African Journal of Biotechnology Vol. 8 (12), pp. 2653-2666, 17 June, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Review

Petroleum biotechnology: Technology trends for the future

Carmina Montiel[1], Rodolfo Quintero[2] and Jorge Aburto[1]*

[1]Instituto Mexicano del Petróleo, Eje Central Lázaro Cárdenas 152, Col. San Bartolo Atepehuacan, 07730 Mexico City, Mexico.
[2]Universidad Autónoma Metropolitana. Unidad Cuajimalpa Avenida Constituyentes 1054, Col. Lomas Altas, 11950, México City, Mexico.

Accepted 19 December, 2008

This paper comes out from the need to provide a general overview of the current biotechnology situation and its future impact on the petroleum industry. This important industrial sector already encounters challenges as the decreasing oil reserves, the fluctuating oil prices, the increasing demand of petroleum, fuels and petrochemicals and finally more strict environmental regulations. These challenges will persist and strengthen in the following years. Biotechnology has come a long way since the 1950's and today impacts different industrial sectors such as food, pharmaceutics, medicine, agriculture, textile, etc. Because of good experiences of the above industries, the petroleum industry interests is now in biotechnology as an alternative technology to resolve the challenges and needs of this worldwide important sector. We described throughout the paper the main factors that drive or restrain research and development (R&D) in biological processes applied to petroleum industry. Moreover, we identified several challenges and opportunities, where R&D in petroleum biotechnology plays an important role to surmount the industrial needs during the following years.

Key words: Technology prospective, biotechnology, petroleum, oil industry, biorefining.

INTRODUCTION

Biotechnology has strengthened its position during last years as the assembly of technologies focusing on the production of goods and services by means of biological systems or its products. The fast technological progression derived from the modification of deoxyribonucleic acid (DNA) allowed the industrialization of new processes. Hence, biotechnology has nowadays a broad and diverse impact at industrial level, e.g. food, pharmaceutical, agrochemical, environmental, chemical, and recently energy (Table 1). Petroleum is a complex mixture of hydrocarbons (paraffins, naphthenes and aromatics), and is at present the largest source of energy followed by natural gas, which is a mixture of methane besides other gases. Both are valuable substrates for microorganisms. Hence, the implementation of biological processes in the oil industry to explore, produce, refine, transform petroleum into valuable derivatives and clean the pollution refers here as petroleum biotechnology.

R&D in oil industry is led by several key drivers, e.g. the intensifying demand of fuel, the need to enhance oil recovery, the increasing stocks of heavy crude, the seeking for adequate profit margins, and the fulfilment of more severe environmental regulations. In Mexico, specific key drivers are the increasing necessity of processing heavy crude -***Mexican Maya*** and ***KU Maloob Zap*** Oil-; the increasing exploitation of fractured oil reservoirs; the transformation of petroleum to high-value petrochemicals and goods; and the cleaning of polluted soils and aquifers. During the last 25 years, an increasing awareness concerning the effect of polluting emissions provoked a more strict environmental regulation, resulting in deep changes in technologies and industrial processses. In order to face these challenges, biotechnology can potentially provide a solution to the need for improved and expanded fuel upgrading worldwide, because bioprocesses for fuel upgrading do not require hydrogen and produce far less carbon dioxide than thermochemical processes and versatility of microbial metabolism and its

*Corresponding author. E-mail: jaburto@imp.mx.

Table 1. Current and future research on petroleum biotechnology and correlated areas.

Industry sector	Impact			Benefits
	Today	Near future	Distant future	
Food and Feed additives	+++	+++	+++	Reduced energy consumption, less corrosive waste, better use of raw materials and safer production. Sweetener and high quality in foods.
Fine chemicals	+	++	+++	Remarkable selectivity and reduced steps of reaction
Personal care products	+	++	++	Reduced phosphate load to rivers and lakes. Reduced energy consumption with lower temperature washing. More user-friendly and/or safer products due to the reduced alkalinity of the product
Petroleum (Sulphur and nitrogen free fuels). Biotechnological products [surfactants, polymers, Biopetrochemicals, microbial enhanced oil recovery (MEOR)]	+	+	++	Versatility of microbial metabolism and its intrinsic ability to mediate transformation of complex raw materials at a wide range of reaction conditions
Energy	+	++	+++	Greater utilization of natural, renewable resources reduced harmful auto emissions.
Textile	+	++	++	Lower discharge of chemicals and wastewater and decreased handling of hazardous chemicals.

intrinsic ability to mediate transformation of complex raw materials at a wide range of reaction conditions, e.g. high-temperature, pressure, and salinity; extreme acidity or alkalinity and hydrophobic media (Le Borgne and Quintero, 2003). Petroleum industry has therefore researched and applied bioprocesses as complementary technologies on diverse platforms to reduce investment and maintenance costs as well as a valuable promise to overcome technological barriers regarding the upgrading of petroleum and gas (Figure 1 and Table 2). Nevertheless, the research efforts have not been all successful because scepticism, lack of funding, competitiveness, cost/efficiency ratio and governmental policy.

Nevertheless, the US Department of Energy (DOE) prepared in 2000 a technology roadmap for the petroleum industry including biodesulfurization of gasoline and diesel as technical areas of greatest concern and where academic and private efforts on research must be increased. Nevertheless, it did not take attention on the potentiality of biotechnology to others fields for the oil industry. This study provides a state-of-the-art as well as the challenges and opportunities for research and development (R&D) in petroleum biotechnology, specifically biorefining, recovery of petroleum and environmental.

TRENDS IN FUELS

Nowadays the crude oil is and will be the major source of energy in the world. It is expected that in 2030 the oil production in the world increase until 107 million barrels per day (EIA, 2006; Figure 2). Historically, estimates of world oil reserves have generally trended upward. As of January 1, 2007, proven world oil reserves, as reported by Oil and Gas Journal (2006) increased by 4% than 2006. The largest net increase in estimated crude oil reserves has been made in Canada, Iran and Kazakhstan and the 56% of world's total proved oil reserves are located in Middle East. In spite of this increment countries like México (16 billions barrels), China (8 billions barrels), Norway (2.9 billions barrels), Australia (1.3 billions barrels) and the United Kingdom (1.3 billions barrels) showed the largest declines in oil reserves between 2000 and 2007. However by the end of this century oil resources will be reduced and the production and use of heavy crude oil will be increase (Figure 3).

Heavy crude oil as fuel source implies an environmental impact because of the high content of sulfur, nitrogen, metal and aromatics compounds. In order to remove those kinds of molecules and obtain cleaner fuels the traditional refineries work under hard conditions of temperature and pressure thereby increasing the cost.

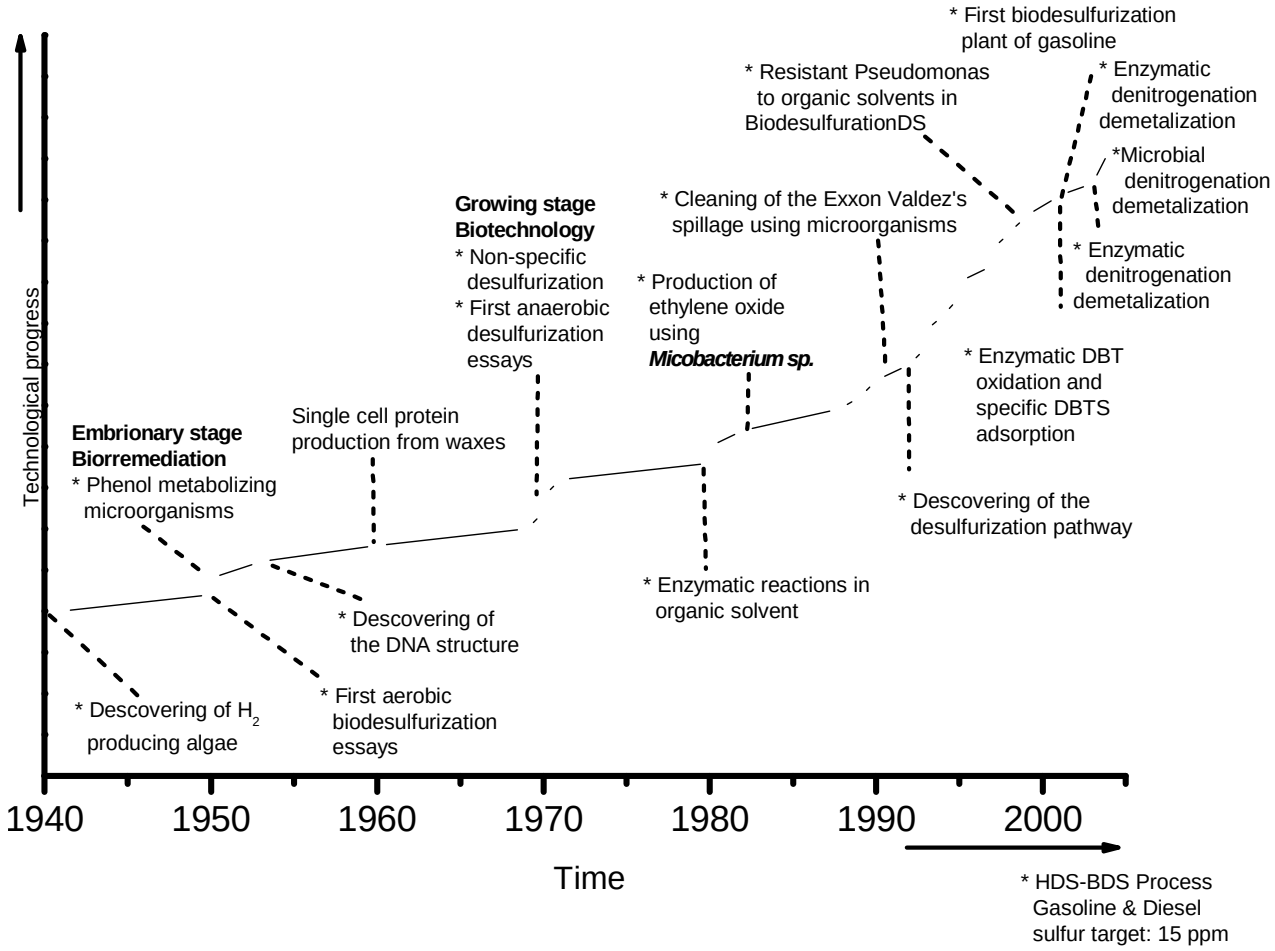

Figure 1. Time line for petroleum biotechnology.

BIOREFINING

Crude oil is rarely used in its raw form but must instead be processed into various products as liquefied petroleum gas (LPG), gasoline, diesel, solvents, kerosene, middle distillates, residual fuel oil and asphalt. The refining process involves the use of various thermal and catalytic processes to convert molecules in the heavier fractions to smaller molecules in fractions distilling at lower temperatures (Speight, 1998). Recalcitrant compounds containing sulphur, nitrogen and metals are mainly associated with the heaviest fraction in petroleum.

The use of fossil fuels for energy generation and petrochemical industry is expected still to increase in the first decades of this century. The demand for low-sulfur fossil fuels has been intensified by the stricter regulatory standards for reduced levels of sulfur-oxides in atmospheric emissions. It can be estimated that in next decades 30% of oil should be desulfurized. Such necessities will increase the cost of physicochemical process of hydrodesulfurization (HDS). There is a need to reduce the severity of refining operations to decrease the costs and make the refining through the development of milder physical and chemical processes. Biorefining is the use of living organism or part of them like enzymes in order to upgrade petroleum, that is, the application of bioprocesses to the fractionation and enhancing of petroleum, which might contribute to mitigate the associated pollution and up-grading of heavy crude. Biotechnology involves the use of wide range of conditions including milder temperature and pressure conditions, cleaner and selective processes, low emission and no generations of undesirable by-products. The microbial and enzymatic catalysis can be manipulated and used for more specific applications where the chemical processing requires several steps. It is being proposed that heavy crude oil and some fractions like gas-oil, gasoline and diesel may be subjected to biorefining to get off most of the sulphur, nitrogen, toxic metals, asphaltenes (Le Borgne and Quintero, 2003). Nevertheless, biorefining of petroleum has been also related in the past to the production of single cell protein (SCP) and reviewed by Hamer and Al-Awadhi (2000). They established that SCP production from waxy n-alkane feedstock (WAF) was a valuable

Table 2. Biotechnologies related with the petroleum industry.

Petroleum platform	Technology	Organization	Sponsor/country
Exploration	Petroleum Geomicrobiological prospecting	Phillips Petroleum Inc.	Private resources/USA
		Geo-microbial Technologies, Inc.	Private resources /USA-Colombia
		Mississipi State University	Public resources/USA
Production	Microbial Enhanced Oil Recovery (MEOR)	California Institute of Technology and private Corporation	Private and public resources/USA
		University of Surrey Guildford UK	Private resources/UK
		Japan National Oil Corporation Kansai Research Institute.	Private resources/Japan
		Centre d´Océanologie de Marseille	Public resources/France
		Csiro Petroleum	Private resources/ Australia
Biorefining	Microbial biodesulfurization of different petroleum streams	Enchira (formerly Energy BioSystems Corp.)/Dep. of Energy (DOE)	Public and own resources/USA
		Petroleum Energy Center of Japan (PECJ)/ King Abdulaziz City for Science and Technology (KACST)	Public and private resources/Japan-Saudi Arabia
		Petro Star/Enchira Biotechnology Corp	Private/USA
		Institute of Microbial Technology, Chandigarh.	Public resources/India
		Paques Bio Systems BV/Shell International Oil Products	Private resources /Netherlands
		Mexican Petroleum Institute (IMP)	Public resources/Mexico
	Biodesulfurization by biocatalysis	Institute of biotechnology (IBT-UNAM)/IMP	Public resources/Mexico
		Idaho National Eng. and Environmental Laboratory/Texaco	Public and private resources/USA
		Lund University.	Public resources/Sweden
	Desulphurization by selective polymeric adsorbents	IMP/IBT-UNAM/Institute of Food Research (Norwich)	Public resources/Mexico-UK
	Biodenitrogenation	Institute of Gas Technology/ Petrobras R&D	Public and Private resources/USA-Brazil-Univ. Federal do Rio de Janeiro
		University of Alberta-Syncrude	Public and private resources/Canada
		Institute of Process Engineering (IPE), Institute of Process Engineering	Public and private resources/China
		Graduate School of Science and Technology, Niigata Engineering Co Japan Bioindustry Association, National Oil Corporation.	Public and private resources/Japan

Table 2. Contd.

Petroleum platform	Technology	Organization	Sponsor/country
Biorefining	Biocraking	Oak Ridge National Laboratory/Chevron/Phi-llips/Texaco	Public and private resources/USA
		Lawrence Berkeley National Laboratory/BP Amoco/Chevron/Natural Gas Center/ Texaco	Public and private resources/USA
		IMP	Public resources/Mexico
Transportation	Pipeline's Biocorrosion	Argonne National Laboratories	Public resources/USA
		Commissariat à l'Énergie Atomique (CEA)	Public resources/France-International partnership
		National Metallurgical Laboratory Madras Centre	Public resources /India
		Scientific and Engineering Center, LUKOIL Oil Company,	Public and private resources/Russia
		IMP	Public resources/Mexico
Biopetrochemistry	Phenolic polymerization	Kyoto University/Japan Chemical Innovation Institute (NIMC)	Public resources/Japan
		University of Maryland at College Park	Public resources/USA
	Alkene oxidation	Japan Energy Corp.	Private resources/Japan
		VTT-biotechnology and Food Res./Nesteoy	Public and private resources/Finland
	Hydroxylation, Epoxidation, Alkenes \ oxidation	Exxon Research and Engineering Co.	Private resources/USA
	Sulphur oxidation, Aromatic oxidation	IBT-UNAM	Public resources/Mexico
	Bioproduction of fats and oils from kerosene and gas	Petroleum Energy Center of Japan (PECJ)	Public and private resources/Japan
Alternative fuels	Ethanol Biodiesel Biogas	Various organizations	Public and private resources/Worldwide
Effluent Treatment and bioremediotion of polluted sites	i. Aerobic and anaerobic treatments ii. Phytoremediation iii. Bioventing iv. Biosparging v. Biopiles vi. Slurry reactors	Various organizations	Public and private resources/Worldwide

manufacturing, e.g. for British petroleum (BP) by the time they have no internal demand for WAF. Today, SCP production has been displaced by energy and petrochemical needs.

We consider that the modern refining industry faces two main key drivers: more stringent environmental regulations and steady depletion of light crude reserves. The former limits the concentration of nitrogen- and sulphur-containing compounds in fuels. The latter urges to compete by enhancing of heavy to light crude and elimination of metals from fossil feedstock.

Sulphur elimination

Sulphur levels in crude oils may range from 1000 - 30000 ppm (Monticello, 2000) and this compound represents the major petroleum pollutants that contribute to air con-

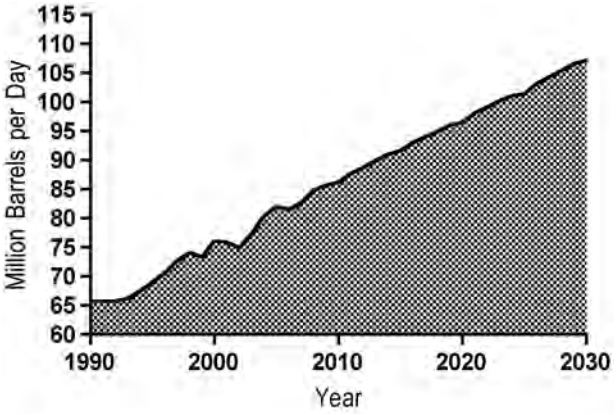

Figure 2. World oil production 1990 - 2030.

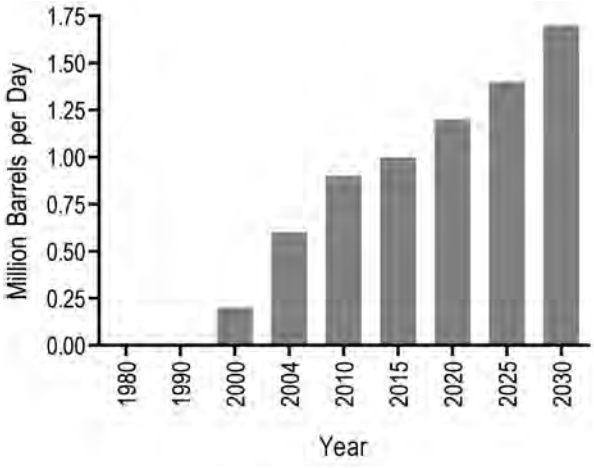

Figure 3. World ultra-heavy crude oil production 1980 - 2030.

tamination, acid rain, equipment corrosion and catalyst poisoning. Moreover, high-sulphur diesel could duplicate NOx emissions and have no severe effect on hydrocarbon emissions (USEPA, 1998). The US Environmental Protection Agency (EPA) proposed, in its Tier 2 Act, a stringent cap for sulphur in diesel and gasoline. By year 2010, diesel in terminals must have no more than 15 ppm in USA, in Mexico in 2006 the production of gasoline premium requires a range of sulphur between 30 - 80 ppm, by 2008 for gasoline magna and 10 ppm for diesel by 2009 (Villalobos-Hiriart, 2006). Hydrodesulfurization (HDS) removes sulphur and nitrogen, as hydrogen sulfide and ammonia, satisfying the current regulation of 500 ppm sulphur in diesel. A deeper HDS (below 200 ppm) requires important investment and consequently high costs because the need to employ higher temperature (265 - 425°C), pressure (75 - 100 kg/cm^2) and larger quantities of hydrogen (290 - 1260 scf/bbl) (Speight, 1998; Gonzalez, 1996; EIA). Moreover, catalyst poisoning requires a regular replacement which provokes higher operational cost.

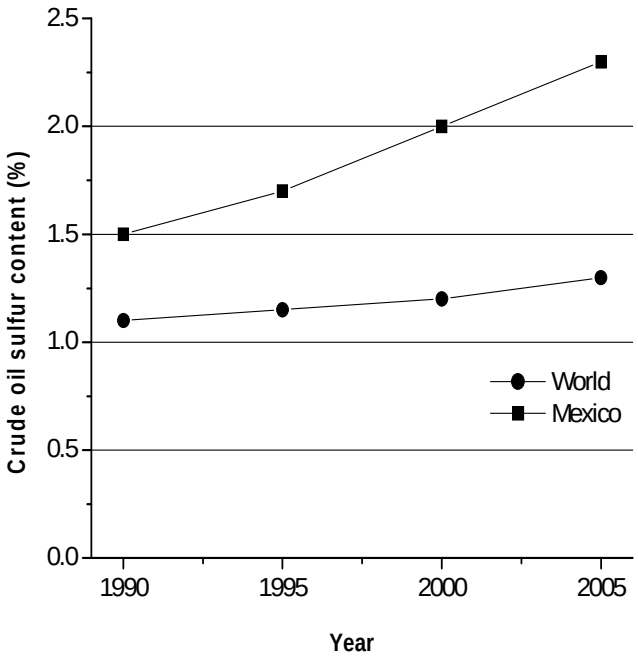

Figure 4. Trend of sulphur content in crude oil from 1990 to 2005.

In spite of low sulphur levels (< 15 ppm) reached by the above conventional and new technologies, they are focused on light crude that contain less organosulphur compounds than heavy crude. Moreover, sulphur in petroleum will increase as a result of a current and future extensive production of heavy crude (Figure 4). Hydrotreating will require higher hydrogen feed a more active catalyst and in some cases a higher temperature to treat high sulphur fuels. On other hand, while the hydrotreating are focusing in reduce sulphur species to H_2S, the oxidizing technologies promotes the oxidizing species formation. For example, organosulfur compounds like dibenzotiophene are transformed into sulfone. In order to obtain a desulfurized product, finally the sulfones are removed in a second step. This process presents some advantages like the omission of hydrogen. However, it is unselective and need large volume of water to form the emulsion (65 - 69%) that must be effectively broken to enhance oil recovery (Ito and Veen, 2006). Hence, refiners will deal in the middle term with high sulphur crude, stringent regulations, implementation delays because of supplying the critical equipment and high-cost technologies (Gonzalez, 1996; EIA).

A sulfur adsorption technology for fuels by Phillips Petroleum named “S-Zorb” reached sulphur content lower than 10 ppm for gasoline; S-Zorb-diesel is currently under development.

The biodesulfurization (BDS) is an alternative technology to HDS that is expected to play a important rol in biorefining (that is, in pre-refining and post-refining) of oil products that involves a microbial or enzymatic system

that selectively oxidizes the sulphur atoms or removes sulfur without attacking the C-C bond and avoiding the lost of oil value (Kilbane, 2003; Furuya et al., 2001). Between the 1970's and 1980's, advances in BDS yielded low-octane values, that is, low quality fuels, because of anaerobic (Kim et al., 1990) or aerobic degradation (Kodama et al., 1973) of carbon backbone by microorganisms that obviously did not satisfy industrial expectations. These bacteria have clearly some drawbacks for the microbial processing of petroleum but they are effective for soil and water remediation treatments. Despite the discovery since 1978 of specific microorganisms (4S pathway) that remove sulphur without affecting the octane value of fuels (Malik, 1978), the refining industry was not concerned because of the flexible environmental rules, high light petroleum reserves and profitability of conventional processes. We consider that a different situation highlighted the resurgence of BDS during the 1990's: a more severe environmental emission regulation, the increasing worldwide fuel demand, the depletion of light crude reserves and the exploitation of new reservoirs of sulphur- and nitrogen-rich oils, such as heavy crude, tar sands and oil shale. Consequently, oil industry closed efforts with academia to research and develops mainly applicability of BDS and less of BDN processes.

Notable advances in BDS have been accomplished hitherto and extensively reviewed (McFarland et al., 1998; Ohshiro et al., 1999; Monticello, 2000; Klein et al., 1999; Kilbane, 2003; Okada et al., 2002). Some sulphur specific strains were discovered e.g. ***Brevibacterium*** (van Afferten et al., 1990), ***Pseudomonas*** (Monticello et al., 1985), ***Gordonia*** (Rhee et al., 1998), and (Kadoma et al., 1973; Gallagher et al., 1993; Kilbane, 1994; Ohshiro and Izumi, 1999; Folsom et al., 1999; Kilbane et al., 2003; Kertesz and Wietek, 2001). These bacteria desulphurize dibenzothiophene (DBT) through the sulphur specific 4S pathway that involves the use of oxidoreductase enzymes encoded by the gene operon Dsz ABC (Castorena et al., 2002).

The characterization of both mono-oxygenases ***DszC*** and ***DszA*** showed the possibility of metal ions at the active center, but there is still lack of studies to elucidate the structure of these enzymes. Refractory compounds to HDS such as dibenzothiophene (DBT) and methylated derivatives (C_1-DBT) were attacked preferentially, followed by more highly alkylated molecules (C_x-DBT). A four-enzymatic system together with coenzyme requirements limits BDS application to the use of complete cells or cell-free extracts. However, a sulphur removal up to 70% has been achieved for model DBTs solutions and diesel fuels (Mcfarland et al., 1998; Folsom et al., 1999; Maghsoudi et al., 2001; Ohshiro and Izumi, 1999). In addition to these mesophilic bacteria, some thermophilic microorganism have been reported to carry out biodesulphurization of oil including ***Paenibalcillus*** sp. ***A11-2*** (Onaka et al., 2001), ***Sulfolobus acidocaldarius*** (Kardinahl et al., 1996), ***Bacillus subtilis*** WU-S2B (Kimura et al., 2001), and ***Mycobacterium phlei*** WU-F1 and WU-0103 (Furuya et al., 2002; Ishii et al., 2005).

A study financed by the Japan Cooperation Center, Petroleum (JCCP) using resting cells from a thermophile strain of ***M. phlei*** WU-F1 catalyzed the complete oxidation of 149 ppm DBT (26 ppm sulphur) at 50°C and 90 min and was also found to possesses desulfurizing ability toward naphtol thiophene present in gas oil and hydrodesulfirized light gas oil at 50 and 45°C, respecttively (Furuya et al., 2002). These bacteria removed 60 to 70% of sulphur content from the hydrodesulfurized gas oil (Furuya et al., 2003). In the same way, ***M. phlei*** WU-0103 was able to reduce 52% of sulfur in a crude straight-run light gas oil fraction not treated with HDS. ***B. subtilis*** WU-S2B is another thermophilic bacteria able to degrade DBT completely at 12 h and its derivatives approximately 50% at 24 h, through selective cleavage of C-S bonds, resulting in the accumulation of 2-hydroxybiphenyl (Kimura et al., 2000). This kind of reaction would be desirable to remove the sulfur atom without C-C bond cleavage, that is, without loss calorific power.

The bioprocessing of fuels takes place regularly into an oil and water emulsion in view to retain microbial viability and activity. The oxidation reaction occurs into the cytoplasm requiring mass transfer of DBT from the oil to the cell, a phenomenon not fully understood. It is suggested, however, that in the case of ***Rhodococcus,*** it assimilates DBTs directly from the oil due to its hydrophobic membrane (Monticello, 2000) or by the bio- or exosurfactant aided uptake of DBTs from the water phase (Ohshiro et al., 2000; Han et al., 2001).

Indeed, preliminary results from the Oak Ridge National Laboratory (ORNL) showed that ***Rhodoccocus*** sp. forms a better water-hexadecane emulsion (surface tension of 0.35e-3 N/m) than ***Escherichia coli*** (0.58e-3 N/m), and is attributed to a non identified biosurfactant and a more hydrophobic cell wall structure (Borole et al., 2002). Because of biosurfactant production and solvent resistance (log P from 3.1 to 3.4) of some ***Pseudomonas*** strains (Isken et al., 1999), several recombinants ***Pseudomonas*** were designed to acquire the desulphurization (***Dsz)*** genes (Gallardo et al., 1997; Macfarland et al, 1998). The recombinant bacteria ***Pseu-domonas aeruginosa*** EGSOX desulphurized 95% of a solution containing 6.4 ppm sulphur (37 ppm DBT) in 24 h more efficiently than the native host ***Rhodoccocus erythropolis*** with accumulation of a known bactericide, i.e. hydroxybiphenyl (HBP). This issue was minimized by the expression of ***Dsz*** enzymes (C and A) from ***R. erythropolis*** and a flavin oxidoreductase from ***Vibrio harveyi*** in a recombinant ***E. coli*** causing an increase in the DBT removal but a decrease in the rate of production of HBP (Reichmuth et al., 2000). There is one point for considering in the use of biphasic system is the oxygen mass transfer limitation in the biphasic media: the oxygenase besides using the oxygen as cosubstrate also uses in their endogenous metabolism. Therefore, in order to allow the oxygenase compete for the oxygen with the endogenous respiration

during biotransformation, it is necessary to increase the oxygen concentration using an oxygen-enriched air and/or an increased pressure.

Recently Vitreoscilla hemoglobin technology, a strategy to improve the transfer, supply and store of oxygen ***in vivo*** was used to enhance the biodesulfurization activity under low aereation in two liquid phase systems introducing the vgb gene into ***R. erytropolis.*** The results show 73% of sulfur reduction in diesel sample oil under hypoxic conditions (Xiong et al, 2007).

Nevertheless, an industrial BDS should be applied to middle distillates containing 200 to 500 ppm sulphur, up to two orders of magnitude higher than described here and above. Poor reaction rate and extension as well as cell inhibition by substrate or metabolites are all issues limiting an industrial application.

BDS processes involving isolated enzymes have been also investigated because of its greater technological utility in organic solvents. An enzymatic desulphurization approach would have at least three advantages compared to the utilization of complete cells, that is, activity at low or null water content, thermomechanical stability, and minimized mass-transfer issues (Klibanov, 2001). Moreover, there are oxidative enzymes, must of them non-coenzyme dependent, with high activity and broad specificity. Several polycyclic aromatic hydrocarbons (PAHs) found in petroleum showed to be substrate to several fungi strains by the involvement of peroxidase- and laccase-type enzymes (Pickard et al., 1999; Johannes, and Majcherczyk, 2000). It is assumed that all radicals formed by laccase oxidation act as mediator compounds, e.g. aniline, methionine, 2,2'–azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT), to transform other non substrate compounds such as PAHs. A fungal laccase promoted DBT oxidation in the presence of a large excess of ABTS or HBT. An enzymatic BDS process using chloroperoxidase (CPO) from ***Caldariomyces fumago*** coupled with distillation at 325°C oxidized and removed preferentially the organosulphur compounds from a diesel containing 16000 ppm sulphur at ambient temperature and atmospheric pressure (Ayala et al., 1998; Ayala et al., 2000). Despite the elimination of up to 83% of organosulphur compounds, 2 μg of diesel was diluted in 1 mL of 20% acetonitrile-acetate buffer system which makes it inappropriate for industrial scale. Recently, the DBT oxidation in a continuous reactor using immobilized CPO on mesoporous material (SBA-15) and polyvinyl alcohol (PVA) reduced more than 50% of DBT from a organic model solution (Montiel et al., data not reported). Another enzymatic system reported is the coupled oxidation and adsorption of sulphur compounds by cytochrome C immobilized on diatomaceous earth (Celite) which reduced more than 80% of DBT from a model solution (Ryu, 2002). Despite the low activity value at 30°C (44e-6 min^{-1}), unmodified and modified horse heart cytochrome C are able to perform biocatalytic reactions at 80°C with an activity of 0.67 and 4.7 min^{-1}, respectively (Vazquez-Duhalt et al., 1999). Furthermore, it is remarkable that selective adsorption of sulfone occurs with high hydrophobic solvents such as hexadecane whilst desorption is favoured by more polar solvents as octanol. A similar approach used the selective separation of DBT by a designed tailored-made adsorbent through molecular imprinting. The removal of DBT by an imprinted divynilbenzene-methacrylic acid polymer was of 18% from a 640 ppm solution at 30°C and 16 h (Castro, 2001). Later, Aburto et al. (2004) in order to remove selectively sulphur compounds as dibenzotiophene sulfone, reported the synthesis of hydrogels of chitosan with conformational memory through the molecular printing process. These hydrogels recognised and adsorbed the dibenzotiophene sulfone against other related compounds found in diesel e.g. dibenzotiophene, fluoreno and 4,6-dimethyl dibenzotiophene. These materials allowed the elimination of the dibenzotiofene sulfone at approximately 40% in 7 h with an initial concentration of 865 ppm. The industrial application of such method needs further research on adsorption capacity, selectivity, adsorbent recycling and regeneration and temperature effect.

A comparative study involving BDS and HDS, developed by Enchira Biotechnology Corp. (EBC, formerly Energy BioSystems), demonstrated the minor energy consumption and CO_2 generation by the bioprocess to produce a 50 ppm sulphur in diesel from two diesel pools containing 2000 and 500 ppm sulphur. Industries such as Arctic Slope Regional Corp. through its Petro Star subsidiary installed and operate a 5,000 b/d diesel BDS pilot plant in conjunction with EBC by the end of the 1990's. Other organizations such as the US National Laboratories, the Petroleum Energy Center of Japan (PECJ) together with the King Abdulaziz City for Science and Technology (KACST, Saudi Arabia) and the Mexican Petroleum Institute (IMP) from Mexico are carrying out studies on BDS processes. Scaling, equipment availability and coupling to conventional infrastructure as well as refiners' mistrust are additional problems to overcome when we look to field applications. Nevertheless, there are biorefinig processes already applied at the oil industry as shown by the sweetening gas BDS process using ***Thiobacillus*** bacteria to eliminate the H_2S from gas streams developed by Paques Bio Systems BV and Shell International Oil Products. The gas cleaning manages gases containing up to 80% H_2S or solutions containing up to 10 ppm and produces up to 15 tonnes/day of sulphur (Anonymous, 1999).

Nitrogen elimination

Like sulphur, nitrogen is typically found in petroleum as non-basic and basic-related compounds, which contribute to acid and atmospheric contamination and also inter-

feres with the refining processes, leading to equipment corrosion and catalyst poisoning. Quinoline and carbazole are the most widely studied as regard biodegradation. Strains like ***Pseudomonas*** sp., ***Xanthomonas*** sp. and ***Mycobacterium*** sp. have been used as carbazole degrading bacteria as a sole source of carbon, nitrogen and energy (Grosser et al., 1991; Fetzer, 1998; Kaiser, 1996; Kilbane et al., 2002). The genes involved in carbazole degradation (***car***) have been identified and ***car*** gene cluster can be induced with a carbazole addition to the growth medium (Shepherd and Lloyd-Jones, 1998) or expressed constitutively in ***Escherichia coli*** (Sato et al., 1997; Sato et al., 1997b). The carbazol degradation pathway start with the oxidative cleavage of the heterocyclic nitrogen ring of the carbazole to produce 2´aminibiphenyl-2,3-diol; then this compound is oxidized through meta cleavage to yield 2-hydroxy-6-oxo-6-hexa-2e,4z-dienoate. The next metabolic step results in the degradation of one of the aromatic rings to release carbon dioxide (Kilbane, 2006).

Researchers from Brazil (Santos et al., 2006) reported a DBT-degrading bacterial strain able to use carbazol and DBT as the only source of nitrogen and sulphur, respectively, and was identified as ***Gordonia*** sp. F.5.25.8. Growing and resting cells from the above strain were capable of removing 90% of DBT and 40% of carbazole.

Recently, the isolation and characterization of a new bacterial strain able to grows at temperatures up to 70^{o}C and to metabolize carbazole and methyl substituted carbazole molecules in biphasic aqueous-organic media has been reported. This ***Burkholderia*** sp. Strain IMP5GC is able to remove 90% of carbazole in a system composed by a real petroleum destilate and water (1:1). The IMP5GC showed an excellent tolerance to organic solvents as well as toxic compounds found in gas oil (Castorena, et al., 2006).

The use of enzymes to remove nitrogen compounds has also been reported. The use of laccase from ***Coriolopsis gallica*** totally accomplished nitrogen removal in a reaction medium containing 15% acetonitrile. Non oxidation product was identified suggesting that the product of carbazole oxidation becomes sufficiently condensed and polymerizes (Bressler, 2000). While the sulphur specific removal has been reasonably investigated, there is little information concerning the removal of N-heterocycle compounds found in petroleum and related streams without affecting their calorific value.

BIOCRACKING OF PETROLEUM AND METAL ELIMINATION

The properties and economic value of petroleum depend on major fractions such as saturates, aromatics, resins and asphaltenes. Although various parameters defined the type of petroleum, the oils can be easily described in terms of light and heavy. In the transition between heavy to light crude, the carbon and the heteroatom content decrease (nitrogen, sulphur, and oxygen). Crude oil also contains metals in the form of salts and metalloporphyrins in the asphaltene fraction, which is the solid material that precipitates when oil is treated with alkane solvents (n-pentane or n-heptane) (Speight, 1998). The salts are readily eliminated during the crude oil desalting process in which they concentrate into the aqueous phase. The removal of metals trapped in metalloporphyrins is more problematic because porphyrins are embedded in complex asphaltenic structures. Heavy metals (mostly nickel and vanadium) are furthermore corrosive, poison cracking catalysts during refining, and are released as highly toxic oxides during fuels combustion to the environment.

The asphaltenes are responsible for sludge formation resulting in flow reduction by plugging down stream equipment and production of less valuable coke in current upgrading of petroleum. Moreover, the utilization of distillation residue, constituted mainly by asphaltenes and entrapped metals, is of high interest because petroleum refiners will deal in the near future with much heavier crude from different sources, e.g. tar sands, oil shales and off-shore reservoirs.

In the last years, biological processes have emerged as a cost-effective and environmentally favourable alternative to break asphaltenic structures (biocracking) in order to obtain high-value light oils from the less valuable heavy oils. Several US national laboratories, the Alberta University and the Mexican Petroleum Institute (IMP) are already carrying out basic research financed with public and private funds (Table 3). Several extremophile bacteria, e.g. ***Achromobacter, Leptospirillum, Pseudomonas, Sulfolobus, Thiobacillus*** sp., are capable of transforming heavy oils into light crudes (Premuzic, 1999; Premuzic, 1999b). The authors suggested that biocatalyst interacts with heavy oil at heteroatoms and organometallic sites redistributing and fragmenting the heavy polar fractions. The heavy oil are converted into lighter fractions, increasing the content of saturated hydrocarbons, and decreasing the content of organic sulphur- and nitrogen-containing compounds and metals. Despite the fact that the fraction content was determined by analytical methods such as GC and mass spectrophotometry, the labor was extensive, time consuming and not related with process aspects.

On the other hand, several studies have demonstrated the possibility of employing biotechnologies in order to disrupt the wrapping structure and release the entrapped metals. Indeed, thermophilic microorganisms has been used in hydrometallurgy to leach valuable ores, such as gold, copper, zinc, from sulfide minerals including pyrite and arsenopyrite, where the chemistry of leaching dominates the system and the microbial activity plays a second role (Huber and Stetter, 1998; Bosecker, 1997). The same approach could be applied to metal removal from asphaltenic structures in petroleum using enzymes as unwrapping oxidative catalysts. Indeed, CPO from ***C. fumago*** demetalized asphaltenes by chlorination and

successive opening of porphyrinic rings attaining a removal ratio of Ni and V of 93 and 53%, respectively (Fedorak et al., 1993). Another approach involving cytochrome C reductases from ***Bacillus megaterium*** and ***Catharanthus roseuse*** has the advantage to liberate metals like nickel and vanadium by oxidation of porphyrinic rings instead of chlorination, avoiding the formation of halogenated products that are undesirable due to environmental concerns (Xu et al., 1998; Arellano et al., 2004). A study showed that biodemetallation using ***Achromobacter*** BNL-4-23 should take into account the kind of crude or stream to be processed. Indeed, the reduction of nitrogen and the asphaltenic fraction was more extensive in heavy crude while steam-treated crude showed a major reduction in aromatics, sulphur and metals (Premuzic, 1999).

This suggests the possibility that future biocatalysis for the simultaneous removal of sulfur, nitrogen and metals from petroleum could be developed.

TECHNOLOGY POTENTIAL

The bioprocessing of petroleum and downstream might be undertaken by a what-how-what process to clarify the approach to the several key drivers: pollutants elimination, upgrading of heavy crude, diminution of catalyst poisoning and infrastructure corrosion as well as enhancement of petroleum recovery, in order to become a reality at the near term for the oil industry (Figure 5). Klein et al. (1999) pointed out that biocatalytic activity, stability and the oil/water ratio are the most important bottlenecks for BDS processes. Indeed, a successful biorefining of petroleum must first resolve several technical drawbacks such as biocatalyst, solvent tolerance and mass-transfer issues of identified strains (***Rhodococcus*** and ***Pseudomonas*** sp.) and enzymes (like ***Dsz***, CPO, cytochrome c, laccases) to be applied at the petroleum industry (Table 3). Furthermore, Dordick et al. (1998) identified three key biotransformation technologies that might impact the future industry: new strains from extreme environments, metabolic engineering, and new Biocatalyst based compound synthesis (Dordick et al., 1998).

The progress reached by BDS research should serve as a model to encourage developments on biodenitrogenation and heavy oil enhancing at two deadlines. The first phase planned at near term must focus to solve basic problems and to generate knowledge. The second phase, considered at middle term, includes the application of the environmental rules proposed by EPA. This phase would emphasize on process engineering, e.g. mass and heat transfer, reengineering of process units, minimization of stream contamination by sulphur at storage and/or pipeline transport.

Enzymatic and solvent tolerance issues should include screening for new substrate specificity and extremophile microorganisms, searching for coenzyme-independent enzymatic systems, biosurfactant production and enhancing the conversion rate and reaction extension. Indeed, biocatalysts, that is, microorganisms and enzymes, must resist contact and/or immersion on high hydrophobic media such as gasoline or diesel with a low Log P, e.g. 8.25 for hexane. Moreover, they should be active and stable at temperatures higher than 50°C currently found at the petroleum industry, sulphur-specific but at a broader spectrum, that is, with a low and equivalent K_m for DBT and derivatives (C_x-DBTs) and nitrogenous compounds. Concerning BDS rate, Monticello proposed in 2000 a biocatalytic rate of 0.20 mmol DBTs*L^{-1}*min^{-1}*g $catalyst^{-1}$ (Monticello et al, 2000).

To desulfurize a diesel in less than an hour, corresponds with residence times currently found in HDS. Nevertheless, this critical value and higher values has already been reported for cytochrome C (Vazquez-Duhalt et al., 1999; Ryu, 2002) or CPO (Ayala et al., 1998; Ayala et al., 2000). The main two challenges to overcome are the stability of the biocatalyst at low or nil water/solvent ratio or the high water/solvent ratio at high specific BDS activities. The progress accumulated in enhancing stability and activity in organic solvents and high temperatures of other enzymatic systems should be applied to the strains and enzymes involved in biorefining (Klibanov, 2001).

The approach to the mass-transfer issue should comprise the enhancing of the water-oil emulsion in order to maximize the substrate migration from the oil to the biocatalyst as well as the expulsion of the metabolite, e.g. a toxic HBP. The water content should be nil or minimum considering activity and stability of the biocatalyst as well as the separation of the oil-water emulsion. The latter issue is less problematic considering the upgrading of heavy crude because large volume of water is employed during its secondary recovery. Surfactant production by the strain or the external addition is another technological issue that must be kept in mind to increase the biotransformation rate, extension and ease of the separation process. The study of the cellular membrane chemistry and engineering process must assist to overcome the mass-transfer issue at two levels: transport from the fossil feedstock into the cell and from oil to the water phase. Special attention should be focused on separation processes allowing for example the efficient and economic breaking of the water-oil emulsion.

Understanding of biological mechanisms will lead to design and production of tailored-made, robust and high active catalyst. Nowadays, polymer matrices are designed through molecular imprinting to show specific recognition and catalysis against a compound template such as enzymes do. Mesoporous novel materials at nanometre size appear as ideal catalyst per se or as biocatalyst support. Hence, biotechnology and nanotechnology might see its borders melt into one.

Biodesulfurization of diesel has been approached from two perspectives: BDS from 2000 to 500 ppm sulphur where BDS replaces HDS; and HDS coupled to BDS

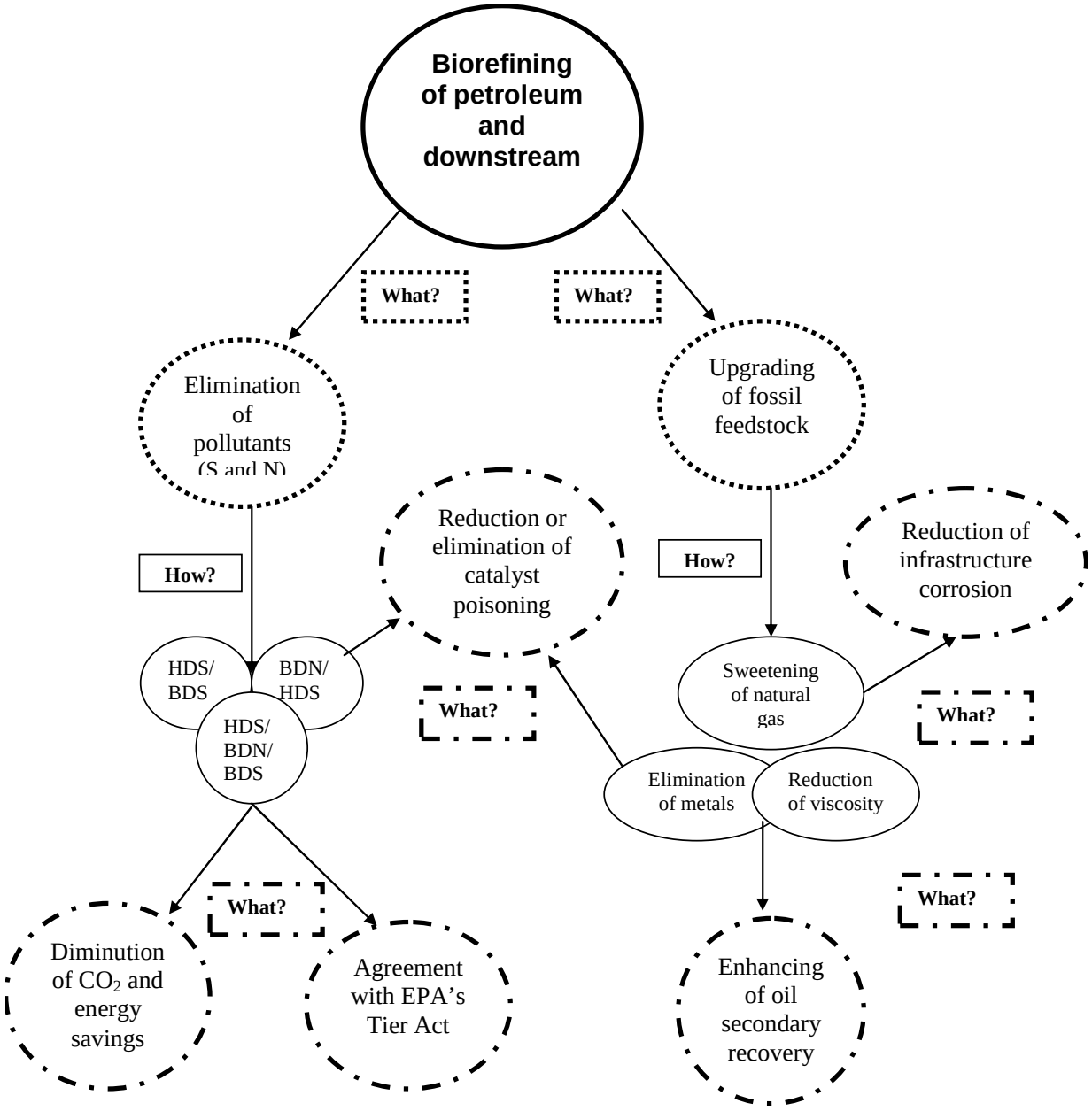

Figure 5. Vision of biorefining beyond 2000.

from 500 to 50 ppm sulphur (Linguist and Pacheco, 1999).

We consider that a HDS-BDS process is more realistic at near term since little reconfiguration of re-fineries will be needed at low costs. Moreover, elimination of organonitrogen compounds using bioprocesses prior to HDS might allow a better efficiency and extension of sulphur compound removal. To our knowledge the latter approach has not been tested, but because of the low nitrogen content in crude, we consider it affordable at the near term.

Future research must be emphasized on as well as specific site mutagenesis and selection of enhanced mutants. Research must focus on the combination of several phenotypes including enhanced desulfurization, high temperature resistant, solvent tolerance and surfactant production. The enzymes must be purified, crystallized, and their structure determined and kinetically characterized. Its physical and chemical modification through enzyme-surfactant interaction and entrapment into a polymer matrix are examples of future research.

Exploitation of heavy crude likes tar sand in Alberta State, Canada requires the injection of light crude or gas oil to diminish viscosity and enhance pumping and

Table 3. Biorefining technology roadmap.

Key driver	Near term	Middle term
Ultra-low sulphur diesel and gasoline (<15 ppm) Reduction of nitrogen compounds on petroleum and downstream Upgrading of heavy crude, tar sands and oil shale: reduction of metals and asphaltenic fraction	**Fossil stream characterization** i. Petroleum chemistry ii. Characterization methods for trace analysis **Biocatalyst** i. Screening for new specific microorganisms including extremophiles ii. Isolation and purification of involved enzymes iii. Search for enzymes non-coenzyme dependent iv. Crystallography: structural studies v. Un- and/or specific mutagenesis for broaden stability, specificity and enhanced activity vi. Kinetic characterization **Solvent tolerance** i. Cell membrane chemistry ii. Un- and/or specific mutagenesis for better solvent resistance iii. Recombinant microorganisms iv. Biocatalyst immobilization v. Protein engineering (immobilization, derivatization, catalytic crystals, catalytic plastics) **Mass-transfer** i. Cell membrane physico-chemistry ii. Surfactant bioproduction or external addition iii. New supports for immobilization: amphipatic core/lipophile shell; mesoporous materials. iv. Separation technology: Liquid-liquid extraction Emulsion breaking technology Solid-liquid extraction Distillation Flash distillation v. Process reengineering	Basic knowledge of biological upgrading of heavy crude and residua Coupling of biological upgrading, BDS and BDN for heavy crude and residua. Coupling of BDS/BDN to downstream petroleum processing Direct BDS/BDN of fuels before and after Hydrotreating BDS/BDN of enriched oxidized sulphur-containing compounds effluents

distribution. Large production of heavy oil in the near future might oblige its transformation into light oil straight-away in reservoirs or during storage. Microorganisms such as fungus might oxidise asphaltenes, responsible for viscosity on heavy crude, enhancing fluidity and pumping (Pickard, personal communication). The same approach could be done using broad specific enzymes such as peroxydases and laccases. Future research must also included the determination of physicochemical properties, valuable in engineering process, of un- and treated heavy oil such as viscosity and API (American Petroleum Institute) gravity.

REFERENCES

van Afferten M, Schacht S, Klein J, Trueper HG (1990). Degradation of dibenzothiophene by ***Brevibacterium*** sp. DO. Arch. Microbiol., 153: 324-328.

Aburto J, Mendez-Orozco A, Le Borgne S (2004). Hydrogels as adsorbents of organosulphur compounds currently found in diesel. Chem. Eng. Proc. 43: 1587-1595.
Ayala M, Tinoco R, Hernández V, Bremuntz P, Vazquez-Duhalt R (1998). Biocatalytic oxidation of fuel as an alternative to biodesulfurization. Fuel Proc. Technol. 57: 101-111.
Ayala M, Robledo NR, Lopez-Munguia A, Vazquez-Duhalt R (2000). Substrate specificity and ionization potential in chloroperoxidase-catalyzed oxidation of diesel fuel. Environ. Sci. Technol., 34: 2804-2809.
Bosecker K (1997). Bioleaching: metal solubilization by microorganisms. FEMS Microbiol. Rev., 29: 591-604.
Le Borgne S, Quintero R (2003). Biotechnological processes for the refining of petroleum. Fuel Proc. Technol. 81: 155-169.
Borole AP, Kaufman EN, Grossman MJ, Minak-Bernero V, Lee MK (2002). Comparison of the emulsion characteristics of Rhodococcus erythropolis and Escherichia coli SOXC-5 cells expressing biodesulfurization genes. Biotechnol. Prog., 18: 88-93.
Bressler DC, Fedorak PM, Pickard MA (2000). "Oxidation of carbazole, p-ethylcarbazole, fluorene and dibenzothiophene by the laccase of***Coriolopsis gallica***". Biotechnol. Lett. 22: 1119-1125.
Castorena G, Mujica V, Le Borgne S, Acuña ME, Bustos Jaimes I, Aburto J (2006). Carbazole biodegradation in gas oil/water biphasic media by a new isolated bacterium Burkholderia sp. Strain IMP5GC. J. Appl. Microbiol. 100: 739-745.
Castorena G, Suarez C, Valdéz I, Amador G, Fernández L, Borgne S (2002). Sulfur-selective desulfurization of dibenzothiophene and diesel oil by newly isolated ***Rhodococcus*** sp. strains. FEMS Microbiol. Lett. 215: 157-161.
Castro B, Whitcombe MJ, Vulfson EN, Vazquez-Duhalt R, Barzana E (2001). Molecular imprinting for the selective adsorption of organosulphur compounds present in fuels. Anal. Chim. Acta, 435: 83-90.
Dordick JS, Khmelnitsky YL, Sergeeva MV (1998). The evolution of biotransformation technologies. Curr. Opin. Microbiol. 1: 311-318.
Fedorak PM, Semple KM, Vazquez-Duhalt R, Westlake DWS (1993). Chloroperoxidase-mediated modifications of petroporphyrins and asphaltenes. Enzyme Microb. Technol., 15: 429-437.
Fetzer S (1998). Bacterial degradation of pyridine, indole, quinoline, and their derivatives under different redox conditions. Appl. Microbiol. Biotechnol., 49: 237-250.
Folsom BR, Schieche DR, DiGrazia PM, Werner J, Palmer S (1999). Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by ***Rhodoccocus erythropolis*** I-19. Appl. Environ. Microbiol., 65(11): 4967-4972.
Furuya T, Kirimura K, Kino K, Usami S (2001). Thermophilic biodesulfurization of dibenzothiophene and its derivatives by ***Mycobacterium phlei*** WU-F1. FEMS Microbiol. Lett., 204: 129-133.
Arellano-Garcia H, Buenrostro-Gonzalez E, Vazquez-Duhalt R (2004). Biocatalytic transformation of petroporphirins by chemical modified cytochrome c Biotecnol. Bioeng. 85: 790-798.
Gallagher R, Olson ES, Stanley DC (1993). Microbial desulphurization of dibenzothiophene. A sulphur specific pathway. FEMS Microbiol. Lett., 107: 31-36.
Hamer G, Al-Awadhi N (2000). Biotechnological applications in the oil industry. Acta Biotechnol. 20: 335-350.
Han JW, Park HS, Kim BH, Shin PG, Park SK, Lim JC (2001). Potential use of nonionic surfactants in the biodesulfurization of bunker-C oil. Energy Fuels, 15(1): 189-196.
Huber H, Stetter KO (1998). Hyperthermophiles and their possible potential in biotechnology. J. Biotechnol., 64: 39-52.
Isken S, Derks A, Wolfes PFG, de Bont JAM (1999). Effect of organic solvents on the yield of solvent-tolerant Pseudomonas putida S12. Appl. Environ. Microbiol., 65(6): 2631-2635.
Gallardo ME, Ferrández A, De Lorenzo V, García JL, Díaz E (1997). Designing recombinant Peudomonas strains to enhance biodesulfurization. J. Bacteriol. 179(22): 7156-7160.
Gonzalez RG (1996). Can you make low-sulphur fuel and remain competitive? Hart's Fuel Technol. Manag. November/December, pp. 56-61.
Johannes C, Majcherczyk A (2000). Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl. Environ. Microbiol., 66: 524-528.
Kaiser JP, Feng Y, Bollag JM (1996). Microbial metabolism of pyridine, quinoline, acridine, and their derivatives under aerobic and anaerobic conditions. Microbiol. Rev., 60: 483-498.
Kardinahl S, Schmidt C, Petersen CL, Schäfer G (1996). Isolation, characterization and crystallization of an iron-superoxide dismutase from the crenarchaeon ***Sulfolobus acidocaldarius***. FEMS Microbiol. Lett. 138: 65-70.
Kilbane JJ (1994). Microbial cleavage of organic C-S bonds. US Patent 535: 88-69.
Kim H, Kim Y, Kim BH (1990). Degradation of organic sulfur compounds and the reduction of dibenzothiophene to biphenyl and hydrogen sulfide. Biotechnol. Lett., 12: 761-764.
Klein J, Catcheside DEA, Fakoussa R, Gazso L, Fritsche W, Hoefer M, Laborda F, Margarit I, Rehm HJ, Reich-Walber M, Sand W, Schacht S, Schmiers H, Setti L, Steinbuechel A (1999). Biological processing of fuels. Appl. Microbiol. Biotechnol., 52: 2-15.
Klibanov AM (2001). Improving enzymes by using them in organic solvents. Nat., 409: 241-246.
Kodama K, Umehara K, Shimizu K, Nakatani S, Minoda Y, Yamada K (1973). Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agric. Biol. Chem., 37: 45-50.
Maghsoudi S, Vossoughi M, Kheirolomoom A, Tanaka E, Katoh S (2001). Biodesulfurization of hydrocarbons and diesel fuels by ***Rhodococcus*** sp. strain P32C1. Biochem. Eng. J., 8: 151-156.
Malik KA (1978). Microbial removal of organic sulphur from crude oil and the environment: Some new Perspectives. Process Biochem., 13: 10-13.
McFarland BL, Boron DJ, Deever W, Meyer JA, Johnson AR, Atlas RM (1998). Biocatalytic sulphur removal from fuels: Applicability for producing low sulphur gasoline. Crit. Rev. Microbiol., 24: 99-147.
Monticello DJ (2000). Biodesulfurization and the upgrading of petroleum distillates**.** Curr. Opin. Biotechnol., 11: 540-546.
Monticello DJ, Baker DT, Finnerty WR (1985). Plasmid-mediated degradation of dibenzothiophene by ***Pseudomonas*** species. Appl. Environ. Microbiol., 49: 756-760.
Ohshiro T, Izumi Y (2000). Purification, characterization and crystallization of enzymes for dibenzothiophene desulfurization. Bioseparations, 9: 185-188.
Onaka T, Kobayashi M, Ishii Y, Konishi J, Maruhashi K (2001). Selective cleavage of the two C unknown; S bonds in asymmetrically alkylated dibenzothiophenes by Rhodococcus erythropolis KA2-5-1. J. Biosci. Bioeng. 92: 80-82.
Pickard MA, Alberta University. Personal communication.
Pickard MA, Roman R, Tinoco R, Vazquez-Duhalt R (1999). Polycyclic aromatic hydrocarbons metabolism by White Rot Fungi and Oxidation by Coriolopsis gallica UAMH 8260 Laccase. Appl. Environ. Microbiol., 65: 3805-3809.
Premuzic ET, Lin MS, Bohenek M, Zhou WM (1999a). Bioconversion reactions in asphaltenes and heavy crude oils. Energy Fuels, 13: 297-304.
Premuzic ET, Lin MS (1999b). Biochemical upgrading of oils. US Patent 585: 87-66.
Reichmuth DS, Hittle JL, Blanch HW, Keasling JD (2000). Biodesulfurization of dibenzothiophene in ***Escherichia coli*** is enhanced by expression of a ***Vibrio harveyi*** oxidoreductase gene. Biotech. Bioeng. 67: 72-79.
Rhee SK, Chang JH, Chang HG (1998). Desulphurization of dibenzothiophene and diesel by a newly isolated ***Gordona*** strain, CYKS1. Appl. Environ. Microbiol., 64: 2327-2331.
Ryu K, Heo J, Yoo IK (2002). Removal of dibenzothiophene and its oxidized product in anhydrous water-immiscible organic solvent by immobilized cytochrome c. Biotechnol. Lett., 24: 143-146.
Sato SI, Nam JW, Kasuga K, Nojiri H, Yamane H, Omori T (1997). Identification and characterization of genes encoding Carbazole 1,9a-dioxygenase in ***Pseudomonas*** sp. strain CA10. J. Bacteriol., 179: 4850-4858.
Sato SI, Ouchiyama N, Kimura T, Nojiri H, Yamane H, Omori T (1997b). Cloning of genes involved in carbazole degradation of ***Pseudomonas*** sp. strain CA10: nucleotide sequence of genes and characterization of meta-cleavage enzymes and hydrolase. J. Bacteriol., 179: 4841-4849.

Speight JG (1998). In The Chemistry and Technology of Petroleum. Third Edition, New York, Marcel Dekker. pp. iii-918.
US Environmental Protection Agency (EPA), Tier 2 Act. July 1998.
Vazquez-Duhalt R (1999). Cytochrome ***c*** as a biocatalyst. J. Mol. Cat. B: Enzymatic, 7: 241-249.
Villalobos-Hiriart A (2006). Una propuesta tecnológica para la elaboración de diesel de ultrabajo azufre en el Sistema Nacional de Refinerías de Petróleos Mexicanos. Tecnol. Ciencia, 21: 5-18.
Xu GW, Mitchell KW, Monticello DJ (1998). Fuel product produced by demetalizing a fossil fuel with an enzyme. US Patent, 562: 48-44.
Xiong X, Xing J, Li X, Bai X, Li W, Li Y, Liu H (2007). Enhancent of biodesulfurization in two-liquid systems by heterogeneous expression of Vitreoscilla haemoglobin. Appl. Environ. Microbiol., 73: 2394-2397.