Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 159 (2021) 210-223 EISSN 2392-2192

Bioremediation Enhancement of Oil-Polluted Water by Bacterial Immobilization: A Review

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ABSTRACT

Oil-spilled and others oil-related pollution is an inevitable events happened almost every year throughout the world's ocean, including Indonesia. To tackle the problems many methods were applied such as physical, chemical and biological or bioremediation. The potential of bioremediation as a method of overcoming oil pollution is very good. However, the current rate and success rate of bioremediation is still relatively low. Immobilization of bioremediation agents is one of the solutions to enhance the removal performance. Here we thoroughly discussed current research in immobilization through several approaches which are covalent immobilization, entrapment, encapsulation and adsorption

***Keywords*:** bioremediation, oil pollution, hydrocarbonoclastic bacteria, immobilization

1. INTRODUCTION

Indonesia is the largest archipelagic country in the world. Therefore, Indonesia is able to use the ocean as a transportation route for commodities, including oil. The high activity of oil

(Received 25 June 2021; Accepted 15 July 2021; Date of Publication 17 July 2021)

transportation in Indonesian seas increases the possibility of accidents, such as oil spills or accidents on *tankers* carrying oil to the high seas or coasts. This is evident from several accidents that caused oil spills, for example 2 *tankers*, namely the Indonesian-flagged Kartika Segara and the Dominican ship, JBB De Rong 19 which collided and spilled at least 300 tons of crude oil in the Singapore Strait which also borders the Indonesian coast. [1]. Based on data from the Special Task Force for Upstream Oil and Gas Business Activities (SKK Migas) summarized by Bisnis Indonesia, the volume of oil spilled in 2013 reached 3,025.6 barrels, 1,113.8 barrels in 2014, 226.4 barrels in in 2015 and 787.2 barrels in 2016. It is even reported that the total oil released to the sea during Montara Well Incident in 2009 was as high as 4.7 million liter [2].

Oil leaks in the seas of neighboring countries also have an impact on Indonesia. This can happen because Indonesia's geographical location is between 2 oceans, so polluted oil can be carried by currents easily to the oceans and coasts of Indonesia. One example of this case is oil pollution that spilled from a refinery in Montara, Australia in 2008. The oil was carried by currents and reached the Timor Sea, NTT, thus damaging seaweed cultivation in Indonesia and the impact is still being felt until 2017 [3].

The high incidence of oil pollution in Indonesian oceans encourages the development of fast and environmental friendly remediation methods. The method used to restore natural conditions from polluted environmental is known as remediation. Oil remediation in the sea can be done in several ways, such as physical, chemical, and biological approaches. In addition to these three approaches, burning is *in-situ* also carried out to eliminate oil pollution on the sea surface quickly [4].

Remediation of oil in the sea with a physical approach is the most commonly used method to control the spread of oil in the sea, without changing its physical and chemical properties. A variety of barriers are used in this technique, such as barrier floats and skimmers. The spread of polluting oil is limited and collected using a barrier float [5], then removed using a skimmer [6]. However, strong waves are able to carry oil through the buoy easily, both from the top of the buoy and the bottom [7]. In addition, the success of using skimmers is limited because skimmers are prone to being clogged by debris in the sea, such as plastic waste, as well as thick oil on the sea surface [7].

Remediation techniques with a chemical approach are usually carried out with mechanical/physical remediation techniques. An example of a chemical remediation technique is the use of dispersants. This compound is used to convert the oil layer into small droplets, so that the oil droplets are able to descend into the sea, become more soluble and more easily degraded [8]. However, the dispersants only turn the oil layer into droplets that descend into the water column. The oil granules can disrupt marine ecosystems. In addition, the content of dispersants that can cause health problems in humans, coupled with the toxic nature of polluting oils that become small grains present a threat to the environment [9].

Under certain conditions, in-situ burning can offer a solution that can minimize damage to the marine environment because polluting oil is lost from the sea surface. However, this method is not environmentally friendly because it produces air pollution in the form of smoke. In addition, the smoke is also able to disrupt marine and coastal ecosystems because the residue produced is able to cover the beach or envelop benthic organisms [10]. Physical and chemical approaches have not been able to solve the problem of oil pollution in the sea effectively due to its safety, relatively high cost, ineffective elimination of pollutant and high degree of secondary waste generation. Therefore, environmentally friendly and sustainable remediation methods need to be developed, such as bioremediation [11].

Two types of bioremediation approaches that are usually recognized are bioaugmentation and biostimulation. Bioaugmentation is the addition of microorganism cultures that have the ability to degrade pollutant compounds, while biostimulation is environmental modification to support the growth of pollutant-degrading microorganisms by adding growth factors, such as phosphorus, nitrogen, oxygen and carbon [12]. Bioremediation offers a method that is cheaper, environmentally friendly, permanent results and is more accepted by the community in an effort to clean up pollutants [13].

The potential of bioremediation as a method of overcoming oil pollution is very good. However, the current rate and success rate of bioremediation is still relatively low. Bioaugmentation techniques generally use microorganism cell culture directly as a bioremediation agent. However, cells that are not immobilized are more susceptible to various inhibitory factors in the environment. One example of a limiting factor in the environment is salinity. Diaz *et al.* [14] explained that microorganisms are generally unable to survive in environments with high salinity, such as water which is produced as a by-product along with oil and gas (*produced water*). The salinity of this water varies depending on the location of the refinery, from 100-300,000 ppm [15].

Pollutant-degrading microorganism cells need to have high viability in polluted areas. Therefore, it is necessary to immobilize cells into a *carrier* to increase the stability of bacteria, thereby increasing their ability to survive and degrade polluting oil. This can happen because macromolecules, such as proteins, or living cells are protected from non-ideal conditions, such as high salinity or the presence of toxic compounds. As a result, the intracellular activity of immobilized live microorganisms is more stable and oil degradation occurs at a faster rate [16]. Here we will discuss the fundamental of bioremediation and hydrocabonoclastic bacteria, which are the primary group of oil degrading bacteria, as well as the implication of its immobilization using various technique including covalent immobilization, entrapment, encapsulation and adsorption.

2. BIOREMEDIATION

Bioremediation is a process in which various biological agents, such as bacteria, fungi, protists, or the enzymes they secrete are used to degrade contaminants in the environment into compounds that are more environmentally friendly [17]. The advantages of the application of bioremediation in dealing with waste compared to other remediation techniques are that the treatment is cheaper, better accepted by the community, permanently degrades pollutants, and can be carried out in polluted places [18]. In addition, anthropogenic chemical compounds, namely chemical pollutants produced directly by human activities, such as *methyl tertiary butyl ether* (MTBE) and *ethyl tertiary butyl ether* (ETBE) which are used as octane enhancers in gasoline, can be degraded by several types of microbes naturally [19].

There are two bioremediation techniques that are commonly used in tackling environmental pollution, namely biostimulation and bioaugmentation. Biostimulation is the addition of nutrients to support the growth of pollutant-degrading microorganisms, while bioaugmentation is the addition of microorganisms from outside the environment that can accelerate the process of pollutant degradation [20].

2. 1. Biostimulation

Biostimulation is the addition of nutrients, such as carbon, nitrogen, phosphorus and others, to a polluted area to support the growth of pollutant-degrading bacteria that are already present in the environment. This principle was developed because local microorganisms found in polluted areas have the ability to naturally convert and mineralize organic compounds [21].

Monitored natural attenuation is a remediation technique that has been accepted in many countries. Natural attenuation, which is the degradation of pollutants by local microbes without human intervention, is the main mechanism of the monitored attenuation technique. Other mechanisms, such as evaporation, dispersion, absorption and dilution contribute to the reduction of pollutant concentrations in the environment [22]. However, adaptation and biodegradation of pollutants by local microbes can be hampered if the environment does not support these microbial activities. Therefore, the addition of nutrients, access to oxygen or other electron acceptors [23], adjustment of pH, temperature [24] and redox conditions were carried out to stimulate microbial activity [22].

Research by Darmayati [25] showed that the concentration of nutrients added in the form of slow-release fertilizer was directly proportional to the rate of oil degradation by hydrocarbonoclastic bacteria. This study uses a sand column mesocosmic system placed in the tidal zone for 3 months. A total of 200 g Arabian Light Crude Oil (ALCO) was added in each mesocosmos. The hydrocarbonoclastic bacteria grown in the control reached a density of 2-3 x 107 cells/g, while the bacteria supported by the addition of nutrients reached a density of 2-3 x 108 cells/g. Natural attenuation degrades 28% of crude oil in the third month (0.65 g/day degradation rate). However, the degradation can be increased by the addition of slow-release fertilizer up to 33-55% in the third month (degradation rate 0.99-1.56 g/day).

The advantage of the application of biostimulation lies in the bioremediation agent, namely local microorganisms that have been acclimatized to the polluted environment, and are evenly distributed. On the other hand, the weakness of the application of biostimulation is in ensuring the distribution of added nutrient sources in the polluted environment is even and can be utilized by local hydrocarbonoclastic microbes optimally. In addition, the addition of nutrient sources can support the growth of heterotrophic microflora that are unable to degrade pollutants, thus competing with hydrocarbonoclastic microbes [26].

2. 2. Bioaugmentation

Bioaugmentation is the addition of microorganisms from outside the polluted environment, both wild-type and those that have undergone genetic engineering that have the ability to accelerate the process of pollutant degradation [27]. This technique was developed because there are types of pollutants that cannot be degraded by local microorganisms, so that these pollutants contaminate water in the long term [28].

Several types of pollutants are resistant to biodegradation because they aretoxic, have low solubility in water, are not bioavailable, are molecularly stable, and are difficult to biodegrade. Some compounds also cannot be used as substrates by enzymes resulting from the metabolism of microorganisms efficiently. The chemical structure of a complex pollutant often requires a consortium of microorganisms not found in the environment to degrade it [28].

Research by Nikolopoulou et al. [29] tested the capability of a local microbial consortium that has been acclimatized (autochtonous bioaugmentation) in remediating petroleum pollution in the sea. The consortium of microorganisms was tested with three types of treatment, namely control (consortium of pollutant degrading microbes), NPKM (consortium of pollutant degrading microbes + KNO_3 + K_2HPO_4), and NPKMR (consortium of pollutant degrading microbes + KNO_3 + K_2HPO_4 + biosurfactant). The control treatment did not show any degradation of the content of alkanes or polycyclic aromatic hydrocarbons (PAHs) until the end of the study. On the other hand, microbial treatment given stimulants (NPKM and NPKMR) was able to degrade short chain alkanes (C14-C30). NPKM degrade alkanes C15 28 times faster than the controls (69 ug / hr cell), while degrading NPKMR 23 times faster (56 mcg / hr cell). Pristane and phytane as biomarkers were also degraded 3 times faster than control through NPKM treatment, and 12 times faster than control through NPKMR. Within 30 days, 90% of the total alkanes had been degraded through the NPKMR treatment, while the NPKM treatment had degraded about 75% of the total alkanes contained. This study also shows that a combination of bioaugmentation and biostimulation is required to achieve optimal degradation effectiveness.

However, the success of bioaugmentation techniques on a laboratory scale does not guarantee the same results when applied in the field. Studies have often seen a decrease in the number of microorganisms added shortly after application to contaminated sites. This can occur due to insufficient substrate, changes in temperature, pH, limited nutrients, competition with other microorganisms, phase infection, shock caused by pollutants, predation by protozoa, and quorum sensing [28].

3. HYDROCARBONOCLASTIC BACTERIA

Bacteria that have the ability to utilize hydrocarbon compounds as a source of nutrition are called hydrocarbonoclastic bacteria. Several bacterial species are known to utilize hydrocarbons as the sole source of nutrients [30]. Most obligate hydrocarbonoclastic bacteria belong to the genera *Oleispira*, *Oleiphilus*, *Thalassolituus*, *Alcanivorax*, and *Cycloclasticus* [31]. This is evidenced from the carbon use test which shows that hydrocarbonoclastic bacteria only use hydrocarbons, such as Tween 40 and 80 [30, 32]. These bacteria have an important role in cleaning the environment after an oil spill, so that it becomes a principle in biologically explaining how ecosystems are able to naturally degrade oil [33].

Schneiker *et al.* [34] performed *sequencing* genome on the hydrocarbonoclastic bacterium *Alcanivorax borkumensis.* This bacterium is very influential in the bioattenuation of oil because its population is able to reach 80% of the total population of bacteria in oil-contaminated waters. To facilitate the utilization of water-insoluble hydrocarbons, these bacteria produce exopolysaccharides extensively and develop pili, so that these bacteria are able to attach to the layer between water and oil. In addition, *A. borkumensis* produces biosurfactants that are able to emulsify alkanes, thereby increasing the surface area of the oil. As a result, the exposed surface of the oil is larger and easier to be degraded by bacteria.

The microbial consortium has the highest biodegradation potential because the degradation of a complex mixture of organic compounds in polluted areas as a whole requires more than one microorganism with different genetic information [35]. Research by Márquez-Rocha *et al.* [36] showed that biodegradation using a consortium of immobilized bacteria was more effective than biodegradation using a single immobilized bacterial culture. The consortium of bacteria tested consisted of *Bacillus brevis*, *Bacillus licheniformis*, *Bacillus sphaericus* and *Pseudomonas aeruginosa*.efficiency degradation *of crude oil* The highest observed in bacterial consortium, ie 81.70%, followed by a single culture with efficiency degradation slightly lower, namely *B.brevis* (75.42%), *P.aeruginosa* KH6 (73.97%), *B.licheniformis* (66.3%) and *B. sphaericus* (63.34%).

4. IMMOBILIZATION

One of the keys to the success of bioremediation is to maintain a large population of bacteria because there is a positive correlation between the cell growth rate of the bioremediation agent and the rate of pollutant degradation [375]. Therefore, bioremediation agents need to be immobilized in a matrix to increase their ability to survive in polluted locations [38].

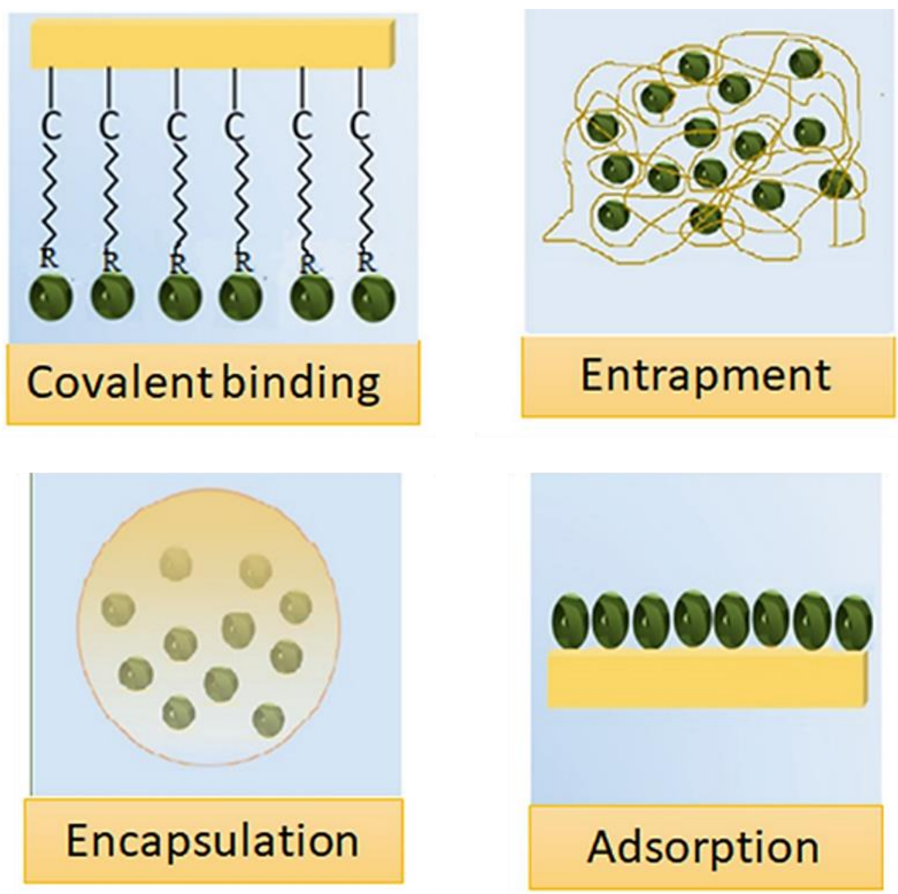

Figure 1. Type of Microbial Cells Immobilization [43]

Cell immobilization is a method of physically trapping or localizing living microbial cells in a space or area to limit migration and provide an environment with different characteristics from the external environment, but still maintain the catalytic activity of these cells [39]. Immobilized cells have a higher shelf life because they are protected from the environment by *carrier* a stable. As the ability of immobilized pollutant-degrading cells increases to survive and adapt, the ability of these cells to remediate the environment also increases [40]. In addition to increasing cell viability, immobilization also simplifies the process of separation and *recovery* of immobilized bacteria, so that immobilized cells can be used repeatedly. This can reduce overall remediation costs [40].

Cell immobilization can be done by several techniques, namely using covalent bonds, *entrapment*, encapsulation, and adsorption [41]. Immobilization techniques can be categorized into two groups, namely passive and active immobilization techniques. Passive immobilization techniques depend on the natural tendency of microorganism cells to adhere and grow on the matrix surface, while active immobilization techniques change the conditions of the matrix or microorganisms to support the attachment of microorganism cells to the matrix surface [42].

Research Diaz *et al.* [14] proved that immobilized hydrocarbonoclastic bacteria (bacteria that metabolize hydrocarbon compounds) were able to survive better and showed 4-7 times higher ability to degrade oil than free cells in marine environments with high salinity levels. In addition, bioremediation agents in the form of immobilized bacteria can be recovered and reused, thereby lowering the overall cost of remediation activities [40].

The bacteria that degrade pollutant compounds are immobilized into a material called a *carrier*. Several properties that need to be possessed by a material to become *carrier* a goodare non-toxic, non-polluting, non- *biodegradable*, able to accommodate high cell density, high stability, long shelf life, inexpensive, have an optimal diffusion distance for the medium to reachcenters *carrier*, cells and *carriers are* easily separated, and easy to re-form [44].

Angelim *et al.* [45] used *chitosan* as abase *carrier* for immobilizing a consortium of bacteria consisting of 12 different genera, namely Xantomonadaceae, *Brachybacterium* sp., *Martellela* sp., *Cytophaga* sp., *Sphingomonas* sp., *Sphingopyxis* sp., *Bhargavaea* sp., *Mesorhizobium* sp., *Gordonia* sp., *Thalassospira* sp., *Pseudomonas* sp., and *Dietzia* sp. In the same study, it can be seen that the test of the viability of the bacteria immobilized showed increased cell count of 4.5×10^6 CFU / g of sediment to 1.1×10^8 CFU / g of sediment after 12 days, while the control showed the number of cells that are not significantly different.

4. 1. Covalent Immobilization

Immobilization using covalent bonds is a technique that is often used to immobilize enzymes, but not for living cells [41]. The working principle of this technique is to form a covalent bond between the *carrier* in the form of an activated inorganic matrix and the cell with the help of a *crosslinking agent* [46]. The functional groups usually involved in covalent cell binding are side chains of lysine (-amino group), cysteine (thiol group), and aspartic acid and glutamic acid (carboxylic, imidazole, and phenol groups) [47].

Research by Scouten [48] succeeded in immobilizing cells *Saccharomyces paradoxus* live on the Separon H 1000 matrix. The matrix was prepared for immobilization by attaching functional groups, such as amino groups, carboxylic groups, and their derivatives, and activated using glutaraldehyde or carbodiimides. Immobilization of *Saccharomyces paradoxus* for 4 days on Separon H 1000 which had added -alanyl and NHgroups$_2$ showed very good results, namely cell density reached 30 mg dry weight per gram of matrix and immobilized stably. In addition, this immobilization technique does not eliminate the ability of cells to divide. Incubation of cells *Saccharomyces cerevisiae* immobilized for 10 hours showed an increase in cell density, from 1.7 mg dry weight per gram matrix to 5.0 mg dry weight per gram matrix. The number of immobilized cells found in the incubation environment increased to 350 *viable count*/mL.

Immobilization using covalent bonds is rarely applied to living cells because the binding agents used to form covalent bonds between cells and *carriers* are usually cytotoxic, thus potentially damaging living cells [49]. In addition, living cells divide, so that new cells will not bind to the *carrier* and contaminate the environment [41].

4. 2. Entrapment

Trapping is an immobilization technique *irreversible*, *in* which microorganism cells trapped in a *carrier* are permanently or matrix. Cells trapped in the matrix pores will interact with the matrix pore walls, so that the cells are fixed. Cells also get protection from the outside environment by *carriers*, so that bacterial cells are able to survive longer [46]. Cells do not interact chemically with the matrix. Therefore, the material used as a *carrier* can be modified to achieve optimal microenvironment conditions [50].

Chen *et al.* [51] compared the biodegradability of petroleum between a consortium of bacteria immobilized by trapping techniques and those that were not immobilized. Five *strains of* bacteria isolated and used to degrade petroleum pollution are *Exiguobacterium* sp. ASW-1, *Pseudomonas aeruginosa* ASW-2, *Alcaligenes* sp. ASW-3, *Alcaligenes* sp. ASS-1, and *Bacillus* sp. ASS-2. The *carrier* material used for the trapping method of the bacterial consortium is calcium alginate. The degradation efficiency of the immobilized bacterial consortium was always higher than that of the immobilized at all levels of petroleum concentration. The maximum degradation efficiency of 58.6% was shown by the consortium of bacteria that were not immobilized, while the consortium of immobilized bacteria reached 63.9% at a concentration of 2% (w/v) petroleum.

4. 3. Encapsulation

Encapsulation is an immobilization method *irreversible* other than entrapment. Similar to the trapping technique, bacteria are immobilized in the core of the *carrier* (in the form of a membrane wall), but the cell does not interact with the membrane pore wall. The membrane wall is semi-permeable, so that substrates and nutrients can still diffuse into the *carrier*, but prevent cells from migrating out [52]. This limited access is one of the advantages of the encapsulation method because the immobilized biocatalyst is protected from non-ideal environmental conditions. In addition, this method also prevents the release of biocatalyst from the *carrier*, thereby maintaining the amount of biocatalyst and its degradation efficiency [53].

Moslemy, Neufeld & Guiot [54] investigated the ability of encapsulated cells to degrade gasoline. The bacterial consortium was isolated from gasoline-contaminated areas and cultured before being encapsulated into *microbeads* gellan gum-based (16-53 m diameter). The encapsulated cells reached a density of 2.6 mg dry weight per gram of *microbead*. Encapsulated cells degraded 90% of 50-600 mg/L gasoline hydrocarbons within 5-10 days at 10 °C Unimmobilized cells showed similar results to encapsulated (90% of 50-400 mg/L). L of gasoline hydrocarbon compounds), but it takes 30 days to degrade pollutants with a concentration of 600 mg/L. Encapsulation provides protection for the bacterial consortium from toxic hydrocarbon compounds, thereby eliminating the adaptation period required for the bacterial consortium if it is not immobilized, which is 1-5 days, depending on the gasoline concentration.

4. 4. Adsorption

Adsorption is an immobilization technique based on physical interaction between microorganisms and the surface *carrier*. This technique is *reversible*, simple, inexpensive and still effective. Immobilization of microorganisms on the right surface is able to stimulate microbial metabolism, protect cells from disruptive agents, and maintain their physiological activity [46]. Cell adsorption on *carriers* can be induced by weak bonds, such as van der Waals bonds, ionic and hydrophobic interactions [52]. One of the things that distinguishes the adsorption technique from the entrapment technique is the direct contact between the immobilized cells and the nutrients. The adsorption technique involves the transfer of cells to surface *carrier*, followed by cell attachment, and then colonization of the surface *carrier* by cells [55].

Wang *et al* [56] investigated the effectiveness of *Sphingomonas* sp. strain XLDN2-5 immobilized on *gellan gum* by adsorption technique to degrade *carbazole*. Iron nanoparticles were also used to support magnetic immobilization. The results showed that degradation of *carbazole* the highest was observed in the magnetically immobilized cells, which was 24.10 mg/g *carrier*. Non-magnetically immobilized cells also showed high effectiveness, namely 12.15 mg/g *carrier*. However, the activity of immobilized cells in degrading *carbazole mobilized* decreased when compared to cells. Immobilized Degraded 3,340 g of *cells carbazole* completely in just 20 hours, whereas immobilized cells took 36 hours.

5. CONCLUSION

Bioremediation is an appealing option for environmental friendly and efficient restoration of polluted water. The development of its process, including use of immobilization technique to better enhance its performance, lead to a more reliable remediation technology. It is necessary to unveil more implication of this immobilization such as the search of most convenient carrier, mechanism of immobilization and its fate and interaction in the environment. This could be accomplished by conducting more study incorporating multi perspective approaches from physical, chemistry, materials as well as biology. Future research are widely open that can bring a Wider group of microorganism such as the use of microalgae and fungi as well as different type of polutant including heavy metals, persistant organic pollutants (POPs) and Dyes [57-62].

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