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Review

Novel Approach of Tackling Wax Deposition Problems in Pipeline Using Enzymatic Degradation Process: Challenges and Potential Solutions

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Abstract: Anthropogenic activities have led to hydrocarbon spills, and while traditional bioremediation methods are costly and time-consuming, recent research has focused on engineered enzymes for managing pollutant. The potential of enzymes for resolving wax flow problems in the petroleum industry remains unexplored. This paper offers a comprehensive review of the current state of research activities related to the bioremediation of petroleum-polluted sites and the biodegradation of specific petroleum hydrocarbons. The assayed enzymes that took part in the degradation were discussed in detail. Lipase, laccase, alkane hydroxylase, alcohol dehydrogenase, esterase, AlkB homologs and cytochrome P450 monooxygenase are among the enzymes responsible for the degradation of more than 50% of the hydrocarbons in contaminated soil and wastewater and found to be active on carbon C8 to C40. The possible biodegradation mechanism of petroleum hydrocarbons was also elucidated. The enzymes' primary metabolic pathways include terminal, subterminal, and ω-oxidation. Next, given the successful evidence of the hydrocarbon treatment efficiency, the authors analyzed the opportunity for the enzymatic degradation approach if it were to be applied to a different scenario: managing wax deposition in petroleum-production lines. With properties such as high transformation efficiency and high specificity, enzymes can be utilized for the treatment of viscous heavy oil for transportability, evidenced by the 20 to 99% removal of hydrocarbons. The challenges associated with the new approach are also discussed. The production cost of enzymes, the characteristics of hydrocarbons and the operating conditions of the production line may affect the biocatalysis reaction to some extent. However, the challenges can be overcome by the usage of extremophilic enzymes. The combination of technological advancement and deployment strategies such as the immobilization of a consortium of highly thermophilic and halotolerant enzymes is suggested. Recovering and reusing enzymes offers an excellent strategy to improve the economics of the technology. This paper provides insights into the opportunity for the enzymatic degradation approach to be expanded for wax deposition problems in pipelines.

Keywords: enzyme; alkane degradation; petroleum hydrocarbon; wax deposition; production line

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1. Introduction

Petroleum hydrocarbons are a blend of aromatic and aliphatic carbon compounds, with the composition changing depending on where the reservoir is found [1]. The composition of petroleum hydrocarbons can be characterized into four primary categories:

saturates, aromatics, asphaltenes (which include phenols, fatty acids, and ketones), and resins (which include pyridines and amides) [2,3]. Saturates are nonpolar hydrocarbons that are also called waxes. Aromatic hydrocarbons contain polarizable rings, while resins and asphaltenes have polar substituents with oxygen, sulfur, and nitrogen [4]. These organic compounds are highly insoluble in water, which limits the hydrocarbon uptake needed for microorganisms to perform degradation [5]. Anthropogenic activities, such as petroleum exploration, transportation and refining accumulated hydrocarbon spillage at sites, consequently harm beaches, animal habitats, mangrove forests and human settlements. Figure 1 visualizes some of the oil spill disasters that have ensued over time. The situation had a major ecological impact on the environment, which must be managed responsibly.

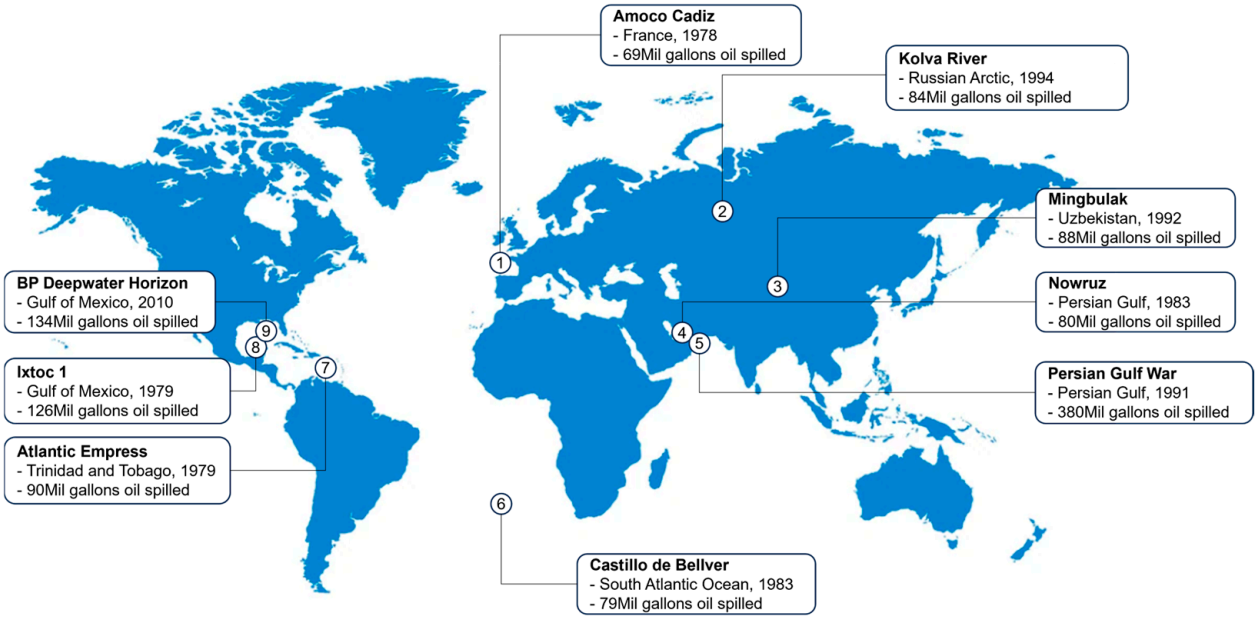

Figure 1. Locations of significant historical oil-spill incidents [6].

Historically, research has focuses more on microbial degradation or the bioremediation of toxic pollutants in water and soil using microbes. This technique was costly, time-consuming, and limited by severe conditions of extreme pH, temperature, and chemical shock [6]. Over the past 15 years, research has focused on the use of enzymes for pollution management.

In the petroleum industry, hydrocarbons are not only managed for their spillage but also from the perspective of flow assurance during the transportation of hydrocarbons in pipelines. When a hydrocarbon is high in wax content, the wax crystallizes and is deposited on the pipeline wall, especially in cold environments where the production is farther offshore [7]. The wax accumulation restricts the flow of hydrocarbon and causes blockages [8]. Oil companies are applying chemicals to reduce the wax, combined with pipeline thermal insulation, and pigging to scrap off the wax deposit [9]. However, these strategies are expensive, inefficient, and less robust due to a lack of understanding of their workability in different fields' operating conditions [7]. This gap creates a greater opportunity for novel discoveries, as conceptualized in Figure 2. To the authors' knowledge, the enzymatic degradation of hydrocarbons for managing wax deposition in production lines has not been widely studied.

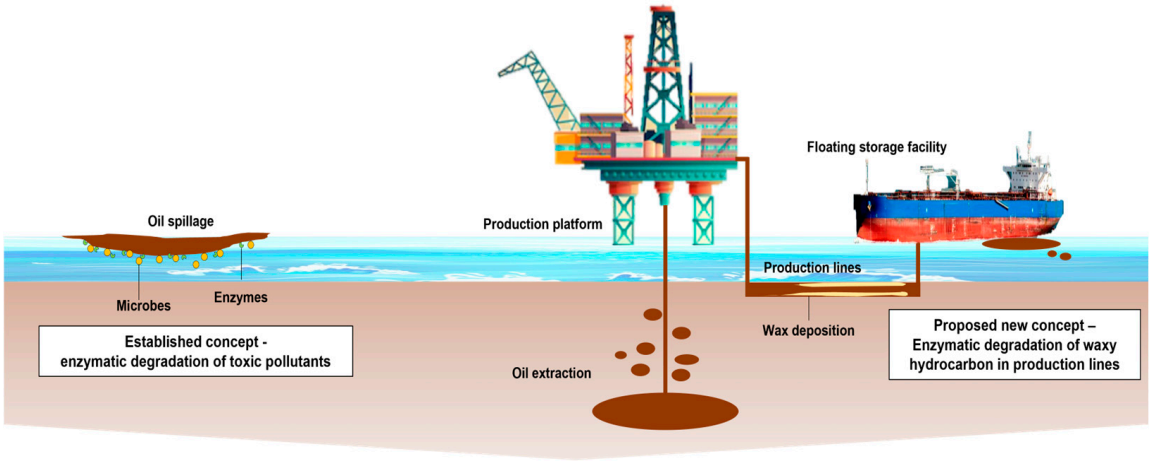

Figure 2. Exploiting biocatalytic degradation of hydrocarbon technique for new purpose.

2. Wax Deposition Scenario and the Limitations of Existing Wax Treatment

Wax deposition occurs when wax precipitates out of the petroleum when its temperature is reduced. This is because alkanes that are heavier than carbon number C18 has a lower solubility in the liquid phase [10]. There are many factors affecting wax deposition in production lines, such as molecular diffusion, operating temperature, pressure, asphaltene composition, hydrocarbon flow velocity and pipeline design [11]. One of the adverse effects of wax deposition in the petroleum industry is the blockage of the pipeline, which causes an increase in pressure drop and decrease in flowability. For petroleum-production lines in cold regions or pipelines located below sea level, the wax build-up issue is very alarming [8]. Substantial expenditures are required for wax deposition control and remediation. The prevention and treatment of the wax deposition problem are conventionally performed using thermal, mechanical, chemical, and biological methods.

2.1. Thermal Treatment

The thermal method involves the installation of a heat tracing device and suitable insulation materials in a transportation pipeline to minimize wax deposition on the walls, and hot fluid circulates to keep the crude oil above its wax appearance temperature [12]. However, electrical heaters are prone to burn-out, which prompts an automatic cut-off system when overheating occurs [13]. The integrated production and transportation system of crude oil will be majorly impacted by this interruption. On the other hand, the limitation of the insulation technique is that it requires a material with suitable conductivity and chemical-resistance properties [12].

2.2. Mechanical Treatment

The mechanical method involves frequently scraping the pipeline using a rubber or metal tool before the wax begins to build up, together with the continuous injection of a solvent or wax inhibitor [14]. This technique has an advantage for a non-heating pipeline whereby the significant energy consumption can be reduced. However, the cutting force of the wax layer is dependent on the tool geometry and the friction at the wax–tool interface. A study that investigates the effect of tool geometry on wax scraping demonstrated that a 45° rake angle removed more of the wax layer than a negative 30° angle [15]. The efficiency of wax removal is also affected by the sliding speed of the scraper, which influences the rubbing force of the wax layer [16]. In addition, there is also the risk of a wax plug forming and the scraper becoming wedged inside the pipeline when handling viscous crude oil [14].

2.3. Ultrasonic and Electromagnetic Wave Propagation

The displacement of wax by ultrasonic and electromagnetic wave propagation is a relatively new approach. It was reported that when the crude oil was treated with

ultrasonic waves, the resin and asphaltene fractions in the crude oil were increased due to the modification of the wax structure, which became a more polar compound. These increased fractions were regarded as natural surfactants in crude oil and caused the wax appearance temperature to reduce by 12 °C [17]. A morphological study has shown that the size of the wax crystals became smaller and rounded after irradiation treatment due to a decrease in wax molecules [18]. However, research has indicated that this technique requires combination with chemical [19–21] or thermal treatment [22] for it to be effective, which risks doubling the treatment cost.

2.4. Chemical Treatment

Chemical injection is by far the most effective and mature method. Chemicals like solvents, surfactants, wax inhibitors and pour-point depressants were injected to alter the surface characteristics and solubilize the wax deposits, leading to reduction in wax appearance temperature and pour point, so the wax would not form in cold environments [23]. It was reported that wax was reduced by 65 to 73% when a polyacrylate polymer-based pour-point depressant was applied to a waxy crude oil [24]. However, chemicals like wax inhibitors and pour-point depressants are typically solids at operating sites, thus requiring dilution with a solvent, which in turn increases the expenses and the number of possible operational hazards [25]. Additionally, the performance of the chemical is not universal. Its effectiveness is chemically specific to the composition of the crude oil [26,27]. Various type of copolymers that functioned as wax crystal modifiers produced different results, and their mechanisms are poorly understood despite many reported studies [28]. Some research has found that asphaltene constituents in crude oil can hinder the effectiveness of the chemicals [29]. Solvents like kerosene, gasoline and benzol are relatively cheap and are used to solubilized wax deposits. A combination of diesel, xylene, toluene, naphthalene and dispersant was formulated at an optimum ratio, which resulted in more than 85% of wax solubilization [23]. However, the usage of solvents posed toxicity and flammability threats to the handlers, and the effectiveness of the solvents also depends on the permeability of the wax deposits [23].

2.5. Biological Treatment

The biological method highlights an efficient and environmentally friendly solution involving hydrocarbon-degrading microbes. Bacteria are readily obtainable and inexpensive, and their handling is convenient. Because of this, bacteria have been utilized successfully for decades in the bioremediation of hydrocarbon-polluted sites [30,31]. The microbial degradation of petroleum was proven successful for ocean clean-up and onshore wastewater treatment. During the oil spill from the Exxon Valdez tanker, the microbial populations sampled from the shoreline of Prince William Sound beach showed the foremost ability to degrade aromatic hydrocarbons; subsequently, alkane degradation became more dominant over time [32]. Another study examined microbial communities from the Gulf of Mexico shoreline contaminated by the Deepwater Horizon blowout, which was also shown to be responsible for the major degradation of polyaromatic hydrocarbons [33]. A bioreactor was used to treat industrial wastewater rich in hydrocarbons through the acclimatization of microbial consortium, resulting in a hydrocarbon degradation of more than 97%, especially on alkanes C10 to C35 [34]. In fact, many advanced bioreactors have been designed to allow for the precise control of biodegradation parameters to increase the mass transfer and reaction rates during the microbial degradation reaction [35]. However, the survivability of bacteria is dependent on nutrients and growth enhancers. This approach may not be suitable for petroleum-production lines due to factors such as the high temperature and pressure of the incoming petroleum, seawater salinity, the availability of microbial substrates, the nutrient solution, dissolved oxygen and nitrogen sources, whereby bacteria are commonly sensitive to the changes of these factors [36]. On top of that, the presence of sulfate-reducing bacteria leads to biofouling, where the microbial growth forms biofilms that can clog and damaged the production lines [37]. The waxy petroleum treatment in production lines requires a

less-complex and faster degradation of the high-molecular-weight hydrocarbons [25], but at the same time, it does not impact the low-molecular-weight hydrocarbons that are valuable to the sale value of the petroleum. However, there is no means to control degradation using the microbial method. Table 1 summarizes the limitations of each wax treatment method.

Table 1. Wax treatment/inhibition strategies and their limitations.

Wax Treatment/Inhibition Strategy	Positive Findings from Previous Experimental Studies	Limitations
Scraping of pipelines using rubber/metal tool before the wax begin to buildup	Suitable for non-heating pipeline, can reduce energy consumption for pipeline transportation [14]	1. The cutting force of the wax layer is dependent on the tool geometry and friction at its surface, e.g., wax removal is better at 45° rake angle than a negative 30° angle [15] 2. The sliding speed of the scraper affects the rubbing force, thus the wax deposit removal [38,39] 3. Formation of wax plug or scrapper became stuck caused by viscous petroleum [14]
Electrical heating, insulation and circulation of hot fluid to keep the oil above wax appearance temperature	Wax removal below wax appearance temperature was possible for wax content below 14% [40] More effective when asphaltene content in oil is high because activated asphaltenes have strong interactions with wax then decrease the wax appearance temperature [41]	4. Electrical heaters frequently burn out thus require an automatic cut off system when overheating occurs [12] 5. The insulation technique requires suitable material with low conductivity and chemical-resistance properties [12]
Ultrasonic and electromagnetic waves propagation to create cavitation and displace the wax	The wax appearance temperature of oil was reduced by 12 °C related to an increased in natural surfactant fractions, i.e., resin and asphaltene caused by modified composition after being treated with ultrasonic wave [17] By morphology, the size of wax crystals was reduced and rounded after irradiation due to decreased in wax molecules [18]	6. Requires combination with chemical [19–21,42] or thermal [22] to increase wax removal efficiency
Injection of chemical, e.g., wax inhibitor or pour-point depressant to reduce wax appearance temperature	65–73% wax reduced using polyacrylate polymer-based pour-point depressant [24]	7. Wax inhibitors are typically solids at the operating site. Dilution with solvent increased expenses and raised possible hazards [12] 8. Not universal, effectiveness is chemically specific to oil composition, varying from different well and over time. Various types of copolymers that functioned as wax crystal modifiers produced different results, and its mechanism is poorly understood despite many reported studies [28] 9. Asphaltene content in oil may hindered chemical performance [29]

Table 1. *Cont.*

Wax Treatment/Inhibition Strategy	Positive Findings from Previous Experimental Studies	Limitations
Injection of solvents, e.g., kerosene, gasoline or benzol to solubilize wax deposits	Solvent formulation consisted of diesel, xylene, toluene, naphthalene and dispersant solubilized >85% of wax without requires heating above 90 °C [23]	10. The effectiveness depends on the permeability of the wax deposit 11. Posed toxicity and flammability risks to handlers [23]
Hydrocarbon-degrading bacteria to cleanly degrade the hydrocarbon	Bacteria is easy to get, inexpensive, easy to handle and biodegradable (environmentally friendly) [12]	12. Dependent on nutrients and growth enhancers, and in the presence of sulfate-reducing bacteria, pipeline corrosion is a showstopper [37]

The advancements in technology and knowledge in protein engineering, recombinant DNA, synthetic biology, and metabolic engineering have enabled the design of novel enzymes with desired properties such as high catalytic activity, substrate specificity, and thermostability, tailored for wax treatment conditions. In this regard, the enzymatic degradation of waxy petroleum in production lines represents a novel approach.

3. Microbial Degradation of Petroleum Hydrocarbon and Enzymes Involved in the Reaction

Since enzymes' potential can be maximized in a lab setting, biocatalysis— a technique that uses enzymes to perform chemical transformation—is a more strategic approach than dealing with the entire microorganism [43]. Within the biocatalytic degradation research, previous studies have described biodegradation pathways, genes, and the characterization of the biodegradative enzymes. The usage of these enzymes as biocatalysts either for making a useful compound or to transform toxic compounds into nontoxic compounds has also been described [44]—for example, the bioremediation of waste materials such as organic and inorganic pollutants and pharmaceutically active compounds in wastewater, industrial chemicals, and pesticides [45–55].

On the other hand, there are also specific studies on the biocatalytic degradation of petroleum, which is the main topic of this paper. For the past 10 years, the trend in research publications related to the specific biocatalytic degradation of hydrocarbon pollutants is more common than for hydrocarbon wax (Figure 3), probably because pollution clean-up is a more pressing matter globally, while the wax issue is specifically managed in the oil and gas sector. Both subjects are elaborated in detail in the next section, focusing on the species of bacteria hosting the enzymes responsible for removing the petroleum hydrocarbons.

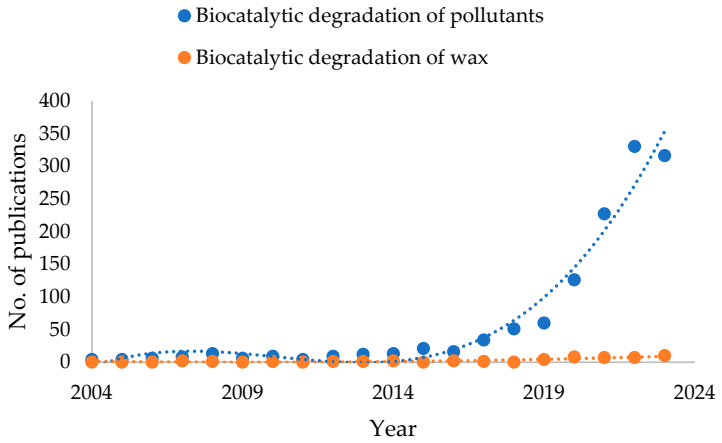

Figure 3. Research publications on "biocatalytic degradation", "pollutants" and "wax", based on data obtained from Google Scholar on 3 August 2024.

3.1. Bioremediation of Petroleum-Contaminated Sites

The effective bioremediation of polluted sites depends on the survivability of the degrading microbes. One study sought to solve the low yield of degradative enzymes and biosurfactants by the intermittent inoculation of microbial consortia *S. chilikensis*, *B. firmus* and *H. hamiltonii*, to maintain a constant level of the degradative enzymes oxidoreductase, lipase, and catalase, and lipoprotein biosurfactants. As a result, a steep decrement of petroleum was observed [56]. Petroleum-polluted sites are commonly associated with high metal and salinity content. The metal-tolerant bacterium *Novosphingobium panipatense* P5:ABC was able to degrade 90% of petroleum, and alkane hydroxylase was responsible for this [57]. A comparison between free *P. stutzeri* and immobilized *P. stutzeri* showed that immobilized bacteria are more efficient in removing hydrocarbons from produced water and were reused repeatedly for 10 cycles, due to the sorbent-degrader synergies [58]. The microbial consortia consisted of salt-tolerant and biosurfactant producers from *Dietzia* sp. CN-3 and *Acinetobacter* sp. HC8–3S had achieved 95.8% degradation efficiency of petroleum, whereby alkane hydroxylase genes alkB and alkM degraded the medium-chain alkane C14-C26 [59]. Immobilized indigenous bacteria with high adaptability to petroleum-refining oil sludge showed a great advantage, with an 88.78% degradation of petroleum due to the high catalytic activity of enzymes [60]. Immobilized microorganisms may suffer from the inconsistency of degradation performance because only the external cells exhibit high metabolic activity, while the cells in the support pores are unable to access the hydrocarbon [61]. These issues were solved with the assistance of a bioemulsifier agent, produced by the biocatalyst from *Aspergillus brasiliensis*, when exposed to the electrical field. The in situ biosurfactant production modified the surface properties and sorption capacity, improving the hydrocarbon attachment [62]. Very recent studies have used transposon mutagenesis technology to increase the biosurfactant production of *Enterobacter xiangfangensis* [63] and *Enterobacter hormaechei* [64] mutants to enhance the bioavailability of petroleum. As a result, the degradation of recalcitrant petroleum in oil sludge was achieved to a high degree.

These studies demonstrate the high efficacy of enzymes as bioremediation agents in polluted sites where the hydrocarbon load is low (Table 2). On the contrary, treating waxy hydrocarbons in production lines is more challenging than the bioremediation of hydrocarbon-polluted sites. The treatment requires enzymes to perform in a non-aqueous, hydrocarbon-rich environment that is probably hostile to the enzymes. In this scenario, petroleum has a higher fraction of high-molecular-weight carbon that makes up the wax composition, limiting the susceptibility of the enzymes to the hydrocarbon. The next section describes the biodegradation of specific waxy petroleum and the enzymes taking part in the reaction.

Table 2. Microbial treatment of petroleum-contaminated sites and the assayed enzymes.

Source of Hydrocarbon	Hydrocarbon-Degrading Microbe	Reported Enzymes	Hydrocarbon Treatment Condition	Hydrocarbon Removal Efficiency	Reference
Contaminated soil	*Pseudomonas aeruginosa*	Not described	Strains inoculated to 500 g of contaminated soil	55–84% in consortium; 25–47% in individual cultures	[65]
Oil sludge from petroleum refinery	Wild-type strains *Enterobacter xiangfangensis* STP-3; mutant strains *Enterobacter Xiangfangensis* M257 and *Enterobacter Xiangfangensi* M916	Lipase, laccase, alkane hydroxylase, alcohol dehydrogenase, esterase	10 g oil sludge and 20 g bacterial culture in 500 mL reactor	82–87.5% in mutant strains; 72.15% in wild-type strains; in 7 days.	[63]

Table 2. *Cont.*

Source of Hydrocarbon	Hydrocarbon-Degrading Microbe	Reported Enzymes	Hydrocarbon Treatment Condition	Hydrocarbon Removal Efficiency	Reference
Polluted seawater	biosurfactant-producing hydrocarbonoclastic bacteria *Enterobacter hormaechei*	Lipase, laccase, alkane hydroxylase, alcohol dehydrogenase, esterase	5 liter polluted seawater with 10 g strains treated in 8 liter tank with bottom aeration.	85% in 10 days	[64]
Oil sludge	*Acinetobacter* sp. SCYY-5	Not described	Incubated at 30 °C at 150 rpm	69.17% in 10 days	[66]
Oil sludge from refinery	*Pseudomonas aeruginosa*; *Staphylococcus* sp.	Not described	Lab scale-anaerobic bioreactors	90% in 14 days	[67]
Contaminated soil from oil processing	Immobilized consortium of *Flavobacterium johnsoniae* BS1 and *Shewanella baltica* BS2	Not described	Incubated at 30 °C at 150 rpm, and pH 7.5	93.32% in 3 days	[68]
Oil sludge from refinery	Indigenous hydrocarbonoclastic bacteria	Lipase, laccase, esterase	5 g oil sludge with 5 g immobilized bacteria	88.78% in 7 days	[60]
Contaminated soil	Consortium of halotolerent and biosurfactant producing bacteria, *Dietzia* sp. CN-3 and *Acinetobacter* sp. HC8-3S	Alkane hydroxylase genes, alkB and alkM	100 g of soil with 5 g of crude oil, inoculated with 10 mL bacteria consortium, mixed and kept at 30 °C	95.8% in 10 days	[59]
Contaminated soil	Indigenous bacteria	Not described	Dual-chamber reactors with membrane, filled with 250 mL saturated soil and artificial groundwater at 22 °C	37.5% in 137 days	[69]
Oil-in-saltwater emulsions	*Pseudomonas stutzeri* immobilized on xerogel microspheres	Not described	0.15 g bacteria inoculated into 15 mL of oil-in-water emulsions cultured at 150 rpm and 35 °C	10% in immobilized bacteria; 61% in free bacteria in 72 h	[58]
Crude oil	*Acinetobacter* sp.; *Bacillus* sp.; *Pantoea* sp. and *Enterobacter* sp.	Not described	Addition of nitrogen, phosphorus and potassium (NPK)	20–40% in 28 days	[70]
Contaminated soil	Heavy metal tolerant bacterium *Novosphingobium panipatense* P5:ABC	Alkane hydroxylase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase	Incubated at 25 °C at 130 rpm	90% degradation in 6 days	[57]

Table 2. *Cont.*

Source of Hydrocarbon	Hydrocarbon-Degrading Microbe	Reported Enzymes	Hydrocarbon Treatment Condition	Hydrocarbon Removal Efficiency	Reference
Oily sludge from refinery	Bacteria consortium of *Shewanalla chilikensis*, *Bacillus firmus* and *Halomonas hamiltonii*	Lipase, catalase, oxidoreductase	Incubated at 37 °C	96% in 30 days	[56]
Contaminated sediment from oil platform	Indigenous bacteria	Not described	10 g oily sediments with additional surfactants, incubated at 30 °C at 120 rpm	51.29% in 28 days	[71]
Polluted water	Consortium of protozoan species, *Aspidisca* sp., *Trachelophyllum* sp. And *Peranema* sp.,	Not described	Bioreactor at 30 °C, shaken continuously at 100 rpm	61–90% by natural attenuation; 22–55% by nutrient supplementation with glucose; 10–67% by biostimulation with surfactant	[72]
Crude oil	*Pseudomonas aeruginosa* NCIM 5514	Not described	Incubated at 37 °C at180 rpm	Oil viscosity reduced from 1883-1002 cp; 61.03% and 60.63% biodegradation of C8–C36+	[73]
Dehydrated crude oil	*Pseudomonas* sp., *Bacillus* sp., *Ochrobactrum* sp.	Not described	10% inoculation, and shaking at 120 rpm	47% in immobilized bacteria; 26% in free bacteria in 5 days	[74]
Hexadecane	*Aspergillus niger*	Not described	Electric field pretreatment	53–86% in 48 h	[75]
Polluted seawater	*Dietzia maris* CBMAI 705 and *Micrococcus* sp. CBMAI 636	Not described	Incubated at 28 °C at 120 rpm	99% in 21 days	[76]
Refinery wastewater	*Alcaligenes odorans*, *Bacillus subtilis*, *Corynebacterium propinquum* and *Pseudomonas aeruginosa*	Not described	37 °C in aerobic condition	70%	[77]

3.2. Biodegradation of Waxy Petroleum Hydrocarbons

Waxy hydrocarbons are associated with long-chain alkane compounds of C17 to C55 that tend to deposit on the cold surface [78]. According to a study, methane-monooxygenase enzymes oxidize short-chain alkanes C1–C4, cytochrome P450 enzymes oxidize medium-chain alkanes C5–C16, and essentially unknown enzyme systems oxidize long-chain alkanes C17+ [79]. However, a study on the biocatalytic degradation of high-paraffinic crude oil has characterized long-chain alkane hydroxylase homologues AlkMa and AlkMb and encoded a putative flavin-binding monooxygenase, AlmA [80]. AlkB-type alkane hydroxylase homologues alkMa and alkMb, from *Alcanivorax borkumensis* SK2 [81,82] and *Acinetobacter* DSM 17874 [83], have been demonstrated to be involved in the degradation of n-alkanes with chain lengths of C10 to C20. It was also reported that AlkB genes in *Anoxybacillus* sp. WJ-4 were responsible for a 58.75% degradation of C8 to C22 [84]. It has been discovered that the cytochrome P450 monooxygenases gene in *Alcanivorax dieselolei* B-5 can grow on

C6 to C12. [85]. The flavin-dependent monooxygenase LadA was discovered in *Geobacillus thermodenitrificans* NG80-2, which degrades C15 to C36 [86]. AlmA is the first cloned gene proven to be involved in the bacterial degradation of long-chain n-alkanes of C32 and longer [83]. Other than alkane hydrolase and monooxygenase, lipase in *Stenotrophomonas maltophilia* was found able to degrade 94.13% of diesel oil, which was mainly composed of C12 to C20 [87]. Asphaltene, a fraction of petroleum, has a complex structure; hence, it is difficult to break down. A study reported that catechol dioxygenase and laccase in *Pestalotiopsis* sp. NG007 played an important role in the 77% degradation of C13-C30 asphalt [88]. Moreover, 82–90% of C32 and 82–88% of long-chain alkane C40 were degraded by thermophile bacteria strains *Geobacillus stearothermophilus* IR2, *Geobacillus thermoparaffinivorans* IR4 and *Bacillus licheniformis* MN6. The enzyme assays showed the presence of alkane hydroxylase, alcohol dehydrogenase and lipase [89]. A study on the degradation rate of motor oil using *Alcanivorax borkumensis* successfully achieved a 75% degradation due to the production of biosurfactants, which allowed the media to mix well with the hydrocarbon sample through reduction of interfacial tension, thus allowing degradation to happen [90]. These studies highlight a few important hydrocarbon-degrading enzymes that can be potentially sourced for the purpose of managing wax deposition in transportation lines (Table 3).

Table 3. Microbial treatment of petroleum hydrocarbons and the assayed enzymes.

Source of Hydrocarbon	Hydrocarbon-Degrading Microbe	Reported Enzymes	Hydrocarbon Treatment Condition	Hydrocarbon Removal Efficiency	Reference
Crude oil	*Bacillus subtilis* SL and *Pseudomonas aeruginosa* WJ-1	Not described	Incubated for 7 days at 37 °C at 180 rpm	32.61% in *Bacillus subtilis* SL; 54.35% in *Pseudomonas* sp. WJ-1; 58.60% in SL and WJ-1	[91]
n-hexadecane	Immobilized *Bacillus thuringiensis* (BTS)	Not described	37 °C at 120 rpm, and pH 7	81.62–86.65% in 192 h	[92]
Alkane C7-C25	*Bacillus subtilis* BL-27	Not described	Incubated at 45 °C at 150 rpm	65% in 5 days	[93]
Crude oil	*Raoultella ornithinolytica* PS, *Bacillus subtilis* BJ11, *Acinetobacter lwoffii* BJ10, *Acinetobacter pittii* BJ6, *Serratia marcescens* PL	Not described	0.2 g crude oil with 5 mL strain, incubated at 30 °C at 180 rpm for 10 days.	70–94% in 10 days	[94]
Crude oil	*Rhodococcus erythropolis* OSDS1, *Serratia proteamaculans* S1BD1, *Alcaligenes* sp. OPKDS2, *Rhizobium erythropolis* OSDS1, *Rhizobium* sp. PNS1, *Pseudomonas* sp. BSS9BS1	Not described	Incubated at 30 °C at 130 rpm	85.26% 15 days	[95]
Crude oil	*Cupriavidus* sp. OPK, *Rhodococcus erythropolis* OSPS1, *Pseudomonas* sp. BSS	Not described	Incubated at 120 rpm in dark room	74–83% in 3 days	[96]
Diesel	*Halomonas* sp. and *Aneurinibacillus* sp.	Not described	Incubated at 151 rpm at 30 °C	82.65% in 12 days	[97]
Bonny light crude oil	*Bacillus* sp. SB4, *Pseudomonas* sp. SC8, *Serratia* sp. SC11, and *Acinetobacter* sp. SC12	Not described	Incubated at 180 rpm at 30 °C	12–36%	[98]

Table 3. *Cont.*

Source of Hydrocarbon	Hydrocarbon-Degrading Microbe	Reported Enzymes	Hydrocarbon Treatment Condition	Hydrocarbon Removal Efficiency	Reference
Petroleum hydrocarbons, hexane, hexadecane and motor oil	*Alcanivorax borkumensis*	Alkane hydroxylase, lipase and esterase	Incubated at 30 °C at 150 rpm	75–81.5% in 72 h	[90]
Hexadecane and BTEX	*Alcanivorax borkumensis* SK2	Alkane hydroxylase, lipase and esterase	Incubated at 32 °C at 120 rpm	79–96.7%	[82]
Long-chain n-alkanes C32 and C40	*Geobacillus thermoparaffinivorans* IR2, *Geobacillus stearothermophillus* IR4 and *Bacillus licheniformis* MN6	Alkane hydroxylase, alcohol dehydrogenase and lipase	Incubated at 50 °C at 120 rpm	87–90% in 20 days (for C32); 82–88% in 20 days (for C40)	[89]
Belayim Mix crude oil	*Lipomyces tetrasporus* RS-Y1 and *Paecilomyces variotii* RS-F3	Not described	Incubated at 28 °C at 150 rpm	58.15–68.3% in 30 days	[99]
Alkane mixture C8–C22	*Anoxybacillus* sp. WJ-4	alkB homologs genes	Incubated at 70 °C at 180 rpm	58.75% in 40 days	[84]
Crude oil (C12-C25) and asphalt (C13-C30)	*Pestalotiopsis* sp. NG007	Catechol 1,2-dioxygenase, catechol 2,3 dioxygenase, laccase, manganese peroxidase, lignin peroxidase	Incubated at 25 °C	77–92% in 30 days	[88]
Crude oil	*Scenedesmus obliquus* and *Chlorella vulgaris*	Not described	Incubated at 25 °C at 80 rpm under dark condition	46–88% in 6 weeks	[100]
Crude oil	*Bacillus atrophaeus* 5-2a, *Bacillus aryabhattai* 6-2a and *Bacillus amyloliquefaciens* 6-2c	Not described	2 g crude oil in 20 mL bacterial suspension, incubated at 40 °C	82.32–94.50%	[101]
Diesel oil and used engine oil	*Stenotrophomonas maltophilia*, *Bacillus cereus* and *Bacillus pumilus*	Lipase	Aerobic conditions at 30 °C	94.13% in diesel oil; 99.77% in used engine oil	[87]
Alkane C8–C36	*Alcanivorax dieselolei* B-5	AlkB homologs (AlkB1 and AlkB2), CYP153 homolog (P450), alkane hydroxylase (AlmA)	Incubated at 28 °C at 200 rpm	p450 was upregulated on C8–C16; AlmA on C22–C36; alkB1 and alkB2 C12–C26	[85]
Alkane C5–C36	*Geobacillus thermodenitrificans* NG80-2	Alkane monooxygenase LadA		LadA was upregulated on C15–C36	[86]

4. Biodegrading Mechanism of Petroleum Hydrocarbons

The degradation of petroleum hydrocarbons is a complex process involving a variety of enzymes that work synergistically to break down the complex hydrocarbon molecules into simpler compounds. Some of the key enzymes in hydrocarbon degradation are the oxygenases, dehydrogenases, hydrolases and reductases. Oxygenases are enzymes that introduce one or two oxygen atoms derived from molecular oxygen in the alkane

substrate [102], which are monooxygenases and dioxygenases, respectively. Alkane hydroxylases and cytochrome P450 monooxygenases are an example of monooxygenases that have been reported widely. Dioxygenases such as catechol dioxygenase and naphthalene dioxygenase incorporated both atoms of molecular oxygen to cause a ring cleavage in an aromatic compound [103]. Dehydrogenases are the enzymes that catalyze the removal of hydrogen atoms from the hydrocarbon molecules, leading to a formation of oxidized products and facilitating the further breakdown of the hydrocarbon. Alcohol dehydrogenase and aldehyde dehydrogenase have been reported in this category [104,105]. Hydrolases catalyze the hydrolysis reaction, which involves the cleavage of a chemical bond using water. For example, esterase breaks down ester linkages present in some of the hydrocarbon derivatives [106]. Finally, reductases catalyze the addition of hydrogen atoms to a certain hydrocarbon intermediate, where the reduction step helps to activate the molecule for further degradation processes. As an example, carbonyl reductase catalyzes the reduction of carbonyl groups (C=O) present in aldehyde and ketones, and the products can be further metabolized by other enzymes [107,108].

Alkanes are also known as paraffins, which translates to the alkanes having low chemical reactivity [109]. To biodegrade a petroleum, the alkane molecules need to be activated to initiate and continue the stepwise metabolisms [110]. The regio- and stereoselective oxidation of non-activated methyl or methylene groups is a challenging, but it is a crucial chemical process [111]. Alkane degradation can be achieved via oxygen-dependent oxygenase, which triggers the initial activation and breaking of C-H bonds in aerobic conditions [112].

Alkane degradation in microorganisms had been described and illustrated by three peripheral metabolic pathways, which include terminal oxidation, subterminal oxidation, and ω-oxidation (Figure 4). The methyl group of alkanes is oxidized by the alkane-activation enzyme alkane monooxygenase (AMO), involved in both the terminal and subterminal pathways. The reactions render a primary alcohol in terminal oxidation and a secondary alcohol in subterminal oxidation. Alcohol dehydrogenase (ADH) converts the alcohol into aldehydes (for terminal) and ketones (for subterminal). Aldehydes are further oxidized to fatty acid by aldehyde dehydrogenase (ADH), while ketones are oxidized by Baeyer–Villiger monooxygenase (BVMO) to render esters, which are subsequently hydrolyzed to alcohol and fatty acid by carboxylesterase (CE). The fatty acids are conjugated to coenzyme A and enter the β-oxidation cycle to generate acetyl-CoA [113,114]. Alkane molecules can also be oxidized at both ends through the ω-hydroxylation of fatty acids at the terminal ω-methyl group, rendering an ω-hydroxy fatty acid that is further converted into a dicarboxylic acid and processed by β-oxidation. The subterminal oxidation of alkanes generates a secondary alcohol, which is converted to the corresponding ketone and then oxidized by a Baeyer–Villiger monooxygenase to render an ester. The ester is hydrolyzed by an esterase, generating an alcohol and a fatty acid, and enters β-oxidation. β-oxidation plays a crucial role in fatty acid metabolism and energy production, but it is not directly involved in the breakdown of alkane molecules during alkane degradation. Both terminal and subterminal oxidations can coexist in the microorganisms [113–115].

The research related to the biodegradation of wax has led to the discovery of many bacteria species hosting enzymes involved in the initial step of aerobic degradation pathways. *Acinetobacter* sp., *Alcanivorax* sp., *Arthrobacter* sp., *Bacillus* sp., *Dietzia* sp., *Geobacillus* sp., *Marinobacter* sp., *Mycobacterium* sp., *Pseudomas* sp., and *Rhodococcus* sp. are among the bacterial strains capable of utilizing C18 and more [116]. A similar discovery is that alkanes are oxidized to the corresponding primary and secondary alcohol by substrate-specific terminal and subterminal monooxygenases or alkane hydroxylases. Alkane hydroxylases are flexible biocatalysts that perform a variety of beneficial oxidation reactions. *Pseudomonas putida* GPo1 encoded the functional alkane hydroxylase that oxidizes C5 to C16 [117]. AlkB-type enzymes work with two electron-transfer proteins—a dinuclear iron rubredoxin and a mononuclear iron rubredoxin reductase—to transfer electrons from NADH to the active site of alkane hydroxylase [113]. *Acinetobacter* sp. Strain M-1 was shown to harbor

two integral membrane alkane hydroxylases responsible for the degradation of C10 to C30 [118]. Interestingly, AlkB, found in *Acinetobacter oleivorans* DR1, was able to utilize long-chain alkanes C12 to C36, but not short-chain alkanes C6 to C10. AlkB1 was responsible for long-chain alkanes C24 to C26, whereas AlkB2 was responsible for medium-chain alkanes C12 to C16 [119]. The recombinant strain *Rhodococcus erythtopolis* AP-expressing AlkB gene cluster was experimentally confirmed to degrade C12 to C24 [120]. The study comprehensively reported that the alkB gene cluster includes an alkB gene encoded for alkane hydroxylase, two rubredoxins (A and B), and rubredoxin reductase. The proteins rubredoxin and rubredoxin reductase are required to shuttle electrons to AlkB, which the enzyme uses for alkane hydroxylation [120]. Five proteins were expressed by *Thalassolitus oleivorans* hosting the terminal oxidation of C14, which were characterized as alkane 1-monooxygenase, oxidoreductase ferredoxin, ferredoxin reductase, alcohol dehydrogenase, and aldehyde hydrogenase. The ferredoxin reductase oxidized NAD(P)H to NAD(P)+, generating electrons that were transferred to ferredoxin, which shuttled the electrons to alkane monooxygenase. Alkane monooxygenase introduces oxygen into alkanes at the terminal site, converting them into primary alcohol. The alcohol is further oxidized to aldehyde and fatty acids by alcohol dehydrogenase and aldehyde dehydrogenase. The pathway was then possibly switched to subterminal oxidation when grown on the longer-chain C28, evidenced by the significant upregulation of Baeyer–Villiger monooxygenase and esterase, responsible for catalyzing ketones and ester metabolism [121]. Long-chain thermophilic alkane monooxygenase was discovered when a genome and proteome analysis of *Geobacillus thermodenitrificans* strain NG80-2, isolated from a deep-subsurface oil reservoir [122], revealed a plasmid-encoded thermophilic enzyme designated as LadA. LadA was determined to be involved in the terminal oxidation of long-chain alkanes able to convert C15 to C36 to primary alcohol. The research highlighted many attributes of LadA: it was a thermophile, able to act on long-chain alkanes, a single-component with no coenzyme requirement, soluble (extracellular), and easily expressed and purified in *E. coli*, making it a great biocatalyst for industrial applications [86].

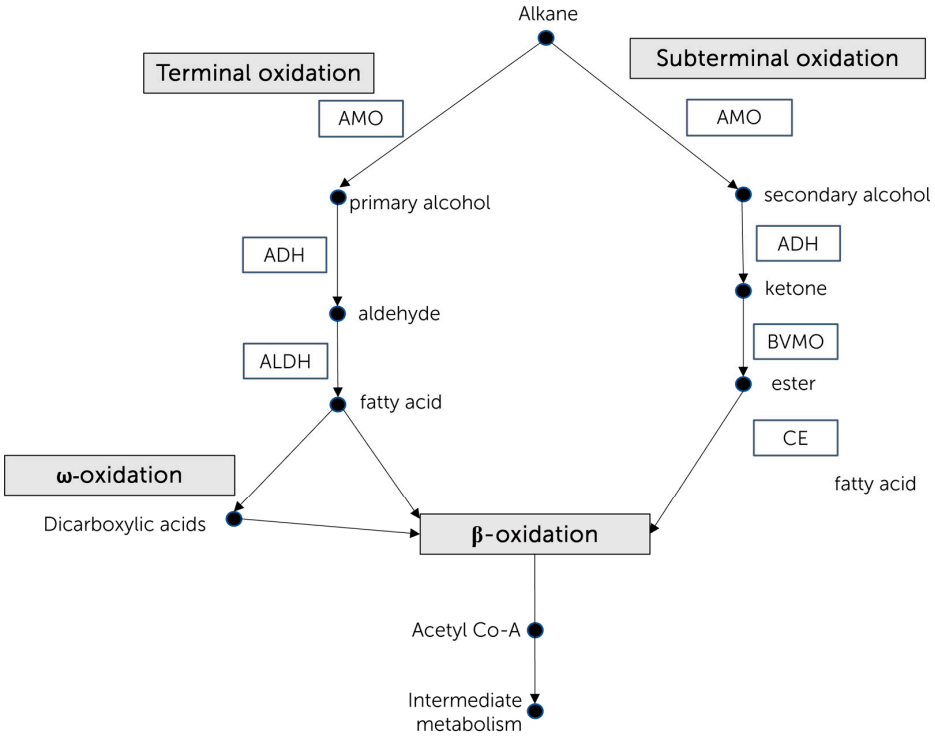

Figure 4. Reported alkane-degrading pathways, adopted and re-illustrated from Beilen et al., 2007 [79].

5. Biocatalytic Degradation of Petroleum Hydrocarbons—Opportunity for Application and Gaps in Know-How

The petroleum industry is looking into energetically efficient processes with minimum environmental impact. A novel and nonconventional technique like enzyme catalysis for hydrocarbon management has been explored. With properties such as high transformation efficiency and high specificity, enzymes can be utilized not only for cleaning up oil-contaminated water and soil but also for the treatment of viscous heavy oil for transportability, as well as for refining petroleum [102].

The direct application of microbial metabolites such as enzyme extracts or recombinant enzymes, in contrast to whole organisms, is advantageous for the petroleum industry. This is attributed to better process control in terms of enzyme concentration, faster reactions, the ability to remediate complex and persistent compounds, and specificities that enable enzymes to perform at wide range of temperature, pH and salinity levels [123,124]. Enzymes are also biodegradable proteins, so there is no environmental persistence problem. Furthermore, recombinant DNA technology has been introduced to improve the stability and activity of enzymes at a larger scale and lower cost [125]. The conversion or transformation of inert nonpolar hydrocarbons is a challenging biochemical reaction for a single enzyme but is tolerable for multi-enzyme systems [126]. Research has recently reported the usage of an enzyme consortium consisting of oxygen-dependent oxygenase, which is able to catalyze the cleavage of the C-H bond in long-chain wax in hydrocarbons into carbon of a shorter chain, resulting in improved flowability [127]. The concept is illustrated in Figure 5.

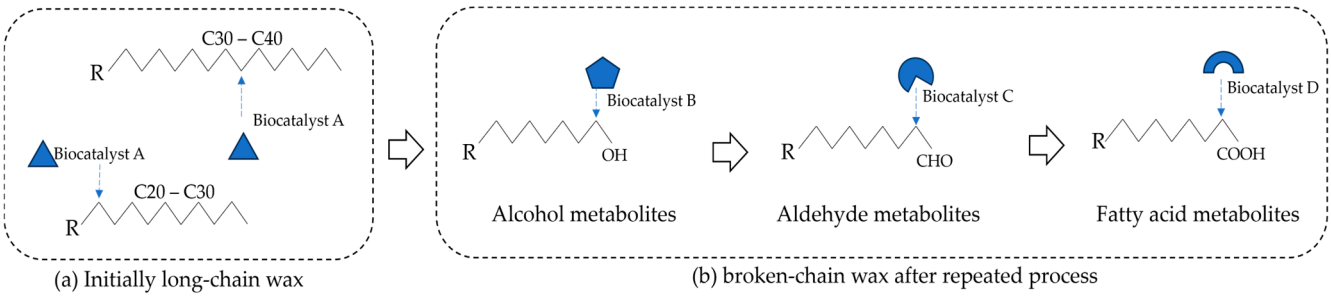

Figure 5. Illustration on using enzyme consortium consisting of different types of biocatalyst for paraffin wax degradation.

Table 4 summarizes the research findings from a reaction system consisting of enzyme consortiums extracted from microbes and their targeted hydrocarbon substrate. It was evidenced from these studies that intracellular and extracellular enzymes are capable of degrading up to 99% of the hydrocarbon.

However, similar conditions were applied in most of the studies: a long treatment time, i.e., more than 24 h; the petroleum substrates used are mostly of short- and medium-chain alkanes, less than C30; and essentially, the partial degradation of wax or asphaltene leads to a reduction in the viscosity of heavy petroleum [128]. However, the analyses mainly discuss the degradation products. Elaborations on the changes in physical or flow properties of the reacted petroleum, i.e., viscosity, morphology, waxiness, are very limited. On contrary, the application of the enzymatic degradation of wax in production lines needs to occur in hydrocarbon-rich environments (non-aqueous system) in a short treatment time, because the petroleum-production process is a continuous operation. In addition, waxy crude oils are composed of a high percentage of chain alkanes more than C30. Therefore, these limitations needed to be studied in detail.

Table 4. Enzymatic degradation of hydrocarbon.

Hydrocarbon-Degrading Enzyme	Substrate	Treatment Condition	Hydrocarbon Removal Treatment Efficiency	Reference
Intracellular crude enzyme consortium from *Providencia rettgeri* L1 supplemented with formate dehydrogenase	Petroleum residual oil	10 mL reaction system containing crude enzymes and residual oil at 30 °C for 8 h	23.8% oil degraded at 1 h; 56.7% at 5 h; 49.5% at 8 h	[129]
Enzyme consortium of alkane hydroxylase and lipase from *Alcanivorax borkumensis* SK2	Soil contaminated with diesel fuel	5000 L enzyme solution injected into 4 injection wells at 14 lpm for 12 weeks	36–99% degradation of C10-C50	[130]
Protease, catalase, lipase, and amylase from peel wastes of sweet orange *Citrus sinensis* Osbeck and watermelon *Citrullus lanatus*	Used motor oil-contaminated soils	Enzyme solutions added to the contaminated soils for 6 weeks	62–74% TOC removal by orange peel enzymes; 39–45% TOC removal by watermelon peel enzymes	[131]
Intracellular enzymes from *Acinetobacter calcoaceticus* 21 and formate dehydrogenase CbFDH from *Candida boidinii*	Oil-contaminated sludge	2 g oily sludge with 10% oil by weight reacted with enzyme 21 and CbFDH at 30 °C at 150 rpm	35.6% oil degraded in 12 h using enzyme 21/CbFDH with the protein ratio of 1:4	[132]
Ligninolytic enzyme Laccase and Manganese Peroxidase microencapsulated in bilayer cross-linked Ca-alginate beads	Polluted sediment	Incubated in the dark at 25–27 °C with vertical mixing at 50 rpm for 70 days	27–28% total petroleum hydrocarbon degraded	[133]
Intracellular and extracellular enzymes from *Aspergillus* sp. RFC-1	Oilfield crude oil	Extracellular and intracellular enzymes inoculated into 20 mg/L crude oil, cultivated at 30 °C at 120 rpm for 7 days	Degradation efficiencies of crude oil by extracellular enzymes were higher (7–25%) than by intracellular enzymes (5–24%)	[134]
Alkane hydroxylase, lipase and esterase from *Alcanivorax borkumensis* crude enzyme preparation	Hexadecane, motor oil, contaminated soil	50 mL Milli-Q water, 10 mg/mL crude enzymes and petroleum sources, incubated at 30 °C for 7 days at 100 rpm	Removal of hexadecane (73.75% to 59.74%); motor oil (74% to 83%); contaminated soil (88.52%)	[37]
Fungal enzymes *Aspergillus fumigatus* (PJ1, PJ2, PJ3 and PJ5), *Aspergillus flavus* (PJ4), and *Aspergillus terreus* (PJ6)	Oil-contaminated soil	2 g crude oil and 30 mL of crude enzyme solution in 100 mL glass bottles sealed with rubber stoppers, incubated statically at 40 °C for 4 days, with regular shaking every 4 h under oxygen deprived conditions	3.70–15.68% removal of alkane, 23.33–40.56% of aromatics, 13.33–35.56% of resins, and 24.69–34.57% of asphaltenes. Oil viscosity reduced by 40.5–59.0%. Total oil removal efficiency of 83.40% to 87.78%	[135]
Dioxygenase immobilized onto single-walled carbon nanotube from *Arthrobacter chlorophenolicus* A6	Aromatic hydrocarbon intermediates catechol, 4-chlorocatechol and 3-methylcatechol)	Free and immobilized enzymes added to substrate solution and H2O2 as oxygen source at molar ratio 1:2, then mixed at 25 °C for 1 min. The precipitate was separated by centrifugation.	Relative activity of immobilized enzyme is the highest at pH 7.5, temperature 40 °C, and 0.6 M salt; and retained 40% of activity after 7 cyles of reusability	[103]

Table 4. *Cont.*

Hydrocarbon-Degrading Enzyme	Substrate	Treatment Condition	Hydrocarbon Removal Treatment Efficiency	Reference
Versatile peroxidase (VP) from white-rot fungus *Bjerkandera adusta*	Antracene in silicone oil	Two-phase partitioning bioreactor with 250 mL reaction medium containing 10% silicone oil saturated with anthracene at 30 °C	61.88% oxidation of anthracene after 38 h	[123]
Intracellular and extracellular enzymes from *Pseudomonas* sp., *Bacillus* sp., *Ochrobactrum* sp	Dehydrated crude oil	Extracellular enzyme inoculated into 20 mg/L of crude oil, cultivated at 30 °C g at 120 rpm for 18 days	6–15% oil degradation in extracellular enzyme; 4–15% in intracellular enzyme	[136]

6. Challenges of Applying Enzymatic Degradation to Petroleum Industry

One of the obstacles that hinders the enzymes from replacing chemicals for industrial applications is the high cost of production and purification. There are a few factors contributing to this. Firstly, enzymes undergo denaturation during production and storage. The unfolding of the enzyme tertiary structure to a disordered polypeptide causes the residues to be unaligned, therefore hindering the interactions in its functional groups. Secondly, the presence of other chemicals in the petroleum-production system causes an irreversible loss of activity [137]. During oil and gas operations, enzymes are prone to denaturation and loss of activity because of three factors: temperature and pressure stability at the hydrocarbon production facilities, e.g., reservoirs, production flowlines and transportation pipelines; high salt stability due to the carry-over of seawater; and stability in non-aqueous media, i.e., a hydrocarbon-rich environment. Research has also highlighted that biocatalysts should be kinetically efficient and have little dependency on enzyme cofactors to be cost-competitive [138]. However, with the advancement of proteomics and molecular biology, enzyme production can be optimized to resist and withstand petroleum-production's operating conditions.

Apart from the cost competitiveness between enzymes and chemicals for wax treatment, the physicochemical characteristics of hydrocarbons are also a challenge. Hydrocarbon wax is nonpolar, while asphaltene and resin are polar constituents that contain both normal and polycyclic aromatic hydrocarbons [139]. A polyaromatic hydrocarbon is chemically stable due to resonance energy and the high number of aromatic rings [75]. Therefore, petroleum hydrocarbons with high asphaltene and resin content may be associated with slower biodegradation. Petroleum hydrocarbons are chemically inert, and high energy barriers must be overcome to initiate the activation and cleavage of carbon–hydrogen bonds. The hydrophobic property of the petroleum is the main obstacle for the enzymatic attack of the carbon backbone [140]. In addition, petroleum hydrocarbons contain crystalline, semi-crystalline and amorphous regions. Only the amorphous and semi-crystalline regions are more susceptible to enzymatic degradability, as illustrated in Figure 6.

For enzymatic degradation to be successful, one must identify the most suitable enzyme for that application. For example, extracellular enzymes with high substrate affinities can produce high-product turnover, and the ability to maintain stable activity under the operational conditions of the intended application is preferable [141]. However, the enzymatic performance depends on the physical conditions of the sites, such as temperature, pH, and salinity, and the enzyme properties, such as catalytic sites and enzyme activity [124]. The enzymes are exposed to the harsh conditions of high temperature, pressure, and fluid turbulence. The movement of petroleum from a reservoir to the surface is a continuous process. The temperature of a freshly produced hydrocarbon can be as high as 60 to 70 °C, while the pH is typically 7 to 9 [1]. Apart from temperature and pH factor, the catalytic activity of the selected enzymes must not be inhibited by metals. Salts, or dissolved metal

ions present in seawater, are produced together with the petroleum from the reservoir containing Na^+, Mg^{2+} and Ca^{2+} as the major constituents, and the composition is varied for different reservoirs [142]. Under an aerobic environment, the C-H bond activation can be catalyzed by the oxygen-dependent oxygenase, which is ubiquitous in the case of petroleum-polluted sites [103]. The dissolved oxygen from the produced water, which is entrained with petroleum during production, is available for the oxygenase during wax degradation [143,144].

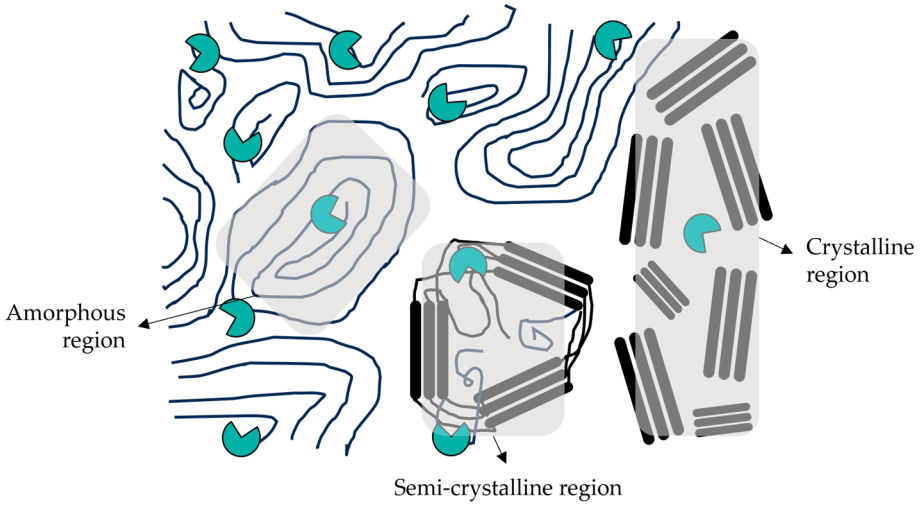

Figure 6. Illustration of how the amorphous region of petroleum is more susceptible to enzyme attack than the crystalline region (re-illustrated from Wei and Zimmerman, 2017 [140]).

7. Current Approaches and Future Outlook of Applying Enzymatic Degradation for Wax Management in Petroleum Operations

Enzyme stability and efficiency are the main challenges for industrial application; therefore, powerful tools of molecular biochemistry should be used for improvement. Enzymes isolated from extremophilic microorganisms that are resistant to pH, high salinity, and organic solvents; thermostable; and thrive in a reduced oxygen environment [145] are the most beneficial for the petroleum industry. Extremophilic enzymes derived from *Halobacterium* sp., *Haloferax* sp., *Haloarcula* sp. and *Pyrococcus* sp. have been reported to have salt-adapted ability, while *Rhodoccus* sp. and *Pseudomonas* sp. are reported to host organic-solvent active enzymes [146]. Enzymes adapted to high-salt environments, known as halophilic enzymes, can retain their functional conformation in the presence of high ionic strength, and they possess stable, multilayered hydration shells that help to preserve their biological functions [146]. The new discovery of thermostable enzymes from microorganisms living in extremely high temperatures of ~100–200 °C, such as deep reservoirs, is pertinent. Enzymes from the genus *Bacillus* sp. are commonly reported as being thermophilic [89,147]. Other than *Bacillus* sp., thermophilic enzymes can also be derived from *Thermomicrobium* sp. and *Thermooleophilum* sp., and dominantly from *Geobacillus* sp., and are typically active at 57 to 80 °C [148]. This is an important physicochemical property for enzyme application as petroleum facilities, from production to refining, typically operate at high temperatures and salinity. In addition, the enzyme catalytic treatment of petroleum performed at high temperatures is beneficial for high-viscosity substrates. At high temperatures, the viscosity of the petroleum is reduced, leading to an increase in diffusion coefficients; therefore, the bioavailability of the enzymes is improved [148]. Enzymes should be able to operate in non-aqueous system due to the hydrophobicity of petroleum, thereby limiting the mass transfer and interaction between the enzymes and the hydrophobic substrates. This probably can be solved using reaction mixtures containing organic solvents to increase the substrate accessibility, but the high enzyme activity must be maintained, as supported by [149]. In addition, the enzyme–wax interaction can be

facilitated by specific structural features of the enzyme, such as hydrophobic pockets or active sites that can accommodate nonpolar molecules [150]. In some cases, enzymes can be immobilized on hydrophobic supports to enhance the interaction with hydrophobic substrates [151]. On top of that, there is also research that provides a positive indication of using enzymes from the genus *Pseudomanas* sp. for the biodegradation of plastic polymers such as polyethylene, polyethylene glycol and polyethylene terephthalate [152]. Furthermore, 95% of polyethylene terephthalate was found to be degraded in a heterogenous enzymatic reaction [153]. In the case of wax treatment in a production line, a similar approach can be adopted due to the resemblance in characteristics between plastic polymers and waxy crude oil, such as hydrophobicity, high molecular weight, and lack of favorable functional groups. To find an adequate solution for petroleum hydrocarbons, the enzyme complexes' behavior, metabolites, and degradation pathways must be explored [154].

The industrial application of biocatalysts is often restricted by the short-term operational stability and difficulty in the recovery of enzymes. However, these limitations are overcome by immobilization, although there is no specific remedy protocol because each enzyme has different properties. The primary objective of enzyme immobilization is to increase the economics of the biocatalytic process. The immobilization of enzymes is a highly valuable technique that was introduced to overcome the limitation of free enzymes. The immobilization technique has evolved to a simple, fast, and efficient process [155]. Covalent binding, physical adsorption, or enzyme entrapment through immobilization lead to high number of enzymes being in contact with the high surface area of support, subsequently discouraging the dissociation of protein into subunits or the formation of inactive intermolecular aggregates [156]. Immobilization supports like silica encapsulation protect microorganisms from lysis and harsh environments of high salinity and organic load, in addition to concentrating their amount, thus accelerating the degradation process [124]. The potential applications of immobilized enzymes are broadened because of their high versatility such as high enzyme/substrate ratio, increased functional efficiency of the enzyme, minimized reaction time, and minimized contamination in products [157]. Immobilized enzymes have an enhanced tolerance to variations in environmental factors, such as temperature, pH, organic solvents, and long storage [158]. Immobilized enzymes are easy to control, whereby the reaction can be stopped by physical removal rather than by heat inactivation when using soluble or free enzymes [159].

The stability of immobilized enzymes concerning temperature and pH is widely reported in the literature to translate the benefit of the immobilization technique for soluble or free enzymes [160]. For example, Mazlan (2017) immobilized laccase on polymer microspheres. They found that the optimum temperature for laccase is extended from 40 °C (in free form) to 50 °C (in immobilized form), while the optimum pH is shifted from 4 (in free form) to 5 (in immobilized form). The reasons is that the covalent bond formation, through the amino groups of the immobilized enzyme, causes the molecule to have a higher activation energy for the better reorganization and conformation of substrate binding. Also, the multipoint attachments of the enzyme support increased enzyme stabilization; thus, an extended optimum temperature and pH were observed [161]. The immobilization of horseradish peroxidase on iron oxide magnetic nanoparticles using physical bonding also produced a similar optimum temperature and pH shifting [162,163]. Oxidoreductases are not stable under various conditions, whereby pressure, temperature and pH may cause a dissociation of the enzyme subunit. Basri (2022) immobilized *Mycobacterium phlei* carboxylic acid reductase (*Mp*CAR) onto polymeric support Seplite LX120 via an adsorption technique to achieve 3 weeks of storage stability at an ambient temperature. The optimum reaction temperature and pH of the *Mp*CAR were shifted from 42 °C and 7.5 (in free enzyme) to 60 °C and 9 (in immobilized form) [164]. An immobilized aldehyde dehydrogenase from *Anoxybacillus geothermalis* D9 exhibited an improvement in temperature stability from 30 to 90 °C in contrast to its free form [104]. Immobilized carboxylesterase from *A. geothermalis* yielded a broad thermal stability and pH tolerance, with an optimal temperature of 80 °C and an optimal pH of 7 (Johan et al., 2023 [165]). Bolivar (2012) elaborated that

under soluble or free conditions, enzyme subunits dissociate under an acidic condition, whilst tertiary and quaternary structures are distorted under an alkaline condition [166]. Through enzyme immobilization, the conformational freedom of the enzyme is narrowed due to the multipoint covalent attachment of the enzyme that causes a reduction in the intermolecular reaction; thus, the pH stability is enhanced [167]. pH affects the ionization of functional groups within enzymes, and when immobilized, the enzyme has a better orientation of active sites and thus a higher affinity towards substrates, as reflected in the higher activity [163]. The rigidity in conformation also enables enzymes to withstand higher temperatures for a longer time [168–170]. Zhou (2001) cross-linked ß-galactosidase to graphite surfaces and studied the good shifting of optimum temperature and pH. They provide a more straightforward explanation centered around diffusional effects, in which the immobilized enzyme is easily contacted by substrates with the increase in temperature and pH [171].

However, the success rate of enzyme immobilization to produce high catalytic activity and the stability of enzymes depends on immobilization time, procedure, surface coverage, surface curves, active sites orientation, mass transfer and chemical bonding [158]. A study has pointed out that the porosity and particle size of the immobilized support could influence the mass transfer kinetics between the reactant (the hydrocarbon) and the enzymes. A maximum efficacy of the reactant diffusivity may exist, related to the particle size of the immobilized enzymes, and need to be determined experimentally [62].

Enzymes are always associated with high costs; therefore, efforts should be made to recover and reuse them instead of continuously putting them in production lines, which is a more suitable practice for chemicals. Enzyme immobilization provides an opportunity for separation and reuse because immobilized enzymes are stationary throughout the application process instead of mobile with the reactant. The recovery of enzymes makes the application cost-attractive because of the reduction in production costs, labor costs and space requirements [172]. Where the productivity over time and volume of hydrocarbons to be treated is high, fluidized bed reactors are preferred (Figure 7). Immobilized enzyme particles can be sustained in suspension by the flowing stream of hydrocarbons [173].

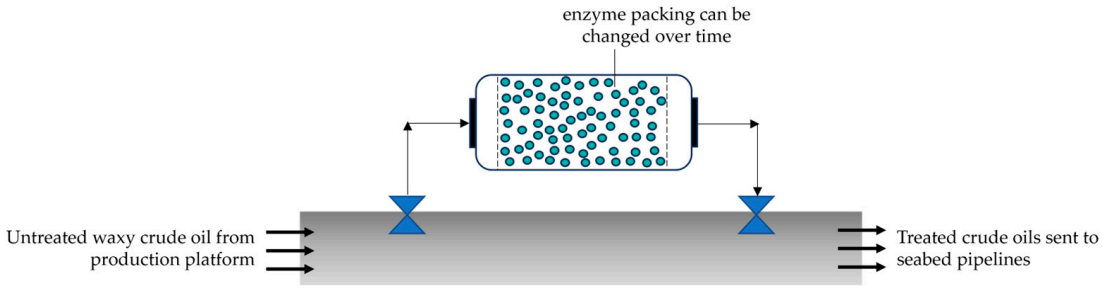

Figure 7. Proposed fluidized bed reactor concept for recovery and reuse of enzyme.

8. Conclusions

This paper reviews the potential of exploiting enzymes to tackle the wax deposition problem in petroleum-production lines. Enzymes' catalytic activity can be enhanced in the laboratory to suit specific applications. Research evidence on the microbial degradation of petroleum-contaminated sites successfully highlighted AlkB homologs, alkane hydroxylases, alkane monooxygenases, lipase, laccase and esterase as highly capable hydrocarbon-degrading enzymes. Based on this precursor, this review narrowed down the research and focused on papers related to the microbial degradation of specific alkane compounds and crude oil. A similar group of enzymes was identified and was reported to be able to remove 12–96% of hydrocarbons in the alkane compound in 3–42 days. Based on these studies, it was evidenced that hydrocarbon-degrading enzymes are a good alternative for wax deposition treatment in transportation lines. This paper then discussed the possible challenges for the enzymes when applied in wax deposition. The costly production of

enzymes is the main factor, attributed to enzyme denaturation and the presence of unexpected chemicals that reduce catalytic activity. The enzymes' dependency on cofactors may also reduce their cost-competitiveness in comparison to chemical treatment. Secondly, the hydrophobicity of petroleum hinders enzymatic degradation. Petroleum hydrocarbons are chemically inert, and high energy barriers must be overcome to initiate the activation and cleavage of the carbon–hydrogen bond. Thirdly, enzymes are subjected to the harsh conditions of high temperature, pressure, and fluid turbulence in production lines; therefore, enzyme performance may be deteriorated. However, the challenges can be overcome by the combination of technological advancement and application strategies. The current approaches and prospects for enzyme application in the petroleum industry were discussed next. Recombinant enzymes with thermophilic and halotolerant properties have been discovered in petroleum degradation, although research is scarce. Hence, efforts should be made to enrich this discovery. The direct application of enzyme extracts, in contrast to whole organisms, is advantageous for the petroleum industry in terms of better process control, faster reactions, and the ability to perform in wide range of temperature, pH and salinity. Additionally, using a consortium of enzymes is favorable since the transformation of inert nonpolar hydrocarbons is a challenging biochemical reaction for a single enzyme but is tolerable for multi-enzyme systems. With the usage of powerful tools of molecular biochemistry, enzyme stability and efficiency can also be improved. Enzymes isolated from extremophilic microorganisms that are resistant to pH and organic solvents, thermostable, and thrive in a reduced-oxygen environment are the most beneficial for the petroleum industry. The advances in the enzyme immobilization technique have significantly improved enzymes' susceptibility to the harsh conditions of the transportation pipeline. This technique also allows for the recovery and reuse of enzymes, making the enzyme cost-competitive with other wax treatment strategies. This paper intended to contribute to sustainability by discussing the potential of the enzymatic degradation approach being expanded to the petroleum-production process.

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