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ENDOPHYTIC REMEDIATION: A BLOOMING APPROACH TOWARDS CLEANER ENVIRONMENT

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ABSTRACT

With the advent of technologies, industrial flourish, agricultural malpractice and human interposing, a lot of toxic pollutants pops up into the environment which are of serious concern. The pollutants may include heavy metals like lead(Pb), cadmium(Cd), cobalt(Co) or can be petroleum based toxic substances or may be water-soluble Triphenylmethane dyes(TPM) which are often persistent in nature. A lot of approaches have been made to treat and detoxify the contaminants. Various chemical treatments, use of inert materials like charcoal or various micro-organisms have been exposed for the same purpose. On contrary, these chemical methods have their own disadvantages. So, apart from those, a whole new ambit have came into focus that deals with endophytes. These are quiet profused in most plant tissues. Endophytes are explored to biodegrade the toxic substances and simultaneously improve the soil and water quality. This chapter deals with the basis of endophytic bioremediation emphasizing on the isolation, identification, sources and probable laboratory approaches followed for bioremediation. This content will also portray the outline on how to achieve enhanced bioremediation and brief discussion of other beneficial potential of endophytes.

KEYWORDS: Endophytes, Bioremediation, Triphenylmethane dyes, Bioaccumulation.

INTRODUCTION

Endophytes are microorganisms that populate in the tissues of host plant inter or intra-cellularly without causing any potent harm to the plant.[1] The host-endophytes interactions can extend from obligate or facultative to complex association of mutualism or antagonism.[2] Several factors influences the endophytic growth including host plant itself.

Nutritional supply by host plant[3], geographic location, seasonal variations also contributes as major factors in endophyte-host relation. To maintain steady symbiotic relation, they show adaptive mechanisms like production of various bioactive compounds or assisting in plant growth by production of IAA, siderophores[4] etc. Endophytes have also been reported to prevent plant pathogens protecting the host plant from diseases. Epichloë endophytic fungus prevents fungal pathogens of some grasses.[5]

Recent explorations have revealed that endophytes have a great potential to bioremediate toxic pollutants from environment. Due to industrialization[6], population growth, urbanization and mining activities[7], there are increasing rate of pollution due to heavy metal intoxication, various dyes[8], several other organic and inorganic metal compounds. Use of extensive metal based fertilizers in the agricultural fields may also add to the pollution. They are generally persistent in nature and thereby leads to biomagnification.[9] The pollutant metals generally include arsenic, cadmium, aluminium, copper, lead, zinc and chromium which severely impact environment as well as human. There are various chemical methods for treatments of these pollutants, however, they seemed to be not so effective. There raised the need of some efficient, ecofriendly, versatile, cost-effective techniques to remove these type of contaminants from the environment. At this point, endophytes emerged a very promising advancement as biological control of heavy metal based pollutants. Not only heavy metals, endophytes reportedly is found capable of detoxicating other pollutants like oil, dyes, pesticides etc. Use of endophytic bacteria in rhizosphere have been reportedly known to lessen the heavy metal and pesticide contamination in the soil by inducing various enzymatic activity.[10]

Bioremediation is a process which incorporates biological entities to treat the contaminants and transform to diminish their toxicity. This highly acknowledged technology deals with the growth of specific microflora or microbial consortium native to that of contaminated site to achieve the desired purpose of ecological detoxification. There are basically two mechanisms involving in the process, viz. Biosorption and bioaccumulation.[11] According to Kousha et al, Biosorption ensnares the pollutants without breaking down and follows sequestration technique of attaching contaminants by their functional group with the cell wall.[12] Bioaccumulation in turn, is an approach involving chemical transformation of pollutants and mineralizations, followed by transportation and amassing within the cells in less harmful forms.

Chlorinated solvents, Phenols, BTEX(benzene, toluene, ethylbenzene, xylene), Polyaromatic hydrocarbons like napthalene, pyrene, various pesticides, heavy metals like Cu, Pb, Zn, Cd and Triphenylmethane(TPM) dyes[13] are some of the prospective pollutants for bioremediation.

There are several factors like soil moisture, soil pH, oxygen content, nutrient availability, temperature, soil type and concentration of contaminants or heavy metals that is required to be considered for bioremediation approach. It is suggested that ph 2-3 and 8-9 as best suitable for biosorption of textile dyes, Iqbal et al stated that controlled temperature and moisture lead to greater bioavailability thereby reducing polycyclic aromatic hydrocarbon(PAH)[14] and phenol which are considerably recalcitrant pollutant.[15]

BIOREMEDIATION BY ENDOPHYTES

Endophytes are found in almost all plants and some of them show high potential to remove heavy metal. Endophytes have been isolated for the bioremediation purpose from various plants starting from cultivated *Brassica napus*[16], various hyperaccumulators like *Pteris vittata*[17], *Brassica junea*[18] to some non-hyperaccumulator like *Arabidopsis napus*[19] and *Glycine max.*[20] The endophytic varieties can be of wide spectrum of varieties commonly fungi or may be bacteria.

Bioremediation process involves breaking of complex compounds into simple forms or may be converting toxic pollutants into bio-feasible forms. *Nicotiana tabacum* inoculated with endophytes was reported to show cadmium stress tolerance.[21] Endophytes isolated from *Solanum nigram* have shown their capability of multi-metal resistance.[22] Lacercat-Didier have isolated endophytes from poplar roots which not only was tolerant to Zn, Cd, Cu and Pb but also have shown increase in the root tip and shoot biomass.[23] Genetically engineered *Burkholderia cepacia* reduced phytotoxicity of *Lupinus luteus* by increasing nickel uptake and root biomass.[24] Acremonium sp. PO997 remediated metal ions Fe^{+2}, Mn^{+2}, Zn^{+2}, Pb^{+2}, Cu^{+2}, Al^{+3}.[25] Table 1 below shows some of the plant-endophyte interaction which is proved to be effective in bioremediation.

Table 1: List of successful endophytic bioremediation.

Endophytes	Category	Plants	Contaminant	Reference
Penicillium sp. FT2G59	Fungi	*Dysphania ambrosoides*	Zn^{+2}, Pb^{+2}, Cd^{+2}	[26]
Pseudomonas putida	Bacteria	*Pisum sativum*	2, 4-D	[27]
Pseudomonas fluorescens G10	Bacteria	*Brassica napus*	Pb	[28]
Bacillus sp L14	Bacteria	Solanum nigram	Cd, Pb, Cu	[22]
Phomposis sp. B3	Fungi	*Oryza sativa*	Phenanthrene	[29]
Penicillium citrinium BTF08	Fungi	*Musa* sp.	Zn^{+2}, Pb^{+2}, $Cd^{+2,}$ Cu^{+2}	[30]
Stenotrophomonas sp. S20	Bacteria	*Tamarix chinensis*	Ni	[31]
Pseudomonas sp. P21	Bacteria			
Sphingobium sp. S42	Bacteria			
Bacillus sp. Fcl1	Bacteria	*Curcuma longa*	Quinalphos(Pesticide)	[32]

2. 1 Sources of endophytes

The term endophyte was originated from the Greek words endon= within and phyte=plants. Carrol 1986 defined endophytes as symptomless mutualistic organisms associating with the aerial parts of the plant. Hallman et al defines endophytes as isolated organisms from surface-sterilized plant explants or from within the plant tissues, that apparently produces no indication of disease in the host plant.[33] Stone et al later stated that they may be colonizing forms of organisms staying in mutually beneficial interaction for a span of their life cycle.[34] The endophytes can be of broad spectrum ranging from fungus, bacteria, protists etc.

The very initial discussion which should pop up revolves around what is the source of endophytes and how do they get associated with the plants. Adam et al. had stated the seeds as the basic origin of endophytes[35], however seed-originating hypothesis is still controversial as endophytes can also enter externally through fine slit in the seed coat. Experimental evidences have shown that seed treatments colonized endophytes in the internal tissues of root radicles newly emanated from the seed coat manifesting the role of spermosphere as a source of endophytes.[36] Vegetative propagation materials[37], rhizosphere soil[38] and phylloplane[39] have also been reported as a source of endophytes. Isolation of endophytes from *Pinus sylvestris* roots by Babu et al.[4], Bruisson et al. worked on isolating 78 potential epiphytes and endophytes from three grapevine cultivers contributing to plant defense mechanism against pathogen intervention[40] gives some sketches of endophytic sources.

There are several other endophytes that have been reportedly isolated. Some of which have been spotted in the Table-2.

However, statistical analysis and observations have converged the rhizosphere as the primary source of endophytic varieties. Kumar et al. reported to observe occurrence of endophytic colonization in the rhizosphere of older and young plants with different densities.[41]

Excluding the seed transmitted endophytes, which are already residing within the plants, potential internal colonizing endophytes can congregate by identifying their host via the chemotaxis, electrotaxis or inadvertently(may be due to plant wounds).[33]

Table 2: Common Plant parts as endophyte source.

Plant part	Endophyte isolated	Plant host	Reference
Root	*Bacillus sp.*	*Pinus sylvestris*	[4]
Needle	*Rhytismataceae*	*Pinus monticola*	[42]
Stem	*Pseudomonas sp.* *Serratia sp.* *Bacillus sp.*	*Piper nigram L. (Piper)*	[43]
Root nodules	*Burkholderia sp* *Ensifer sp* *Devosia sp.*	*Acacia*	[44]
Root nodules	*Bradyrhizobium sp.*	*Cicer arientum*(Chick pea)	[45]
Nodules	*Burkholderia sp.*	*Mimosa pudica*	[46]
Leaves	*Bacillus cereus* *Bacillus subtilis* *Penicillium chrysogenum* *Penicillium crustosum*	*Teucrium polium L.*	[47]
Bulbs	*Paenibacillus polymyxa SK1*	*Lilium lancifolium.*	[48]
Leaves	*Penicillium sp* *Alternaria sp.* *Aspergillus sp.*	*Ephedra pachyclada*	[49]
Seeds	*Bacillus velezensis*	*Oryza sativa L.*	[50]
Fruit	*Alternaria* GFAV15	*Tinospora cordifolia*	[51]

2. 2 Isolation and identification of endophytes

Endophytic organisms are extensively found in many plant species, inhabiting quiescently or dynamically colonizing forms in plant tissues. It has been found that endophytes are isolated from meristem, resin ducts, leaves, roots, stem, bark, petiole and buds.[52][53][54] They may dwell locally or within the physiological system internally by penetrating the plant tissues using hydrolytic enzymes. Endophytes can be sourced from either rhizosphere or endophyte-pestered seeds or other plant materials. Isolation procedure of endophytes is crucial for exploring these highly potent organisms. Though isolation of plant surface-inhabiting endophytic organisms are comparatively convenient from practical experimentals, however, non-extractable internal plant colonizing organisms create challenge in isolation process.

There are several techniques have been availed for isolation of endophytic organisms. All the techniques have their own advantages and limitations. Depending on the objective and ground of study, researchers may employ the most suitable approach. Commonly, endophytes are isolated by surface sterilization followed by culturing the pulverized plant tissue extract[55] or directly culturing the plant tissue[56] in suitable media. Surface sterilization is an important step to ensure the possibility of isolating endophytes without epiphytic desecration.[57] The process often involves Ethanol and mercuric chloride wash of the plant tissue, however, sodium hypochlorite and sodium thiosulphate are also used for extensive sterilization.[58] Few other techniques have been discussed in Table 3.

Table 3: Isolation techniques for endophytes.

Technique	Basic procedure	Reference(s)	Advantage	Disadvantage
Trituration and surface disinfestation	Surface disinfection followed by trituration of plant samples manually or mechanically	[59][60]	Can be used for any plant tissue including seeds, fruits, leaves	Complete surface sterilization is difficult Chance of loss in endophytic community
Vacuum and pressure extraction	Vacuum technique to extract plant sap	[61]	1. System-colonizing endophytic organisms can be isolated selectively. 2. Lacks surface-dis infestations	1. Not suitable for soft tissues like seedlings or herbs. 2. Pressure should be optimized.
Centrifugation	Surface sterilization followed by centrifugation and microscopy	[62]	Multiple sample handling at a time. Suitable for soft plant tissues	Tedious process.

The success rate of isolation process may be influenced by several factors starting from the nature of endophytes depending on whether it is extractable or nonextractable, point of their localization etc. There are chances of loss of endophytic community due to extensive use of toxic surface-disinfectant like Mercuric chloride or use of uncontrolled pressure as described earlier. So critical optimization of the isolation approaches is also required depending on the research work and the specific objectives adopted. Healthy plants source without any demarcating disease symptom may also contribute to the isolation success as the probability of obtaining unwanted pathogens is reduced.[63]

Endophytes, being flourished as current research interest due to its wide potential, isolation has become the important step to explore about these microorganism group. Several isolation of endophytes have been reported. Verma et al have prepared four culture media to isolate endophytes. They have used Malt Yeast agar(MYA), Mycological agar(MCA), Potato dextrose agar(PDA) and Nutrient agar(NA). About 272 isolates were recovered and MCA media was found to be most effective(95 isolates) with most variety richness isolated.[64] Sun et al to isolated endophytic species from Cu-tolerant plants over LB agar media supplemented with Cu. The source plant was surface sterilized with the regular process of ethanol-mercuric chloride-distilled water wash.[65] The isolates are further enumerated by colony counting grown on the media, taking into account the dilution factors or the most probable number(MPN). Some more media used for isolations have been listed in Table 4. However, some obligate fungus becomes non-culturable for not responding to synthetic media, hindering to the exploration.

Table 4: Media for isolation of endophytes from host plant tissue.

Sl. No.	Host Plant	Plant parts	Media	Organism	Isolates	Reference
1	*Miquelia dentata Bedd.*	Leaf, fruit	Nutrient agar plate	Bacteria	*Bacillus sp., Lysinibacillus sp.*	[66]
2	*Oryza sativa(Rice)*	Stems, Roots	Tryptic soy agar(Difco)	Bacteria		[67]
3	*Glycine sp. (Soybean)*	Stem, Root nodules	Potato dextrose agar(PDA)/Trypticase soy agar	Bacteria	*Bacillus sp., Staphylococcus sp.*	[68]
4	*Alyssum bertolonii Desv.*	Root, Stem, leaves	Trypton soy broth	Bacteria	*Staphylococcus sp., Pseudomonas sp., Microbacterium sp.*	[69]
5.	*Helianthus annuus L. (Sunflower)*	Roots with rhizospheric soil	Yeast manitol medium(YEM)/Red congo(RC)/ Nitrogen free base(NfB)	Bacteria	*Bacillus sp.*	[70]
6.	*Taxus sp.*	Woody branch, shoots, leaves	Malt agar/Malt agar+50ppm rose bengal and tetracycline HCl	Fungi	*Alternaria sp, Fusarium sp., Mycelia sp.*	[71]
7	*Azadirachta indica(Neem)*	Leaves	Potato dextrose agar(PDA)	Fungi		[72]

8.	*Pasania edulis*	Leaves	2% Malt extract agar(Difco)	Fungi	*Phyllosticta sp., Colleotrichus sp.*	[73]
9.	*Cascinium fenestratum*	Stem, Leaves	Potato dextrose agar(PDA)	Fungi	*Phomopsis sp, Alternaria sp., Aspergillus sp.*	[74]

The identification of endophytes is another critical step for gaining extensive knowledge on them. Earlier, the identifications were mostly done on the basis of morphological features. The colony, vegetative hyphae, conidial aspects plays important identifying attributes.[75] Later to this, viable staining along with light microscopy were introduced for identifications. 2, 3, 5-triphenyltetrazolium dichloride conversion to red colored formazon[76] or use of acridine orange[61] to determine endophytes were reported. Nenkateswarlu et al have performed morphological identification using crystal violet staining under inverted microscopy.[77] However, taxonomic study could not be done using this staining techniques. With further progression, electron microscopic study was taken into account. Turner et al have used scanning electron microscopy(SEM) and cryo-SEM to detect endophytes in plants like corn.[78] But there is a possibility of contamination while sample preparation. This problem was overcome by transmission electron microscopy where sample is fixed prior to sectioning. This approach was successfully utilized to identify endophytes in rice[79], potato[80] etc. But drawback lies in the point of sectioning such thin sample tissue preparation along with the inadequate presence of endophytes within the sample(For example, detection limit is $1X10^5$ CFU/ml in root[81]). Besides these, laser scanning electron microscopy also contributed in endophytes isolation. Some other methodologies for endophtye identifications have also been reported. Immunoassay[82], nucleic acid hybridization and autoradiography to name a few. Antibody cross reaction followed by immunofluroscence detection by microscopy, tissue printing[83], enzyme-linked immunosorbent assay(ELISA) are some of the approaches bracketing immunoassay technique. Autoradiography may involve labelling of microbes(endophyte) and culturing and then studied using mass spectroscopy or autoradiography.[81]

With headway through limitations for different detection procedures, molecular biology gave rise to modern tools for a more advanced way to identify endophytes. Various genetic markers, sequencing and bioinformatics tools have taken the identification to next level. These approaches not only helped to study vividly on both culturable and non-culturable endophytes, but it also gives a detailed data on phylogeny by genetic analyses. Diaz et al have isolated genomic DNA and amplified internal trancribed spacer(ITS) region by polymerase chain reaction(PCR) to generate DNA bar-coding.[84] Sun et al have also reported to use DNA bar coding approach to identify endophytes.[85] Kulinisky et al have identified *Pseudomonas, Burkholdriaeceae*, *Enterobacteriaeceae* using partial 16s rDNA sequence analysis.[86] Luo et al have put forward a protocol to identify endophytes using 16s r RNA technique.[87] These molecular techniques are proved to be very helpful in cases where endophytes are morphologically difficult to identify. Similar case have been reported by Crozier et al where they successfully identified 59 morphologically unidentifiable isolates with the help of DNA extraction and sequential analysis of nuclear ribosomal DNA(rDNA).[88] The genetic sequences are further analysed for phylogenetic study or comparative analysis using bioinformatics tools.

A successful bioremediation may include all round checks starting from optimalization of conditions, determing tolerance of endophytes to pollutants (heavy metals), contribution of endophytes to host plant growth promoting traits and checking the stability of remediation by endophytes at varied range of pH and temperatures. The plant surface(Expected source of endophytes) is surface sterilised[89] and cultured. Negative growth rate assures the isolates to be endophytes[90] and indicates successful surface sterilization. The isolated endophytes are subjected to heavy metal tolerance tests using agar-dilution method where microbes are cultured in LB agar medium supplemented with various concentrations of heavy metals. Zafar et al suggested that growth and tolerance can be tested by tolerance index(TI) test or MIC.[91] Endophytes are also known to promote plant growth by assisting in generation of metabolites. So in laboratory, plant growth promoting traits of endophytes were tested by IAA, siderophore production[92] on Chrome Azurol S agar(CAS) and ACC deaminase activity study. Effect of heavy metals on the growth of endophytes are tested by incorporating heavy metals in media, generally at late exponential phases. Atomic adsorption spectroscopy(AAS) was involved to analyse the residual heavy metals after treating endophytic culture under a range of pH(pH 6-9) and temperature(30-37°C).

For further advanced study, different bioinformatics tools can be utilized to explore more about the genetic background responsible for bioremediation. Ali et al have used bioinformatical approach to study the endophytic nature of *Burkholderia* sp. In their work[93], they have employed Reciprocal small distance algorithm[94], Artemis genome browser and annotation tool to perform comparative genome analysis study.[95]

STRATEGIES FOR ENHANCED BIOREMEDIATION BY ENDOPHYTES

As already discussed, endophytes assist in bioremediation either by biosorption or by bioaccumulation process. They may be co-cultured with some hyperaccumulator plants to aid to their growth and metal uptake capacity by altering metabolism of the plant, hence boosting the phytoremediation process. However, it is always appreciated for betterment of whole bioremediation by enhancing the bioactivity of the organisms employed. This is a very recent blooming area of research for the environmentalists. Though not much strategies have been explored to enhance the bioremediation capacity of the endophytes. However, to point the discussion, some of the reported methods have been sketched out.

There are various pre-treatments to alter the functional group on cell wall, improving the binding capability for metals on the surface.[96] Pre-treatments include physical and chemical procedures. Boiling or autoclaving, cold shock can be listed in physical pre-treatments. Different acid and alkali treatments, use of detergents can be follow up procedures under chemical treatments. Khalil et al have applied sodium carbonate to treat cultured endophytes which prominently increased the enhancement rate by 91. 2% for treated isolates.[97] Citric acid treated endophyte *Fusarium* sp. enhanced thorium metal remediation of treated samples which effaced metal of 75. 47 mg/g whereas untreated samples were found to remove only 11. 35mg/g of metal.[98]

Polymerisation and controlled grafting is another technique followed to enhance endophytic bioremediation. Altering the cell wall by cross-linking leads to the modification of functional group & increased bioadsorption. Luo et al have used grafting on cell wall of *Pseudomonas* sp Lk9, endophyte on *Solanaum nigram* L. with poly(allylamine hydrochloride)(PAA) crosslinked with glutaraldehyde. An increased bioremediation was achieved for the tailored bacteria.[99]

Another effective strategy that can be incorporated is immobilization. In this process, cells are encapsulated or trammeled in some natural or synthetic matrix. The matrix assists in better biosorption elevating the remediation process. Alginate[100], polyurethane are some of the materials that can be used as matrix. This method is advantageous over free cell in terms of greater biocompatibility, cost effective and can be reused.

In laboratories, it is pretty predictable to study single metal interaction with individual microbial strain. In contrary, amidst natural condition, single microbial species existence is almost impossible. Moreover, targeted pollutants for bioremediation may not be accessible in purely bio-available forms or may be mixed with other contaminants when seen in native environment. In this case, an approach of using microbial consortia instead of single microbe system may help in better enhancement effect. Some researchers have gone a step forward to involve multi-metal system in laboratories to resemble the natural condition. Bilal et al tested combined aluminium(Al)-zinc(Zn) stress tolerance of *Paecilomyces formosus* LHL10 and *Sphingomonas* sp. LK11 when co-inoculated in Al-Zn distressed *Glycine max L*. A. This co-inoculation significantly contributed to growth and stress tolerance of *Glycine max* L.[101] Mastretta et al. used *Pseudomonas* sp., *Sanguibacter* sp., *Stenotrophomonas* sp., *Enterobacter* sp., that reportedly enhanced three times higher Cd^{2+} accumulation.[102]

CONCLUSION

Bioremediation by endophytes is a sprouting field in environmental advancements. Though quiet a number of works have been done on bioremediation and endophytes individually, however, exploring the potential of endophytes in bioremediation promises a great prospect. Endophytes are reportedly worked amazing in reducing heavy metal pollutants, dyes and pesticides from the soil. It has also shown capability in aiding plants to abiotic stress tolerance and promoting growth. However, insistence are to be made on enhancing the bioremediation capability of the endophytes. For this, an elaborative study needs to be done on adaptive mechanism of endophytes detouring plant defense mechanism. Exhaustive swotting needs to be done on plant-endophyte interaction, utilizing the bioactive compounds produced by them or genetic sequence analysis. From all the points discussed earlier, it is worth noticeable that endophytes are the cynosure in the field of bioremediation, which is not only versatile, but keeps a good impact for futuristic environmental applications.

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