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Biomineralization of toxicants, recalcitrant and radioactive wastes in the environment using genetically modified organisms

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ABSTRACT

Aim: The study was aimed to evaluate biomineralization of toxicants, recalcitrant and radioactive wastes in the environment using genetically modified organisms**.**
Materials and Methods: CRISPR/Cas 9 technology was projected for genetic engineering of organisms for better performance and increased biological efficiency in waste management.
Results: Some plants showed high sensitivity to pollution in the environment, whereas, bacteria like *Pseudomonas spp., Bacillus spp., Achromobacter, Flavobacterium, Acinetobacter. Citrobacter koseri and Serratia ficaria* effectively degraded spilled petrol, diesel, crude oil, oil-based paints and other toxic polycyclic aromatic hydrocarbons. White rot fungi also degraded spilled *crude oil,* plastics, radioactive and E-wastes.
Conclusion: It was concluded that rapid elimination of wastes in the environment using biologically engineered entities is the surest way to a cleaner and healthier environment.

Keywords: Bioremediators; biosensors; CRISPR/Cas9 technology; recalcitrant wastes; waste management.

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Introduction

The massive production and accumulation of wastes in the environment is a consequential recipe for total or partial dysfunction of the ecosystem(s) with such visible evidence like global climate change, increased global warming, loss of endangered species (fauna and flora), rapid destruction of wild and aquatic life, encroachment of productive lands for agriculture etc., if left unchecked, the world may someday become submerged by wastes or possibly go extinct. This situation is further worsened by epileptic or ineffective waste management strategies used all over the world, therefore, an appraisal or a retrospect of all possible options for timely detection and effective remediation of pollutants (wastes) in the environment is imminent (Zaghloul *et al*., 2020).

The use of indicator species such as bacteria, planktons, plants and animals as proactive "Biosensors" to monitor the type and level of pollutants in the environment *vis-a-vis* the application of bacteria and fungi in the mineralization, transformation or absorption of these pollutants is a promising approach to this problem (EL Hanafy, 2016). Microorganisms are ubiquitous, unfastidious, resilient and cosmopolitan in their nutrient and environmental requirements for survival (Abatenh, 2017). The nutritional versatility of these organisms can also be exploited for mineralization of pollutants, while genetic modifications can be conducted to produce highly beneficial strains of organisms with high propensity for adaptation and zero potential for mutation, solely for the purpose of cleaning the environment. This kind of process is termed "Bioremediation". Bioremediation is based on the ability of certain microbes and mushrooms to degrade or remove, alter or immobilize, decontaminate and transform physical wastes, or neutralize various chemicals spilled in the environment such as insecticides, herbicides, pesticides, fungicides etc., or convert, modify or detoxify, absorb and utilize toxic pollutants from the environment facilitated by their exogenously produced enzymes which act

as biocatalysts for energy production and biomass accumulation in the process (Feng, 2004). The involvement of microbes in large scale degradation, detoxification or removal of recalcitrant pollutants in the environment, such as organic compounds (Wang *et al*., 2014; Cao *et al*., 2015), hydrocarbons and heavy metals (Alisi *et al*., 2009, Plociniczak *et al*., 2013) etc., have been investigated and documented by various researchers (EL Hanafy, 2016). The diverse abilities of many bacterial strains to degrade recalcitrant compounds found in petroleum have been reported too and some of these organisms play crucial roles as primary degraders of spilled oil in the environment (EL Hanafy, 2016). These bacteria can produce bio-surfactants that can emulsify these hydrocarbons (Calvo *et al*., 2004; Magdalena *et al*., 2014; Pan *et al*., 2014). Some species from the genera *Acinetobacter, Burkholderia, Gordonia, Dietzia, Brevibacterium, Aeromicrobium, Celeribacter, Mycobacterium* and *Sphingomonas*, isolated from petroleum-contaminated soil have high affinity for hydrocarbon degradation (Daugulis *et al*., 2003; Chaillan *et al*., 2004; Cao *et al*., 2015; EL Hanafy, 2016). Microorganisms are effective pollutant removal tools in soil, water and sediments (Abatenh, 2017), therefore, genetic engineering and optimization of nature's choicest cleansing agents (Bacteria, Planktons, Fungi, Plants and Animals) for better performance and increased biological efficiency is the key to effective waste management.

Pollutants and Wastes: "Waste" is an inevitable by-product of most human activities and its introduction to the environment is called "Pollution", therefore, wastes are "Pollutants" since they contaminate the environment at varying degrees. The definition provided here is simplistic rather than holistic since there are other sources of pollution that are not necessarily "by-products" of human activities. Key sources of wastes introduction into the environment was presented. One of the most influential sources of environmental pollution is "Oil spillage". Oil spillage has been one of the major environmental issues in the last decades (EL Hanafy, 2016). There is geometric increase in the number or incidence of oil spillage around the world. One such example is the incidence of oil spillage from the Taylor Energy well in the Gulf of Mexico (USA), which was caused by a hurricane on September 16, 2004 (EL Hanafy, 2016). On January 21, 2011, another oil spillage happened in Mumbai (India), resulting in the leakage of about 55 tons of crude oil in the Arabian Sea. Then, in December 2014, another oil spillage was recorded in Sundarbans (Bangladesh) caused by the collision of a cargo vessel with an oil tanker. Oil spillage can occur at any time and can cause immense damage to the environment (EL Hanafy, 2016). Therefore, an effective cleanup mechanism described as imperative, since it is bio- and eco-friendly.

Biological agents for detection and mineralization of wastes in the environment

Biosensors: Planktons and plants are excellent pollution detectors in both aquatic and terrestrial environments, as they respond abruptly to certain changes in the environment which might be chemical or gaseous pollutants. Some examples of biosensors were presented. It included *Eichhornia crassipes, Wolffia globosa* (Plants), *Euglena elastica* and *Phacus tortus* (Phytoplanktons) as shown.

Bacteria: Bacteria are ubiquitous and they can survive almost on anythng, thus making them excellent candidates for biodegradation and bioremediation. Below are several specific bacteria species known to participate in bioremediation. Some notable examples include: *Achromobacter* sp., *Acinetobacter* sp., *Bacillus cereus, B. coagulans, B. subtilis, Burkholderia cepacia, Citrobacter koseri, flavobacterium* sp., *Pseudomonas aeruginosa, P. alcaligenes, P. putida, P. mendocina, P. veronii, Serratia liquefaciens* etc.

Fungi (Mushrooms): Fungi have an advantage over bacteria not just in metabolic versatility but also their environmental resilience. They are able to oxidize a diverse amount of chemicals and survive in harsh environmental conditions such as low moisture and high concentrations of pollutants. Therefore, fungi are potentially an extremely powerful tool in soil bioremediation and some versatile species such as White Rot Fungi had been a hot topic of research (Gadd, 2001; Harms *et al*., 2011).

Definition of terminologies

Biodegradation: This is simply the process by which large organic substances are broken down by microorganisms into smaller units with or without altering the initial toxic nature of the substance.

Biomineralization: This is the conversion of organic substance into its constituent inorganic components by the actions or metabolic activities

of microorganisms such as Archaea or bacteria, or the chemical mediation of the exogenously secreted extracellular enzymes from Fungi (Mushrooms) as shown.

Biotransformation: This is defined as any metabolically induced change in the chemical composition of a compound aided by the metabolic activities of some microbial entities (King *et al*., 1998).

Bioremediation: This refers to the use of microorganisms and some other higher forms of life to degrade contaminants that poses serious threat to the environment, or health hazard to plants, animals and humans. This process was clearly illustrated.

Bioavailability**:** This is referred to as the proportion of degradable organic or inorganic substances (pollutants or waste) readily assessable by the microorganisms during the process of bioremediation.

Biostimulation: This involves the modification of the environment (by addition of nutrient, water, molasses, phosphorus, nitrogen etc. to the soil) to stimulate the process of bioremediation by natural flora (bacteria, protozoans etc.) of the environment.

Bioaugmentation: It is the process whereby organisms (Archaea or Bacteria) with high potentials or capacity for degradation are inoculated into the contaminated site to increase the rate of biodegradation (Sylvia *et al*., 2005).

Genome engineering of biological entities

The following procedure was sacrosanct, imperative and pertinent in the development of resilient, efficient and viable strains of organisms for effective cleaning of the environment. The procedure and laboratory protocols adapted in this report were excerpts from Liu *et al*. (2015).

Synthesis of sgRNA fragment and recognition of target loci

The sgRNA is the principal driver for gene insertion or deletion. It consists primarily of a customized crRNA sequence attached to the scaffold or backbone structure known as tracrRNA sequence. The sgRNA fragment can be designed manually or synthesized *in vivo* or *in vitro*. In principle, the specificity of the Cas9 nuclease is determined by the 20-nt guide sequence within the sgRNA. A potential target sequence must instantaneously precede the protospacer-adjacent motif "PAM" (5′-NGG-3′) to be recognized by the first 20-nt of sgRNA via Watson-Crick base pairing. The recognition between sgRNA and its target will lead to Cas9 cleavage at 3bp upstream from the PAM.

Note*: "N" represents specific nucleotides like Adenine, Thymine, Cytosine, Guanine etc.*

Synthesis of oligonucleotides used as guide

Once a 20-nt target site is selected, a pair of DNA oligonucleotides (oligos) can be synthesized as follows,

Forward oligo:

5′-gattGNNNNNNNNNNNNNNNNN NGG-3′

Reverse oligo:

5′-ctaaCNNNNNNNNNNNNNNNNN NCC-3′

Here the successive "N"s in the forward oligo correspond to the 5′ 20-nt preceding the PAM (5′-NGG-3′) and those in the reverse oligo are just the reverse complementary sequence.

Vector construction:

To construct a single strand (1×) sgRNA-Cas9 plasmid

1. Phosphorylate and anneal each pair of oligos.
2. Digest 0.5µg of psgR-Cas9-At with BbsI nuclease for at least 2hrs at 37 °C.
3. Ligate the BbsI-digested vector with the oligo duplex.
4. Transform *E. coli* with the ligation product.
5. Identify positive clones of *E. coli* by colony PCR.

To construct a double strand (2×) sgRNA-Cas9 plasmid

1. Construct two separate single strands of sgRNA-Cas9 plasmid using the initial procedure stated above.
2. Amplify the double strand sgRNA module from the single strands sgRNA- Cas9 plasmids by PCR using the primer pair.
3. Digest the PCR product of the double strand sgRNA module and the single strands sgRNA-Cas9 plasmids.
4. Ligate the digested products to obtain a double strand sgRNA-Cas9 plasmid.
5. Transform the ligation product into *E. coli*.
6. Identify positive clones of *E. coli* by colony PCR.

Sub-clone the sgRNA-Cas9 cassette into the plant expression vector

1. Digest the pCAMBIA1300 vector and 1× or 2× sgRNA-Cas9 plasmid with HindIII and EcoRI in separate reactions at 37 °C for 2hrs.
2. Run the reactions on a 1% agarose gel, cut the digested band of 1× sgRNA-Cas9 (5.7 kb) or 2× sgRNAs-Cas9 (6.2 kb) and the band of linearized pCAMBIA1300. Purify the gel slices using gel and PCR clean-up system (Wizard®, Promega, USA).

3. Set up the ligation reaction and incubate at 16 °C for 2hrs.
4. Transform the ligation product into *E. coli*.
5. Identify positive clones of *E. coli* by colony PCR.
6. Purify plasmid from the culture of the positive clone and transform the plasmid into Agrobacterium tumefaciens GV3101 using the freeze-thaw (Weigel and Glazebrook, 2006a) or any other equivalent method.

Plant transformation and growth conditions

A detailed procedure for plant transformation under optimal growth conditions was given by Weigel and Glazebrook (2006b).

Transient expression of the CRISPR/Cas9 system

The expression of genes was rated using the method of Wu *et al*. (2009).

Examination of sgRNA-Cas9-mediated gene modifications

The examination of the target genes to ascertain if they were modified during the process can be aided by the following methods:

a. Detection of targeted gene modification in T1 generation

Since the leaves of plants and other organisms in the T1 generation mostly contain chimeric DNAs, detection of targeted gene modifications can be performed with a single piece of leaf or single units of the individual organism. Genomic DNAs of the leaf samples and mushrooms were extracted using CTAB method (Springer 2010), while that of bacteria and other protists were carried out using the procedure described by Marmur (1961). At least 24 representative samples were analyzed for each T1 population.

b. Detection of targeted gene modifications by RFLP

A prerequisite for the use of this assay is that the selected cleavage sites for CRISPR/Cas9 (3bp upstream of PAM) should be found within the restriction endonuclease site, so that PCR amplicons from gene-modified samples are resistant to restriction enzyme digestion.

- Create DNA amplicons from the genetically modified samples using PCR
- Digest the PCR products
- Digested products should be analyzed by electrophoresis in a 1.5% agarose gel.

***Note**: PCR amplicons should be resistant to restriction enzyme digestion by showing uncleaved bands.*

c. Detection of targeted gene modifications by dCAPs assay

For targeted gene sites that contain no restriction enzyme sites for RFLP assay, dCAPs assay can be used as an alternative choice for mutation detection (Neff *et al*., 1998).

d. Detection of targeted gene modifications by Surveyor assay

Surveyor nuclease is a mismatch-specific DNA endonuclease that cleaves with high specificity at the 3′ side of any mismatch site in both DNA strands, including base substitutions, insertions or deletions (Qiu *et al*., 2004).

- Design of primers used for Surveyor assay: Specific PCR primers are designed to amplify ~ 500bp DNA fragment surrounding the genomic target site.
- Amplify the DNA fragments using PCR
- Analyze 5.0 µL of the PCR product by electrophoresis on a 1.5% agarose gel to ensure that no unspecific bands are present.

e. Detection of targeted gene deletion by AFLP

In a situation whereby two adjacent genomic sites (within a range from 100-bp to 1-kb) are targeted, long fragment deletions between the two targeted sites occur frequently, which facilitate the detection of gene deletion by AFLP. Primers used for AFLP assay are designed to amplify DNA fragment containing both target sites.

f. Detection of targeted gene modification by DNA sequencing

PCR primers are designed to amplify a specific DNA fragment surrounding the genomic target site. PCR products are purified and sequenced using either primer. A typical chromatogram for chimera shows unique peaks before the sgRNA target site but, immediately after the target site, multiple peaks start to appear in each nucleotide position.

Selection of stable genes in T2 generation inherited from the T1 generation

T2 seedlings from those T1 lines are then transplanted to soil and used for further screening for the desired gene modifications. The selected lines are kept for further cultivation to produce seeds. In cases of low efficiency in detection of the target genes or in a situation where multiplex gene modifications are expected, the population size used for mutation analysis should be increased.

Selection of stably inherited gene modifications in T3 generation

Twenty T3 seeds were planted for each of the selected T2 lines for segregation and identification of T-DNA free plants with stable inherited gene modifications. PCR analyses for T-

DNA sequence are performed using the following primers for the Cas9 gene:
Cas9-3451F: 5′-CCCAAGAGGAACAGCGATAAG-3′
Cas9-4115R:5′-GGTCGATGGTGGTGTCAAAG-3′

Discussion

Majority of the pollutants found in the environment are difficult to detect without specialized equipment. Although, most of the sophisticated methods used for detecting the presence of various pollutants can actually quantify certain chemicals/gases in the environment, sadly, no monitoring device can estimate the level of potential toxicity or the rising change in the chemical nature of the toxicants (Kovalchuk and Kovalchuk, 2008). Therefore, a comprehensive analysis of environment using biosensors to assess the quality of soil, water or air is absolutely essential. A number of different biosensors were successfully used in the past including bacterial, animal (mammals, fish, and worms) and plants (Kovalchuk and Kovalchuk, 2008). Plants as bio-monitors can be an appealing alternative to animals. Plants are energy producers and a food source for many organisms. Therefore, their role in transferring contaminants to higher trophic levels is difficult to overestimate (McVey and Macler, 1993; Notten *et al*., 1995). For decades, they were used as a part of ecological risk assessment of agricultural and industrial chemicals, solid wastes, food additives, and chemically and radioactively polluted soil and water.

The most common factors used for studying mutagenicity of various pollutants in plants are based on the detection of chromosomal aberrations in *Allium cepa* (Fiskesjo, 1995; Ma *et al*., 2005), *Tradescantia* sp. (Ichikawa, 1992), *Vicia faba* plants (Kanaya *et al*., 1994) or *Zea mays* (Grant and Owen, 2006). Due to its sensitivity, the *Allium cepa* test was the first of nine plant assay systems evaluated by the Gene-Tox Program of the US Environmental Protection Agency (Grant, 1994). The assay is based on the evaluation of the percentage of aberrant mitotic events and different fractions of chromosomal aberrations (Fiskesjo, 1988). *Tradescantia* sp. has been used to analyze air quality in high traffic areas (Ma and Harris, 1985), municipal waste storages and the quality of air from a landfill vent pipe (Ma *et al*., 1996). This plant has also been used for the analysis of water and soil pollution, and even for the evaluation of bioremediation effectiveness at hazardous waste sites (Sandhu *et al*., 1989; Ruiz *et al*., 1992; Baud-Grasset *et al*., 1993). *Eichhornia crassipes*, *Wolffia globosa, Euglena elastica*, *Phacus tortus*, and *Trachelon anas*, also showed high sensitivity to pollution in the aquatic ecosystem (Parmar *et al*., 2016).

Most treatment systems rely on microbial activity to remove unwanted mineral nitrogen compounds (i.e. ammonia, nitrite, nitrate). The removal of nitrogen is a two stage process that involves nitrification and denitrification. During nitrification, ammonium is oxidized to nitrite by organisms like *Nitrosomonas europaea*, while nitrite is further oxidized to nitrate by microbes like *Nitrobacter hamburgensis*. In anaerobic conditions, nitrate produced during ammonium oxidation is used as a terminal electron acceptor by microbes like *Paracoccus denitrificans* (MicrobeWiki, 2018). The result is N_2 gas. Through this process, ammonium and nitrate, two pollutants responsible for eutrophication in natural waters, are remediated. *Pseudomonas putida* is a gram-negative soil bacterium that is involved in the bioremediation of toluene, a component of paint thinner. It is also capable of degrading naphthalene, a product of petroleum refining, in contaminated soils (MicrobeWiki, 2018). *Dechloromonas aromatica* is a rod-shaped bacterium which can oxidize aromatic hydrocarbons including benzoate, chlorobenzoate, and toluene, combining the reaction with the reduction of oxygen, chlorate, or nitrate. It is the only organism able to oxidize benzene anaerobically. Due to the high propensity of benzene contamination, especially in ground and surface water, *D. aromatica* is especially useful for *in situ* bioremediation of this substance (MicrobeWiki, 2018).

Deinococcus radiodurans is a radiation-resistant extremophile bacterium that is genetically engineered for the bioremediation of solvents and heavy metals. An engineered strain of *D. radiodurans* has been shown to degrade ionic mercury and toluene from radioactive wastes (Hassam *et al*., 2008). Phosphomannose Isomerases (PMI) from the Archaea "*Aeropyrum pernix* and *Thermoplasma acidophilum*" degrades Methyl tert-Butyl Ether (MTBE) by using the contaminant as the sole carbon and energy source (Jessica *et al*., 1999). *Alcanivorax borkumensis* is a marine rod-shaped bacterium which consumes hydrocarbons, such as the ones found in fuel, and

produces carbon dioxide. It grows rapidly in environments damaged by oil, and has been used to aid the cleaning of more than 830,000 gallons of oil from the Deepwater Horizon oil spilled in the Gulf of Mexico (Biello, 2010). *Pseudomonas alcaligenes, P. mendocina and P. putida P. veronii, Achromobacter, Flavobacterium, Acinetobacter* are effective degraded spilled *petrol, diesel and other polycyclic aromatic hydrocarbons like toluene, P. cepacia, Bacillus cereus, B. coagulans, Citrobacter koseri and Serratia ficaria* were used to clean up spilled diesel and crude oil, *B. subtillis* was used to clean up oil-based paints *(*Abatenh *et al*., 2017*)*. Using fungi as potential treatment of contaminants began in 1985 when the white rot fungi, *Phanerochaete chrysosporium,* was discovered to metabolize multiple key environmental pollutants (MicrobeWiki, 2018). The most important feature of these fungi is their enzymatic functional ability to metabolize complex chemicals such as lignin. Similar abilities were later discovered in other white rot fungi. In addition, white rot fungi are highly advantageous because they degrade lignin extracellularly through its hyphal extension. This allows them to access soil contaminants that not bioavailable to other organisms. These inexpensive fungi can tolerate extreme environmental conditions, such as pH, temperature, and moisture (MicrobeWiki, 2018). While many microbial organisms that are used for bioremediation require pre-conditioning of the environment for them to survive, white rot fungi can directly be applied into most systems because they degrade based upon nutrient deprivation (Fragoeiro, 2005). *Pleurotus pulmonarius (Edible mushroom) degraded spilled crude oil,* radioactive and E-wastes, and *P. ostreatus degraded* plastics (Kulshreshtha *et al*., 2014).

The potential for gene therapy to address human disease has been evident for some years, and much progress has been made in its applications (Cox *et al*., 2015; Naldini, 2015; NCBI Bookshelf, 2018). Gene therapy refers to the replacement of faulty genes, or the addition of new genes as a means to cure disease or improve the ability to fight disease. Genome editing is one aspect of gene therapy. Established approaches to gene therapy have been based on the results of extensive prior laboratory research on individual cells and on nonhuman organisms, establishing the means to add, delete, or modify genes in living organisms. Key advances include the development of techniques for generating molecular tools for cutting the DNA of genomes in specific places to allow targeted alterations in the DNA sequence. Over recent years, several such methods have been introduced and used effectively in clinical applications. Within the past 5 years, a completely novel system has been developed based on fundamental research on bacterial systems of immunity to viral infections. The first such system to be developed for use in genome editing of human cells, known as CRISPR/Cas9, is based on RNA-guided targeting and is much simpler, faster, and cheaper than earlier methods. The ease of design, together with the remarkable specificity and efficiency of the CRISPR/Cas9 system has revolutionized the field of genome editing and reignited interest in the potential for editing of the human genome. The development of the CRISPR/Cas9 system as a programmable genome-editing tool was built on a firm foundation of earlier research.

Genomes and their constituent genes are made of double-stranded DNA; this DNA can be broken accidentally (e.g., by radiation) or purposefully, using proteins called endonucleases (often called nucleases) that can generate double-strand breaks (DSBs) in DNA. Cells have mechanisms to repair DSBs in DNA, and these mechanisms can be used to generate alterations in the DNA sequence. Groundbreaking work in bacteria, yeast, and mammalian systems shows that DSBs dramatically stimulate the rate of DNA repair by non-homologous end joining (NHEJ), in which the broken ends are reattached. Such NHEJ repair often results in the deletion or insertion of DNA sequences of varying length, which can disrupt gene function (Rouet *et al*., 1994). However, if a homologous stretch of DNA is introduced into the cell as a donor template, homology-directed repair (HDR) can lead to more accurate repair or, if specific alterations are included in the homologous stretch, it can introduce specific precise changes into the recipient genomic DNA. These cellular DNA repair mechanisms have been used to develop several methods that allow genes or the genome to be edited in a very precise manner.

Conclusion

Genetic engineering of organisms for better performance and increased efficiency in the

detection and remediation of waste and pollutants in the environment using CRISPR/Cas9 technology is exclusive and novel in Biological Science for gene/genome editing of very important cell lines. By optimizing gene editing vectors and processes, the efficiency in gene-cutting, insertion, deletion and recombination using CRISPR/Cas9 technology is higher and more efficient than other conventional methods. As it was earlier stated, if proper attention is not given to the detection and remediation of wastes and other harmful pollutants in the environment and indeed around the world, the world may someday become overshadowed by wastes or possibly go extinct based on the action of harmful chemicals or gases disrupting the proper functioning of the global system. Therefore, early detection and rapid remediation of wastes in the environment using biologically engineered entities is the surest way to a cleaner and healthier global environment.

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