved in chloroform/methanol (2:1, v/v). The extracts were applied on 10 x 10 cm glass DC-Fertigplatten plates, precoated with 0.25 mm silica gel 60 with fluorescent indicator UV254 (60F254 silica gel plates - Merck, Darmstadt, Germany). The TLC chromatogram was performed applying the following developing solvent system: hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as mobile phase for TAG analysis (Wältermann *et al.*, 2000).

Neutral lipids were visualized on plates after brief exposure to iodine vapor. Olive oil, oleic acid, and oleyl oleate were used as standard substances for TAG, fatty acids and WE, respectively.

3.2.3.3 TAG quantification

After evaporation of iodine, TAG bands were scraped from the TLC plates and filtered using a Pasteur pipet containing glass wool. TAG fraction was eluted from Silica Gel by adding three times 1 mL chloroform and transferred to a previously weighted glass flask. Chloroform was evaporated in the fume hood and TAG amounts were determined gravimetrically (Santala *et al.*, 2011b).

3.2.4 Analysis of cellular fatty acid content and composition

Approximately 15 mg of whole lyophilized cells or 5 mg of TAG fraction obtained from TLC (depending on the strain) were submitted to methanolysis in the presence of 15 % (v/v) sulfuric acid (Brandl *et al.*, 1988; Timm *et al.*, 1990). Briefly, samples were added to teflon screw-capped glass test tubes, mixed with 1.5 mL chloroform to which 3 ml of a 85:15 methanol/sulphuric acid solution was added. The tubes were heated at 100°C for 3.5 hours. Subsequently, 1 mL of ultra-pure water was added, and the samples were vortexed for 1 min, and after phase separation (approximately 30 min), the organic phase was collected, containing the fatty acid methyl esters. Heptanoic acid (C7:0) and pentadecanoic acid (C15:0) were used as internal standards, added to the samples before methanolysis takes place (1.5 mL). Methyl esters were quantified in a gas chromatograph (Varian 3800) equipped with a flame ionization detector (GC-FID). FAMEs were separated on a CP-Sil 52 CB 30 m x 0.32 mm x 0.25 µm capillary column (Teknokroma, TR-WAX). One microliter portion of the organic phase was analyzed using a splitless mode. Helium was used as carrier gas at a flow rate of 1.0 mL min-1. A temperature program was established for an

efficient separation of the methyl esters. Initial oven temperature was set at 50 °C for 2 min with an increase of 10 °C min-1 to a final temperature of 225 °C. Injector and detector temperatures were set at 220 °C and 250 °C, respectively. The fatty acids were identified by comparison of the respective retention factor values (Rf) of standard fatty acid methyl esters.

3.2.5 Statistical analysis

Significant differences between biological samples, cultivated on different carbon sources and on different accumulation periods, were evaluated using two factor analyses of variances (ANOVA), using SPSS 22.0.0 statistical software. Statistical significance was established at the $P < 0.05$ level.

3.3 Results

3.3.1 TLC profile of neutral lipid storage compounds in *R. opacus* B4 and *R. opacus* PD630

The patterns of neutral lipid produced by *R. opacus* B4 and by *R. opacus* PD630 are represented in Figure 3.2. This is the first report on accumulation of storage lipids in *R. opacus* B4. The most intense TLC band observed in all tested conditions corresponds to triacylglycerols (TAG). Furthermore, fatty acids (FA) and wax esters (WE) were also present but in minor amounts. Three additional faint bands of unknown identity were visualized: located below FA, immediately below TAG bands and between TAG and WE.

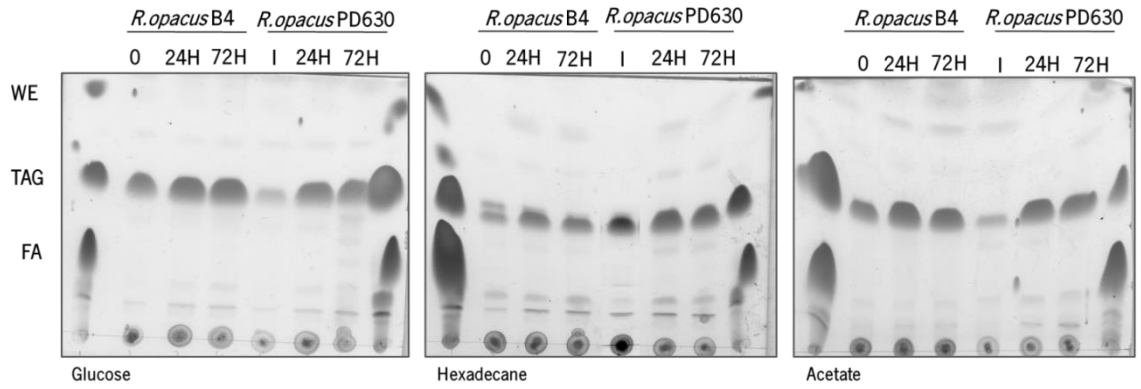


Figure 3.2 TLC analysis of storage lipid accumulation in *R. opacus* B4 and *R. opacus* PD630. Cells were cultivated on 4 % glucose (w/w), 0.6 % acetate (w/w) and 0.1 % hexadecane (w/w). After growth, cells were transferred to MS medium with a molar ratio of C/N 300 during 24 h and 72 h.

3.3.2 Growth and substrate degradation

In both strains, maximum biomass density was obtained with glucose, whereas the minimum was achieved with hexadecane as carbon source (Table 3.1). A significant increase in biomass production by increasing the incubation period (from 24 to 72 h) was observed when cells of *R. opacus* B4 were cultivated on glucose, reaching the highest value observed for this strain, namely 3.3 ± 0.01 g L^{-1} ($p<0.05$). On the other hand, the effect of incubation time in *R. opacus* PD630 was not verified, although it was able to reach higher cell densities than *R. opacus* B4 at 24 and 72 h, namely 1.5-fold and 1.3-fold more ($p<0.05$).

Using hexadecane as carbon and energy source, *R. opacus* B4 presented higher growth when compared to *R. opacus* PD630. Throughout time, *R. opacus* B4 doubled biomass concentration, with the highest density obtained at 72 h (0.837 ± 0.281 g L^{-1}), while *R. opacus* PD630 presented similar values in both incubation times, but about half of the ones obtained for *R. opacus* B4 ($p<0.05$). Acetate had a different behavior when compared to the other carbon sources. For both strains, there was a 35 % decrease in CDW from 24 h to 72 h incubation period ($p<0.05$).

Regarding substrate consumption, there was a general increase with incubation time for both strains and for all carbon sources (Table 3.1) Cells of *R. opacus* B4 consumed less glucose in the first twenty four hours (8.1 ± 4.1 %) when compared to *R. opacus* PD630 (22.8 ± 0.8 %) ($p<0.05$). When cultivated on hexadecane, *R. opacus* B4 degraded more substrate at 24 h (70.5 ± 5.1 %) than *R. opacus* PD630 (52.6 ± 9.6 %) ($p<0.05$). Acetate was almost all consumed by both strains during the first 24 h incubation time (97.3 % in *R. opacus* B4 and 81.1 % in *R. opacus* PD630), reaching full depletion at 72 h.

3.3.3 TAG accumulation and yields

The highest and the lowest TAG levels were obtained by *R. opacus* PD630 cultivated for 72 h in acetate and hexadecane, respectively (Table 3.1). In glucose, cultivation periods of 24 h and 72 h did not affect significantly TAG levels in *R. opacus* B4, but an increase of 34 % was observed in *R. opacus* PD630. *R. opacus* PD630 was able to accumulate more TAG than *R. opacus* B4 especially at 72 h ($p<0.05$). On acetate, *R. opacus* B4 suffered a decrease of more than 50 % of TAG levels from 24 h to 72 h. On the other hand, *R. opacus* PD630 increased TAG production with incubation time, achieving a maximum of 0.444 g g^{-1} CDW at 72 h ($p<0.05$), which was 3.8-fold higher ($p<0.05$) than the one obtained with *R. opacus* B4.

For hexadecane, the amount of TAG was not affected by incubation time in *R. opacus* B4 whereas *R. opacus* PD630 had a 35 % decrease in TAG content ($p<0.05$).

Regarding TAG yields on substrate, maximum and minimum values were achieved by *R. opacus* B4 grown on glucose for 24 h (0.203 g g^{-1} substrate consumed) and acetate during 72 h (0.016 g g^{-1} substrate consumed), respectively (Table 3.1). For *R. opacus* PD630 the highest yield was obtained at 72 h using glucose as carbon source, whereas hexadecane was the most unsuitable carbon source, resulting in the lowest yield. As for the TAG content, increasing the accumulation period length from 24 to 72 h also influenced TAG yield in *R. opacus* B4 growing on acetate, resulting in a 3.4-fold decrease. TAG yield, in *R. opacus* PD630 was not affected in the same conditions.

With hexadecane, *R. opacus* B4 presented similar yields for both accumulation periods, 2 and 5 fold higher yields than *R. opacus* PD630 ($p<0.05$) for 24 h and 72 h respectively. In *R. opacus* PD630 a 47 % decrease was observed after 72 h.

Table 3.1 Cell dry matter, triacylglycerols (TAG) content, % of substrate degradation and yields in cells of *R. opacus* B4 and *R. opacus* PD630 cultivated in different carbon sources under nitrogen limiting conditions (C/N=300) during 24 h and 72h. The means are the result of at least two independent experiments.

Strain	Carbon source	Time (h)	CDW (g L^{-1})	TAG (g g^{-1} CDW)	Substrate consumed (%)	TAG Yield (g g^{-1}substrate consumed)
R. opacus B4	Glucose	Inoculum	2.2 *	0.044 *		
		24	2.5 ± 0.04	0.230 ± 0.045	8.1 ± 4.3	0.203 ± 0.068
		72	3.3 ± 0.01	0.217 ± 0.014	37.4 ± 5.6	0.054 ± 0.015
	Acetate	Inoculum	0.45 *	0.081 *		
		24	1.1 ± 0.1	0.252 ± 0.040	97.3 ± 0.5	0.054 ± 0.005
		72	0.724 ± 0.017	0.117 ± 0.061	100.0 ± 0.0	0.016 ± 0.002
	Hexadecane	Inoculum	0.398 *	0.079 *		
		24	0.459 ± 0.134	0.186 ± 0.021	70.5 ± 5.1	0.147 ± 0.069
		72	0.837 ± 0.281	0.170 ± 0.034	85.2 ± 17.7	0.194 ± 0.112
R. opacus PD630	Glucose	Inoculum	2.4 *	0.111 *		
		24	3.9 ± 0.4	0.287 ± 0.003	22.8 ± 0.8	0.076 ± 0.001
		72	4.1 ± 0.5	0.384 ± 0.051	26.5 ± 5.8	0.125 ± 0.024
	Acetate	Inoculum	0.620*	0.169 *		
		24	1.1 ± 0.01	0.246 ± 0.015	81.1 ± 10.4	0.054 ± 0.003
		72	0.726 ± 0.040	0.444 ± 0.179	100.0 ± 0.0	0.059 ± 0.033
	Hexadecane	Inoculum	0.250 *	0.063 *		
		24	0.321 ± 0.168	0.141 ± 0.013	52.6 ± 19.8	0.070 ± 0.049
		72	0.468 ± 0.062	0.092 ± 0.008	94.5 ± 4.2	0.037 ± 0.004

3.3.4 Fatty acid composition of biomass and TAG produced

Fatty acid composition of intact cells was not influenced by incubation time but drastically affected by the substrate used (Table 3.2). With glucose and acetate, and for both strains, fatty acids varied from 14 to 18 carbon atoms, being heptadecanoic acid (C17:0) the predominant one. In *R. opacus* B4 the fraction of saturated fatty acids was about 90 % and odd numbered fatty acids accounted for about 80 %, whereas in *R. opacus* PD630 these fractions were 85 % and 70 %, respectively.

For hexadecane, fatty acids with lower carbon chains (C10-C16) were mainly detected, and hexadecanoic acid (C16:0) was predominant. In both strains, even-numbered fatty acids were 100 %.

Comparing both strains for glucose and acetate, the main differences were obtained for the percentage of C16:1 that was three-fold higher in *R. opacus* PD630 than in *R. opacus* B4, and of C17:0, that corresponded to around 63 % to 76 % of the fatty acids present in *R. opacus* B4 and around 49 % to 62 % in *R. opacus* PD630 ($p<0.05$). With hexadecane, C16:1 percentage was two-fold higher in *R. opacus* PD630 than in *R. opacus* B4, and C16:0 was 42 % to 55 % in *R. opacus* B4 and 35 % to 38 % in *R. opacus* PD630 ($p<0.05$).

The fatty acid patterns of produced TAG fraction showed some important differences, depending on the strain, accumulation period length and carbon source used (Table 3.3). In both strains cultivated on glucose and acetate the most dominant fatty acids were C16:0 (35 % to 55 %) and C17:0, ranging between 15 % and 40 % for glucose, and between 35 % and 50 % for acetate. For hexadecane, the predominant fatty acids were C10:0 (almost 30 %) and C16:0 (55 %) in *R. opacus* B4, and C16:0 (85 %) in *R. opacus* PD630. In *R. opacus* B4, the fraction of saturated fatty acids was similar for all carbon sources, reaching 90 % to 96 %. Even-numbered fatty acids were 55 % to 65 % for glucose and acetate, and 100 % for hexadecane. In *R. opacus* PD630, with hexadecane, no odd-numbered fatty acids were detected as well, and 60 % to 70 % of even-numbered fatty acids were found with glucose and acetate. In both strains cultivated on glucose, there was a transition between saturated and unsaturated fatty acids from 24 h to 72 h incubation length. C10:0 and C12:0 were only detected in *R. opacus* B4. TAG from *R. opacus* B4 cultivated on glucose and acetate had a higher percentage of C17:0 (39 % and 48 %, respectively) than from *R. opacus* PD630 (16 % and 38 %). In contrast, in *R. opacus* PD630 TAG contained higher percentages of C14:0 (13 %); C16:0 (86 %) and C16:1 (39 %) when compared to those of *R. opacus* B4 in hexadecane.

Table 3.2 Total fatty acid synthesis and composition in cells of *R. opacus* B4 and *R. opacus* PD630 cultivated in different carbon sources under nitrogen limiting conditions (C/N=300) during 24 h and 72 h. The means are the result of at least two independent experiments.

Strain	Substrate	Time	FA	Relative proportion of fatty acids [%, w/w]								
		(h)	(g g^{-1} CDW)	C10:0	C12:0	C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1
R. opacus B4	Glucose	Inoculum	0.364 *	—	—	5.9	7.7	1.3	78.3	3.7	1.0	2.3
		24	0.528 ± 0.049	—	—	8.8 ± 2.05	9.9 ± 0.90	1.7 ± 0.11	72.0 ± 1.15	2.6 ± 0.20	0.9 ± 0.17	4.6 ± 0.02
		72	0.613 ± 0.051	—	—	6.9 ± 0.3	10.0 ± 0.73	1.6 ± 0.04	72.2 ± 2.89	3.0 ± 0.48	1.3 ± 0.32	5.3 ± 0.08
	Acetate	Inoculum	0.235 *	—	—	16.3	14.9	1.4	49.4	8.2	5.3	4.9
		24	0.664 ± 0.013	—	—	6.4 ± 0.21	10.3 ± 0.16	1.3 ± 0.01	75.9 ± 0.02	1.8 ± 0.30	0.3 ± 0.02	4.2 ± 0.21
		72	0.517 ± 0.014	—	—	6.7 ± 0.21	9.2 ± 0.01	1.4 ± 0.03	75.0 ± 1.03	1.6 ± 0.07	—	5.3 ± 0.08
	Hexadecane	Inoculum	0.087 *	—	—	20.8	62.7	12.9	—	—	—	4.2
		24	0.273 ± 0.088	—	—	16. ± 0.35	72.1 ± 0.89	10.9 ± 0.51	—	—	—	2.9 ± 0.01
		72	0.285 ± 0.037	4.8 ± 0.60	0.6 ± 0.03	18.1 ± 0.80	60.2 ± 1.47	13.2 ± 0.93	—	—	—	3.1 ± 0.12
R. opacus PD630	Glucose	Inoculum	0.525 *	—	—	6.2	10.5	3.3	72.5	3.2	1.3	2.8
		24	0.507 ± 0.013	—	—	8.9 ± 0.99	16.1 ± 0.29	5.8 ± 0.19	57.2 ± 0.36	3.3 ± 0.52	1.1 ± 0.43	7.0 ± 0.14
		72	0.580 ± 0.014	—	—	7.7 ± 0.22	12.6 ± 0.15	5.6 ± 0.15	63.3 ± 0.24	3.0 ± 0.10	1.2 ± 0.05	6.5 ± 0.08
	Acetate	Inoculum	0.238 *	—	—	14.6	18.3	8.3	49.3	3.5	—	7.0
		24	0.505 ± 0.006	—	—	5.7 ± 0.67	15.3 ± 0.19	2.5 ± 0.74	65.8 ± 3.48	3.7 ± 0.33	3.0 ± 0.42	4.4 ± 0.61
		72	0.449 ± 0.001	—	—	4.9 ± 0.86	10.4 ± 1.21	3.6 ± 0.26	63.4 ± 4.95	5.8 ± 0.45	7.0 ± 0.20	4.9 ± 0.57
	Hexadecane	Inoculum	0.116 *	—	—	22.5	47.7	28.2	—	—	—	2.4
		24	0.218 ± 0.003	—	—	11.4 ± 1.58	53.3 ± 2.04	35.2 ± 0.11	—	—	—	—
		72	0.254 ± 0.041	—	0.5 ± 0.03	16.1 ± 0.57	54.0 ± 0.21	27.5 ± 1.45	—	—	—	2.0 ± 0.31

Table 3.3 Fatty acid profile of TAG fraction in *R. opacus* B4 and *R. opacus* PD630 cultivated in different carbon sources under nitrogen limiting conditions (C/N=300) during 24 hours and 72 hours. The means are the result of at least two independent experiments.

Strain	Substrate	Time (h)	Relative proportion of fatty acids [%, w/w]								
			C10:0	C12:0	C14:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1
R. opacus B4	Glucose	Inoculum	—	6.7	7.6	41.7	—	44.0	—	—	—
		24	—	1.2 ± 0.09	3.2 ± 0.69	49.7 ± 0.12	—	34.3 ± 1.79	4.3 ± 1.46	3.3 ± 0.46	4.0 ± 1.42
		72	—	1.4 ± 0.42	3.1 ± 0.03	38.3 ± 1.13	3.2 ± 0.21	24.4 ± 0.32	14.4 ± 0.37	2.0 ± 0.01	13.2 ± 0.26
	Acetate	Inoculum	—	12.8	15.1	27.0	16.7	—	—	—	28.4
		24	—	—	2.9 ± 1.17	46.1 ± 0.99	—	47.6 ± 1.98	—	—	3.4 ± 2.13
		72	—	—	5.3 ± 0.57	38.8 ± 3.57	—	52.0 ± 1.30	—	—	3.9 ± 0.16
	Hexadecane	Inoculum	—	0.8	4.8	70.4	16.2	—	—	—	7.8
		24	27.5 ± 0.8	1.3 ± 0.08	5.7 ± 0.17	54.0 ± 1.12	9.5 ± 0.29	—	—	—	2.0 ± 0.21
		72	28.9 ± 1.36	1.1 ± 0.51	6.1 ± 0.41	55.0 ± 5.01	6.8 ± 0.14	—	—	—	2.1 ± 0.68
R. opacus PD630	Glucose	Inoculum	—	—	4.2	50.2	2.9	31.4	5.2	5.1	2.0
		24	—	—	2.8 ± 0.52	48.4 ± 4.43	6.1 ± 2.7	18.7 ± 1.25	12.7 ± 0.43	3.1 ± 0.07	8.2 ± 3.27
		72	—	—	2.0 ± 0.04	35.3 ± 2.73	6.3 ± 2.28	16.3 ± 1.00	11.9 ± 2.31	2.5 ± 0.28	25.7 ± 11.6
	Acetate	Inoculum	—	—	7.3	68.6	—	24.1	—	—	—
		24	—	—	3.8 ± 0.19	53.1 ± 6.72	—	34.5 ± 1.88	8.6 ± 1.16	—	—
		72	—	—	4.5 ± 0.47	53.9 ± 2.92	—	35.1 ± 0.01	6.5 ± 1.4	—	—
	Hexadecane	Inoculum	—	—	28.4	71.6	—	—	—	—	—
		24	—	—	13.3± 3.91	86.7 ± 4.25	—	—	—	—	—
		72	—	—	7.3 ± 2.86	70.4 ± 7.79	19.7 ± 7.12	—	—	—	2.6 ± 0.43

3.4 Discussion

In this study we demonstrate for the first time the capability of *R. opacus* B4, a bacterium isolated from a gasoline contaminated soil, to accumulate lipid storage compounds from glucose, acetate and hexadecane. *R. opacus* PD630 was considered for comparison.

Data gathered in terms of cell density (as CDW), TAG content, fatty acids composition, and TAG yield, allowed to assess the potential of both strains to produce TAG from each carbon source. The differences observed in the values obtained under the assessed conditions, are a result of different dynamics of growth, substrate consumption and accumulation rate of carbon storage compounds. For practical applications, however, it is important to analyze global volumetric TAG productions and to extract objective conclusion with interest for industrial and environmental biotechnological applications.

R. opacus B4 showed a TAG production capability of 0.09 g L^{-1} and 0.14 g L^{-1} at 24 h and 72 h, with hexadecane as carbon source, which was 2 and 3.3 fold higher than the volumetric production obtained by *R. opacus* PD 630 (0.045 g L^{-1} and 0.043 g L^{-1} for the same periods). The ability of *R. opacus* B4 to transform and degrade several types of hydrocarbons and to stabilize water-oil phases was previously reported (Na *et al.*, 2005; Yamashita *et al.*, 2007; Honda *et al.*, 2008; Sameshima *et al.*, 2008), but not its capacity to produce TAG from these carbon sources.

For acetate, both strains showed similar maximal TAG productions of about 0.3 g L^{-1}. In *R. opacus* B4 acetate consumption was faster, and, the exhaustion of the external carbon source likely induced the decrease observed in TAG levels for the longest incubation period (72 h), due to its mobilization for cell maintenance. TAG's use as internal carbon and energy source has been also reported by (Alvarez et al., 2000) in *R. opacus* PD630 and in *R. ruber* NCIMB 40126 cultivated on gluconate and glucose, after depletion of these carbons sources.

With glucose, *R. opacus* PD630 showed higher TAG volumetric productions, 1.1 g L^{-1} and 1.6 g L^{-1}, about two-fold the production obtained for *R. opacus* B4 in the same incubation periods. The type of carbon source is one of the most determinant factor influencing fatty acid content and composition in bacteria. *R. opacus* B4 presented a higher level of total fatty acids (intact cells) when cultivated on glucose (53 % to 61 % of cellular dry weight) or acetate (52 % to 66 %) than in hexadecane (27 % to 29 %), and exhibited higher percentage than *R. opacus* PD630. Taking into account these results, *R. opacus* B4 can also be considered an oleaginous bacterium, since it can accumulate more than 20 % of biomass as lipids. There are several studies in literature reporting fatty acid accumulation in several *Rhodococcus* species using these substrates, ranging between

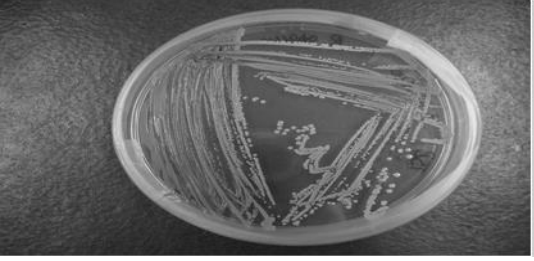
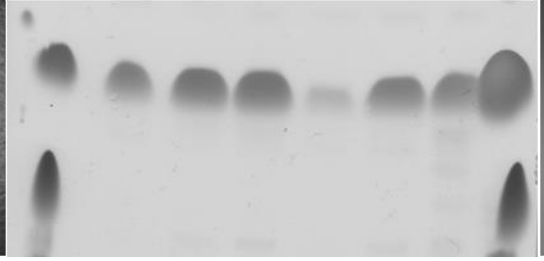
21 % and 31 % for acetate (Hernandez*, et al.*, 2008, Hori*, et al.*, 2009) and between 13 % and 22 % for hexadecane (Alvarez *et al.*, 1997a; Alvarez, 2003; Silva *et al.*, 2010), but none study has been performed before with *R opacus* B4.

In general, the results obtained for the produced TAG fraction are in agreement with the ones obtained for intact cells and a high variability of fatty acids was obtained, influenced by the type of carbon source used. When cells were cultivated on hexadecane, only even-numbered fatty acids were detected in produced TAG, being C16:0 the dominant one. These results are in accordance with several works (Alvarez *et al.*, 1996; Alvarez *et al.*, 1997a; Alvarez, 2003; Gouda *et al.*, 2008; Hernandez*, et al.*, 2008; Silva *et al.*, 2010), revealing that the fatty acids produced were directly related to the chain length of the carbon source and to ß-oxidation pathway. Interestingly, in *R. opacus* B4 shorter even-numbered fatty acids were detected in TAG, which indicated a more efficient ß-oxidation of hexadecanoic acid than in *R. opacus* PD630. In the case of acetate and glucose, C16:0 and C17:0 were the dominant fatty acids, with higher percentage in *R. opacus* B4. These results, in particular dominance of hexadecanoic acid, corroborated the ones previously reported (Alvarez *et al.*, 1996; Alvarez,*et al.*, 1997a; Alvarez, 2003; Hernandez *et al.*, 2008; Holder *et al.*, 2011), where several *Rhodococcus* species exhibited the same pattern. It also suggested that these substrates must be converted into acetyl-CoA which is the precursor of de novo fatty acid synthesis pathway. Presence of odd-numbered fatty acids can result from the presence of an effective methylmalonyl-CoA production through succinyl-CoA (derived from TCA cycle), resulting in the formation of propionyl-coA, a 3-carbon molecule, which is a precursor for the synthesis of odd-numbered fatty acids. Similar results were found in previous works (Alvarez *et al.*, 1997a; Alvarez, 2003; Hernandez *et al.*, 2008; Holder *et al.*, 2011).

Although focus on current research has been much directed to increase TAG production in *R opacus* PD630, such as running the process at a higher scale (Kurosawa *et al.*, 2010), as well as to perform metabolic engineering studies (Hetzler & Steinbüchel, 2013; Kurosawa, 2013), new strains of *Rhodococcus opacus* should also be considered and explored for similar abilities. In particular the strain studied in this work, *R. opacus* B4, shows relevant interest in TAG production from liquid hydrocarbons, herein demonstrated with hexadecane. Combining TAG production with hydrocarbons degradation is a powerful strategy to achieve environmental remediation while producing added value compounds.

3.5 Conclusions

This work demonstrated for the first time the production and accumulation of TAG in *R. opacus* B4 using glucose, acetate and hexadecane, under unbalanced growth conditions. When compared to *R. opacus* PD630, known as the best accumulating TAG bacterium, *R. opacus* B4 revealed higher TAG content and yield for hexadecane and also higher TAG yield for shorter incubation times (24 h) with glucose. This strain also produced more TAG, 0.09 g L^{-1} and 0.14 g L^{-1} at 24 h and 72 h, with hexadecane as carbon source, which was 2 and 3.3 fold higher than the volumetric production obtained by *R. opacus* PD630. Additionally, a high variability in fatty acids profile of TAG for all carbon sources was detected, increasing the range of potential biotechnological applications. The obtained results open new perspectives for the use of *R. opacus* B4 to produce triacylglycerol with fatty acids composition relevant for industrial purposes, in particular using oily (alkane-based) waste or wastewater as cheap raw-material.


Chapter 4

Neutral lipid production from hydrocarbon-contaminated cork sorbents using *R. opacus* B4

The exponential increase in exploitation of crude oil resources consequently promoted the development of petroleum-based industries. In this way, several oil spillages have been reported in land as well as in aquatic systems, with tremendous negative impacts on human health and several ecosystems.
In order to minimize these accidents, cork has been used as oil biosorbent as a sustainable alternative to other conventional remediation techniques. Management of the subsequent residue involves a significant cost associated to its treatment/disposal, as no economic valorization of this residue is currently performed.
In this work, a biological, environmental friendly solution is proposed to valorize hydrocarbon-contaminated cork sorbents. It was demonstrated that *R. opacus* B4 is able to decontaminate hexadecane-contamined cork sorbents while producing neutral lipids that can serve as raw material for biodiesel and biomethane production. *R. opacus* B4 was able to degrade up to 96 % of the hexadecane impregnated in natural and thermally treated cork sorbents. Triacylglycerol (TAG) was the main neutral lipid produced in all tested conditions. In particular, when *R. opacus* B4 was cultivated with natural cork contaminated with hexadecane a yield of 0.59 g ± 0.06 g TAG per gram of hexadecane consumed and a TAG content of 0.60 ± 0.06 g of TAG per gram of biomass was achieved whereas for treated cork a yield of 0.69 g ± 0.06 g TAGs per gram of hexadecane consumed and a TAG content of 0 .97 g ± 0.04 g of TAG per gram of biomass was obtained. Palmitic acid was the predominant fatty acid present in the produced TAG (60 % - 70 % w/w) followed by palmitoleic acid (15 % - 20 % w/w). In alternative to TAG extraction, whole cell lipid-rich biomass obtained from *R. opacus* B4 cultivated on hexadecane contaminated cork can be efficiently converted to methane at a yield of about 0.4 mL CH_4 per miligram of added substrate.

4.1 Introduction

During the last decade, due to the increasing demand for liquid petroleum, several industrial activities related to petroleum exploration have dramatically increased. During routine operations, transportation and storage processes, inevitably there are oil spillages, which can occur in land and in marine systems, accidentally or deliberately (Gogoi *et al.*, 2003; Lucas & MacGregor, 2006). Crude oil is mainly composed by a mixture of hydrocarbons, with several millions of chemical components (Marshall & Rodgers, 2003). Hydrocarbon molecules can be divided in four main categories, namely alkanes with different chain lengths and branch points, cycloalkanes, mono-aromatic and polycyclic aromatic hydrocarbons; resins and asphaltenes (Balba *et al.*, 1998; Harayama, 1999; Speight, 1999) and many of them are persistent and recalcitrant organic pollutants (Killops & Al-Juboori, 1990; Oudot *et al.*, 1998) due to their hydrophobic nature (low water solubility) and low volatility. Therefore, their release to the environment has become a serious pollution threat, resulting in a high negative impact on the biotic and abiotic components of the ecosystems (Mueller *et al.*, 1992; Margesin *et al.*, 2003; Peterson *et al.*, 2003; Head *et al.*, 2006; Heintz, 2007). In order to mitigate this problem, several technologies have been developed to treat hydrocarbon contaminated environments, employing biological, physical, chemical and thermal processes (Lessard & DeMarco, 2000; Lundstedt *et al.*, 2003; Mullin & Champ, 2003; Fingas & Fieldhouse, 2011). Among those, physical containment and recover, using a variety of equipment such as booms, barriers, skimmers, as well as natural and synthetic absorbent materials, are usually in the primary line of defense against oil spills. They present some advantages, since can be applied to all types of oils, no maintenance is required and are simple to use (Adebajo *et al.*, 2003; Dave, 2011).

Cork is a good natural absorbent due to their hydrophobic nature (Silva *et al.*, 2005) and is being used as biosorbent material in the removal of hydrocarbonsin oil spills and leaks (Silva, 2007). After use, oil contaminated cork residues are treated by conventional physical-chemical methods, having an associated cost. Therefore, alternative and less expensive treatment techniques should be explored.

Members of the genus *Rhodococcus* are aerobic gram-negative bacteria widely distributed through different environments such as soils, sediments and water (Langdahl *et al.*, 1996; Heald *et al.*, 2001; Alvarez *et al.*, 2004; Luz *et al.*, 2004; Peng *et al.*, 2008) and particularly in hydrocarbon-contaminated ecosystems (Whyte et al., 1998; Peressutti *et al.*, 2003; Van Hamme *et al.*, 2003).

This genus is, in fact, known by the ability to transform and/or degrade several types of pollutants, mostly due to the presence of many catabolic genes in their genomes (Larkin *et al.*, 2005; Martinkova *et al.*, 2009; Kuyukina & Ivshina, 2010; Larkin *et al.*, 2010). Consequently, several *Rhodococcus* strains are being study for bioremediation of hydrocarbon-contaminated sites and industrial wastewaters (Bell *et al.*, 1998; de Carvalho, 2012; Auffret *et al.*, 2014; Zampolli *et al.*, 2014). In addition, another attractive property of *Rhodococcus* species is the production of storage compounds, especially TAG, during cultivation on several carbon sources. Particularly, there are some reports describing TAG synthesis by *Rhodococcus* cells using single hydrocarbons (Alvarez, 2003; Gouda *et al.*, 2008). In this work, the potential of using *R. opacus* B4 to produce lipids from different hexadecane contaminated cork sorbents was assessed in order to evaluate the feasibility of applying this approach to valorize these materials.

4.2 Materials and Methods

4.2.1 Cork sorbents

Two types of cork used as absorbents were tested: natural cork and thermally treated cork. Natural cork can absorb five times its weight in oil while thermally treated cork, due to a thermal treatment at 450°C can absorb ten times its weight in oil (Silva, 2007). Both materials were provided by Cortiçeira Amorim, S.A., Portugal.

4.2.2 Bacterial strain, media and cultivation conditions

4.2.2.1 Strain and media

R. opacus B4 (NBRC 108011) was purchased from the National Institute of Technology and Evaluation, Biological Resource Center, Japan (NBRC). This strain was selected due to the ability to produce lipid storage compounds, namely TAG when cultivated on hexadecane ($C_{16}H_{34}$), as demonstrated in Chapter 3. Hexadecane was chosen as a model contaminant since it is usually used as the main representative compound of aliphatic hydrocarbons, one of the most dominant groups found in crude oil and derivatives.

The culture media used for maintenance and growth of cells are described in section 3.2.1.1 of Material Methods of Chapter 3.

4.2.2.2 Preparation of seed culture

Cells from a single colony of *R. opacus* B4 grown on 802 medium agar plates at 30ºC during 4 days were inoculated in 50 ml of 802 medium in a 250 ml flask. The seed culture was incubated on a rotary shaker (150 rpm) at 30°C until the middle of the exponential growth phase was reached (48 hours).

Growth of the seed cultures was determined by measuring optical density at 600 nm wavelength with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan).

4.2.2.3 Growth and lipid accumulation experiments

The experiments were divided in two stages. In stage I, culture conditions promoted high cell densities (growth conditions) while in stage II neutral lipid accumulation (storage conditions) was promoted (see Figure 4.1 for a schematic representation of the experimental procedure). The two-stage experiments were carried out under sterile conditions in duplicate using 250 ml conical flasks containing 50 ml of defined medium incubated on a rotary shaker (250 rpm) at 30 ºC.

Cells of seed culture were harvested, washed with sterile sodium chloride solution (0.9 %, w/v), and re-suspended in fresh MS medium. Then, cells were used to inoculate flasks to an optical density at 600 nm (OD_{600nm}) of 0.1 and cultivated in MS medium supplemented with hexadecane (0.1 %, w/v) as sole carbon source at a molar carbon to nitrogen ratio of 4 (C/N=4). Cells were grown until the middle of the exponential growth phase which corresponds to 150 hours of cultivation (stage I).

Considering the maximum absorption capacity of both cork sorbents, 0.01 g of natural cork or 0.005 g of thermally treated cork were weight together with 50 mg of hexadecane (final concentration of 1g L^{-1} in the culture medium) to new flasks. After 24 hours of contact between cork and hexadecane, MS medium was added in order to obtain a molar carbon to nitrogen ratio of 300 (storage lipid accumulation conditions). Cells from the grown inoculum (stage I) were collected by centrifugation (4°C; 10 min., 10000 g), washed twice with sterile sodium chloride solution (0.9 %, w/w) and transferred to the previously prepared fresh MS medium with hexadecane contaminated cork. In parallel, several control experiments were performed: 1) *R. opacus* B4 growing on MS medium supplemented with hexadecane 0.1 % (w/v) and without previous addition

of cork – B4.C16; 2) MS medium supplemented with hexadecane 0.1 % (w/v) and natural cork (NC) – NC.C16; 3) MS medium supplemented with hexadecane 0.1 % (w/v) and thermally treated cork (TTC) – TTC.C16; 4) MS medium supplemented with natural cork but without the addition of hexadecane – B4.NC and 5) MS medium supplemented with thermally treated cork (TTC) but without the addition of hexadecane – B4.TTC. After 48 hours of cultivation, cells were harvested, washed and kept at -80°C until further lyophilization.

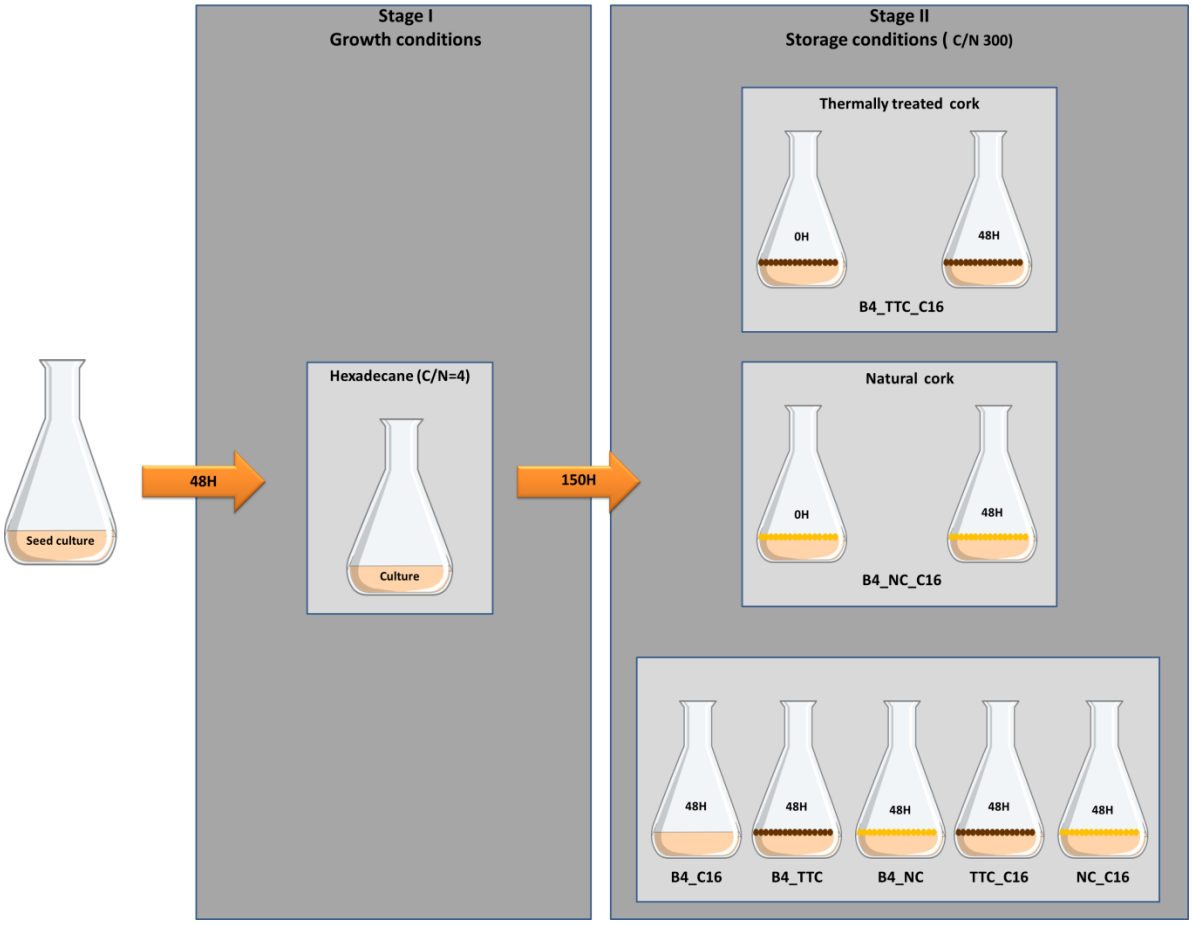


Figure 4.1 Schematic representation of the experimental procedure adopted for assessing accumulation of lipid storage compounds in cells of *R. opacus* B4 cultivated in the presence of hexadecane-contaminated cork sorbents.

4.2.3 Analytical methods

4.2.3.1 Chemical oxygen demand (COD)

Chemical oxygen demand (COD) was determined using the cuvette-test Lck414 (Hach-Lange, Germany). These measurements were made in triplicate, using the manufacturer's procedures.

4.2.4 Hexadecane extraction and quantification

The experimental procedure adopted for extraction and quantification of hexadecane is described in section 3.2.2.2 of Material Methods of Chapter 3.

4.2.5 Extraction and analysis of neutral lipids

4.2.5.1 Total lipid extraction

The experimental procedure adopted for total lipid extraction from cells of *R. opacus* B4 is described in section 3.2.3.1 of Material Methods of Chapter 3.

4.2.5.2 Neutral lipid analysis

The experimental procedure adopted for analysis of neutral lipid profiles in cells of *R. opacus* B4 is described in section 3.2.3.2 of Material Methods of Chapter 3.

4.2.5.3 TAG quantification

The experimental procedure adopted for quantification of TAG in cells of *R. opacus* B4 is described in section 3.2.3.3 of Material Methods of Chapter 3.

4.2.6 Analysis of fatty acid composition

The experimental procedure adopted for analysis of fatty acid composition of TAG isolated from cells of *R. opacus* B4 is described in section 3.2.4 of Material Methods of Chapter 3.

4.2.7 Biochemical methane productions (BMP) assays

4.2.7.1 Inoculum and substrate

An inoculum consisting of disrupted anaerobic granular sludge, obtained from a brewery wastewater treatment plant, and manure (1:1, v/v) was used in all biodegradability assays. The sludge contained 31.9 g L^{-1} of total solids and 22.4 g L^{-1} of volatile solids (VS). The specific methanogenic activity (SMA) in the presence of acetate (30 mM) was 79.1 mL $CH_{4\,(STP)}$ g^{-1} VS d^{-1}.

SMA was determined according to (Costa *et al.*, 2012). Lipid-rich biomass from *R. opacus* B4 cultivated in hexadecane contaminated natural cork (B4_NC_C16) an in hexadecane thermally treated cork (B4_TTC_C16) were used as substrates. Each sample was characterized in terms of chemical oxygen demand (COD) and TAG content.

4.2.7.2 Experimental design

The anaerobic biomethane production (BMP) assays were performed according to Angelidaki, 2009. A working volume of 60 mL and 20 % (v/v) of inoculum were used and the assays were performed at 37°C. The lipid-rich biomass was added to 120 mL serum bottles containing basal medium. Basal medium pH was adjusted to 7.0 using sodium hydroxide or hydrochloric acid 2 mol L^{-1}. The vials were sealed and the headspace flushed with N_2/CO_2 (80:20 v/v). Prior to the assays,Na_2S9H_2O was added to basal medium, to a final concentration of 1 mmol L^{-1}. All the assays were performed in triplicate. A blank assay was performed in order to subtract the residual substrate present in the inoculum. Avicel, mainly composed by crystalline cellulose (average particle size 50 μm) was used as control assay. The methane accumulated in closed serum bottles headspace was measured by gas chromatography (GC) equipped with a flame ionization detector (FID) using a gas tight syringe to sample 500 μL. Methane production was corrected for standard temperature and pressure (STP) conditions (0 °C and 1 bar). Biochemical methane potential (BMP) was defined as the volume of methane produced per gram of substrate added to the assays, as expressed in Eq. (4.1):

$$BMP = CH_4/g\ substrate\ = \frac{Kg\ COD - CH_4\ \times 350(L\ CH_4/Kg\ COD)}{g\ substrate\ added} \qquad (4.1)$$

4.2.8 Statistical analysis

Significant differences between samples were evaluated using two factor analysis of variance (ANOVA), using SPSS 22.0.0 statistic software. Statistical significance was established at the $P < 0.05$ level.

4.3 Results and Discussion

4.3.1 Cork characterization

Cork sorbents used in this study were characterized in terms of neutral lipid composition (Table 4.1). TAG content in both materials was low, corresponding to about 7 % and 9 % of its weight in natural and thermally treated cork, respectively. Regarding fatty acid composition, short to medium length chain saturated and even-numbered fatty acids were mainly detected in both materials, namely caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0) and myristic acid (C14:0). In addition, oleic acid (C18:1), a long-chain unsaturated fatty acid, was also detected in natural cork. The presence of fatty acids in cork was already demonstrated by other authors, since they are one of the principal components of the cork extractive fraction, solubilized in low-polarity solvents, and also of suberine (Domingues *et al.*, 2007; Şen *et al.*, 2010; Olivella *et al.*, 2013).

Table 4.1 Characterization of cork sorbents used in this study

Material	Relative proportion of fatty acids (% , w/w)								COD ($g\ g^{-1}$)	TAG content ($g\ g^{-1}$)
	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1		
Natural cork (NC)	16.6	29.6	17.9	13.1	-	-	-	22.8	0.55	0.072
Thermally treated cork (TTC)	20.5	37.5	24.4	17.6	-	-	-	-	0.45	0.091

- Not detected.

4.3.2 Removal of hexadecane from cork-contaminated sorbents by *R. opacus* B4

The ability of *R. opacus* B4 to use hexadecane from contaminated-cork sorbents was assessed. The results of hexadecane consumption by *R. opacus* B4 in the tested conditions are described in Table 4.2. Since cork has a high affinity for hydrocarbons, a set of control experiments were performed to evaluate hexadecane retention by each type of cork (NC_C16 for natural cork and TTC_C16 for thermally treated cork). For natural cork, it was found that approximately 16 % (± 3 %) of the hexadecane initially added could not be recovered with the extraction procedure, while for thermally treated cork practically all hexadecane was recovered (98 % ± 3 % recovery). This difference may be related to the existence of larger pores in thermally treated cork, since high

temperatures caused the expansion of cork cells, increasing its porosity and consequently lowering diffusion limitations (Pintor *et al.*, 2012). No changes were detected in hexadecane concentration added to both cork types (experiments NC_C16 and TTC_C16) after de 48 hours period of the experiment; the differences observed were within the percentages of hexadecane unrecovered from the cork with the extraction procedure (Table 4.2).

In the control experiments containing cork (without hexadecane) and cells of *R. opacus* B4 from stage I (used as inoculum), hexadecane in concentrations of 0.256 g L^{-1} and 0.199 g L^{-1} was detected in thermally treated cork (B4_TTC) and in natural cork (B4_NC), respectively. These amounts of hexadecane derived from the growth stage and were probably attached to *R. opacus* B4 cells. In the presence of hydrocarbons, cells of several strains of *Rhodococcus* tend to aggregate and consequently retain the hydrocarbon between cells due to their high hydrophobicity (Alvarez *et al.*, 1996; Binazadeh *et al.*, 2009). After 48 hours of cultivation, almost all residual hexadecane, derived from the growth stage, was consumed by *R. opacus* B4 in the experiment containing natural cork (97.6 %), whereas in the one with thermally treated cork, only 42.6 % was degraded.

In the assays performed with contaminated cork sorbents (B4_NC_C16 and B4_TTC_C16) *R. opacus* B4 was able to consume high levels of hexadecane, namely 94 % degradation in B4_NC_C16 and 96 % degradation in B4_TTC_C16. Without cork (B4_C16), a 93 % hexadecane degradation was observed. These results clearly revealed that both types of cork did not affect hexadecane degradation by *R. opacus* B4, indicating that this strain can effectively be used in the treatment of hydrocarbon-contaminated cork residues. The ability to access the hexadecane impregnated in the cork can be related to the production of surfactants. These compounds are produced by several strains of *Rhodococcus* (Lang & Philip, 1998; Rosenberg & Ron, 1999; Iwabuchi *et al.*, 2002; Philp *et al.*, 2002; Pal *et al.*, 2009; Pirog *et al.*, 2013) and are known to emulsify hydrocarbons, reducing surface tensions and thereby increasing mobility and solubility of hydrocarbons (Zhang & Miller, 1992; Zhang & Miller, 1995; Al-Tahhan *et al.*, 2000). In fact, it was previously demonstrated that *R. opacus* B4 is able to stabilize water-in-oil emulsion, promoting the bioconversion of water insoluble compounds, such as hydrocarbons (Honda *et al.*, 2008).

Table 4.2 Hexadecane consumption by cells of *R. opacus* B4 cultivated on hexadecane (B4_C16); on hexadecane contaminated natural cork (B4_NC_C16); on hexadecane contaminated thermally treated cork (B4_TTC_C16); on natural cork (B4_NC) and on thermally treated cork (B4_TTC) without the addition of hexadecane. Experiments with thermally treated cork contaminated with hexadecane (CTT_C16) and with natural cork contaminated with hexadecane (CN_C16) were performed to determine hexadecane retention in cork sorbents

Experiment	Hexadecane concentration (g L^{-1})		Hexadecane consumption (%)
	0 h	48 h	
B4_C16	1.072 ± 0.003	0.081 ± 0.040	92.45 ± 7.39
B4_CTT_C16	0.924 ± 0.006	0.015 ± 0.002	96.04 ± 0.90
B4_CN_C16	0.812 ± 0.187	0.037 ± 0.007	93.94 ± 0.44
B4_CTT	0.256*	0.147 ± 0.003	42.55 ± 2.00
B4_CN	0.199*	0.004 ± 0.001	97.62 ± 0.37
CTT_C16	0.824± 0.0283	0.715 ± 0,050	13.51 ± 3.38
CN_C16	1.022±0.268	0.887 ± 0.378	4.79 ± 3.24

* hexadecane from growth stage

4.3.3 Production of neutral lipids from hexadecane-contaminated cork sorbents by *R. opacus* B4

The capability of *R. opacus* B4 to accumulate lipid storage compounds at the expense of hexadecane-contaminated cork sorbents was evaluated. Figure 4.2 shows the profiles of neutral lipid present in the cells of *R. opacus* B4 under the tested conditions. Generally, TAG were the main storage lipid compounds present, whereas no wax esters were detected. Free fatty acids were also present in all conditions. Interestingly, a band of unknown identity between TAG and WE standards was detected, especially when cells of *R. opacus* B4 were cultivated with thermally treated cork and with natural cork not contaminated with hexadecane (lanes 5 and 6, respectively).

Table 4.3 presents TAG contents of *R. opacus* B4 cells in all tested conditions. It was observed that when cells of *R. opacus* B4 were transferred to MS medium supplemented with 1 g L^{-1} of hexadecane under storage conditions (B4_C16 initial time) TAG production amounted to 0.34 g g^{-1} CDW. This result indicated that TAG accumulation occurred already in the growth stage (stage I, C/N=4) and approximately 0.94 mg of TAG were transferred to the accumulation stage (stage II). After 48 hours of cultivation under nitrogen limiting conditions (stage II, C/N=300) there was a significant increase of TAG production, reaching 0.47 g g^{-1} CDW ($p < 0.05$).

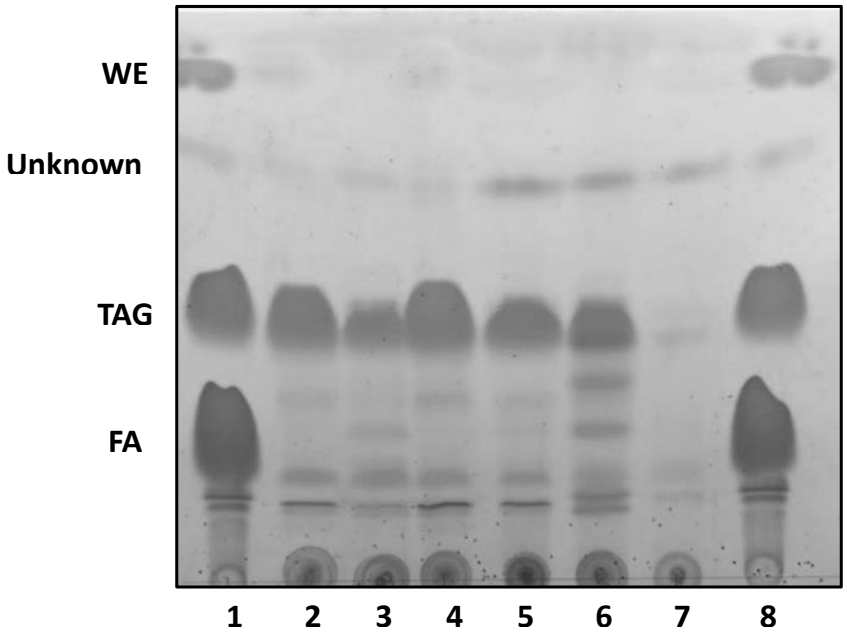


Figure 4.2 TLC analysis of neutral lipid compounds present in cells of *R. opacus* B4. Cells were firstly grown on MS medium supplemented with 1 g L^{-1} hexadecane at a molar C/N ratio of 4. After growth, cells were transferred to fresh MS medium supplemented with hexadecane /1 g L^{-1}) contaminated cork residues with a molar ratio of C/N 300 during 48 hours. 1 and 8 – neutral lipids standards; 2 – B4_NC_C16; 3 – B4_C16 (initial time); 4 – B4_C16; 5 – B4_TTC_C16; 6 – B4_NC; 7 – B4_TTC.

When *R. opacus* B4 was cultivated in the presence of each type of cork without the addition of hexadecane, lower amounts of TAG were detected, namely 0.25 g g^{-1} in B4 _NC and 0.08 in B4_TTC ($p<0.05$), indicating that this strain was able to preferentially consume the previously stored TAG instead of consuming the remaining hexadecane (57.4 %). These results are in agreement with other works that demonstrated that strains of *Rhodococcus* can mobilize and use the accumulated TAG in the absence of an external carbon source (Alvarez *et al.*, 2000).

Cells of *R. opacus* B4 cultivated in the presence of thermally treated cork contaminated with hexadecane showed significantly higher TAG levels, namely 0.97 g g^{-1} (B4_TTC_C16), than for natural cork contaminated with hexadecane (B4_NC_C16) which accumulated 0.60 g g^{-1} ($p< 0.05$).

TAG yields, expressed as grams of TAG formed per gram of hexadecane consumed by *R. opacus* B4 are described in Table 4.3. The highest yields were obtained in the presence of hexadecane-contaminated cork sorbents, with higher yield in the presence of contaminated thermally treated cork (0.69 g g^{-1}) than with contaminated natural cork (0.59 g g^{-1}) ($p<0.05$). These results are probably related to the higher porosity of thermally treated cork, hence leading to an easier hexadecane access by *R. opacus* B4 (Pintor *et al.*, 2012). The lowest TAG yields were obtained for

R. opacus B4 cultivated on cork sorbents, since the only residual amount of hexadecane available was the one transferred from the growth stage, therefore blocking TAG production.

Table 4.3 TAG content and yields in cells of *R. opacus* B4 cultivated in MS medium at a molar C/N ratio of 300 supplemented with 1 g L^{-1} hexadecane (B4_C16); hexadecane contaminated natural cork (B4_NC_C16); hexadecane contaminated thermally treated cork (B4_TTC_C16); natural cork (B4_NC) and thermally treated cork (B4_TTC) after 48 hours

Experiment	TAG content (g g^{-1} CDW)	TAG formed (g)	TAG yield (g TAG g^{-1} C16 consumed)
B4_C16 (initial time)	0.34 ± 0.15	—	—
B4_C16	0.47 ± 0.04	22.6	0.47
B4_NC _C16	0.60 ± 0.06	25.0 ± 0.9	0.59 ± 0.04
B4_TTC_C16	0.97 ± 0.04	32.0 ± 7.8	0.69 ± 0.06
B4_NC	0.25 ± 0.03	4.22 ± 0.3	0.38 ± 0.03
B4_TTC	0.08 ± 0.02	0	0

4.3.4 Fatty acid composition of TAG produced by *R. opacus* B4

The quantitative and qualitative composition of TAG in terms of fatty acids is a determinant factor in several biotechnological and industrial applications. The fatty acid composition of TAG fraction in cells of *R. opacus* B4 is presented in Table 4.4. In all tested conditions, palmitic acid (C16:0) was the main fatty acid present, with values ranging from 56 % to 83 %. These results are in accordance with several works (Alvarez *et al.*, 1996; Alvarez *et al.*, 1997a; Alvarez, 2003; Gouda *et al.*, 2008; Hernandez *et al.*, 2008; Silva *et al.*, 2010), revealing that the fatty acids produced were directly related to the chain length of the carbon source. The fatty acid composition of TAG fraction produced from hexadecane-contaminated cork showed some differences according to the type of cork. In the case of natural cork contaminated with hexadecane (B4_NC_C16), *R. opacus* B4 did not produce fatty acids shorter than C12:0, whereas the longer chain fatty acids (C14 to C18) were most predominant, accounting to 85 % of the total fatty acids. On the other hand, in the experiment performed with hexadecane-contaminated thermally treated cork (B4_TTC_C16) shorter chain fatty acids, namely C8:0; C10:0 and C14:0 were present in trace amounts.

Table 4.4 Fatty acid profile of TAG fraction in *R. opacus* B4 cultivated in hexadecane (B4_C16); hexadecane contaminated natural cork (B4_NC_C16); hexadecane contaminated thermally treated cork (B4_TTC_C16); natural cork (B4_NC) and thermally treated cork (B4_TTC) under nitrogen limiting conditions (C/N=300) during 48 hours.

Experiment	Relative proportion of fatty acids [%, w/w]							
	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1
B4_C16_t0	——	——	*	2.6 ± 0.5	67.4 ± 4.8	8.1 ± 2.3	35.2 ± 5.9	7.8 ± 3.1
B4_C16	——	——	*	3.7 ± 0.3	58.6 ± 1.7	21.6 ± 3.4	7.2 ± 1.2	2.8 ± 0.02
B4_NC_C16	——	*	10.5 ± 2.2	4.5 ± 1.2	67.7 ± 9.7	13.5 ± 1.5	7.8 ± 2.4	1.8 ± 0.4
B4_TTC_C16	1.5±0.2	2.6 ± 0.7	*	5.5 ± 1.7	71.5 ± 11.6	21.0 ± 9.6	20.9 ± 3.7	2.8 ± 0.7
B4_NC	6.2±1.2	1.7± 0.4	1.5 ± 0.4	6.5 ± 1.1	82.8 ± 8.8	10.4 ± 5.0	——	1.3 ± 0.2
B4_TTC	——	——	1.4 ± 0.6	2.0 ± 0.3	55.5 ± 0.6	8.5 ± 2.8	3.8 ± 0.6	3.5 ± 0.6

Biodiesel is mainly composed by fatty acid methyl esters (FAME) obtained from vegetable and animal oil sources through a process of TAG transesterification. Thus it has been considered a promising alternative to the fossil fuels (Papanikolaou *et al.*, 2008; Easterling *et al.*, 2009). More recently, a considerable interest has been given to oleaginous bacteria as a potential source of lipids to be applied in oil industries. Several bacteria are characterized by the production of high levels of fatty acids, incorporated in TAG and WE (Wältermann & Steinbüchel, 2005). In fact, bacterial TAG have similar characteristics when compared to those from plant and animal origins, but at the same time had their own features. Namely, it is possible to change bacterial TAG composition by altering the carbon source used for cultivation (Alvarez & Steinbuchel, 2002). Some of the most important characteristics that significantly affect the quality of biofuels are the length of the fatty acids chain and also the level of unsaturation (Knothe, 2005). In this study, both hexadecane contaminated cork sorbents promoted accumulation of fatty acids with longer chains by *R. opacus* B4. In particular, natural cork contaminated with hexadecane led to the accumulation of palmitic acid (C16:0); palmitoleic acid (C16:1) and oleic acid (C18:0), making these TAG suitable options for biodiesel production, since these fatty acids are found in plants and animal oils and improves oxidative stability (Meng *et al.*, 2009). Some reports found in the literature corroborate these results, revealing the importance of TAG produced by strains *of Rhodococcus*, using different carbon sources, in biofuels industry (Gouda *et al.*, 2008; Kurosawa *et al.*, 2010; Kosa & Ragauskas, 2012; Cortes & de Carvalho, 2015).

4.3.5 Biomethane production from *R. opacus* B4 lipid-rich biomass

After TAG accumulation, the biochemical methane potential of the resulting fatty biomass (TAG containing cells + cork) was evaluated in batch assays. Figure 4.3 presents the cumulative methane production as a function of time from *R. opacus* B4 lipid-rich biomass cultivated in hexadecane contaminated cork sorbents. Methane production started to occur after the first day of digestion in all the assays and maximum methane production was achieved after 7 days of incubation.

Table 4.5 shows biomethane production and conversion yields of *R. opacus* B4 lipid-rich biomass cultivated in hexadecane contaminated cork sorbents. The highest biomethane production and conversion yield in terms of COD was obtained for *R. opacus* B4 biomass cultivated on natural cork (B4_NC_C16), reaching 0.42 mL CH_4 per gram of added substrate and 0.815 g COD produced g^{-1} COD added, demonstrating that about 81 % of this biomass was converted to methane. *R. opacus* B4 biomass from hexadecane contaminated thermally treated cork (B4_TTC_C16) reach similar biomethane production values (0.40 g CQO produced g^{-1} substrate added). However, conversion yield was relatively lower than B4_NC_C16, where only 71.1 % of COD was converted to methane.

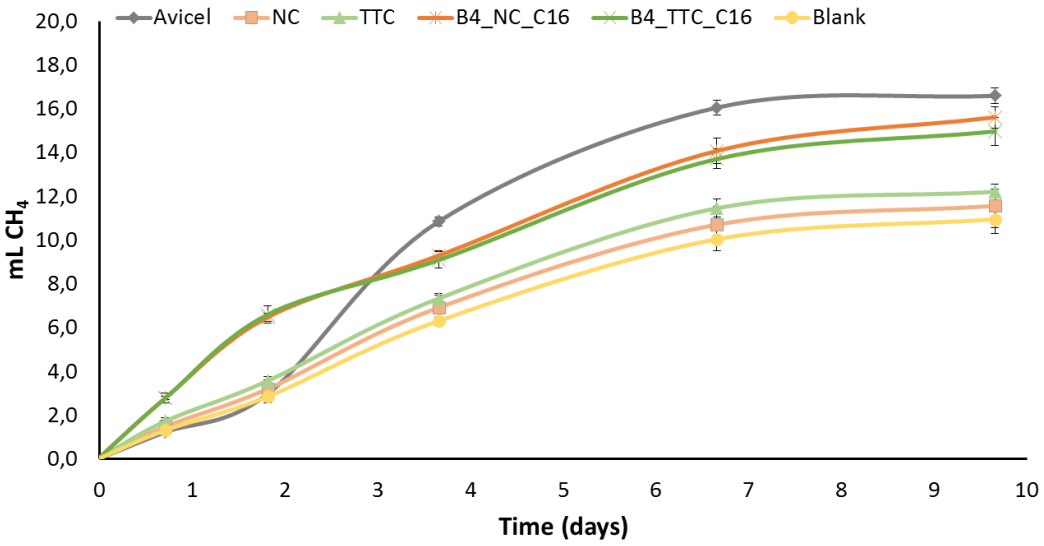


Figure 4.3 Cumulative methane production of *R. opacus* B4 lipid-rich biomass cultivated in hexadecane contaminated thermally treated cork sorbent (B4_TTC_C16) and in hexadecane contaminated natural cork sorbent (B4_NC_C16), of natural cork (NC) andthermally treated cork (TTC). Blank: sludge used as inoculum, Avicel: control assay.

The effect of the two cork sorbents on biomethane production was also evaluated. Biomethane production from natural cork (NC) was very low, 0.034 mL CH_4 g^{-1} substrate. Methane production from thermally treated cork (TTC) was approximately two fold higher when compared to NC. Both cork sorbents presented very low conversion yields, where only 16 % and 32 % of COD were converted to methane in NC and TTC, respectively. These results reveal that bacterial lipid-rich biomass has a high energetic potential in terms of methane production, due to its high TAG content. In fact, lipids are good candidates for biomethane production, since higher levels of methane can be produced when compared to low energetic compounds, namely carbohydrates or proteins (Alves *et al.*, 2009).

Table 4.5 Biomethane production and conversion yields in *R. opacus* B4 lipid-rich biomass cultivated in hexadecane contaminated cork sorbents.

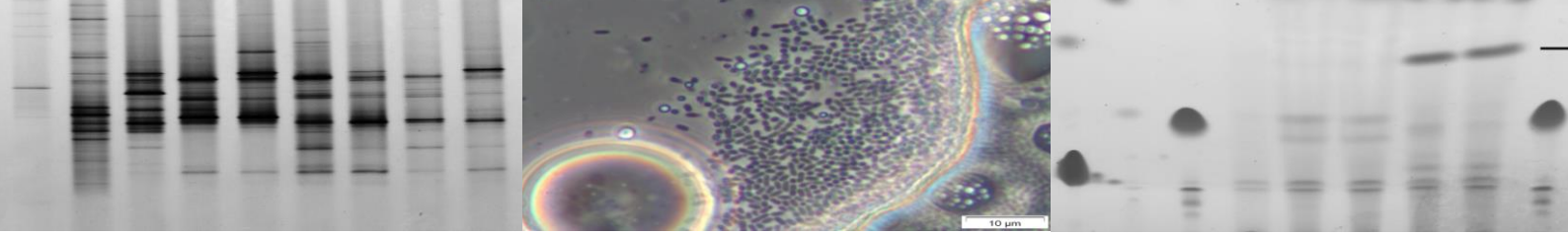
Experiment	Biomethane production* (mL CH_4 mg^{-1} substrate)	Conversion yield (g COD produced g^{-1} COD added)
B4_NC_C16	0.42 ± 0.023	0.815 ± 0.04
B4_TTC_C16	0.40 ± 0.023	0.711 ± 0.04
NC	0.034 ± 0.004	0.158 ± 0.01
TTC	0.058 ± 0.005	0.323 ± 0.02
Avicel	0.51 ± 0.011	1.05 ± 0.01

*Biomethane production from blank assay was discounted

4.4 Conclusions

In this work it was demonstrated that cork used as oil-spill sorbents, contaminated with liquid hydrocarbons, herein demonstrated with hexadecane, can be biologically treated by *R. opacus* B4 with concomitant valorization by production of neutral lipid storage compounds, mainly triacyglycerols. *R. opacus* B4 was able to consume the majority of hexadecane impregnated in natural cork and thermally treated cork, revealing that both types of cork did not affect hexadecane degradation. The hexadecane consumed by *R. opacus* B4, in nitrogen limiting conditions, was redirected to the production and accumulation of TAG. Fatty acid composition of TAG fractions obtained showed that both cork residues promoted the accumulation of fatty acids of 16 and 18 carbon long chains by *R. opacus* B4, demonstrating the feasibility of using this TAG as raw material for several industrial applications, namely in biodiesel industry. Additionally, the obtained lipid-rich

biomass (whole cells) can be efficiently used for biomethane production. The obtained results support a novel approach for the management of oil-spill contaminated cork sorbents through its valorization by production of bacterial lipids that can be used as raw materials for biofuels production, minimizing economical costs and environmental impacts, when compared to conventional treatment technologies. As future work, this type of approach should be applied to real and complex hydrocarbon residues and the economic viability of this type of lipid-rich biomass further assessed.



Chapter 5

Selection of hydrocarbonoclastic communities towards production of carbon storage compounds of biotechnological relevance

Hydrocarbonoclastic bacteria are important players in bioremediation of hydrocarbon contaminated ecosystems with additional potential for application in biological treatment of industrial wastewaters. Synthesis and accumulation of storage lipids such as triacylglycerols (TAG) and wax esters (WE), as well as polyhydroxyalkanoates (PHA), has been reported in this group of bacteria when submitted to growth-limiting conditions (e.g. nitrogen limitation). These compounds are relevant raw materials for the oleochemical industry. Its biosynthesis in combination with industrial wastewater treatment can contribute to make the process more economic and environmentally sustainable. In this work, sludge collected at the wastewater treatment plant from a heavy machinery maintenance service unit was enriched in carbon storage producing bacteria. Wastewater collected from the same service unit, containing lubricant and engine oil waste, was used as carbon source. The selective pressure was applied in the form of alternating periods of presence of the carbon substrate (feast conditions) followed by its absence (famine conditions). Cells having stored sufficiently amounts of reserve materials can survive the starvation period, whereas non-accumulating bacteria were not able to survive. Throughout the enrichment, biomass samples were collected and characterized in terms of lipid storage profiles and bacterial diversity by 16S rRNA-based techniques (DGGE, cloning and sequencing).The analysis of bacterial 16S rRNA gene V6-V8 region profiles obtained by DGGE, revealed a decrease of bacterial diversity along the enrichment process, until establishment of a nearly stable profile. Most dominant DGGE-bands in the final community corresponded to sequences with high similarity (99 %) to those of members of the genus *Rhodococcus*, *Acinetobacter* and *Pseudomonas* which are known for their ability to produce TAG, WE and PHA, respectively. In the applied conditions, the enriched community was able to accumulate small amounts of TAG (< 5 % of cell dry weight), and a non-identified lipidic compound (29 % ± 5.7 % cell dry weight), as well as PHA (3.8 % ±1.05 %). The results obtained in this work show the potential of using feast and famine conditions as a selective pressure to obtain a hydrocarbonoclastic community able to produce several types of lipidic storage compounds from real hydrocarbon-based wastewaters.

5.1 Introduction

Nowadays due to the increasing development of several types of oil industries, high levels of by-products, wastes and effluents are being formed. These industries are mainly related to petroleum extraction and transportation, automotive repair and maintenance garages, lubrication and maintenance of industrial machinery and car washing. These types of residues are mainly composed by complex mixtures of alkanes, aromatic hydrocarbons, phenols, asphaltenes, emulsified oils, solvents and lubricants (Bhattacharya *et al.*, 2003). Therefore, there is a general concern about the disposal and treatment of the resulting contaminated residues. The release of these wastes into the environment without previous treatment can lead to dramatic consequences, namely to ecosystems as well as to public human health (Baheri & Meysami, 2002). Conventional remediation techniques, such as landfarming and incineration are often applied (Vidali, 2001). However, these techniques are expensive and environmentally unfriendly and most of the times cannot be considered a definitive solution (Ouyang *et al.*, 2005; Sood & Lal, 2009). One attractive alternative treatment for oil contamination is bioremediation (Bragg *et al.*, 1994; Prince *et al.*, 1994).

Bioremediation processes consist in the application of microorganisms to reduce and transform injurious compounds into less harmful forms (Prince & Sambasivam, 1993; Lal & Khanna, 1996) and is considered one of the most efficient, economical and innocuous methods to decontaminate hydrocarbon contaminated environments (Gogoi *et al.*, 2003; Morelli *et al.*, 2005). Due to its complex nature, a single microorganism is usually not able to degrade all constituents of oil wastes. In last years, several works were performed in the biodegradation of oily residues, using pure cultures and also bacterial enrichments (Mishra *et al.*, 2001; Chaillan *et al.*, 2004). Mixed bacterial consortia can combine actions being more efficient in hydrocarbon bioremediation processes (Boopathy, 2000; Revellame *et al.*, 2012). This type of wastes promotes the dominance of hydrocarbon-degrading bacterial populations, able to degrade the pollutants. On the other hand, oil-rich wastewaters are characterized by high carbon concentrations and very low amounts of nitrogen, which favors the occurrence of carbon storing bacteria. These bacteria produce lipid storage compounds, namely triacylglycerols (TAG), wax esters (WE) and polyhydroxyalkanoates (PHA). These compounds have important biotechnological applications, namely in the biodiesel, oleochemical and bioplastics industries (Steinbüchel, 2001; Wahlen *et al.*, 2009; Röttig *et al.*,

2010). Therefore, the establishment of a selective enrichment process to obtain indigenous lipid accumulating and hydrocarbon degrading bacterial community is of major importance, since it combines two processes: treatment of oil wastes and production of lipids that can be used as feedstocks for biotechnological purposes. In this work, an enrichment process was established by using wastewater containing lubricant and engine oil waste as carbon source and sludge collected from the same location as inoculum. The selective pressure was applied in the form of alternating periods of presence of the carbon substrate (feast conditions) followed by its absence (famine conditions).

5.2 Material and Methods

5.2.1 Sludge and wastewater source

Sludge collected at a wastewater treatment plant from a heavy machinery maintenance service unit (ALSTOM Portugal, S.A., Maia) was enriched in carbon storage producing bacteria. This inoculum was in a medium poor in nutrients but rich in hydrocarbons, conditions that are favorable to the existence of bacteria with ability to store carbon in intracellular compounds. Wastewater collected from the same service unit, containing lubricant and engine oil waste, was used as carbon source for the enrichment process. Only the liquid fraction of the wastewater was used.

5.2.2 Multistage selective enrichment

The selective pressure was applied in the form of alternating "feast" (carbon rich, nitrogen poor) and "famine" (carbon poor, nitrogen poor) periods (Figure 5.1). Cells having stored sufficient amounts of carbon reserves during the "feast" period (accumulation stage) were able to survive in the "famine" period, whereas bacteria that had no carbon reserves were not able to survive (selection stage). This process was performed on MS medium which contained (per liter of distilled water) 9 g of $Na_2HPO_4.12H_2O$, 1.5 g of KH_2PO_4, 0.2 g of $MgSO_4.7H_2O$, 1.2 g of Fe-NH_4-citrate, 20 mg of $CaCl_2$, 2 mL of Hoagland solution and 0.5 g of $NaHCO_3$ (Schlegel *et al.*, 1961). The assays were performed at 30°C with shaking (150 rpm). Cyclosporine was used to inhibit fungal growth. In the accumulation stages, carbon was provided by the wastewater, at a CQO concentration of 5 g L^{-1}, and no nitrogen source was added. This resulted in a high C/N ratio, stimulating the

accumulation of storage compounds by competent microorganisms. In the selection stages, neither carbon nor nitrogen was provided. The total process lasted 13 cycles, corresponding to 7 months. In order to have enough biomass, growth stages of 4 days were performed at the end of cycles 6, 10 and 13. In the first period (6 cycles), the accumulation and selection stages lasted 7 days each and in the second period (7 cycles), the accumulation stages lasted 3 days. Biomass samples were collected at the end of accumulation stages of cycles 3, 5, 6, 10, 11 and 13, for further evaluation of the neutral lipids profiles. Samples were collected at the end of selection and growth stages of cycles 3, 6, 10 and 13 for molecular characterization of bacterial community over time.

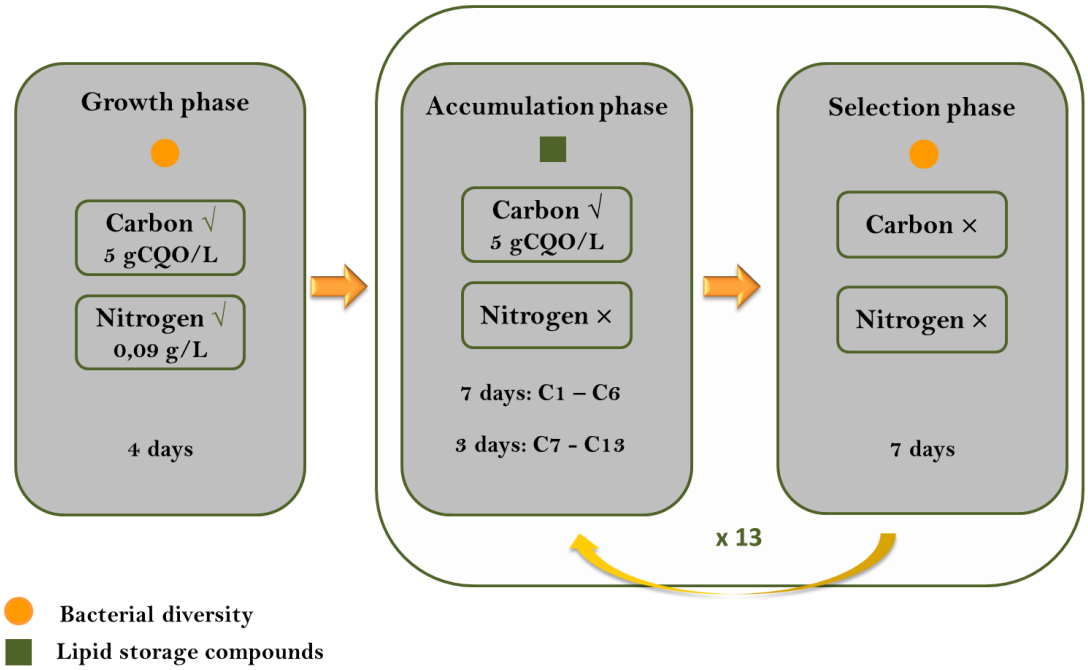


Figure 5.1 Schematic representation of the enrichment process applied.

5.2.3 Analytical Methods

Chemical oxygen demand (COD), total nitrogen (TN), ammonium (NH_4^+), phosphate (PO_4^{3-}), nitrates, nitrites and total ammonia nitrogen TAN present in the wastewater were determined using the cuvette-tests Lck914, Lck338, Lck386, Lck305, Lck340, Lck341 and Lck303, respectively (Hach-Lange, Germany). These measurements were made in triplicate, according to the manufacturer's procedures.

5.2.4 Neutral lipids extraction, analysis and quantification

The experimental procedures for the extraction, analysis and quantification of neutral lipid in the enriched bacterial community are described in sections 3.2.3.1; 3.2.3.2 and 3.2.3.3 included in Material and Methods of Chapter 3.

5.2.5 PHA extraction and quantification

The accumulation of two types of PHA, PHB (polyhydroxybutyrate) and PHV (polyhydroxyvalerate), by the microbial community were determined according to the procedure described in (Vieira, 2005). Freeze-dried biomass of each sample was weighed (8 to 20 mg) into digestion tubes. A blank digestion tube and digestion tubes with PHB and PHV standards in a range between 0.4 and 2.7 mg of these polymers were also prepared, with intervals of approximately 0.3 mg. These standards were used to plot the standard curves. To the digestion tubes were added 1.5 mL of internal standard benzoic acid in dichloromethane (1 mg mL^{-1}) and 1.5 mL of 1-propanol:hydrochloric acid (75:25 v/v). The tubes were closed and shaken vigorously in vortex to facilitate the initial contact between the reactants and the biomass or the standards and placed in a digester block at 100°C for 3.5 hours. At the end of the digestion, the tubes were removed from the digester and allowed to cool at room temperature. Then, the content of the digestion tubes was transferred to 10 mL glass flasks. The tubes were washed with 2 mL of ultrapure water, agitated and then transferred to the glass flasks. The flasks were sealed and shaken vigorously for 1 minute. Thus, it was ensured that the two phases came in full contact, optimizing extraction.

Thereafter, the bottles were inverted for 20 minutes to allow phase separation. The desired phase is the heavier one, which contains the ester. The organic phase was transferred to 1.5 mL glass vials to which was added Na_2SO_4 to ensure the complete dehydration of the organic phase. Finally, the vials were shaken on vortex and then stored at 0 °C.

The samples, standards and blanks were analyzed by gas chromatography (GC) with flame ionization detector (GD-FID). The GC equipment was a GC Varian 3800, Varian Inc. USA with a TR-WAX (Teknokroma, Spain) column, 30m x 0.32 mm x 0.25 mm. The injector and detector temperatures were 220°C and 250°C, respectively. Column temperature was 50°C for 2 minutes, with a 15 °C min^{-1} ramp to 225 °C, and a final hold at 225 °C for 5 minutes. Helium was used as carrier gas at 50 KPa and 1 mL min^{-1}.

5.2.6 Total hydrocarbons extraction and analysis

Hydrocarbon content in the wastewater was analysed at SGS Portugal (Sociedade Geral de Superintendência, S.A.) according to DIN EN 14039/EN ISO 16703.

Extraction of the hydrocarbons present in the culture media was performed based on procedures disclosed in NJDEP EPH 10/08 (NJDEP, 2010) and began with the acidification of media with hydrochloric acid to a pH below 2.0. Acidified media were transferred to 50 mL separatory funnels. Then, 7.5 mL of chloroform: methanol (3:1, v/v) was added to each flask and transferred to the corresponding separatory funnel (Koma*, et al.*, 2001). The separatory funnels were vigorously stirred for 2 minutes and then allowed to rest for 5 minutes to allow the organic and aqueous phase separation and the organic phases were carefully collected. This process was performed three times. This process was also performed in control flasks, corresponding to the beginning of the assays.

The extracts were cleaned on Florisil Sep-Pak columns 6cc (Waters, USA) in a vacuum manifold. The columns were equilibrated with 6 mL of methanol:chloroform (3:1) and the samples were passed through columns to new flasks. Then, additional 6 mL of solvent were passed through the columns to remove any remaining hydrocarbons. The samples were allowed to evaporate in the hood, suspended in 1.5 mL of hexane and were analyzed by GC-FID.

The gas chromatograph equipment (GC Varian® star 3400CX) was adjusted with the following parameters values for accurate and wide hydrocarbon range analysis. The injector temperature was set to 285ºC. The column model was VF-1ms, 30 m long and 0.25 mm internal diameter, and made from fused silica coated with dimethylpolysiloxane as stationary phase. The column temperature was held a 60ºC for 1 minute and then programmed up to 290ºC at 8ºC min^{-1}. The carrier gas was helium and its flow rate of 1 ml min^{-1}. The detector was operated at 300ºC. Each run took 47.5 minutes. Total hydrocarbons (TH) concentration was calculated by determining the total area, resolved peaks and unresolved complex mixture (UCM), between C10 and C40 standards, and comparing to a calibration made with mineral oil standard mixture type A (diesel fuel) and B (lubricant oil) for EN 14039 and ISO 16703 (Fluka Analytical).

5.2.7 DNA extraction and amplification

Aliquots of well-homogenized sludge were immediately frozen at the time of sampling and stored at −20°C. Total genomic DNA was extracted using a FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad,

CA, USA) according to the manufacturer's instructions. 16S rRNA genes were amplified by PCR using a Taq DNA Polymerase kit (Life Technologies, Gaithersburg, MD, USA). All primers used were synthesized commercially by Invitrogen (Life Technologies) and are listed in Table 5.1. Size and yield of PCR products were estimated using a 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) via 1,5 % (wt/vol) agarose gel electrophoresis and SYBRSafe (Invitrogen - Life Technologies) staining. Primer set 968-f/1401-r (Muyzer *et al.*, 1993; Nübel *et al.*, 1996) was used for amplification of the regions V6 to V8 of the 16S rRNA gene, for denaturing gradient gel electrophoresis (DGGE) purpose. Primer 968-f was modified by the addition of a 40-bp GC clamp at the 5′ end of the sequence. The thermocycling program used for this amplification was: predenaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40 s and elongation at 72°C for 90 s, and postelongation at 72°C for 5 min. The reactions were subsequently cooled to 4°C. Primer set Bact27-f/Uni 1492-r was used for 16S rRNA gene amplification, for sequencing purposes (Lane, 1991). The thermocycling program used for this amplification was: predenaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s and elongation at 72°C for 90 s, and postelongation at 72°C for 5 min. The reactions were subsequently cooled to 4°C.

Table 5.1 Oligonucleotides used in this study

Oligonucleotide	Sequence (5'–3')	Specificity	Reference or source
Bact27-f	GTT TGA TCC TGG CTC AG	Bacterial 16S	Lane (1991)
Uni1492-r	CGG CTA CCT TGT TAC GAC	Universal 16S	Lane (1991)
968-f	AAC GCG AAG AAC CTT AC	Bacterial 16S	Nübel *et al.*, (1996)
1401-r	CGG TGT GTA CAA GAC CC	Bacterial 16S	Nübel *et al.*, (1996)
GC clamp	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G		Muyzer *et al.*, (1993)
PG2-r	GGC CGC GAA TTC ACT AGT G	pGEM-T	Promega Corp.
PG1-f	TGG CGG CCG CGG GAA TTC	pGEM-T	Promega Corp.
T7	TAA TAC GAC TCA CTA TAG GG	pGEM-T	Promega Corp.
SP6	GAT TTA GGT GAC ACT ATA G	pGEM-T	Promega Corp.

5.2.8 DGGE analysis

DGGE analysis of the amplicons was performed in a Dcode system (Bio-Rad, Hercules, CA, USA) with 8 % (vol/vol) polyacrylamide gels and a denaturant gradient of 30 to 60 % or 40 to 60 %. A 100 % denaturing solution was defined as 7 M urea and 40 % formamide. Electrophoresis was performed for 16 hours at 85 V in 0.5× TAE buffer at 60 °C. DGGE gels were stained with $AgNO_3$ according to the procedure previously described by (Sanguinetti CJ, 1994).

5.2.9 Cloning and sequencing of PCR-amplified products

PCR products of 16S rRNA gene were purified with Nucleo Spin Extract II kit (Clontech Laboratories) and cloned into E. coli® 10G Electrocompetent Cells (Lucigen® Corporation) by using Promega pGEM-T Easy vector system (Promega, Madison, WI, USA). PCR was performed on cell lysates of ampicillin-resistant transformants by using pGEM-T-specific primers PG1-f and PG2-r (Table 4.1) to confirm the size of the inserts. The thermocycling program used for this amplification was the same that was used with Bact27-f/Uni 1492-r primer set. Clones with the correct size insert were further amplified with primer set 968-f/1401-r for DGGE comparison with original sample profiles. PCR products of transformants resolving at the same position of predominant bands in the DGGE community fingerprint were chosen for further analysis. Selected clones were amplified with using pGEM-T vector-targeted primers SP6/T7 (Table 4.1), purified using the Nucleo Spin Extract II kit (Clontech Laboratories), and subjected to DNA sequence analysis. The thermocycling program used for the amplification with SP6/T7 primer set was: predenaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s and elongation at 72°C for 90 s, and postelongation at 72°C for 5 min. The reactions were subsequently cooled to 4°C. Sequencing reactions were performed at Eurofins MWG, Germany. Partial sequences were assembled using the CAP application included in the BioEdit v7.0.9 software package (Hall, 1999). Consensus sequences obtained were checked for potential chimera artifacts using Mallard v1.02 (Ashelford *et al.*, 2006) and Pintail v1.1 (Ashelford *et al.*, 2005) software.

5.2.10 Phylogenetic analysis

Similarity searches for the 16S rRNA gene sequences derived from the clones were performed using the NCBI BLAST search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul*, et al.*, 1990).

5.3 Results and Discussion

5.3.1 Wastewater characterization

Table 5.2 describes several chemical parameters of the oily wastewater used as carbon source in the selective enrichment process. This wastewater is characterized by a high COD, which reflects an elevated organic load and also by high amounts of total hydrocarbons. The nitrogen-containing compounds were present in very low amounts. These conditions are optimal for the development of a microbial community rich in lipid accumulating bacteria. The wastewater pH is slightly acid, which can inhibit bacteria that normally grow at neutral pH.

Table 5.2 Chemical charaterization of the wastewater used in this study

Parameter	Concentration
COD (g L^{-1})	307 ± 14
Total hydrocarbons (g Kg^{-1} DW)	720
TN (mg L^{-1})	172 ± 18
Nitrates (mg L^{-1})	94 ± 9
Nitrites (mg L^{-1})	0
TAN (mg L^{-1})	130 ± 40
Organic nitrogen (mg L^{-1})	77 ± 9
pH	5.5

5.3.2 Molecular characterization of the enriched bacterial community

During the development of the selective enrichment process, several biological samples were collected in order to analyse bacterial diversity by DGGE. The bacterial profiles are presented in Figure 5.2. The obtained DGGE profiles showed a decrease in bacterial diversity throughout time until the establishment of a relatively stable profile at the end of cycle 13 (C13f). In fact, while in inoculum profile (I) several bands of different intensities were visible, in further bacterial profiles there was a decrease in the number and intensity of some bands.

At the end of the enrichment process (C13f) only few bands were present. In particular, two predominant bands were detected (bands 1 and 3), which probably corresponds to the most dominant bacteria. Therefore, these results suggested that the conditions applied were effective in the establishment of a stable enriched bacterial community.

A significant shift in bacterial diversity was observed between C6 and C10 profiles. In fact, C3 and C6 bacterial profiles were very similar and considerably different from those of cycles 10 and 13, which in turn were also similar between them. These results are possibly related to the differences applied in terms of the duration of accumulation phases between cycles, since until cycle 10 the accumulation stage lasted 7 days and in the further cycles the selective pressure was intensified, with a decrease to 3 days of the accumulation stage. Therefore, the duration of accumulation steps may drastically affect bacterial diversity of the enriched community.

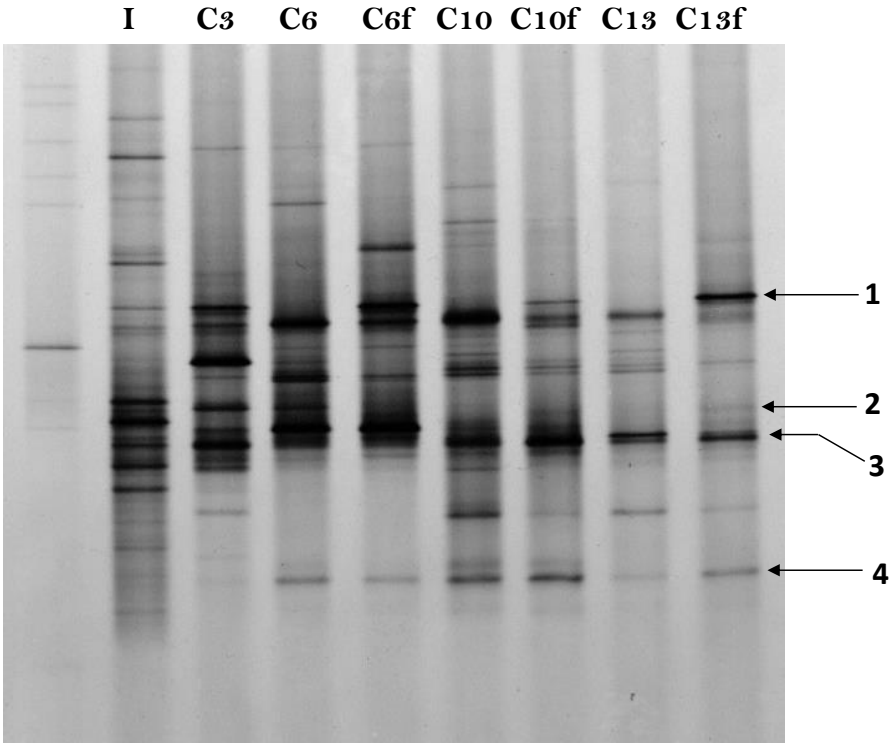


Figure 5.2 DGGE profiles of bacterial PCR-amplified 16S rRNA gene fragments obtained from the biomass collected during the enrichment process.; I – Initial inoculum; C3 - cycle 3; C6 – cycle 6; c6f – final phase of cycle 6; C10 – cycle 10 ; C10f – final phase of cycle 10; C13 – cycle13; C13f – final phase of cycle 13.

A significant shift in bacterial diversity was observed between C6 and C10 profiles. In fact, C3 and C6 bacterial profiles were very similar and considerably different from those of cycles 10 and 13, which in turn were also similar between them. These results are possibly related to the differences applied in terms of the duration of accumulation phases between cycles, since until cycle 10 the accumulation stage lasted 7 days and in the further cycles the selective pressure was intensified, with a decrease to 3 days of the accumulation stage. Therefore, the duration of accumulation steps may drastically affect bacterial diversity of the enriched community.

The identification of bacterial species present in the microbial community at the end of the enrichment process (cycle 13) was performed by 16S rRNA gene sequencing. Clones whose bands were not visible in the DGGE profile of the bacterial community (C13f) were also identified to obtain more information about the phylogeny of the species that are present probably in minor amounts in the community. From the results presented in Table 5.3 it can be seen that the majority of 16S rRNA gene sequences retrieved belong to the phylum Poteobacteria and to orders Pseudomonadales and Burkholderiales. The three most intense bands present in the DGGE profile (bands 1, 3 and 4) corresponded to sequences with high similarity to members of the genus *Acinetobacter*, *Pseudomonas* and *Rhodococcus* (99 % similarity) which are known for their ability to produce WE, PHA and TAG, respectively (Waltermann *et al.*, 2000; Ishige *et al.*, 2002; Tobin & O'Connor, 2005). The bands corresponding to *Acinetobacter* sp. WJ07 and *Pseudomonas extremaustralis* CT14-3 showed a significantly higher intensity when compared to the band corresponding to *Rhodococcus* sp. MN9-3. The remaining sequences showed high similarity with members of the genera *Opitutus* (93 %), *Pelomonas* (95 %), *Zoogloea* (98 %) and *Acidovorax* (99 %). Some species of *Acidovorax* and *Zoogloea* are described as capable of producing polyhydroxyalkanoates (PHA) (Fukui *et al.*, 1976; Renner *et al.*, 1996).

5.3.3 Neutral lipid storage compounds

The profile of neutral lipid storage compounds accumulated by the enriched microbial community was evaluated through thin layer chromatography (TLC) (Figure 5.3). In the initial phase of the enrichment (cycle 3), it was only possible to detect scarce bands at the level of fatty acids. However throughout the enrichment two distinct bands corresponding to TAG became visible (C5 and C6). This results are in agreement with the results obtained in DGGE profile, where there was an increase in intensity of the band that corresponds to *Rhodococcus* sp. 3-MN9, probably a TAG producer. The presence of two bands corresponding to TAG can possibly mean the presence of TAG with different fatty acid compositions, namely in chain length, level of polarity and degree of fatty acids saturation (Christie, 1989). However in the next stages there was a shift in TAG bands and only one single band was detected (C11 and C13). This can be related to the imposed decrease of the accumulation phase duration (from 7 to 3 days).

Table 5.3 Phylogenetic affiliation of the retrieved 16SrRNA gene sequences and correspondent band position in the DGGE profiles of enriched bacterial community throughout time

PB[a]	Phylum/Order[b]	Afilliation[c]	Similarity (%)
1	*Proteobacteria/ Pseudomonadales*	Uncultured *Acinetobacter* sp. clone AtlantisII_b (HQ530522)	99
		Acinetobacter sp. WJ07 (HM045831)	99
2	*Proteobacteria/ Burkhoderiales*	Uncultured bacterium clone S25_1600 (EF575256)	99
		Acidovorax sp. BSB421 (Y18617	99
3	*Proteobacteria/ Pseudomonadales*	*Pseudomonas extremaustralis* strain CT14-3 (AJ583501)	99
4	*Actinobacteria/ Actinomycetales*	*Rhodococcus* sp. MN9-3 (JQ396610)	99
Nd	*Proteobacteria/ Burkholderiales*	Uncultured *Comamonadaceae* bacterium clone D25_14 (EU266893)	99
		Acidovorax sp. isolate G8B1 (AJ012071)	99
Nd	*Verrucomicrobia/ Opitutales*	Uncultured bacterium clone 4y-121 (FJ444768)	98
		Opitutus terrae PB90-1 (NR_028890)	93
Nd	*Proteobacteria/ Burkholderiales*	*Acidovorax delafieldii* (AF078764)	99
Nd	*Proteobacteria/ Burkholderiales*	Uncultured bacterium clone Q7593-HYSO (JN391994)	96
		Pelomonas saccharophila strain DSM 654 (NR024710)	95
Nd	*Proteobacteria/ Rhodocyclales*	Uncultured bacterium SJA-21 (AJ009455)	99
		Zoogloea sp. EMB 62 (DQ413151)	98

[a]Bands marked in Fig.1
[b]Classified using the RDP Naive Bayesian Classifier (Wang *et al.*, 2007)
[c]Closest relatives in GenBank with accession number
nd – position on DGGE not detected

The content of the different microbial storage compounds was measured in the final phase of the enrichment and are described in Table 5.4. TAG content produced by the microbial community was very low, namely below 5 %. At this stage, among the identified community species only one belonged to the genus *Rhodococcus*, which is reported as specialist in TAG production (*Rhodococcus* sp. MN9-3) (Alvarez, 2013). According to the literature, most of the *Rhodococcus* species can accumulate TAG to up to 30 % of its cellular dry weight when are cultivated on hydrocarbons (Alvarez *et al.*, 1997a). The low levels of TAG accumulated by this community can be possibly explained by the presence of other bacteria that, in the conditions applied, do not produce

TAG or produce other types of storage compounds, limiting TAG accumulation as well as by an inadequate C/N ratio applied. Some authors reported a C/N ratio ranging between 50 and 500 as optimal for TAG accumulation in *Rhodococcus* species grown on hydrocarbons and sugars (Alvarez *et al.*, 1996; Alvarez *et al.*, 1997a; Alvarez, 2003; Gouda *et al.*, 2008; Kurosawa *et al.*, 2010). However, these studies were done with simple substrates, making it impossible to directly compare these values with the values used in this work. Interestingly, in the last cycles of the enrichment (C11 and C13, Figure 5.3) an intense band was detected between TAG and WE standards, which corresponded to 29 % of the cellular dry weight (Table 5.4).

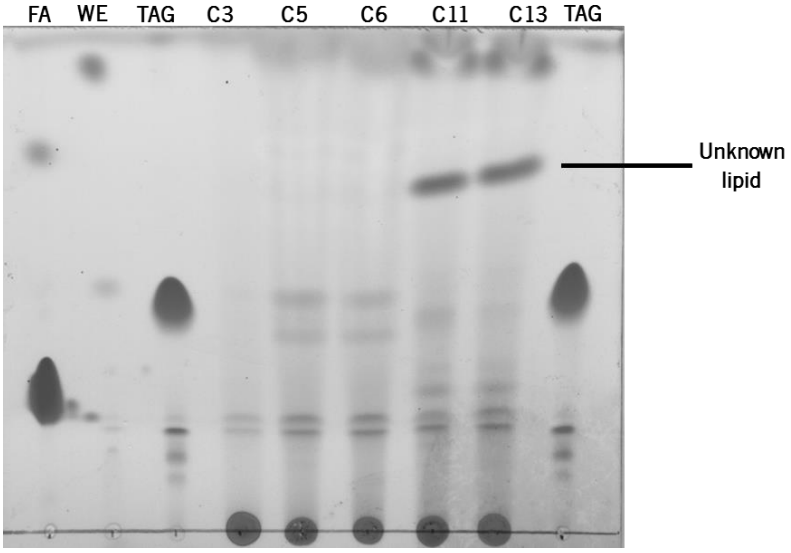


Figure 5.3 Neutral lipid profiles accumulated by the microbial community throughout the enrichment process. As solvent system was used hexane: diethyl ether: acetic acid (80:20:1 v / v / v). FA – Fatty acids; WE – Wax ester TAG – Triacylglycerol, C3 - cycle 3, C5 - Cycle 5, C6 - Cycle 6, C11 - Cycle 11; C13 - Cycle 13.

This unknown lipid may be a wax diester-like compound, as previously found by Kalscheuer & Steinbuchel (2003). According to this author, *Acinetobacter calcoaceticus* strain ADP1 is able to accumulate wax diesters under nitrogen limiting conditions, which resolve at similar TLC zone of the unknown lipid detected in this work. This result is supported by the phylogenetic identification of bacteria present at the end of the enrichment, where one of the most dominant bands corresponds to a member of the genus *Acinetobacter.* PHA content was also low, around 4 % of cellular dry weight. This result can be related to the presence of oxygen during the enrichment process. It is known that PHA accumulation in mixed microbial cultures increased if in the enrichment process anaerobic conditions with alternating aerobic condition are applied (Reis *et al.*, 2003).

Table 5.4 Neutral lipid content accumulated by the microbial community at the end of the enrichment process (C13)

Storage compound	TAG	PHB + PHV	Unknown lipid
(% CDW)	<5	3.8 ± 1.05	29 ± 1.3

5.3.4 Wastewater degradation

The enriched bacterial community ability to degrade hydrocarbons present in wastewater was assessed. Figure 5.4 illustrates the total hydrocarbon profiles at the beginning of the degradation assay (time 0) (A) and after 24 hours (B). The wastewater is mainly composed by medium chain hydrocarbons, between C9 and C12, and a complex mixture in the range between C17 and C40 hydrocarbons characterized by the presence of two significant GC-unresolved humps. At the end of degradation assay, the microbial enriched community was able to fully degrade short chain hydrocarbons, while longer chain hydrocarbons were also degraded, but in a minor extent. These results clearly demonstrated the feasibility of the developed enriched community to treat hydrocarbon-based wastewaters, supporting the phylogenetic identification, indicating the presence of hydrocarbon degrading-bacteria such as *Pseudomonas*, *Acinetobacter* and *Rhodococcus* genus (Larkin *et al.*, 2005; Palleroni *et al.*, 2010; Kostka *et al.*, 2011).

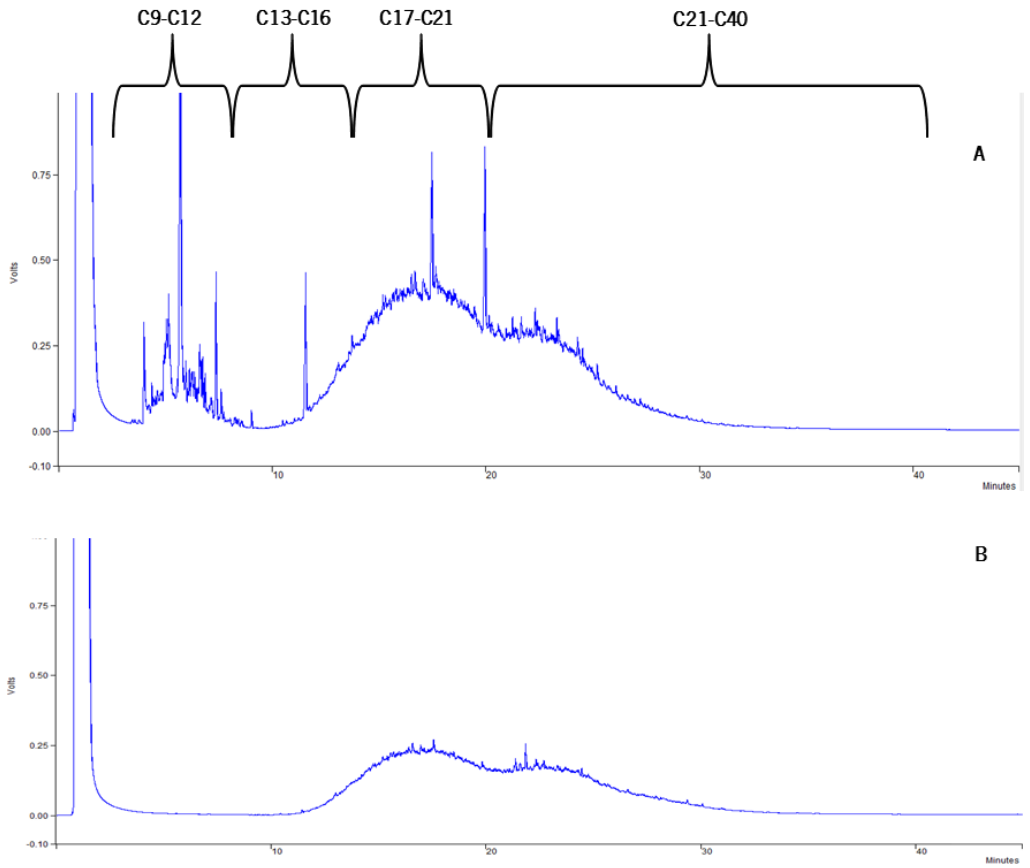
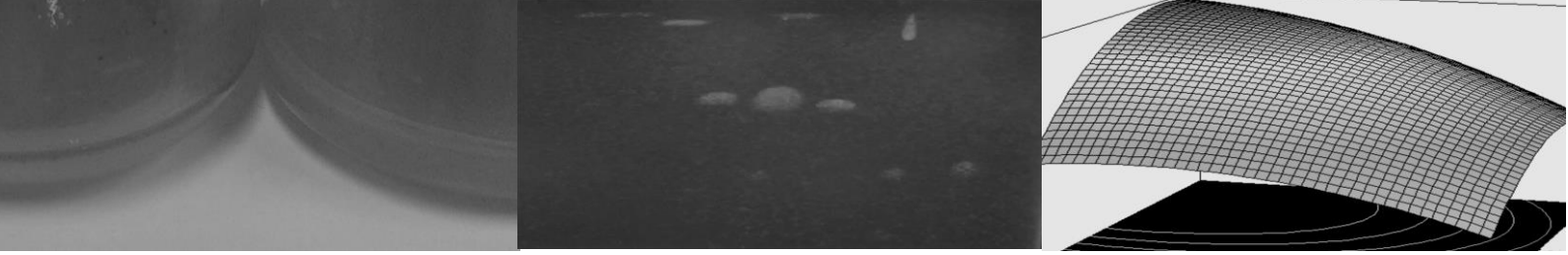


Figure 5.4 Total hydrocarbon profile of the enriched bacterial community in the beginning of the degradation assay (A) and after 24 hours degradation (B).

5.4 Conclusions

In this work, a multistage enrichment process was developed to obtain an indigenous microbial community enriched in hydrocarbon-degrading and carbon storage-accumulating bacteria, using a real oily wastewater as carbon source. In the conditions applied the bacterial enriched community was dominated by member of the genus *Rhodococcus*, *Pseudomonas* and *Acinetobacter* and accumulated, although in low levels, TAG (less than 5 % of the CDW) and PHA (4 % of the CDW) as well as an unknown lipid (29 % of the CDW), presumably a wax ester-like compound. The obtained mixed commutity was able to degrade almost all wastewater hydrocarbons. The application of this type of approach (successive cycles of feast and famine conditions) and further optimization of several operational parameters is a promising way to combine in a more economic and environmentally sustainable way two biotechnological processes: pollutant decontamination and bioenergy production.



Chapter 6

Influence of cultivation conditions on the production of carbon storage compounds in mixed hydrocarbonoclastic communities

The significant increase of global industrialization has been promoting the generation of large amounts of residues and wastewaters. In particular, oily wastewaters (contaminated with hydrocarbons) must be considered, since their disposal into the surrounding environments can represent a serious threat to several types of environmental resources. Simultaneously, the drastic depletion of fossil fuel resources demands for search of alternative feedstocks with environmental and economic advantages. Therefore, the production of bacterial lipids using inexpensive substrates, as wastes, has attracted much attention. The present work aims at identify the influence of several cultivation parameters on storage compound accumulation by an enriched bacterial community at the expense of a hydrocarbon-based wastewater. By applying a five-level-three factor central composite circumscribed design based on surface response methodology it was found that the interaction between carbon and nitrogen concentrations positively influenced polyhydroxyalkanoates (PHA) accumulation whereas interaction between carbon and nitrogen concentrations with cultivation time affected PHA in a negative way. Regarding neutral lipids production, nitrogen concentration and the interaction between carbon and nitrogen were the significant parameters. Neutral lipids produced were essentially triacylglycerol (TAG), presenting a highly diversity of chemical structures composed by a narrow range of fatty acids. Therefore, the mixed microbial community enriched in hydrocarbonoclastic and storage compound accumulating bacteria can be an effective inoculum to establish a more cost-effectively and ecofriendly biotechnological process combining valuable compounds production and treatment of hydrocarbon contaminated wastewater.

6.1 Introduction

Hydrocarbonoclastic bacteria have the ability to degrade a wide range of hydrocarbon compounds: branched, unbranched and cyclic alkanes as well as aromatics namely monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAH) and can be found in several distinct ecosystems, including marine waters, soils and sediments, especially in oil-polluted ones (Bragg *et al.*, 1994; Harayama *et al.*, 2004; Head *et al.*, 2006) Some of the most representative genera are *Alcanivorax*, *Pseudomonas, Acinetobacter* and *Rhodococcus* (Koma *et al.*, 2001; Larkin *et al.*, 2005; Cappello *et al.*, 2007; Palleroni *et al.*, 2010). In addition to biodegradation capabilities, members of this group can produce lipid storage compounds, namely TAG, WE and PHA (Alvarez & Steinbuchel, 2002; Manilla-Pérez *et al.*, 2011; Chen *et al.*, 2014a), which can be used as raw materials for the production of feed additives, cosmetics, lubricants, biofuels and bioplastics (Steinbüchel & Füchtenbusch, 1998; Alvarez, 2010).

The process of bacterial storage lipid production and accumulation is complex and the amount and type of carbon storage is highly dependent on several parameters, such as the species itself, carbon source type and cultivation conditions. Lipid accumulation is promoted under unbalanced growth conditions, when an excess of carbon is present but low levels of nitrogen occur, resulting in a high ratio of carbon to nitrogen in culture medium (Olukoshi & Packter, 1994; Alvarez, *et al.*, 2000; Manilla-Pérez*, et al.*, 2011). In general, cells in the initial and exponential phase of growth do not produce significant amounts of lipids, whereas in the stationary phase, there is a drastic increase (Packter & Olukoshi, 1995; Wältermann *et al.*, 2005). Wältermann *et al.* (2005) described that under conditions of cell proliferation *R. opacus PD630* produced low levels of TAG and *Acinetobacter calcoaceticus* ADP1 accumulated low TAG and WE. However, when transferred to lipid accumulation conditions, both bacteria increased significantly TAG and WE content after 24 hours. Several works reported differences occurring between species in terms of quantity and type of carbon storage compounds production. Several *Rhodococcus* strains grown on glucose and gluconate accumulated TAG as a major storage compound and PHAs in lower amounts, whereas with hexadecane only TAG were produced (Alvarez *et al.*, 1997a; Alvarez, 2003; Hernandez *et al.*, 2008). Interestingly, *R. ruber* is a peculiar strain since accumulates considerable PHA and TAG levels, but PHA production occurs already in exponential growth phase and TAG are only produced after, when cells enter the stationary growth phase (Alvarez *et al.*, 2000). Members of the genera

Alcanivorax and *Acinetobacter* presents different neutral lipids profiles, when compared to *Rhodococcus*. In a general way, WE are the most dominant storage lipid in these bacteria, whereas TAG are produced in lower amounts, depending on the carbon source used. *A. borkumensis* SK2 accumulated predominantly TAG when cultivated on pyruvate and similar levels of TAG and WE with hexadecane (Manilla-Pérez *et al.*, 2011). On the other hand, *Acinetobacter* sp. produced essentially WE from sugars and organic acids, while accumulated higher amounts when grown on hexadecane (Fixter *et al.*, 1986). Furthermore, magnesium, ammonium, phosphate and carbon source concentrations as well as pH medium, and osmolarity influence TAG accumulation in *R. opacus* PD630 (JanSzen *et al.*, 2013). Due to the complexity of factors influencing storage compounds production, several works regarding the optimization of lipid storage compounds and the influence of cultivation conditions were published (Gouda *et al.*, 2008; Kurosawa*, et al.*, 2010; Kurosawa, 2014). However, the use of pure bacterial cultures and defined carbon sources to obtain lipid storage compounds make the process quite expensive, due mainly to the high prices of substrates and also to the lower product yields (Reddy *et al.*, 2003). Additionally, there are several bacteria able to accumulate storage compounds at the expense of several types of residues and municipal wastewaters (Gouda *et al.*, 2008: Hall *et al.*, 2011; Revellame *et al.*, 2012).

In this work, the influence of several culture conditions, namely carbon and nitrogen concentrations (C/N ratio) and cultivation time, on storage compounds produced from a microbial community previously enriched in hydrocarbon degrading and storage compound accumulating bacteria, using an oil rich wastewater, was assessed. The enriched community was dominated by member of the genus *Rhodococcus*, *Pseudomonas* and *Acinetobacter* (Chapter 5), known for their ability to produce TAG, WE and PHA, respectively.

6.2 Material and methods

6.2.1 Sludge and wastewater source

The microbial community enriched in hydrocarbonoclastic lipid accumulating bacteria was obtained from sludge collected at a wastewater treatment plant from a heavy machinery maintenance service unit (ALSTOM Portugal, S.A., Maia) as previously described in section 5.2.2 included in Material and Methods of Chapter 5. Wastewater collected from the same service unit, containing lubricant and engine oil waste, was used as carbon source. Wastewater characterization was

performed as described in sections 5.2.3 and 5.2.5 included in Material and Methods of Chapter 5 and is presented in Table 5.2 of the same Chapter.

The enriched bacterial community was grown on autoclaved MS medium (composition described in section 5.2.2 included in Material and Methods of Chapter 5) supplemented with a COD concentration of 5 g L^{-1} of wastewater and 0.5 g L^{-1} of NH_4Cl to obtain high biomass levels. This process was performed in 7 cycles of 2 days each. Cells were transferred to new MS medium at the end of each cycle. Cellular growth was monitored by optical density at 600 nm.

6.2.2 Factorial experimental design and statistical analysis

To study the effects of several cultivation conditions and possible interaction effects on storage compounds production by the enriched bacterial community, a factorial central composite circumscribed design (CCCD) was used based on surface response methodology. The three factors (independent variables) selected were COD concentration, N concentration and accumulation time. For each independent variable five levels were chosen (: −a, −1, 0, +1, +a, where a = 2n/4; n=number of parameters and 0 corresponded to the central level), resulting on a total of 32 experiments (including duplicates and a quadruplicate central point). The experimental levels were selected by varying the parameters above and below the respective central level. The coded and uncoded levels of the independent variables are given in Table 6.1. The assays were performed in a random way. The software package Design-Expert ® (Stat-Ease, Inc., Minneapolis) was used to perform the design matrix of the experiments subjected to regression analysis and their statistical evaluation.

Table 6.1 Independent variables and levels used in central composite circumscribed design (CCC) for the production of storage compounds by enriched bacterial community

		Level				
		Coded values				
Variable	**Units**	-1.68 (-)	-1	0	+1	+1.68 (+)
		Real values				
COD	**g L^{-1}**	10.0	14.05	20.0	25.95	30.0
N	**g L^{-1}**	0.083	0.056	0.129	0.16	0.250
Time	**Hours**	12	29	54	79	96

Additionally, the software program was used to obtain three-dimensional response surface curves and to display the interaction among independent variables, maintaining one of the independent

variables constant. The experimental results obtained were analyzed to assess the effects of each variable as well as their interaction effects on storage compounds production by the response surface regression procedure using the following equation (Eq. 5.1):

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \beta_{ij} X_i X_j$$

(Eq. 5.1)

Where Y_i is the response variable, β_0 is the constant, β_i is the linear effect, β_{ii} is the quadratic effect, β_{ij} is the interactive effect and X_i is the coded factor level. The results were analyzed by using ANOVA, i.e. analysis of variance, suitable for the experimental design. ANOVA was applied to identify the statistically significant terms. Statistical significance was established at the $P < 0.05$ level.

6.2.3 Experimental assays

The assays were performed in 200 mL flasks containing 50 mL of MS medium supplemented with ammonium chloride as nitrogen source at room temperature (22°C) under agitation (150 rpm).

6.2.4 Neutral lipids analysis

6.2.4.1 Total lipid extraction

The experimental procedure for the extraction of total lipids produced by the enriched bacterial community is described in section 3.2.3.1 included in Material and Methods of the Chapter 3.

6.2.4.2 Fractionation of different lipid classes

Total cell extracts were subjected to a separation process based on lipid class using a solid phase extraction technique (Revellame *et al.*, 2012). The extract was reconstituted in n-hexane (1 mL) and applied on a SPE silica column (SiOH) Chromabond 1000-mg, 6 mL, (Macherey-Nagel, Düren, Germany), previously conditioned with n-hexane. The sample was then separated in four main fractions: fraction A, composed by hydrocarbons; fraction B, composed by wax esters and sterol esters; fraction C composed by triacylglycerols and fraction D, composed by free fatty acids, monoacyglycerols and diacylglycerols. Fraction A was eluted by applying 3.7 ml of 94/6 (v/v) n-

hexane/diethyl ether; fraction B was obtained by applying 4.5 ml of 94/6 (v/v) n-hexane/diethyl ether; fraction C was eluted by applying 10 ml of 94/6 (v/v) n-hexane/diethyl ether; fraction D was obtained applying 15 ml of 85/15/2 (v/v/v) n-hexane/diethyl ether/acetic acid. All fractions were collected, evaporated to dryness and weighted. The fraction volumes were optimized based on TLC analysis.

6.2.4.3 Neutral lipids quantification

Fractions A, B and C, corresponding to total neutral lipid compounds, were gravimetrically quantified.

6.2.4.4 Neutral lipid detection by TLC

Lipid factions were dissolved in a mixture of chloroform: methanol (2:1 v/v) and analysed by TLC as described in section 3.2.3.2 included in Material and Methods of Chapter 3.

6.2.4.5 Neutral lipids analysis by GC-MS

Fractions B, C and D were analysed by mass spectrometry through electrospray ionization (ESI) obtained in a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) and in an ion trap mass spectrometer Finnigan LXQ linear ion trap mass spectrometer (Thermo Fisher).

ESI conditions in the ESI-Q-TOF mass spectrometry were as follows: electrospray voltage was 3 kV in the positive ion mode with a cone voltage of 30 V. The source temperature was 80°C and the desolvation temperature was 150 °C. Data acquisition and treatment of results was carried out with MassLynx™ software V4.0 (Waters).

ESI-MS and MS/MS conditions obtained on a Finnigan LXQ linear ion trap mass spectrometer (Thermo Fisher) were the following: electrospray voltage 5 kV; capillary temperature 275 °C, and the sheath gas (He) flow rate 25 units. The normalized collision energy (CE) varied between 33 and 40 (arbitrary units) for MS/MS. Data acquisition and treatment of results were carried out with the Xcalibur® Data System 2.0 (Thermo Scientific, San Jose, CA, USA).

6.2.5 PHA extraction and quantification

The experimental procedure for extraction and quantification of the PHA produced by the enriched bacterial community is described in section 5.2.5 included in Material and Methods of Chapter 5.

6.3 Results and Discussion

6.3.1. Influence of cultivation conditions on storage compounds accumulation

The microbial community enriched in hydrocarbonoclastic and reserve compounds accumulating bacteria obtained in Chapter 5 was cultivated in different concentrations of carbon and nitrogen (resulting in different C/N ratios) and different incubation time. A statistical design strategy together with a response surface methodology (central composite circumscribed design) was used to assess the interactive effects of the three independent variables, (COD concentration (A), N concentration (B) and cultivation time (C)), in PHA and neutral lipid production and accumulation (response variable). From the statistical design, four replicates of the central point and fourteen star points (including duplicates) were determined, resulting in a total of thirty two assays with five different coded levels (high (+1.682), medium (0), low (-1.682)). The range of COD concentrations of the wastewater varied between 10 to 30 g L^{-1}, the N concentration between 0.083 to 0.250 g L^{-1} (resulting in a theoretical C/N ratio variation between 23 to 241) and cultivation time between 12 h and 96 h.

6.3.1.1 PHA accumulation

Table 6.2 presents the conditions determined by the experimental design for each assay and the correspondent values of PHA (PHB+PHV) accumulation. Among the tested conditions, the assays performed with 20 g COD L^{-1}, 0.1058 g N L^{-1} and 12 hours incubation time, assays 15 and 22, gave the lowest PHA content (< 1 % w/w) whereas the highest levels of PHA were obtained in assay 14, with 14 g COD L^{-1}; 0.1559 g N L^{-1} and 79 hours, and assay 2, with 14 g COD L^{-1}, 0.0557 g N L^{-1} and 29 hours, reaching about 9 % (w/w) of PHA (8.2 % PHB and 1 % PHV). Several studies

showed bacterial PHA accumulation from different types of wastewaters (Ganzeveld *et al.*, 1999; Salehizadeh & Van Loosdrecht, 2004; Dias *et al.*, 2006; Verlinden *et al.*, 2011; Bhuwal *et al.*, 2014). Overall the PHA content obtained in this work was still quite low. Similar results were obtained in other works, where sludge and industrial wastewaters were used as carbon source. Species of the genus *Rhizobium* were cultivated but the PHB production was only up to 10 % (Ben Rebah *et al.*, 2009). In another work, an enriched community from activated sludge was only able to accumulated 14 % PHA on a CDW basis under nitrogen-limiting conditions (Mockos *et al.*, 2008). However, the PHA content obtained in this work was still quite low when compared to the literature, focusing on the optimization of different operational conditions (Vargas *et al.*, 2014) and on the use of other types of complex wastes, mainly composed by sugars and/or organic acids, such as different fermented molasses (Carvalho *et al.*, 2014; Albuquerque *et al.*, 2010; Pisco *et al.*, 2009); biodiesel and municipal wastewaters (Dobroth *et al*; 2011; Coats *et al.*, 2007) and palm oil, olive oil mill polmace, paper mill and cannery effluents (Sudesh *et al.*, 2011; Waller *et al.*, 2012 Bengtsson *et al.*, 2008; Gurieff *et al.*, 2007).

The experimental data described in Table 6.2 were used in the statistic Design-Expert software to perform multiple regression analysis and to generate a model representing the relation between independent and response variables. The generated second-order polynomial equation was established as follows:

$$\boldsymbol{Y = 0.063 - (3.45 \times 10^{-3}) \times A - (5.342 \times 10^{-3}) \times B + 5.459 \times 10^{-3} \times C + 0.011 \times A \times B - (6.301 \times 10^{-3}) \times A \times C - (7.208 \times 10^{-3}) \times B \times C - (7.312 \times 10^{-3}) \times A^2 + 1.687 \times 10^{-3} \times B^2 - (0.014) \times C^2}$$

(Eq. 5.2)

In this equation Y represents the PHA content obtained by the enriched bacterial community (% CDW) and the terms A, B and C are the coded values of the independent variables for carbon content (g COD L^{-1}), nitrogen content (g L^{-1}) and cultivation time (h), respectively.

Table 6.2 Experimental design to assess the effects of independent variables (COD concentration – A; nitrogen concentration – B and cultivation time - C) on PHA production

Assay	Coded values			Actual values			C/N *	% PHA (g g^{-1} CDW)
	A:COD (g L^{-1})	B:N (g L^{-1})	C: t (h)	A:COD (g L^{-1})	B: N (g L^{-1})	C: t (h)		
15	0	0	-1.682	20	0.1058	12	47	0.50 %
22	0	0	-1.682	20	0.1058	12	47	0.78 %
2	-1	-1	-1	14	0.0557	29	63	9.15 %
24	-1	-1	-1	14	0.0557	29	63	3.60 %
6	1	-1	-1	26	0.0557	29	116	2.78 %
5	1	-1	-1	26	0.0557	29	116	3.95 %
7	-1	1	-1	14	0.1559	29	23	3.60 %
18	-1	1	-1	14	0.1559	29	23	2.25 %
29	1	1	-1	26	0.1559	29	42	4.93 %
27	1	1	-1	26	0.1559	29	42	6.35 %
20	-1.682	0	0	10	0.1058	54	24	6.11 %
9	-1.682	0	0	10	0.1058	54	24	4.98 %
31	1.682	0	0	30	0.1058	54	71	3.40 %
8	1.682	0	0	30	0.1058	54	71	3.14 %
32	0	-1.682	0	20	0.0216	54	231	1.31 %
21	0	-1.682	0	20	0.0216	54	231	8.57 %
10	0	1.682	0	20	0.1900	54	26	5.20 %
26	0	1.682	0	20	0.1900	54	26	6.36 %
12	0	0	0	20	0.1058	54	47	5.19 %
25	0	0	0	20	0.1058	54	47	5.62 %
16	0	0	0	20	0.1058	54	47	8.02 %
21	0	0	0	20	0.1058	54	47	6.26 %
28	-1	-1	1	14	0.0557	79	63	2.26 %
13	-1	-1	1	14	0.0557	79	63	2.91 %
11	1	-1	1	26	0.0557	79	116	3.60 %
17	1	-1	1	26	0.0557	79	116	3.17 %
23	-1	1	1	14	0.1559	79	23	6.11 %
14	-1	1	1	14	0.1559	79	23	9.17 %
3	1	1	1	26	0.1559	79	42	3.27 %
1	1	1	1	26	0.1559	79	42	4.49 %
30	0	0	1.682	20	0.1058	96	47	3.97 %
4	0	0	1.682	20	0.1058	96	47	4.32 %

CDW – Cell dry weight

* molar carbon was estimated considering a ratio between COD and total organic carbon of about 4, based on the theoretical values calculated for saturated hydrocarbons with the general formula of CnH2n + 2.

In order to investigate the adequacy of the model, multiple regression analyses on the data were applied and the statistical significance of the model was evaluated by means of ANOVA. Table 6.3 displays the coefficients and p-values of the independent variables as well as of their interaction on PHA accumulation. The p-values were used as a tool to check the significance of each coefficient. The smaller the p-value, the more significant is the corresponding factor or the interaction between

factors itself. A p-value lower than 0.05 revealed that the model terms are significant. Analysis of ANOVA of the quadratic model indicated that the model is significant (p-value<0.001), suggesting that the model was statistically significant with a confidence interval of 99.99 % and suitable for revealing the relation of carbon concentration, nitrogen concentration and cultivation time and predict the response values in terms of PHA production. The regression analysis of the experimental design revealed that although the carbon concentration (A) was not significant (p=0.1253), the self-interaction (A^2) significantly influenced PHA production (p<0.05). The nitrogen concentration (B) and interaction with carbon concentration (AB) exerted a significant negative (p=0.0339) and positive (p=0.0010) influence respectively, whereas self-interaction (B^2) did not affect PHA content. The cultivation time (C), the interaction with carbon (AC) and ammonium concentrations (BC) and cultivation time self-interaction (C^2) induced a significant effect but with a negative influence in the accumulation of PHA. Therefore, non-significant terms with p-values higher than 0.05 were eliminated from the model, namely the nitrogen concentration self-interaction (B^2).

Table 6.3 Degree of positive or negative effects of the experimental variables on PHA production

	PHA	
Factor	**Coefficient**	**P-value**
Model	_____	<0.0001*
A – COD	3.41×10^{-3}	0.1276
B – N	-5.13×10^{3}	0.0339*
C – t	-5.50×10^{-3}	0.0206*
AB	0.011	0.0010*
AC	-6.36×10^{-3}	0.0413*
BC	-7.27×10^{3}	0.0214*
A^2	-7.89×10^{3}	0.0103*
B^2	-1.69×10^{3}	0.5444
C^2	-0.014	<0.0001*

* p value < 0.05

The effects of the three parameters (carbon concentration, nitrogen concentration and cultivation time) on PHA production were represented by 3D response surface graphs, as shown in Figure 6.1.

Higher levels of PHA were obtained (8.2 %) by decreasing carbon and nitrogen concentrations, resulting in an estimated C/N ratio of 63. PHA is a type of intracellular carbon reserve material and energy reserve produced under carbon and nitrogen imbalance conditions, so the ratio of C/N is an important factor in the biosynthesis of these compounds, namely through nitrogen limitation conditions (Doi*, et al.*, 1995).In the literature similar C/N ratios (between 20 and 40) are routinely applied to obtain higher PHA levels (Grothe *et al.*, 1999; Tian *et al.*, 2000; Amirul *et al.*, 2008). However, there are some reports where high levels of PHA were obtained at higher C/N ratios, namely between 50 and 70 by species of *Pseudomonas*, *Azotobacter* and *Chelatococcus* growing on different sugars (Kato *et al.*, 1996; Quagliano & Miyazaki, 1997; Xu *et al.*, 2014).

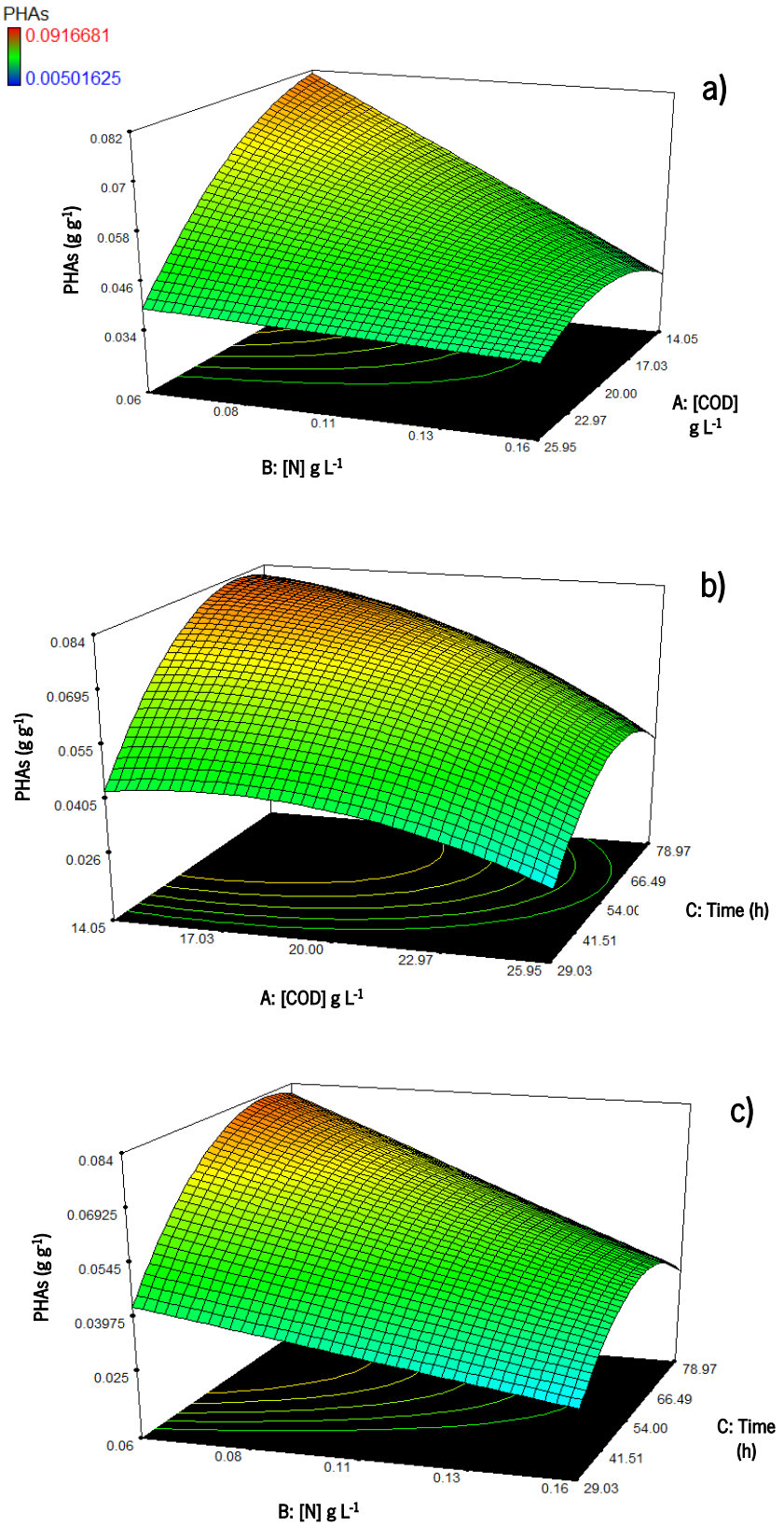


Figure 6.1 Three-dimensional response surface plots showing the effect of interactions between the independent variables in PHA accumulation. a) Interaction between carbon and nitrogen concentrations; b) Interaction between carbon concentration and cultivation time; c) Interaction between nitrogen concentration and cultivation time.

Lower PHA levels (4.0 %) were found mainly in two conditions, namely when carbon concentration increased and nitrogen concentration decreased, resulting in an estimated C/N ratio of 116 and also by decreasing carbon concentration and increasing nitrogen concentration (C/N=23). These results indicated that PHA accumulation in the enriched bacterial community is negatively affected by using extreme C/N ratios. In the case of low C/N ratio (C/N=23), obtained by low carbon and high nitrogen concentrations, the amount of nitrogen must be sufficient to promote growth instead of PHA production, whereas for high C/N ratio (C/N=116), the low amount of PHA may be due to the excessive discrepancy between carbon (26 g COD L^{-1}) and nitrogen (0.06 g N L^{-1}), inhibiting PHA accumulation. The same phenomenon was reported in previous works, where PHA levels increased by increasing C/N ratio to an intermediate value and then decreased by further increasing C/N ratio (Kulpreecha *et al.*, 2009).

The interaction between cultivation time and carbon and nitrogen concentrations on PHA synthesis is shown in Figure 6.1 b and c, respectively. For both situations, a decrease in carbon and nitrogen concentrations (C/N=63) and increase in incubation time led to an increase in PHA content, indicating a positive effect of time on biosynthesis of PHA. Therefore, longer cultivation times in the presence of low carbon and nitrogen concentrations promoted the production of PHA in the enriched bacterial community. These results are in agreement with the ones obtained by several authors, where it was found an increase of the PHA content until the stationary phase of growth (Bhuwal *et al.*, 2014; Xu*, et al.*, 2014). Consequently, in order to obtain higher PHA levels it is important to increase cultivation time as well as to decrease proportionally the carbon and nitrogen concentrations, keeping the C/N ratio around 60.

6.3.1.2 Neutral lipids accumulation

Neutral lipids accumulation in the assays performed in the conditions determined by the experimental design is described in Table 6.4. The lowest neutral lipid content measured was between 1 and 5 % of the CDW for 79 hours of cultivation at a concentration of COD and nitrogen of 26 g L^{-1} and 0.0557 g L^{-1}, respectively (C/N ratio =116). The highest values of neutral lipids detected in the enriched bacterial community was around 33 % of the CDW cultivated on 20 g COD L^{-1}, 0.0216 g N L^{-1} (C/N ratio =231) after 54 hours of cultivation. This result is in the range of values reported in other works, including the ones obtained in oleaginous bacterial pure cultures proposed for neutral lipid production (more than 20 % of lipids in cellular dry weight) (Alvarez *et al.*,

1996, Hernandéz & Alvarez, 2010; Manilla-Pérez, *et al.*, 2011; Revellame *et al.*, 2012; Tamis, 2015).

Table 6.4 Experimental design to assess the effects of independent variables (COD concentration – A; nitrogen concentration – B and cultivation time - C) on neutral lipid production

Assay	Coded values			Actual values			C/N*	% Neutral lipids (g g^{-1} CDW)
	A: COD (g L^{-1})	B: N (g L^{-1})	C: t (h)	A: COD (g L^{-1})	B: N (g L^{-1})	C: t (h)		
15	0	0	-1.682	20	0.1058	12	47	23.9
22	0	0	-1.682	20	0.1058	12	47	24.1
2	-1	-1	-1	14	0.0557	29	63	15.4
24	-1	-1	-1	14	0.0557	29	63	15.5
6	1	-1	-1	26	0.0557	29	116	10.5
5	1	-1	-1	26	0.0557	29	116	10.8
7	-1	1	-1	14	0.1559	29	23	7.5
18	-1	1	-1	14	0.1559	29	23	10.2
29	1	1	-1	26	0.1559	29	42	6.9
27	1	1	-1	26	0.1559	29	42	14.7
20	-1.682	0	0	10	0.1058	54	24	5.9
9	-1.682	0	0	10	0.1058	54	24	8.3
31	1.682	0	0	30	0.1058	54	71	11.0
8	1.682	0	0	30	0.1058	54	71	9.3
32	0	-1.682	0	20	0.0216	54	231	33.5
21	0	-1.682	0	20	0.0216	54	231	30.0
10	0	1.682	0	20	0.1900	54	26	10.4
26	0	1.682	0	20	0.1900	54	26	3.9
12	0	0	0	20	0.1058	54	47	14.6
25	0	0	0	20	0.1058	54	47	7.0
16	0	0	0	20	0.1058	54	47	12.5
21	0	0	0	20	0.1058	54	47	7.6
28	-1	-1	1	14	0.0557	79	63	8.6
13	-1	-1	1	14	0.0557	79	63	14.7
11	1	-1	1	26	0.0557	79	116	1.2
17	1	-1	1	26	0.0557	79	116	4.6
23	-1	1	1	14	0.1559	79	23	8.8
14	-1	1	1	14	0.1559	79	23	4.6
3	1	1	1	26	0.1559	79	42	8.1
1	1	1	1	26	0.1559	79	42	15.1
30	0	0	1.682	20	0.1058	96	47	12.3
4	0	0	1.682	20	0.1058	96	47	21.4

CDW – Cell dry weight

*molar carbon was estimated considering a ratio between COD and total organic carbon of about 4, based on the theoretical values calculated for saturated hydrocarbons with the general formula of CnH2n + 2.

The experimental data described in Table 6.4 were used in the statistic Design-Expert software to perform multiple regression analysis and to generate a model representing the relation between

independent and response variables. The generated second-order polynomial equation was established as follows:

$$\boldsymbol{Y = 9.81 + 0.14 \times A\ (-2.97) \times B\ (-1.57) \times C + 2.98 \times A \times B + 0.033 \times A \times C + 1.72 \times B \times C\ (-1.68) \times\ A^2\ + 2.15 \times B^2 + 2.50 \times C^2}$$

(Eq 5.3)

In this equation Y represents the neutral lipid content obtained by the enriched bacterial community (% CDW) and the terms A, B and C are the coded values of the independent variables for carbon content (g COD L^{-1}), nitrogen content (g L^{-1}) and cultivation time (hours), respectively. In order to investigate the adequacy of the model, multiple regression analyses on the data were applied and the statistical significance of the model was evaluated by means of ANOVA. Table 6.5 displays the coefficients and p-values of the independent variables as well as of their interaction on neutral lipids accumulation. Analysis of ANOVA of the quadratic model indicated that the model is significant (p-value<0.05), suggesting that the model was statistically significant with a confidence interval of 95 % and suitable for revealing the relation of carbon concentration, nitrogen concentration and cultivation time and predicting the response values in terms of neutral lipid production. The regression analysis of the experimental design revealed that only nitrogen concentration and the interaction between carbon and nitrogen concentrations are significant for neutral lipid biosynthesis (p<0.05).

Figure 6.2 shows the effect of the interaction between carbon and nitrogen concentrations on neutral lipid accumulation at different cultivation times. Highest neutral lipid levels (33 %) were found for 29 hours cultivation time with the lowest carbon and nitrogen concentrations analyzed (C/N = 126). The lowest levels of neutral lipids (less than 1 %) were obtained by decreasing carbon concentration and increasing nitrogen concentration (C/N=43). A similar behavior was observed when cultivation time was increased, namely to 54 and 79 hours (Figure 6.2 b and c, respectively), however, the highest amounts of neutral lipids decreased to approximately 26 % (for a C/N ratio of 126). Therefore, it seems that higher levels of neutral lipids are produced by the enriched bacterial community for shorter cultivation times, at high C/N ratios. Previous reports have demonstrated that neutral lipid accumulation is highly dependent on several culturing conditions, being C/N one of the most relevant in several types of bacteria (Riesenberg & Guthke, 1999). Some literature described the same behavior, where strains of *Rhodococcus*, *Alcanivorax* and *Marinobacter*

accumulated more TAG at high C/N ratios (Gouda *et al.*, 2008; Kurosawa *et al.*, 2010; Manilla-Pérez *et al.*, 2011).

Table 6.5 Degree of positive or negative effects of the experimental variables on neutral lipids production

	Neutral lipids	
Factor	**Coefficient**	**P-value**
Model	_____	< 0.0429*
A – COD	0.14	0.9105
B – N	-2.97	0.0215*
C – t	-1.57	0.2050
AB	2.98	0.0408*
AC	0.33	0.8364
BC	1.72	0.2834
A^2	-1.68	0.3000
B^2	2.15	0.1872
C^2	2.50	0.1278

* p value < 0.05

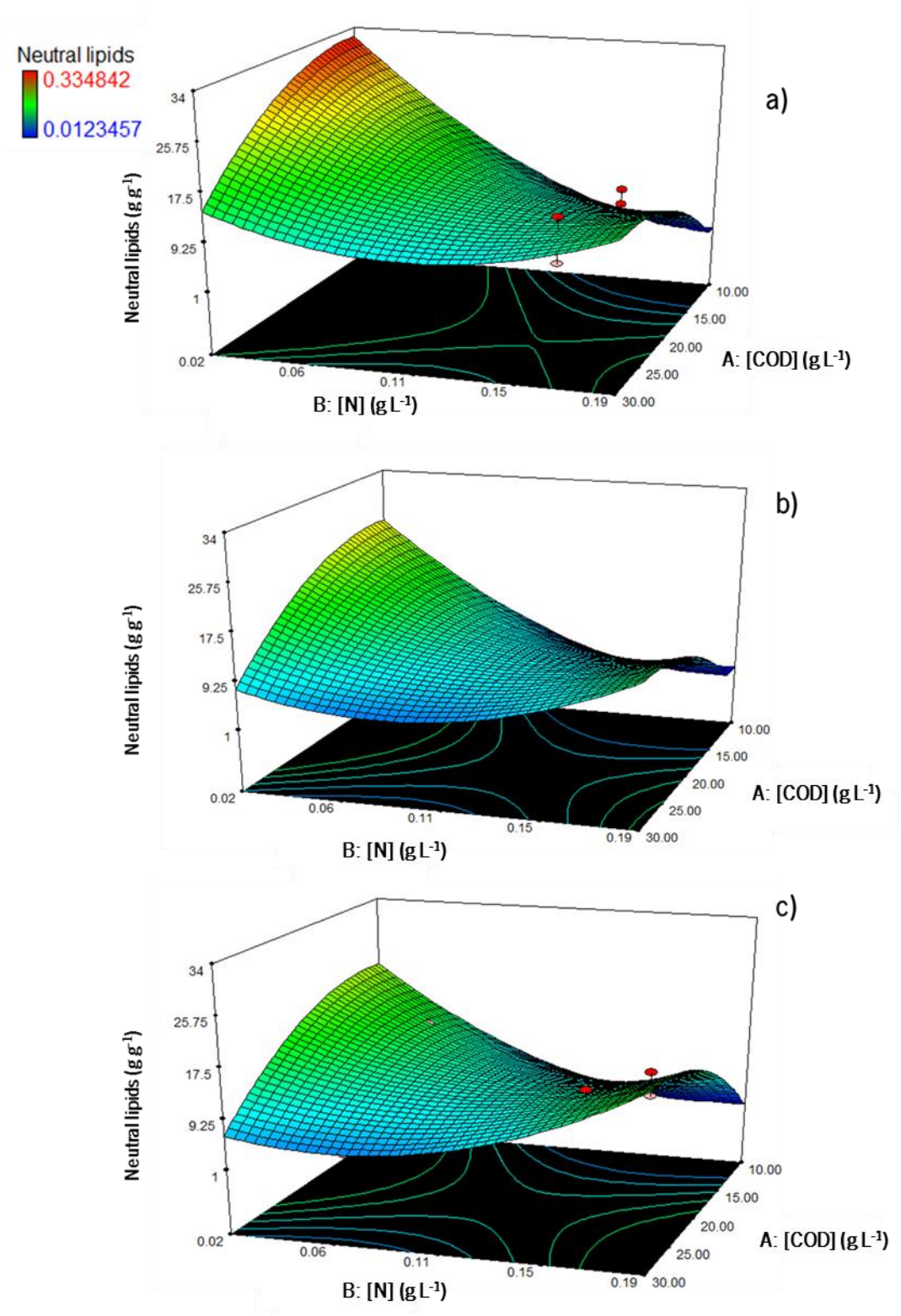


Figure 6.2 Three-dimensional response surface plots showing the effect of interactions between the independent variables in PHA accumulation showing the interaction between the independent variables in the FAEE production. a) Interaction between carbon and nitrogen concentrations at 29 hours of cultivation; b) Interaction between carbon and nitrogen concentrations at 54 hours of cultivation; c) Interaction between carbon and nitrogen concentrations at 79 hours of cultivation .

The classes of neutral lipids produced by the enriched bacterial community cultivated in conditions promoting maximum neutral lipid content (about 30 % of CDW) was further analysed by thin layer chromatography (TLC) (Figure 6.3). TAG were the most abundant lipids, representing 69 % (w/w) of total neutral lipid content. Fatty acids (FA), monoacylglycerols (MAG) and diacylglycerols (DAG) were also found, accounting for 22 % of total neutral lipids, whereas WE were produced in lower amounts, corresponding to 7 % of total neutral lipids.

The composition of each cell extract fraction (A to D, Figure 6.3) was analysed by GC-MS. The mass spectrometry analysis of fraction B showed no molecular species corresponding to wax esters or sterol esters. The analysis of these molecules and consequent fragmentation might have been hindered by the very low WE amounts, almost undetected in the TLC. In Table 6.6 are described the several TAG structures identified in fraction C.

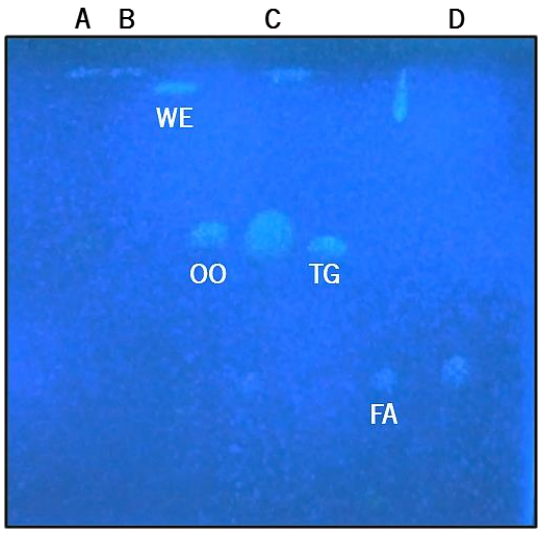


Figure 6.3. TLC profile of neutral lipid compounds produced by the enriched bacterial community cultivated at a concentration of COD and nitrogen of 20 g L^{-1} and 0.0216 g L^{-1}, respectively, after 54 hours of cultivation (maximum lipid production). The cell extract was separated in 4 fractions: A – hydrocarbons; B – wax esters and steryl esters; C – triacylglycerols; D – fatty acids; monoacylglycerols and diacylglycerols. WE – wax ester standard (oleyl oleate); OO and TG– triacylglycerol standards (olive oil and tripalmitin, respectively); FA – fatty acid standard (oleic acid).

Table 6.6 Molecular species of triacylglycerols identified as $[M+NH_4]^+$ ions detected by ESI-Q-TOF-mass spectrometry in fraction C of the cell extract obtained from the enriched community cultivated in conditions promoting maximum lipid content.

m/z $[M+NH_4]^+$	Triacylglycerol (C:N)	Fatty acyl chains
822.9	48:1	16:0/16:0/16:1
		14:0/16:0/18:1
		16:0/16:1/18:1
848.9	50:2	16:0/16:0/18:2
		18:1/18:1/14:0
		16:1/16:1/18:0
		14:0/18:0/18:2
850.9	50:1	16:0/16:0/18:1
		14:0/18:0/18:1
		16:0/16:1/18:0
		14:0/18:0/18:2
874.9	52:3	16:0/18:1/18:2
		16:1/18:1/18:1
876.9	52:2	16:0/18:1/18:1
902.9	54:3	18:1/18:1/18:1

The most abundant TAG ions were found at *m/z* 850.9, 876.8 and 902.8 assigned to $[M+NH_4]^+$ adducts of TAG(50:1), TAG(52:2) and TAG(54:3), respectively. The fatty acids detected in TAG molecules ranged between myristic acid (C14:0) and linoleic acid (C18:2), being the most abundant, palmitic acid (C16:0), palmitoleic acid (C18:0) and oleic acid (C18:1). The free fatty acids profile of the enriched community is presented in Table 6.7. A wide range of fatty acids are produced by the enriched bacterial community, with carbon chain length ranging between 14 and 25 carbon atoms. Interestingly, odd-chain fatty acids, namely C15:0 and C17:0, and unsaturated fatty acids between one and five double bonds were also detected.

Table 6.7 Molecular species of free fatty acids identified as $[M+NH_4]^+$ ions detected by ESI-Q-TOF-mass spectrometry in fraction C of the cell extract obtained from the enriched community cultivated in conditions promoting maximum lipid content.

m/z $[M+NH_4]^+$	Fatty acyl chains
227.2	14:0
241.2	15:0
253.2	16:0
255.2	16:1
269.2	17:0
281.3	18:0
283.3	18:1
301.2	20:0
303.2	20:1
307.2	20:2
309.2	20:3
311.3	20:4
325.2	20:5
337.2	21:0
339.2	22:0
367.3	24:0
381.3	25:0

The structures of MAG and DAG produced by the enriched community are listed in Table 6.8. MAG are mainly composed by palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2), whereas DAG are composed by a mixture of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3).

This wide range of TAG, free fatty acids, MAG and DAG structures possibly reflects the complex mixture of hydrocarbon types present in the wastewater used as carbon and energy source. In fact, this results are in agreement with previous reports, where a wide range of fatty acids were produced by a mixed microbial population present in municipal activated sludge and also by pure bacterial cultures, namely *R. opacus* PD630 and *Gordonia* sp. DG, when grown on real and complex agro-industrial wastes and municipal wastewaters (Gouda *et al.*, 2008; Revellame *et al.*, 2012).

Table 6.8 Molecular species of monoacylglycerols and diacylglycerols identified as $[M+NH_4]^+$ ions detected by ESI-Q-TOF-mass spectrometry in fraction C of the cell extract obtained from the enriched community cultivated in conditions promoting maximum lipid content.

m/z $[M+NH_4]^+$	Monoacyglycerols	Diacylglycerols	
	C/N	C/N	Fatty acyl chains
348.3	16:0		
372.4	18:2		
376.4	18:0		
608.6		34:3	16:0/18:3
610.6		34:2	16:0/18:2
612.6		34:1	16:0/18:1
630.6		36:6	18:3/18:3
632.6		35:5	18:2/18:3
634.6		36:4	18:2/18:2

6.3.2. Relation between PHA and neutral lipid accumulation

Several microorganisms have the ability to produce simultaneously more than one type of storage compounds (Alvarez *et al.*, 2000; Alvarez, 2003; Hernandéz & Alvarez, 2010; Manilla-Pérez *et al.*, 2011). Since the enriched bacterial community used in this study was able to produce PHA and neutral lipids, it is important to evaluate the optimal culturing conditions to obtain maximum amounts of each compound. After knowing the relevant interactions between variables that led to the maximum levels of storage compounds synthesis, optimization was performed using the ''point optimization'' technique. Model simulations to predict maximum and minimum neutral lipids and PHA levels by the enriched bacterial community and the corresponding cultivation parameters are represented in Table 6.9.

When only neutral lipids were maximized, a value of 36.9 % was obtained when the microbial community was cultivated on 11.92 g COD L^{-1}, 0.02 g N L^{-1} (resulting in an estimated C/N ratio of 151) during approximately 15 hours. Similar culturing conditions, except cultivation time, were obtained by maximize neutral lipids and minimize PHA content, with PHA levels reaching only 1.6 %. By maximizing PHA production, a value of 9.7 % was obtained for similar conditions of maximum TAG production, but for longer cultivation period, namely 76 hours. Similar values of

PHA and neutral lipid were observed when PHA were maximized and neutral lipid minimized (7.6 % and 11.1 %, respectively) at a higher C/N ratio and 72 hours of cultivation.

Table 6.9 Model simulations to predict maximum and minimum neutral lipids and PHA levels by the enriched bacterial community and the corresponding cultivation parameters.

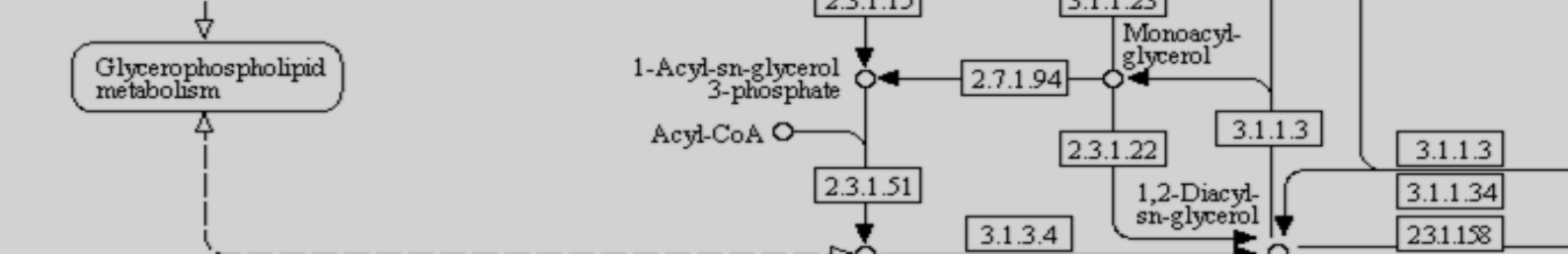
Simulation		Predicted values (% CDW)		COD	N	C/N ratio	Time
Neutral lipids	PHA	Neutral lipids	PHA				
Maximize	———	36.9	———	11.92	0.02	151	14.97
———	Maximize	———	9.7	15.98	0.03	135	75.66
Maximize	Minimize	33.7	1.6	10.24	0.04	65	12.52
Minimize	Maximize	11.1	7.6	10.00	0.07	36	72.17
Maximize	Maximize	25.4	9.2	11.14	0.02	141	51.6

For maximization of both types of storage compounds, 25.4 % of neutral lipids and 9.2 % of PHA were detected for an intermediate cultivation time of 51 hours. These results can be explained, at least partially, by the fact that metabolic pathways of PHA, TAG and WE synthesis shared common precursors, thereby resulting in a competition for the same metabolites under accumulation conditions, namely acetyl-coA and propionyl-coA. Propionyl-CoA can be utilized for biosynthesis of 3-HV in poly(3HB-co-3HV) and of odd-numbered fatty acids, whereas acetyl-coA can be used directly for the production of fatty acids which are incorporated in TAG and WE synthesis and indirectly through the production of succinyl-coA by TCA cycle, which results in the further synthesis of propionyl-coA metabolic pathway (Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000; Bequer Urbano*, et al.*, 2013). Additionally, in a whole bacterial community perspective, it is possible that the differences in storage compounds accumulation for different cultivation time might be due to differences in carbon source uptake and consequent reserve material production rates. The mixed bacterial community is mainly composed by members of the genera *Rhodococcus*, *Acinetobacter* and *Pseudomonas* (Chapter 5). For short cultivation times, TAG accumulating bacteria were possibly more efficient in the synthesis of neutral lipid compounds, whereas for longer cultivation times, the higher PHA accumulation can be related to the dominance of species of the genus *Pseudomonas*. Despite the increasing number of works regarding individual neutral lipids and PHA production by pure cultures, there is still a gap in more specific knowledge concerning the simultaneous production of these reserve compounds by mixed communities. To our knowledge this is the first report describing several storage compounds production by a mixed community

enriched in lipid accumulating bacteria, using hydrocarbon-based wastewater. These results demonstrated that by altering culturing conditions it is possible to direct the distribution of metabolites to different storage compounds to achieve higher PHA or neutral lipid levels in bacterial communities, which could increase the range of industrial applications.

6.4 Conclusions

This work evaluated the effect of cultivation conditions on PHA and neutral lipid compounds synthesis in an indigenous mixed bacterial community grown on wastewater contaminated with lubricants and motor oil. The developed experimental design, using the response surface methodology, showed that the interactions between the carbon and nitrogen concentrations as well as cultivation time heavily influenced PHA accumulation. A PHA content of 8.2 % of CDW was obtained using lower carbon and nitrogen concentrations at longer cultivation time, namely 14 g COD L^{-1} and 0.056 g N L^{-1} at 79 hours of cultivation. On the other hand, only the interaction between carbon and nitrogen concentrations and cultivation time influenced neutral lipid production. The mixed bacterial community produced 33.5 % (CDW basis) of neutral lipids after 29 hours of cultivation using lower carbon and nitrogen concentrations, namely 10 g COD L^{-1} and 0.02 g N L^{-1}, being mainly composed by TAG with a wide range of chemical structures and fatty acid composition. Although neutral lipid and PHA amounts achieved in this work were lower than those obtained by pure cultures using simple carbon sources, it showed that enriched bacterial communities obtained from activated sludge are potential resources for obtaining high-valued storage compounds. Additionally, the use of hydrocarbon-rich wastewaters, which are cheap raw materials, can significantly reduce the production and commercialization costs as well as to mitigate environmental pollution, by decreasing the amount of wastes that have to be treated. In this way, this study opens new perspectives to develop a more economic and environmentally sustainable process.



Chapter 7

Tracking metabolic pathways for biofuels production in hydrocarbonoclastic bacteria

The high demand for more sustainable and economically feasible oil feedstocks led to an increasing exploration of bacterial lipid storage compounds, namely triacylglycerol (TAG), wax ester (WE) and polyhydroxybutyrates (PHB). Hydrocarbonoclastic bacteria are able to degrade different types of hydrocarbons, usually present in oily wastewaters and residues. Some members of this group have the capability to produce storage compounds, which makes these bacteria ideal candidates for biotechnological processes combining pollution reduction and production of valuable compounds. However, in order to improve these capabilities, it is fundamental to get a deeper knowledge on the pathways involved in the production of lipid storage compounds.

In this study, a genome-based comparative analysis of genes and metabolic reactions responsible for TAG, WE and PHB biosynthesis of four hydrocarbonoclastic bacterial genera with recognized capabilities in lipid storage compounds production was performed. A bioinformatics approach applied to the bacterial sequenced genomes revealed the presence of genes coding for complete metabolic pathways in all species, particularly for TAG and PHB biosynthesis. *R. opacus* strains are highly enriched in genes involved in TAG and PHB metabolism, whereas *Alcanivorax*, *Acinetobacter* and *Pseudomonas* have a high number of genes coding for enzymes involved in PHB production. A more detailed research on metabolic pathways involved in WE biosynthesis will be required, mainly focused on genes and enzymes already identified in other bacterial species. This study represents the first attempt to unravel and compare the genomic machinery mediating storage lipid products in a wide range of hydrocarbonoclastic bacterial species.

7.1 Introduction

During the last decade, the importance of bacterial lipid storage compounds has significantly increased, mainly due to their potential applications in industrial and biotechnological processes. Triacylglycerols (TAG) are feedstocks for biodiesel production, while wax esters (WE) can be used in oleochemical industry and polyhydroxyalkanoates are applied in bioplastics manufacturing (Verlinden *et al.*, 2007; Rontani, 2010; Kosa & Ragauskas, 2011). Among hydrocarbonoclastic bacteria, *Rhodococcus*, *Acinetobacter*, *Alcanivorax* and *Pseudomonas* genera are considered as some of the main producers of storage lipids. Members of *Rhodococcus* genus are widely known for the remarkable ability to produce and accumulate TAG. Several works reported TAG accumulation under different carbon sources and different cultivation parameters in nitrogen limiting conditions (Alvarez *et al.*, 1996; Alvarez *et al.*, 1997a; Voss & Steinbuchel, 2001; Alvarez, 2003; Hernandez *et al.*, 2008; Silva *et al.*, 2010; Bequer Urbano*, et al.*, 2013; JanSzen*, et al.*, 2013; Cortes & de Carvalho, 2015; Shields-Menard *et al.*, 2015) and its fatty acid composition and chemical structure (Wältermann *et al.*, 2000; Schreiberová *et al.*, 2010). In addition to TAG, the presence of PHA and WE, although in minor amounts, was detected in some species (Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000; Hernandez *et al.*, 2008; Hori *et al.*, 2009). In fact, *R. opacus* PD630 stands out from the other *Rhodococus* species, since it accumulates lipids up to 87 % of the cellular dry weight when cultivated on olive oil (Alvarez *et al.*, 1996) and therefore it is assumed as model organism to study TAG metabolism in bacteria. Several efforts have been performed, aiming to unravel the metabolic and genomic bases of this phenomenon. Several works reported the presence of several genes coding for wax ester synthase-diacylgycerol-acyltransferase (WS/DGAT enzyme), responsible for the last reaction in TAG and WE synthesis (Alvarez*, et al.*, 2008; Hernandez *et al.*, 2008; Hernández *et al.*, 2013) and also proteins associated to TAG formation (MacEachran *et al.*, 2010; MacEachran & Sinskey, 2013). Holder and co-workers developed a working model based on a metabolic reconstruction based on genome of *R. opacus* PD630 to go deeper on metabolic pathways for TAG accumulation (Holder *et al.*, 2011). More recently, a comparative study using different *Rhodococcus* species was undertaken to assess the presence of genes and enzymes involved in TAG biosynthesis (Villalba, 2013).

The *Acinetobacter* genus is an efficient WE producer, although it can accumulate low levels of TAG. The composition of WE produced in *Acinetobacter baylyi* ADP1 is mainly determined by the type of

carbon source used, particularly alkanes (Ishige *et al.*, 2002; Ishige *et al.*, 2003). In the proposed metabolic pathway for WE synthesis (Reiser & Somerville, 1997), the critical reaction is the esterification reaction between fatty alcohol and fatty acyl-CoA molecules catalyzed by the bifunctional enzyme WS/DGAT (Kalscheuer & Steinbuchel, 2003). This enzyme was further purified and extensively biochemically characterized, namely in terms of substrate specificity, important amino acids and residues and *in vivo* and *in vitro* ability to produce WE (Kalscheuer *et al.*, 2003; Stöveken *et al.*, 2005; Röttig & Steinbüchel, 2013b). The sequenced genome of *Acinetobacter baylyi* ADP1 led to the development of a genome-scale metabolic model and a collection of single knock out mutants (de Berardinis *et al.*, 2008; Durot *et al.*, 2008). These works were the basis for the metabolic engineering studies to improve TAG and WE biosynthesis (Santala *et al.*, 2011b, Santala *et al.*, 2014). *Alcanivorax* species belong to obligate oil degrading bacteria and the synthesis of neutral lipid compounds, such as TAG and WE, was recently reported in different marine hydrocarbonoclastic genera (Kalscheuer *et al.*, 2007). However the genetic and metabolic dynamics of lipid storage compounds are poorly known in these organisms. The sequenced genome of *Alcanivorax borkumensis* SK2 allowed the identification of two genes coding for WS/DGAT with high similarity to those of *A. bailyi* ADP1 and the possible existence of an alternative metabolic pathway for neutral lipids production (Kalscheuer *et al.*, 2007). TAG accumulation and mobilization processes were analysed and TAG physiological role on survival in natural environments was assessed in *A. borkumensis* SK2 (Manilla-Pérez *et al.*, 2010b). Furthermore, the production and export of wax esters in different *Alcanivorax* species and other hydrocarbonoclastic bacteria was reported and the C/N ratio considered a fundamental requisite for lipid accumulation processes (Manilla-Pérez *et al.*; 2010a; Manilla-Pérez *et al.*, 2010c; Manilla-Pérez *et al.*, 2011).

Accumulation of PHA is a common feature among species belonging to *Pseudomonas* genus. In fact, *P. putida*, *P. oleovorans*, *P. aeruginosa*, and *P. stutzeri* are PHA producers (Lageveen *et al.*, 1988; Huijberts*, et al.*, 1992; He *et al.*, 1998; Silva-Queiroz *et al.*, 2009). The general metabolic pathway for PHA synthesis in *Pseudomonas* is based on ß-oxidation and *de novo* fatty acid synthesis to provide precursors for the formation of monomers of hydroxyacyl-coAs. The condensation reaction between different monomers is performed by PHA synthase (Prieto *et al.*, 2007). The regulation of these metabolic pathways is extremely complex since it is dependent on several factors, such as dependence of central carbon metabolism pathways, composition of the used carbon source and nitrogen concentrations. The genome sequence and the subsequent

reconstruction of a genome-scale metabolic model in *P. putida* KT2440 led to a more comprehensive knowledge on PHA metabolism and to select suitable strategies for enhancing PHA production (Nogales *et al.*, 2008; Puchałka *et al.*, 2008). Consequently, some metabolic engineering and system biology studies were recently performed aiming the improvement of PHA synthesis (Escapa *et al.*, 2012; Poblete-Castro *et al.*, 2012; Poblete-Castro *et al.*, 2013; Borrero-de Acuna *et al.*, 2014; Poblete-Castro, 2014) Moreover, *P. putida* has been extensively studied regarding PHA production by using organic fatty acids as carbon sources (Hartmann *et al.*, 2006; Sun *et al.*, 2007; Chung *et al.*, 2009; Escapa *et al.*, 2012). Nevertheless, scarce information is available about PHA synthesis using other carbon sources such as polycyclic aromatic hydrocarbons.

Coupled to lipid storage compounds production, these bacterial genera also have the ability to degrade a wide range of hydrocarbons, being used in bioremediation of oily wastes. Although a vast body of information on metabolic pathways of lipid reserve materials was already discovered, the genome sequences will allow the identification and elucidation of the role of genes and enzymes involved and ultimately will allow the increase of storage lipids yields, using inexpensive substrates as carbon sources. In this work, a comparative genome-based analysis of metabolic pathways involved in TAG, WE and PHB synthesis in hydrocarbonoclastic bacteria with different accumulation capabilities will be performed.

7.2 Materials and methods

7.2.1 Bacteria and genomes

The following hydrocarbonoclastic bacterial strains were studied in this work: *R. opacus* B4 (Na, et al., 2005); *R. opacus* PD630 (Alvarez *et al.*, 1996); *Alcanivorax borkumensis* SK2 (Yakimov *et al.*, 1998); *Acinetobacter baylyi* ADP1 (Vaneechoutte *et al.*, 2006) and *Pseudomonas putida* KT2440 (Franklin *et al.*, 1981). Information about the bacterial genomes, namely genome size, number of genes and reference source are represented in Table 7.1. The gene and protein sequences of each bacterium were obtained from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

Table 7.1 Genomic information of the five hydrocarbonoclastic species used in this study

Genome size (Mb)	Species	Nr genes	Genome/BioProject reference
8.83	*R. opacus* B4	8259	http://www.ncbi.nlm.nih.gov/genome/1328; PRJNA13791, PRJDA34839
9.42	*R. opacus* PD630	8632	http://rhodocyc.broadinstitute.org/RHOTITCHED/organism-summary; PRJNA182034, PRJNA30413
3.12	*A. borkumensis* SK2	2816	http://www.ncbi.nlm.nih.gov/genome/1765; PRJNA58169, PRJNA13005
3.61	*A. baylyi* ADP1	3320	http://www.ncbi.nlm.nih.gov/genome/14166; PRJNA201584, PRJNA221007
6.18	*P. putida* KT2440	5516	http://www.ncbi.nlm.nih.gov/genome/174; PRJNA57843, PRJNA267

7.2.2 Metabolic pathways and gene/enzyme sequences

The metabolic pathways involved in different lipid storage compounds production were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa & Goto, 2000). For each metabolic pathway, EC numbers were selected and for each EC number, the corresponding orthologous genes were identified. This set of genes was filtered by an automatic procedure, where genes of the organisms taxonomically most closely related to the ones studied were obtained. The corresponding protein sequences were run against the ones obtained from genomes of each strain, using BLASTP, in order to identify homologous proteins present in the selected hydrocarbonoclastic species. The search was performed based on the Smith-Waterman algorithm and the results were grouped based on degree of similarity: very high similarity (more than 90 %); high similarity (between 50 % and 90 %); moderate similarity (between 10 % and 50 %) and low similarity (less than 10 %).

7.3 Results and Discussion

7.3.1 Triacylglycerol synthesis

Generally, the metabolic pathway responsible for TAG production is mainly composed by three successive acylation reactions using acyl-CoA and glycerol as donor and receptor molecules, respectively. The first two reactions consist in the addition of two acyl-coA molecules to the

positions *sn* 1 and 2 of glycerol-3-phosphate (G3P), catalyzed by glycerol-3-phosphate acyltransferase (G3P acyltransferase) and 1-acylglycerol 3-phosphate acyltransferase (1-acyl-G3P acyltransferase), respectively. The formed 1,2 diacyl-sn glycerol 3-phpsphate/ phosphatidic acid is then desphosphorylated by phosphatidic acid phosphatase (PAP). The last acylation reaction is catalyzed by diacylglycerol acyl transferase (DGAT), adding an acyl group to diacylglycerol (DAG), forming triacylglycerol (TAG). This set of sequential reactions is designated as Kennedy pathway, being extensively studied in plants and yeasts, but poorly in bacteria. Table 7.2 shows TAG synthesis enzymes and the corresponding orthologous genes found in the genomes of representative members of hydrocarbonoclastic bacteria. Several genes coding for G3P acyltransferase (2.3.1.15) and 1-acyl-G3P acyltransferase (2.3.1.51) were found in genomes of *R. opacus* B4 and *R. opacus* PD630, some of those presenting high similarity percentage (more than 90 %).

Table 7.2 Genes and the corresponding metabolic pathways involved in the TAG biosynthesis pathways present in the genomes of selected hydrocarbonoclastic bacteria. Green color represents genes with more than 90 % similarity; blue color represents genes with similarity between 50 % and 90 %; orange color represents genes with similarity between 10% and 50%; Pink color represents genes with similarity lower than 10 %.

Enzyme	EC number	Metabolic reaction	Orthologous	*R. opacus* B4	*R. opacus* PD630	*A. borkumensis* SK2	*A. baylyi* ADP1	*P. putida* KT2440
Glycerol kynase	2.7.1.30	ATP + glycerol = ADP + sn-glycerol 3-phosphate	K00864	ROP_63250	OPAG_03267T0	ABO_0714	ACIAD0930	PP_1075
glycerol-3-phosphate O-acyltransferase	2.3.1.15	acyl-CoA + sn-glycerol 3-phosphate = CoA + 1-acyl-sn-glycerol 3-phosphate	K00631	ROP_57110	OPAG_04306T0	ABO_1132	ACIAD3232	PP_1520
			K08591	ROP_15270	OPAG_08398T0	ABO_2058		PP_0391
			K03621		OPAG_06704T0	ABO_1067		PP_1912
1-acyl-sn-glycerol-3-phosphate acyltransferase	2.3.1.51	acyl-CoA + 1-acyl-sn-glycerol 3-phosphate = CoA + 1,2-diacyl-sn-glycerol 3-phosphate	K00655	ROP_41110	OPAG_05898T0	ABO_0006	ACIAD3002	PP_1844
				ROP_08430		ABO_0683		PP_0923
						ABO_1917		PP_0058
			K14674			ABO_1581	ACIAD1335	
			K13509	ROP_39260	OPAG_05898T0	ABO_0006	ACIAD0567	
					OPAG_06417T0	ABO_1917	ACIAD3002	
					OPAG_04305T0	ABO_0683	ACIAD2050	
					OPAG_06418T0	ABO_1112	ACIAD2964	
					OPAG_06288T0	ABO_2492	ACIAD1665	
Phosphatidate phosphatase	3.1.3.4	1,2-diacylglycerol 3-phosphate + H2O = a 1,2-diacyl-sn-glycerol + phosphate	K01080	ROP_50370	OPAG_02574T0	ABO_0363	ACIAD0554	PP_3315
					OPAG_07226T0		ACIAD2266	PP_3105
					OPAG_00617T0		ACIAD2942	PP_2429
					OPAG_06641T0		ACIAD0237	
							ACIAD1005	
							ACIAD3665	

Table 7.2 (Continued)

Enzyme	EC number	Metabolic reaction	Orthologous	*R. opacus* B4	*R. opacus* PD630	*A. borkumensis* SK2	*A. baylyi* ADP1	*P. putida* KT2440
Diacylglycerol O-acyltransferase	2.3.1.20	acyl-CoA + 1,2-diacyl-sn-glycerol = CoA + triacylglycerol	K00635	ROP_68400	OPAG_03807T0	ABO_2742	ACIAD0832	
				ROP_63930	OPAG_03334T0	ABO_1804		
				ROP_13050	OPAG_00138T0			
				ROP_54550	OPAG_07648T0			
				ROP_26950	OPAG_07266T0			
				ROP_02100	OPAG_03918T0			
				ROP_57120	OPAG_01553T0			
				ROP_04650	OPAG_07257T0			
				ROP_29070	OPAG_03922T0			
					OPAG_07212T0			
					OPAG_05208T0			
					OPAG_06225T0			
					OPAG_07267T0			
					OPAG_05286T0			
					OPAG_06690T0			
			K14456			ABO_1410		
			K11160			ABO_1410		
			K18851	ROP_39370	OPAG_09203T0			
				ROP_50250	OPAG_08440T0			
				ROP_39360	OPAG_02587T0			
					OPAG_02587T0			
					OPAG_09203T0			

Table 7.2 (Continued)

Enzyme	EC number	Metabolic reaction	Orthologous	*R. opacus* B4	*R. opacus* PD630	*A. borkumensis* SK2	*A. baylyi* ADP1	*P. putida* KT2440
phospholipid:diacylglycerol acyltransferase	2.3.1.58	oxalyl-CoA + L-2,3-diaminopropanoate = CoA + N3-oxalyl-L-2,3-diaminopropanoate	K00679					
Phosphatidate cytidylyltransferase	2.7.7.41	CTP + phosphatidate = diphosphate + CDP-diacylglycerol	K00981	ROP_66160	OPAG_03566T0	ABO_1148	ACIAD1375 ACIAD1664	PP_1596
CDP-diacylglycerol—serine O-phosphatidyltransferase	2.7.8.8	CDP-diacylglycerol + L-serine = CMP + (3-sn-phosphatidyl)-L-serine	K00998					PP_3664
			K17103	ROP_18760	OPAG_00719T0	ABO_0486	aci:ACIAD3149	PP_0731 PP_4677
Phosphatidylserine decarboxylase	4.1.1.65	phosphatidyl-L-serine = phosphatidylethanolamine + CO2	K01613	ROP_18750	OPAG_00718T0	ABO_2213	ACIAD3560	PP_4908
Phospholipase C	3.1.4.3	a phosphatidylcholine + H2O = 1,2-diacyl-sn-glycerol + phosphocholine	K01114	ROP_22030	OPAG_01064T0			
CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase	2.7.8.5	CDP-diacylglycerol + sn-glycerol 3-phosphate = CMP + 3(3-sn-phosphatidyl)-sn-glycerol 1-phosphate	K00995	ROP_06460 ROP_67400 ROP_68590	Pd630_LPD03374 Pd630_LPD03506 Pd630_LPD04996	ABO_1306	ACIAD0342 ACIAD1669	PP_4097
Phosphatidylglycero phosphatase GEP4	3.1.3.27	phosphatidylglycerophosphate + H2O = phosphatidylglycerol + phosphate	K01095			ABO_2168	ACIAD3574	PP_0520

These results corroborate the ones obtained by Villalba and co-authors, who performed a genome-based comparative analysis of TAG metabolism in six different *Rhodococcus* strains (Villalba, 2013). Different genes with very high similarity coding for G3P acyltransferase and 1-acyl-G3P acyltransferase were also found in *A. borkumensis* SK2, *A. baylyi* ADP1 and *P. putida* KT2440 genomes, although more genes with moderate similarity percentage were detected in *A. borkumensis* and *A. baylyi*. Phosphatidic acid phpsphatase (PAP) enzyme (3.2.3.4) which catalyzes the desphosphorilation of 1,2-sn-G3P/phopshatidic acid, is encoded by genes present in all bacterial species, being *A. baylyi* the one with the highest number of genes, although with similarity levels below 10 % (Table 7.2). The presence of this enzyme in bacterial genomes is poorly studied. Only recently it was analysed and characterized in *Streptomyces coelicolor*, revealing the presence of two genes, lppα (SCO1102) and lppβ (SCO1753), catalysing DAG formation in TAG metabolic pathways (Comba*, et al.*, 2013). The last step in TAG biosynthesis is performed by the diacylgycerol acyltransferase.(DGAT) enzyme, coded by the atfA gene family, being the only that is unique to TAG synthesis (Cases *et al.*, 1998).

A high number of genes coding for this enzyme (2.3.1.20) was found in *Rhodococci* strains (Table 7.2). Three and five genes with similarity percentages higher than 90 % were found in the genomes of *R. opacus* B4 and *R. opacus* PD630, respectively. Three genes with similarity levels ranging between 50 % and 90 % were found in both strains and finally five and twelve genes with 10 % to 50 % similarity were found in *R. opacus* B4 and *R. opacus* PD630. This genomic abundance has already been detected in these strains when compared to other *Rhodococccus* species (Alvarez *et al.*, 2008; Alvarez & Steinbuchel, 2010; Holder *et al.*, 2011; Hernandéz *et al.*, 2013; Villalba , 2013). In comparison to previous studies, where 9 DGAT genes were found in the genome of *R. opacus* B4 (Villalba , 2013) and 17 genes were detected in *R. opacus* PD630 (Holder*, et al.*, 2011, Villalba MS, 2013), in the present work 3 and 2 additional DGAT enzymes were detected in *R. opacus* B4 and *R. opacus* PD630, respectively. Despite this ubiquity, currently only two DGAT genes (atfA1 and atfA2) were demonstrated to have an active role in TAG synthesis in *R. opacus* PD630. (Alvarez *et al.*, 2008; Hernandéz *et al.*, 2013). Interestingly, a double mutant strain in these 2 genes still revealed the ability to accumulate TAG, suggesting the presence of additional genes responsible for TAG synthesis, as demonstrated in the present work. In *A. borkumensis* SK2 two genes with more than 90 % similarity and one gene with very low similarity (less than 10 %) were detected. These results are in agreement with the ones obtained by Kalscheuer and co-workers, where two genes, atfA1 and atfA2, were found in *A. borkumensis* and their role in TAG

synthesis was confirmed (Kalscheuer *et al.*, 2007). Furthermore, atfA1 and atfA2 single and double mutants produced lower but substantial TAG formation in *A. borkumensis* SK2, suggesting the presence of additional genes responsible for TAG synthesis. *A. baylyi* ADP1 is known for its ability to produce and accumulate TAG and WE, although several studies reported a higher synthesis of WE when compared to TAG (Makula *et al.*, 1975; Ishige *et al.*, 2003; Kalscheuer & Steinbuchel, 2003; Santala *et al.*, 2011b). Only one gene (ACIAD0832) coding for the DGAT enzyme (2.3.1.20) was found in *A. baylyi* ADP1. Interestingly, this gene codes for a new bacterial class of acyltransferase, presenting both DGAT and acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WS) activities (WS/DGAT) (2.3.1.75) (AtfA), which is very different from those occurring in eukaryotes (Kalscheuer & Steinbuchel, 2003; Stöveken *et al.*, 2005; Wältermann *et al.*, 2007). The DGAT activity was confirmed by the construction of AtfA knock-out mutants, where a dramatic decrease of TAG levels was detected (Kalscheuer & Steinbuchel, 2003; Röttig & Steinbüchel, 2013b). However, low levels of TAG accumulated were found, suggesting the presence of additional pathway for TAG synthesis, only activated under unknown conditions.

1,2-diacyl-sn-glycerol-3-phosphate/phosphatidic acid is a common precursor for TAG and membrane phospholipid synthesis (Zhang & Rock, 2008), suggesting an alternative route for TAG synthesis in bacteria. This compound can be used for the synthesis of two types of diacylglycerols: 1,2 diacyl-sn-glycerol and CDP-diacylglycerol. Genes coding for putative enzymes involved in phospholipid synthesis with high similarity percentages were detected in all bacteria: phosphatidate cytidylyltransferase (2.7.7.41), CDP-diacylglycerol—serine-O-phosphatidyltransferase (2.7.8.8), phosphatidylserine decarboxylase (4.1.1.65). Curiously, no genes coding for phospholipase C (3.1.4.3) were found in the genomes of *A. borkumensis*, *A. baylyi* and *P. putida*. Using CDP-diacylglycerol as substrate, two sequential enzymes can catalyze the synthesis of 1,2-diacylglycerol, further consumed to form TAG by the Kennedy pathway. Three genes coding for CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase (2.7.8.5) were detected in both *Rhodococcus* strains and only one gene in the other species. No genes coding for phosphatidylglycerol phosphatase GEP4 (3.1.3.27) in *Rhodococcus* strains were present, whereas one gene coding for this enzyme is present in *A. borkumensis*, *A baylyi* and *P. putida*. The last reaction in phospholipid formation is catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) (2.3.1.58). PDAT is present in several eukaryotes (plants and yeasts) but until now no homologous genes were identified in bacterial genomes. However, in *S. coelicolor* PDAT activity was detected, indicating that an alternative pathway for TAG synthesis may be present (Arabolaza,

et al., 2008). In this work, no genes coding for PDAT enzyme were found in in the genomes of the five hydrocarbonoclastic bacteria analysed.

A comprehensive comparative analysis of genome-based metabolic pathways involved in TAG synthesis in different hydrocarbonoclastic bacteria is represented in Figure 7.1.

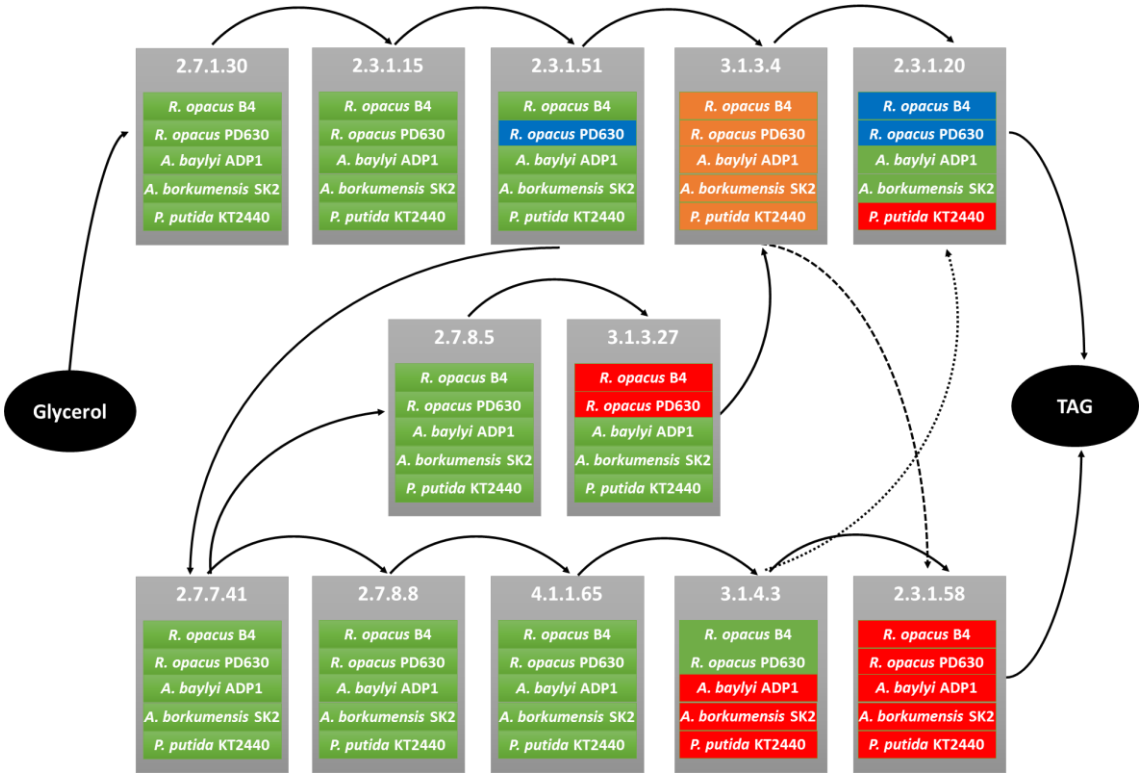


Figure 7.1 Proposed metabolic pathway for TAG synthesis in hydrocarbonoclastic bacteria. Each EC number represented is characterized in Table 7.2. The same color code as in Table 7.2 is applied. Red color represents absence of genes.

There are evident differences in the metabolic reactions catalysing the formation of TAG, depending essentially on the bacterial species itself. *R. opacus* B4 and *R. opacus* PD630 share the same enzymatic profiles, where glycerol-3-phoshpate is sequentially acylated to 1,2-diacyl-sn-gycerol-3-phosphate through the Kennedy pathway. Consequently, 1,2-diacyl-sn-glycerol-3-phosphate is channeled to glycerophospholipid metabolism and converted to CDP-diacylglycerol which in turn is transformed into 1.2-diacylglycerol through a set of sequential reactions catalyzed by enzymes 2.7.8.8; 4.1.1.65 and 3.1.4.3. Finally, the last acylation reaction to produce triacylglycerol is performed by 1,2-diacylglycerol acyltransferase, the only enzyme that belongs exclusively to TAG synthesis pathway. By contrast, *A. borkumensis* SK2 and *A. baylyi* ADP1 possess a different metabolic route. Similarly to *Rhodococcus* strains, glycerol-3-phosphate is converted to 1,2-diacyl-

sn-gycerol-3-phosphate by means of the Kennedy pathway. This compound can then be converted to 1,2 diacyl-sn-glycerol/phosphatidic acid by two ways: the first consists in the direct transformation catalysed by phosphatidic acid phosphatase (3.1.3.4); the second way consists in its conversion to CDP-diacylgycerol, followed by phosphatidylglycerol formation through the action of 2.7.8.5 and 3.1.3.27 and subsequent conversion to 1,2 diacyl-sn-glycerol by phosphatidic acid phosphatase (3.1.3.4). However, no gene coding for phopsphatidic acid phosphatase was found in the genomes of *Alcanivorax* and *Acinetobacter* strains. This result does not mean a total enzyme absence, since it can have a different gene/protein sequence and further research should be performed using taxonomically more closely related sequences, for instance, *S. coelicolor* phosphatidic acid phosphatase sequences (Comba *et al.*, 2013). Finally, the synthesis of triacylglycerol is once more performed by 1,2-diacylglycerol acyltransferase. The genome of *P. putida* KT2440 has genes coding for all enzymes involved in TAG synthesis, except for the enzymes that catalyse the last step, 1,2-diacylglycerol acyltransferase (2.3.1.20) and phospholipid:diacylglycerol acyltransferase (2.3.1.58). These results suggest that no diacylglycerol acyltransferase activity is present, which is confirmed by the absence of reports in literature regarding TAG production and accumulation in the genus *Pseudomonas*.

7.3.2 Wax ester synthesis

The general metabolic pathway involved in WE synthesis in bacteria, using acyl-coA as precursor, consists in two sequential reduction reactions producing aldehyde and alcohol intermediates, followed by a final acylation reaction. Acyl-coA is first converted to a fatty aldehyde by an acyl-CoA reductase which is further transformed into the corresponding fatty alcohol by an NADPH-dependent fatty aldehyde reductase. The final reaction in WE synthesis is catalyzed by an acyltransferase enzyme, mediating a esterification reaction between fatty a acyl-coA and the alcohol molecule (Wältermann *et al.*, 2007; Kalscheuer, 2010; Röttig & Steinbüchel, 2013a). Table 7.3 describes putative WE synthesis enzymes and the corresponding orthologous genes found in the genomes of representative members of hydrocarbonoclastic bacteria. Genes coding for the enzymes fatty acyl-CoA reductase (1.2.1.50) and NADPH-dependent fatty aldehyde reductase (1.2.1.-) were not detected in the genomes of any of the hydrocarbonoclastic bacteria analysed.

Table 7.3 Genes and the coreesponding metabolic reactions involved in the WE biosynthesis pathways present in the genomes of selected hydrocarbonoclastic bacteria. Green color represents genes with more than 90 % similarity; blue color represents genes with similarity between 50% and 90 %; orange color represents genes with similarity between 10 % and 50 %; Pink color represents genes with similarity lower than 10 %

Enzyme	EC number	Metabolic reaction	Orthologous	*R. opacus* B4	*R. opacus* PD630	*A. borkumensis* SK2	*A. baylyi* ADP1	*P. putida* KT2440
long-chain-fatty-acyl-CoA reductase	1.2.1.50	a long-chain aldehyde + CoA + NADP+ = a long-chain acyl-CoA + NADPH + H+	K03400					
fatty aldehyde reductase	1.2.1.-		K13356					
wax ester synthase	2.3.1.75	acyl-CoA + a long-chain alcohol = CoA + a long-chain ester	K15406	ROP_54550	OPAG_07648T0		ACIAD0832	
				ROP_26950	OPAG_03918T0			

This can be related to the fact that the metabolic pathways for WE synthesis present in KEGG, which was used as reference for metabolic screening, belong to the metabolism of plants.

In fact, no metabolic pathways responsible for synthesis of WE in bacteria has been fully described. However, some experimental evidences points out the presence of these enzymes in bacteria. Using *Acinetobacter calcoaceticus* BD413, several mutants defective in WE production were constructed, where one of the mutants was able to produce WE when cultivated on alcohols and aldehydes but no WE were detected in the presence of the respective alkane, suggesting the presence of an acyl-coA reductase (Reiser & Somerville, 1997). In recent studies, it was suggested the presence of a NADPH-dependent alcohol dehydrogenase AlrA in *Acinetobacter* strains as the main responsible for the conversion of fatty aldehydes into the corresponding fatty alcohols (Tani *et al.*, 2000; Uthoff & Steinbüchel, 2012). Additionally, different alcohol and aldehyde reductase enzymes were found in *Marinobacter aquaeolei* VT8 (Wahlen*, et al.*, 2009; Willis *et al.*, 2011; Lenneman *et al.*, 2013). Although with a very low similarity percentage (less than 10 %), genes coding for wax ester synthase (2.3.1.75) were identified in the genomes of *R. opacus* B4, *R. opacus* PD630 and *A. baylyi* ADP1. In fact, this bacterial enzyme represents a novel type of acyltrasnferase family which is not phylogenetically related to those present in eukaryotes, revealing dual activity as WE synthase and acyl-coenzyme A:diacylglycerol acyltransferase (WS/DGAT) (Kalscheuer & Steinbuchel, 2003; Wältermann *et al.*, 2007; Röttig & Steinbüchel, 2013a). WS/DGAT was firstly identified in *A. baylyi* ADP1 (Kalscheuer & Steinbuchel, 2003). Afterwards, several studies were performed to extensively characterize this enzyme in terms of biochemical parameters, substrate range, cellular localization, active site and catalytic mechanism (Stöveken *et al.*, 2005; Wältermann *et al.*, 2005; Stöveken *et al.*, 2009; Röttig & Steinbüchel, 2013b). Several WS/DGAT homologous sequences were found in *Rhodococcus opacus* strains and some of them revealed WS activity (Alvarez *et al.*, 2008; Holder *et al.*, 2011; Villalba, 2013). *A. borkumensis* SK2 is able to produce both TAG and WE compounds, depending on the carbon source used for growth (Manilla-Pérez *et al.*, 2011). This metabolic capability was confirmed by the presence of two WS/DGAT homologous genes in the genome of *A. borkumensis*, namely atfA1 and atfA2. The corresponding enzymes, AtfA1 and AtfA2 revealed WS activity (Kalscheuer *et al.*, 2007; Manilla-Pérez *et al.*, 2011).

7.3.2 Polyhydroxyalkanoate synthesis

Polyhydroxyalkanoates (PHA) are the main storage compounds produced by mostbacteria. Depending on the monomer composition, PHA can be classified in three main categories: short-chain-length (scl) PHA, composed by monomers ranging between 3 and 5 carbons (3-hydroxybutyrate (3HB), and 3-hydroxyvalerate (3HV)); medium-chain-length (mcl) PHA, composed by monomers ranging between 6 and 16 carbons (for instance, 3-hydroxyhexanoate (3HHx), 3-hydroxyheptanoate (3HHp), and 3-hydroxytetradecanoate (3HTD)) and also a mixture of scl and mcl monomers (scl-mcl) (Steinbüchel & Valentin, 1995; Sudesh *et al.*, 2000; Tokiwa *et al.*, 2009; Keshavarz & Roy, 2010). Among PHA, Polyhydroxybutyrate (PHB) is the most common and studied class in bacteria (Verlinden *et al.*, 2007). The generic bacterial metabolic pathways involved in PHB consist in three sequential reactions, using acetyl-CoA as precursor (Steinbüchel *et al.*, 1992). The first reaction is mediated by acetyl-CoA acyltransferase (PhaA), condensing two acetyl-CoA molecules with subsequent formation of acetoacetyl-CoA. Subsequently, the acetoacetyl-CoA molecule is converted in (R)-3-hydroxybutyryl-CoA by the action of NADPH-dependent acetoacetyl-CoA reductase (PhaB). Finally, the third reaction is catalysed by PHA synthase (PhaC), which promotes the polymerization of (R)-3-hydroxybutyryl-CoA monomers, forming PHB. Table 7.4 describes the genes coding for PHB synthesis identified in the genomes of selected hydrocarbonoclastic bacteria. Genes encoding for acetyl-CoA acyltransferase (EC 2.3.1.9) with very high similarity percentage (more than 90 %) were detected in the genomes of all bacterial species. However, significant differences were found between them. *Rhodococcus* strains present a high number of genes (23 genes in *R. opacus* B4 and 26 genes in *R. opacus PD630*), whereas *A. borkumensis*, *A. baylyi* and *P. putida* have between 4 and 7 genes. Using acetoacetyl-CoA as precursor, 2 possible alternative ways to produce(R)-3-hydroxybutyryl-CoA were identified: 1) direct reduction of acetoacetyl-CoA by means of acetoacetyl-CoA reductase (1.1.1.36); 2) two-step dehydrogenation and epimerization, forming as (S)-3-hydrozybutanoyl-CoA as intermediate by the action of 3-hydroxyacyl-CoA dehydrogenase (1.1.1.35) and 3-hydroxybutyryl-CoA epimerase (5.1.2.3), respectively.

Table 7.4 Genes and the correponding netabolic reactions involved in the PHB biosynthesis pathways present in the genomes of selected hydrocarbonoclastic bacteria. Green color represents genes with more than 90 % similarity; blue color represents genes with similarity between 50 % and 90 %; orange color represents genes with similarity between 10 % and 50 %; Pink color represents genes with similarity lower than 10 %

Enzyme	EC number	Metabolic reaction	Orthologous	*R. opacus* B4	*R. opacus* PD630	*A. borkumensis* SK2	*A. baylyi* ADP1	*P. putida* KT2440
Acetyl-CoA C-acetyltransferase	2.3.1.9	2 acetyl-CoA = CoA + acetoacetyl-CoA	K00626		OPAG_03903T0 OPAG_04490T0 OPAG_02287T0 OPAG_06267T0 OPAG_06657T0 OPAG_00428T0			
Hydroxymethylglutaryl-CoA synthase	2.3.3.10	acetyl-CoA + H2O + acetoacetyl-CoA = (S)-3-hydroxy-3-methylglutaryl-CoA + CoA	K01641	ROP_52560	OPAG_02164T0 OPAG_06888T0 OPAG_02333T0 OPAG_06179T0			
Acetoacetyl-CoA reductase	1.1.1.36	(R)-3-hydroxyacyl-CoA + NADP+ = 3-oxoacyl-CoA + NADPH + H+	K00023	ROP_46150	OPAG_01262T0	ABO_1069 ABO_2534 ABO_0632 ABO_0960 ABO_2053 ABO_0656 ABO_0959 ABO_0990 ABO_0954 ABO_0663 ABO_1265 ABO_1918	ACIAD0871 ACIAD0582 ACIAD2509 ACIAD1565 ACIAD1022 ACIAD2676 ACIAD1824 ACIAD0995 ACIAD2416 ACIAD1691 ACIAD0989 ACIAD1439	PP_1914 PP_2783 PP_1852 PP_3547 PP_3073 PP_1953 PP_3926 PP_1946 PP_1817 PP_2794 PP_2784 PP_2002

Table 7.4 (Continued)

Enzyme	EC number	Metabolic reaction	Orthologous	*R. opacus* B4	*R. opacus* PD630	*A. borkumensis* SK2	*A. baylyi* ADP1	*P. putida* KT2440
Acetoacetyl-CoA reductase	1.1.1.36	R)-3-hydroxyacyl-CoA + NADP+ = 3-oxoacyl-CoA + NADPH + H+	K00023					PP_3972 PP_1171 PP_0738 PP_2608
3-hydroxy-3-methylglutaryl-CoA lyase	4.1.3.4	(R)-3-hydroxyacyl-CoA + NADP+ = 3-oxoacyl-CoA + NADPH + H+	K01640	ROP_49950 ROP_45850 ROP_51390	OPAG_02624T0 OPAG_01292T0 OPAG_07354T0 OPAG_02298T0 OPAG_02470T0	ABO_2665	ACIAD2820	PP_3394 PP_3540
poly (3-hydroxyalkanoate) synthase	2.3.1.-		K03820	ROP_43060 ROP_36330	OPAG_07900T0 OPAG_05039T0 OPAG_03189T0	ABO_1418	ACIAD0415	PP_5003 PP_5005
3-hydroxybutyrate dehydrogenase	1.1.1.30	(R)-3-hydroxybutanoate + NAD+ = acetoacetate + NADH + H+	K00019	ROP_48680	OPAG_02763T0	ABO_1479	ACIAD2509	PP_3073
hydroxybutyrate-dimer hydrolase	3.1.1.22	(R)-3-((R)-3-hydroxybutanoyloxy)butanoate + H2O = 2 (R)-3-hydroxybutanoate	K07518					
Poly(3-hydroxybutyrate) depolymerase	3.1.1.75	[(R)-3-hydroxybutanoate]n + H2O = [(R)-3-hydroxybutanoate]n-x + [(R)-3-hydroxybutanoate]x; x = 1—5	K05973			ABO_1418		
3-hydroxybutyryl-CoA epimerase	5.1.2.3	(S)-3-hydroxybutanoyl-CoA = (R)-3-hydroxybutanoyl-CoA	K01825			ABO_1652	ACIAD0335	PP_2136
			K01782	ROP_25300 ROP_41320	Pd630_LPD00606 Pd630_LPD02756	ABO_1566	ACIAD2989	
3-hydroxyacyl-CoA dehydrogenase	1.1.1.35	(S)-3-hydroxyacyl-CoA + NAD+ = 3-oxoacyl-CoA + NADH + H+	K01825			ABO_1652	ACIAD0335	PP_2136
			K01782	ROP_25300 ROP_41320	Pd630_LPD00606 Pd630_LPD02756	ABO_1566	ACIAD2989	
			K07516	ROP_26130	Pd630_LPD04727			

One gene with more than 90 % similarity coding for acetoacetyl-CoA reductase (1.1.1.36) was found in the genomes of *Rhodococcus* strains. On the other hand, several genes with 10 % to 50 % similarity were identified in the other species, namely 21, 24 and 30 genes in *A. borkumensis*, *A. baylyi* and *P. putida*, respectively. In the second alternative way, genes with very high similarity coding for both enzymes were detected in the genomes of all bacteria. The last reaction of PHB synthesis is performed by polyhydroxyalkanoate synthase (2.3.1-). In all hydrocarbonoclastic bacteria analysed, genes presenting more than 90 % similarity for this enzyme were found. In KEGG database, one additional pathway composed by 5 sequential reactions that leads to PHB synthesis was identified. The first reaction is catalyzed by 3-hydroxy-3-methylglutaryl CoA synthetase (2.3.3.10), using acetyl-CoA and acetoacetyl-coA as precursors. However, few genes with less than 10 % similarity coding for this enzyme were identified in the genomes of *R. opacus* B4 and *R. opacus* PD630 and no genes were found in the other bacteria. The two following reactions are mediated by 3-hydroxy-3-methylglutaryl-CoA lyase (4.1.3.4) and 3-hydroxybutyrate dehydrogenase (1.1.1.30), forming (R)-3-hydroxylbutanoate. In all genomes of hydrocarbonclastic bacteria analysed, genes coding for these two enzymes are present with a high similarity percentage (more than 90 %). However, the enzymes catalyzing the last two reactions, namely hydroxybutyrate-dimer hydrolase (3.1.1.22) and poly(3-hydroxybutyrate) depolymerase (3.1.1.75) were not detected in the genomes of any bacterial species. A comprehensive comparative analysis of genome-based metabolic pathways involved in PHB synthesis in different hydrocarbonoclastic bacteria is represented in Figure 7.2. *R. opacus* B4 and *R. opacus* PD630 synthesize PHB compounds by the typical metabolic pathway, where two acetyl-CoA moelcules are condensed, by the action of acetyl-coA acyltransferase (2.3.1.9), forming acetoacetyl-CoA which is further converted in (R)-3-hydroxy-butanoyl-CoA by acetoacetyl-CoA reductase (1.1.1.36). Finally, poly(3-hydrozyalkanoate) synthase (2.3.1-) acts on the polymerization of (R)-3-hydroxybutyryl-CoA monomers, forming PHB. The genus *Rhodococcus* is widely known as being the main TAG producer among hydrocarbonoclastic bacterial genera. However, some members of the *Rhodococcus* genus, including *R. opacus* species, have the ability to accumulate PHB. When cultivated on different carbon sources, such as glucose gluconate, acetate and toluene, under nitrogen limiting conditions, *R. aetherivorans*, *R. fascians*; and *R. erythropolis* accumulated significant PHA amounts (Haywood *et al.*, 1991; Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000; Alvarez, 2003; Hori *et al.*, 2009). These PHA compounds vary in their composition, being accumulated in the form of copolyester formed by 3-hydroxybutyrate and 3-hydroxyvalerate

monomers or homopolyester composed by 3-hydroxybutyrate monomer (Alvarez & Steinbuchel, 2002; Alvarez, 2003; Hernandez *et al.*, 2008). In addition, *R. opacus* PD630, *R. jostii* RHA1 and *R. ruber* are able to produce PHA in addition to TAG and other storage compounds (Alvarez *et al.*, 1997a; Alvarez *et al.*, 2000; Hernandez *et al.*, 2008; Hernandéz & Alvarez, 2010). Despite the vast body of reports describing PHA accumulation by different *Rhodococcus* species, it remains to explore the genomic and metabolic machinery responsible for this storage capability. The key enzymes responsible for PHA synthesis are PHA synthases. Until now, the genes coding for this enzyme were only identified in *R. ruber* and *R. jostii* (Pieper & Steinbuchel, 1992; Hernandez *et al.*, 2008). In *R. ruber*, the PHA synthase (PhaCRr) enzyme produces polymers of 3-hydroxy fatty acids of short chain length, ranging between 3 to 5 carbons. More recently, it was demonstrated the presence of 3 sets of genes encoding PHA synthases in *R. jostii* RHA1, which are approximately 40 % similar to the one identified in *R. ruber* (Hernandez *et al.*, 2008). Additionally, mutants of *R. opacus* PD630 defective in TAG synthesis suffered an increase in PHA levels, demonstrating that the carbon flow was channeled to PHA metabolism (Hernandéz & Alvarez, 2010).

A. *borkumensis*, *A. baylyi* and *P. putida* seem to present slight differences in PHB synthesis metabolic pathways when compared to *Rhodococcus opacus*. Acetoacetyl-CoA is converted to PHB by means of two alternative enzymes: 3-hydroxyacyl-CoA dehydrogenase (1.1.1.35) and 3-hydroxybutyryl-CoA epimerase (5.1.2.3). Although genes encoding for all the enzymes necessary to PHB production are present in the genomes of both species, there are very few reports describing the synthesis of PHB and the presence of PHB synthase genes in *A. borkumensis* and *A. baylyi* (Rees *et al.*, 1993; Schembri *et al.*, 1994; Sabirova *et al.*, 2006b). This can be related to the focus on the ability of these species to accumulate TAG and WE (Manilla-Pérez *et al.*; 2010a; Santala *et al.*, 2011a; Santala *et al.*, 2011b). *Pseudomonas* is one of the most significant genera towards PHA production and accumulation. In fact, PHA is considered the main storage compound in this genus and there is an extensive number of reports describing PHA synthesis in different *Pesudomonas* species using different substrates as carbon and energy sources as well as characterizing their metabolic pathways (Matsusaki *et al.*, 1998; Ren *et al.*, 2005; Ayub *et al.*, 2006; Tan *et al.*, 2010; Poblete-Castro *et al.*, 2012; Poblete-Castro *et al.*, 2013; Yang *et al.*, 2013; Borrero-de Acuna *et al.*, 2014).

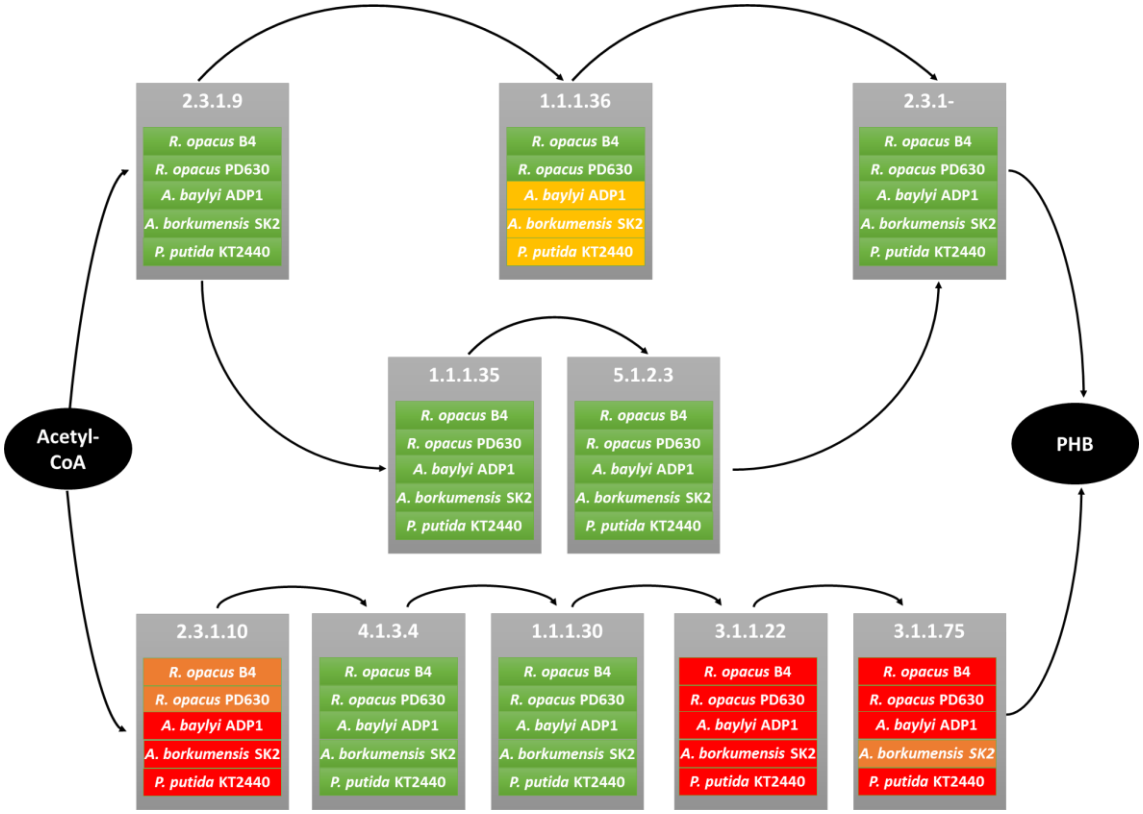


Figure 7.2 Proposed metabolic pathways for PHB synthesis in hydrocarbonoclastic bacteria. Each EC number represented is characterized in Table 7.4. The same color code as in Table 7.2 is applied. Red color represents absence of genes.

7.4 Conclusions

In this work a genome-based comparative analysis of metabolic pathways involved in TAG, WE and PHB synthesis of different hydrocarbonoclastic bacterial species was performed. Although most of bacteria present complete sets of genes responsible for TAG and PHB biosynthesis, there are differences in the metabolic routes and in the number of genes among the different species. In *R. opacus* strains, TAG production can occur through a set of reactions from the glycerolipid and glycerophoshoplipid metabolism, being highly enriched with genes encoding for diacylglycerol O-acyltransferase (EC 2.3.1.20), mediating the last reaction in TAG synthesis. In *Alcanivorax* and *Acinetobacter* this synthesis mainly occurs through glycerolipid metabolism, whereas *Pseudomonas* does not possess any gene encoding for diacylglycerol O-acyltransferase.

For PHB biosynthesis three main metabolic routes were identified. *Rhodococcus* can produce PHB by using three and five step sequential reactions, whereas *Alcanivorax*, *Acinetobacter* and *Pseudomonas* may use preferentially the five step reaction metabolic route. Interestingly, *Rhodococcus* genomes are highly enriched in genes coding for Acetyl-CoA C-acetyltransferase (EC

2.3.1.9) whereas *Alcanivorax*, *Acineotbacter* and *Pseudomonas* have a high number of genes coding for Acetoacetyl-CoA reductase (EC 1.1.1.36).

This comparative study represents a general and comprehensive platform to elucidate about metabolic pathways present in hydrocarbonoclastic bacteria with different abilities in TAG, WE and PHB production. In this context, the obtained results can be used to go deeper on the identification of key genes for the development of engineered strains with enhanced metabolic capability in storage lipid compounds for biotechnological and industrial purposes.

Chapter 8

Final conclusions and perspectives for future work

8.1 Final conclusions

In this thesis it was explored the production of bacterial lipid storage compounds using hydrocarbon substrates. This ability was demonstrated by pure bacterial cultures using hexadecane as model hydrocarbon compound in liquid and solid contaminated wastes and also by mixed enriched bacterial cultures using real and complex hydrocarbon-based wastewaters. A deeper knowledge on genomic basis of lipid storage metabolism of hydrocarbonoclastic bacteria was obtained, using a comparative bioinformatics approach. The main conclusions of this thesis were:

1) *R. opacus* B4 was able to produce lipid storage compounds (mainly TAG) under nitrogen limiting conditions from different carbon sources, namely glucose, acetate and hexadecane. When compared with the best known TAG producer *R. opacus* PD630, revealed a better performance regarding TAG content, yield and volumetric production for hexadecane (Chapter 3). It was also demonstrated that *R. opacus* B4 is able to decontaminate different hexadecane impregnated cork sorbents, natural cork and thermally treated cork, producing TAG as main lipid storage compound. The most dominant fatty acids present in TAG were palmitic and palmitoleic acids. The obtained lipid-rich biomass was efficiently converted to biomethane (Chapter 4). These results demonstrated the use of *R. opacus* B4 as potential feedstock, either as extracted TAG or whole lipid-rich biomass, for biodiesel and biomethane production.

2) A microbial community enriched in hydrocarbon degrading carbon storage producing bacteria was successfully developed by a multistage enrichment process under carbon and feast and famine conditions, using sludge and hydrocarbon-rich wastewater as inoculum and carbon sources, respectively. The enriched community was mainly composed by hydrocarbon-degrading bacteria, belonging to *Rhodococcus*, *Acinetobacter* and *Pseudomonas* genera, known for their ability to synthesize TAG, WE and PHA. Additionally, it was able to degrade the majority of hydrocarbons present in the wastewater, while producing TAG, PHA and an unknown lipid compound in substantial amounts (Chapter 5). The influence of carbon and nitrogen concentrations as well as cultivation time on storage compound production by the obtained microbial community was evaluated. The community produced lower PHA amounts (9%) when compared to TAG (33%), being TAG mainly composed by a wide range of chemical structures and fatty acid composition. Interactions between carbon and nitrogen

concentrations as well as cultivation time heavily significantly influenced PHA accumulation, whereas only the interaction between carbon and nitrogen concentrations affect neutral lipid compounds production (Chapter 6). These results demonstrated that indigenous enriched microbial communities can be obtained from oily wastewaters and are potential resources for producing high-value storage compounds. Additionally, the use of this type of wastewaters which are cheap raw materials, can significantly reduce production and commercialization cost as well as mitigate environmental pollution by decreasing the amount of wastes that has to be treated.

3) A genome-based comparative analysis of metabolic pathways involved in TAG, WE and PHB synthesis of different hydrocarbonoclastic bacterial species was performed. Although most of bacteria present complete sets of genes responsible for TAG and PHB biosynthesis, there are important differences in the metabolic routes and in the number of genes among the different species. In *Rhodococcus* strains, TAG production can occur through a set of reactions from the glycerolipid and glycerophoshoplipid metabolism, being highly enriched in genes encoding for diacylglycerol O-acyltransferase (EC 2.3.1.20), mediating the last reaction in TAG synthesis. In *Alcanivorax* and *Acinetobacter* this synthesis mainly occurs through glycerolipid metabolism, whereas *Pseudomonas* does not possess any gene encoding for diacylglycerol O-acyltransferase. For PHB biosynthesis three main metabolic routes were identified. *Rhodococcus* can produce PHB by using three and five step sequential reactions, whereas *Alcanivorax*, *Acinetobacter* and *Pseudomonas* may use preferentially the five step reaction metabolic route. These results constituted a comprehensive platform to elucidate about storage compound production metabolic pathways present in hydrocarbonoclastic bacteria with different abilities in TAG, WE and PHB compounds.

8.2 Perspectives for future work

The work presented in this thesis significantly contributes to an increased knowledge on the use of hydrocarbonoclastic bacteria for two biotechnological processes: treatment of hydrocarbon-based wastes and production of storage compounds, with recognized properties as feedstocks for biofuels and bio-based chemicals industries. Although bacterial treatment of complex residues and carbon storage compounds production processes are emergent and some progress has been made in this subject, the scientific research is still in its infancy. Therefore, further efforts should be made regarding different fundamental and applied aspects on these bacterial processes in order to efficiently develop and improve a dual bacterial-based biotechnological process combining hydrocarbon pollutants decontamination and production of bacterial feedstocks for industrial applications.

The capability of *R. opacus* B4 to produce TAG with fatty acids composition relevant for biofuels and biogas production purposes using hydrocarbon-based liquid and solid wastes opens new perspectives on the use of hydrocarbonclastic bacteria for production of bio-based compounds with industrial applications. In this context, further investigation on the isolation of novel hydrocarbon-degrading bacteria able to produce lipid storage compounds as well as exploring these capabilities in the existing ones should be performed. Additionally, this type of approach should be applied to a wider ranger of real and complex hydrocarbon residues as well as assess the economic viability of this type of lipid-rich biomass.

The development of indigenous mixed cultures enriched in hydrocarbon degrading carbon storage accumulating bacteria at the expense of oily wastewaters is a promising way to combine in a more economic and environmentally sustainable way two biotechnological processes: pollutant decontamination and production of bacterial lipids. However, in order to efficiently develop this type of process is mandatory to perform more research focusing in further improvement of reserve compound production process, namely by optimization of operational conditions and technology development and also to assess changes in bacterial diversity throughout the process.

The genome-based comparative study developed in this thesis represents a general and comprehensive platform to elucidate about metabolic pathways present in hydrocarbonoclastic bacteria with different abilities in TAG, WE and PHB compounds. In this context, this type of approach should further be applied to other bacteria and the obtained results can be used to go deeper on the identification of key genes for the development of engineered bacterial strains with enhanced metabolic capability in storage lipid compounds for biotechnological and industrial applications.

References

Adamczak M, Bornscheuer UT & Bednarski W (2009) The application of biotechnological methods for the synthesis of biodiesel. *Eur J Lipid Sci Technol* **111**: 808 - 813.

Adebajo MO, Frost RL, Kloprogge JT, Carmody O & Kokot S (2003) Porous materials for oil spill cleanup: a review of synthesis and absorbing properties. *Journal of Porous Materials* **10**: 159-170.

Ageitos J, Vallejo J, Veiga-Crespo P & Villa T (2011) Oily yeasts as oleaginous cell factories. *Applied Microbiology and Biotechnology* **90**: 1219-1227.

Akaraonye E, Keshavarz T & Roy I (2010) Production of polyhydroxyalkanoates: the future green materials of choice. *Journal of Chemical Technology & Biotechnology* **85**: 732-743.

Akpor OB OU, Olaolu TD, Aderiye, BI (2014) Remediation of polluted wastewater effluents: hydrocarbon removal. *Trends in Apllied Siences Research* **9**: 160-173.

Al-Husseini M (2009) World production of conventional petroleum liquids to 2030: A comparative overview. *GeoArabia* **14**: 215-267.

Al-Mailem DM, Kansour MK & Radwan SS (2015) Moderately thermophilic, hydrocarbonoclastic bacterial communities in Kuwaiti desert soil: enhanced activity via Ca2+ and dipicolinic acid amendment. *Extremophiles* **19**: 573-583.

Al-Tahhan RA, Sandrin TR, Bodour AA & Maier RM (2000) Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates. *Applied and Environmental Microbiology* **66**: 3262-3268.

Alberti F, Bienati B, Bottino A, Capannelli G, Comite A, Ferrari F & Firpo R (2007) Hydrocarbon removal from industrial wastewater by hollow-fibre membrane bioreactors. *Desalination* **204**: 24-32.

Albuquerque MGE, Torres CAV & Reis MAM (2010) Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: Effect of the influent substrate concentration on culture selection. *Water Research* **44**: 3419-3433.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Alvarez A, Alvarez H, Kalscheuer R, Waltermann M & Steinbuchel A (2008) Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus PD630*. *Microbiology* **154**: 2327 - 2335.

Alvarez H (2003) Relationship between beta-oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria. *International Biodeterioration and Biodegradation* **52**: 35 - 42.

Alvarez H (2006) Bacterial Triacylglycerols. *Triglycerides and Cholesterol Research* **6**: 159 - 176.

Alvarez H & Steinbuchel A (2002) Triacylglycerols in prokaryotic microorganisms. *Applied Microbiology and Biotechnology* **60**: 367 - 376.

Alvarez H & Steinbüchel A (2010) Physiology, biochemistry, and molecular biology of triacylglycerol accumulation by *Rhodococcus*. *Biology of Rhodococcus,* Vol. 16 (Alvarez HM) 263-290. Springer Berlin Heidelberg.

Alvarez H, Kalscheuer R & Steinbuchel A (1997a) Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. *Fett/Lipid* **99**: 239 - 246.

Alvarez H, Kalscheuer R & Steinbuchel A (2000) Accumulation and mobilization of storage lipids by Rhodococcus opacus PD630 and *Rhodococcus ruber* NCIMB 40126. *Applied Microbiology Biotechnology* **54**: 218 - 223.

Alvarez H, Mayer F, Fabritius D & Steinbuchel A (1996) Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. *Archives in Microbiology* **165**: 377 - 386.

Alvarez H, Souto M, Viale A & Pucci O (2001) Biosynthesis of fatty acids and triacylglycerols by 2,6,10,14-tetramethyl pentadecane-grown cells of *Nocardia globerula* 432. *FEMS Microbiology Letters* **200**: 195 - 200.

Alvarez HM (2010) Biotechnological Production and Significance of Triacylglycerols and Wax Esters. *Handbook of Hydrocarbon and Lipid Microbiology* (Timmis K) 2995-3002. Springer Berlin Heidelberg.

Alvarez HM, Pucci OH & Steinbüchel A (1997b) Lipid storage compounds in marine bacteria. *Applied Microbiology and Biotechnology* **47**: 132-139.

Alvarez HM, Silva RA, Haro PA, Alvarez AF, Perez JD & Villalba MS (2007) From basic research to application: Use of *Rhodococcus* spp. for environmental biotechnology in semiarid Patagonia, Argentina. *Journal of Biotechnology* **131**: S243-S244.

Alvarez HM, Luftmann H, Silva RA, Cesari AC, Viale A, Wältermann M & Steinbüchel A (2002) Identification of phenyldecanoic acid as a constituent of triacylglycerols and wax ester produced by *Rhodococcus opacus* PD630. *Microbiology* **148**: 1407-1412.

Alvarez HM, Silva RA, Cesari AC*,* Zamit AL, Peressutti SR, Teichelt R, Keller U, Malkus U, Rasch C, Maskow T, Mayer F, Steinbüchel A (2004) Physiological and morphological responses of the soil bacterium *Rhodococcus opacus* strain PD630 to water stress. *FEMS Microbiology Ecology* **50**: 75-86.

Alvarez HM SR, Herrero M, Hernández MA, Villalba MS (2013) Metabolism of triacylglycerols in *Rhodococcus* species: insights from physiology and molecular genetics. *Journal of Molecular Biochemistry* **2**: 69-78.

Alvarez PJ & Vogel TM (1991) Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Applied and Environmental Microbiology* **57**: 2981-2985.

Alves MM, Pereira MA, Sousa DZ, Cavaleiro AJ, Picavet M, Smidt H & Stams AJM (2009) Waste lipids to energy: how to optimize methane production from long-chain fatty acids (LCFA). *Microbial Biotechnology* **2**: 538-550.

Alzate ME, Muñoz R, Rogalla F, Fdz-Polanco F & Pérez-Elvira SI (2014) Biochemical methane potential of microalgae biomass after lipid extraction. *Chemical Engineering Journal* **243**: 405-410.

Amirul AA, Yahya ARM, Sudesh K, Azizan MNM & Majid MIA (2008) Biosynthesis of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA1020 isolated from Lake Kulim, Malaysia. *Bioresource Technology* **99**: 4903-4909.

Amouric A, Quéméneur M, Grossi V, Liebgott PP, Auria R & Casalot L (2010) Identification of different alkane hydroxylase systems in *Rhodococcus ruber* strain SP2B, an hexane-degrading actinomycete. *Journal of Applied Microbiology* **108**: 1903-1916.

Anderson AJ & Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews* **54**: 450-472.

Andrady AL & Neal MA (2009) Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**: 1977-1984.

Andreoni V, Bernasconi S, Colombo M, Van Beilen JB & Cavalca L (2000) Detection of genes for alkane and naphthalene catabolism in *Rhodococcus* sp. strain 1BN. *Environmental Microbiology* **2**: 572-577.

Angelidaki I AM, Bolzonella D, Borzacconi L, Campos JL, Guwy AJ, Kalyuzhnyi S, Jenicek P, van Lier JB (2009) Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays *Water Science and Technology* **59**: 927-934.

Antonio RV, Steinbüchel A & Rehm BHA (2000) Analysis of in vivo substrate specificity of the PHA synthase from *Ralstonia eutropha*: formation of novel copolyesters in recombinant *Escherichia coli*. *FEMS Microbiology Letters* **182**: 111-117.

Arabolaza A, Rodriguez E, Altabe S, Alvarez H & Gramajo H (2008) Multiple pathways for triacylglycerol biosynthesis in *Streptomyces coelicolor*. *Applied and Environmental Microbiology* **74**: 2573 - 2582.

Arikan EB (2015) A review: investigation of bioplastics. *Journal of Civil Engineering and Architecture* **9**: 188-192.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Applied and Environmental Microbiology* **71**: 7724-7736.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environmental Microbiology* **72**: 5734-5741.

Asperger O, Naumann A & Kleber HP (1981) Occurrence of cytochrome P-450 in *Acinetobacter* strains after growth on N-hexadecane. *FEMS Microbiology Letters* **11**: 309-312.

Atabani AE, Silitonga AS, Badruddin IA, Mahlia TMI, Masjuki HH & Mekhilef S (2012) A comprehensive review on biodiesel as an alternative energy resource and its characteristics. *Renewable and Sustainable Energy Reviews* **16**: 2070-2093.

Atagana HI (2014) Biological degradation of crude oil refinery sludge with commercial surfactant and sewage sludge by co-composting. *Soil and Sediment Contamination: An International Journal* **24**: 494-513.

Atlas RM (1995) Petroleum biodegradation and oil spill bioremediation. *Marine Pollution Bulletin* **31**: 178-182.

Auffret M, Labbé D, Thouand G, Greer CW & Fayolle-Guichard F (2009) Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis*. *Applied and Environmental Microbiology* **75**: 7774-7782.

Auffret M, Yergeau E, Labbé D, Fayolle-Guichard F & Greer C (2014) Importance of *Rhodococcus* strains in a bacterial consortium degrading a mixture of hydrocarbons, gasoline, and diesel oil additives revealed by metatranscriptomic analysis. *Applied Microbiology and Biotechnology* 1-12.

Ayub ND, Pettinari MJ, Méndez BS & López NI (2006) Impaired polyhydroxybutyrate biosynthesis from glucose in *Pseudomonas* sp. 14-3 is due to a defective β-ketothiolase gene. *FEMS Microbiology Letters* **264**: 125-131.

Babu R, O'Connor K & Seeram R (2013a) Current progress on bio-based polymers and their future trends. *Progress in Biomaterials* **2**: 1-16.

Babu V, Thapliyal A & Patel GK (2013b) *Biofuels Production*. Wiley.

Baheri H & Meysami P (2002) Feasibility of fungi bioaugmentation in composting a flare pit soil. *Journal of Hazardous Materials* **89**: 279-286.

Balat M (2011) Potential alternatives to edible oils for biodiesel production – A review of current work. *Energy Conversion and Management* **52**: 1479-1492.

Balba MT, Al-Awadhi N & Al-Daher R (1998) Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *Journal of Microbiological Methods* **32**: 155-164.

Ball AS, Stewart RJ & Schliephake K (2012) A review of the current options for the treatment and safe disposal of drill cuttings. *Waste Management & Research* **30**: 457-473.

Banchio C & Gramajo H (2002) A stationary-phase acyl-coenzyme A synthetase of *Streptomyces coelicolor* A3 (2) is necessary for the normal onset of antibiotic production. *Applied Environmental Microbiology* **68**: 4240 - 4246.

Bansode RR, Losso JN, Marshall WE, Rao RM & Portier RJ (2004) Pecan shell-based granular activated carbon for treatment of chemical oxygen demand (COD) in municipal wastewater. *Bioresource Technology* **94**: 129-135.

Barbe V, Vallenet D, Fonknechten N, Kreimeyer A, Oztas S, Labarre L, Cruveiller S, Robert C, Duprat S, Wincker P, Ornston LN, Weissenbach J, Marliére P, Cohen NG, Médique C (2004) Unique features revealed by the genome sequence of *Acinetobacter* sp. ADP1, a versatile and naturally transformation competent bacterium. *Nucleic Acids Research* **32**: 5766-5779.

Bargiela R, Mapelli F, Rojo D, Chouaia B, Tornés J, Borin, S, Richter M, Del Pozo MV, Cappello M, Genovese M, Gertler C, Martínez-Martínez, M, Denaro R, Fodelianakis S, Amer RA, Bigazzi D, Han X, Chen J, Chernikova TN, Golyshina OV, Mahjoubi M, Jaouanil A, Benzha F, Magagnini M, Hussein E, Al-Horani F, Cherif A, Blaghen M, Abdel-Fattah YR, Kalogerakis N, Barbas C, Malkawi HI, Golyshin PN, Yakimov MM, Daffonchio D, Ferrer M (2015) Bacterial population and biodegradation potential in chronically crude oil-contaminated marine sediments are strongly linked to temperature. *Scientific Reports* **5**: 11651.

Barksdale L & Kim K (1977) *Mycobacterium*. *Bacteriological Reviews* **41**: 217-232.

Barney BM, Wahlen BD, Garner E, Wei J & Seefeldt LC (2012) Differences in substrate specificities of five bacterial wax ester synthases. *Applied and Environmental Microbiology* **78**: 5734-5745.

Barupal D, Kind T, Kothari S, Lee D & Fiehn O (2010) Hydrocarbon phenotyping of algal species using pyrolysis-gas chromatography mass spectrometry. *BMC Biotechnology* **10**: 1-9.

Battikhi MN (2014) Bioremediation of Petroleum Sludge. *Journal of Microbiology & Experimentation* **1**: 11-14.

Beccari M, Bertin L, Dionisi D, Fava F, Lampis S, Majone M, Valentino F, Vallini G, Villano M (2009) Exploiting olive oil mill effluents as a renewable resource for production of biodegradable polymers through a combined anaerobic–aerobic process. *Journal of Chemical Technology & Biotechnology* **84**: 901-908.

Bej AK, Saul D & Aislabie J (2000) Cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica. *Polar Biology* **23**: 100-105.

Bell, Philp, Aw & Christofi (1998) The genus *Rhodococcus*. *Journal of Applied Microbiology* **85**: 195-210.

Ben Rebah F, Prévost D, Tyagi R & Belbahri L (2009) Poly-β-hydroxybutyrate production by fast-growing Rhizobia cultivated in sludge and in industrial wastewater. *Applied Biochemistry and Biotechnology* **158**: 155-163.

Bengtsson S, Werker A, Christensson M, Wellander T (2008) Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater *Bioresource technology* **99**: 509-516.

Bequer Urbano S, Albarracín VH, Ordoñez OF, Farías ME & Alvarez HM (2013) Lipid storage in high-altitude Andean Lakes extremophiles and its mobilization under stress conditions in *Rhodococcus* sp. A5, a UV-resistant actinobacterium. *Extremophiles* **17**: 217-227.

Bhattacharya D, Sarma PM, Krishnan S, Mishra S & Lal B (2003) Evaluation of genetic diversity among *Pseudomonas citronellolis* strains isolated from oily sludge-contaminated Sites. *Applied and Environmental Microbiology* **69**: 1435-1441.

Bhuwal A, Singh G, Aggarwal N, Goyal V & Yadav A (2014) Poly-beta-hydroxybutyrate production and management of cardboard industry effluent by new *Bacillus* sp. NA10. *Bioresources and Bioprocessing* **1**: 9.

Binazadeh M, Karimi IA & Li Z (2009) Fast biodegradation of long chain n-alkanes and crude oil at high concentrations with *Rhodococcus* sp. Moj-3449. *Enzyme and Microbial Technology* **45**: 195-202.

Bioplastics E (2014) Bioplastics - facts and figures. (Institute for Bioplastics and Biocomposites).

Boopathy R (2000) Factors limiting bioremediation technologies. *Bioresource Technology* **74**: 63-67.

Borrero-de Acuna J, Bielecka A, Haussler S, Schobert M, Jahn M, Wittmann C, Jahn D, Pobelte-Castro I (2014) Production of medium chain length polyhydroxyalkanoate in metabolic flux optimized *Pseudomonas putida*. *Microbial Cell Factories* **13**: 88.

Bosco F & Chiampo F (2010) Production of polyhydroxyalcanoates (PHAs) using milk whey and dairy wastewater activated sludge: Production of bioplastics using dairy residues. *Journal of Bioscience and Bioengineering* **109**: 418-421.

Bossert I, Kachel WM & Bartha R (1984) Fate of hydrocarbons during oily sludge disposal in soil. *Applied and Environmental Microbiology* **47**: 763-767.

Bragg JR, Prince RC, Harner EJ & Atlas RM (1994) Effectiveness of bioremediation for the Exxon Valdez oil spill. *Nature* **368**: 413-418.

Brandl H, Goss RA, Lenz RW & Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(beta-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Applied and Environmental Microbiology* **54**: 1977 - 1982.

Bredemeier R, Hulsch R, Metzger JO & Berthe-Corti L (2003) Submersed culture production of extracellular wax esters by the marine bacterium *Fundibacter jadensis*. *Marine Biotechnology* **5**: 579-583.

Brennan L & Owende P (2010) Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14**: 557-577.

Brigham CJ KK, Rha C, Sinskey AJ (2011) Bacterial carbon storage to value added products. *Microbial & Biochemical Technology* **S3**: 002.

Bryn K, Jantzen E & Bøvre K (1977) Occurrence and patterns of waxes in Neisseriaceae. *Microbiology* **102**: 33-43.

Bucci DZ, Tavares LBB & Sell I (2005) PHB packaging for the storage of food products. *Polymer Testing* **24**: 564-571.

Burken JG, Vroblesky DA & Balouet JC (2011) Phytoforensics, dendrochemistry, and phytoscreening: new green tools for delineating contaminants from past and present. *Environmental Science & Technology* **45**: 6218-6226.

Caplan JA (1993) The worldwide bioremediation industry: prospects for profit. *Trends in Biotechnology* **11**: 320-323.

Cappello S, Denaro R, Genovese M, Giuliano L & Yakimov MM (2007) Predominant growth of *Alcanivorax* during experiments on "oil spill bioremediation" in mesocosms. *Microbiological Research* **162**: 185-190.

Carman GM & Han G-S (2006) Roles of phosphatidate phosphatase enzymes in lipid metabolism. *Trends in Biochemical Sciences* **31**: 694-699.

Carvalho G,Oehmen A, Albuquerque MGE, Reis, MAM (2014) The relationship between mixed microbial culture composition and PHA production performance from fermented molasses. *New Biotechnology* **31**: 257-263.

Cases S, Smith SJ, Zheng Y-W, Myers HM, Lear SR, Sande E, Novak S, Collins C, Welch CB, Lusis AJ, Erickson SK, Farese RV (1998) Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proceedings of the National Academy of Sciences* **95**: 13018-13023.

Castaldi FJ (2003) Tank-based bioremediation of petroleum waste sludges. *Environmental Progress* **22**: 25-36.

Castilho LR, Mitchell DA & Freire DMG (2009) Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology* **100**: 5996-6009.

Castillo-Carvajal L, Sanz-Martín J & Barragán-Huerta B (2014) Biodegradation of organic pollutants in saline wastewater by halophilic microorganisms: a review. *Environmental Science and Pollution Research* **21**: 9578-9588.

Chaillan F, Le Flèche A, Bury E, Phantavong Yh, Grimont P, Saliot A & Oudot J (2004) Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in Microbiology* **155**: 587-595.

Chanprateep S (2010) Current trends in biodegradable polyhydroxyalkanoates. *Journal of Bioscience and Bioengineering* **110**: 621-632.

Chavan A & Mukherji S (2008) Treatment of hydrocarbon-rich wastewater using oil degrading bacteria and phototrophic microorganisms in rotating biological contactor: Effect of N:P ratio. *Journal of Hazardous Materials* **154**: 63-72.

Chen G-Q (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chemical Society Reviews* **38**: 2434-2446.

Chen G-Q (2010) Plastics Completely Synthesized by Bacteria: Polyhydroxyalkanoates. *Plastics from Bacteria,* Vol. 14 (Chen GG-Q) 17-37, Springer Berlin Heidelberg.

Chen G-Q & Wu Q (2005) The application of polyhydroxyalkanoates as tissue engineering materials. *Biomaterials* **26**: 6565-6578.

Chen M, Xu P, Zeng G, Yang C, Huang D & Zhang J (2015) Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. *Biotechnology Advances* **33**: 745-755.

Chen Y, Huang Y-C & Lee C-Y (2014a) Production and characterization of medium-chain-length polyhydroxyalkanoates by *Pseudomonas mosselii* TO7. *Journal of Bioscience and Bioengineering* **118**: 145-152.

Chen Y, Ding Y, Yang L, Yu J, Liu G, Wang X, Zhang S, (2014b) Integrated omics study delineates the dynamics of lipid droplets in *Rhodococcus opacus* PD630. *Nucleic Acids Research* **42**: 1052-1064.

Chisti Y (2007) Biodiesel from microalgae. *Biotechnology Advances* **25**: 294-306.

Choi J-i & Lee SY (1997) Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation. *Bioprocess Engineering* **17**: 335-342.

Christie WW (1989) Fatty acids and Lipids: Structures, Extraction and Fractionation into Classes. *Gas Chromatography and Lipids,* Vol. 1) Oily Press Ltd.

Chronopoulou P-M, Sanni GO, Silas-Olu DI, van der Meer JR, Timmis KN, Brussaard CPD & McGenity TJ (2015) Generalist hydrocarbon-degrading bacterial communities in the oil-polluted water column of the North Sea. *Microbial Biotechnology* **8**: 434-447.

Chung A, Liu Q, Ouyang S-P, Wu Q & Chen G-Q (2009) Microbial production of 3-hydroxydodecanoic acid by pha operon and fadBA knockout mutant of *Pseudomonas putida* KT2442 harboring tesB gene. *Applied Microbiology and Biotechnology* **83**: 513-519.

Coats ER, Loge FJ, Wolcott MP, Englund K,Mcdonald AG (2007) Synthesis of polyhydroxyalkanoates in municipal wastewater treatment. Water *Environment Research* **79**: 2396-2403.

Coelho A, Castro AV, Dezotti M & Sant'Anna Jr GL (2006) Treatment of petroleum refinery sourwater by advanced oxidation processes. *Journal of Hazardous Materials* **137**: 178-184.

Coleman J (1990) Characterization of *Escherichia coli* cells deficient in 1-acyl-sn-glycerol-3-phosphate acyltransferase activity. *Journal of Biological Chemistry* **265**: 17215-17221.

Comba S, Menendez-Bravo S, Arabolaza A & Gramajo H (2013) Identification and physiological characterization of phosphatidic acid phosphatase enzymes involved in triacylglycerol biosynthesis in *Streptomyces coelicolor*. *Microbial Cell Factories* **12**: 9.

Cormack WPM & Fraile ER (1997) Characterization of a hydrocarbon degrading psychrotrophic Antarctic bacterium. *Antarctic Science* **9**: 150-155.

Cortes MALRM & de Carvalho CCCR (2015) Effect of carbon sources on lipid accumulation in *Rhodococcus* cells. *Biochemical Engineering Journal* **94**: 100-105.

Costa JC, Gonçalves PR, Nobre A & Alves MM (2012) Biomethanation potential of macroalgae *Ulva* spp. and *Gracilaria* spp. and in co-digestion with waste activated sludge. *Bioresource Technology* **114**: 320-326.

Coulon F, McKew B, Osborn A, McGenity T & Timmis K (2007) Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environmental Microbiology* **9**: 177 - 186.

da Silva LJ, Alves FC & de França FP (2012) A review of the technological solutions for the treatment of oily sludges from petroleum refineries. *Waste Management & Research* **30**: 1016-1030.

Dahlqvist A, Ståhl U, Lenman M, (2000) Phospholipid:diacylglycerol acyltransferase: An enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proceedings of the National Academy of Sciences* **97**: 6487-6492.

Dalal J, Sarma PM, Mandal AK & Lal B (2013) Response surface optimization of poly (3-hydroxyalkanoic acid) production using oleic acid as an alternative carbon source by *Pseudomonas aeruginosa*. *Biomass and Bioenergy* **54**: 67-76.

Daniel J, Deb C, Dubey V, Sirakova T, Abomoelak B, Morbidoni H & Kolattukudy P (2004) Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. *Journal of Bacteriology* **186**: 5017 - 5030.

Das K & Mukherjee AK (2007) Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresource Technology* **98**: 1339-1345.

Das N & Chandran P (2011) Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview. *Biotechnology Research International* **2011**.

Dashti N, Ali N, Eliyas M, Khanafer M, Sorkhoh NA & Radwan SS (2015) Most hydrocarbonoclastic bacteria in the total environment are diazotrophic, which highlights their value in the bioremediation of hydrocarbon contaminants. *Microbes and Environments* **30**: 70-75.

Dave DG, A.E. (2011) Remediation technologies for marine oil spills: a critical review and comparative analysis. *American Journal of Environmental Science* **7**: 423-440.

Davis JB (1967) *Petroleum Microbiology*. Elesevier Publishing Company.

De-qing S, Jian Z, Zhao-long G, Jian D, Tian-li W, Murygina V & Kalyuzhnyi S (2007) Bioremediation of oil sludge in Shengli oilfield. *Water, Air, and Soil Pollution* **185**: 177-184.

de Andrés C, Espuny MJ, Robert M, Mercadé ME, Manresa A & Guinea J (1991) Cellular lipid accumulation by *Pseudomonas aeruginosa* 44T1. *Applied Microbiology and Biotechnology* **35**: 813-816.

de Berardinis V, Vallenet D, Castelli V, Banas A, Lee M, Sandager L, Ronne H, Stymne S (2008) A complete collection of single-gene deletion mutants of Acinetobacter baylyi ADP1. *Molecular Systems Biology* **4**: 174.

de Carvalho CCCR (2012) Adaptation of *Rhodococcus erythropolis* cells for growth and bioremediation under extreme conditions. *Research in Microbiology* **163**: 125-136.

de Carvalho CCCR & da Fonseca MMR (2005) Degradation of hydrocarbons and alcohols at different temperatures and salinities by *Rhodococcus erythropolis* DCL14. *FEMS Microbiology Ecology* **51**: 389-399.

de Carvalho CCR, Wick L & Heipieper H (2009) Cell wall adaptations of planktonic and biofilm *Rhodococcus erythropolis* cells to growth on C5 to C16 n-alkane hydrocarbons. *Applied Microbiology and Biotechnology* **82**: 311-320.

de Carvalho CCR, Fatal V, Alves S & da Fonseca MM (2007) Adaptation of *Rhodococcus erythropolis* cells to high concentrations of toluene. *Applied Microbiology and Biotechnology* **76**: 1423-1430.

Desai C, Pathak H & Madamwar D (2010) Advances in molecular and "-omics" technologies to gauge microbial communities and bioremediation at xenobiotic/anthropogen contaminated sites. *Bioresource Technology* **101**: 1558-1569.

Di Gennaro P, Terreni P, Masi G, Botti S, De Ferra F & Bestetti G (2010) Identification and characterization of genes involved in naphthalene degradation in *Rhodococcus opacus* R7. *Applied Microbiology and Biotechnology* **87**: 297-308.

Di Gennaro P, Zampolli J, Presti I, Cappelletii M, D'Ursi P, Orro A, Mezzelania A, Milanesi L (2014) Genome sequence of *Rhodococcus opacus strain* R7, a biodegrader of mono- and polycyclic aromatic hydrocarbons. *Genome Announcements* **2**.

Di Martino C, López NI & Raiger Iustman LJ (2012) Isolation and characterization of benzene, toluene and xylene degrading *Pseudomonas* sp. selected as candidates for bioremediation. *International Biodeterioration & Biodegradation* **67**: 15-20.

Diallo MS, Cagin T, Faulon JL & Goddard Iii WA (2000) Chapter 5 Thermodynamic Properties of Asphaltenes: A Predictive Approach Based on Computer Assisted Structure Elucidation and Atomistic Simulations. *Developments in Petroleum Science,* Vol. Volume 40, Part B (Teh Fu Y & George VC), 103-127. Elsevier.

Dias JML, Lemos PC, Serafim LS, Oliveira C, Eiroa M, Albuquerque MG, Ramos AM, Oliveira R, Reis MA (2006) Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: from the substrate to the final product. *Macromolecular Bioscience* **6**: 885-906.

Dillon DA, Wu W-I, Riedel B, Wissing JB, Dowhan W & Carman GM (1996) The *Escherichia coli* pgpB gene encodes for a diacylglycerol pyrophosphate phosphatase activity. *Journal of Biological Chemistry* **271**: 30548-30553.

DIN EN 14039:2004: Characterization of waste. Determination of hydrocarbon content in the range of C10 to C40 by gas chromatography.

Diya'uddeen BH, Daud WMAW & Abdul Aziz AR (2011) Treatment technologies for petroleum refinery effluents: A review. *Process Safety and Environmental Protection* **89**: 95-105.

Dobroth ZT, Hu S, Coats ER, McDonald AG (2011) Polyhydroxybutyrate synthesis on biodiesel wastewater using mixed microbial consortia. *Bioresource Technology* **102:** 3352-3359.

Doggett T, Rascoe A (2009). Global energy demand seen up 44 percent by 2030. Washington, DC: REUTERS. Available from: http://www.reuters.com/article/2009/05/27/us-eia-global-demand-idUSN2719528620090527 Accessed: 16.09.2015.

Doi Y, Kitamura S & Abe H (1995) Microbial synthesis and characterization of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromolecules* **28**: 4822-4828.

Domingues VF, Priolo G, Alves AC, Cabral MF & Delerue-Matos C (2007) Adsorption behavior of α -cypermethrin on cork and activated carbon. *Journal of Environmental Science and Health, Part B* **42**: 649-654.

Doong R-a & Lei W-g (2003) Solubilization and mineralization of polycyclic aromatic hydrocarbons by *Pseudomonas putida* in the presence of surfactant. *Journal of Hazardous Materials* **96**: 15-27.

Durot M, Le Fevre F, de Berardinis V*,* Kreimeyer A, Vallenet D, Combe C, Smidtas S, Salanoubat M, Weisseinbach J, Schachter V (2008) Iterative reconstruction of a global metabolic model of *Acinetobacter baylyi* ADP1 using high-throughput growth phenotype and gene essentiality data. *BMC Systems Biology* **2**: 85.

Dyksterhouse SE, Gray JP, Herwig RP, Lara JC & Staley JT (1995) *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *International Journal of Systematic and Evolutionary Microbiology* **45**: 116-123.

Easterling ER, French WT, Hernandez R & Licha M (2009) The effect of glycerol as a sole and secondary substrate on the growth and fatty acid composition of *Rhodotorula glutinis*. *Bioresource Technology* **100**: 356-361.

El-Naas MH, Al-Zuhair S, Al-Lobaney A & Makhlouf S (2009) Assessment of electrocoagulation for the treatment of petroleum refinery wastewater. *Journal of Environmental Management* **91**: 180-185.

EN ISO 16703:2004 Soil quality – Determination of content of hydrocarbon in the range C10 to C40 by gas chromatography.

EPA (2015) Water Quality Standards and the Water Quality-based Approach to Pollution Control. http://water.epa.gov/scitech/swguidance/standards/handbook/chapter07.cfm.

Escapa I, Morales V, Martino V, Pollet E, Avérous L, García J & Prieto M (2011) Disruption of β-oxidation pathway in *Pseudomonas putida KT2442* to produce new functionalized PHAs with thioester groups. *Applied Microbiology and Biotechnology* **89**: 1583-1598.

Escapa IF, García JL, Bühler B, Blank LM & Prieto MA (2012) The polyhydroxyalkanoate metabolism controls carbon and energy spillage in *Pseudomonas putida*. *Environmental Microbiology* **14**: 1049-1063.

Essaid HI, Bekins BA, Godsy EM, Warren E, Baedecker MJ & Cozzarelli IM (1995) Simulation of aerobic and anaerobic biodegradation processes at a crude oil spill site. *Water Resources Research* **31**: 3309-3327.

Etuk CU, John RC, Ekong UE & Akpan MM (2012) Growth study and hydrocarbonoclastic potential of microorganisms isolated from aviation fuel spill site in Ibeno, Nigeria. *Bulletin of Environmental Contamination and Toxicology* **89**: 727-732.

Fan XB, R.; Zhou, Y. (2010) Glycerol (byproduct of biodiesel production) as a source for fuels and chemicals – Mini Review. *The Open Fuels & Energy Science Journal* **3**: 17-22.

Fathepure BZ (2014) Recent studies in microbial degradation of petroleum hydrocarbons in hypersaline environments. *Frontiers in Microbiology* **5**: 173.

Felizardo P, Neiva Correia MJ, Raposo I, Mendes JF, Berkemeier R & Bordado JM (2006) Production of biodiesel from waste frying oils. *Waste Management* **26**: 487-494.

Fernández-Martínez J, Pujalte MJ, García-Martínez J, Mata M, Garay E & Rodríguez-Valera F (2003) Description of *Alcanivorax venustensis* sp. nov. and reclassification of *Fundibacter jadensis* DSM 12178T (Bruns and Berthe-Corti 1999) as Alcanivorax jadensis comb. nov., members of the emended genus *Alcanivorax*. *International Journal of Systematic and Evolutionary Microbiology* **53**: 331-338.

Fernández D, Rodríguez E, Bassas M*,* Vinas M, Solanas AM, Llorens J, Marqués AM, Manresa A (2005) Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa* NCIB 40045: Effect of culture conditions. *Biochemical Engineering Journal* **26**: 159-167.

Ferrarese E, Andreottola G & Oprea IA (2008) Remediation of PAH-contaminated sediments by chemical oxidation. *Journal of Hazardous Materials* **152**: 128-139.

Fingas M & Fieldhouse B (2011) Chapter 22 - Review of Solidifiers. *Oil Spill Science and Technology,* (Fingas M) 713-733. Gulf Professional Publishing, Boston.

Finnerty WR (1992) The biology and genetics of the genus *Rhodococcus*. *Annual Reviews in Microbiology* **46**: 193 - 218.

Fixter LM, Nagi MN, Mccormack JG & Fewson CA (1986) Structure, distribution and function of Wax Esters in *Acinetobacter calcoaceticus*. *Microbiology* **132**: 3147-3157.

Folch J, Lees M & Sloane Stanley G (1957) A simple method for the isolation and purifcation of total lipides from animal tissues. *Journal of Biological Chemistry* **226**: 497-509.

Fountoulakis MS, Terzakis S, Kalogerakis N & Manios T (2009) Removal of polycyclic aromatic hydrocarbons and linear alkylbenzene sulfonates from domestic wastewater in pilot constructed wetlands and a gravel filter. *Ecological Engineering* **35**: 1702-1709.

Franklin FC, Bagdasarian M, Bagdasarian MM & Timmis KN (1981) Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. *Proceedings of the National Academy of Sciences* **78**: 7458-7462.

Fritsche W & Hofrichter M (2008) Aerobic Degradation by Microorganisms. *Biotechnology,* 144-167 Wiley-VCH Verlag GmbH.

Füchtenbusch B & Steinbüchel A (1999) Biosynthesis of polyhydroxyalkanoates from low-rank coal liquefaction products by *Pseudomonas oleovorans* and *Rhodococcus ruber*. *Applied Microbiology and Biotechnology* **52**: 91-95.

Fukui T, Yoshimoto A, Matsumoto M, Hosokawa S, Saito T, Nishikawa H & Tomita K (1976) Enzymatic synthesis of poly-β-hydroxybutyrate in *Zoogloea ramigera*. *Archives of Microbiology* **110**: 149-156.

Galbe M & Zacchi G (2007) Pretreatment of Lignocellulosic Materials for Efficient Bioethanol Production. *Biofuels,* Vol. 108 (Olsson L), 41-65. Springer Berlin Heidelberg.

Ganesh Kumar A, Vijayakumar L, Joshi G, Magesh Peter D, Dharani G & Kirubagaran R (2014) Biodegradation of complex hydrocarbons in spent engine oil by novel bacterial consortium isolated from deep sea sediment. *Bioresource Technology* **170**: 556-564.

Ganzeveld KJ, van Hagen A, van Agteren MH, de Koning W & Schoot Uiterkamp AJM (1999) Upgrading of organic waste: production of the copolymer poly-3-hydroxybutyrate-co-valerate by Ralstonia eutrophus with organic waste as sole carbon source. *Journal of Cleaner Production* **7**: 413-419.

Gao H-J, Wu Q & Chen G-Q (2002) Enhanced production of D-(−)-3-hydroxybutyric acid by recombinant *Escherichia coli*. *FEMS Microbiology Letters* **213**: 59-65.

Gao X, Gao W, Cui Z, Han B, Yang P, Sun C & Zheng L (2015) Biodiversity and degradation potential of oil-degrading bacteria isolated from deep-sea sediments of South Mid-Atlantic Ridge. *Marine Pollution Bulletin* **97**: 373-380.

Gargouri B, Karray F, Mhiri N, Aloui F & Sayadi S (2011) Application of a continuously stirred tank bioreactor (CSTR) for bioremediation of hydrocarbon-rich industrial wastewater effluents. *Journal of Hazardous Materials* **189**: 427-434.

Gargouri B, Karray F, Mhiri N, Aloui F & Sayadi S (2014) Bioremediation of petroleum hydrocarbons-contaminated soil by bacterial consortium isolated from an industrial wastewater treatment plant. *Journal of Chemical Technology & Biotechnology* **89**: 978-987.

Gavrilescu M & Chisti Y (2005) Biotechnology—a sustainable alternative for chemical industry. *Biotechnology Advances* **23**: 471-499.

Geiselbrecht A, Hedlund B, Tichi M & Staley J (1998) Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cycloclasticus* strains. *Applied Environmental Microbiology* **64**: 4703 - 4710.

Geißdörfer W, Kok RG, Ratajczak A, Hellingwerf KJ & Hillen W (1999) The genes rubA and rubB for alkane degradation in *Acinetobacter* sp. strain ADP1 are in an operon with estB, encoding an esterase, and oxyR. *Journal of Bacteriology* **181**: 4292-4298.

Genovese M, Crisafi F, Denaro R, Cappello S, Russo D, Calogero R, Santisi S, Catalfamo M, Modica A, Smedile F, Genovese L, Golyshin PN, Giuliano L, Yakimov MM (2014) Effective bioremediation strategy for rapid in situ cleanup of anoxic marine sediments in mesocosm oil spill simulation. *Frontiers in Microbiology* **5**: 162.

Gertler C, Näther DJ, Cappello S, Gerdts G, Quilliam RS, Yakimov MM & Golyshin PN (2012) Composition and dynamics of biostimulated indigenous oil-degrading microbial consortia from the Irish, North and Mediterranean Seas: a mesocosm study. **81**: 520-536.

Gibb A CA, Wong RCK, Goodman RH (2001) Bioremediation kinetics of crude oil at 5°C. *Journal of Environmental Engineering* **127**: 818-824.

Gibson DT & Parales RE (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Current Opinion in Biotechnology* **11**: 236-243.

Gnanaprakasam A SV, Surendhar A, Thirumarimurugan M, Kannadasan T (2013) Recent strategy of biodiesel production from waste cooking oil and process influencing parameters: a review. *Journal of Energy* **2013**: 1-10.

Gogoi BK, Dutta NN, Goswami P & Krishna Mohan TR (2003) A case study of bioremediation of petroleum-hydrocarbon contaminated soil at a crude oil spill site. *Advances in Environmental Research* **7**: 767-782.

Golyshin PN, Chernikova TN, Abraham W-R, Lünsdorf H, Timmis KN & Yakimov MM (2002) Oleiphilaceae fam. nov., to include *Oleiphilus messinensis* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. *International Journal of Systematic and Evolutionary Microbiology* **52**: 901-911.

Golyshin PN, Martins Dos Santos VAP, Kaiser O, Ferrer M, Sabirova YS, Lünsdorf H, Chernikova TN, Golyshina OV, Yakimov MM, Pühler A, Timmis KN (2003) Genome sequence completed of *Alcanivorax borkumensis*, a hydrocarbon-degrading bacterium that plays a global role in oil removal from marine systems. *Journal of Biotechnology* **106**: 215-220.

Gouda M, Omar S & Aouad L (2008) Single cell oil production by *Gordonia* sp. DG using agro-industrial wastes. *World Journal of Microbiology and Biotechnology* **24**: 1703-1711.

Gouveia L & Oliveira A (2009) Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology & Biotechnology* **36**: 269-274.

Grossi V, Yakimov MM, Al Ali B, Tapilatu Y, Cuny P, Goutx M, La Cono V, Giuliano L, Tamburini C (2010) Hydrostatic pressure affects membrane and storage lipid compositions of the piezotolerant hydrocarbon-degrading *Marinobacter hydrocarbonoclasticus* strain #5. *Environmental Microbiology* **12**: 2020-2033.

Grothe E, Moo-Young M & Chisti Y (1999) Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic. *Enzyme and Microbial Technology* **25**: 132-141.

Grund E, Denecke B & Eichenlaub R (1992) Naphthalene degradation via salicylate and gentisate by *Rhodococcus* sp. strain B4. *Applied and Environmental Microbiology* **58**: 1874-1877.

Gurieff N, Lant P (2007) Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production. *Bioresource Technology* **98**: 3393-3403.

Habe H & Omori T (2003) Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Bioscience, Biotechnology, and Biochemistry* **67**: 225-243.

Hall J, Hetrick M, French T, Hernandez R, Donaldson J, Mondala A & Holmes W (2011) Oil production by a consortium of oleaginous microorganisms grown on primary effluent wastewater. *Journal of Chemical Technology & Biotechnology* **86**: 54-60.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hamada T, Sameshima Y, Honda K, Omasa T, Kato J & Ohtake H (2008) A comparison of various methods to predict bacterial predilection for organic solvents used as reaction media. *Journal of Bioscience and Bioengineering* **106**: 357-362.

Hara A, Baik S-h, Syutsubo K, Misawa N, Smits THM, Van Beilen JB & Harayama S (2004) Cloning and functional analysis of alkB genes in *Alcanivorax borkumensis* SK2. *Environmental Microbiology* **6**: 191-197.

Harayama S, Kasai Y & Hara A (2004) Microbial communities in oil-contaminated seawater. *Current Opinion in Biotechnology* **15**: 205-214.

Harayama SK, Kasai Y, Shutsubo K (1999) Petroleum biodegradation in marine environments. *Journal of Molecular Microbiology and Biotechnology* **1**: 63-70.

Hartmann R, Hany R, Pletscher E, Ritter A, Witholt B & Zinn M (2006) Tailor-made olefinic medium-chain-length poly[(R)-3-hydroxyalkanoates] by *Pseudomonas putida* GPo1: Batch versus chemostat production. *Biotechnology and Bioengineering* **93**: 737-746.

Hassanshahian M, Emtiazi G, Caruso G & Cappello S (2014) Bioremediation (bioaugmentation/biostimulation) trials of oil polluted seawater: A mesocosm simulation study. *Marine Environmental Research* **95**: 28-38.

Haywood G, Anderson A, Williams D & Dawes E (1991) Accumulation of poly(hydroxyalkanoates) copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate by *Rhodococcus* sp. NCIMB 40126. *International Journal of Biological Macromolecules* **13**: 83 - 88.

He W, Tian W, Zhang G, Chen G-Q & Zhang Z (1998) Production of novel polyhydroxyalkanoates by *Pseudomonas stutzeri* 1317 from glucose and soybean oil. *FEMS Microbiology Letters* **169**: 45-49.

He Y-M, Duan X-G & Liu Y-S (2014) Enhanced bioremediation of oily sludge using co-culture of specific bacterial and yeast strains. *Journal of Chemical Technology & Biotechnology* **89**: 1785-1792.

Head IM, Jones DM & Roling WFM (2006b) Marine microorganisms make a meal of oil. *Nature Reviews in Microbiology* **4**: 173-182.

Heald S, Brandão PB, Hardicre R & Bull A (2001) Physiology, biochemistry and taxonomy of deep-sea nitrile metabolising *Rhodococcus* strains. *Antonie Van Leeuwenhoek* **80**: 169-183.

Heintz RA (2007) Chronic exposure to polynuclear aromatic hydrocarbons in natal habitats leads to decreased equilibrium size, growth, and stability of pink salmon populations. *Integrated Environmental Assessment and Management* **3**: 351-363.

Hejazi RF, Husain T & Khan FI (2003) Landfarming operation of oily sludge in arid region—human health risk assessment. *Journal of Hazardous Materials* **99**: 287-302.

Hempel FB, Lindenkamp N, Klingl A, Zauner S, Linne U, Steinbüchel A, Maier UG (2011) Microalgae as bioreactors for bioplastic production. *Microbial Cell Factories* **10**: 81-86.

Hernandez M, Mohn W, Martinez E, Rost E, Alvarez A & Alvarez H (2008) Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. *BMC Genomics* **9**: 600.

Hernández M, Arabolaza A, Rodríguez E, Gramajo H & Alvarez H (2013) The atf2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous *Rhodococcus opacus* PD630. *Applied Microbiology and Biotechnology* **97**: 2119-2130.

Hernández M, Comba S, Arabolaza A, Gramajo H & Alvarez H (2015) Overexpression of a phosphatidic acid phosphatase type 2 leads to an increase in triacylglycerol production in oleaginous *Rhodococcus* strains. *Applied Microbiology and Biotechnology* **99**: 2191-2207.

Hernandéz MA & Alvarez HM (2010) Glycogen formation by *Rhodococcus* species and the effect of inhibition of lipid biosynthesis on glycogen accumulation in *Rhodococcus opacus* PD630. *FEMS Microbiology Letters* **312**: 93-99.

Hetzler S & Steinbüchel A (2013) Establishment of cellobiose utilization for lipid production in *Rhodococcus opacus* PD630. *Applied and Environmental Microbiology* **79**: 3122-3125.

Hisano T, Tsuge T, Fukui T, Iwata T, Miki K & Doi Y (2003) Crystals structure of the (R)-specific enoyl-CoA hydratase from *Aeromonas caviae* Involved in polyhydroxyalkanoate biosynthesis. *Journal of Biological Chemistry* **278**: 617-624.

Ho S-H, Huang S-W, Chen C-Y, Hasunuma T, Kondo A & Chang J-S (2013) Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource Technology* **135**: 191-198.

Hoffmann N, Steinbüchel A & Rehm BHA (2000) Homologous functional expression of cryptic phaG from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. *Applied Microbiology and Biotechnology* **54**: 665-670.

Hoffmann N, Amara AA, Beermann BB, Qi Q, Hinz H-J & Rehm BHA (2002) Biochemical characterization of the *Pseudomonas putida* 3-Hydroxyacyl ACP:CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. *Journal of Biological Chemistry* **277**: 42926-42936.

Holder J, Ulrich J, DeBono A*,* Godfrey P, Desjardins C, Zucker J, Zeng Q, Leach A, Ghiviriga I, Dancel C, Abeel T, Gevers D, Kodira C, Desani B, Affourtit J, Birren B, Sinskey A (2011) Comparative and functional genomics of *Rhodococcus opacus* PD630 for biofuels development. *PLoS Genetics* **7**: e1002219.

Holliger C, Gaspard S, Glod G, Heijman C, Schumacher W, Schwarzenbach RP & Vazquez F (1997) Contaminated environments in the subsurface and bioremediation: organic contaminants. *FEMS Microbiology Reviews* **20**: 517-523.

Holtzapple E & Schmidt-Dannert C (2007) Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: identification and characterization of isoprenoid coenzyme A synthetase and wax ester synthases. *Journal of Bacteriology* **189**: 3804-3812.

Honda K, Yamashita S, Nakagawa H, Sameshima Y, Omasa T, Kato J & Ohtake H (2008) Stabilization of water-in-oil emulsion by *Rhodococcus opacus* B-4 and its application to biotransformation. *Applied Microbiology and Biotechnology* **78**: 767-773.

Hori K, Abe M & Unno H (2009) Production of triacylglycerol and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by the toluene-degrading bacterium *Rhodococcus aetherivorans* IAR1. *Journal of Bioscience and Bioengineering* **108**: 319-324.

Hu G, Li J & Zeng G (2013) Recent development in the treatment of oily sludge from petroleum industry: A review. *Journal of Hazardous Materials* **261**: 470-490.

Huijberts GN, Eggink G, de Waard P, Huisman GW & Witholt B (1992) *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. *Applied and Environmental Microbiology* **58**: 536-544.

Huijberts GNM & Eggink G (1996) Production of poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2442 in continuous cultures. *Applied Microbiology and Biotechnology* **46**: 233-239.

Icho T & Raetz CR (1983) Multiple genes for membrane-bound phosphatases in *Escherichia coli* and their action on phospholipid precursors. *Journal of Bacteriology* **153**: 722-730.

Isaac P, Martínez FL, Bourguignon N, Sánchez LA & Ferrero MA (2015) Improved PAHs removal performance by a defined bacterial consortium of indigenous *Pseudomonas* and actinobacteria from Patagonia, Argentina. *International Biodeterioration & Biodegradation* **101**: 23-31.

Ishige T, Tani A, Sakai Y & Kato N (2003) Wax ester production by bacteria. *Current Opinion in Microbiology* **6**: 244-250.

Ishige T, Tani A, Takabe K, Kawasaki K, Sakai Y & Kato N (2002) Wax Ester oroduction from n-alkanes by *Acinetobacter* sp. Strain M-1: ultrastructure of cellular inclusions and role of acyl Coenzyme A reductase. *Applied and Environmental Microbiology* **68**: 1192-1195.

Ismail S & Dadrasnia A (2015) Biotechnological potential of *Bacillus salmalaya* 139SI: a novel strain for remediating water polluted with crude oil waste. *PLoS One* **10**: e0120931.

Iwabuchi N, Sunairi M, Urai M, Itoh C, Anzai H, Nakajima M & Harayama S (2002) Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. *Applied and Environmental Microbiology* **68**: 2337-2343.

JanSzen H, Ibrahim MH, Broker D & Steinbuchel A (2013) Optimization of macroelement concentrations, pH and osmolarity for triacylglycerol accumulation in *Rhodococcus opacus* strain PD630. *AMB Express* **3**: 38.

Jetter R & Kunst L (2008) Plant surface lipid biosynthetic pathways and their utility for metabolic engineering of waxes and hydrocarbon biofuels. *The Plant Journal* **54**: 670-683.

Jiménez JI, Miñambres B, García JL & Díaz E (2002) Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environmental Microbiology* **4**: 824-841.

Johnson K, Jiang Y, Kleerebezem R, Muyzer G & van Loosdrecht MCM (2009) Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. *Biomacromolecules* **10**: 670-676.

Jones DM, Douglas AG, Parkes RJ, Taylor J, Giger W & Schaffner C (1983) The recognition of biodegraded petroleum-derived aromatic hydrocarbons in recent marine sediments. *Marine Pollution Bulletin* **14**: 103-108.

Juang L-C, Tseng D-H & Lin H-Y (2007) Membrane processes for water reuse from the effluent of industrial park wastewater treatment plant: a study on flux and fouling of membrane. *Desalination* **202**: 302-309.

Juni E (1978) Genetics and physiology of *Acinetobacter*. *Annual Review of Microbiology* **32**: 349-371.

Kalscheuer R (2010) Genetics of Wax Ester and Triacylglycerol Biosynthesis in Bacteria. *Handbook of Hydrocarbon and Lipid Microbiology* (Timmis K) 527-535. Springer Berlin Heidelberg.

Kalscheuer R & Steinbuchel A (2003) A novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *Journal of Biological Chemistry* **278**: 8075 - 8082.

Kalscheuer R, Stölting T & Steinbüchel A (2006a) Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology* **152**: 2529-2536.

Kalscheuer R, Wältermann M, Alvarez H & Steinbüchel A (2001) Preparative isolation of lipid inclusions from *Rhodococcus opacus* and *Rhodococcus ruber* and identification of granule-associated proteins. *Archives of Microbiology* **177**: 20-28.

Kalscheuer R, Uthoff S, Luftmann H & Steinbüchel A (2003) In vitro and in vivo biosynthesis of wax diesters by an unspecific bifunctional wax ester synthase/acyl-CoA:diacylglycerol

acyltransferase from *Acinetobacter calcoaceticus* ADP1. *European Journal of Lipid Science and Technology* **105**: 578-584.

Kalscheuer R, Stöveken T, Luftmann H, Malkus U, Reichelt R & Steinbüchel A (2006b) Neutral lipid biosynthesis in engineered *Escherichia coli:* jojoba oil-like Wax Esters and Fatty Acid Butyl Esters. *Applied and Environmental Microbiology* **72**: 1373-1379.

Kalscheuer R, Stöveken T, Malkus U, Reichelt R, Golyshin PN, Sabirova JS, Ferrer M, Timmis KN, Steinbüchel A (2007) Analysis of storage lipid accumulation in *Alcanivorax borkumensis*: evidence for alternative triacylglycerol biosynthesis routes in bacteria. *Journal of Bacteriology* **189**: 918-928.

Kanehisa M & Goto S (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research* **28**: 27-30.

Karmakar A, Karmakar S & Mukherjee S (2010) Properties of various plants and animals feedstocks for biodiesel production. *Bioresource Technology* **101**: 7201-7210.

Kasai Y, Kishira H & Harayama S (2002) Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. *Applied Environmental Microbiology* **68**: 5625 - 5633.

Kasai Y, Kishira H, Syutsubo K & Harayama S (2001) Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker oil-spill accident. *Environmental Microbiology* **3**: 246-255.

Kasai Y, Shindo K, Harayama S & Misawa N (2003) Molecular characterization and substrate preference of a polycyclic aromatic hydrocarbon dioxygenase from *Cycloclasticus* sp. strain A5. *Applied and Environmental Microbiology* **69**: 6688-6697.

Kato M, Bao HJ, Kang CK, Fukui T & Doi Y (1996) Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. *Applied Microbiology and Biotechnology* **45**: 363-370.

Keshavarz T & Roy I (2010) Polyhydroxyalkanoates: bioplastics with a green agenda. *Current Opinion in Microbiology* **13**: 321-326.

Kesseler M, Dabbs ER, Averhoff B & Gottschalk G (1996) Studies on the isopropylbenzene 2,3-dioxygenase and the 3-isopropylcatechol 2,3-dioxygenase genes encoded by the linear plasmid of *Rhodococcus erythropolis* BD2. *Microbiology* **142**: 3241-3251.

Khanna S & Srivastava AK (2005) Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry* **40**: 607-619.

Khardenavis AA, Suresh Kumar M, Mudliar SN & Chakrabarti T (2007) Biotechnological conversion of agro-industrial wastewaters into biodegradable plastic, poly β-hydroxybutyrate. *Bioresource Technology* **98**: 3579-3584.

Killops SD & Al-Juboori MAHA (1990) Characterisation of the unresolved complex mixture (UCM) in the gas chromatograms of biodegraded petroleums. *Organic Geochemistry* **15**: 147-160.

Kim D, Chae J-C, Zylstra GJ, Kim YS, Kim SK, Nam MH, Kim YM, Kim E (2004) Identification of a novel dioxygenase involved in metabolism of o-xylene, toluene, and ethylbenzene by *Rhodococcus* sp. strain DK17. *Applied and Environmental Microbiology* **70**: 7086-7092.

Kim S-J, Choi DH, Sim DS & Oh Y-S (2005) Evaluation of bioremediation effectiveness on crude oil-contaminated sand. *Chemosphere* **59**: 845-852.

King GM, Kostka JE, Hazen TC & Sobecky PA (2015) Microbial responses to the deepwater Horizon oil spill: from coastal wetlands to the deep sea. *Annual Review of Marine Science* **7**: 377-401.

Kingston P (2002) Long-term environmental impact of oil spills. *Spill Science & Technology Bulletin* **7**: 53-61.

Kirchmann H & Ewnetu W (1998) Biodegradation of petroleum-based oil wastes through composting. *Biodegradation* **9**: 151-156.

Klein B, Grossi V, Bouriat P, Goulas P & Grimaud R (2008) Cytoplasmic wax ester accumulation during biofilm-driven substrate assimilation at the alkane–water interface by *Marinobacter hydrocarbonoclasticus* SP17. *Research in Microbiology* **159**: 137-144.

Knothe G (2005) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology* **86**: 1059-1070.

Koma D, Hasumi F, Yamamoto E, Ohta T, Chung S-Y & Kubo M (2001) Biodegradation of long-chain n-paraffins from waste oil of car engine by *Acinetobacter* sp. *Journal of Bioscience and Bioengineering* **91**: 94-96.

Koma D, Sakashita Y, Kubota K, Fujii Y, Hasumi F, Chung S-Y & Kubo M (2003) Degradation of car engine base oil by *Rhodococcus* sp. NDKK48 and *Gordonia* sp. NDKY76A. *Bioscience, Biotechnology, and Biochemistry* **67**: 1590-1593.

Koma D, Sakashita Y, Kubota K, Fujii Y, Hasumi F, Chung SY & Kubo M (2004) Degradation pathways of cyclic alkanes in *Rhodococcus* sp. NDKK48. *Applied Microbiology and Biotechnology* **66**: 92-99.

Kosa M & Ragauskas AJ (2011) Lipids from heterotrophic microbes: advances in metabolism research. *Trends in Biotechnology* **29**: 53-61.

Kosa M & Ragauskas A (2012) Bioconversion of lignin model compounds with oleaginous Rhodococci. *Applied Microbiology and Biotechnology* **93**: 891-900.

Kosa M & Ragauskas AJ (2013) Lignin to lipid bioconversion by oleaginous Rhodococci. *Green Chemistry* **15**: 2070-2074.

Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, Delgardio J, Norton N, Hazen TC, Huettel M (2011) Hydrocarbon-degrading Bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the deepwater Horizon oil spill. *Applied and Environmental Microbiology* **77**: 7962-7974.

Koul SF, M.H. (2013) Petrochemical industrial waste: bioremediation techniques - an overview. *International Journal of Advancements in Research & Technology* **2**: 211-257.

Kourmentza C, Mitova E, Stoyanova N, Ntaikou I & Kornaros M (2009) Investigation of PHAs production from acidified olive oil mill wastewater (OOMW) by pure cultures of Pseudomonas spp. strains. *New Biotechnology* **25,** Supplement: S269.

Kulakov LA, Chen S, Allen CCR & Larkin MJ (2005) Web-type evolution of *Rhodococcus* gene clusters associated with utilization of naphthalene. *Applied and Environmental Microbiology* **71**: 1754-1764.

Kulpreecha S, Boonruangthavorn A, Meksiriporn B & Thongchul N (2009) Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*. *Journal of Bioscience and Bioengineering* **107**: 240-245.

Kumar P, Barrett DM, Delwiche MJ & Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research* **48**: 3713-3729.

Kumar S, Gupta N & Pakshirajan K (2015) Simultaneous lipid production and dairy wastewater treatment using *Rhodococcus opacus* in a batch bioreactor for potential biodiesel application. *Journal of Environmental Chemical Engineering* **3**: 1630-1636.

Kumar T, Singh M, Purohit HJ & Kalia VC (2009) Potential of *Bacillus* sp. to produce polyhydroxybutyrate from biowaste. *Journal of Applied Microbiology* **106**: 2017-2023.

Kunihiro N, Haruki M, Takano K, Morikawa M & Kanaya S (2005) Isolation and characterization of *Rhodococcus* sp. strains TMP2 and T12 that degrade 2,6,10,14-tetramethylpentadecane (pristane) at moderately low temperatures. *Journal of Biotechnology* **115**: 129-136.

Kurosawa K, Anthony Debono C & Sinskey AJ (2015a) Lignocellulose-derived inhibitors improve lipid extraction from wet *Rhodococcus opacus* cells. *Bioresource Technology* **193**: 206-212.

Kurosawa K, Boccazzi P, de Almeida N & Sinskey A (2010) High-cell-density batch fermentation of *Rhodococcus opacus* PD630 using a high glucose concentration for triacylglycerol production. *Journal of Biotechnology* **147**: 212 - 218.

Kurosawa K, Plassmeier J, Kalinowski J, Rückert C & Sinskey AJ (2015b) Engineering l-arabinose metabolism in triacylglycerol-producing *Rhodococcus opacus* for lignocellulosic fuel production. *Metabolic Engineering* **30**: 89-95.

Kurosawa K LJ, Sinskey AJ (2015c) Tolerance and adaptive evolution of triacylglycerol-producing *Rhodococcus opacus* to lignocellulose-derived inhibitors. *Biotechnology for Biofuels* **8**: 76-90.

Kurosawa K, Plassmeier JK, Sinskey AJ (2015d) Improved glycerol utilization by a triacylglycerol producing *Rhodococcus opacus* strain for renewable fuels. *Biotechnology for Biofuels* **8**: 31-42.

Kurosawa K WS, Sinskey JA (2013) Engineering xylose metabolism in triacylglycerol producing *Rhodococcus opacus* for lignocellulosic fuel production. *Biotechnology for Biofuels* **6**: 134-147.

Kurosawa K WS, Sinskey JA (2014) Triacylglycerol production from corn stover using a xylose-fermenting *Rhodococcus opacus* strain for lignocellulosic biofuels. *Microbial & Biochemical Technology* **6**: 254-259.

Kuyukina M & Ivshina I (2010) Application of *Rhodococcus* in bioremediation of contaminated environments. *Biology of Rhodococcus,* Vol. 16 (Alvarez HM) 231-262. Springer Berlin Heidelberg.

Kwon KK, Oh JH, Yang S-H, Seo H-S & Lee J-H (2015) *Alcanivorax gelatiniphagus* sp. nov., a marine bacterium isolated from tidal flat sediments enriched with crude oil. *International Journal of Systematic and Evolutionary Microbiology* **65**: 2204-2208.

Lageveen RG, Huisman GW, Preusting H, Ketelaar P, Eggink G & Witholt B (1988) Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of Poly-(R)-3-Hydroxyalkanoates and Poly-(R)-3-Hydroxyalkenoates. *Applied and Environmental Microbiology* **54**: 2924-2932.

Lai Q, Wang J, Gu L, Zheng T & Shao Z (2013) *Alcanivorax marinus* sp. nov., isolated from deep-sea water. *International Journal of Systematic and Evolutionary Microbiology* **63**: 4428-4432.

Lai Q, Wang L, Liu Y, Fu Y, Zhong H, Wang B, Chen L, Wang J, Sun F, Shao Z (2011) *Alcanivorax pacificus* sp. nov., isolated from a deep-sea pyrene-degrading consortium. *International Journal of Systematic and Evolutionary Microbiology* **61**: 1370-1374.

Lal B & Khanna S (1996) Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. *Journal of Applied Bacteriology* **81**: 355-362.

Lane D (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics,* Vol. 4 (Goodfellow ESaM), John Wiley and Sons, Chichester.

Lang S & Philip JC (1998) Surface-active lipids in Rhodococci. *Antonie Van Leeuwenhoek* **74**: 59 - 70.

Langdahl BR, Bisp P & Ingvorsen K (1996) Nitrile hydrolysis by *Rhodococcus erythropolis* BL1, an acetonitrile-tolerant strain isolated from a marine sediment. *Microbiology* **142**: 145-154.

Larkin M, Kulakov L & Allen CR (2010) Genomes and Plasmids in *Rhodococcus*. *Biology of Rhodococcus,* Vol. 16 (Alvarez HM), 73-90. Springer Berlin Heidelberg.

Larkin MJ, Kulakov LA & Allen CCR (2005) Biodegradation and *Rhodococcus* – masters of catabolic versatility. *Current Opinion in Biotechnology* **16**: 282-290.

Lau N-S & Sudesh K (2012) Revelation of the ability of *Burkholderia* sp. USM (JCM 15050) PHA synthase to polymerize 4-hydroxybutyrate monomer. *AMB Express* **2**: 1-9.

Leahy JG & Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews* **54**: 305-315.

Lee SY (1996a) Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering* **49**: 1-14.

Lee SY (1996b) Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends in Biotechnology* **14**: 431-438.

Lefebvre O & Moletta R (2006) Treatment of organic pollution in industrial saline wastewater: A literature review. *Water Research* **40**: 3671-3682.

Lehner R & Kuksis A (1996) Biosynthesis of triacylglycerols. *Progress in Lipid Research* **35**: 169-201.

Leman J (1997) Oleaginous Microorganisms: An Assessment of the Potential. *Advances in Applied Microbiology,* Vol. Volume 43 (Saul LN & Allen IL) 195-243. Academic Press.

Lenneman EM, Ohlert JM, Palani NP & Barney BM (2013) Fatty alcohols for wax esters in *Marinobacter aquaeolei* VT8: two optional routes in the wax biosynthesis pathway. *Applied and Environmental Microbiology* **79**: 7055-7062.

Lennen RM & Pfleger BF (2013) Microbial production of fatty acid-derived fuels and chemicals. *Current Opinion in Biotechnology* **24**: 1044-1053.

Lessard RR & DeMarco G (2000) The significance of oil spill dispersants. *Spill Science & Technology Bulletin* **6**: 59-68.

Leys NM, Ryngaert A, Bastiaens L, Top EM, Verstraete W & Springael D (2005) Culture independent detection of *Sphingomonas* sp. EPA 505 related strains in soils contaminated with Polycyclic Aromatic Hydrocarbons (PAHs). *Microbial Ecology* **49**: 443-450.

Li H, Cann, A. F., and Liao, J. C. (2010) Biofuels: biomolecular engineering fundamentals and advances. *Annual Review of Chemical and Biomolecular Engineering* **1**: 19-36.

Li P, Chakraborty S & Stubbe J (2009) Detection of covalent and noncovalent Intermediates in the polymerization reaction catalyzed by a C149S class III polyhydroxybutyrate synthase. *Biochemistry* **48**: 9202-9211.

Li Q, Du W & Liu D (2008) Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology* **80**: 749-756.

Liang M-H & Jiang J-G (2013) Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Progress in Lipid Research* **52**: 395-408.

Likhoshvay A, Lomakina A & Grachev M (2014) The complete alk sequences of *Rhodococcus erythropolis* from Lake Baikal. *SpringerPlus* **3**: 621.

Lin T-C, Pan P-T & Cheng S-S (2010) Ex situ bioremediation of oil-contaminated soil. *Journal of Hazardous Materials* **176**: 27-34.

Liu C & Shao Z (2005) *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. *International Journal of Systematic and Evolutionary Microbiology* **55**: 1181-1186.

Liu CW, Chang WN & Liu HS (2009) Bioremediation of n-alkanes and the formation of biofloccules by *Rhodococcus erythropolis* NTU-1 under various saline conditions and sea water. *Biochemical Engineering Journal* **45**: 69-75.

Liu G-h, Ye Z, Tong K & Zhang Y-h (2013) Biotreatment of heavy oil wastewater by combined upflow anaerobic sludge blanket and immobilized biological aerated filter in a pilot-scale test. *Biochemical Engineering Journal* **72**: 48-53.

Liu W, Luo Y, Teng Y, Li Z & Ma L (2010) Bioremediation of oily sludge-contaminated soil by stimulating indigenous microbes. *Environmental Geochemistry and Health* **32**: 23-29.

Liu Z, Wang Y, He N, Huang J, Zhu K, Shao W, Wang H, Yuan W, Li Q (2011) Optimization of polyhydroxybutyrate (PHB) production by excess activated sludge and microbial community analysis. *Journal of Hazardous Materials* **185**: 8-16.

López-Lara IM, Sohlenkamp C & Geiger O (2003) Membrane lipids in plant-associated bacteria: their biosyntheses and possible functions. *Molecular Plant-Microbe Interactions* **16**: 567-579.

Lozada M, Riva Mercadal J, Guerrero L, Di Marzio W, Ferrero M & Dionisi H (2008) Novel aromatic ring-hydroxylating dioxygenase genes from coastal marine sediments of Patagonia. *BMC Microbiology* **8**: 50.

Lucas Z & MacGregor C (2006) Characterization and source of oil contamination on the beaches and seabird corpses, Sable Island, Nova Scotia, 1996–2005. *Marine Pollution Bulletin* **52**: 778-789.

Lundstedt S, Haglund P & Öberg L (2003) Degradation and formation of polycyclic aromatic compounds during bioslurry treatment of an aged gasworks soil. *Environmental Toxicology and Chemistry* **22**: 1413-1420.

Luz AP, Pellizari VH, Whyte LG & Greer CW (2004) A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil. *Canadian Journal of Microbiology* **50**: 323-333.

Luz Paje M & Couperwhite I (1996) Short communication: Benzene metabolism via the intradiol cleavage in a *Rhodococcus* sp. *World Journal of Microbiology and Biotechnology* **12**: 653-654.

MacEachran D & Sinskey A (2013) The *Rhodococcus opacus* TadD protein mediates triacylglycerol metabolism by regulating intracellular NAD(P)H pools. *Microbial Cell Factories* **12**: 104.

MacEachran D, Prophete M & Sinskey A (2010) The *Rhodococcus opacus* PD630 heparin-binding hemagglutinin homolog TadA mediates lipid body formation. *Appl Environ Microbiol* **76**: 7217 - 7225.

Madison LL & Huisman GW (1999) Metabolic engineering of Poly(3-Hydroxyalkanoates): from DNA to plastic. *Microbiology and Molecular Biology Reviews* **63**: 21-53.

Maeng JH, Sakai Y, Tani Y & Kato N (1996) Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in *Acinetobacter* sp. strain M-1. *Journal of Bacteriology* **178**: 3695-3700.

Mahjoubi M, Jaouani A, Guesmi A, Ben Amor S, Jouini A, Cherif H, Najjari A, Boudabous A,Koubaa N, Cherif A (2013) Hydrocarbonoclastic bacteria isolated from petroleum contaminated sites in Tunisia: isolation, identification and characterization of the biotechnological potential. *New Biotechnology* **30**: 723-733.

Makkar RS & Rockne KJ (2003) Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry* **22**: 2280-2292.

Makula RA, Lockwood PJ & Finnerty WR (1975) Comparative analysis of the lipids of *Acinetobacter* species grown on hexadecane. *Journal of Bacteriology* **121**: 250-258.

Malviya R & Chaudhary R (2006) Factors affecting hazardous waste solidification/stabilization: A review. *Journal of Hazardous Materials* **137**: 267-276.

Mandal S & Mallick N (2009) Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Applied Microbiology and Biotechnology* **84**: 281-291.

Manilla-Pérez E, Lange A, Hetzler S & Steinbüchel A (2010a) Occurrence, production, and export of lipophilic compounds by hydrocarbonoclastic marine bacteria and their potential use to produce bulk chemicals from hydrocarbons. *Applied Microbiology and Biotechnology* **86**: 1693-1706.

Manilla-Pérez E, Lange AB, Luftmann H, Robenek H & Steinbüchel A (2011) Neutral lipid production in *Alcanivorax borkumensis SK2* and other marine hydrocarbonoclastic bacteria. *European Journal of Lipid Science and Technology* **113**: 8-17.

Manilla-Pérez E, Lange AB, Hetzler S, Wältermann M, Kalscheuer R & Steinbüchel A (2010b) Isolation and characterization of a mutant of the marine bacterium *Alcanivorax borkumensis* SK2 defective in lipid biosynthesis. *Applied and Environmental Microbiology* **76**: 2884-2894.

Manilla-Pérez E, Reers C, Baumgart M, Hetzler S, Reichelt R, Malkus U, Kalscheuer A, Waltermmann M, Steinbüchel A (2010c) Analysis of lipid export in hydrocarbonoclastic bacteria of the genus *Alcanivorax*: identification of lipid export-negative mutants of *Alcanivorax borkumensis* SK2 and *Alcanivorax jadensis* T9. *Journal of Bacteriology* **192**: 643-656.

Mansur A, Adetutu E, Kadali K, Morrison P, Nurulita Y & Ball A (2014) Assessing the hydrocarbon degrading potential of indigenous bacteria isolated from crude oil tank bottom sludge and hydrocarbon-contaminated soil of Azzawiya oil refinery, Libya. *Environmental Science and Pollution Research* **21**: 10725-10735.

Margesin R & Schinner F (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology* **56**: 650-663.

Margesin R, Labbé D, Schinner F, Greer CW & Whyte LG (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Applied and Environmental Microbiology* **69**: 3085-3092.

Marín J, Moreno J, Hernández T & García C (2006) Bioremediation by composting of heavy oil refinery sludge in semiarid conditions. *Biodegradation* **17**: 251-261.

Marin JA, Hernandez T & Garcia C (2005) Bioremediation of oil refinery sludge by landfarming in semiarid conditions: Influence on soil microbial activity. *Environmental Research* **98**: 185-195.

Markou G & Nerantzis E (2013) Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. *Biotechnology Advances* **31**: 1532-1542.

Marshall AG & Rodgers RP (2003) Petroleomics: the next grand challenge for chemical analysis. *Accounts of Chemical Research* **37**: 53-59.

Martínez-Morales F, Schobert M, López-Lara IM & Geiger O (2003) Pathways for phosphatidylcholine biosynthesis in bacteria. *Microbiology* **149**: 3461-3471.

Martinkova L, Uhnakova B, Patek M, Nesvera J & Kren V (2009) Biodegradation potential of the genus *Rhodococcus*. *Environment International* **35**: 162 - 177.

Maruyama A, Ishiwata H, Kitamura K, Sunamura M, Fujita T, Matsuo M & Higashihara T (2003) Dynamics of microbial populations and strong selection for *Cycloclasticus pugetii* following the Nakhodka oil spill. *Microbial Ecolology* **46**: 442 - 453.

Maruyama T, Ishikura M, Taki H, Shindo K, Kasai H, Haga M, Inomata Y, Misawa N (2005) Isolation and characterization of o-xylene oxygenase genes from *Rhodococcus opacus* TKN14. *Applied and Environmental Microbiology* **71**: 7705-7715.

Matsumoto Ki, Takase K, Yamamoto Y, Doi Y & Taguchi S (2009) Chimeric enzyme composed of polyhydroxyalkanoate (PHA) synthases from *Ralstonia eutropha* and *Aeromonas caviae* enhances production of PHAs in recombinant *Escherichia coli*. *Biomacromolecules* **10**: 682-685.

Matsusaki H, Manji S, Taguchi K, Kato M, Fukui T & Doi Y (1998) Cloning and molecular analysis of the Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) biosynthesis genes in *Pseudomonas* sp. Strain 61-3. *Journal of Bacteriology* **180**: 6459-6467.

McCool GJ & Cannon MC (2001) PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. *Journal of Bacteriology* **183**: 4235-4243.

McCurdy AT, Higham AJ, Morgan MR, Quinn JC & Seefeldt LC (2014) Two-step process for production of biodiesel blends from oleaginous yeast and microalgae. *Fuel* **137**: 269-276.

McGenity T, Folwell B, McKew B & Sanni G (2012) Marine crude-oil biodegradation: a central role for interspecies interactions. *Aquatic Biosystems* **8**: 10.

McGenity TJ, Whitby C & Fahy A (2010) Alkaliphilic Hydrocarbon Degraders. *Handbook of Hydrocarbon and Lipid Microbiology* (Timmis K) 1931-1937. Springer Berlin Heidelberg.

McKew BA, Coulon F, Osborn AM, Timmis KN & McGenity TJ (2007) Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. *Environmental Microbiology* **9**: 165-176.

Medina-Bellver JI, Marín P, Delgado A, Rodríguez-Sánchez A, Reyes E, Ramos JL & Marqués S (2005) Evidence for in situ crude oil biodegradation after the Prestige oil spill. *Environmental Microbiology* **7**: 773-779.

Medipally SRY, F.M.; Banerjee, S.; Shariff, M. (2015) Microalgae as sustainable renewable energy feedstock for biofuel production. *BioMed Research International* **2015**: 1-13.

Megharaj M, Ramakrishnan B, Venkateswarlu K, Sethunathan N & Naidu R (2011) Bioremediation approaches for organic pollutants: A critical perspective. *Environment International* **37**: 1362-1375.

Mekonnen T, Mussone P, Khalil H & Bressler D (2013) Progress in bio-based plastics and plasticizing modifications. *Journal of Materials Chemistry A* **1**: 13379-13398.

Meng X, Yang J, Xu X, Zhang L, Nie Q & Xian M (2009) Biodiesel production from oleaginous microorganisms. *Renewable Energy* **34**: 1-5.

Metting FB, Jr. (1996) Biodiversity and application of microalgae. *Journal of Industrial Microbiology* **17**: 477-489.

Mishra S, Jyot J, Kuhad RC & Lal B (2001) In situ bioremediation potential of an oily sludge-degrading bacterial consortium. *Current Microbiology* **43**: 328-335.

Mockos G, Smith W, Loge F & Thompson D (2008) Selective enrichment of a methanol-utilizing consortium using pulp and paper mill waste streams. *Applied Biochemistry and Biotechnology* **148**: 211-226.

Moosai R & Dawe RA (2003) Gas attachment of oil droplets for gas flotation for oily wastewater cleanup. *Separation and Purification Technology* **33**: 303-314.

Morelli IS, Del Panno MT, De Antoni GL & Painceira MT (2005) Laboratory study on the bioremediation of petrochemical sludge-contaminated soil. *International Biodeterioration & Biodegradation* **55**: 271-278.

Moser B (2009) Biodiesel production, properties, and feedstocks. *In Vitro Cellular & Developmental Biology - Plant* **45**: 229-266.

Mrayyan B & Battikhi MN (2005) Biodegradation of total organic carbons (TOC) in Jordanian petroleum sludge. *Journal of Hazardous Materials* **120**: 127-134.

Mueller J, Resnick S, Shelton M & Pritchard P (1992) Effect of inoculation on the biodegradation of wathered Prudhoe Bay crude oil. *Journal of Industrial Microbiology* **10**: 95-102.

Muhr A, Rechberger EM, Salerno A*,* Reiterer A, Malli K, Strohmeier K, Mittelbach M, Koller M (2013) Novel description of mcl-PHA biosynthesis by *Pseudomonas chlororaphis* from animal-derived waste. *Journal of Biotechnology* **165**: 45-51.

Mullin JV & Champ MA (2003) Introduction/Overview to In Situ Burning of Oil Spills. *Spill Science & Technology Bulletin* **8**: 323-330.

Murphy DJ (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. *Progress in Lipid Research* **32**: 247-280.

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.

Na KS, Kuroda A, Takiguchi N, Ikeda T, Ohtake H & Kato J (2005) Isolation and characterization of benzene-tolerant *Rhodococcus opacus* strains. *Journal of Bioscience and Bioengineering* **99**: 378-382.

Naether DJ, Slawtschew S, Stasik S, Engel M, Olzog M, Wick LY, Timmis KN, Heipieper HJ (2013) Adaptation of the hydrocarbonoclastic bacterium *Alcanivorax borkumensis* SK2 to alkanes and toxic organic compounds: a physiological and transcriptomic approach. *Applied and Environmental Microbiology* **79**: 4282-4293.

Narancic T, Kenny ST, Djokic L, Vasiljevic B, O'Connor KE & Nikodinovic-Runic J (2012) Medium-chain-length polyhydroxyalkanoate production by newly isolated *Pseudomonas* sp. TN301 from a wide range of polyaromatic and monoaromatic hydrocarbons. *Journal of Applied Microbiology* **113**: 508-520.

Narasimharao K, Lee A & Wilson K (2007) Catalysts in production of biodiesel: a review. *Journal of Biobased Materials and Bioenergy* **1**: 19-30.

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VA, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Chris Lee P, Holtzapple E, Scanlan D, Tran K, Moazzez A, Utterback T, Rizzo M, Lee K, Kosack D, Moestl D, Wedler H, Lauber J, Stjepandic D, Hoheisel J, Straetz M, Heim S, Kiewitz C, Eisen JA, Timmis KN, Düsterhöft A, Tümmler B, Fraser CM (2002) C omplete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environmental Microbiology* **4**: 799-808.

Nhi-Cong LT, Mikolasch A, Klenk H-P & Schauer F (2009) Degradation of the multiple branched alkane 2,6,10,14-tetramethyl-pentadecane (pristane) in *Rhodococcus ruber* and *Mycobacterium neoaurum*. *International Biodeterioration & Biodegradation* **63**: 201-207.

Niepceron M, Portet-Koltalo F, Merlin C, Motelay-Massei A, Barray S & Bodilis J (2010) Both *Cycloclasticus* spp. and *Pseudomonas* spp. as PAH-degrading bacteria in the Seine estuary (France). *FEMS Microbiology Ecology* **71**: 137-147.

Nigam PS & Singh A (2011) Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science* **37**: 52-68.

NJDEP (2010) Analysis of Extractable Petroleum Hydrocarbon Compounds (EPH) in Aqueous and Soil/Sediment/Sludge Matrices. (Protection E), 1-19.

Nogales J, Palsson B & Thiele I (2008) A genome-scale metabolic reconstruction of *Pseudomonas putida* KT2440: iJN746 as a cell factory. *BMC Systems Biology* **2**: 79.

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amman RI, Ludwig W, Backhaus H(1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* **178**: 5636-5643.

Nunal S, Al SN, Santander-De Leon S, Bacolod E, Koyama J, Uno S, Hidaka M, Yoshikawa T, Maeda H (2014) Bioremediation of heavily oil-polluted seawater by a bacterial consortium immobilized in cocopeat and rice hull powder. *Biocontrol Science* **19**: 11-22.

Obayori OS, Adebusoye SA, Adewale AO, Oyetibo GO, Oluyemi OO, Amokun RA & Ilori MO (2009) Differential degradation of crude oil (Bonny Light) by four *Pseudomonas* strains. *Journal of Environmental Sciences* **21**: 243-248.

Obuekwe CO, Hourani G & Radwan SS (2001) High-temperature hydrocarbon biodegradation activities in Kuwaiti desert soil samples. *Folia Microbiologica* **46**: 535-539.

Oh Y-S, Sim D-S & Kim S-J (2001) Effects of nutrients on crude oil biodegradation in the upper intertidal zone. *Marine Pollution Bulletin* **42**: 1367-1372.

Okoh AI (2006) Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. *Biotechnology and Molecular Biology Review* **1**: 38-50.

Olajire AAE, J.P. (2014) Aerobic degradation of petroleum components by microbial consortia. *Petroleum & Environmental Biotechnology Advances* **5**: 195-227.

Olivella MÀ, Jové P, Bianchi A, Bazzicalupi C & Cano L (2013) An integrated approach to understanding the sorption mechanism of phenanthrene by cork. *Chemosphere* **90**: 1939-1944.

Oller I, Malato S & Sánchez-Pérez JA (2011) Combination of advanced oxidation processes and biological treatments for wastewater decontamination—a review. *Science of The Total Environment* **409**: 4141-4166.

Ollivier B & Magot M (2005) *Petroleum Microbiology*. ASM Press.

Olukoshi ER & Packter NM (1994) Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. *Microbiology* **140**: 931-943.

Oudot J, Merlin FX & Pinvidic P (1998) Weathering rates of oil components in a bioremediation experiment in estuarine sediments. *Marine Environmental Research* **45**: 113-125.

Ouyang W, Liu H, Murygina V, Yu Y, Xiu Z & Kalyuzhnyi S (2005) Comparison of bio-augmentation and composting for remediation of oily sludge: A field-scale study in China. *Process Biochemistry* **40**: 3763-3768.

Packter NM & Olukoshi ER (1995) Ultrastructural studies of neutral lipid localisation in *Streptomyces*. *Archives in Microbiology* **164**: 420-427.

Paisse S, Duran R, Coulon F & Goñi-Urriza M (2011) Are alkane hydroxylase genes (alkB) relevant to assess petroleum bioremediation processes in chronically polluted coastal sediments? *Applied Microbiology and Biotechnology* **92**: 835-844.

Pal M, Vaidya B, Desai K, Joshi R, Nene S & Kulkarni B (2009) Media optimization for biosurfactant production by *Rhodococcus erythropolis* MTCC 2794: artificial intelligence versus a statistical approach. *Journal of Industrial Microbiology & Biotechnology* **36**: 747-756.

Pala DM, de Carvalho DD, Pinto JC & Sant'Anna Jr GL (2006) A suitable model to describe bioremediation of a petroleum-contaminated soil. *International Biodeterioration & Biodegradation* **58**: 254-260.

Palanisamy NR, J.; Kumar, S.; NS Vasanthi, N.S.; Chandran, P.; Khan, S. (2014) Diesel biodegradation capacities of indigenous bacterial species isolated from diesel contaminated soil. *Journal of Environmental Health Science & Engineering* **12**: 142.

Palleroni NJ, Pieper DH & Moore ERB (2010) Microbiology of Hydrocarbon-Degrading *Pseudomonas*. *Handbook of Hydrocarbon and Lipid Microbiology* (Timmis K) 1787-1798. Springer Berlin Heidelberg.

Papanikolaou S, Fakas S, Fick M, Chevalot I, Galiotou-Panayotou M, Komaitis M, Marc I, Aggelis G (2008) Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. *Biomass and Bioenergy* **32**: 60-71.

Parsons JB & Rock CO (2013) Bacterial lipids: Metabolism and membrane homeostasis. *Progress in Lipid Research* **52**: 249-276.

Patel S, Nelson DR & Gibbs AG (2001) Chemical and physical analyses of wax ester properties. *Journal of Insect Science* **1**: 7.

Pei LMS, M.; Wei, W. (2011) Conversion of Biomass into Bioplastics and Their Potential Environmental Impacts. *Biotechnology of Biopolymers* (Elnashar PM) 57-74.

Pelech SL & Vance DE (1989) Signal transduction via phosphatidylcholine cycles. *Trends in Biochemical Sciences* **14**: 28-30.

Peng F, Wang Y, Sun F, Liu Z, Lai Q & Shao Z (2008) A novel lipopeptide produced by a Pacific Ocean deep-sea bacterium, *Rhodococcus* sp. TW53. *Journal of Applied Microbiology* **105**: 698-705.

Peoples OP & Sinskey AJ (1989a) Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (phbC). *Journal of Biological Chemistry* **264**: 15298-15303.

Peoples OP & Sinskey AJ (1989b) Poly-beta-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding beta-ketothiolase and acetoacetyl-CoA reductase. *Journal of Biological Chemistry* **264**: 15293-15297.

Peressutti SR, Alvarez HM & Pucci OH (2003) Dynamics of hydrocarbon-degrading bacteriocenosis of an experimental oil pollution in Patagonian soil. *International Biodeterioration & Biodegradation* **52**: 21-30.

Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE & Irons DB (2003) Long-term ecosystem response to the Exxon Valdez oil spill. *Science* **302**: 2082-2086.

Philip S, Keshavarz T & Roy I (2007) Polyhydroxyalkanoates: biodegradable polymers with a range of applications. *Journal of Chemical Technology & Biotechnology* **82**: 233-247.

Philippe VA, Mendez MB, Huang IH, Orsaria LM, Sarker MR & Grau RR (2006) Inorganic phosphate induces spore morphogenesis and enterotoxin production in the intestinal pathogen *Clostridium perfringens*. *Infection Immunology* **74**: 3651 - 3656.

Philp J, Kuyukina M, Ivshina I, Dunbar S, Christofi N, Lang S & Wray V (2002) Alkanotrophic Rhodococcus ruber as a biosurfactant producer. *Applied Microbiology and Biotechnology* **59**: 318-324.

Pieper U & Steinbuchel A (1992) Identification, cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthase gene of the gram-positive bacterium *Rhodococcus ruber*. *FEMS Microbiol Letters* **75**: 73 - 79.

Pijuan M, Casas C & Baeza JA (2009) Polyhydroxyalkanoate synthesis using different carbon sources by two enhanced biological phosphorus removal microbial communities. *Process Biochemistry* **44**: 97-105.

Pintor AMA, Ferreira CIA, Pereira JC, Correia P, Silva SP, Vilar VJ, Botelho CM, Boaventura RA (2012) Use of cork powder and granules for the adsorption of pollutants: A review. *Water Research* **46**: 3152-3166.

Pirog T, Sofilkanych A, Shevchuk T & Shulyakova M (2013) Biosurfactants of *Rhodococcus erythropolis* IMV Ac-5017: synthesis intensification and practical application. *Applied Biochemistry and Biotechnology* **170**: 880-894.

Pisco AR, Bengtsson S, Werker A, Reis MAM, Lemos PC (2009) Community structure evolution and enrichment of glycogen-accumulating organisms producing polyhydroxyalkanoates from fermented molasses. *Applied Environmental Microbiology* **14**: 4676-4686.

PlasticsEurope (2013) The Compelling Facts About Plastics.

Płaza G, Jangid K, Łukasik K, Nałęcz-Jawecki G, Berry C & Brigmon R (2008) Reduction of petroleum hydrocarbons and toxicity in refinery wastewater by bioremediation. *Bulletin of Environmental Contamination and Toxicology* **81**: 329-333.

Poblete-Castro I, Binger D, Rodrigues A, Becker J, Martins dos Santos VAP & Wittmann C (2013) In-silico-driven metabolic engineering of *Pseudomonas putida* for enhanced production of poly-hydroxyalkanoates. *Metabolic Engineering* **15**: 113-123.

Poblete-Castro I, Escapa I, Jager C, Puchalka J, Lam CM, Schomburg D, Prieto MA, Martins dos Santos VA (2012) The metabolic response of *P. putida* KT2442 producing high levels of

polyhydroxyalkanoate under single- and multiple-nutrient-limited growth: Highlights from a multi-level omics approach. *Microbial Cell Factories* **11**: 34.

Poblete-Castro I RA, Lam CMC, Kessler W (2014) Improved production of medium-chain-length polyhydroxyalkanoates in glucose-based fed-batch cultivations of metabolically engineered *Pseudomonas putida* strains. *Journal of Microbiology and Biotechnology* **24**: 59-69.

Prieto M, Eugenio Ld, Galàn B, Luengo J & Witholt B (2007) Synthesis and Degradation of Polyhydroxyalkanoates. *Pseudomonas,* (Ramos J-L & Filloux A), 397-428. Springer Netherlands.

Prince M & Sambasivam Y (1993) Bioremediation of petroleum wastes from the refining of lubricant oils. *Environmental Progress* **12**: 5-11.

Prince RC (2005) *Petroleum Microbiology,*(Ollivier BM), 317-336. American Society for Microbiology Press, Washington DC.

Prince RC, Elmendorf DL, Lute JR*,* Hsu CS, Haith CE, Senius JD, Dechert GJ, Douglas GS, Butler EL (1994) 17.alpha.(H)-21.beta.(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. *Environmental Science & Technology* **28**: 142-145.

Puchałka J, Oberhardt MA, Godinho M*,* Bielecka A, Regenhardt D, Timmis KN, Papin JA, Martins dos Santos VA (2008) Genome-scale reconstruction and analysis of the *Pseudomonas putida* KT2440 metabolic network facilitates applications in Biotechnology. *PLoS Computational Biology* **4**: e1000210.

Purdy G, Pacheco S, Turk J & Hsu F-F (2013) Characterization of mycobacterial triacylglycerols and monomeromycolyl diacylglycerols from *Mycobacterium smegmatis* biofilm by electrospray ionization multiple-stage and high-resolution mass spectrometry. *Analytical and Bioanalytical Chemistry* **405**: 7415-7426.

Qu X-H, Wu Q, Liang J, Qu X, Wang S-G & Chen G-Q (2005) Enhanced vascular-related cellular affinity on surface modified copolyesters of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx). *Biomaterials* **26**: 6991-7001.

Quagliano JC & Miyazaki SS (1997) Effect of aeration and carbon/nitrogen ratio on the molecular mass of the biodegradable polymer poly-β-hydroxybutyrate obtained from *Azotobacter chroococcum* 6B. *Applied Microbiology and Biotechnology* **48**: 662-664.

Queirós D, Rossetti S & Serafim LS (2014) PHA production by mixed cultures: A way to valorize wastes from pulp industry. *Bioresource Technology* **157**: 197-205.

Raghavan PUM & Vivekanandan M (1999) Bioremediation of oil-spilled sites through seeding of naturally adapted *Pseudomonas putida*. *International Biodeterioration & Biodegradation* **44**: 29-32.

Rahul K, Sasikala C, Tushar L, Debadrita R & Ramana CV (2014) *Alcanivorax xenomutans* sp. nov., a hydrocarbonoclastic bacterium isolated from a shrimp cultivation pond. *International Journal of Systematic and Evolutionary Microbiology* **64**: 3553-3558.

Rai R, Keshavarz T, Roether JA, Boccaccini AR & Roy I (2011) Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. *Materials Science and Engineering: R: Reports* **72**: 29-47.

Ramaswamy B, Kar DD & De S (2007) A study on recovery of oil from sludge containing oil using froth flotation. *Journal of Environmental Management* **85**: 150-154.

Rashid N, Rehman MSU, Memon S, Ur Rahman Z, Lee K & Han J-I (2013) Current status, barriers and developments in biohydrogen production by microalgae. *Renewable and Sustainable Energy Reviews* **22**: 571-579.

Ratajczak A, Geißdörfer W & Hillen W (1998) Alkane hydroxylase from *Acinetobacter* sp. strain ADP1 Is encoded by alkM and belongs to a new family of bacterial integral-membrane hydrocarbon hydroxylases. *Applied and Environmental Microbiology* **64**: 1175-1179.

Ratledge C (2013) Microbial oils: an introductory overview of current status and future prospects. *OCL* **20**: D602.

Rattray JM (1984) Biotechnology and the fats and oils industry — An overview. *Journal of the American Oil Chemists' Society* **61**: 1701-1712.

Reddy CSK, Ghai R, Rashmi & Kalia VC (2003) Polyhydroxyalkanoates: an overview. *Bioresource Technology* **87**: 137-146.

Rees G, Vasiliadis G, May J & Bayly R (1993) Production of polyhydroxybutyrate in *Acinetobacter* spp. isolated from activated sludge. *Applied Microbiology and Biotechnology* **38**: 734-737.

Rehm B (2003) Polyester synthases: natural catalysts for plastics. *Biochemical Journal* **376**: 15 - 33.

Rehm B & Steinbuchel A (1999) Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. *International Journal of Biological Macromolecules* **25**: 3 - 19.

Rehm BHA (2010) Bacterial polymers: biosynthesis, modifications and applications. *Nature Reviews Microbiology* **8**: 578-592.

Reis MAM, Serafim LS, Lemos PC, Ramos AM, Aguiar FR & Van Loosdrecht MCM (2003) Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioprocess and Biosystems Engineering* **25**: 377-385.

Reiser S & Somerville C (1997) Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme A reductase. *Journal of Bacteriology* **179**: 2969-2975.

Ren Q, Beilen Jv, Sierro N, Zinn M, Kessler B & Witholt B (2005) Expression of PHA polymerase genes of *Pseudomonas putida* in *Escherichia coli* and its effect on PHA formation. *Antonie Van Leeuwenhoek* **87**: 91-100.

Renault F, Sancey B, Badot PM & Crini G (2009) Chitosan for coagulation/flocculation processes – An eco-friendly approach. *European Polymer Journal* **45**: 1337-1348.

Renner G, Haage G & Braunegg G (1996) Production of short-side-chain polyhydroxyalkanoates by various bacteria from the rRNA superfamily III. *Applied Microbiology and Biotechnology* **46**: 268-272.

Resnick SM, Lee K & Gibson DT (1996) Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp strain NCIB 9816. *Journal of Industrial Microbiology* **17**: 438-457.

Reva ON, Hallin PF, Willenbrock H, Sicheritz-Ponten T, Tümmler B & Ussery DW (2008) Global features of the *Alcanivorax borkumensis* SK2 genome. *Environmental Microbiology* **10**: 614-625.

Revellame ED, Hernandez R, French W, Holmes WE, Benson TJ, Pham PJ, Forks A, Callahan R (2012) Lipid storage compounds in raw activated sludge microorganisms for biofuels and oleochemicals production. *RSC Advances* **2**: 2015-2031.

Riesenberg D & Guthke R (1999) High-cell-density cultivation of microorganisms. *Applied Microbiology and Biotechnology* **51**: 422-430.

Rivas R, García-Fraile P, Peix A, Mateos PF, Martínez-Molina E & Velázquez E (2007) *Alcanivorax balearicu*s sp. nov., isolated from Lake Martel. *International Journal of Systematic and Evolutionary Microbiology* **57**: 1331-1335.

Rocha LL, Colares GB, Angelim AL, Grangeiro TB & Melo VMM (2013) Culturable populations of *Acinetobacter* can promptly respond to contamination by alkanes in mangrove sediments. *Marine Pollution Bulletin* **76**: 214-219.

Rodrigues EM, Kalks KHM & Tótola MR (2015) Prospect, isolation, and characterization of microorganisms for potential use in cases of oil bioremediation along the coast of Trindade Island, Brazil. *Journal of Environmental Management* **156**: 15-22.

Rojo F (2009) Degradation of alkanes by bacteria. *Environmental Microbiology* **11**: 2477-2490.

Röling WFM (2010) Hydrocarbon-Degradation by Acidophilic Microorganisms. *Handbook of Hydrocarbon and Lipid Microbiology,*(Timmis K), 1923-1930. Springer Berlin Heidelberg.

Rontani J-F, Bonin PC & Volkman JK (1999) Production of wax esters during aerobic growth of marine bacteria on isoprenoid compounds. *Applied and Environmental Microbiology* **65**: 221-230.

Rontani J-F, Mouzdahir A, Michotey V, Caumette P & Bonin P (2003) Production of a Polyunsaturated Isoprenoid Wax Ester during Aerobic Metabolism of Squalene by *Marinobacter squalenivorans* sp. nov. *Applied and Environmental Microbiology* **69**: 4167-4176.

Rontani JF (2010) Production of Wax Esters by Bacteria. *Handbook of Hydrocarbon and Lipid Microbiology,*(Timmis K), 459-470. Springer Berlin Heidelberg.

Rosenberg E & Ron EZ (1999) High- and low-molecular-mass microbial surfactants. *Applied Microbiology and Biotechnology* **52**: 154-162.

Röttig A & Steinbüchel A (2013a) Acyltransferases in Bacteria. *Microbiology and Molecular Biology Reviews* **77**: 277-321.

Röttig A & Steinbüchel A (2013b) Random mutagenesis of atfA and screening for *Acinetobacter baylyi* mutants with an altered lipid accumulation. *European Journal of Lipid Science and Technology* **115**: 394-404.

Röttig A, Wenning L, Bröker D & Steinbüchel A (2010) Fatty acid alkyl esters: perspectives for production of alternative biofuels. *Applied Microbiology and Biotechnology* **85**: 1713-1733.

Rude MA & Schirmer A (2009) New microbial fuels: a biotech perspective. *Current Opinion in Microbiology* **12**: 274-281.

Russell NJ & Volkman JK (1980) The effect of growth temperature on Wax Ester composition in the psychrophilic bacterium *Micrococcus cryophilus* ATCC 15174. *Microbiology* **118**: 131-141.

Sabirova JS, Chernikova TN, Timmis KN & Golyshin PN (2008) Niche-specificity factors of a marine oil-degrading bacterium *Alcanivorax borkumensis* SK2. *FEMS Microbiology Letters* **285**: 89-96.

Sabirova JS, Ferrer M, Regenhardt D, Timmis KN & Golyshin PN (2006a) Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis* Induced by alkane utilization. *Journal of Bacteriology* **188**: 3763-3773.

Sabirova JS, Ferrer M, Lünsdorf H, Wray V, Kalscheuer R, Steinbüchel A, Timmis KN, Golyshin PN. (2006b) Mutation in a "tesB-Like" hydroxyacyl-Coenzyme A-specific thioesterase gene causes hyperproduction of extracellular polyhydroxyalkanoates by Alcanivorax borkumensis SK2. *Journal of Bacteriology* **188**: 8452-8459.

Sakai Y, Maeng JH, Tani Y & Kato N (1994) Use of Long-chain n-Alkanes (C13–C44) by an Isolate, *Acinetobacter* sp. M-1. *Bioscience, Biotechnology, and Biochemistry* **58**: 2128-2130.

Salehizadeh H & Van Loosdrecht MCM (2004) Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnology Advances* **22**: 261-279.

Sameshima Y, Honda K, Kato J, Omasa T & Ohtake H (2008) Expression of *Rhodococcus opacus* alkB genes in anhydrous organic solvents. *Journal of Bioscience and Bioengineering* **106**: 199-203.

Sanguinetti CJ DNE, Simpson AJ (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques* **17**: 914-921.

Santala S, Efimova E, Karp M & Santala V (2011a) Real-Time monitoring of intracellular wax ester metabolism. *Microbial Cell Factories* **10**: 75.

Santala S, Efimova E, Koskinen P, Karp MT & Santala V (2014) Rewiring the wax ester production pathway of *Acinetobacter baylyi* ADP1. *ACS Synthetic Biology* **3**: 145-151.

Santala S, Efimova E, Kivinen V, Larjo A, Aho T, Karp M & Santala V (2011b) Improved triacylglycerol production in *Acinetobacter baylyi* ADP1 by metabolic engineering. *Microbial Cell Factories* **10**: 36.

Schedler M, Hiessl R, Valladares Juarez A, Gust G & Muller R (2014) Effect of high pressure on hydrocarbon-degrading bacteria. *AMB Express* **4**: 77.

Schembri MA, Bayly RC & Davies JK (1994) Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from an *Acinetobacter* sp.: Evidence that the gene is both plasmid and chromosomally located. *FEMS Microbiology Letters* **118**: 145-152.

Schenk P, Thomas-Hall S, Stephens E*,* Marx UE, Mussgnug JH, Posten C, Kruse O, Hankamer B (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. *BioEnergy Research* **1**: 20-43.

Schlegel HG, Kaltwasser H & Gottschalk G (1961) Ein submersverfahren zur kultur wasserstoff oxidierender bakterien: wachstumsphysiologische untersuchungen. *Arch Mikrobiol* **28**: 209 - 222.

Schneiker S, dos Santos VAPM, Bartels D*,* Bekel T, Brecht M, Buhrmester J, Chernikova TN, Denaro R, Ferrer M, Gertler C, Goesmann A, Golyshina OV, Kaminski F, Kachane AN, Lang S, Linke B, McHardy AC, Meyer F, Nechitaylo T, Puhler A, Regenhardt T, Rupp O, Selbitschka W, Yakimov MM, Sabirova JS, Timmis KN, Vorholter FJ, Weidner S, Kaiser O, Golyshin PN (2006) Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. *Nature Biotechnology* **24**: 997-1004.

Schreiberová O, Krulikovská T, Sigler K, Čejková A & Řezanka T (2010) RP-HPLC/MS-APCI analysis of branched chain TAG prepared by precursor-directed biosynthesis with *Rhodococcus erythropolis*. *Lipids* **45**: 743-756.

Şen A, Miranda I, Santos S, Graça J & Pereira H (2010) The chemical composition of cork and phloem in the rhytidome of *Quercus cerris* bark. *Industrial Crops and Products* **31**: 417-422.

Serafim L, Lemos P, Albuquerque ME & Reis MM (2008) Strategies for PHA production by mixed cultures and renewable waste materials. *Applied Microbiology and Biotechnology* **81**: 615-628.

Serafim LS, Lemos PC, Oliveira R & Reis MAM (2004) Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. *Biotechnology and Bioengineering* **87**: 145-160.

Shabir G, Afzal M, Tahseen R & Khalid SIQMKZM (2013) Treatment of oil refinery wastewater using pilot scale fed batch reactor followed by coagulation and sand filtration. *American Journal of Environmental Protection* **1**: 10-13.

Shah AA, Hasan F, Hameed A & Ahmed S (2008) Biological degradation of plastics: A comprehensive review. *Biotechnology Advances* **26**: 246-265.

Shao Z & Wang W (2013) Enzymes and genes Involved in aerobic alkane degradation. *Frontiers in Microbiology* **4**.

Shi S, Valle-Rodriguez J, Khoomrung S, Siewers V & Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. *Biotechnology for Biofuels* **5**: 7.

Shields-Menard S, Amirsadeghi M, Sukhbaatar B, Revellame E, Hernandez R, Donaldson J & French WT (2015) Lipid accumulation by *Rhodococcus rhodochrous* grown on glucose. *Journal of Industrial Microbiology & Biotechnology* **42**: 693-699.

Shimada K, Itoh Y, Washio K & Morikawa M (2012) Efficacy of forming biofilms by naphthalene degrading *Pseudomonas stutzeri* T102 toward bioremediation technology and its molecular mechanisms. *Chemosphere* **87**: 226-233.

Silva-Queiroz SR, Silva LF, Pradella JGC, Pereira EM & Gomez JGC (2009) PHAMCL biosynthesis systems in *Pseudomonas aeruginosa* and *Pseudomonas putida* strains show differences on monomer specificities. *Journal of Biotechnology* **143**: 111-118.

Silva EJ, Rocha e Silva NMP, Rufino RD, Luna JM, Silva RO & Sarubbo LA (2014) Characterization of a biosurfactant produced by *Pseudomonas cepacia* CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. *Colloids and Surfaces B: Biointerfaces* **117**: 36-41.

Silva RA, Grossi V, Olivera NL & Alvarez HM (2010) Characterization of indigenous *Rhodococcus* sp. 602, a strain able to accumulate triacylglycerides from naphthyl compounds under nitrogen-starved conditions. *Research in Microbiology* **161**: 198-207.

Silva SP, Sabino MA, Fernandes EM, Correlo VM, Boesel LF & Reis RL (2005) Cork: properties, capabilities and applications. *International Materials Reviews* **50**: 345-365.

Silva SPR, R. (2007) Meio de Absorção/Adsorção à Base de Derivados de Cortiça para Absorção/Adsorção de Óleos. Vol. PT103492 (Indústria CA), Portugal.

Simon-Colin C, Gouin C, Lemechko P, Schmitt S, Senant A, Kervarec N & Guezennec J (2012) Biosynthesis and characterization of polyhydroxyalkanoates by *Pseudomonas guezennei* from alkanoates and glucose. *International Journal of Biological Macromolecules* **51**: 1063-1069.

Simon-Colin C, Alain K, Raguénès G, Schmitt S, Kervarec N, Gouin C, Crassous P, Costa B, Guezennec JG (2009) Biosynthesis of medium chain length poly(3-hydroxyalkanoates) (mcl PHAs) from cosmetic co-products by *Pseudomonas raguenesii* sp. nov., isolated from Tetiaroa, French Polynesia. *Bioresource Technology* **100**: 6033-6039.

Singer ME, Tyler SM & Finnerty WR (1985) Growth of *Acinetobacter* sp. strain HO1-N on n-hexadecanol: physiological and ultrastructural characteristics. *Journal of Bacteriology* **162**: 162-169.

Singh AVK, V.; Srivastava J.N. (2013) Assessment of bioremediation of oil and phenol Contents in refinery waste water via bacterial consortium. *Petroleum & Environmental Biotechnology Advances* **4**: 1-4.

Singh Saharan B, Grewal A & Kumar P (2014) Biotechnological production of polyhydroxyalkanoates: a review on trends and latest developments. *Chinese Journal of Biology* **2014**: 18.

Singh SN, Kumari B & Mishra S (2012) Microbial Degradation of Alkanes. *Microbial Degradation of Xenobiotics,* (Singh SN), 439-469. Springer Berlin Heidelberg.

Sirakova T, Dubey V, Deb C, Daniel J, Korotkova T, Abomoelak B & Kolattukudy P (2006) Identification of a diacylglycerol acyltransferase gene involved in accumulation of triacylglycerol in *Mycobacterium tuberculosis* under stress. *Microbiology* **152**: 2717 - 2725.

Song X, Xu Y, Li G, Zhang Y, Huang T & Hu Z (2011) Isolation, characterization of *Rhodococcus* sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. *Marine Pollution Bulletin* **62**: 2122-2128.

Sood N & Lal B (2009) Isolation of a novel yeast strain *Candida digboiensis* TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions. *Journal of Environmental Management* **90**: 1728-1736.

Sorek A, Atzmon N, Dahan O, Gerstl Z, Kushisin L, Laor Y, Mingelgrin U, Nasser A, Ronen D, Tsechansky L, Weisbrod Nm Graber ER (2008) "Phytoscreening": the use of trees for

discovering subsurface contamination by VOCs. *Environmental Science & Technology* **42**: 536-542.

Sorkhoh NA, Ghannoum MA, Ibrahim AS, Stretton RJ & Radwan SS (1990) Crude oil and hydrocarbon-degrading strains of *Rhodococcus rhodochrous* isolated from soil and marine environments in Kuwait. *Environmental Pollution* **65**: 1-17.

Speight JG (1999) The chemical and physical structure of petroleum: effects on recovery operations. *Journal of Petroleum Science and Engineering* **22**: 3-15.

Spence MJ, Bottrell SH, Thornton SF, Richnow HH & Spence KH (2005) Hydrochemical and isotopic effects associated with petroleum fuel biodegradation pathways in a chalk aquifer. *Journal of Contaminant Hydrology* **79**: 67-88.

Spiecker PM, Gawrys KL, Trail CB & Kilpatrick PK (2003) Effects of petroleum resins on asphaltene aggregation and water-in-oil emulsion formation. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **220**: 9-27.

Staley JT (2010) Cycloclasticus: A Genus of Marine Polycyclic Aromatic Hydrocarbon Degrading Bacteria. *Handbook of Hydrocarbon and Lipid Microbiology,* (Timmis K), 1781-1786. Springer Berlin Heidelberg.

Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB, Keasling JD (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* **463**: 559-562.

Steinbüchel A (2001) Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. *Macromol Biosc* **1**: 1-24.

Steinbüchel A & Valentin HE (1995) Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiology Letters* **128**: 219-228.

Steinbüchel A & Füchtenbusch B (1998) Bacterial and other biological systems for polyester production. *Trends in Biotechnology* **16**: 419-427.

Steinbüchel A & Hein S (2001) Biochemical and Molecular Basis of Microbial Synthesis of Polyhydroxyalkanoates in Microorganisms. *Biopolyesters,* Vol. 71 (Babel W & Steinbüchel A), 81-123. Springer Berlin Heidelberg.

Steinbüchel A, Hustede E, Liebergesell M, Pieper U, Timm A & Valentin H (1992) Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. *FEMS Microbiology Letters* **9**: 217-230.

Steinbüchel A HE, Liebergesell M, Pieper U, Timm A, Valentin H (1993) Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. *FEMS Microbiology Reviews* **10**: 347-350.

Stöveken T & Steinbüchel A (2008) Bacterial acyltransferases as an alternative for lipase-catalyzed acylation for the production of oleochemicals and fuels. *Angewandte Chemie International Edition* **47**: 3688-3694.

Stöveken T, Kalscheuer R & Steinbüchel A (2009) Both histidine residues of the conserved HHXXXDG motif are essential for wax ester synthase/acyl-CoA:diacylglycerol acyltransferase catalysis. *European Journal of Lipid Science and Technology* **111**: 112-119.

Stöveken T, Kalscheuer R, Malkus U, Reichelt R & Steinbüchel A (2005) The wax ester synthase/acyl Coenzyme A:diacylglycerol acyltransferase from *Acinetobacter* sp. Strain ADP1: characterization of a novel type of acyltransferase. *Journal of Bacteriology* **187**: 1369-1376.

Striebich RC, Smart CE, Gunasekera TS, Mueller SS, Strobel EM, McNichols BW & Ruiz ON (2014) Characterization of the F-76 diesel and Jet-A aviation fuel hydrocarbon degradation profiles

of *Pseudomonas aeruginosa* and *Marinobacter hydrocarbonoclasticus*. *International Biodeterioration & Biodegradation* **93**: 33-43.

Subramaniam R, Dufreche S, Zappi M & Bajpai R (2010) Microbial lipids from renewable resources: production and characterization. *Journal of Industrial Microbiology & Biotechnology* **37**: 1271-1287.

Sudesh K, Abe H & Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science* **25**: 1503-1555.

Sudesh K, Bhubalan K, Chuah J-A, Kek Y-K, Kamilah H, Sridewi N & Lee Y-F (2011) Synthesis of polyhydroxyalkanoate from palm oil and some new applications. *Applied Microbiology and Biotechnology* **89**: 1373-1386.

Suja F, Rahim F, Taha MR, Hambali N, Rizal Razali M, Khalid A & Hamzah A (2014) Effects of local microbial bioaugmentation and biostimulation on the bioremediation of total petroleum hydrocarbons (TPH) in crude oil contaminated soil based on laboratory and field observations. *International Biodeterioration & Biodegradation* **90**: 115-122.

Sun Z, Ramsay J, Guay M & Ramsay B (2007) Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440. *Applied Microbiology and Biotechnology* **74**: 69-77.

Suriyamongkol P, Weselake R, Narine S, Moloney M & Shah S (2007) Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants — A review. *Biotechnology Advances* **25**: 148-175.

Suzuki T, Yamane T & Shimizu S (1986) Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. *Applied Microbiology and Biotechnology* **24**: 370-374.

Tahhan RA, Ammari TG, Goussous SJ & Al-Shdaifat HI (2011) Enhancing the biodegradation of total petroleum hydrocarbons in oily sludge by a modified bioaugmentation strategy. *International Biodeterioration & Biodegradation* **65**: 130-134.

Takei D, Washio K & Morikawa M (2008) Identification of alkane hydroxylase genes in *Rhodococcus* sp. strain TMP2 that degrades a branched alkane. *Biotechnology Letters* **30**: 1447-1452.

Takizawa N, Iida T, Sawada T, Yamauchi K, Wang Y-W, Fukuda M & Kiyohara H (1999) Nucleotide sequences and characterization of genes encoding naphthalene upper pathway of *Pseudomonas aeruginosa* PaK1 and *Pseudomonas putida* OUS82. *Journal of Bioscience and Bioengineering* **87**: 721-731.

Tamis JS, D.Y.; Jiang, Y.; van Loosdrecht, M.C.M.; Kleerebezem, R. (2015) Lipid recovery from a vegetable oil emulsion using microbial enrichment cultures. *Biotechnology for Biofuels* **8**: 39.

Tan G-YA, Chen C-L, Ge L, Li L, Tan SN & Wang J-Y (2015) Bioconversion of styrene to Poly(hydroxyalkanoate) (PHA) by the new bacterial strain *Pseudomonas putida* NBUS12. *Microbes and Environments* **30**: 76-85.

Tan Y, Neo P-C, Najimudin N, Sudesh K, Muhammad TST, Othman AS & Samian R (2010) Cloning and characterization of poly(3-hydroxybutyrate) biosynthesis genes from *Pseudomonas* sp. USM 4-55. *Journal of Basic Microbiology* **50**: 179-189.

Táncsics A, Benedek T, Szoboszlay S, Veres PG, Farkas M, Máthé I, Marialigeti K, Kukolya J, Lányi S, Kriszt B (2015) The detection and phylogenetic analysis of the alkane 1-monooxygenase gene of members of the genus *Rhodococcus*. *Systematic and Applied Microbiology* **38**: 1-7.

Tani A, Sakai Y, Ishige T & Kato N (2000) Thermostable NADP+-dependent medium-chain alcohol dehydrogenase from *Acinetobacter* sp. strain M-1: purification and characterization and gene expression in *Escherichia coli*. *Applied and Environmental Microbiology* **66**: 5231-5235.

Tani A, Ishige T, Sakai Y & Kato N (2002) Two acyl-CoA dehydrogenases of *Acinetobacter* sp. strain M-1 that uses very long-chain n-alkanes. *Journal of Bioscience and Bioengineering* **94**: 326-329.

Tavassoli T, Mousavi SM, Shojaosadati SA & Salehizadeh H (2012) Asphaltene biodegradation using microorganisms isolated from oil samples. *Fuel* **93**: 142-148.

Teira E, Lekunberri I, Gasol JM, Nieto-Cid M, Álvarez-Salgado XA & Figueiras FG (2007) Dynamics of the hydrocarbon-degrading *Cycloclasticus* bacteria during mesocosm-simulated oil spills. *Environmental Microbiology* **9**: 2551-2562.

Teramoto M, Queck SY & Ohnishi K (2013) Specialized hydrocarbonoclastic bacteria prevailing in seawater around a port in the Strait of Malacca. *PLoS One* **8**: e66594.

Teramoto M, Suzuki M, Hatmanti A & Harayama S (2010) The potential of *Cycloclasticus* and *Altererythrobacter* strains for use in bioremediation of petroleum-aromatic-contaminated tropical marine environments. *Journal of Bioscience and Bioengineering* **110**: 48-52.

Teramoto M, Ohuchi M, Hatmanti A, Darmayati Y, Widyastuti Y, Harayama S & Fukunaga Y (2011) *Oleibacter marinus* gen. nov., sp. nov., a bacterium that degrades petroleum aliphatic hydrocarbons in a tropical marine environment. *International Journal of Systematic and Evolutionary Microbiology* **61**: 375-380.

Thevenieau F & Nicaud J-M (2013) Microorganisms as sources of oils. *OCL* **20**: D603.

Thorn KA & Aiken GR (1998) Biodegradation of crude oil into nonvolatile organic acids in a contaminated aquifer near Bemidji, Minnesota. *Organic Geochemistry* **29**: 909-931.

Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar H-K & Zotchev SB (2007) Identification of novel genes Involved in long-chain n-alkane degradation by *Acinetobacter* sp. strain DSM 17874. *Applied and Environmental Microbiology* **73**: 3327-3332.

Throne-Holst M, Markussen S, Winnberg A, Ellingsen T, Kotlar H-K & Zotchev S (2006) Utilization of n-alkanes by a newly isolated strain of *Acinetobacter venetianus*: the role of two AlkB-type alkane hydroxylases. *Applied Microbiology and Biotechnology* **72**: 353-360.

Tian W, Hong K, Chen G-Q, Wu Q, Zhang R-q & Huang W (2000) Production of polyesters consisting of medium chain length 3-hydroxyalkanoic acids by P*seudomonas mendocina* 0806 from various carbon sources. *Antonie Van Leeuwenhoek* **77**: 31-36.

Timm A, Byrom D & Steinbüchel A (1990) Formation of blends of various poly(3-hydroxyalkanoic acids) by a recombinant strain of *Pseudomonas oleovorans*. *Applied Microbiology and Biotechnology* **33**: 296-301.

Tobin KM & O'Connor KE (2005) Polyhydroxyalkanoate accumulating diversity of *Pseudomonas species* utilising aromatic hydrocarbons. *FEMS Microbiology Letters* **253**: 111-118.

Tokiwa Y, Calabia B, Ugwu C & Aiba S (2009) Biodegradability of Plastics. *International Journal of Molecular Sciences* **10**: 3722.

Tomás-Gallardo L, Gómez-Álvarez H, Santero E & Floriano B (2014) Combination of degradation pathways for naphthalene utilization in *Rhodococcus* sp. strain TFB. *Microbial Biotechnology* **7**: 100-113.

Touzé T, Blanot D & Mengin-Lecreulx D (2008) Substrate specificity and membrane topology of *Escherichia coli* PgpB, an undecaprenyl pyrophosphate phosphatase. *Journal of Biological Chemistry* **283**: 16573-16583.

Tsuge T, Hisano T, Taguchi S & Doi Y (2003) Alteration of chain length substrate Specificity of *Aeromonas caviae* R-enantiomer-specific enoyl-Coenzyme A hydratase through site-directed mutagenesis. *Applied and Environmental Microbiology* **69**: 4830-4836.

Ubani OA, H. I.; Thantsha, M.S. (2012) Biological degradation of oil sludge: A review of the current state of development. *African Journal of Biotechnology* **12**: 6544-6567.

Ulrici W (2008) Contaminated Soil Areas, Different Countries and Contaminants, Monitoring of Contaminants. *Biotechnology*, 5-41. Wiley-VCH Verlag GmbH.

USEPA (2015a) Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2013, U.S. Environmental Protection Agency, Washington, DC.

USEPA (2015b) Short-Term Energy Outlook.

Uthoff S & Steinbüchel A (2012) Purification and characterization of an NAD+-dependent XylB-Like aryl alcohol dehydrogenase identified in *Acinetobacter baylyi* ADP1. *Applied and Environmental Microbiology* **78**: 8743-8752.

Uthoff S, Broker D & Steinbuchel A (2009) Current state and perspective of producing biodiesel-like compounds by biotechnology. *Microbial Biotechnology* **2**: 551 - 565.

Uthoff S, Stöveken T, Weber N, Vosmann K, Klein E, Kalscheuer R & Steinbüchel A (2005) Thio wax ester biosynthesis utilizing the unspecific bifunctional wax ester synthase/acyl Coenzyme A:diacylglycerol acyltransferase of *Acinetobacter* sp. strain ADP1. *Applied and Environmental Microbiology* **71**: 790-796.

Valappil SP, Misra SK, Boccaccini AR & Roy I (2006) Biomedical applications of polyhydroxyalkanoates, an overview of animal testing and in vivo responses. *Expert Review of Medical Devices* **3**: 853-868.

van Beilen J & Funhoff E (2007) Alkane hydroxylases involved in microbial alkane degradation. *Applied Microbiology and Biotechnology* **74**: 13-21.

van Beilen JB, Kingma J & Witholt B (1994) Substrate specificity of the alkane hydroxylase system of *Pseudomonas oleovorans* GPo1. *Enzyme and Microbial Technology* **16**: 904-911.

Van Beilen JB, Marín MM, Smits THM, Röthlisberger M, Franchini AG, Witholt B & Rojo F (2004) Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. *Environmental Microbiology* **6**: 264-273.

Van Hamme JD, Singh A & Ward OP (2003) Recent advances in petroleum microbiology. *Microbiology and Molecular Biology Reviews* **67**: 503-549.

Vaneechoutte M, Young DM, Ornston LN, De Baere T, Nemec A, Van Der Reijden T, Carr E, Tjernberg I, Dijkshoorn L (2006) Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*. *Applied and Environmental Microbiology* **72**: 932-936.

Vargas A, Montaño L, Amaya R (2014) Enhanced polyhydroxyalkanoate production from organic wastes via process control. *Bioresource Technology* **156:** 248-255.

Vázquez S, Nogales B, Ruberto L, Hernández E, Christie-Oleza J, LoBalbo A, Bosch R, Lalucat J, Mac Cormak W (2009) Bacterial community dynamics during bioremediation of diesel oil-contaminated antarctic soil. *Microbial Ecology* **57**: 598-610.

Venkateswar Reddy M, Nikhil GN, Venkata Mohan S, Swamy YV & Sarma PN (2012) *Pseudomonas otitidis* as a potential biocatalyst for polyhydroxyalkanoates (PHA) synthesis using synthetic wastewater and acidogenic effluents. *Bioresource Technology* **123**: 471-479.

Venosa AD & Zhu X (2003) Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. *Spill Science & Technology Bulletin* **8**: 163-178.

Verlinden R, Hill D, Kenward M, Williams C, Piotrowska-Seget Z & Radecka I (2011) Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB Express* **1**: 11.

Verlinden RAJ, Hill DJ, Kenward MA, Williams CD & Radecka I (2007) Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology* **102**: 1437-1449.

Vidali M (2001) Bioremediation. An overview. *Pure and Applied Chemistry* **73**: 1163-1172.

Vieira M (2005) Estudo da cinética de acumulação e consumo de polímeros de reserva num reactor de biofilmes para a remoção do azoto. Thesis, University of Minho, Braga, Portugal.

Vila J, Tauler M & Grifoll M (2015) Bacterial PAH degradation in marine and terrestrial habitats. *Current Opinion in Biotechnology* **33**: 95-102.

Villalba MS HM, Silva RA, Alvarez HM (2013) Genome sequences of triacylglycerol metabolism in *Rhodococcus* as a platform for comparative genomics. *Journal of Molecular Biochemistry* **2**: 94-105.

Villano M, Valentino F, Barbetta A, Martino L, Scandola M & Majone M (2014) Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process. *New Biotechnology* **31**: 289-296.

Voss I & Steinbuchel A (2001) High cell density cultivation of *Rhodococcus opacus* for lipid production at a pilot-plant scale. *Applied Microbiology and Biotechnology* **55**: 547 - 555.

Wackett LP (1996) Co-metabolism: is the emperor wearing any clothes? *Current Opinion in Biotechnology* **7**: 321-325.

Wahlen BD, Oswald WS, Seefeldt LC & Barney BM (2009) Purification, characterization, and potential bacterial wax production role of an NADPH-dependent fatty aldehyde reductase from *Marinobacter aquaeolei* VT8. *Applied and Environmental Microbiology* **75**: 2758-2764.

Wahlen BD, Morgan MR, McCurdy AT*,* Willis RM, Morgan MD, Dye DJ, Bugbee B, Wood BD, Seefeldt LC (2013) Biodiesel from microalgae, yeast, and bacteria: engine performance and exhaust emissions. *Energy & Fuels* **27**: 220-228.

Wake H (2005) Oil refineries: a review of their ecological impacts on the aquatic environment. *Estuarine, Coastal and Shelf Science* **62**: 131-140.

Waller JL, Green PG, Loge FJ (2012) Mixed-culture polyhydroxyalkanoate production from olive oil mill pomace. *Bioresource Technology* **120**: 285-289.

Walter U, Beyer M, Klein J & Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. *Applied Microbiology and Biotechnology* **34**: 671-676.

Wältermann M, Luftmann H, Baumeister D, Kalscheuer R & Steinbuchel A (2000) *Rhodococcus opacus* strain PD630 as a new source of high-value singel-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. *Microbiology (SGM)* **146**: 1143 - 1149.

Wältermann M & Steinbüchel A (2005) Neutral lipid bodies in prokaryotes: recent insights into structure, formation, and relationship to eukaryotic lipid depots. *Journal of Bacteriology* **187**: 3607-3619.

Wältermann M & Steinbüchel A (2006) Wax Ester and Triacylglycerol Inclusions. *Inclusions in Prokaryotes,* Vol. 1 (Shively J),137-166. Springer Berlin Heidelberg.

Wältermann M, Stöveken T & Steinbüchel A (2007) Key enzymes for biosynthesis of neutral lipid storage compounds in prokaryotes: Properties, function and occurrence of wax ester synthases/acyl-CoA:diacylglycerol acyltransferases. *Biochimie* **89**: 230-242.

Wältermann M, Luftmann H, Baumeister D, Kalscheuer R & Steinbüchel A (2000) *Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. *Microbiology* **146**: 1143-1149.

Wältermann M, Hinz A, Robenek H*,* Troyer D, Reichelt R, Malkus U, Galla HJ, Kalscheuer R, Stöveken T, von Landenberg P, Steinubüchel A (2005) Mechanism of lipid-body formation in prokaryotes: how bacteria fatten up. *Molecular Microbiology* **55**: 750-763.

Wang B, Rezenom YH, Cho K-C*,* Tran JL, Lee do G, Russell DH, Gill JJ, Young R, Chu KH (2014) Cultivation of lipid-producing bacteria with lignocellulosic biomass: Effects of inhibitory compounds of lignocellulosic hydrolysates. *Bioresource Technology* **161**: 162-170.

Wang J, Yin J, Ge L, Shao J & Zheng J (2010) Characterization of oil sludges from two oil fields in China. *Energy & Fuels* **24**: 973-978.

Wang LK, Hung YT, Lo HH & Yapijakis C (2004) *Handbook of Industrial and Hazardous Wastes Treatment*. CRC Press.

Wang X, Wang Q, Wang S, Li F & Guo G (2012) Effect of biostimulation on community level physiological profiles of microorganisms in field-scale biopiles composed of aged oil sludge. *Bioresource Technology* **111**: 308-315.

Wang Z, Fingas M, Blenkinsopp S*,* Sergy G, Landriault M, Sigouin L, Foght J, Semple K, Westlake DWS (1998) Comparison of oil composition changes due to biodegradation and physical weathering in different oils. *Journal of Chromatography A* **809**: 89-107.

Weber WJ & Kim HS (2005) Optimizing contaminant desorption and bioavailability in dense slurry systems. 1. Rheology, mechanical mixing, and PAH desorption. *Environmental Science & Technology* **39**: 2267-2273.

Wei Z, Zeng G, Kosa M, Huang D & Ragauskas A (2015a) Pyrolysis oil-based lipid production as biodiesel feedstock by *Rhodococcus opacus*. *Applied Biochemistry and Biotechnology* **175**: 1234-1246.

Wei Z, Zeng G, Huang F, Kosa M, Huang D & Ragauskas AJ (2015b) Bioconversion of oxygen-pretreated Kraft lignin to microbial lipid with oleaginous *Rhodococcus opacus* DSM 1069. *Green Chemistry* **17**: 2784-2789.

Wentzel A, Ellingsen T, Kotlar H-K, Zotchev S & Throne-Holst M (2007) Bacterial metabolism of long-chain n-alkanes. *Applied Microbiology and Biotechnology* **76**: 1209-1221.

Westfall PJ & Gardner TS (2011) Industrial fermentation of renewable diesel fuels. *Current Opinion in Biotechnology* **22**: 344-350.

Whyte LG, Hawari J, Zhou E, Bourbonnière L, Inniss WE & Greer CW (1998) Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. *Applied and Environmental Microbiology* **64**: 2578-2584.

Whyte LG, Smits THM, Labbé D, Witholt B, Greer CW & van Beilen JB (2002) Gene cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* strains Q15 and NRRL B-16531. *Applied and Environmental Microbiology* **68**: 5933-5942.

Whyte LG, Slagman SJ, Pietrantonio F*,* Bourbonièrre L, Koval SF, Lawrence JR, Inniss WE, Greer CW (1999) Physiological adaptations involved in alkane assimilation at a low remperature by *Rhodococcus* sp. strain Q15. *Applied and Environmental Microbiology* **65**: 2961-2968.

Wilkinson WOB, R.M. (1997) sn-Glycerol-3-phosphate acyltransferase from *Escherichia coli*. *Biochimica et Biophysica Acta* **1348**: 3-9.

Willis RM, Wahlen BD, Seefeldt LC & Barney BM (2011) Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: A bacterial enzyme catalyzing the reduction of fatty Acyl-CoA to fatty alcohol. *Biochemistry* **50**: 10550-10558.

Wu Y, Lai Q, Zhou Z, Qiao N, Liu C & Shao Z (2009) *Alcanivorax hongdengensis* sp. nov., an alkane-degrading bacterium isolated from surface seawater of the straits of Malacca and Singapore, producing a lipopeptide as its biosurfactant. *International Journal of Systematic and Evolutionary Microbiology* **59**: 1474-1479.

Xu F, Huang S, Liu Y, Zhang Y & Chen S (2014) Comparative study on the production of poly(3-hydroxybutyrate) by thermophilic *Chelatococcus daeguensis* TAD1: a good candidate for large-scale production. *Applied Microbiology and Biotechnology* **98**: 3965-3974.

Xu N, Wang W, Han P & Lu X (2009) Effects of ultrasound on oily sludge deoiling. *Journal of Hazardous Materials* **171**: 914-917.

Yakimov MM, Timmis KN & Golyshin PN (2007) Obligate oil-degrading marine bacteria. *Current Opinion in Biotechnology* **18**: 257-266.

Yakimov MM, Golyshin PN, Lang S, Moore ERB, Abraham W-R, Lünsdorf H & Timmis KN (1998) *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-

producing marine bacterium. *International Journal of Systematic and Evolutionary Microbiology* **48**: 339-348.

Yakimov MM, Giuliano L, Gentile G, Crisafi E, Chernikova TN, Abraham WR, Lünsdorf H, Timmis KN, Golyshin PN (2003) *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. *International Journal of Systematic and Evolutionary Microbiology* **53**: 779-785.

Yakimov MM, Giuliano L, Denaro R, Crisafi E, Chernikova TN, Abraham WR, Luensdorf, H, Timmis KN, Golyshin PN (2004) *Thalassolituus oleivorans* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. *International Journal of Systematic and Evolutionary Microbiology* **54**: 141-148.

Yakimov MM, Denaro R, Genovese M, Cappello S, D'Auria G, Chernikova TN, Timmis KN, Golyshin PN, Giuliano L (2005) Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons. *Environmental Microbiology* **7**: 1426-1441.

Yam K, van der Geize R & Eltis L (2010) Catabolism of aromatic compounds and steroids by *Rhodococcus*. *Biology of Rhodococcus,* Vol. 16 (Alvarez HM), 133-169. Springer Berlin Heidelberg.

Yamashita S, Satoi M, Iwasa Y, Honda K, Sameshima Y, Omasa T, Kato J, Ohtake H (2007) Utilization of hydrophobic bacterium *Rhodococcus opacus* B-4 as whole-cell catalyst in anhydrous organic solvents. *Applied Microbiology and Biotechnology* **74**: 761-767.

Yang C, Zhang W, Liu R, Zhang C, Gong T, Li Q, Wang S, Song C (2013) Analysis of polyhydroxyalkanoate (PHA) synthase gene and PHA-producing bacteria in activated sludge that produces PHA containing 3-hydroxydodecanoate. *FEMS Microbiology Letters* **346**: 56-64.

Yang S-T (2007) Chapter 1 - Bioprocessing—from Biotechnology to Biorefinery. *Bioprocessing for Value-Added Products from Renewable Resources,*(Yang S-T), 1-24. Elsevier, Amsterdam.

Yang Y, Chen RF & Shiaris MP (1994) Metabolism of naphthalene, fluorene, and phenanthrene: preliminary characterization of a cloned gene cluster from *Pseudomonas putida* NCIB 9816. *Journal of Bacteriology* **176**: 2158-2164.

Yang Y, Wang J, Liao J, Xie S & Huang Y (2015) Abundance and diversity of soil petroleum hydrocarbon-degrading microbial communities in oil exploring areas. *Applied Microbiology and Biotechnology* **99**: 1935-1946.

Yemashova N, Murygina V, Zhukov D, Zakharyantz A, Gladchenko M, Appanna V & Kalyuzhnyi S (2007) Biodeterioration of crude oil and oil derived products: a review. *Reviews in Environmental Science and Bio/Technology* **6**: 315-337.

Yen C-LE, Stone SJ, Koliwad S, Harris C & Farese RV (2008) Thematic review series: Glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *Journal of Lipid Research* **49**: 2283-2301.

Yoo C, Jun S-Y, Lee J-Y, Ahn C-Y & Oh H-M (2010) Selection of microalgae for lipid production under high levels carbon dioxide. *Bioresource Technology* **101**: S71-S74.

Yu J & Stahl H (2008) Microbial utilization and biopolyester synthesis of bagasse hydrolysates. *Bioresource Technology* **99**: 8042-8048.

Yu L, Han M 6& He F (2013) A review of treating oily wastewater. *Arabian Journal of Chemistry*.

Zampolli J, Collina E, Lasagni M & Di Gennaro P (2014) Biodegradation of variable-chain-length n-alkanes in *Rhodococcus opacus* R7 and the involvement of an alkane hydroxylase system in the metabolism. *AMB Express* **4**: 73.

Zhang Q, Wang D, Li M, Xiang W-N & Achal V (2014) Isolation and characterization of diesel degrading bacteria, *Sphingomonas* sp. and *Acinetobacter junii* from petroleum contaminated soil. *Frontiers of Earth Science* **8**: 58-63.

Zhang X, Agrawal A & San K-Y (2012) Improving fatty acid production in *Escherichia coli* through the overexpression of malonyl coA-Acyl carrier protein transacylase. *Biotechnology Progress* **28**: 60-65.

Zhang Y-M & Rock CO (2008) Membrane lipid homeostasis in bacteria. *Nature Review Microbiology* **6**: 222-233.

Zhang Y & Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). *Applied and Environmental Microbiology* **58**: 3276-3282.

Zhang Y & Miller RM (1995) Effect of rhamnolipid (biosurfactant) structure on solubilization and biodegradation of n-alkanes. *Applied and Environmental Microbiology* **61**: 2247-2251.

Zhang Z, Hou Z, Yang C, Ma C, Tao F & Xu P (2011) Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. *Bioresource Technology* **102**: 4111-4116.

Zhang Z, Gai L, Hou Z, Yang C, Ma C, Wang Z, Sun B, He X, Tang H, Xu P (2010) Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils. *Bioresource Technology* **101**: 8452-8456.

Zhao W & Chen G-Q (2007) Production and characterization of terpolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) by recombinant *Aeromonas hydrophila* 4AK4 harboring genes phaAB. *Process Biochemistry* **42**: 1342-1347.

Zhao X, Wang Y, Ye Z, Borthwick AGL & Ni J (2006) Oil field wastewater treatment in Biological Aerated Filter by immobilized microorganisms. *Process Biochemistry* **41**: 1475-1483.

Zheng C, Yu L, Huang L, Xiu J & Huang Z (2012) Investigation of a hydrocarbon-degrading strain, *Rhodococcus ruber* Z25, for the potential of microbial enhanced oil recovery. *Journal of Petroleum Science and Engineering* **81**: 49-56.

Zheng LZ, Li Z, Tian H-L, Li M & Chen G-Q (2005) Molecular cloning and functional analysis of (R)-3-hydroxyacyl-acyl carrier protein:coenzyme A transacylase from *Pseudomonas mendocina* LZ. *FEMS Microbiology Letters* **252**: 299-307.

Zhou L, Jiang X & Liu J (2009) Characteristics of oily sludge combustion in circulating fluidized beds. *Journal of Hazardous Materials* **170**: 175-179.

Zhuang X, Han Z, Bai Z, Zhuang G & Shim H (2010) Progress in decontamination by halophilic microorganisms in saline wastewater and soil. *Environmental Pollution* **158**: 1119-1126.

Zinn M & Hany R (2005) Tailored material properties of polyhydroxyalkanoates through biosynthesis and chemical modification. *Advanced Engineering Materials* **7**: 408-411.

Zou C, Wang M, Xing Y, Lan G, Ge T, Yan X & Gu T (2014) Characterization and optimization of biosurfactants produced by *Acinetobacter baylyi* ZJ2 isolated from crude oil-contaminated soil sample toward microbial enhanced oil recovery applications. *Biochemical Engineering Journal* **90**: 49-58.