First Report on Marine Actinobacterial Diversity around Madras Atomic Power Station (MAPS), India

P. Sivaperumal1* • T. Lakshmi2 • S. Rajeshkumar3

1Department of Pharmacology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical & Technical Sciences, Chennai, Tamil Nadu, India. E-mail: marinesiva86@gmail.com
2Department of Pharmacology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical & Technical Sciences, Chennai, Tamil Nadu, India.
3Department of Pharmacology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical & Technical Sciences, Chennai, Tamil Nadu, India.

ABSTRACT
Baseline assessments of marine microbial studies are very limited around ecologically sensitive areas of Nuclear Power Plant (NPP) site with respect to their occurrence, distribution, role in adaptation. Distribution and diversity of marine microbes are largely dependent on the physico-chemical parameters relating to specific area, especially spore producing marine actinobacteria are a source for different application. Marine actinobacterial diversity with conventional and 16S rRNA gene analysis was done around Madras Atomic Power Station. Totally, 60 different strains are identified in genera level and it’s belongs to 10 genera with dominant by Streptomyces sp. (8 species) Nocardiopsis (8), Microbispora (7) and Rhodococcus (5). This is the first report on marine actinobacterial diversity and the results could be act as baseline inventory in terms of microbial diversity around NPP sites.

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Introduction
The marine environment is complex, dynamic and it’s mainly characterized by the presence of saline and alkaline conditions that affect the way of life of organisms living in Sea. The environmental factors such as topography, water movement and stratification, temperature, pH, salinity, light availability, nutrients and sediment texture will be determined the composition of the biota around marine ecosystem (Karande, 1991). These marine areas are colonized with rich marine life owing to the nutrient sources which are necessary for the growth of the microbes that mediate the environmental factors, which of central importance in the ocean including microbes (ICMAM, 2002).

Among all the marine microbes, the marine actinobacteria is unique and one of the most striking ecologically important group. These bacteria inhabit an extraordinary array of habitats, from those that offer an ideal condition for most living creatures to support most marine life forms. They found of relatively benign and nutrient-rich environments of oceans and also survive in extreme environments such as hot springs (Brock 1978), salt brines (Anton et al., 2005), acid mine waters at pHs near zero (Baker and Banfield, 2003), deep in Antarctic ice (Christner et al., 2001; Price, 2000) and kilometres below the Earth’s surface (White et al., 1995) coastal and mangrove environment (Sivakumar, 2005). Further, it could play an important role in nutrient cycle in marine environment by re-calcitrating the material, nitrogen fixation and breakdown of organic matter in to more readily assimilated nutrient (Jensen et al., 1991).
Isolation and identification of actinobacteria has been difficult because of their slow growth as compared to other bacteria. When they were cultivated in nutrient rich media, some of them took double the normal time period; similarly few of them took half of time for their growth (Feller and Gerday, 2003). To obtain more and rare actinobacteria, a variety of pre-treatment techniques are used (Goodfellow and Haynes, 1984; Cho et al., 1994; Kamala et al., 2011& 2013). Further, identification of actinobacteria also very difficult as compare with other bacteria. Yet, one characteristic alone would be inadequate especially in the identification of a genera as well as species (Burkholder et al., 1954; Krasilnikov, 1960). Application of molecular techniques has given us cutting edge knowledge for identification and phylogenetic determination of microorganisms (Edwards-Ingram et al., 2004). In recent decades, application of rRNA gene sequence analysis facilitated to bring out some new order of this phylum in to the taxonomy (Edwards-Ingram et al., 2004). However, marine microbial diversity around ecologically importance area is very limited. Hence, the present study was carried out to obtain baseline inventory of marine actionbacteria around MAPS.

**Materials and Methods**

**Sampling Site**

Marine sediment samples were collected around Madras Atomic Power Station, India. Five locations were selected around NPP site comprising of two different marine environment viz., inshore (intertidal) and offshore respectively. The offshore and inshore sediment samples were collected by Van Veen grab of 0.04 m² and corer in sterile polythene bags and transported to the laboratory.

**Isolation Method**

The sediment samples were dried in room temperature and grind aseptically with mortar and pestle. To reduce the contaminants and support the actinobacterial growth, several methods of pre-treatments have been proposed which includes dry heat, phenol, calcium carbonate, phenol with heat, and calcium carbonate and heat with calcium carbonate (Baskaran et al., 2011; Kamala et al., 2011). After pre-treatment the samples were serially diluted with sterile sea water up to $10^4$ to $10^6$ dilution. One ml of diluted suspension was spread on starch Casein Agar (SCA), Kuster’s Agar (KUA) and Actinomycete Isolation Agar (AIA) without antibiotics supplement. The actinobacterial colonies were counted from 7th day onwards up to 28 days and the colonies were picked up and grown separately by streaking in petriplates.

**Identification of Marine Actinobacteria**

The colour of mature sporulation aerial mycelium, soluble pigment (Tresner et al., 1961), formation of melanoid pigment, colour of substrate mycelium or reverse side pigment and spore chain morphology (Shirling and Gottlieb, 1966) using Cell wall amino-acid (Cummins and Harris 1958) and whole cell sugar patterns (Lechevalier and Lechevalier, 1970) were studied. Molecular identification of marine actinobacteria was done and phylogenetic analysis and evolutionary relationship were done by MEGA 6 software (Tamura et al., 2011).

**Results and Discussion**

**Isolation of Marine Actinobacteria**

Physico chemical parameters of the coastal environment depend on the regional environmental condition such as rainfall, fresh water inflow, tidal movement and other biological activities (Satpathy et al., 2010). Depending on human activities, consumption pattern and industrial area like power plant etc., water and sediment quality criteria have been specified to determine its suitability for particular purpose. Hence, in the present study, the analyse of different physico chemical parameters such as atmospheric and surface temperature, water and sediment pH, salinity, dissolved oxygen, nutrients in water like, nitrate, nitrate and inorganic phosphate, macronutrient viz., nitrogen, phosphorous and potassium in sediment sample, organic carbon in sediment and sediment texture were performed. In addition, the actinobacterial population densities from various locations in different NPP sites were also studied. Actinobacterial populations in the estuarine and marine sediments vary in density with varying regions and even among sites within an ecosystem and actinobacteria are being reported from the marine sub habitats such as marine sediments (Ellaiah et al., 2002; Okazaki, 2006) of almost all parts of the world. Thus, they have worldwide distribution which indicates their plasticity and adaptability to extremely varied environmental conditions. In the present study, marine actinobacterial population density of onshore and offshore sediment samples varied from $10^3$ to $10^9$ CFUg⁻¹.

**Conventional and molecular Identification of marine actinobacteria from TAPS**

A total of 60 marine actinobacterial strains were isolated from inshore and offshore sediment samples of MAPS which belonged to 8 genera viz., *Streptomyces* spp. (23), *Nocardiosis* sp. (17), *Micromonospora* sp. (7), *Nocardia* sp. (4), *Rhodococcus* sp. (4), *Saccharomonospora* sp. (2), *Actinopolyspora* sp. (2) and *Pseudonocardia* sp. (1). From onshore sediment sample twelve strains were isolated and it produce aerial mycelia were grey (5), yellow (3), pink (1), red (1), white (1) and cream (1) and that of substrate mycelia were yellow (4), red (2), brown (2) and green (1) colour respectively. Similarly, five isolates were produced melanoid pigment and soluble pigments, which were brown, yellow and red in colour (Table 1). All twelve isolates were identified as *Streptomyces* through cell wall aminoacid, sugar pattern and three types of spore chain morphology namely spiral (9), spiral with rectiflexibles (2) and spiral with rectinuclapierti (1). The phylogenetic analysis involved 17 nucleotide sequences. All twelve isolates belonged to *Streptomyces* sp. and the phylogentic tree had IV clusters; cluster I had 5 isolates with three reference
sequences (NR043504, AB184397 and AB184430) at 89% bootstrap level; cluster II had five isolates and reference strain NR115673 at 93% bootstrap level; One isolate M156 at 92% bootstrap level was in cluster III while another isolate M152 had 73% bootstrap level was in cluster IV with reference strain (NR115673) (Fig.1).

| Isolat es | A.m. co lor | M pigm ent | Spigm ent | Pigm ent | Spore chain morphol ogy | Cell wall aminoacids | Whole cell sugars | Cell wall type & sugar pattern | Index |
|-----------|-------------|------------|-----------|----------|-------------------------|----------------------|------------------|-------------------------------|-------|
| M151      | WY          | 0          | 1(Y)      | 0        | SRF                     | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M152      | P           | 1          | 1(R)      | 1(R)     | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M153      | Gy          | 0          | 0         | 0        | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M154      | Gy          | 1          | 1(Br)     | 1 (Br)   | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M155      | Gy R        | 0          | 0         | 0        | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M156      | WY          | 0          | 0         | 1 (Y)    | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M157      | Gy          | 1          | 1(Br)     | 1 (br)   | SRA                     | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M251      | W           | 1          | 1(Br)     | 1 (R)    | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M252      | WGY         | 1          | 0         | 1(Y)     | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M253      | Cr          | 0          | 0         | 1(Y)     | SRF                     | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M254      | WY          | 0          | 0         | 1(Y)     | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |
| M255      | Gy          | 0          | 0         | 1(Gr)    | S                       | LL- AZP              | -                | -                             | I (N.C) Streptom ycessp. |

Morphological analysis: 1 - Present; 0 - absent; W - white; R - red; Cr - cream; Br - brown; Y - yellow; P - pink; Blk - black; Or - orange; Ly - ivory; Gy - gray; Bl - blue; Gr - green;Bg - beige. Spore chain morphology: RF - rectiflexibles; RA - rectinaculaperti; S - spiral; St - straight; Vs - Verticillate; RARF - rectinaculaperti and rectiflexibles; SRF - Spiral and rectiflexibles; SRA - spiral and rectinaculaperti.

Cell wall analysis: + Present; - Absent; DLA^{+}- meso dianaminopimelic acid; ^+m- minor amount was detected; N.C - Non characteristic.

A.m.color- Aerial mass color, Mel. Pigment- Melanine pigment, Sol. pigment- Soluble pigment, Rev. pigment- reverseside pigment.

![Fig. 1 Neighbor-joining dendrogram showing the phylogenetic relationship of 16S rDNA sequences from the stations M1 and M.](image)

A total of 48 strains were isolated from the offshore sediment samples of MAPS sites. The colours of spore masses of the isolates were grey (13), white (9), red (5), yellow (7), ivory (21), orange (3), green (3), blue (2), pink (2), cream (1), brown (1) and beige (1) colour on aerial mycelia and yellow (10), orange (9), pink (3), red (6), brown (9), beige (3), ivory (1) and green (1) on substrate mycelia. However out of 48 isolates, fourteen isolates showed melanoid pigments and 11 isolates produced soluble pigments of red, brown, yellow, blue, grey and orange colour. All 48 isolates showed four (I, II, III & IV) of cell wall types and three (A, D & N.C) of sugar patterns (Table 2). The isolates belonged to eight genera viz., Streptomyces (11), Nocardiopsis (7), Micromonospora (7), Nocardia (4), Rhodococcus (4), Saccharomonospora (2), Actinopolyspora (2) and Pseudonocardia (1). The evolutionary history was inferred
Table 2. Morphological and cell wall analysis of marine actinobacteria from MAPS offshore sediment samples

| Isolates | A.m. color | M pigment | S pigment | Rpi pigment | Cell wall amino acids | Whole cell sugars | Cell wall type & sugar pattern | Index |
|----------|------------|-----------|-----------|-------------|-----------------------|------------------|-----------------------------|-------|
| M351     | WGY        | 0 0       | 19        | 1(Y)        | Mono spores           | -                | +                          | II     |
| M352     | W          | 0 0 0     | 0         | 19          | Long chain            | -                | +                          | (N.C)  |
| M353     | Gy         | 1 0 1     | 1(Or)     | Mono spores | -                     | +                | +                          | (N.C)  |
| M354     | W          | 1 0       | 1(Y)      | S           | +                     | +                | -                          | I      |
| M355     | R          | 1 0 1(P)  | 0         | Short chains | -                     | +                | -                          | IV     |
| M356     | Y          | 0 1 1(Or) | 1(Br)     | RA          | +                     | +                | -                          | (N.C)  |
| M357     | WGY        | 0 1 1(Y)  | 1         | Long chain  | -                     | +                | -                          | III    |
| M358     | Or         | 0 0 1(Or) | 1         | Mono spores | -                     | +                | -                          | II     |
| M359     | R          | 1 0 1(R)  | 0         | S           | +                     | -                | -                          | I      |
| M360     | GyY        | 0 0 1(Y)  | 1         | Long chain  | -                     | +                | -                          | III    |
| M361     | Y          | 0 0 1(Br)| SRA       | +           | +                     | +                | -                          | I      |
| M362     | W          | 0 0 1(Br)| 1(Br)     | Long chain  | -                     | +                | -                          | III    |
| M363     | Or         | 0 0 1(Or)| Short rods| -           | +                     | -                | -                          | IV     |
| M364     | Gr         | 0 1(Or)   | 0         | Single spore | -                     | +                | -                          | IV     |
| M365     | Gy         | 0 0 1(Y)  | 1         | Short cocci | -                     | +                | -                          | IV     |
| M366     | Y          | 1 0 1(Bg)| 1         | Nocardiops   | -                     | +                | -                          | IV     |
| M367     | YR         | 0 0 1(Or)| Mono spore| -           | +                     | -                | -                          | IV     |
| M368     | Gy         | 0 0 0      | S         | +           | +                     | -                | -                          | I      |
| M369     | W          | 0 0 1(Bg)| Single spore| -           | +                     | -                | -                          | IV     |
| M370     | Bl         | 0 0 1(Or)| Mono spore| -           | +                     | -                | -                          | III    |
| M371     | Gy         | 0 0 0      | RF        | +           | +                     | -                | -                          | I      |
| M372     | R          | 0 0 1(Or)| Short cocci| -           | +                     | -                | -                          | IV     |
| M373     | R          | 0 0 1(R)  | Mono spore| -           | +                     | -                | +                          | II     |
| M374     | R          | 0 0 1(R)  | Short cocci| -           | +                     | -                | +                          | IV     |
| M375     | P          | 1 1(Or)   | 1(P)      | Long chain  | -                     | +                | -                          | III    |
| M376     | Gy         | 0 0 1(Or)| Mono spore| -           | +                     | -                | +                          | II     |
| M377     | W          | 1 0 1(Y)  | 1         | Short chain  | -                     | +                | -                          | IV     |
| M378     | Gy         | 1 0 1(Br)| Spiral    | +           | +                     | -                | -                          | I      |
| M379     | P          | 0 0 1(Or)| Mono spore| -           | +                     | -                | +                          | II     |
| M380     | W          | 0 0 1(Y)  | 1         | Long chain  | -                     | +                | -                          | III    |
| M381     | Gy         | 1 0 0      | Long spore| -           | +                     | -                | -                          | I      |

Using the Neighbor-Joining method and the optimal tree with the sum of branch length was shown to be 1.7981 (Fig. 2). The phylogenetic analysis involved 57 nucleotide sequences and there were a total of 818 nucleotide positions in the final dataset. The phylogenetic tree had six clusters at 50 to 90% bootstrap level. Cluster I had Nocardiopsis, cluster II had Streptomyces, cluster III has Pseudonocardia and Saccharomonospora, cluster IV had Actinopolyspora, cluster V had Nocardia and Rhodococcus and cluster VI had Micromonospora.
Morphological analysis: 1 - Present; 0 - absent; W - white; R - red; Cr - cream; Br - brown; Y - yellow; P - pink; Blk - black; Or - orange; Iy - ivory; Gy - gray; Bl - blue; Gr - green; Bg - beige.

Spore chain morphology: RF - rectiflexibles; RA - rectinaculapierti; S - spiral; St - straight; Vs - Verticillate; RARF - rectinaculapierti and rectiflexibles; SRF - Spiral and rectiflexibles; SRA - spiral and rectinaculapierti. Cell wall analysis: + Present; - Absent; DLA2P - meso diaminopimelic acid; +m - minor amount was detected; N.C - Non characteristic. A.m.color - Aerial mass color, Mel. Pigment - Melanin pigment, Sol. pigment - Soluble pigment, Rev. pigment - reverse side pigment.

Fig. 2 Neighbor-joining dendrogram showing the phylogenetic relationships of 16S rDNA sequences from the station M3, M4 and M5
Streptomyces are the dominant genera of actinobacteria in marine environment. From little Andaman and Nicobar group island, 32 and 52 actinobacterial strains were isolated and all of them are assigned to Streptomyces, respectively (Swarnakumar, 2010). In the present study Streptomyces was the dominant genus represented by a total of 98 strains which were isolated from inshore and offshore sediment samples. The previous study, 124 marine actinobacteria were isolated from the sediment samples collected from the intertidal zone in the Republic of Palau. These isolates are belonged to the family Brevibacteriaceae, Corynebacterium, Dermacoccaceae, Dietziaceae, Geodermatophilaceae, Gordoniaceae, Intrasporangiaceae, Microbacteriaceae, Micrococccaceae, Micromonosporaceae, Mycobacteriaceae, Nocardiaceae, Nocardialaceae, Nocardiopsaceae, Nocardiopsaceae, Romicronosporaceae, Pseudonocardia and Streptomyces (Gontang et al. 2007). Moreover, 30 isolates from six marine sediment samples collected from Gulf of Mexico and they belonged to the following genera Actinomadura, Dietzia, Gordania, Micromonospora, Nonomuraea, Rhodococcus, Saccharomonospora, Saccharopolyspora, Salinospora, Streptomyces, Solwaraspora and Verrucosispora (Maldonado et al. 2008). Additionally, 64 isolates were identified from eight different marine sediment samples from Kerala and those were allocated to the genus of Streptomyces, Glycomyces, Nocardiopsis, Nocardiodes, Actinpolyspora, Nocardia, Kibdelosporangium, Actinosynema, Actinomadura, Thermoactinomycetes, Kineospora and Saccharopolyspora (Remya and Vijayakumar, 2008). Furthermore, 20 actinobacterial strains which belonged to Streptomyces and Nocardiopsis were isolated from Mediterranean Sea (Oner et al., 2014). In the present study, Streptomyces, Nocardiopsis, Rhodococcus, Nocardia and Saccharopolyspora showed wide distribution in coastal environment especially in inshore sediments. Additionally, the predominant number of Streptomyces (38%) (Fig.3) is in agreement with earlier reported by Swarnakumar, 2010; Karthikeyan et al., 2014.

Fig. 3 Percentage composition of marine actinobacterial genera from MAPS

Conclusions
The baseline assessment of marine actinobacterial diversity were done around the proposed and running MAPS, India. This is a first kind of study around ecologically important area, besides, Streptomyces sp. Nocardiopsis sp., Microbispora and Rhodococcus kind of novel genus were isolated. These appear to be an indigenous part of microbial communities in the respective marine environments. This primary data will be useful in future ecological assessment and might be useful to analysis of diversity differ in future.

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References
Purnama, A., Al-Barwani, H.H., and Smith, R. (2005). Calculating the environmental cost of seawater desalination in the Arabian marginal seas. Desalination, 185(1-3), 79-86.
Baker, B.J., and Banfield, J.F. (2003). Micobial communities in acid mine drainage. FEMS microbiology ecology, 44 (2): 139-152.
Baskaran, R., Vijayakumar, R., and Mohan, P.M. (2011). Enrichment method for the isolation of bioactive actinomycetes from mangrove sediments of Andaman Islands, India. Malaysian Journal of Microbiology, 7(1), 26-32.
Brock, T.D. 1978. The habitats. In Thermophilic Microorganisms and Life at High Temperatures. Springer, New York, NY, 12-38.
Burkholder, P.R., Sun, S.H. Ehrlich J., and Anderson, L.E. (1954). Criteria of speciation in the genus Streptomyces. Ann. N.Y. Acad. Sci., 60: 102 - 123.
Christner, B.C., Mosley-Thompson, E., Thompson, L.G., and Reeve, J.N. (2001). Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. Environmental Microbiology, 3(9), 570-577.
Cho, S.H., Hwang, C.W., Chung, H.K., and Yang, C.S. (1994). A new medium for the selective isolation of soil actinomycetes. Microbiology and Biotechnology Letters, 22(5), 561-563.
Edwards- Ingram, L.C., Gent, M.E., Hoyle, D.C., Hayes, A., Stateva, L.I., and Oliver, S.G. (2004). Comparative genomic hybridization provides new insights into the molecular taxonomy of the Saccharomyces sensu stricto complex. Genome research, 14(6), 1043-1051.
Ellaiah, P., Adinarayana, K., Babu, A.N. Thaer, B., Srinivasulu, T., and Prabhakar, T. (2002). Bioactive actinomycetes from marine sediments of Bay of Bengal near Machilipatnam. Geobios, 29: 97-100.
Lekha, L., Raja, K.K., Rajagopal, G., and Easwaramoorthy, D., 2014. Synthesis, spectroscopic characterization and antibacterial studies of lanthanide (III) Schiff base complexes containing N, O donor atoms. *Journal of Molecular Structure*, 1056, 307-313.

Panda, S., Doraiswamy, J., Malaiappan, S., Varghesse, S.S., and Del Fabbro, M., 2016. Additive effect of autologous platelet concentrates in treatment of intrabony defects: a systematic review and meta-analysis. *Journal of investigative and clinical dentistry*, 7(1), 13-26.

Putchala, M.C., Ramani, P., Sherlin, H.J., Premkumar, P., and Natesan, A., 2013. Ascorbic acid and its pro-oxidant activity as a therapy for tumours of oral cavity—a systematic review. *Archives of oral biology*, 58(6), 563-574.

Danda, A.K., 2010. Comparison of a single noncompression miniplate versus 2 noncompression miniplates in the treatment of mandibular angle fractures: a prospective, randomized clinical trial. *Journal of oral and maxillofacial surgery*, 68(7), 1565-1567.

Kavitha, M., Subramanian, R., Narayanan, R., and Udhayabanu, V., 2014. Solution combustion synthesis and characterization of strontium substituted hydroxyapatite nanocrystals. *Powder technology*, 253, pp.129-137.

Venu, H., Subramani, L., and Raju, V.D., 2019. Emission reduction in a DI diesel engine using exhaust gas recirculation (EGR) of palm biodiesel blended with TiO2 nano additives. *Renewable energy*, 140, 245-263.

Neelakantan, P., Varughese, A.A., Sharma, S., Subbarao, C.V., Zehnder, M., and De-Deus, G., 2012. Continuous chelation irrigation improves the adhesion of epoxy resin-based root canal sealer to root dentine. *International endodontic journal*, 45(12), 1097-1102.

Samuel, M.S., Bhattacharya, J., Raj, S., Santhanam, N., Singh, H., and Singh, N.P., 2019. Efficient removal of Chromium (VI) from aqueous solution using chitosan grafted graphene oxide (CS-GO) nanocomposite. *International journal of biological macromolecules*, 121, 285-292.

Danda, A.K., Muthusekhar, M.R., Narayanan, V., Baig, M.F., and Siddareddi, A., 2010. Open versus closed treatment of unilateral subcondylar and condylar neck fractures: a prospective, randomized clinical study. *Journal of oral and maxillofacial surgery*, 68(6), 1238-1241.

Gopalakannan, S., Senthilvelan, T., and Ranganathan, S., 2012. Modeling and optimization of EDM process parameters on machining of Al 7075-B4C MMC using RSM. *Procedia Engineering*, 38, 685-690.

Venu, H., Raju, V.D., and Subramani, L., 2019. Combined effect of influence of nano additives, combustion chamber geometry and injection timing in a DI diesel engine fuelled with ternary (diesel-biodiesel-ethanol) blends. *Energy*, 174, 386-406.

Lekha, L., Raja, K.K., Rajagopal, G., and Easwaramoorthy, D., 2014. Schiff base complexes of rare earth metal ions: Synthesis, characterization and catalytic activity for the oxidation of aniline and substituted anilines. *Journal of Organometallic Chemistry*, 753, pp.72-80.

Krishnamurthy, A., Sherlin, H.J., Ramalingam, K., Natesan, A., Premkumar, P., Ramani, P., and Chandrasekar, T., 2009. Glandular odontogenic cyst: report of two cases and review of literature. *Head and neck pathology*, 3(2), 153-158.

Parthasarathy, M., Lalvani, J.I.J., Dhinesh, B., and Annamalai, K., 2016. Effect of hydrogen on ethanol-biodiesel blend on performance and emission characteristics of a direct injection diesel engine. *Ecotoxicology and environmental safety*, 134, 433-439.

PradeepKumar, A.R., Shemes, H., Jothilatha, S., Vijayarabharathi, R., Jayalakshmi, S., and Kishen, A., 2016. Diagnosis of vertical root fractures in restored endodontically treated teeth: a time-dependent retrospective cohort study. *Journal of Endodontics*, 42(8), 1175-1180.

Neelakantan, P., Grotra, D., and Sharma, S., 2013. Retreatability of 2 Mineral Trioxide Aggregate-based Root Canal Sealers: A Cone-beam Computed Tomography Analysis. *Journal of endodontics*, 39(7), 893-896.

Sajan, D., Lakshmi, K.U., Erdogdu, Y., and Joe, I.H., 2011. Molecular structure and vibrational spectra of 2, 6-bis (benzylidene) cyclohexanone: A density functional theoretical study. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 78(1), 113-121.

Uthra Kumar, R., Vesta, C., Raj, C.J., Krishnan, S., and Das, S.J., 2010. Bulk crystal growth and characterization of non-linear optical bishiothiourea zinc chloride single crystal by unidirectional growth method. *Current Applied Physics*, 10(2), 548-552.

Neelakantan, P., Cheng, C.Q., Mohanraj, R., Sriraman, P., Subbarao, G., and Sharma, S., 2015. Antibiofilm activity of three irrigation protocols activated by ultrasonic, diode laser or Er: YAG laser in vitro. *International endodontic journal*, 48(6), 602-610.

Neelakantan, P., Sharma, S., Shemes, H., and Wesselink, P.R., 2015. Influence of irrigation sequence on the adhesion of root canal sealers to dentin: a Fourier transform infrared spectroscopy and push-out bond strength analysis. *Journal of endodontics*, 41(7), 1108-1111.

Prathiba, K.M., Johnson, P., Ganesh, M., and Subhashini, A.S., 2013. Evaluation of salivary profile among adult type 2 diabetes mellitus patients in South India. *Journal of clinical and diagnostic research: JCDR*, 7(8), 1592.