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Potential Approaches to Improving Biodegradation of Hydrocarbons for Bioremediation of Crude Oil Pollution

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ABSTRACT

With increasing demands of fossil fuel energy, extensive exploration of natural sources has caused a number of large scale accidental spills of crude oil and resulted in some significantly environmental disasters. The consequence of oil pollution to environment and human health has brought a serious challenge to environmental scientists. Physical and chemical approaches to cleanup oil spills are too expensive and create adverse effects. Bioremediation has shown a great potential and competitive privilege because of environment friendly and cost effective. A number of efficient microbial strains have been identified and isolated, which can effectively degrade various components of petroleum oil. However, the biodegradation efficiency is usually limited by abiotic factors, such as temperature and pH, which are hardly to be controlled in the in situ condition but adequate oxygen supply and nutrient balancing are of importance to impact microbial functions. Therefore, this review especially addresses potential approaches to improving bioremediation of crude oil by supplying solid oxygen and adjusting C: N: P ratio to optimize microbial activities in order to improve the effectiveness and efficacy of bioremediation of crude oil pollutants. In addition, it also elucidates advantages of bioremediation, isolation of selective microbial strains, and evaluation of the biodegradation rates.

Keywords*: *Biodegradation*, *Bioremediation*, *Crude Oil*, *Hydrocarbon*, *Nutrient Balance*, *Oxygen

1. Critical Importance and Advantages of Bioremediation

Oil contamination with petroleum and petroleum-based hydrocarbons in accidental spills has caused critical concerns in environment, ecological systems, human health, tourism and recreation activities. Such pollution to water and soil has become often in recent decades with the increasing demand of fossil fuel energy requiring offshore drilling due to the global population growth and persistent persuasion for an increase of GDP. Indeed oil spill has resulted in some environmental disasters throughout the world. For example, the Lakeview Gusher spill in California reached up to 1 200 000 tons of crude oil from May 1910 to September 1911, which was the largest spill in the world history [1]. Since the late of 1970s, there has been a number of large spills [2,3] around the world (**Table 1**) and the consequence of oil spill pollution can last decades. For instance, the smallest spill among 13 major spills occurred in 1989 by the Exxon Valdez in Prince William Sound of Alaska caused about 100 tons of oil still remained in the beaches of Prince William Sound as of 2001 [4]. The incident of oil spill from BP in the Gulf Coast of Mexico from April to July, 2010, has caused almost 600 000 tons of crude oil spilled along the Gulf Coast. The total amount of all major spills was as much as 37 billion barrels of crude oil, which exceeds the total amount of crude oil consumption for the entire world annually (30 billion barrels in 2006) [5]. All these spills have caused tremendous damage to ecological and environmental systems, especially to many plant species, a wide array of animals, human health, which resulted in the alteration of the coast aesthetics for tourism and recreations.

Oil spills can leave a legacy for decades, even centuries into the future. The deaths of marine animals and migratory birds and impacts of their losses on marine ecosystems, and on human health effects by oil spills are difficult to evaluate [6]. Cleanup efforts will require decades of dedicated work, and conversion of a toxic environment to a healthy one will need long time and

Table 1. Major oil spills in the world by order of quantity [2].

Date	Location	Spill type	Tons of crude oil	Barrels
5/14/1910–9/10/1911	U.S. Kern County, California	Lakeview Gusher	1 200 000	9 000 000
4/20–7/15, 2010	U.S. Gulf of Mexico	Deepwater horizon	560 000 – 585 000	4 100 000 – 4 300 000
1/23/1991	Iraq, Persian Gulf and Kuwait	Gulf War oil spill	270 000 – 820 000	2 000 000 – 6 000 000
6/3/1979–3/23/1980	Mexico, Gulf of Mexico	Ixtoc I	454 000 – 480 000	3 329 000- 3 520 000
7/19/1979	Trinidad and Tobago	Atlantic Empress/Aegean Captain	287 000	2 105 000
3/2/1992	Uzbekistan	Fergana Valley	285 000	2 090 000
2/4/1983	Iran, Persian Gulf	Nowruz Field Platform	260 000	1 907 000
5/28/1991	Angola	ABT Summer offshore	260 000	1 907 000
8/6/1983	South Africa, Saldanha Bay	Castillo de Bellver	252 000	1 848 000
3/16/1978	France, Brittany	Amoco Cadiz	223 000	1 635 000
4/11/1991	Italy, Mediterranean Sea Near Genoa	MT Haven	144 000	1 056 000
11/10/1988	Canada	Odyssey	132 000	968 000
3/24/1989	U.S. Prince William Sound, Alaska	Exxon Valdez oil tanker	35 065 – 103 896	257 000 – 750 000 [3]

cost tremendously. Toxicity of crude oil often includes necrosis and congestion of the liver, fat degeneration, and dissociation of hepatocytes. Birds and animals in oil contaminated area usually have black emulsion in the digestive tract with a petroleum odor, which leads to decrease in the absorption of nutrients and eventually to death due to a series of consequences, such as rupture of capillaries and hemorrhage, hepatocellular dissociation, hemosiderosis, renal tubular necrosis, and anemia [7]. Crude oil consists of a number of rather complicated components, which are toxic and can excert side effects on the environment and ecological systems. For instance, the aromatics in crude oil produce particular adverse effects to the local microbial flora. It was found that α-pinene, limonene, camphene, and isobornyl acetate were inhibitory to microorganisms. Phenolic and quinonic naphthalene derivatives inhibited the growth of the microbial cells [8]. Increase of naphthalene, 2-methylnaphthalene and pyrene can cause prolonged lag phase and reduce the growth rates of two bacteria [9]. In terms of the toxic effects of cyclohexane on the energy transduction in *saccharomyces cerevisiae*, cyclohexane inhibited oxygen uptake in intact cells and isolated mitochondria [10]. Therefore, immediate actions should be taken to remove or remediate the contaminant after an accidental spill. Physical cleanup is expensive, and the disruption to the habitats may result in an even worse impact than the oil pollution itself, and such cleanup on the floating water or within plant communities is almost impossible. Chemical approach, such as application of dispersion would cause environmental side effects. For example, coral reef can be affected by crude oil and dispersants. Early developmental forms (like coral larvae) are particularly sensitive to such toxic effects, and oil slicks can significantly reduce larvae development and viability [11]. Natural oxidation by weathering takes decades due to the lack of oxygen and nutrients in the water for microbial organisms.

Bioremediation through hydrocarbon biodegradation using selected microbial organisms has provided a favorable opportunity because it is environmentally friendly and cost effective. Those microbial species or particular strains can digest hydrocarbons and utilize the resulting compound carbon as food and energy sources for growth and reproduction. Simultaneously the hydrocarbons are hydrolyzed from toxic and complicated organic compounds into nontoxic and simple inorganic compounds, such as CO_2 and H_2O along with microbial biomass accumulation, through oxidation under aerobic conditions. Under certain circumstances, some anaerobic microbial organisms can degrade hydrocarbons as well through reduction. For instance, a benzene-tolerant strain, *Flavobacterium* sp. DS-711 isolated from deep-sea sediments of 1 945 m had as much as more than 90% of *n*-alkanes in kerosene degraded [12]. However, oxidation by aerobic microbial organisms has been commonly considered to be a predominant and effective approach to the hydrocarbon degradation. In addition, under anaerobic conditions, some intermediate materials, such as fermentation products, ethanol or methane (CH_4) can be produced [13]. A large number of related studies, such as specific microbial strain screen and isolation, bench- or lab-scale trials under different environmental conditions, *i.e.*, pH and temperature, have been conducted with

promising results. However, the implementation of such technology in a field scale is still very limited. A pilot *in situ* trial by application of *P. aeruginosa* to degrade hydrocarbons for cleanup of the oil spilled from the Exxon Valdez in Prince William Sound, Alaska [14] seemed unsuccessful due to the low temperature and a lack of essential nutrients for microbial growth and activities.

2. Isolation and Identification of Microbial Strains to Selectively Degrade Hydrocarbons

The petroleum crude oil usually consists of 83 - 87% carbon and 10 - 14% hydrogen, 0.1 - 2% nitrogen, 0.1 - 1.5% oxygen, 0.5 - 6% sulfur, and < 0.1% metals [15]. The predominant hydrocarbons are theoretically degradable but the components are rather complicated. It contains aliphatic and polycyclic aromatic hydrocarbons (PAH), for example, crude oil consists of paraffins 15 - 60%, naphthenes 30 - 60%, aromatics 3-30% and asphaltics 6% by weight [16]. Therefore, the specific microbial strain screen and isolation need to be performed by supplying petroleum crude oil or a particular component, if the selective strain is going to be utilized to degrade that component, as carbon for food and energy source with mineral nutrients essentially to build up the microbial biomass.

To isolate a microbial hydrocarbon degrader, a common approach is to use an enrichment culture system in which the candidate strain from oil contaminated water or soil samples is cultured in mineral salts medium (MSM) consisting of essential macro- and micro-nutrients (0.4% NH_4NO_3, 0.47% KH_2PO_4, 0.0119% $N_{a2}HPO_4$, 0.001% $CaCl_2{\cdot}2H_2O$, 0.1% $MgSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$, and 0.0015 $FeSO_4{\cdot}4H_2O$ at pH 7.0 with phosphate buffer) [17]. Basically, microbial organisms are transferred from samples collected to the above MSM medium and cultured at 30˚C in a rotary shaker at 150 rpm with 0.1% yeast extract until turbid growth is observed. The bacterial culture is diluted and spread on MSM agar plates containing crude oil (1%) as carbon source for selective isolation of petroleum degrader. The plates are sealed and incubated at 30˚C until appearance of several colonies. Individual colonies can be purified by repeating the culture on MSM agar plates containing 1% crude oil. Identification of the candidate strains will be performed based on physiological and biochemical tests, or 16S rRNA sequencing.

Physiological and biochemical tests: using phenotypic and biochemical characterizations to identify specific strains can be performed as described by Yumoto *et al.* [18].

DNA base composition and DNA-DNA hybridization: The DNA content isolated from bacterial cells can be determined fluorometrically using photo-biotin-labeled DNA probes and microplates [19] and compared with strains in the national collection of microorganisms. For such an approach, *Pseudomonas aeruginosa* JCM5962^T and *Serratia marcescens* JCM 1239^T can serve as reference strains.

Phylogenetic analysis using 16S rRNA gene sequence comparison: Almost the full length of 16S rRNA genes of bacteria has been amplified by PCR with following sets of primers 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCC-3' corresponding to the positions 9 to 27 and 1525 to 1541, respectively [17].

3. Monitoring and Evaluation of Biodegradation Rate of Hydrocarbons

To evaluate the biodegradation process of various hydrocarbons, cultured media can be extracted at certain time intervals with dichloromethane. The extract can be analyzed by a gas chromatography (GC), GC-MS (mass spectrometer), gas-liquid chromatography (GLC), or high performance liquid chromatography (HPLC).

With GC analysis, the carrier gas is helium, the injector and detector temperatures are set at 250˚C and 300˚C, respectively, for analysis of total petroleum hydrocarbons (TPH). However, for gasoline analysis, Wongsa *et al.* [17] suggested that the column temperature is first maintained at 35˚C for 5 min, then increased to 220˚C; for diesel oil analysis, the column temperature is set at 50˚C and then ramped to 270˚C with a regime of $5^{\circ}C{\cdot}min^{-1}$. In the case of lubricating oil, 320˚C is needed for both injector and detector temperatures, and the column temperature can be set at 100˚C initially and ramped to 320˚C at a rate of $10^{\circ}C{\cdot}min^{-1}$.

For GC-MS analysis, resulting chromatograms can be analyzed by various particular software packages, such as Saturn Software GC/MS Workstation Version 5.52, to identify petroleum components after the spectra have been obtained at temperatures similar to those described in the GC analysis. Detailed information has been provided elsewhere by other researchers [20].

4. Potential Approaches to Improving Biodegradation of Hydrocarbons and Bioremediation of Oil Pollutants

4.1. Performances of Various Bacterial Strains

Bioremediation approaches, *i.e.* using selected indigenous microbial organisms to degrade hydrocarbons, are currently receiving favorable publicity because bioremediation is environment friendly and cost effective. Among those microorganisms, the genus *Pseudomnas,* particularly *P. putida* F1 is one of the most well-studied hydrocarbon degrading bacterial strains, this strain and

several others are commercially available (ATCC, The Essentials of Life Science Research), having approved the capability to utilize organic compounds from the generic group aliphatic, cyclo-aliphatic, aromatic and/or polynuclear aromatic hydrocarbons. *Pseudomnas* can facilitate the degradation and utilization of carbon derived from complicated compounds as its food and energy sources with metabolic plasmids. The strains isolated from contaminated sites [14,21,22] have shown promising potential in biodegradation of hydrocarbons and bioremediation of contaminated sites mostly in bench scale studies. For instance, the bioremediation rate of naphthalene using *P. putida* can reach as high as 61 mg L^{-1} hr^{-1} [23]. Strains of *P. aeruginosa* WatG and *Serratia marcescens* HokM, isolated in Japan [17], showed that about 90-95% of diesel oil and kerosene can be degraded within 2 and 3 weeks, and petroleum hydrocarbon (TPH) can be degraded by 72% in 4 weeks (**Figure 1**). Moriya and Horikoshi [12] reported that a strain of *Flavobacterium* sp. named DS-711 isolated from deep sea sediment degraded 90% of *n*-alkane in kerosene, which is greater than toluene-tolerant *P. putida*. A strain of *Bacillus subtilis* is a good degrader of both hydrocarbons with degradability of 98% *n*-hexadecane and 75% naphthalene [24].

4.2. Biosurfactant Production

To effectively degrade hydrocarbons of crude oil, emulsification with a surfactant is of importance due to their low water solubility, especially polyaromatic components in solid and liquid discharges of petroleum. Some strains, such as *P. putida*, and *B. subtilis*, can produce rhamnolipid biosurfactant, which can dramatically enhance aqueous dispersion via emulsification, and stimulate the biodegradation of organic compounds [25,26]. The emulsification plays an important role in the degradation of organic compounds, especially for polyaromatic hydrocarbons, such as naphthalene and n-paraffin fractions, and such emulsification usually can be observed in 24-48 hrs after inoculation with some effective microbial strains [27]. Other researchers indicated that the dissolution rates for hydrophobic particles into the culture media during the bioremediation process were up to 4 times greater compared to mass transfer rates into abiotic controls due to the production of biosurfactant by *P. putida* [23].

4.3. Application of Bacterial Consortium

Recent research indicates that use of mixed bacterial consortium is more efficient in biodegradation of crude oil than individual bacterial strains. For example, according to Sathish Kumar *et al.* [28], the mixed consortium of four bacterial strains degraded a maximum of 77% crude oil, followed by 69% by *Pseudomonas sp.* BPS1-8, 64% by *Bacillus* sp. ISS1-7, 45% by *Pseudomonas* sp. HPS2-5, and 41% by *Gorynebacterium* sp. BPS2-6 at 1% crude oil concentration. Increasing crude oil concentration from 1 to 12%, the degradation rate by the same consortium was decreased but still reached up to 45%.

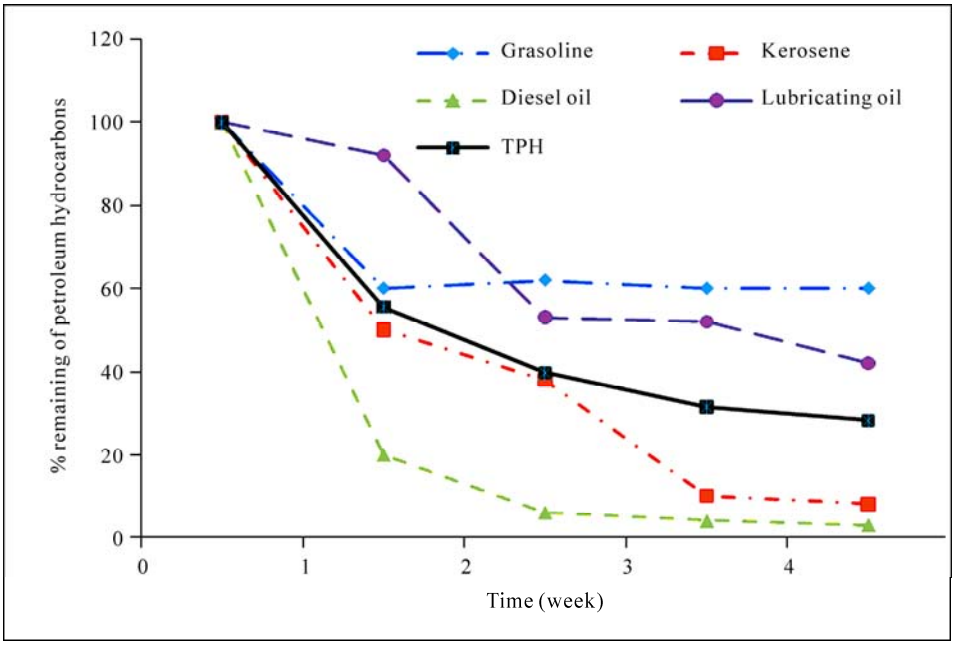

Figure 1. Biodegradation of different hydrocarbon compounds of petroleum products by a selective strain, WatG of *Pseudomonas aeruginosa* (adapted from Wongsa *et al.*) [17].

4.4. Biodegradation Time and Efficiency

Regarding the degradation time, it varies from one researcher to the others. For instance, Atlas and Bartha [29] observed that the degradation started after a 2 to 4 day lag period, and reached its maximum within 2 weeks with their continuous monitoring on CO_2 evolution. At this time of degradation, up to 60% of the crude oil and 75% of the model hydrocarbon mixture were degraded by *Flavobacterium* sp. and *Brevibacterium* sp. isolated from coastal waters after each was added at the final level of 1 ml per 100 ml artificial sea water. Berwick [30] indicated that about 98% of the solvent (carbon tetrachloride) extractable oil was degraded over 83 days and the degradation in percent was in the following order: aromatics > saturates > heterocyclics > asphalts, but the degradation rate for any of these fractions was above 94%.

4.5. Immobilizing Microbial Cells

Hydrocarbon biodegradation can be significantly improved by immobilizing microbial cells. For example, using the bacterial consortium MPD-M isolated from sediments associated with Colombian mangrove roots can effectively degrade hydrocarbons in water with salinities varying from 0 to 180 g/L. However, the effectiveness was 4 and 7 times greater with immobilized cells on polypropylene fibers compared to free living cells [31]. Moslemy *et al.* [32] demonstrated that an enriched bacterial consortium encapsulated in gellan gum microbeads (16-53 μm dia.) at the rate of 2.6 mg·cells/g bead degraded over 90% gasoline hydrocarbons within 5-10 days for the initial concentration of 50-600 mg/L. However, degrading the same amount of gasoline hydrocarbons by free cells at equivalent levels required as long as 30 days. The improved effectiveness of encapsulation may result from potentially reducing biotic and abiotic stresses, providing a number of advantages, including protecting cells from the toxic effects of hazardous compounds [33-35], and increasing their survival and metabolic activities [36,37].

4.6. Potential Implementation and in Situ Application

Successful implementations of crude oil biodegradation in the field scale have been reported. Bacterial strains such as *P. aeruginosa* have been applied to degrade hydrocarbons since 1989 for cleanup of the oil spilled from the Exxon Valdez in Prince William Sound, Alaska [14]. Efficient enzymes extracted from the bacterium *Bacillus cereus* DQ01 isolated from oil fields have been proven to digest the hydrocarbon, *n*-hexadecane [38]. *Alcanivorax borkumensis* is a recently discovered hydrocarbonoclastic bacterium. According to Martin dos Santos *et al.* [39], it might be the most important global oil degrader discovered up to date.

4.7. Impacts of Environmental Conditions and/or Abiotic Factors on Biodegradation

The mechanism whereby microorganisms degrade hydrocarbons has not been fully elucidated. It is known that some selective strains possess a capability to tolerate a certain concentration of hydrocarbons and can utilize them as their carbon and energy sources for growth and reproduction. Therefore, the end products of hydrocarbon biodegradation under aerobic conditions are simply CO_2, H_2O and the accumulation of microbial biomass. Since the oxidation or hydrolysis of those hydrocarbons is accomplished by microbes, any factors influencing microbial growth and activities can definitely impact the biodegradation rate and the effectiveness. Moreover certain abiotic conditions such as temperature, pH, oxygen supply and nutrient balance, have been proved to play a crucial role in the oxidation and hydrolysis involved. For instance, temperature of 35˚C and pH 7 were found to be optimum for maximum degradation of crude oil by a consortium of *Pseudomonas* strains [28]. Mittal and Singh [40] observed that using degrading consortia of *Pseudomonas* sp. for bioaugmentation of polycyclic aromatic hydrocarbon (PAH) of polluted soil with addition of nutrients and other environmental factors, *i.e.*, tilling (aeration) resulted in 79% removal of PAH in 60 days, while only 30% removal was achieved by indigenous microflora alone. The result was obtained from the nutrient ratio of C:N:P in 120:10:1 based on Gibb's formula (assuming crude oil contains 78% carbon) but the optimal ratio is unknown. Oxygen and nutrient supply are important to optimize the microbial activity because inhibition of biodegradation by nutrient or oxygen limitation or toxic effects exerted by volatile hydrocarbons may occur due to high concentrations of undispersed hydrocarbons in water [28].

The evidence in improvement of hydrocarbon or crude oil biodegradation by aeration or oxygen supply has been observed by a number of researchers [41-44]. The addition of nutrients adjusts the essential nutrient balance for microbial growth and reproduction, and oxygen supply can maintain the aerobic environmental condition to stimulate the microbial oxidation and hydrolysis of hydrocarbon compounds. As matter of fact, in soil or water with high levels of hydrocarbon contamination, the oxygen demand often exceeds the supply, which is the reason that the amount of oxygen supply was the most important single factor affecting biodegradation of petroleum [45].

4.8. Oxygen Supply and Nutrient Balancing to Improve Hydrocarbon Biodegradation for Bioremediation of Oil Pollutants

Both biotic and abiotic conditions play a crucial role in improvement of the bioremediation efficiency due to optimization of function of indigenous microbial strains. With application of efficient strains isolated, abiotic condition improvement can definitely increase the biodegradation efficiency and effectiveness. Among which, temperature and pH are adjustable factors in a bench scale trial but unrealistic to be implemented with an *in situ* approach. Nutrient balancing, especially the supply of essential nutrients such as N and P can improve the biodegradation efficiency by optimizing the bacterial C:N:P ratio. However, appropriate rate and application method are of great importance as insufficient rate cannot balance the nutrient ratio but excess amount will produce toxic effects to microbial organisms. In addition, the impact of nutrient supply on water quality may bring public concerns on a possible eutrophication risk. The limited quantity of supply is required only to provide an appropriate rate to microbial organisms for their growth and reproduction. Therefore, it is urgently needed to study the bacterial consumption rate and the optimum ratio of such nutrients required by efficient microbial strains. Nutrient fate and consequences of biodegradation of hydrocarbons associated with nutrient supply to influence the environment and water quality should be monitored. Also, to prevent toxic effects on microbial organisms by abrupt supply of these nutrients, slow release controlled by polymers can be an ideal approach as it provides prolong and constant supply of nutrients to microbes. Oxygen supply has been evidently proved to be a stimulating approach to assist microbial degradation of hydrocarbons [46] because oxidation and hydrolysis are primary procedures to break down the complicated hydrocarbon compounds to be utilized by microbial organisms for food.

Regarding oxygen source, it requires a slow but a consistent supply to create an aerobic environment for the microbial strains. The introduction of solid peroxygen materials provides a viable alternative for meeting the oxygen demand by microbial organisms [46]. These materials are primarily peroxide salts of calcium and magnesium: CaO_2 and MgO_2, which can release oxygen at enhanced levels over extended time periods as described below:

$$CaO_2 + 2\ H_2O \rightarrow Ca\ (OH)_2 + H_2O_2$$
$$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$$
$$MgO_2 + H_2O \rightarrow 1/2\ O_2 + Mg\ (OH)_2$$

Indeed, calcium or magnesium peroxide is a powerful oxidizing agent and it breaks down into lime, water and oxygen, which does not form any persistent, toxic residual compounds. The other ideal oxide material is calcium superoxide, CaO_4 or $Ca(O_2)_2$, which contains higher percentage of stored oxygen than CaO_2, which has been used in emergency breathing apparatus for miners and as auxiliary oxygen sources for astronauts. Both of them have similar physical and chemical characteristics but the former can release a double amount of O_2 with water compared to the latter with the following reaction [47, 48]:

$$CaO_4 + xH_2O \rightarrow CaO_2{\cdot}xH_2O + O_2$$
$$\downarrow$$
$$Ca(OH)_2 + xH_2O_2$$
$$\downarrow$$
$$O_2 + xH_2O$$

From which, double amount of oxygen can be released from CaO_4 as compared to CaO_2 to meet the microbial requirement for oxygen but there is no toxic substance produced. In addition, to avoid possible toxic effects by CaO_4, polymers can be applied to separate each component to prevent direct contacts of microbial organisms with calcium superoxide. The production of CaO_4 can be accomplished by the reaction of potassium superoxide (KO_2), which is commercially available, with calcium chloride ($CaCl_2$) in accordance with the following equation [49]:

$$2KO_2 + CaCl_2 \rightarrow CaO_4 + 2KCl$$

Therefore, to improve the hydrocarbon degradation efficiency and effectiveness, it is important to utilize indigenous microbial strains isolated specifically for crude oil degradation that are commercially available, or identified and isolated from enrichment culture and screening. Apply solid oxygen materials, such as calcium superoxide, and adjust nutrient balance, especially to optimize C:N:P ratio for efficient degradation of hydrocarbons of crude oil under optimum environmental conditions, such as appropriate temperature and pH values in the media.

Regarding the nutrient supply, the controlled release substrate with optimal ratio can provide the microbial organisms a slow and constant nutrient supply under the controlled conditions. Previous studies have displayed that the addition of essential metabolic nutrients, N and P, to oil contaminated beaches is an effective approach for stimulating bioremediation of oil pollutants by indigenous microbial biomass [50-52]. However, the application of excessive amount of nutrients over metabolic needs by microbes can result in extra bioremediation costs and potential marine eutrophication impacts [53]. The use of slow release nutrients with an appropriate rate may provide a continuous nutrient supply by maintaining a sufficient nutrient status for the perpetuation of microbial metabolic activities without causing environmental

concerns and save the cost [54]. Xu *et al.* [55] found out that an addition of 0.8% of slow-release fertilizer, Osmocote^{TM} consisting of 18, 4.8, and 8.3% N-P-K (w/w) to oil polluted sediments was sufficient to maximize metabolic activity of the microbial biomass and the biodegradation of straight-chain alkanes (C_{10}-C_{33}); and application of 1.5% rate resulted in optimal biodegradation of recalcitrant branched-chain alkanes, such as pristine and phytane. The application of soluble nutrients to the oil contaminated sites has shown an especially promising potential of the indigenous microbial organisms for stimulating biodegradation of petroleum hydrocarbons in the tropical environment of Singapore with high temperature and humidity [56]. Therefore, to improve the biodegradation efficiency and implementation, integrating various components, such as microbial strains in consortium, solid oxygen source, appropriate rate of nutrients with controlled release pattern, into a granule formulation with an oleophilic matrix, may provide an ideal approach to improving bioremediation of crude oil pollutants.

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