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Article

# Actinobacteria Isolation from Metal Contaminated Soils for Assessment of their Metal Resistance and Plant Growth Promoting (PGP) Characteristics

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**Heavy metals and metalloids removal can be considered as one of the most important world challenges because of their toxicity and direct impact on human health. Many processes have been introduced but biological processes of remediation seem to offer the most suitable solution in terms of efficiency and low cost. Actinobacteria constitute one of the major microbial populations in soil, and this can be attributed to their adaptive morphological structure as well as their exceptional metabolic power. Among microbes, actinobacteria are morphologic intermediate between fungi and bacteria. Studies on microbial diversities in metal contaminated lands have shown that actinobacteria may constitute a dominantly active microbiota in addition to α Proteobacteria. Furthermore, isolation studies have shown metal removal mechanisms which are reminiscent of notable multiresistant strains, such as *Cupriavidus metallidurans*. Apart from members of genus *Streptomyces*, which produce more than 90% of commercialized antibiotics, and the nitrogen fixing *Frankia*, little attention has been given to other members of this phylum. This is because of difficult culture condition requirements and maintenance. In this review, we focused on specific isolation of actinobacteria and their potential applications in metal bioremediation and plant growth promotion.**

**Key words:** Actinobacteria, Metal resistance, Bioremediation, PGP.

## Introduction

The earth's surface is affected by around 240,000 square kilometers of mining operations, with widespread consequences that directly alters the biotopes (Furrer et al., 2002). Transmission of heavy metals and metalloids in nature is noxious for micro and macroorganisms, including humans (De Boer et al., 1999). Among those affected, soil microorganisms play key roles in the global biogeochemical cycle of elements. Disruption of such ecological niches from metal contamination can have an injurious impact on the biosphere stability.

As much as metal contamination shows deleterious effects on microbes, it also leads to rise of resistance in order to survive in such hostile environments. Microorganisms can survive at certain metal concentrations through biosorption activities, bioprecipitation, extracellular sequestration, transport mechanisms and chelation. Microorganisms that exhibit such traits hold great potential applications in bioremediation programs (Haferburg and Kothe, 2007).

Results of previous works dealing with microbial diversity in metal contaminated soils in Korea showed abundance of different taxonomic groups predominated by α proteobacteria (Tipayno et al., 2012; Karelova et al., 2011). Members of phylum proteobacteria are relatively well documented, however, other less studied phyla have also been found in these metal contaminated soils, including members of actinobacteria. This group holds a very interesting population from which 90% of practical antibiotics, two third of all biologically active substances from microbial origin come from (Hamaki et al., 2005). Actinobacteria includes many extremophilic, and stress-resistant genera like *Geodermatophilus*, *Blastococcus* and *Modestobacte*r as well as the well known *Streptomyces* known as with many other actinobacteria for biosynthesis of pigments in response to stress conditions.

Interest in the study of plant growth promoting (PGP)

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traits among actinobacteria is largely due to their reputation as the best secondary metabolite producers, with generally spectacular results. *Micromonospora, Nocardia, Rhodococcus, Streptosporangium* and *Oerskovia* have been shown to increase dry weight in corn, soybean, cucumbers, tomatoes and sorghum (El-Tarabily and Krishnapillai, 2006) Other actinobacteria have also been shown to increase root and shoot weight of soybean in soil infested with *Phytophtora megasperma*. Aside from their plant growth promotion qualities, actinobacteria are good and efficient biocontrol agents that act via antibiosis or hyperparasitism, as well as other possible ways (Doumbou et al., 2001).

This review was made essentially to show the possible applications of actinobacteria in remediation and growth promotion of plants in metal contaminated soils in Korea. In this review, we will cover their capacity for metal resistance and as PGP candidates, after giving an overview of their taxonomy. Specific conditions and requirements for isolation and growth are also discussed here.

## Actinobacteria: Taxonomy and properties

The term actinomycete was first generated from *Actinomyces bovis* the causative agent of actinomycosis (Stackbrandt and Schumann, 2006). Within the 18 recognized lineages of bacteria; the phylum of actinobacteria is considered one of the largest taxonomic units containing 1 classis, 5 subclasses and 14 suborders (Stackebrandt et al., 1997b). Stackebrandt et al (1997a) classified actinobacteria under the prokaryotic kingdom with as a classis holding orders Actinomycetales, *Micrococcales*, members of the Brevibactereae, Micrococceae as well as other microorganisms included in this lineage through phylogenetic approaches (Fig.1). This phylum is composed of gram positive bacteria known for their high G+C content, reaching as high as 70% among *Streptomyces* and *Frankia* species. Their morphologies are very diversified. For instance, *Micrococcus* and *Arthrobacter* are respectively coccoid and rod-coccoid, while *Nocardia* are presenting fragmenting filamentous forms. The genus *Streptomyces* expresses a permanent and highly filamentous mycelium (Ventura et al., 2007). This genus has been reported as the largest source of antibiotics producing 70 to 80% of the total pharmaceutically useable forms (Bérdy, 2005). Based on 16s rRNA sequencing, 39 families and 130 genera were identified. Because of the

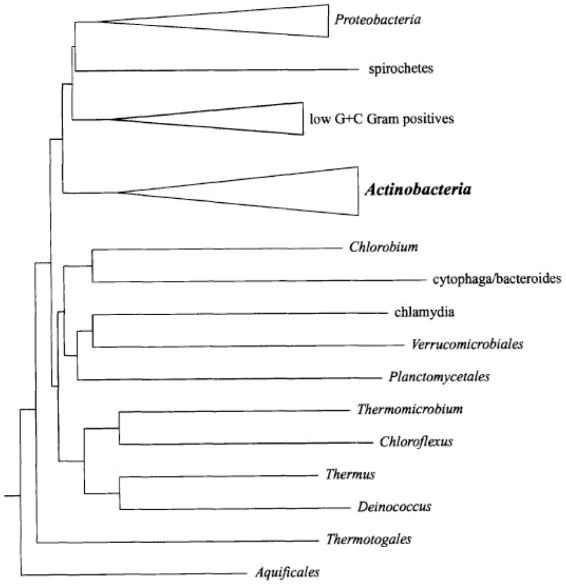


**Fig. 1. Phylogenetic tree based on 16s rDNA/rRNA showing the position of actinobacteria within the domain bacteria (Stackebrandt et al, 1997a).**

ancient divergence of Actinobacteria from other groups, it is difficult to define their phylogenetically closest group.

Phylum Actinobacteria contains a panoply of known species involved in a variety of ecological functions, from the probiotic B*ifidobacterium spp*. to the dreaded *Mycobacterium tuberculosis*. These species have been subjects of many scientific studies resulting to their entire genome sequences being made available in databases. On the other hand, interesting mycelial genera playing important roles in nitrogen fixation and carbon cycling in soil have received less attention from researchers, with the exception of *Thermobifida*, *Frankia* and *Streptomyces* (Ventura et al., 2007). Species under *Streptomyces* were first identified for their antibiotic production, which lead to overspeciation in the taxon, from an initial number of 40 species to more than 3000. Only after the launching of the International *Streptomyces* Project (ISP) which introduced keys identification criteria based on morphological aspect and numerical taxonomy by Williams et al., (1983) was this overspeciation limited and redescription of more than 450 species was done (Annaliesa and Wellington, 2001). Attention to nitrogen fixation among actinobacteria was focused only on genus *Frankia*, which are able to make symbiotic relationships with a range of actinorhizal plants (Benson and Silvester, 1993). The nitrogen fixing ability of *Frankia* was confirmed through a number of analytical tools, such as the quantification of total nitrogen by Kjeldhal analysis, acetylene reduction assay, $^{15}N$ in-

**Table 1. Media for Actinobacterial isolation (cited in articles).**

| Medium | Properties | References |
|---|---|---|
| Luedmann Medium | Rich medium. Slow growing, melanotrophic actinobacteria | (Essoussi et al., 2010) |
| Actinobacteria Isolating Agar (AIA) | Minimal medium Fast and slow growing actinobacteria | (Baskaran et al., 2011) |
| Defined Propionate Medium (DPM) and Buffered Ammonium Propionate (BAP) | Specific for *Frankia* species | (Gtari et al., 2004) |
| International Streptomyces Project (ISP) Media (1 to 9) | Morphological, pigmentation and physiological changes | (Wink et al., 2003) |

corporation and growth in N-free media (Gtari et al., 2012). However, difficulties in culture requirements for growing genus *Frankia* lead to some drawbacks in its applicability. Thus, interest is now turned on non-*Frankia* nitrogen fixing actinobacteria. *Mycobacterium flavum* was identified as nitrogen fixing based on its capacity to grow in nitrogen-free medium and ability to reduce acetylene, but only under low oxygen level (Biggins and Postgate, 1969). *Corynebacterium autotrophicum* was also shown to fix molecular nitrogen under autotrophic and heterotrophic conditions (Berndt et al., 1978).

## Actinobacteria Isolation

Many works have been conducted for actinobacteria isolation. As generally slow growing organisms, compared to other bacteria, they require specific conditions for isolation and maintenance. Hamaki et al., (2005) reported that new actinomycetes can be isolated just by using soil extract agar medium and selection of microscopic colonies. In opposition to the concept supporting the power of cultivation-independent molecular ecological techniques in the study of bacteria in their natural habitat, Shayne et al. (2003) identified 10 new families of actinobacteria by classic isolation procedures.

Luedemann medium has been reported to be efficient for isolation of slow growing actinobacteria belonging to Geodermatophilacea group (Essoussi et al., 2010) as well as for total soil actinobacteria screening (Ara et al., 2012). Actinobacteria Isolating Agar (AIA) has also been suggested as a selective medium for actinobacteria in sediments (Baskaran et al., 2011). The ISP (International *Streptomyces* Project) family media have been used for studying physiological characteristics of already isolated actinobacteria. Slow growing nitrogen fixing actinobacterial taxa belonging to genus *Frankia* that were found associated with actinhorizal plants, can be isolated in liquid media like buffered ammonium propionate (BAP) or defined propionate medium (DPM) (Gtari et al., 2004). Compared to Luedemann or AIA, these media are minimal and thus, limit colonization by fast growing bacteria. Colonization by fungi and fast growing prokaryotes are considered major obstacles in isolation because actinomycetes require longer time for incubation. Use of antibiotics and antifungal substances to limit such growth has often been reported in literature. Porter et al. (1959) suggested the combination of cycloheximide and sodium propionate (already a component of DPM and AIA media) as efficient in inhibiting fungal growth. Addition of potassium dichromate ($K_2Cr_2O_7$) has also been cited to limit fungal development (Zhang et al., 2006). To discourage growth of other prokaryotes, especially gram negative bacteria, nalidixic acid is being added to culture media (Zhang et al., 2006; Hamdali et al., 2012). Older studies involved use of a multitude of other antibiotics for bacteria inhibition. A combination of polymyxin B and sodium penicillin was used to inhibit a wide range of bacteria including *Pseudomonas fluorescens, Bacillus licheniformis* and *Mycobacterium phlei* without affecting actinobacterial population. This treatment however, spares certain common fast growing bacteria namely *Bacillus subtilis and Bacillus mycoides* which invade plates very fast after sample inoculation (Williams and Davies, 1965). These bacilli persist even after oven treatment consisting for 15min at 65°C, which supposedly eliminates non sporulating bacteria and actinobacterial spores (Baskaran et al., 2011) (Table 1).

## Actinobacteria and metals

There is no official definition for heavy metals. The term encompasses metals and semi metals associated with

contamination, as well as human and ecological toxicity (ecotoxicity). The most known definition of heavy metals is with reference to the density of their elemental form, which is generally above 7 g $ml^{-1}$. Definitions suggested by many authors based on physicochemical concepts do not make logical sense because of the lack of connection between those physicochemical properties and density, toxicity or ecotoxicity (Colin et al., 2012). Selenium and arsenic, regarded as semi metals or metalloids have often been included also in the group of heavy metals because of their toxicity, having physical properties similar to metals but chemical behavior similar to non-metals (Duffus, 2002). Heavy metals are divided into two categories. One includes those essential for life, generally incorporated into enzymes and cofactors, but which become toxic in higher doses by militantly binding to enzymes and DNA and production of oxygen radicals via Fenton reaction (e.g., Fe, Zn, Cu, Mn,Co, Ni, Cr) The non essential metals are always toxic even in low concentrations (e.g., Hg, Cd, Pb) (Valls and Lorenzo, 2002). Presence of high concentrations of these heavy metals can directly affect the soil fertility and cause serious ecological problems, affecting the food chain, as well as exposing humans to health risks (Machado et al., 2010). Removal of these heavy metals from the environment using physicochemical processes is expensive, requiring a lot of energy and material, so that resorting to biological means, like the use of microorganisms offers an efficient substitute.

Iwamoto and Nasu (2001) described the application of microorganisms for heavy metals removal, as well as the advantages of *in situ* application of bioremediation agents. In fact, they enumerated three categories for *in situ* bioremediation: (i) bioattenuation consisting of the monitoring of natural degradation to ensure the decrease of the contaminant with time; (ii) biostimulation which consists of stimulation of the biodegradation process with nutrients, substrates or electron acceptors and (iii) bioaugmentation which consist of inoculation of contaminated sites with external effective micro-organisms so that biodegradability increase. Another advantage of using microbes is that they do not produce toxic intermediates when degrading pollutants. Heavy metals biodegradation may involve myriads of mechanisms such as active transport through efflux pumps, transformation to less toxic chemical compounds using enzymatic transformations via redox reactions (intracellular sequestration), methylation and alkylation/dealkylation (Colin et al., 2012).

They reported the importance of the actinobacteria phylum for heavy metal bioremediation. The filamentous nature, which place them as intermediate between bacteria and fungi, their metabolic power and ability for fast colonization of selective substrates can suggest possibility for widespread metal resistance among them. Despite all of these properties, however, data on the actinobacterial resistance to metals are still insufficient comparing to gram negative bacteria. For example, mechanisms for resistance to high concentrations of copper are well explained among *E.coli* and related genera (Munson et al., 2000). But these have not been well elucidated for the majority of known actinobacteria and have only just recently started attracting scientists' interest. Karelova et al., (2011) defined the actinobacteria phylum as the second well represented group after proteobacteria (α, β and γ), in heavy metal contaminated farm land in southwest Slovakia, and furthermore, isolated nine new species and genera in the same study. Another study examining 16s rDNA and 16s rRNA in metal contaminated bulk and rhizosphere soils in the goal to compare diversity of existing and metabolically active populations in those sites, showed that the most predominant phylotypes in both sites belong to actinobacteria compared to α proteobacteria (Gremion et al., 2003). It is actually known through genomic analysis, but not yet shown at the functional level, that actinobacterial cells possess efflux transporters that excrete toxic and excess levels of metals, keeping a low cytoplasmic metal concentration level, the same mechanism elucidated in the famous *Cupriavidus metallidurans* (Nies, 2003). It has also been shown that some of the ATP binding cassette transporters, involved in antibiotic resistance among actinobacteria work as metal efflux pumps. In fact, works on actinobacteria resistance to heavy metals found strong correlation between secondary metabolite production and the ability of the bacteria to cope with stress factors, including high concentrations of heavy metals. Micro-organisms including actinobacteria, regulate their homeostasis by maintaining low, non-toxic metal concentrations within the cells. Since they consist of reactive heavy metals, such abilities are logically encountered more readily among microbes living in contaminated environment than in others because bacteria may activate and adapt a mechanism of detoxification to ensure survival (Eitinger and Mandrand-Berthelot, 2000). Schmidt et al.

(2005) isolated actinobacterial strains belonging to the genus *Streptomyces* showing multitude of responses to metal contamination. One spectacular trait is the release of diffusible substance (not characterized in the published work) that allows not only resistance of the producer strain, but also of adjacent strains in the culture media. This process may explain the phenomenon of in vivo co-adaptation to high metals concentrations.

Genus *Streptomyces* has been the primary focus of scientists when dealing with actinobacteria and heavy metals, because of its wide reputation for secondary metabolite and pigment production. It has been shown by many authors that heavy metal contamination negatively affects production of antibiotics and pigments by *Streptomyces* species (Abbas and Edward, 1989). Schmidt et al. (2005) reported inhibition by cadmium of antibiotic production and total growth of *Streptomyces* species. The same result was previously reported by Abbas and Edward (1989), showing high toxicity to *streptomyces* of not only cadmium but also mercury, nickel, copper and lead. The same study reported that manganese, cobalt, zinc and chromium showed complex effects on *Streptomyces* strains by inhibiting their antibiotic production, but interestingly, enhancing their growth yield. These contradictory results, maybe due to continuous process of microbial evolution, genome instability and lateral gene transfer. Plasmids reminescent of those found in multiresistant *Cupriavidus metallidurans*, but allowing resistance only for mercury, were described for the genus *Streptomyces* (Ravel et al., 1998; Haferburg and Kothe, 2007). Two other strains showing cadmium resistance were also characterized from marine soil samples in India (Lakshmipathy and Kannabiran, 2010). It is reasonable to assume that tolerance to high concentrations of metals, which in low amounts are essentials for cell metabolism, is widely more encountered than resistance to non essential metals like cadmium or mercury. It is known that microorganisms interact with toxic metals through three possible mechanisms: biosorption, bioaccumulation and enzymatic reduction (Srinath et al., 2002). Marta et al., (2011) showed bioaccumulation of Cr(III) and reduction of Cr(VI) to Cr(III) by *Streptomyces sp.* The rate of reduction depends on the carbon source used by the microorganism. Chromium reduction to bioaccumulative state increases in *Streptomyces* termocarboxydus when glycerol is used as a carbon source instead of glucose. Comparative results were published showing biosorption of cadmium by an isolate identified as *Streptomyces tendae*, which reached exponential phase of growth in 4 day in absence of any $Cd^{2+}$ pressure, with a 1.492 g of biomass but 0.304 g within 8 days in presence of 8 mg $l^{-1}$ of $Cd^{2+}$ .Transmission electron microscopy (TEM) showed that the highest amount of biosorbed cadmium was in the cell wall (Sineriz et al., 2009).

Literature dealing with metal resistance of non-*streptomyces* actinobacteria and their applications is scarce This is maybe due to in vitro culture difficulties as well as difficulties in the actual application for bio-remediation process. *Frankia* strains, as a major actinobacteria clade for example, have been examined for heavy metal resistance, but as bio-marker to check possible gene circulation among them and their potential actinorhizal plant host, being used for land reclamation in strip-mined areas (Richards et al., 2002).

## Actinobacteria as a PGP candidates

**Streptomyces PGP candidates** As a potential source of bioactive metabolites, actinobacteria are also efficient source of agroactive products, with *Streptomyces* accounting for about 60% of insecticides and herbicides produced (Doumbou et al., 2001). For example, *Streptomyces kasugaensis* which produces the antibiotic, kasugamycin that inhibit protein synthesis in many microorganisms has been commercialized for the control of rice blast agent *Pyricularia orizae* as well as *Pseudomonas* diseases in many crops (Schluenzen et al., 2006) *Streptomyces cacaoi*, which also produces the antibiotic, polyoxin is used against a range of fungal phytopathogens (Copping and Duke, 2007). Unlike the data provided on secondary metabolite patterns of *Streptomyces*, relatively less documentation is available about their PGP traits, despite them being the most abundant microflora in the soil. This is probably due to their capacity for spore formation which imposes unfavorable growth conditions to the plant (Doumbou et al., 2001). Although, most studies on symbiotic plant actinomycetes have been intensively done on nitrogen fixing *Frankia*, Sardi et al. (1992) were able to isolate and characterize 482 endophytic *streptomyces* from 13 roots of healthy randomly chosen plants. These *Streptomyces* species isolated from plant root nodules synthesized zeatine, indol acetic acid and giberellic acid and showed antagonistic activities against plant pathogens like *Pse-*

**Table 2. Summary of some PGP traits encountered among actinobacteria (cited in articles).**

| Actinobacterial strains | PGP trait | References |
|---|---|---|
| *Streptomyces kasugaensis* | Antagonism against *Pyriculariaorizae* | (Schluenzen et al., 2006) |
| *Streptomyces cacaoi* | Antagonism against fungi | (Copping and Duke, 2007) |
| *Streptomyces spp.* | Zeatine, IAA, Giberellic acid, antagonism against Pseudomonas savastonii | (Ghodhbane-Gtari et al., 2010; Solans, 2007) |
| *Streptomyces olivaceoviridis* and *Streptomyces rochei* | auxins, gibberellins and cytokinins production | (Aldesuquy et al., 1998) |
| *Nocardiopsis dassonvillei* | Hyperparasitism against *Fusarium oxysporum* | (El-Tarabily and Krishnapillai, 2006) |
| *Micromonospora globosa* | Hyperparasitism against *Fusarium oxysporu* | (El-Tarabily and Krishnapillai, 2006) |
| *Micromonospora carbonacea* | Cell wall degradation of *Sclerotinia minor* | (El-Tarabily et al., 2000) |
| *Micromonospora endolithica* | Phosphate solubilization | (El-Tarabily et al., 1997) |

*udomonas savastonii* (Ghodhbane-Gtari et al., 2010; Solans, 2007). One of the first reports on plant growth promotion by *Streptomyces* (aside their biocontrol features) showed ability to increase shoot length and shoot fresh mass in wheat. Hormone analysis revealed that two strains, *Streptomyces olivaceoviridis* and *Streptomyces rochei* produce considerable amounts of auxins, gibberellins and cytokinins (Aldesuquy et al., 1998).

**Non-Streptomyces PGP candidates** Less attention has been given to non-*streptomyces* actinomycetes and *Frankia* for their interaction with plants as growth promoters or biocontrol agents. No much information is available with regards to their production of antifungal volatile compounds compared to the *Streptomyces* group (El-Tarabily et al., 2000). There is a dearth of literature dealing with hyperparasitism among non- streptomycetes. El-Tarabily and Krishnapillai (2006) analyzed widespread hyperparasitism among *Streptomyces*, but cited only two references dealing with this phenomenon among other actinomycetes. These included, *Nocardiopsis dassonvillei* which exhibits mycolytic and parasitic activities against the vegetative hyphae of *Fusarium oxysporum*, and *Micromonospora globosa* which parasitize *Fusarium udum* through coiling, penetration and branching leading to granulation, coagulation of the cytoplasm and hyphal lysis. The importance of cell wall degrading enzymes produced by non-*streptomyces* actinomycetes has also been shown, such as that of chitinase and β-1,6 glucosidase (Gacto et al., 2000; Nawani et al., 2002). *Micromonospora carbonacea*, high producer of chitinase has been shown to suppress the pathogen of lettuce *Sclerotinia minor*. Massive hyphal plasmolysis was observed when the two microorganisms were grown together with the fungus as the only carbon source (El-Tarabily et al., 2000). Data on other biocontrol mechanisms employed by non-*streptomyces* actinomycetes are still too little considering the potential of these microorganisms. There is likewise, not much information available concerning plant growth promoting substances produced by actinomycetes, although reports on the ability of *Streptoverticillium netropsis, Actinomadura rubra, Actinoplanes philippiniensis, Micromonospora carbonacea* and *Streptosporangium albidum* to increase root weight of carrot even in presence of the pathogen *Pseudomonas coloratum* (El-Tarabily et al., 1997) have been made. The same author underlined the role of *Micromonospora endolithica* in increasing of phosphorus availability to the plant compared to *Micromonospora olivasterospora* which failed to promote plant growth because of absence of phosphate solubilization faculty (Table 2).

## Conclusion

Actinobacteria are interesting prokaryotic phylum holding diverse genera ranging from human pathogens to the nitrogen fixing strains (Stackebrandt et al., 1997a). Their morphological characteristics place them as evolutionary intermediate between bacteria and fungi. They can be encountered in many habitats, but especially abundant in soil. They are known as strong secondary metabolite producers, owing to a rich pool of metabolic enzymes and as pigment producers. These enable them to colonize hostile habitats, including soils contaminated with heavy metals and metalloids. In fact, biodiversity studies have shown the high abundance and ubiquity of actinobacteria in such soils, in addition to the most

expanded clade of α Proteobacteria (Karelova et al., 2011). Furthermore, isolation works have shown that many strains can survive high metal concentration using panoply of mechanisms. Some strategies are similar to those observed in the multiresistant *Cupriavidus metallidurans*, but works here need more consolidation for complete elucidation. Because of their abundance in soils, actinobacteria constitute a major group of rhizospheric microorganisms, and colonization of plant roots systems is highly probable. However, there is not much data on this topic perhaps due to difficulties in field application of these slow growing microorganisms. And this likely explains why most of works are concentrated on *Streptomyces*, known for their fast growth and as source of more than 60% of commercialized herbicides and insecticides. These, make them good candidates for biocontrol and plant growth promotion (Doumbou et al., 2001). Furthermore, isolation and cultivation of this group is easy even with competition from other bacteria, which is not the case for other members of this phylum. As such, a section of this review suggests optimization of specific culture media for actinobacteria growth. This may offer an opportunity for isolation and exploitation of non-*streptomyces* actinobacteria, which can provide more answers to plant growth promotion and bioremediation in metal contaminated soils.

Previous studies on actinobacteria as biocontrol and PGP candidates provide sufficiently encouraging results to consider them as possible substitutes for chemical pesticides. However, more studies are needed to understand and make applications of their ability to control plant pathogens (El-Tarabily et al., 2000; Schluenzen et al., 2006; Copping and Duke, 2007). Developing commercialized biocontrol formulations has usually been met with problems in practice, and improvement of the consistency of their performance must be improved, for this purpose, molecular genetics and mutants generation through transposon mutagenesis offer a good alternative (Doumbou et al., 2001).

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