REVIEW ARTICLE

Bioremediation: A review of applications and problems to be resolved*

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Abstract This review article describes the factors affecting bioremediation processes including: goals of bioremediation and the optimal ecological conditions required; inoculation of microorganisms; co-metabolism; bioavailability and its improvement; biological evolution and its utilization; monitoring and control of bioremediation processes; identification of bioremediation effectiveness and ecological remediation and its key elements. The current progress in bioremediation techniques is summarized. The direction of future development, research and applications is also examined.

Keywords: **polluted environment, bioremediation, ecological process, new research progress.**

Bioremediation is a form of biotechnology having the characteristics of low energy consumption, high efficiency and high environmental safety, which can degrade and remove environmental pollutants by the physical metabolism of the bacteria, fungi, higher plants and cellular free enzymes. In a broad sense, bioremediation consists of three main categories, namely: the microbial remediation, the phytoremediation and the cellular free enzyme remediation. As a challenging leading edge technology[1], bioremediation has moved into a flourishing era. It is anticipated that bioremediation will be one of the key development areas in environmental technology during the early 21st century. In order to enhance the research on bioremediation in China, ensure its proper development, and transform research findings into successful commercial applications, great attention must be given to the key issues that are reviewed in the following sections.

1 Goals and optimal ecological conditions required for bioremediation

The goal of bioremediation is to reduce pollutants in soils, ground or surface water to a level lower than the standard value for environmental safety[2]. As a biotechnical measure, successful bioremediation is determined by many factors, and in particular, the following technical parameters should be considered.

(1) Microorganisms: Miracle microorganisms with special activities should be screened. These miracle microorganisms should have the ability to degrade pollutants from an initial high concentration to a level lower than the stipulated standard value without the generation of any toxic metabolites at a reasonable rate.

(2) Treatment sites[3]: On-site chemical pollutants and their concentrations should not inhibit degradable activities of specific microorganisms and enzymes, and the absorption of hyperaccumulative plants. Otherwise, the dilution should be considered[4]. Chemical pollutants should be bioavailable and conditions in an on-site reactor should be suitable for organisms to live. Therefore some knowledge about treatment sites and treatment processes as well as required ecological conditions should be necessary.

(3) Moisture: Many data show that moisture is one of key factors to regulate and control living activities of microorganisms, plants and cellular free enzymes[3, 5], since moisture is the medium through which nutrients and organic constituents diffuse into living cells and metabolites are discharged from living organisms. Moisture has a great impact on the bioremediation of contaminated soils and ground water by

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affecting the soil permeability, characteristics and quantity of soluble materials, the osmotic pressure, the pH value of the soil solution and the hydraulic conductivity rate of unsaturated soils. Some studies[3, 6] have shown that 25% ~ 85% of the water-holding capacity or −0.01 MPa may be the optimal level for the soil moisture validity.

(4) Nutrients: The growth of special organisms will be inhibited when the concentration of N, P or other nutrients is not high enough. The sufficiency of nutrition supply, the co-oxidation substrate and other parameters (including delivery methods, timing and doses) which can facilitate the growth of microorganisms and plants will be another limiting factor for the bioremediation. With regard to optimal ecological conditions, many researchers[3, 7] pointed out that the optimal concentration ratio of C∶N∶P is 100∶10∶1. The remediation of surface soils will be easier to achieve in terms of the easiness of nutrition supply and control. However, surface remediation does not necessarily mean the success of bioremediation processes, and maybe subsurface remediation or even deep layer medium remediation does. Therefore the regulation and control technique of the substratum, namely the technique of necessary materials required for bioremediation which are injected to the substratum, is an important component of bioremediation process control. At present, commonly used dosing systems include gravity or hydraulic feeding devices and orifice systems, while circulating pumps, radius borers and the hydraulics fracturing systems for the low permeable zone are still under development[8, 9].

(5) Oxygen and electron acceptors: Sufficient oxygen supply is a key factor in bioremediation processes[9]. In the phytoremediation, for the respiration of plants, a certain minimum amount of oxygen is required. In microbial remediation, the degradation speed of organic pollutants is more often than not determined by the supply rate of terminal electron acceptors. Most of soil microorganism populations take oxygen as the terminal electron acceptor. The oxidation-reduction potential also has some effects on metabolic processes of microorganism populations in subsurface soils. Many researchers[5, 9, 10] pointed out that the optimal DO level and the lowest air-filled pore volume for the aerobic metabolism are higher than 0.2 mg/L and 10%, respectively, under single factor conditions. For anaerobic metabolisms, the optimal DO level is less than 1% (aerobic and facultative anaerobic organisms > 50 mV, anaerobic organisms < 50 mV). Investigations into the combined action of different ecological conditions will be a main research trend in the future.

(6) Rhizosphere effects: In view of phytoremediation, rhizosphere plays a significant role[11, 12]. The activity of rhizospheric microorganisms, total biomass, physical sizes such as the ratio of root to stem and the ratio of root surface area to root volume and development conditions of plant root systems have a direct relationship with pollutant degradation and accumulation rates. Different types of plants and different rhizosphere functions have different degradation capabilities. Studies[12, 13] showed that virgate acrogenous roots of most monocotyledonous plants are delicately thin, usually less than 100 μm, and have a larger surface area than that of dicotyledonous plants. For example, the average diameter of wheat roots is 0.1 mm, while its average coverage area is bigger than 6 m^2. Those monocotyledonous plants with fine roots can flourish even in impoverished low nutrition soils. Dicotyledonous plants have thick and strong roots, normally 0.6 ~ 1.0 mm in diameter, which are suitable for the growth in tight soils. When compared to dicotyledonous plants, monocotyledonous plants have a higher rate of pollutant degradation or accumulation in soils. Furthermore, there are many oxidase systems in the rhizosphere of monocotyledonous plants, which have some special degradation capabilities for organic pollutants and can promote degradation processes of organic pollutants. In view of the significant role of the rhizosphere in phytoremediation, this will be an important researching field to undertake, both on the combined action of microorganisms and plants and on the relationship between the microorganism activity and the healthy root growth[14].

(7) Soil chemical and physical factors[15, 16]: Bioremediation processes are also affected by soil chemical and physical factors including the content of organic material and clay particles, cationic exchangeable capacity (CEC) and pH value, soil temperature and climate changes which can affect soil temperature and bioavailability of phosphate and calcium fertilizers. Studies[3, 12] have shown that the optimal pH is between 5.5 and 8.5 and the optimal temperature range is between 15 ℃ and 45 ℃ for bioremediation processes. With regard to other factors, the optimal ranges are still not available, and further studies need to be carried out to determine these important parameters.

2 Inoculation of microorganisms

A number of microorganisms have some unique or obligate metabolism functions and are correlated with indigenous microorganism community. The inoculation of microorganisms means to introduce those microorganisms into treatment sites. This is a key step in microbial remediation processes. The efficiency of microbial remediation processes can be assessed by the following criteria: the increase of microorganism biomass, the improvement of pollutant bioavailability, the optimization of microbial community structures and the favorable control of degradation processes, the enhancement of the activity of indigenous microorganism communities, especially significant impacts of inoculated microorganism on eco-chemical behavior, and the final concentration of pollutants[17, 18].

The unavailability of substrates for microorganism growth, the competition of indigenous microorganism communities, the presence of antibiotic substances, and the inhibition of predators all have great impacts on the growth and reproduction of introduced microorganisms. The biomass of big inoculums can solve this problem to some extent, yet the basic application equipment which can produce and disperse large quantities of bacteria biomass is required[9]. The important factor is that when inoculums are mixed with onsite environmental medium, only the consistency between original living conditions and onsite ones can ensure the avoidance of a large impact on the cell growth rate of inoculums caused by ecological conditions that differ from cultivation conditions and the identification of the misused microorganism during the cultivation of inoculums. When returned or applied to onsite treatment sites, those microorganisms acquired by the laboratory sifting with high growth rate and high metabolism rate are prone to compete with indigenous microorganisms. The future development will be sifting to the domestication of living microorganisms under laboratory conditions to enhance their degradation abilities under low substrate and low inorganic nutrition conditions, and to their abilities of being adsorbed to the surface with many pollutants, their abilities of migration or chemotaxis and their ability to maintain a high survival rate under different ecological conditions. It is also hoped that more potential inoculums can be obtained[9, 20].

The addition of chemicals that can promote the removal or degradation of pollutants can increase the ratio of successful inoculation of microorganisms[21, 22]. Special chemicals can facilitate the growth of onsite inoculums, and thus by significantly multiplying the biomass of inoculums, pollutants can be removed more rapidly. In contrast with the biomass of separated inoculums, special chemicals are more ready to integrate with the environmental medium. After the substrate is consumed by inoculums or removed from supply, a large amount of microorganism population will utilize target pollutants as their carbon sources[21].

Data[3, 21] have shown that peat moss may be the ideal carrier for the inoculation of rhizobia. Only bran, sawdust, infertile soils and clay have been tested as the biomass carrier so far. More studies and applications on this issue are still greatly needed.

3 Co-metabolizable mechanisms

Generally speaking, co-metabolism in bioremediation means pollutants in environment undertake some metabolic transformations while microorganism populations are utilizing some other chemicals as their carbon or energy source. During this process, the removal and detoxification of pollutants are all indirect or incidental events.

Nowadays, this special action has become one of the most important components for the bioremediation strategy. Unfortunately, the onsite regulation and control of this process has met with great difficulties, and research expenditure has been fairly considerable. The successful bioremediation of phenanthrene (PAH) is considered to be involved in this expensive co-metabolism process. Studies[23] showed that some enzymes which can be used by some special bacteria to degrade PAHs can oxidise other PAHs. Some researchers[22, 23] segregated the bacteria out of *Pseudomonas paucimoblis*, while taking fluoranthene as the only carbon and energy source. They can make some biotransformations to PAHs including naphthalene, fluorine, acenaphthene, phenanthrene, anthracene, 2-methylnaphthalene, 2, 6- dimethylnaphthalene, benzo [b] fluorine, biphenyl, and benzo [a] pyrene. Data[3, 24] showed that the degradation of DDT is a co-metabolism process under anaerobic conditions. In addition, the co-metabolite can be degraded by the aerobic microorganisms.

A notable factor is that under the predefined ecological settings which are supposed to promote the degradation of PAHs by indigenous microorganisms, co-metabolism could never be maximized. Therefore there are some risks associated with reducing PAHs to a level lower than the cleaning level by the co-metabolism[23] alone. Normally it is unknown which PAHs exactly are serving as the main carbon and energy source. It is impossible to keep the concentration of the right PAHs which is acting as the carbon and energy source at the optimum level at which the co-metabolism is supposed to be optimized. Sometimes the addition of low concentration of PAHs can stimulate the microorganism community to degrade high molecular weight PAHs to an even lower level through the co-metabolism.

It is also important to identify induced products by the co-metabolism. Laboratory studies[25, 26] showed that the strain cultivated by biphenyls can co-metabolize dibenzofuran. The oxidation of butane can cause the co-metabolism of chlorinated solvents. Field experiments[27] showed that the introduction of some substrates into a treatment site can promote the enrichment of microorganism communities which can degrade methane and phenol, and trichloroethene (TCE) can be degraded by a co-metabolism bypass. Some calculations showed that during the degradation of methane, 30% of TCE can be degraded, while 90% of TCE can be removed during the degradation of phenol. If ecological conditions in an onsite treatment site can be carefully controlled, the complete removal of target pollutants can be achieved. Studies[24] of Sun et al. demonstrated that isoprenoid-alkyl, alkyl phenyl group and amino oxidation bacteria can also co-metabolize TCE.

The bioremediation of many pollutants such as petroleum hydrocarbons and organic dyestuffs can produce some chemicals much more toxic than their matrixes due to the co-metabolism[28, 29]. In other words, although concentrations of original pollutants are reduced, converted products can exert much more toxicity acting on ecosystems. Additionally, some oxidation end-products by the co-metabolism tend not to be degraded by indigenous microorganisms. From the toxicological point of view, biological evaluations about biodegradable processes are necessary. Presently the existing methods in use are: the higher plant ecotoxicological methods (such as inhibition test of plant root elongation, test of seed germination, the test of the early stage seedling growth and the toxic test of horsebean root tips), the acute toxicity tests of earthworms, the subacute toxicity and the chronic toxicity, the protozoan ecotoxicological methods, the fish embryo indication test, the Ames mutagenesis evaluation method. Initially these tests were mostly used to undertake toxicity tests of pure chemicals[30]. With the development of research on environmental problems and increasing demand for the ecological quality of the environment, these methods have been applied to the evaluation of waste dumps, environmental pollution sites and the evaluation of the co-metabolism in bioremediation of contaminated soils and groundwater, etc.

4 Bioavailability and its improvement

Even under the optimal ecological conditions, immediate contacts between specific microorganisms, enzymes, plants and pollutants are often reduced owing to adsorption and immobilization of pollutants in environmental medium such as soil, sediments, and lithometeor. Therefore the biodegradability of pollutants and the availability of nutrients for specific microorganisms, enzymes and plants are decreased[31, 32]. In this sense, the observed bio-concentration of chemical pollutants in environmental systems could be defined as the effectiveness of biodegradability during bioremediation.

Two factors are often ignored when bioavailability is considered. One factor is that in many cases, especially at microbial remediation level, the effective concentrations based on individual cells are normally quite low, which to a large extent owe much to the combination of pollutants with special surface and the separation of bacteria from biological membranes[18]. Until now bioremediation processes and their onsite applications have not taken this key issue into account. In some cases, the application of surfactants to bioremediation can improve the bioavailability and the rate of biodegradation processes. Loeser et al. provided evidence[33] that the improvement in bioavailability can increase the biodegradation rate and the biological exploitability. Researches[28, 33] on using surfactants to biodegrade petroleum hydrocarbon and PAHs revealed that there was some unknown biodegradability by soil microorganism communities. For example, when bacteria using fluoranthene as the only carbon and energy source were applied to the remediation of petroleum-contaminated soils, they showed an ability to attack other PAHs.

Surfactants will play a significant role in the future bioremediation engineering[34, 35]. In particular, the development of biosurfactants can significantly reduce treatment expenses. Therefore in the future, studies on surfactants promoted biodegradation processes and the mechanism involved, and the engineering policy of using surfactants or other approaches that can improve transferring characteristic must be carried out. The removal of chemicals that can enhance bioavailability should also be considered to avoid negative effects caused by onsite distribution of pollutants, for example, the infiltration of pollutants into a non-polluted area and impacts of secondary pollution.

For hydrophobic organic pollutants, although the total concentration is fairly high, inhabited bacteria population in a water-droplet interface is fairly small due to the hydrophobic characteristics. Wastes from petroleum products, kreosote, coal tar and PCBs belong to this category. So far there is no research on how the bacteria can envelop and consume hydrophobic pollutants, although some data[3] showed that bacteria can generate emulsifiers. When added into an onsite treatment site, these emulsifiers can facilitate the biodegradation of hydrophobic pollutants. The application of those bioemulsifiers including their application in bioreactors may also improve the bioavailability of hydrophobic pollutants and lead to a final biodegradation. Unfortunately, bioemulsifiers are prone to be biodegraded themselves. In other words, they cannot be the substitute of chemosynthetic surfactants in bioremediation processes in a certain period. Therefore, the main task of the application of bioremediation processes will be to determine how to prevent bioemulsifiers from being biodegraded themselves.

5 Biological evolution and its utilization

Contaminated environments can result in a tolerance to organisms. It will be easy to screen some microorganisms or plants with high degradability or hyperaccumulative characteristic of some pollutants[12, 20, 36]. Conversely, in a clean environment, it will always be difficult to obtain miracle microorganisms or the hyperaccumulative plants needed in bioremediation processes. With regard to the identification of specific microorganisms and hyperaccumulative plants, from the viewpoint of biological evolution, contaminated environment has some positive effects.

On the one hand the need is to identify biodegradation and bioaccumulation processes in the contaminated environment. At the same time it is important to lay the foundation for the technical perfection of bioremediation by developing microorganisms or plants with even stronger biodegradability and bioaccumulation characteristics through the intentional, long term breeding with respect to biological evolution as well as by applying those biological evolution mechanisms[37, 38] including the modulation and utilization of transcriptional factors. On the other hand, the purposeful control of introduced specific microorganisms should be taken under the principle of biological evolution, including the decrease in introduced microorganism population with the disappearance of pollutants and the method of extracting specific microorganisms to apply to some polluted spots elsewhere.

With the globalization of environmental pollution and the long term exposure of many organisms to contaminated environment, the tolerance of living components in an ecosystem to pollutants is generally enhanced. Meanwhile the ecosystem has evolved itself[39]. From the viewpoint of economic benefits and resources saving, when enacting judging standards of bioremediation effectiveness, one should take biological evolution into account, and some pertinent researches on biological evolution under bioremediation conditions should be undertaken.

6 Monitoring and control of bioremediation

How can the effectiveness of contaminated soil bioremediation be evaluated? Chemical analyses are the traditional method, and they alone, can only perform either qualitative or quantitative tracing on target pollutants. It is difficult to give a correct evaluation on metabolite pollutants and their ecological toxicity that are generated during bioremediation processes. Ecotoxicological methods including the structure-toxicity correlation analysis of pollutants can be used to complement the chemical analyses. With these new methods it is also possible to predict the progress of special bioremediation and to reveal underlying problems. These factors are helpful to enable some engineering reconstruction to be undertaken to improve bioremediation processes.

In recent years biotechnology has been widely applied to the monitoring of bioremediation[40~42]. The method using the nucleic acid probe can detect specific microorganisms with biodegradability based on the cloned requisite gene, for example, the nucleic acid probes identifying monooxygenase and dioxygenase genes which can initially oxidize aromatics have been developed; the method of gene expression can establish the correlation between the disappearance of a certain pollutant and environmental expression of a certain gene responsible for the initial metabolism of pollutants by virtue of separating the special gene probe from marked RNA, such as the gene expression of monooxygenases and dioxygenases which are responsible for the initial metabolism; the method of stressing the activation of start-up genes can induce special metabolism bypass to degrade some pollutants by environmental activation of start-up genes, such as the start-up gene activated by the low nutrition level or different temperature; in combination with the spectrophotometry, the method of stable isotope can determine fluxes of organic or inorganic pollutants affected by bioremediation processes; by using the stable isotope method of carbon and nitrogen, degradation products of some pollutants such as CO_2 can be traced. So far the biotechnological methods which can be applied to this kind of monitoring also include the biological diversity identification, the immunological test, the informative gene and the chemical species design.

The application of biotechnology enables a better understanding of many microbiological and botanic ecological issues. Most of bioremediation studies are focused on research and development of new ecological processes; and so far there are few concerns about process control and long-term bioavailability of bioremediation processes. It is anticipated that molecular biology and biotechnology will finally be the most efficient, the cheapest and the most sensitive method for the process monitoring and control[41].

7 Evaluation of bioremediation effectiveness

It is difficult to tell whether a bioremediation process application is successful or not[43]. Generally speaking, if concentrations of target pollutants are reduced to a lower level than the expected one, it can be said that it is a successful or efficient application. Nevertheless one thing that must be pointed out is that it must be biodegradation instead of volatilization or dilution that causes the reduction in concentrations of pollutants. In some cases volatilization, dilution and redistribution of pollutants are far more effective than biodegradation[9, 44], and more often than not these mechanisms are mistaken for biodegradation.

Many methods and steps can strengthen bioremediation of contaminated soils and its application to contaminated sites. However, the strengthened treatment may cause potential ecological risks which discount the successful application of bioremediation processes. Some experiments[5, 9] have shown that the optimization of ecological conditions has some negative impacts on specific microorganisms. For example[45], although the application of microorganism inoculums in kreosote-contaminated soil remediation seems very successful, the nutrients weaken degradation of pollutants by inoculums because the nutrients which are added along with specific microorganism inoculums have stronger stimulations on the growth and the reproduction of indigenous microorganisms than that of the specific microorganism inoculum itself.

The short remediation time and the corresponding affordable expenditure should also be considered as the component of evaluating effectiveness of bioremediation. The low degradation rate of high molecular weight PAHs, the decrease in the activity of microorganisms, and the concentration determination of mixture pollutants are main limiting factors for the applications of bioremediation[9, 46]. Bioremediation processes are bound to fail if the costs of reinoculation and the addition of nutrients are not affordable. Therefore the successful application of bioremediation has an immediate relationship with the remediation time and the expenditure control.

Although we can accurately identify pollutants which are heterogeneously distributed in environment, it is still possible to underestimate the heterogeneity in many cases. In the case of *in situ* bioremediation applications, developing proper sampling and analytical methods to identify loading of pollutants in environment[47] is more important than bioremediation itself. Less advanced sampling and analytical methods may lead to incorrect evaluation of the environmental complexity and finally may lead to the failure of bioremediation processes. For example, in the bioremediation of soil contaminated with petroleum hydrocarbons, the degradation of hydrocarbons is enhanced by the addition of manure, oxygen and biological materials degrading hydrocarbons. It is not possible to judge the success of bioremediation according to the degradation of hydrocarbons because we cannot determine the degradation rate of PAHs using outdated detection methods. Simulation studies on their chemical behavior and their migration and transformations in different environmental mediums[48] to scientifically evaluate the success of bioremediation application will be an important part of future researches and applications of bioremediation.

It is not difficult to understand that the success of bioremediation technology under laboratory conditions does not necessarily lead to the success of the onsite applications[9,43]. Similarly the successful application at one site cannot automatically lead to success at another site. With the application of biodegradation-enhanced processes, the issue of the relationship between the "amplification scale" and the effectiveness of bioremediation arises.

8 Ecological remediation and its elements

For bioremediation of combined pollution, individual remediation methods normally do not work. The effective combination of various cleanup methods, namely ecological remediation, can degrade and remove pollutants more efficiently[49]. In order to improve the efficiency of remediation processes during the consolidated remediation, several remediation technologies can be used simultaneously or respectively in different stages. At present the combination of miracle microorganisms with special plants is a relatively mature bioremediation technology[14]. The contribution of special plants to bioremediation processes can be summarized as two aspects: (1) the absorption and accumulation of pollutants by a hyperaccumulative plant itself[50]; (2) the promotion of degrading pollutants by way of improving ecological conditions favorable to miracle microorganisms[51].

The absorption and accumulation of pollutants by a plant itself are affected by many ecological and chemical conditions such as air temperature, precipitation, medium pH, soil clay, CEC, organic matter and the toxicity of chemicals. Some studies[12,51] showed that some plants can compete with soil organic matter in the process of absorbing lipophilic compounds. Roots and vessels of some plants can even accumulate highly concentrated organic pollutants such as 2, 3, 7, 8-tetrachloinated dibenzo-p-dioxins (TCDD). Phytorhizosphere can generate secretions such as saccharides, organic acids, amides and enzymes. These secretions can improve micro-ecological conditions of soils, and accelerate microbiological degradation. The acceleration of microbial degradation of pollutants by phytorhizosphere secretions can be explained as follows[12,52]: (1) secretions from a rhizosphere contain necessary nutrients and other materials for the growth of microorganisms, thereby facilitating the growth and reproduction of microorganisms by improving the availability of nutrients; (2) secretions from a rhizosphere can coordinate microbial metabolisms; (3) the rhizosphere can provide a good habitat for microorganism populations to degrade pollutants; (4) some plants can transfer oxygen to the rhizosphere, which can facilitate aerobic degradation processes.

The main advantage of the joint plant-microorganism remediation approach is that no soil excavations are required, which reduces the exposure extent and the exposure time of pollutants. With regard to large area soil cleanup projects, this approach may be more practicable than others. Nevertheless some pollutants may be toxic to plants, and sometimes redisposal of hyperaccumulative plants may be required. Meanwhile the cleanup cycling time is relatively long[12].

In a word, the goal of bioremediation processes is to use biosystems to efficiently clean up pollutants caused by human activities. Successful bioremediation applications need the cooperation of many disciplines including pollution ecology, molecular biology, biotechnology, soil chemistry, botany, microbiology and environmental engineering. One factor that must be emphasised is that deep understanding of biotechnology microbial principles will benefit the further development of bioremediation technology, and facilitate more efficient and a greater number of bioremediation applications. By assimilating, referencing and learning from both successful and unsuccessful bioremediation applications, the bioremediation of contaminated soil, groundwater and surface water will enter a new era.

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