REVIEW
OF
LITERATURE
Actinomycetes represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world and especially significant for their role in the recycling of organic matter (Srinivasan et al., 1991). When conventional isolation techniques were applied, most of the isolates recovered on agar plates have been identified as *Streptomyces* species which are the dominant actinomycetes in soil. For the purpose of screening novel bioactive molecules, several factors must be considered like choice of screening source, pretreatment, selective medium, culture conditions etc. The pre-treatment techniques including enrichment, physical and selective media may be used to study the ecology of actinomycetes in natural habitats such as soil or water samples (Jensen et al., 2005). Since actinomycetes are slow growers compared to bacteria and fungi, they get easily masked in culture plates when grown on ordinary nutrient media. Hence, some special measures are to be taken for the selective isolation of actinomycetes from the soil samples.

### 2.1. Isolation of actinomycetes

Goodfellow and Haynes (1984) reported that actinomycetes found in the marine ecosystem are relatively neglected and suggested the availability of methods which permit the isolation of specific group of microorganisms makes the exploitation of marine microbes a desirable goal. An alternative approach was to make the isolation medium more selective by adding antibacterial and antifungal compounds.

Treatment of soil suspension with 1.4% (w/v) phenol solution was recommended by Lawrence (1956). Rehacek (1959) centrifuged soil suspension at selected speeds to separate actinomycete spores from other propagules. Tsao et al., (1960) suggested that treatment of soil sample with calcium carbonate was not only effective for obtaining high counts of actinomycetes but also reduced the relative
counts of bacteria and molds. El Nakeeb and Lechevalier (1963) found high colony counts in soils treated with calcium carbonate when compared with untreated samples.

Agate and Bhat (1963) made an attempt to suppress bacterial and fungal contaminants by pre heating the soil sample at 110°C. Nonomura and Ohara (1969) reported that heating air dried soil samples at 120°C reduced the numbers of filamentous bacteria and streptomycetes resulting in the selective isolation of rare actinomycetes. Heat treatment of soil sample at 40 to 45°C for 2-16 h reduced the bacterial population considerably without affecting the colony counts of *Streptomyces* (Williams *et al.*, 1972). Sorokina *et al.* (1991) employed lime treatment of soil sample for the isolation of actinomycetes.

Bulina *et al.* (1997) reported that irradiation of soil samples with 2460 MHz microwaves at 80 W for 30 sec augmented the recovery of actinomycetes and the population of rare genera *Actinomadura, Actinoplanes, Amycolatopsis, Micromonospora, Microtetraspora, Nocardiopsis, Promicromonospora, Saccharopolyspora, Saccharothrix and Streptosporangium* were increased by three fold. Some of the soil samples are directly plated on chitin agar medium (Hsu and Lockwood, 1975) or subjected to filtration through cellulose ester membrane filters followed by isolation on modified Bennett agar (Hirsch and Christinsen, 1983). The calcium carbonate treated soil plated onto the nutrient media proved to be the most efficient technique for the isolation of actinomycetes (Alferova *et al.*, 1989).

Plating the soil samples on starch-casein agar supplemented with rifampicin eliminated the contaminating microorganisms (Pisano *et al.*, 1989). Treatment of soil sample with 1.5% phenol at 30°C for 30 min prior to inoculation on humic acid vitamin agar was reported to be selective for the isolation of soil actinomycetes
belonging to *Streptomyces violaceusniger* phenotypic cluster (Hayakawa et al., 2004). For isolating *Streptomyces* spp., Narayana et al. (2004) employed calcium carbonate treatment followed by drying of soil samples at 45°C for 1 h. Takizawa et al. (1993) reported that culture medium amended with nalidixic acid which proved to be more effective than pretreatment of samples with heat for the isolation of actinomycetes from soils of Chesapeake Bay. Kyung and Yong (1996) recorded enhanced population of actinomycetes in the soil samples pretreated with peptone (6%) and sodium lauryl sulphate (0.05%) at 50°C for 10 min.

Semedo et al. (2001) employed differential centrifugation technique by which the isolation rate of actinomycetes increased up to 5 times as compared to the conventional dilution plate technique. Irradiation of soil suspensions within wavelength bands of 3.8–5.8 and 8–11.5 mm considerably augmented the total number of actinomycetes and increased the fraction of the rare genera (Li et al., 2002). Yamamura et al. (2003, 2005) proposed sucrose gradient centrifugation for the selective isolation of actinomycetes.

### 2.2. Use of selective media and antibiotics for the isolation of actinobacteria

Crook et al. (1950) employed sodium propionate as medium additive which could act as an effective fungal inhibitor while isolating actinomycetes from soil. Corke and Chase (1956) suggested that incorporation of sodium propionate and cycloheximide to the media used for the isolation of actinomycetes. Antibiotics such as actidione (Dulaney et al., 1955; Corke and Chase, 1956, 1964; Corbaz et al., 1963), nystatin and pimaricin (Porter et al., 1960) were successfully employed for the selective isolation of actinomycetes from soil.
Bendict et al. (1955) suggested the use of aminoacids particularly L-arginine for the selective isolation of actinomycetes. A method of combining an antifungal antibiotic with glycerol-arginine medium which selectively encourages the development of aerobic, soil inhabiting actinomycetes was described by Porter et al. (1960). Kuster and Williams (1964) examined several carbon and nitrogen sources for the selective isolation of actinomycetes and found starch and casein as the best mixture. Use of antibiotics like Nystatin (50 µg/ml), Actidione (50 µg/ml), Polymyxin B sulphate (5.0 µg/ml) and Penicillin (1.0 µg/ml) were suggested by Williams and Davies (1965), while Novobiocin (100 µg/ml), Gentamicin (2-5 µg/ml) and Rifampicin (25 µg/ml) were used by Cross (1968), Chormanova (1978) and Shearer (1987) respectively for the preferential isolation of actinomycetes. Palleroni (1980) described a novel technique using capillaries containing KCl inserted into the soil for the isolation of Actinoplanes strains taking the advantage of the positive chemotaxis of actinoplanete zoospores towards KCl. Hirsch and Christensen (1983) introduced the membrane filter technique that selects actinomycetes on the basis of their characteristic mode of mycelial growth.

Selective media could also be employed for the isolation of rare actinomycete genera from soils. Hsu and Lockwood (1975) proposed colloidal chitin agar as suitable medium for intensive isolation of actinomycetes. Humic acid vitamin agar was proposed by Hayakawa and Nonomura (1987) for the selective isolation of Micromonospora, Microbispora, Streptosporangium, Dactylosporangium and Thermomonospora. Bovine bone powder was used for enhancing the population of actinomycetes by Yokoyama and Mino (1990). Suzuki et al. (2001) suggested gellan gum media for the isolation of rare actinomycetes along with Streptomyces spp.

2.3. Screening of marine actinobacteria for antimicrobial activity

Cross-streak method was used for testing the antimicrobial activity of *Streptomyces* sp. by Lakshmanaperumalsamy (1978). Perpendicular streak method is the widely accepted method for the preliminary screening of actinobacteria producing antimicrobial metabolites (Egorov, 1985). Several researchers also reported perpendicular screening technique for assessing the antimicrobial activity of actinobacteria against various human pathogens (Krishnakumari et al., 2006; Thangapandian et al., 2007).

During primary screening, Saadoun and Gharabeh (2002) obtained 54 *Streptomyces* isolates exhibiting remarkable antibacterial activity out of 90 strains tested. A modified cross streak method in which a 5 mm plug of actinomycete strain was inoculated and incubated for 5 days followed by the inoculation of 4mm plugs of four day old fungal cultures cross plugged 15 mm away from the actinomycete culture was used for the primary screening of 106 marine actinomycetes strains isolated by Kathiresan et al. (2005).

Aaron et al. (2008) used liquid-liquid extraction method with DMSO to extract the antimicrobial metabolite produced by the isolate G0655 and the extracts were tested against *Bacillus subtilis*. Ramesh and Mathivanan (2009) selected 111 isolates from 208 marine actinomycetes through secondary screening using ethyl
acetate extracts of the fermentation broths against human pathogens. Borisova (2011) screened 47 *Rhodococcus* strains for antibiotic production using a growth inhibition assay and selected one strain possessing high antimicrobial activity for further study. Siva kumar *et al.* (2011) selected 22 strains from 78 actinomycetes exhibiting antibacterial activity by cross streak method. They reported that among the 22 strains, 12 exhibited antifungal activity and 11 showed both antibacterial and antifungal activities. Gulve and Deshmukh (2012) reported the antibacterial activity of actinomycetes isolates by using perpendicular streak method and 13 isolates out of 107 strains were selected for further study.

### 2.4. Optimization of process parameters for enhanced production of bioactive compounds by actinomycetes

Gupta and Chaudhary (1971) reported the effect of twenty nitrogen compounds on growth and production of antibiotic produced by *S. nigrifaciens*. Utilization of carbon and nitrogen sources by *S. kanamyceticus* for kanamycin production was reported by Basak and Majumdar (1973). Sahay and Srivastava (1977) reported the impact of carbon and nitrogen sources on antibiotic and pigment production by six strains of *Streptomyces* along with their growth pattern. Consumption of different carbon and nitrogen sources for the production of oxytetracycline by *S. rimosus* was studied by Mandal *et al.* (1981).

Vinogradova *et al.* (1985) recorded an inverse relationship between culture growth rate and heliomycin production by *S. oligocinereus* with different carbon sources. Bhattacharya *et al.* (1998) reported that nutrient medium containing arginine and glycerol with initial pH 7.0 supported maximum antibiotic production by *S. hygroscopicus* D1.5. Influence of pH, temperature, oxygen supply and concentration
of inoculum on the production of natamycin by *S. natalensis* was recorded by El-Enhancy *et al.* (2000).

Optimal conditions were tested for the enhanced production of a potent antimicrobial metabolite by *S. sannanensis* RTJ-1 active against Gram positive bacteria (Vasadeva *et al.*, 2006). Anupama *et al.* (2007) recorded that YMD proved to be the best medium for the production of antimicrobial metabolites by *S. purpeofuscus*. Culture medium amended with maltose and soybean meal with initial pH 7 greatly supported the production of antimicrobial metabolites by *S. albidoflavus* cultured at 35°C (Narayana *et al.*, 2008). Singh *et al.* (2008) optimized the nutrient levels of *S. pristinaespiralis* CGMCC 0956 for the production of pristinamycins in submerged fermentation.

Yu *et al.* (2008) reported optimized conditions for the growth and antimicrobial metabolite production by *Streptomyces rimosus* MY02. Kavitha *et al.* (2009) reported that the secondary metabolites obtained from *Nocardia levis* MK-VL_113 grown under optimized cultural conditions showed strong antimicrobial activity against a variety of Gram positive and Gram negative bacteria as well as fungi. Glucose (1 %) and tryptone (0.25 %) were found to be the most suitable carbon and nitrogen sources respectively for optimum production of bioactive metabolites produced by *Pseudonocardia* sp. VUK-10 (Usha kiranmayi *et al.*, 2011).

2.5. *Rhodococcus*

*Rhodococcus* species are Gram-positive, aerobic, non-sporulating and nonmotile bacteria closely related to Mycobacteria and Corynebacteria. They are widely distributed in aquatic and terrestrial habitats (Goodfellow and Williams, 1983; Jingyao and Xu, 1996; Brandao *et al.*, 2002). They have been isolated from soil, fresh
water and marine sediments (Colquhoun et al., 1998; Zhang et al., 2002). Some are opportunistic pathogens of animals including humans (Weinstock and Brown, 2002).

Most strains grow well between 15 and 40°C on media such as glucose-yeast extract agar (Gordon and Mihm, 1962), modified Bennets’ agar (Jones, 1949), glycerol agar (Gordon and Smith, 1953), glycerol-asparagine agar (Shirling and Gottlieb, 1996) and modified Sautons’ agar (Mordarska et al., 1972). They use a wide range of organic compounds as sole sources of carbon for growth and energy (Goodfellow et al., 1982). Colonies of rhodococci may be rough, smooth or mucoid. They are 0.25-2 mm in diameter and may be opaque, buff, coral, cream, orange, pink, red or yellow. Colourless variants may also occur. In all strains, the growth cycle begins with the coccus which may germinate into short rods, form filaments with side projections and show elementary branching. Extensively branched hyphae are found in the most differentiated forms. Fragmentation of the rods, filaments and hyphae results in the formation of cocci and short rods to start next generation (Goodfellow, 1989).

Most rhodococci are sensitive to antibacterial antibiotics such as aminoglycosides, cephalosporins, macrolides, penicillins and tetracyclines. They are less sensitive to sulfonamides and resistant to most antitubercular compounds (Helmenke and Weyland, 1984; McNeil and Brown, 1994). The remarkable metabolic diversity exhibited by rhodococci makes them ideal candidates for enhancing the bioremediation of contaminated sites and as biocatalysts for a wide range of biotransformations (Finnerty, 1992; Warhurst and Fewson, 1994). The cells and enzymes of Rhodococcus erythropolis are extensively used in bioremediation and biocatalysis studies as they carry out innumerable bioconversions and biodegradations
Rhodococcus species are known for bioconversion using biological systems to convert cheap starting material into more valuable compounds due to their ability to metabolize harmful environmental pollutants such as toluene, naphthalene, PCBs and herbicides (Lee et al., 2010; Li et al., 2012).

The genus *Rhodococcus* is classified in the order corynebacteriales based on 16S rRNA gene sequence. It belongs to the family Nocardiaceae which also includes *Nocardia* (Trevisan, 1889) and *Smaragdicoccus* (Adachi et al., 2007). Rhodococci are classified into 30 validly published species (Jones and Goodfellow, 2012).

### 2.6. Some representative species of the genus *Rhodococcus*

- *Rhodococcus rhodochrous*
- *Rhodococcus aetherivorans*
- *Rhodococcus baikonurensis*
- *Rhodococcus coprophilus*
- *Rhodococcus corynebacteriodes*
- *Rhodococcus equi*
- *Rhodococcus erythropolis*
- *Rhodococcus fascians*
- *Rhodococcus globerulus*
- *Rhodococcus gordoniae*
• Rhodococcus jostii
• Rhodococcus koreensis
• Rhodococcus kroppenstedtii
• Rhodococcus kunmingensis
• Rhodococcus kyotonensis
• Rhodococcus maanshanensis
• Rhodococcus marinocens
• Rhodococcus opacus
• Rhodococcus percolates
• Rhodococcus phenolics
• Rhodococcus pyridinivorans
• Rhodococcus qingshengi
• Rhodococcus rhodnii
• Rhodococcus ruber
• Rhodococcus triatomae
• Rhodococcus tukisamuensis
• Rhodococcus wratislaviensis
• Rhodococcus yunnanensis
• Rhodococcus zopfii
2.7. Taxonomic features of *Rhodococcus* sp.

- **Kingdom**: Bacteria
- **Phylum**: Actinobacteria
- **Order**: Actinomycetales
- **Suborder**: Corynebacterineae
- **Family**: Nocardiaceae
- **Genus**: *Rhodococcus*

The genus *Rhodococcus* was first proposed by Zopf (1891) to describe two species of red-pigmented bacteria. It was later viewed as a coherent taxon encompassing species of veterinary, clinical and industrial importance (Finnerty and Annu, 1992). The existence of six distinct groups (clades) within the genus *Rhodococcus* was suggested by Rainey *et al.* (1995) and Goodfellow *et al.* (1998). The four groups that are of particular taxonomic interest include *Rhodococcus equi*, *R. rhodnii*, *R. corynebacteroides* and *R. zopfii*. It is still not clear whether *R. equi* should be placed in the genus *Nocardia*, to which it may be more closely related than to other members of the genus *Rhodococcus*, or whether it merits recognition as a genus in its own right. The latter proposal is supported by evidence suggesting that *R. equi* isolates from veterinary, human and environmental sources are phylogenetically very diverse (Colquhoun *et al.*, 1974; Atalan *et al.*, 1977; Colquhoun *et al.*, 1977; McMinn *et al.*, 1978). It also appears that *R. rhodnii* is more closely related to the genus *Tsukamurella* than to other rhodococci.
Among the *Rhodococcus* species that have been analysed there are three species that have 16S rDNA sequences acquired from several isolates sharing the same species name but which cluster with different species. These are: (a) *R. zopfii*, one isolate of which clusters with *G. bronchialis* and the other with *R. coprophilus*; (b) *R. opacus*, where one isolate clusters with *R. jostii* but another seven with *R. koreensis* and two others with *R. wratislaviensis*; (c) *R. rhodnii*, one isolate which clusters closest to *T. paurometabola* and the other clusters with *R. rhodochrous*. In each case, one or more of these isolates cluster with a member of the same previously described species, but the others cluster distally from these. This suggests that the taxonomic status of these strains requires reassessment (Jones and Goodfellow, 2012).

2.8. Conventional methods

Characteristics such as study of aerial mass colour, substrate mycelium, melanoid pigments, soluble pigments, spore chain morphology, spore surface and assimilation of carbon source are commonly employed in taxonomy of actinobacteria. Classical approaches for classification make use of morphological, physiological and biochemical characters. The classical methods described in the identification key by Nonomura (1974) and Bergey’s Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) are very much useful for the identification of *Rhodococcus*.

2.9. Molecular characterization

The current systematics of the genus *Rhodococcus* is unclear, partly because many members were originally included before the application of a polyphasic taxonomic approach. With the advent of 16S rRNA sequencing, a new hierarchic classification system Actinomycetales was proposed (Embley and Stackebrandt, 1994; Stackebrandt *et al.*, 1997). Included in this class are the bacteria that form spore bodies, motile or non-motile spores and which are capable of fragmenting to produce
coccal shaped forms during their life cycles (Rhodococcus and Dietzia). The 16S rRNA-based phylogenetics (Stackebrandt et al., 1997) and the 100nt 23S rRNA insertion shared by all Actinomycetales (Roller et al., 1992) enabled the systematics of this group that were previously based mainly on morphological variation (Stackebrandt et al., 1997).

2.10. Bioactive compounds from Rhodococci

The genus Rhodococcus is an industrially important taxon that includes many strains that degrade a variety of toxic organic and aromatic compounds. They exhibit diverse metabolic activities including the degradation of various aromatic hydrocarbons (Warhust and Fewson, 1994), chlorinated polycyclic aromatics such as polychlorinated biphenyls, nitroaromatics and other recalcitrant toxic pollutants (Bell et al., 1998). Rhodococci are notable for their ability to degrade a variety of natural and xenobiotic compounds but not much is known about the bioactive metabolite production from Rhodococcus spp.

Chiba et al. (1999) purified and elucidated a novel cyclic tetrapeptide, Rhodopeptins (Mer-N1033) from Rhodococcus sp. isolated from a soil sample collected at Mt Hayachine, Iwate Prefecture, Japan showing high in vitro antifungal activity against Candida albicans and Cryptococcus neoformans. Iwatsuki et al. (2006) elucidated two antimycobacterial agents, lariatins A and B from the culture broth of Rhodococcus sp. K01–B0171 by spectral analysis and advanced protein chemical methods. Kitawaga and Tamura (2008) isolated a new quinoline antibiotic, aurachin RE from culture broth of Rhodococcus erythropolis JCM 6824. The aurachin RE structure determined by NMR and mass spectrometric analysis was similar to that of aurachin C antibiotic that have been identified from Stigmatella aurantiaca. Compared to aurachin C, however, aurachin RE exhibited a wide and strong
antimicrobial spectrum against both high- and low-GC Gram-positive bacteria. They also studied 15 *Rhodococcus erythropolis* strains and classified them into three groups of antibiotic producers producing three different types of antibiotics with molecular weight of 200 Da, 350 Da and 400 Da.

Kurosawa *et al.* (2008) isolated a new class of aminoglycosides named rhodostreptomycin A and B from culture broths of *Rhodococcus fascians* 307CO which were purified by a combination of cation exchange (CM-Sephadex) and reversed-phase HPLC (Lichrospher 60RP-select B). Abdel-Megeed *et al.* (2011) purified, characterized and tested the antimicrobial activity of glycolipids produced by *Rhodococcus erythropolis* isolated from soils of Riyadh area, Saudi Arabia. Borisova (2011) isolated *Rhodococcus opacus* from soils of East Tennessee State University which produced a large zone of inhibition against *R. erythropolis* and a large number of closely related species. The MW of the antimicrobial compound was determined as 911.5452 Da.