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Review

Biotechnology in the petroleum industry: An overview

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ABSTRACT

A significant quantum of crude oil is trapped in reservoirs and often unrecoverable by conventional oil recovery methods. Further downstream, the petroleum industry is facing challenges to remove sulfur, metal, nitrogen as well as undesirable organic compounds from the crude.

Conventional secondary recovery methods such as water and gas injections helped to increase the productivity of the well, while chemical and physical refinery processes such as hydrodesulfurization, desalting, and high-pressure high-temperature hydrotreating remove most inorganic impurities. The increasing demand for oil in the world coupled with very stringent environmental laws piled economical and technical pressure upon the refinery industry to further improve crude oil recovery as well as reduce sulfur, metal and nitrogen concentration to the low ppm levels.

In the search for economical and environmentally friendly solutions, growing attention has been given to biotechnology such as the use of microbial enhanced oil recovery (MEOR). MEOR is an alternate recovery method that uses microorganisms and their metabolic products. In addition, the emerging field of crude oil refining and associated industrial processes such as biodesulfurization, biodemetallation, biodenitrogenation and biotransformation are also covered.

This review aims to provide an overview on MEOR and biorefining relevant to the petroleum industry and highlights challenges that need to be overcome to become commercially successful. Literature pertaining to laboratory experiments, field trials and patents are included in view of industrial applications and further developments.

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1. Introduction

Most economies rely currently on products from crude oil, and inadequate oil resources can jeopardize a countries development and elevate living costs. With the increased consumption of oil by developing countries has increased the demand and price for oil in the world market. The forecast of global energy use by the OECD (Organisation for Economic Co-operation and Development) and Non-OECD countries between 2006 and 2030 (USA EIA, 2009) shows an increase of 15.5% and 73% respectively for oil with the existing energy resources. With the steady increase in demand for oil, the prospective alternatives are exploration of new sources of energy or utilization of enhanced oil recovery (EOR) techniques in poor performing and depleted oil wells.

Different EOR processes are currently employed in the oil industry for the extraction of trapped oil. The EOR method used depends on the characteristics of the crude oil in the oil reservoir. EOR processes fall under the broad classification of thermal (steam flood, combustion and hot water injection), chemical (injection of polymers, surfactants and alkali) and gas injection (CO_2, N_2 and flue gas) (Sen, 2008). In the thermal recovery process heat is added to the reservoir to decrease the oil viscosity and/or to vaporize the oil. The natural temperature of oil reservoirs reported in literature varies from 10 °C in the on average 27 m deep Canadian Athabasca oil sands (Harner et al., 2011) to 124 °C (Brown, 2010) with the majority between 40 and 80 °C (Li et al., 2002; Hao et al., 2004; Ghojavand et al., 2012; Zhang et al., 2012a). Reservoir temperature will rise by 10s–100s of degree Celsius depending on the method used, reservoir characteristics, prevailing heat transport mechanism, duration and temperature of heat supply. The application of thermal techniques, particularly fire flooding, can, however, produce polar compounds such as carbenes and carboids which are incompatible with asphaltenes and capable of causing blockages of the pores and channels through which the oil must move during recovery (Speight, 1999). In the chemical recovery process chemicals are used to extract oil from pores in the oil-bearing rocks and alter its characteristics. The gas injection method utilizes various types of gases miscible or immiscible to displace oil to the extraction point. The major drawbacks of thermal and chemical methods are the high energy requirement and chemical costs. In the case of gas injection, availability of gases at high pressure needs to be considered. Recently in the US there is an increasing trend towards thermal and gas injection EOR projects compared to chemical methods (Alvarado and Manrique, 2010).

A promising alternative oil recovery approach is microbially enhanced oil recovery (MEOR). This has been suggested as early as 1926 by Beckman (Donaldson et al., 1989) and involves the use of microorganisms to enhance oil production. The MEOR constitute injection of microorganisms with essential nutrients into the oil well, and under favorable environmental conditions microbial population grows exponentially and their metabolic products mobilize the residual oil. With the availability of favorable microbes in-situ, injection of nutrients is feasible as reviewed by Gao and Zekri (2011). These microbes are capable of producing a broad range of metabolic products, and their growth and effects depend on factors such as: (1) porosity and permeability, pressure, temperature, dissolved solids, pH and salinity of the reservoir, (2) nutrients provided to the bacteria and (3) specific type of microorganisms injected into the reservoir (Doghaish, 2008; Wang et al., 2008). Metabolic products and applications of microbes in oil reservoirs are given in Fig. 1.

Biotechnological applications can be further extended to MEOR from oil sands (Harner et al., 2011) and the petroleum refinery industry, where microorganisms may be used in refining crude oil (biorefining, Fig. 2). MEOR from oil sands is still in the research stage due to its novelty and unique environmental challenges (low water availability, high hydrocarbon concentration) (Harner et al., 2011).

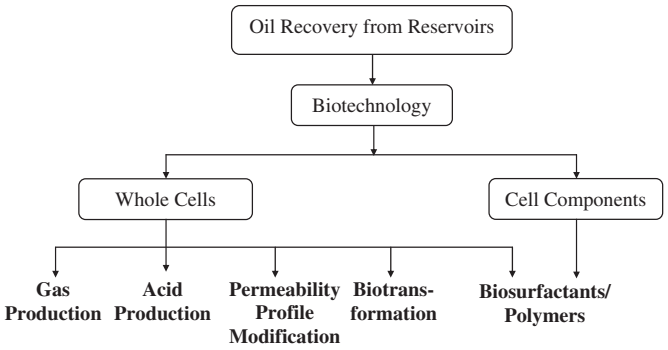

Fig. 1. Potential applications of biotechnology in the recovery of oil from reservoirs.

This review aims to provide an insight and critically discuss the potential and commercial applications of biotechnology in the petroleum industry with particular focus on oil recovery from reservoirs and downstream biorefining.

2. Oil recovery from reservoirs

The majority of energy requirements in the world are currently met by non-renewable fossil fuels. The dwindling rate of discovery of new oil fields makes it necessary to maximize the oil recovery from existing or abandoned fields (Brown and Vadie, 2000; Kerr, 2000; Mehta and Gair, 2001; Giles, 2004).

Primary recovery is the first oil that is produced under natural pressure, which causes the oil to flow from the site of formation to the surface. It is the least expensive method of production and accounts for 20% of original oil in place (OOIP) in reservoirs worldwide (Sandrea and Sandrea, 2007). Secondary recovery is used when there is a fall in the reservoir's natural pressure. The pressure is commonly increased by either water or gas injection. These methods are more expensive than the primary recovery (Table 1) and accounts for additional recovery of 45–50% of OOIP (Sandrea and Sandrea, 2007). It is estimated that approximately 58% of total oil available in the USA is unrecoverable by deploying current technologies (Table 2) and offers a large potential to improve or develop new oil recovery methods. Tertiary or EOR methods include thermal and chemical processes applying solvents, surfactants, micro-emulsions (Santanna et al., 2009), starch/cyclodextrin based synthetic polymers (Leslie et al., 2005) and microbiologically produced polymers such as Xanthan gum (McInerney et al., 2003) to produce an additional 7–15% of OOIP (Sandrea and Sandrea, 2007). From the techniques available or currently under development, MEOR is environmentally friendly

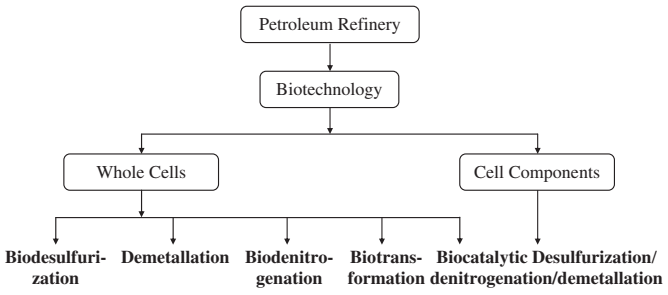

Fig. 2. Potential applications of biotechnology in petroleum refinery.

Table 1
Development and production cost of oil recovery.

Oil recovery	Development capex [USD/barrel]	Production cost [USD/barrel]	Reference
Deepwater field with gas and water injection	4–6	3–4	Sandrea and Sandrea (2007)
Non-conventional Canadian and Orinoco oil sand	4.3–6.3	6 (cold) – 17 (steam)	Sandrea and Sandrea (2007)
EOR	2	>10 (steam or CO_2)	Sandrea and Sandrea (2007)
MEOR	N/A	1.4–2.4 (hydrocarbon degrading anaerobic-facultative microorganisms)	Maure et al. (2001)
	N/A	2.9 (bacterial flooding)	Strappa et al. (2004)
	N/A	0.4–3.9 (biosurfactant)	McInerney et al. (2003)

and sustainable. It is worthwhile mentioning that the principle methods for MEOR from reservoirs have already been proposed 60 years ago by Zobell (1946, 1947) who obtained a patent on the 'Bacteriological Process for the Treatment of Fluid-Bearing Earth Formations' (USA Patent 2,413,278).

All whole-cell applications in MEOR have in common that microbes have to be tolerant to the conditions prevailing in the oil reservoirs, i.e. lack of oxygen, high pressure, increased salinity, and elevated temperature (Denger and Schink, 1995; Cameotra and Makkar, 1998). For example, temperatures and pressures have been reported to approximate 100 °C and 2×10^4 kPa in the North Sea Forties Field, and 125 °C and 5×10^4 kPa in the Ninian Field (Shennan and Levi, 1987). A combination of thermo-, baro- and halophilic microorganisms, therefore, appears to be specially suited to carry out MEOR. Denger and Schink (1995), for example, isolated two halo- and thermo tolerant fermenting bacteria from oily sludge under conditions of enhanced salt concentration (approx. 8% w/v) and temperature (50 °C) producing surface-active compounds (glycolipids).

Banat et al. (2000) emphasized that the strategies involved in MEOR depend on the prevalent oil reservoir conditions, including temperature, pressure, pH, porosity, salinity, geologic make-up of the reservoir, available nutrients and the presence and diversity of indigenous microorganisms. This view was re-iterated by Evans and Furlong (2003) for the biotech market in general stating that "often the complexities of individual projects make the application of 'standard' off-the-shelf approaches very difficult, the upshot being that much what is done must be significantly customized". This can be a real impediment to applying biotechnology, where developmental time and costs are restricted by deadlines and budget. It follows that a generic approach, i.e. data collection (bacterial identification, geological make-up of the site and nutritional status to name but a few), development of the best nutrient medium and/or microorganism (Zhang et al., 2012a), optimal bioreactor design, laboratory, pilot and field tests may not be effective. Instead, a modular approach using off-the-shelf components, building on previous work, and limiting testing to confirmatory studies (need for rapid and accurate screening tests) can all help to reduce this lead time (Lichtfield, 1991). However, more conceptual and empirical techno-economical feasibility studies supported with quantitative data are required to identify the most successful strategies for MEOR.

Table 2
Breakup of oil reserves in the USA (USA DOE, 2008).

Oil reserves	%
Discovered but unrecoverable (potential for improved recovery technologies)	58.1
Cumulative production	28.2
Undiscovered and unrecoverable (potential for advanced exploration technologies)	5.7
Undiscovered but recoverable (potential for advanced exploration technologies)	4.6
Proved reserves	3.4

2.1. Oil recovery by microbial gas production

The method of microbial gas production for MEOR was originally formulated by ZoBell (1946). Zobell's principal idea was to inject or encourage indigenous bacteria to produce CO_2 and/or methane (Converse et al., 2003; Wang et al., 2011) to help re-pressurize the reservoir, decrease oil viscosity and, in the case of limestone or carbonaceous sandstone, to leach out calcite and siderite thus liberating adsorbed oil (Sayyouh and Al-Blehed, 1993).

Behlülgil et al. (1992) injected the anaerobic bacterium *Clostridium acetobutylicum* into a 1D model reservoir of limestone grains with a shut-in period of approximately 45 h, and found an overall increase of 12% in MEOR effectiveness, compared to controls. This increase was attributed to a reduction in viscosity of Raman crude oil from 1096 to 843 ± 86 cP and an increase in pH. The authors also observed that a doubling in shut-in period resulted in no additional oil recovery compared to 45 h while, interestingly, the oil viscosity increased 31%, and pH decreased (more acidic than control). While the authors had no explanation for this phenomenon at that time it is suggested that *C. acetobutylicum* started to produce organic acids which dissolved part of the limestone thus releasing the heavier fractions of the crude oil. Ten years later, the same authors showed that the reduction in Garzan crude oil viscosity from 80 cP to 50 cP was mainly due to dissolved CO_2 produced by *C. acetobutylicum* (Behlülgil and Mehmetoğlu, 2002). Similar gas (acid- and solvent-based) mechanisms were attributed to *C. acetobutylicum* in reviews by Van Hamme et al. (2003) and Youssef et al. (2009), while other authors did acknowledge the additional capability of *C. acetobutylicum* and *Clostridium* sp. to produce biosurfactants (Jack, 1993; Al-Sulaimani et al., 2011). In order to further clarify the relative contribution of organic acids and biosurfactants produced by *C. acetobutylicum* on MEOR additional experiments should be carried out.

Denger and Schink (1995), Giles (2004) and McInerney et al. (2003) mentioned the microbial gas production method in passing, but did not discuss it in further detail. Kobayashi et al. (2012) showed that indigenous microorganisms in the reservoir could be utilized for production of methane for reservoir re-pressurization. The stimulation of methane production for oil recovery, however, should be treated with caution. Methane is not only ignitable but also a potent greenhouse gas which may escape from shallow reservoirs into the atmosphere and affect global climate (Milkov, 2011). Alternatively, nitrogen could also be produced using denitrifying bacteria (Nuryadi et al., 2011).

On-shore gas flooding of oil fields with CO_2 supplied from natural gas reservoirs rich in CO_2 and/or flue gases from power plants have successfully been implemented by oil companies (Blunt et al., 1993; Goodyear et al., 2003). There is also considerable public interest in this strategy, since it offers the dual benefits of additional domestic crude oil production and an economical opportunity to sequester greenhouse gases. The interest in this approach may be exemplified by the US government, which launched the "Enhanced Oil Recovery/CO_2 Injection" program in 2004 (US DOE, 2004).

Microbial gas production, on the contrary, has not attracted significant interest of the scientific community or industry at this stage.

Literature on CO_2 flooding reconfirmed that CO_2 does lead to the dissolution of sandstone components under field conditions (Goodyear et al., 2003). The question, however, whether bacteria are able to produce sufficient amounts of CO_2 in a naturally anaerobic environment to achieve similar effects, remains to be answered. A minimum miscibility pressure may also be required to notice EOR. In addition, field experience has shown that HCO_3^- concentration increased in the aqueous phase, resulting in a drop in pH and $CaCO_3$ scaling problems at the production well, where pressure and acidity of the solution is decreased (Goodyear et al., 2003). Furthermore, relative low pressure zones in the reservoir will allow compressed CO_2 to expand thus cooling the surroundings. This may, depending on temperature and pressure, cause the precipitation of the paraffin/asphaltene fraction with detrimental effect on oil recovery (Srivastava et al., 1997; Brown, 2010). Another issue of concern is the complexity involved in using CO_2 for (M)EOR. Up to 5 phases (all P, T, gas composition dependent) can co-exist in a CO_2 flood, which complicates the understanding, modeling and prediction of expected oil recovery (Goodyear et al., 2003):

- Aqueous phase
- Liquid hydrocarbon phase
- Liquid CO_2 phase
- Gaseous CO_2 phase
- Solid asphaltene (precipitate) phase

It should also be noted that more CO_2 is required in shallow, water-flood cooled reservoirs owing to CO_2 density increase; however, density differences between CO_2, oil and water are reduced thus improving the sweep (Goodyear et al., 2003). This may have a significant bearing on microbial gas flooding. For example, the indigenous microbial population metabolic rate and gas production (including CO_2) can be expected to decrease (due to non-optimal growth conditions), whereas the required CO_2 production has to go up in order to improve oil recovery.

Two strategies for whole-cell MEOR may be pursued in the context of oil recovery by microbial gas production: (i) Stimulation of indigenous or added biomass to utilize the heavy hydrocarbon fraction in the reservoir as carbon source in order to produce gas (and biosurfactants) with the added benefits of reducing crude oil viscosity and the tendency of paraffin/asphaltene deposition, or (ii) supply of selective and cheap nutrients to indigenous or added biomass as carbon source thus avoiding the reduction of the calorific value of the crude oil.

From a practical point of view following challenges have to be addressed:

- Composition of indigenous microorganisms and their metabolic pathways must be known to ensure that unwanted growth, degradation, gas production does not take place;
- Transport of growth stimulant/inhibitor must be efficient;
- Cost of growth stimulant/inhibitor must be low;
- Microbial gas production must be sufficient to achieve desired effect(s);
- Bacterial growth must not result in total blockage of the reservoir.

2.2. Geological reservoir profile modification

After primary crude oil recovery, water is conventionally injected into the well to drive out secondary crude oil. In the highly permeable zones the expected secondary oil recovery should be lower due to loss of water and pressure (Al-Dhafeeri et al., 2005). Saidi (1983) estimated that over 20% of the world's oil reservoirs are naturally fractured highlighting the importance of developing solutions to reduce zone permeability and thus water and pressure loss.

Table 3
USA Patents issued on the modification of the permeability zone to facilitate MEOR (USA Patent Office, 2012).

Year	Patent holder	Patent title	US patent no.
1994	Societe Nationale Elf Aquitaine	Process for transporting particles in a porous medium	5,299,638
1992	Biocarbons Corporation	Method for controlling oil reservoir permeability using biomass oil	5,115,084
1990	The University of Kansas	Subterranean permeability modification by using microbial polysaccharide polymers	4,941,533

Perhaps with this in mind, Buller et al. (1990) proposed the use of polyglucans produced by *Alkaligenes faecalis* (ATCC 31749), *Agrobacterium radiobacter* (ATCC 21679), and *Cellulomonas* sp., as a secondary recovery sweep liquid (Table 3). The polymer is first solubilised in an alkali solution such as sodium hydroxide, and pumped into the injection well. Within the formation, the polymer may be precipitated by the subsequent injection of an acid solution or CO_2 forming HCO_3^- subsequently. Under ideal conditions, the precipitation of biopolymer in the neutralized solution should take place in the higher permeability zones of the formation. By such precipitation, the high permeability zones are selectively blocked off ensuring that subsequent water flooding to push out more oil is diverted to the oil-bearing, low-permeability zones. This property was used to increase the oil recovery by 35% (Vossoughi and Putz, 1994). Other biopolymers such as Xanthan gum, produced by *Xanthomonas campestris*, were also applied to increase the viscosity of water which decreases the mobility ratio and thus improves the sweep efficiency of the water flood (Akit et al., 1989). Stewart and Fogler (2001) proposed the use of exopolymer-producing bacteria to modify geological reservoir profiles for enhanced oil recovery. *Leuconostoc mesenteroides*, gram-positive lactic acid bacteria, was used for the production of exopolymer dextran to generate a pressure drop across the porous media. Three pressure phases were identified as exopolymer-induction phase, plugging phase, and plug propagation phase.

Other approaches to selectively plug subterranean strata and/or oil sand was reported by Hitzman (1962), Cusack et al. (1988), Lappin-Scott et al. (1988) and Lappin-Scott and Costerton (1992). Porous rock contains large pores (pore bodies) and small pores (pore throats) which often have sizes in the micron range (Gao and Zekri, 2011). Since microbes for MEOR often have sizes around 1 micron they can face difficulties in penetrating the subterranean strata prior to reaching their target zone. In order to avoid this problem, the spherical size of the microbes needs to be less than 20% of the pore throat (Jack et al., 1991). Alternatively, Hitzman (1962) proposed the usage of bacterial spores instead of whole cells, while Lappin-Scott et al. (1988) suggested the usage of ultramicrobacteria (UMB) which have a diameter < 0.3 μm. The authors found that starvation of bacteria resulted in decreasing the size of bacterial cells and that such UMBs flowed into and through high permeability zones in earth formations and oil sands under lab conditions, without any unwanted permeability reduction. Plugging occurred only when nutrients were introduced, resulting as a

consequence of the growth-associated increase in size and number of the bacterial cells.

When microorganisms where injected in a field reservoir along with water flooding operations it not only caused the intended plugging of wells but also corrosion problems due to the production of hydrogen sulfide by anaerobic sulfate reducing bacteria (SRB) (Brown, 2010). SRB activity may be inhibited by addition of SRB-specific inhibitory chemicals such as gentamicin (Tanimoto et al., 1989; Yen, 1990). H_2S gas production in the reservoir can also be reduced by addition of nitrate to the injection water (Reinsel et al., 1996) which increases the redox potential of the reservoir and stimulates the growth of sulfide oxidising denitrifiers (Myhr et al., 2002).

Another approach suggested by Chang and Yen (1984) is the usage of lysogenic bacteriophage for bacterial growth control and prevention of reservoir plugging. Commercial phage technology (e.g. from Ecolyse Inc.) is now available for combating the growth of organisms producing H_2S (Summer et al., 2011).

2.3. Biotransformation of crude oil in reservoirs

A significant quantum of crude oil is trapped in reservoirs and difficult to recover due to high crude oil viscosity (Lazar et al., 2007). Decrease in oil viscosity can be achieved by at least two microbial mechanisms (Soudmand-asli et al., 2007): (i) microbial conversion of heavier oil components to light oil components (Purwasena et al., 2010; Gudiña et al., 2012), and (ii) microbial production of products that alters the physical properties of the oil (Li et al., 2002; Gudiña et al., 2012) (Section 2.4).

Gudiña et al. (2012) screened 4 Brazilian oil field wells with a reservoir temperature of 40 °C for extracellular biosurfactant-producing and hydrocarbon-degrading bacteria. Three *Bacillus subtilis* strains out of 58 oil field isolates degraded higher n-alkanes (>C27) under anaerobic conditions, and the percentage of n-alkanes with chains containing less than 25 carbons increased relatively to the control sample. Similar n-alkene degrading capabilities by *Bacilli* species were reported by Kato et al. (2001), Wang et al. (2006) and She et al. (2011) in Japanese and Chinese oil fields suggesting *Bacilli* species to be suitable candidates for MEOR application, either through stimulation of indigenous *Bacilli* species or industrial-scale production of exogenous inocula for well injection. Other groups found similar biosurfactant-producing, hydrocarbon-degrading capabilities in *Pseudomonas* strains albeit under aerobic conditions (Hasanuzzaman et al., 2007; Zhang et al., 2012b). However, since most of the oil reservoirs are either anoxic (Barman Skaare et al., 2007) or anaerobic (Gudiña et al., 2012) the microbial degradation of long-chain n-alkanes under anaerobic conditions appears to be more realistic and relevant for MEOR. Despite a significant amount of evidence that in-situ biotransformation of crude oil takes place quantitative data on improved oil recovery are still lacking.

Besides reduced flow of highly viscous crude oil within reservoirs, precipitated paraffinic and asphaltic fractions from the crude oil can block the main drainage routes. Microbial transformation of precipitated crude oil fractions can therefore be expected to help re-establish oil flow. This technology was reported to successfully improve oil production from several individual wells (Pelger, 1991; Nelson and Schneider, 1993; Gray et al., 2010; Zahner et al., 2010). Paraffin deposit problems are treated in situ using hydrocarbon metabolising microorganisms and is well covered by Youssef et al. (2009). The hydrocarbon degraders are injected into the well along with nutrients, and the wells are shut for several weeks to months for microbial growth and metabolism to proceed. A field trial by Jinfeng et al. (2005) using a mixture of indigenous microorganisms *Arthrobacter* sp, *Pseudomonas* sp and *Bacillus* sp showed a reduction of paraffin from 29.8 to 25.5% in 9 months. Lazar et al. (1999) used strains of *Pseudomonas* in a laboratory study on solid and semi-solid paraffins and demonstrated that significant degradation of paraffins occurred. Another study by Hao et al. (2004) showed that the use of indigenous thermophilic strain TH-2 degraded paraffin in the

Table 4
Patents issued on the application of biosurfactants for MEOR by USA Patent Office (2012).

Year	Patent holder	Patent title	US patent no.
2011	E. I. du Pont de Nemours and Company	Methods for improved hydrocarbon and water compatibility	7,992,639
2011	International Business Machines Corporation	System and method for preparing near-surface heavy oil for extraction using microbial degradation	7,922,893
2009	Savannah River Nuclear Solutions, LLC.	Biological enhancement of hydrocarbon extraction	7,472,747
2006	Baker Hughes Incorporated	Bacteria-based and enzyme-based mechanisms and products for viscosity reduction breaking of viscoelastic fluids	7,052,901
2003	ExxonMobil Upstream Research Company	Process for stimulating microbial activity in a hydrocarbon-bearing, subterranean formation	6,543,535
2000	BHP Minerals International Inc.	Biochemical treatment of bitumen froth tailings	6,074,558
2000	EniTecnologie S.p.A.	Lipopolysaccharide biosurfactant	6,063,602
2000	Universidad Simon Bolivar	Production of oily emulsions mediated by a microbial tenso-active agent	6,060,287
1999	BHP Minerals International Inc.	Extraction of bitumen from bitumen froth and biotreatment of bitumen froth tailings generated from tar sands	5,968,349
1999	Universidad Simon Bolivar	Production of oily emulsions mediated by a microbial tenso-active agent	5,866,376
1998	RAMOT University Authority for Applied Research & Indfustrial	Bioemulsifiers	5,840,547
1994	Nikko Bio Technica Co., Ltd.	Biosurfactant cyclopeptide compound produced by culturing a specific *Arthrobacter* microorganism	5,344,913
1993	Eniricerche S.p.A.	Method of producing surfactin with the use of mutant of *Bacillus subtilis*	5,227,294
1992	Phillips Petroleum Company	Nutrient injection method for subterranean microbial processes	5,083,611
1992	B. W. N. Live-Oil Pty. Ltd.	Recovery of oil from oil reservoirs	5,083,610
1991	Her Majesty the Queen in right of Canada, as represented by the National	Enhanced p roduction of biosurfactant through the use of a mutated *B. subtilis* strain	5,037,758
1990	B.W.N. Live-Oil Pty. Ltd.	Recovery of oil from oil reservoirs	4,971,151
1990	IIT Research Institute	Microbial enhanced oil recovery and compositions therefore	4,905,761
1989	Petroleum Fermentations N.V.	Bioemulsifier production by *Acinetobacter calcoaceticus* strains	4,883,757
1985	The Board of Regents for the University of Oklahoma	Biosurfactant and enhanced oil recovery	4,522,261

crude oil by 16–30%. Commercially proprietary mixtures of hydrocarbon-degrading bacteria are available (Nelson and Schneider, 1993). This makes it difficult for scientific study of these cultures and modifications for improvement. According to Bailey et al. (2001) a predetermined mixture of microorganisms selectively degrades saturated paraffins to unsaturated olefins, where primary alcohol is produced first followed by aldehyde and mono-carboxylic acid. The carboxylic acid is further degraded by beta oxidation which forms fatty acids and acetyl coenzyme A with liberation of CO_2. In addition Lazar et al. (1999) suggested that bacteria not only metabolize the paraffin deposits by partially digesting the paraffin by breaking chemical bonds but also produce biosolvents and biosurfactants until it becomes mobile liquid.

2.4. Biosurfactants for enhanced oil recovery

Of all the biotechnologies proposed for MEOR, applications of biosurfactants have received the greatest attention. This may be reflected by the number of review papers (Hayes et al., 1986; Finnerty, 1994; Banat, 1995a; Desai and Banat, 1997; Cameotra and Makkar, 1998; Banat et al., 2000; Makkar and Cameotra, 2002; Singh et al., 2012) and patents issued by the USA Patent Office (Table 4). The most promising applications of biosurfactants in the petroleum industry are in the cleaning of oil-contaminated tankers, transportation of heavy crude, MEOR and recovery of oil from sludge (Dua et al., 2002). Some of the field applications of MEOR are presented in Table 5. Banat et al. (2000) reported and discussed three main strategies for the use of biosurfactants in MEOR:

1. Production in batch or continuous culture under industrial conditions followed by addition to the reservoir along with the water flood (ex situ MEOR).
2. Production of surface-active compounds by microorganisms at the cell–oil interface within the reservoir formation, implying penetration of metabolically active cells into the reservoir.
3. Injection of selected nutrients (and growth inhibitors for unwanted growth) into a reservoir, thus stimulating the growth of desired indigenous biosurfactant-producing microorganisms.

Biosurfactants are surface-active, degradable organic compounds produced by microorganisms when grown on water-immiscible substrates. They help to reduce surface and interfacial tension (Pacheco et al., 2010) thus forming stable water-oil emulsions which is important for maximum oil extraction. Biosurfactant solubility and activity may be affected by salt concentration and pH with effective pH ranging between 4 and 10 (Amani et al., 2010; Al-Bahry et al., 2013). At pH $\leq$ 4 many biosurfactants were found to precipitate (Amani et al., 2010; Al-Bahry et al., 2013) probably because their isoelectric point is near pH 4. In addition, some authors found an increase in surfactant activity from 0 to 8% NaCl concentration (Darvishi et al., 2011; Al-Bahry et al., 2013) arguably due to salting-in effects while others reported no considerable impact on activity (Freitas et al., 2009; Shavandi et al., 2011).

Microbial cells themselves play a significant role in the surface interaction between oil and water. It has been reported by Kaster et al. (2012) that the oil–water emulsion formed is proportional to the total biomass that is produced and the quality of emulsion increases with the quantity of the biomass. Microbes tend to be at the interface between oil and water with mixed surface wettability. Nielsen et al. (2010) developed a 1D mathematical model that simulated the partitioning of microbial surfactant between the oil and water phase. Interfacial tension was reduced primarily due to lowering the residual oil saturation. The final recovery was not quantified but predicted to depend on the distance from the injection well, maximum growth rate and injection concentration of bacteria and substrate.

The most common biosurfactants are glycolipids and the more complex lipopeptides, lipoproteins and heteropolysaccharides (Dua et al., 2002). Shavandi et al. (2011) reported that *Rhodococcus* sp. strain TA6, isolated from an Iranian oil contaminated soil, produced a mix of extracellular lipids and glycolipids capable of reducing surface tension of a hydrocarbon-based growth medium from 68 to <30 mN/m while enhancing the residual oil recovery of oil-saturated sand packs by up to 70%. The biosurfactant mix was stable during exposure to high salinity (10% NaCl), elevated temperatures (120 °C for 15 min) and within pH 4.0–10.0. Xia et al. (2011) isolated *Pseudomonas aeruginosa*, *B. subtilis* and *Rhodococcus erythropolis*, from the Chinese Xinjiang oil reservoir which produced biosurfactants that reduced the surface tension from 72 to < 30 mN/m and allowed a residual oil recovery of 7–14% using a coreflooding system filled with sandstone. Although experiments were carried out in triplicate, statistical analysis was not provided. The biosurfactants, which were not identified further, remained stable at 2% NaCl, pH > 5 and 120 °C. Amani et al. (2010) demonstrated that biosurfactants produced by *B. subtilis*, *P. aeruginosa*, and *Bacillus cereus* were able to withstand harsh reservoir conditions (120 °C, pH 4, 25 g/L salinity) while greatest reduction in surface tension from 72 to about 26 mN/m was found for *B. subtilis* produced biosurfactants. Although a biosurfactant analysis was carried

Table 5
Field applications of MEOR.

Type	Microorganisms	Depth (m)	Pressure (MPa)	Temp. (°C)	Salinity (mg/l)	Effect of treatment	Reference
Indigenous	Rhodococcus ruber Z25	800–1000	8.3–11.3	35–45	6300–7000	Biosurfactant production	Zheng et al. (2012)
Indigenous mixed culture	–	929.4–1183.5	11.5	46	6461.3 mg/l sodium bicarbonate	Activation of indigenous microbes	Ting Sheng et al. (2009)
Indigenous mixed culture	–	1367–1450	10.79	66	20,000	Activation of indigenous microbes	Bao et al. (2009)
Exogenous mixed culture	Arthrobacter sp Pseudomonas sp Bacillus sp	1906.6–1960	18.89	73	16,790	High temperature MEOR	Jinfeng et al. (2005)
Indigenous mixed culture	–	1930–2030	15.75–23.24	77–82	–	Average increase in oil recovery by 66%	Maure et al., 1999
Indigenous	Bacillus licheniformis	600–2200	6.5–21	40–75	170,000–230,000	Production of various metabolites	Yakimov et al. (1997)
Exogenous mixed culture	–	750–800	9.12	36–47	3000–6000	Increase in residual oil recovery	Lazar et al., 1993
Exogenous + Indigenous mixed culture	–	1151–1171	6.5–14	45	7000	Increase in oil recovery by 11%	Wang et al., 1993

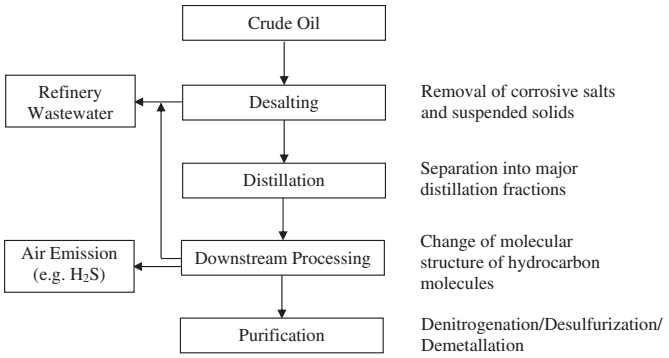

Fig. 3. Simplified flow chart of crude oil refinery processes.

out results (e.g. type of biosurfactant, concentration produced) were not discussed in detail. Biosurfactant flooding experiments using *B. subtilis* showed 25% oil recovery. Lipopeptidic biosurfactants produced by *Bacillus mojavensis* (PTCC 1696) reduced the interfacial tension from 65 to 26.7 mN/m under highly saline conditions (Ghojavand et al., 2012). Field test conducted by Youssef et al. (2013) on a limestone petroleum reservoir illustrated that with lipopeptide biosurfactants produced in-situ by two bacilli strains, oil production increased by 10%. Other Bacilli species and strains isolated from various sources used for producing MEOR biosurfactants include *Bacillus licheniformis* ACO1 (Dastgheib et al., 2008), *B. subtilis* (Gudiña et al., 2012), *B. subtilis* B20 (Al-Bahry et al., 2013) and *B. subtilis* subsp. *subtilis spizizenii* NRRL B-23049 (Youssef et al., 2013). Bacilli strains appear to be good candidates to be used for biosurfactant production in-situ or ex-situ.

In-situ applications appear to be cost effective (Zheng et al. (2012) although no actual figures were provided. Almost three decades ago, Cooper and Paddock (1984) reported production costs for various biosurfactants to range from CA-$ 2.75–152/kg, compared to CA-$ 3.25/kg for Span 60, a biodegradable, commercial synthetic surfactant. Kosaric (1992) estimated total biosurfactant production costs to be about CA-$ 3.00/kg, which was twice as high as commercial synthetic surfactants such as non-ionic alcohol ethoxylate and alkylphenoletoxylate. In both reports material costs comprised approximately 33% of the overall biosurfactant production costs. More recently, Das and Mukherjee (2007) and Banat et al. (2010) suggested in their reviews that industrial-scale usage of biosurfactants for MEOR appears to be limited due to high off-site production costs (Das and Mukherjee, 2007; Banat et al., 2010) although actual data on material and production costs, without reporting actual figures on material and production costs. Mukherjee et al. (2006) and Makkar et al. (2011) suggested three strategies to reduce production costs through: (1) usage of waste or cheap substrate for culture media; (2) efficient bioprocess technologies; (3) development of mutant or recombinant strains for increased yields. For example, date molasse waste (Al-Bahry et al., 2013), sugarcane molasses and sugarcane juice alcohol stillage (Reis et al., 2004), oil palm sludge (Wan Nawawi et al., 2010), glycerol by-product from the biodiesel industry (Freitas et al., 2009) and wastewater biosolids (Becerra et al., 2009) were shown to be suitable low-cost sources of or substrates for biosurfactant production. Publications on estimates of tangible biosurfactant production costs for MEOR are lacking thus highlighting the need for quantitative techno-economical feasibility studies.

Banat (1995a, 5b) reviewed the effectiveness of MEOR by biosurfactants in field studies carried out in former Czechoslovakia, Hungary, the Netherlands, Poland, Romania, the United States and the former USSR, with a significant increase in oil recovery noted in some but not all cases. This re-emphasizes McInerney et al. (2003) point that there is no average oil reservoir, and the chemical and physical properties of oil reservoirs vary considerably, as do the factors that entrap oil. Thus, a generic microbial process will probably not be successful when applied to a specific reservoir.

3. Refining of petroleum

Conventional petroleum refining is the physical, thermal and chemical separation of crude oil, a mixture of many different hydrocarbons and small amounts of impurities, into its major distillation fractions which are then further processed through a series of separation and conversion steps into finished petroleum products (EPA, 1995) (Fig. 3).

As will be discussed in the following sections, biotechnology has the potential to be applied in the transformation of heavy crudes into light crudes, depolymerization of asphaltenes, hydrocarbon cracking, isomerization polymerization, alkylation, product purification (e.g. removal of sulfur, nitrogen, heavy metals), and liquid and gaseous emission treatment. Most petroleum biorefining applications are still at the level of fundamental research with one exception, biodesulfurization, for which pilot plant trials with Total Raffinage and Petro Star were carried out (McFarland, 1999; Le Borgne and Quintero, 2003).

3.1. Biodesulfurization

The removal of sulfur from petroleum is of environmental and economical importance. Most oil reserves contain sulfur. Both organic and inorganic sulfur containing compounds are naturally found in crude oil. Organic sulfur compounds are aromatic or saturated forms of thiols, sulfides and heterocycles. Inorganic compounds include elemental sulfur, hydrogen sulphide and pyrite (Soleimani et al., 2007). Estimates of the sulfur content in crude oil vary from 400 to 60,000 ppm (Speight, 1980; Abbad-Andaloussi et al., 2003) to 1000–30,000 ppm (Monticello, 2000). Direct combustion of sulfur-contaminated hydrocarbons such as diesel, gasoline and kerosene would lead to the gaseous emission of vast amounts of sulfur oxides (SO_x) into the atmosphere (Monticello, 2000). Emissions of SO_x have long been known as the primary source of acid rain and to inactivate catalytic converters in automobile exhaust systems. Significant reduction of sulfur-induced corrosion and slower acidification of engine lubricating oil, which lead to lower maintenance costs and longer maintenance intervals, are additional benefits of using ultralow sulfur diesel in diesel powered vehicles (Stanislaus et al., 2010). Current EU legislation prescribes sulfur levels in diesel to be 50 ppm or below. In 2011, targeted sulfur levels for diesel in the EU and US will have to be further reduced to 10–15 ppm (Ohshiro and Izumi, 1999; Monticello, 2000; Abbad-Andaloussi et al., 2003; Stanislaus et al., 2010).

Conventional chemical processes such as the well-established hydrodesulfurization process (HDS) are able to desulfurize most of the sulfur compounds at high pressure (>100 bar) and temperatures (300 °C) (Konishi et al., 1997; Ohshiro and Izumi, 1999; Stanislaus et al., 2010). However, ultralow sulfur levels as required by current legislation are difficult to achieve due to the presence of recalcitrant aromatic organosulfur compounds (Konishi et al., 1997; Ohshiro and Izumi, 1999; Johnson et al., 2000; Gray et al., 2003). One possible approach to reduce the sulfur level further after HDS is biodesulfuration, a concept known for over 50 years (ZoBell, 1953).

One of the best documented and understood petroleum purification biotechnologies is biocatalytic desulfurization (BDS). The BDS technology was developed by the USA based ENCHIRA Biotechnology Corporation (ENBC) (formerly Energy Biosystems

Corporation) and patented in 1998. There has been worldwide interest in biodesulfurisation as demonstrated by the number of patents and patent holders listed in Appendix (Supplementary Information). The top five patent holders are ENBC (21 patents), the Japanese Petroleum Energy Centre (4), US based Institute of Gas Technology (4), the Korean Advanced Institute of Science and Technology (3), and Exxon Research & Engineering Company (2).

Various reviews on biodesulfurization have been published over the past decade (Grossman, 1996; McFarland, 1999; Ohshiro and Izumi, 1999; Monticello, 2000; Gray et al., 2003; Le Borgne and Quintero, 2003; Srivastava, 2012; Nuhu, 2013) focusing on fundamental and applied aspect of biodesulfurization processes. Of historical importance for our fundamental understanding of the biodesulphurization processes has been the study of alkylated dibenzothiopenes (DBTs), representatives of the organosulfur compounds in the middle distillate fraction (i.e. diesel fuels) (Monticello and Finnerty, 1985).

These compounds have been used as a carbon source in the screening for and metabolic pathway studies of DBT degrading microorganisms (Konishi et al., 1997; Folsom et al., 1999; Grossman et al., 2001; Bhatia and Sharma, 2010). Of particular interest are microorganisms capable of carbon-sulfur (C–S) bond-targeted DBT degradation, since they do not alter the calorific value of the fuel. It was established that 4 genes are involved in the complete C–S bond-targeted DBT degradation (McFarland, 1999; Abbad-Andaloussi et al., 2003; Gray et al., 2003):

dszC – encoding a DBT-monooxygenase
dszA – encoding a DBT-sulfone monooxygenase
dszB – encoding a 2-hydroxybiphenyl sulfinate desulfinase
dszD – encoding a NADH-flavin mononucleotide oxidoreductase

The proprietary BDS process relies on these four genes. Following sulfur removal efficiencies have been determined for various distillate fractions:

30–70% – mid-distillates (Grossman et al., 1999; Pacheco et al., 1999)
65–70% – for partially HDS-treated mid-distillates (Folsom et al., 1999)
≈ 90% – for extensively HDS-treated mid-distillates (Grossman et al., 2001)
20–60% – for light gas oils (Chang et al., 1998; Pacheco et al., 1999; Noda et al., 2003)
75–90% – for cracked stocks (Pacheco et al., 1999)
25–60% – for crude oils (Premuzic and Lin, 1999)

Grossman et al. (1999) reported that despite the significant removal efficiencies achieved, the level of desulfurization is still insufficient to meet the required sulfur levels for all fuels. This indicates that highly substrate-specific reactions are not convenient if the petroleum contains a large number of species of organosulfur compounds (Konishi et al., 1997). Consequently, the biodesulfurization process requires enzymes with a very broad substrate range of the dsz system (Monticello, 2000) to degrade organosulfur compounds oxidized otherwise by sulfur oxidation enzymes (McFarland, 1999). In addition, all metabolites in the DBT degradation pathway are practically immiscible in water, particularly DBT, which has the lowest water solubility (Seymour et al., 1997).

The most important development in the enzymatic DBT degradation is the application of directed evolution techniques (gene shuffling) to the dsz system, creating hybrid genes with better rates and extents of biodesulfurization (Monticello, 2000; Gray et al., 2003). This technique has been used for the removal of DBT and benzothiophene using improved biocatalyst (Kilbane, 2006). Noda et al. (2003) used 22 rhodococcal and mycobacterial strains, capable of growth in hydrophobic medium, as host for the dsz desulfurization gene cluster from *R. erythropolis* (strain KA 2-5-1) and found recombinant *Mycobacterium* strain MR 65 to desulfurize 68 mg/L of sulfur in light gas oil containing 126 mg/L sulfur in the presence of 4,6-dipropyl DBT. *Mycobacterium goodii* X7B was also found to convert DBT to 2-hydroxybiphenyl via the 4S pathway and benzothiophene (Li et al., 2005). Ma et al. (2006) investigated methods to produce biodesulfurization catalysts and found that cultivation of *Rhodococcus* sp. in 1 mM dimethylsulfoxide was most cost-effective. The microbial culture was then used in a bi-phasic system (water-oil ratio 9:1) and found to remove 78% of sulfur (200 ppm initial concentration) from a HDS treated diesel oil. In another study that aimed to overcome fuel toxicity to sulfur-removing microorganisms in bi-phasic systems, Tao et al. (2011) combined the solvent tolerance exhibited by *Pseudomonas putida* (DS23) with biodesulfurization capability through inserting gene cluster dszABCD into a solvent-responsive expression vector. The authors reported a 56% reduction of 0.5 mM DBT within 12 h in a bi-phasic system containing 33.3% (v/v) n-hexane, while another strain induced by isopropyl b-D-1-thiogalactopyranoside could only degrade 26%. In an attempt to reduce the often high water-to-oil ratios (>200) in bi-phasic systems, Kawaguchi et al. (2012) metabolically engineered the hydrophobic microorganism *Rhodococcus opacus* so that it was capable of expressing the dszABC operon constitutively. Maximum DBT consumption rate of 9.5 μmol/(hr · $g_{wet\ cell}$) was observed at a water-to-oil ratio of 4 containing an initial DBT concentration of 0.5 mM. The DBT consumption rate marginally declined with increasing oil content, while in another experiment conducted at an oil–water-ratio of 4, an increase in DBT consumption rate was noted with increasing DBT concentration. In order to enhance the liquid–liquid interfacial area and thus organic sulfur mass transfer in the interfacial region, while simultaneously reducing the problems associated with the use of organic solvents and surfactants in bi-phasic systems, Tang et al. (2013) sonicated a 1 g bunker oil – 45 mL BSA solution mixture at different amplitude ratios and times. After 7 days of incubation using a mixed culture from an oil-contaminated soil, the authors reported an 18.4% sulfur reduction in ultrasound pretreated bunker oil compared to 13.8% sulfur reduction in the mechanically stirred and surfactant-supplemented positive control experiment. While this experiment proved the concept of ultrasound pretreatment greater sulfur removal efficiencies in a shorter period of time are required to render this process industrially viable. Pan et al. (2013) pursued a novel strategy to improve the performance of the BDS process by increasing the sulfur demand of desulfurization-competent cultures to force increased desulfurization activity via modification of the dsz operon so that it encodes a sulfur-rich polypeptide (Sulpeptide1 S1). This strategy was followed up by directed evolution, i.e. subjecting the transformant to repeated passages in a medium with DBT as sole sulfur source. After selection for 40 passages, both the dszABC and dszAS1BC expressing *R. opacus* strains exhibited a >20-fold increase in specific desulfurization ability, and exceeded the specific activity of the control, desulfurization positive strain *Rhodococcus erythropolis* IGTS8.

Another biocatalyst proposed by Ayala et al. (2007) are the extracellular peroxidases. It has been reported that peroxidase treatment combined with distillation can reduce the sulfur content from 1.6 to 0.27% in diesel fuel.

Different configurations of bioreactors have been used by various researchers for biodesulfurization. Mukhopadhyaya et al. (2006) used trickle bed reactor with HSD feed with *Rhodococcus*

sp and achieved 99% of sulfur conversion. Continuous two phase (organic and aqueous) bioreactor was used for the removal of DBT using *Rhodococcus globerulus* DAQ3 which resulted in 12% sulfur removal (Yang et al., 2007). Airlift bioreactors were used by Nandi (2010) and Irani et al. (2011) with different microorganisms and operating conditions and achieved 100% and 50% sulfur removal.

It has already been established that the CO_2 emissions and energy requirements of a BDS based process are reduced compared to the energy-intensive HDS process due to the milder (and safer) process conditions (Linguist and Pacheco, 1999; Singh et al., 2012). Atlas et al. (2001) estimated the HDS process cost of lowering the sulfur content from 500 to 200 ppm to be approximately 0.26 cent per liter, while desulfurization cost increase by a factor of four if the sulfur content is further lowered from 200 to 50 ppm. In addition, the capital costs to set up a BDS process were reported to be 50% of that for HDS (Atlas et al., 1998; Pacheco et al., 1999; Linguist and Pacheco, 1999).

Given the amount of research directed towards biodesulfurization of diesel fuels and the understanding of the fundamental mechanisms gained, further improvements in the cost of biocatalyst production, mass transfer, reactor design and DBT metabolic rate can be expected in the near future.

3.2. Biodemetallation

Crude oil contains metals in two forms, inorganic salts and organometallic compounds. The latter usually comprises of petroporphyrine and other complexes entrapped in the asphaltenes (Le Borgne and Quintero, 2003). Vanadium (V) and Nickel (Ni) containing metalloporphyrins are the most prevalent and are found almost exclusively in the resin and asphaltene fraction of crude oil, whereby Ni and vanadyl porphyrins concentrations up to 120 and 1500 ppm, respectively, have been reported (Speight, 1990). The two metals have been reported to shortening the lifetime of hydrotreating catalysts (Callejas et al., 2001). In addition, many metals (incl. Ni and V) form oxides during fuel combustion (Le Borgne and Quintero, 2003), which are primarily concentrated in the ash and pose a disposal problem. Le Borgne and Quintero (2003) reported that the crude oil desalting process (Fig. 3) effectively removes soluble heavy metal salts. However, metals trapped in petroporphyrins are usually not removed and therefore pose a significant risk to certain downstream processes such as catalytic cracking.

The biological removal of metals from petroleum has attracted relatively little attention in the past, which is evidenced by the low number of publications and patents found on this topic. Lloyd (2003) reviewed the dissimilatory reduction of a number of metals and radionucleotides and concluded that the molecular basis of respiratory metal reduction processes are not fully understood yet, although rapid advances are expected in this area with the imminent availability of complete genome sequences for key metal-reducing bacteria, in combination with genomic and proteomic tools.

In the late 20th century, Energy BioSystems Corporation filed two patents (Xu et al., 1997; Xu et al., 1998) on the demetallization of liquid fossil fuels (e.g. petroleum) by the biocatalytical degradation of porphyrine molecules (incl. metalloporphyrines). It is claimed that the patented method is able to remove nickel, vanadium, cobalt, copper, iron, magnesium, and zinc. The biocatalyst can be an enzyme from heme oxygenase and cytochrome C reductase, such as cytochrome C reductase from *Bacillus megaterium*, *Catharanthus roseuse*, *Escherichia coli*, animal cells (such as liver or kidney cells), plant cells (e.g. *Arabidopsis thaliana*) or yeast cells (e.g. *Candida tropicalis*). Mogollon et al. (1998) reported the biocatalytic removal of nickel and vanadium from petroporphyrins and asphaltenes. Garcia-Arellano et al. (2004) demonstrated the successful modification of Cytochrome C, which not only showed improved activities in a ternary solvent mixture (solvo-tolerance is an important criteria for use in process line), but also the ability to transform the highly recalcitrant asphaltenes.

Earlier research by Fedorak et al. (1993) on demetallation has shown that the enzyme chloroperoxidase from *Caldariomyces fumago* was able to remove 93% of Ni and 53% of V contained in porphyrins and asphaltenes. However, the catalyzed destruction of the porphyrinic ring also resulted in the formation of undesirable chlorinated byproducts.

Table 6
Patents issued on biodenitrogenation of fossil fuels (USA Patent Office, 2012).

Year	Patent holder	Patent title	US patent no.
2010	E.I. du Pont de Nemours and Company	Method for identification of novel anaerobic denitrifying bacteria utilizing petroleum components as sole carbon source	7,740,063
2003	Petroleo Brasileiro S.A.-Petrobras	Bacterial cleavage of only organic C–N bonds of carbonaceous materials to reduce nitrogen content	6,541,240
2001	Same	*Pseudomonas ayucida* useful for cleavage of organic C–N bonds	6,221,651

3.3. Biodenitrogenation

The nitrogen-containing organic compounds found in crude oils may be grouped in two classes – the 'nonbasic' molecules include pyrroles, indoles, and mixed alkyl derivatives of carbazole (structural equivalent to DBT), while the 'basic' molecules are largely derivatives of pyridine and quinoline (structural equivalent to benzothiophene) (Benedik et al., 1998). Benedik et al. (1998) reported that the total nitrogen content of crude oils averages around 0.3%, of which the nonbasic compounds comprise approximately 70–75%. Speight (1980, 1998) reported variations of the nitrogen content in petroleum from 0.1 to 2% according to the source, with high content in heavy oils. Most of the nitrogen-containing aromatic compounds contained in crude petroleum oil can be removed by the chemical and physical refinery processes (e.g. high-pressure, high-temperature hydrotreating) (Benedik et al., 1998). However, the physico-chemical removal of recalcitrant heterocyclic organonitrogen compounds such as quinolines and carbazoles is expensive and hazardous, and modifies many other constituents of petroleum. Their removal is advantageous from an environmental (reduction of NO_x emission upon combustion) and operational (catalyst deactivation by quinolines; equipment corrosion, chemical instability of refined petroleum) point of view (Vazquez-Duhalt et al., 2002; Li et al., 2004). Biodenitrogenation of petroleum could be beneficial for a deep denitrogenation in which the classical hydroprocessing methods are costly and non-selective (Vazquez-Duhalt et al., 2002).

Biodegradation of quinolines (Grant and Al-Najjar, 1976; Shukla, 1986; Schwarz et al., 1989; O'Loughlin et al., 1996) and carbazoles (Fedorak and Westlake, 1984; Ouchiyama et al., 1993; Gieg et al., 1996; Shotbolt-Brown et al., 1996) has been reasonably well studied. Carbazoles are somewhat resistant to microbial degradation and different types of microbes have been used for its degradation (Bai et al., 2010). Some of the bacterial strains used are *Klebsiella* sp. LSSE-H2 (Li et al., 2008), *Burkholderia* sp. strain IMP5GC (Castorena et al., 2008) and *Pseudomonas* sp. (Zhao et al., 2011). However, the use of such cultures in petroleum biorefining applications ideally requires carbon-nitrogen (C–N) bond-targeted organosulfur degradation, since they do not alter the calorific value of the fuel.

Table 7
Patents issued on biotransformation of fossil fuels (USA Patent Office, 2012).

Year	Patent holder	Patent title	US patent no.
2010	E.I. du Pont de Nemours and Company	Ring reduction via natural anaerobic microbiological activity for transformation of crude oil-associated aromatic chemicals	7,677,305
2006	Statoil ASA	Method of treating a hydrocarbon bearing formation	7,124,817
2005	Microbes, Inc.	Microbial-induced controllable cracking of normal and branched alkanes in oils	6,905,870
2000	Exxon Research & Engineering Company	Biological activation of aromatics for chemical processing and/or upgrading of aromatic compounds, petroleum, coal, resin, bitumen and other petrochemical streams	6,156,946

The economic importance of nitrogen-containing heterocycle loss remains to be evaluated (Vazquez-Duhalt et al., 2002). Kilbane et al. (2003) (Table 6) showed that microorganisms such as *Pseudomonas ayucida*, *Aneurinibacillus* sp, *Pseudomonas stutzeri*, *Yokenella* sp. and *Pseudomonas nitroreducens* can be useful in the cleavage of organic C–N bonds. Continuous growth of *Pseudomonas ayucida* in shale oil at mesophilic temperatures (25–35 °C) resulted in the removal of more than 50 %-wt of the organically bound nitrogen as quinoline. Similar to the BDS process, the presence of nitrogen compounds other than the targeted organosulfur compounds may result in the suppression of the C–N bond cleavage. Research on the elucidation of the enzymatic system involved in the cleavage of C–N bonds is still ongoing. More work such as the screening for, selection and engineering of thermophilic bacteria and enzymes able to biodegrade a variety of aromatic nitrogen compounds, function efficiently and for prolonged periods of time at high solvent concentrations, reactor design and the generation of valuable byproducts (e.g. Anthranilic acid) remains to be done before this technology becomes economically viable.

3.4. Biotransformation/bioupgrading of petroleum

The efficient recovery, separation, or processing of heavy oils and bitumens is often hampered by the presence of asphaltenes, which is also reported to increase the viscosity of the oils, enhance the propensity to form emulsions, polymers, and coke (Vazquez-Duhalt et al., 2002). The (bio)transformation or upgrading of these and other aromatic compounds contained in petrochemical and other heavy hydrocarbon streams is therefore of interest to maximize the usage of crude oil and minimize waste. Efforts in (bio) transformation of asphaltenes, for example, have partly been hampered by the highly variable and complex structure of asphaltenes. Asphaltenes may be described as condensed aromatic cores containing alkyl and alicyclic moieties as well as nitrogen-, sulfur- and metal-containing non- and heterocyclic groups (Vazquez-Duhalt et al., 2002).

As briefly mentioned before, Garcia-Arellano et al. (2004) were able to modify asphaltene molecules by the biocatalytic action of a modified Cytochrome C. The biotransformation of aromatic rings such as biphenyls in petroleum was reported by Coyle et al., (2000) (Table 7). The proprietary process involves the hydroxylation of aromatic rings by microorganisms or other biocatalysts. The activated molecules are then processed by conventional hydrogenation and/or hydrogenolysis processes producing cracked and ring-opened products. Peroxidase-catalyzed reactions have the potential to be used for asphaltene transformation (Ayala et al., 2007). Commercial applications have not been reported so far and their successful implementation in the petroleum refinery industry will depend on similar issues as discussed for the other biorefining techniques.

4. Conclusions

The application of biotechnological processes in the petroleum industry is still scant and fragmented. Nonetheless, the literature review and survey also revealed a few exceptions, amongst those are the use of Xanthan gum to enhance oil recovery (Halliburton), the removal of paraffin from oil producing wells by a patented bacterial 'cocktail' (Micro-Bac). In some cases, biotechnology may completely substitute, in others only supplement physico-chemical processes. The vast majority of in-situ and ex-situ processes and applications, however, are still in the R & D stage for a variety of reasons. The economical viability of in-situ applications such as MEOR crucially depends on the knowledge of the composition of indigenous microbial populations and their metabolical pathways, and the ability to efficiently deliver economic nutrients, specific growth inhibitors, enzymes, polymers, chemicals, etc. to the point of application.

Ex-situ applications such as biorefining must be able to fit in-line with the upstream process (e.g. pH, temperature, pressure), should produce value-added by-products, avoid the production of toxic by-products and allow for easy separation of cells/biocatalyst and product. If biocatalysts are to be used they must be stable under the actual process conditions, be active for several hundred hours, have a high reaction rate and be produced economically. Off-the shelf solutions are desirable for widespread use but are currently hampered by the complexity of individual projects.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2013.09.011.

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