

1 **Metagenomic Insights Unveil the Dominance of Undescribed Actinobacteria**
2 **in Pond Ecosystem of an Indian Shrine**

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Abstract

Metagenomic analysis holds immense potential for identifying rare and uncharacterized microorganisms from many ecological habitats. Actinobacteria have been proved to be an excellent source of novel antibiotics for several decades. The present study was designed to delineate and understand the bacterial diversity with special focus on Actinobacteria from pond sediment collected from Sanjeeviraya Hanuman Temple, Ayyangarkulam, Kanchipuram, Tamil Nadu, India. The sediment had an average temperature (25.32%), pH (7.13), salinity (0.960 mmhos/cm) and high organic content (10.7%) posing minimal stress on growth condition of the microbial community. Subsequent molecular manipulations, sequencing and bioinformatics analysis of V3 and V4 region of 16S rRNA metagenomics analysis confirmed the presence of 40 phyla, 100 classes, 223 orders, 319 families and 308 genera in the sediment sample dominated by Acidobacteria (18.14%), Proteobacteria (15.13%), Chloroflexi (12.34), Actinobacteria (10.84%), Cyanobacteria (5.58%), Verrucomicrobia (3.37%), Firmicutes (2.28%), and Gemmatimonadetes (1.63%). Among the Actinobacteria phylum, *Acidothermus* (29.68%) was the predominant genus followed by *Actinospica* (17.65%), *Streptomyces* (14.64%), *Nocardia* (4.55%) and *Sinomonas* (2.9%). Culture-dependent isolation of Actinobacteria yielded all strains of similar morphology to that of *Streptomyces* genus which clearly indicating that the traditional based technique is incapable of isolating majority of the non-*Streptomyces* or the so called rare Actinobacteria. Although Actinobacteria were among the dominant phylum, a close look at the species level indicated that only 15.2% within the Actinobacterial phylum could be assigned to cultured species. This leaves a vast majority of the Actinobacterial species yet to be explored with possible novel metabolites have special pharmaceutical and industrial application. It also indicates that the microbial ecology of pond sediment is neglected fields which need attention.

66 **Keywords:** *Metagenomics, sediment, rare Actinobacteria, Acidothermus and novel secondary*
67 *metabolites*

68 INTRODUCTION

69 It is estimated that only 1% of microbes in the natural environment are culturable under
70 laboratory conditions [46]. More than 88% of culturable isolates belong to four phyla known as
71 *Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes* [47]. However, uncultivated
72 bacteria possess a great diversity of enzymes adaptable to different environmental conditions
73 which could serve as a prolific source of novel bioactive compounds with industrial and
74 pharmaceutical applications. Metagenomics is a tool to study massive microbial communities
75 prevailing in the environment in its natural state irrespective of its uncultivable properties. This
76 technique has not only helped to overcome the barriers of culture dependent techniques but also
77 identified rare microorganisms [23]. It also provides a wide and unbiased view of the potential
78 microbial functions in a single snapshot with high resolution contributing immensely to the
79 global microbial diversity studies from different ecosystem and their potential ecological
80 functions [27]. On the other hand, there have been several reports on the widespread distribution
81 and occurrence of antibiotic resistance genes [24]. One of the most straightforward ways to
82 tackle the issue is to discover novel antibiotics. *Actinobacteria* are one of the largest phyla
83 ubiquitous to both aquatic and terrestrial ecosystems having high G+C content [48,49] which
84 have produced over 10,000 known antibiotics [15, 25]. However, emergences of multidrug
85 resistance have triggered the need for more potent antibiotics. Isolation of rare Actinobacteria
86 could increase the chances of discovering such potentially novel bioactive compounds i.e.
87 Vancomycin was discovered from the rare Actinobacteria strain *Amycolatopsis orientalis* [42,
88 43]. However, rare Actinobacteria are relatively difficult to isolate and maintain due to the
89 difficulties the lack of appropriate selection media, appropriate isolation procedures and
90 mimicking their natural environment [41]. Antibiotic discovery by conventional approach have

91 the drawback of frequent rediscovery of known metabolites. Hence, focus has to be set on rare
92 microorganisms to have a higher potential of novel metabolites [39, 40, 44]. As Actinobacteria
93 are the well-known antibiotic producers and due to the difficulties of rare Actinobacteria
94 isolation under normal laboratory conditions, metagenomic approach was set to systematically
95 assess the Actinobacterial diversity with respect to the overall microbial structure from pond
96 sediment.

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98 **MATERIALS AND METHODS**

99 **Sampling and physico-chemical characterization of sediment**

100 Sediment samples were collected from the oldest pond of Sanjeeviraya Hanuman Temple,
101 Ayyangarkulam, Kanchipuram district, Tamil Nadu, India (Latitude 79.67°N Longitude 12.78°E)
102 in the month of January, 2018. The sediment samples were pooled and submitted to Omega
103 Laboratories (Analytical Testing and Research Centre, Tamil Nadu, India) for physico-chemical
104 analysis.

105 **DNA extraction and next generation sequencing**

106 Total metagenomic DNA was extracted from freshly collected sediment sample using
107 PowerWater® DNA Isolation Kits (MoBio Laboratories) following manufacturer's protocol.
108 DNA quantification was performed using a Qubit™ 3.0 Fluorimeter (Life Technology Ltd.,
109 Paisley, UK) and verified by gel electrophoresing on 2% agarose gel. The 260/280 ratio was
110 measured using a Biophotometer (Eppendorf, Hamburg, Germany). DNA samples were stored at
111 -20°C until sequencing was performed. Samples were sequenced at the UAMS Sequencing Core
112 Facility. Bacterial 16S rRNA gene V3 and V4 regions were amplified using primers containing
113 Illumina adapters following Illumina's 16S metagenomics protocol. Briefly, Kapa Library
114 Amplification Kit was used for PCR and products were cleaned using Beckman Coulter
115 Agencourt AMPure XP Beads according to the 16S Metagenomics protocol. The universal 16S

116 forward and reverse primer sequences (**Supplementary data 1**) were implemented to create a
117 single amplicon of approximately 250bp. Concentrations were adjusted to 4uM and prepared for
118 loading on the Illumina Miseq according to Illumina's 16S metagenomics protocol. Samples
119 were pooled, denatured, and loaded on the Illumina Miseq at 8pM and sequenced paired end (2 x
120 300) using a MiSeq® Reagent Kitv3.

121 **Bioinformatics and Statistical Analysis**

122 QIIME 1.9.1 [31] pipeline was used for the entire downstream analysis. Quality check was
123 performed using FastQC0.11.7 [36] and PHRED score reads with >Q30 were considered for
124 further analysis. High quality reads were adapter trimmed and the paired-end reads were stitched
125 using FLASH 1.2.11 [32] to make consensus FASTA sequences. Consensus reads were formed
126 with 0 mismatch having an average contig length of 350 to 450bp and queried to UCHIME [33]
127 to remove all the chimeric sequence which was subsequently pooled and clustered into
128 Operational Taxonomic Units (OTUs) based on their sequence similarity using Uclust (similarity
129 cutoff = 0.97) [35]. Representative sequence was identified for each OTU against SILVA OTUs
130 database using PyNAST [34]. We analyze the microbial diversity within the samples by
131 calculating Shannon, Chao1 and observed species metrics. The chao1 metric estimates the
132 species richness while Shannon metric is the measure to estimate observed OTU abundances,
133 and accounts for both richness and evenness. The observed species metric is the count of unique
134 OTUs identified in the sample.

135 **Culture dependent isolation of Actinobacteria**

136 Isolation and enumeration of Actinobacteria were performed on selective media Starch Casein
137 Agar (SCA) and Kusters Agar (KUA) supplemented with Nalidixic acid 50µg/ml and Nystatin
138 20µg/ml. Soil sample was serially diluted up to 10⁻⁵ and one milliliter of the serially diluted
139 samples was spread into above mentioned media and incubated at room temperature for 7 days.
140 Microscopic characteristics such as the presence of aerial mycelium, substrate mycelium and

141 mycelial fragmentation were observed under the bright field microscope at 40x magnification.
142 Cultural characteristics such as growth, colony consistency, aerial mass colour, reverse side
143 pigment and soluble pigment was recorded by growing the culture on ISP2 (International
144 Streptomyces Project Medium- 2) agar medium.

145 **Antimicrobial activity**

146 Antimicrobial activity of Actinobacteria cultures were tested by adopting agar plug method
147 against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and
148 *Klebsiella pneumoniae*. The bacterial strains were grown in nutrient agar medium at 37°C.
149 Actinobacterial cultures were grown on YEME (Yeast Extract-Malt Extract) agar plates for 10
150 days at 28°C. Bacterial pathogens were spread on LB (Luria bertani) modified agar plate using
151 sterile cotton swab. Agar plug with 5mm diameter which contains the secreted Actinobacteria
152 metabolites were cut from the YEME agar medium in sterile condition and placed over LB
153 modified agar seeded with test pathogens. All the plates were incubated at 37°C for 24 hours.
154 Zone of inhibition was measured after incubation and expressed in millimetre in diameter.

155

156 **RESULTS**

157 **Pond sediment physico-chemical properties**

158 The physico-chemical property of the studied sediment sample is summarized in **Table 1**. The
159 overall physico-chemical properties of the sample was moderate with an electrical conductivity
160 (EC) and total salinity of the sediment of 0.952 mmhos/cm and 0.960 mmhos/cm respectively
161 and the water content of the sediment at the sampling moments was medium. Although calcium
162 and zinc were the dominant cation in sample, the sampling area showed to be dynamic with
163 respect to salinity contributions from different anions and cations from one year to the other. We
164 observed a neutral pH (7.13) and higher organic matter (10.7%) content in the sample retrieved
165 in January 2018.

166 **Microbial community structure**

167 The AKTS-1 microbiota resulted in 514249 high-quality reads with an average read length of
168 250 bp and 55.09 % total GC content. Base quality score of each cycle in more than 80% of the
169 total reads had phred score greater than 30 (>Q30; error-probability ≥ 0.001). We observed
170 513710 reads passed mismatch filter which were further processed for removal of chimera and
171 singletons. Rarefaction curve indicated that a reasonable number of individuals were sampled
172 (**Figure 1A-C**). All the resulting fragments were classified into 40 phyla, 100 classes, 223
173 orders, 319 families and 308 genera. The represented phyla were dominated by unknown phylum
174 (26.8%) followed by Acidobacteria (18.14%), Proteobacteria (15.13%), Chloroflexi (12.34%),
175 Actinobacteria (10.84%), Cyanobacteria (5.58%), Verrucomicrobia (3.37%), Firmicutes
176 (2.28%), and, Gemmatimonadetes (1.63%) (**Figure 2A**). Further affiliation revealed that the
177 most abundant genus was affiliated to phylum Actinobacteria which included genus
178 Acidothermus (3.21%), Actinospica (1.91%) and Streptomyces (1.58%). Remaining major genera
179 included *Candidatus solibacter*(3.05%) and *Candidatus koribacter*(1.32%) of Acidobacteria
180 phylum, *Anaerolinea* (2.74%) of Chloroflexi phylum, *Bradyrhizobium* (1.7%) of Proteobacteria
181 phylum. However, most of the genres were unidentified and uncultured (**Figure 3**). Within the
182 Actinobacteria phyla, although the uncultured (5.28%) and unclassified (17.84%) OTUs were
183 high, it was dominated by several known genus such as Acidothermus (29.68%), Actinospica
184 (17.65%), Streptomyces (14.64%), Uncultured Actinomycete (5.28%), Nocardia (4.55%),
185 Sinomonas (2.90%), Catenuispora (2.57%), Geodermatophilus (1.60%), and, Mycobacterium
186 (0.95%) (**Figure 2B**).

187 **Isolation and antimicrobial screening of Actinobacterial strains**

188 Eighteen morphologically different Actinobacterial strains were isolated from sediment sample
189 (**Table 2**). On ISP2 agar medium, Actinobacterial strains produced powdery growth (n 10)
190 whereas the remaining eight strains produced rough (n 5) and leather (n 3) growth. All the

191 cultures showed the presence of aerial and substrate mycelium under bright field microscopic
192 observation confirming that *Streptomyces* genus dominated in this isolation. Further, in agar plug
193 method, nine out of 18 Actinobacterial strains showed inhibition against at least one of the five
194 clinical pathogens tested. Notably, strains AKTS3 and AKTS11 were found to be active against
195 all the five tested pathogens (**Table 2**).

196

197 **DISCUSSION**

198 Freshwater bodies account merely 1% of the total water on earth with uneven distribution around
199 the globe. However, freshwater is necessary for majority of plants, animals and for our survival.
200 Microbes play a major role in maintaining water health; its diversity can also indicate a number
201 of parameters regarding water health. Hence, understanding the microbial diversity of the ponds
202 is important. Temple ponds in India serve as a reservoir of freshwater for religious rituals as well
203 as a source for drinking, bathing, washing and daily use. Such ponds are also exposed to various
204 anthropogenic and natural activities giving rise to different carbon sources. Previous studies have
205 found that the water quality as well as microbial community of pond is effected by carbon source
206 [19]. In natural ecosystems, physiochemical conditions such as pH, soils type, temperature,
207 salinity and other organic contents have high impact on metabolic activity and distribution of
208 microbiota [20]. The presence of average electrical conductivity, salinity, and, ion concentration
209 in the sediment sample within the study indicated positive microbial activity and a minimal stress
210 on the microbial community.

211 **Pond sediment microbial community structure**

212 Community structure of pond sediment is known to be extremely complex and only a few
213 dominant phyla are capable of thriving under such severely competitive environmental
214 conditions. Hence, the dominant bacterial phyla in pond sediment were identified and explored

215 as a part of this study. Dominant phyla included Acidobacteria (18.14%), Proteobacteria
216 (15.13%), Chloroflexi (12.34%) and Actinobacteria (10.84%) (**Figure 2A**). However, a major
217 part of the reads were assigned to unknown phylum (26.8%). Previous reports have reported
218 contrasting dominance of phylum in pond sediments. Actinobacteria were seen to be the most
219 dominated phylum in freshwater agricultural pond, mine area pit pond, rainforest soil and
220 Freshwater lake surface [1, 6, 14, 17]. In case of aquaculture pond sediments and ponds located
221 at the vicinity to hexachlorocyclohexane (HCH) production, Proteobacteria were the most
222 dominant [16, 19]. However, in this report, Acidobacteria was the most dominant phylum in
223 freshwater pond sediment. Although the race for the most dominate phylum might not show a
224 clear pattern, it is interesting to note that Actinobacteria stands among one of the most abundant
225 phylum in the entire freshwater pond. Interestingly, the most abundant genus *Acidothermus*
226 (3.21%) fell under phylum Actinobacteria which is rather unusual since *Acidothermus* was
227 originally isolated from Yellowstone National Park acidic hot spring [7]. *Acidothermus* is a
228 monospecific genus described as thermophilic, acidophilic, and cellulolytic bacterium which can
229 also degrade chitin [2, 3]. However, there are previous reports of genus *Acidothermus* being
230 enriched in the salinized rhizosphere of *Tamarixparviflora* [4]. It was also observed that the pond
231 sediment exhibited 308 genera (**Supplementary data 2**) out of which 46.9% of the genus were
232 unclassified indicating the potential novel genus yet to be discovered. In addition, 26.39% of the
233 genus were uncultured. This shows that more than 70% of the reads mapped to genera have
234 never been insolated in pure culture. The isolation of microbes in its pure culture form is of
235 outmost importance in order to study its ecological roles and the potential array of metabolites
236 that can have huge industrial and pharmaceutical applications.

237 **Actinobacterial community**

238 Actinobacterial genus such as *Acidothermus* (3.21%), *Actinospica* (1.91%), and, *Streptomyces*
239 (1.58%) dominated the bacterial genera indicating that the Actinobacterial genus are a dominated
240 member of bacterial Kingdom in pond sediment (**Figure 3**). These genera are also found
241 abundantly in various soil specimens and are possibly enriched in the pond sediment due to
242 presence of aromatic hydrocarbons and other similar factors [16]. A closer look at genus level
243 specifically within the Actinobacterial community highlighted eight predominant genera namely
244 *Acidothermus* (29.6%), *Actinospica* (17.65%), *Streptomyces* (14.64%), *Nocardia* (4.55%),
245 *Sinomonas* (2.90%), *Catenulispora* (2.57%) and *Geodermatophilus* (1.6%) dominated in varying
246 proportions throughout the study period (**Figure 2B**). This is in sharp contrast to freshwater
247 river, lake and agricultural pond being dominated by *Actinomycetales* and *Streptomyces* [17, 10].
248 Although *Streptomyces* was not the most dominated in this study, it occupied the 3rd most
249 abundant genus within Actinobacterial phylum indicating its predominant nature. Interestingly,
250 *Streptomyces* is the largest genus within Actinobacterial phylum and accounts for over 75% of
251 the bioactive compounds production from Actinobacterial phylum [8]. Within species level, a
252 vast majority of the reads (abundance) were either unclassified (56.6%) or uncultured (28.13%)
253 (**Figure 2C**). This is a clear indication that a large section of Actinobacterial species are
254 unexplored and untapped of its full potential. The large proportion of Actinobacterial phylum
255 within bacterial kingdom, however, with substantial unclassified OTUs at species level also
256 suggests that those undescribed bacterial groups with unknown metabolic capabilities are an
257 important part of the pond microbiota, a matter which warrants further investigation. In fact,
258 looking at the Actinobacterial species diversity, only 15 out of 32 OTUs were classified into
259 known species (**Figure 2C**) accounting for merely 15.2% of the Actinobacterial abundance
260 which indicates the dominance of yet-to-be cultured Actinobacterial species. Since majority of
261 microbes are not culturable under laboratory conditions, it is no wonder that there have been an
262 exponential decline of novel bioactive compounds discovery from Actinobacteria [9].

263 Actinobacteria as a promising reservoir of potential novel antibiotics is far from exhausted [46]
264 and research has been extensively performed from academic to industrial groups in quest of
265 novel metabolites [29]. Actinobacteria is a major reservoir of bioactive compounds housing over
266 45% of all microbial origin. Hence, in this study we have isolated 18 Actinobacterial strains in
267 pure culture using SCA selective media. Half of the isolated colonies showed antimicrobial
268 activity against at least one of the five different clinical pathogens tested. Strains AKTS3 and
269 AKTS11 exhibited antimicrobial activity against all the five different clinical pathogens tested
270 (**Table 2**). It is apparent that the discovery of novel rare Actinobacteria can be expected to
271 provide new bioactive compounds [26, 37, 42].

272
273 In the past years, focus on natural product discovery from Actinobacteria shifted from
274 extensively investigated soil-dwelling strains towards underexplored habitats of rare
275 Actinobacteria from unusual ecosystems [30]. This approach can give a significant impact on the
276 discovery platform for novel compounds with promising bioactivities from rare Actinobacteria
277 [45]. However, it is clear that the soil dwelling Actinobacteria from freshwater pond harbors a
278 significant amount of unexplored Actinobacterial species. There were also several bacterial rare
279 species, below 0.001%, (n 186; **Supplementary data 3**) of a great diversity with low abundance,
280 16 belonging to either unknown or uncultured Actinobacteria phylum. It has been suggested that
281 members of the rare biosphere may play important roles in the bacterial community [22, 28].
282 Freshwater ecosystem, especially stagnant water bodies such as lakes have shown some
283 promising results for rare Actinobacterial genus [10, 17]. Development of new selective
284 techniques and expression based metagenomics would enable screening, isolation, and
285 discoveries of rare antibiotic genes which could lead to useful bioactive compounds.

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288

289 **CONCLUSION**

290 The study shows the presence of unexplored rare and uncultured Actinobacteria through
291 metagenomics. The work also clearly demonstrates that most of the actinobacteria strains have
292 high antimicrobial activity. However, the current traditional selective media is not suitable for
293 harvesting rare and uncultured Actinobacteria community. Hence, potential novel secondary
294 metabolite from rare Actinobacteria, especially from exotic environmental habitats, is still
295 underexplored. Focus has to be highlighted to develop novel isolation strategies and media to
296 isolate such rare microbes. Neglecting the rare isolates would understate the immense potential
297 of novel bioactive compounds and their role as a keystone species in the environment.

298

299 **Author contributions**

300 VG; MK performed the experiments, RM conceived and designed the experiments,
301 analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or
302 tables, Wrote the paper: RK RM, MI, MK, AV, VG, TS, JJ, RB, All authors gave critical
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309 **Conflicts of Interest**

310 The authors declare no conflict of interest.

311 **Ethical approval**

312 This article does not contain any studies with pathogenic organisms or anthropogenic
313 activities performed by any of the authors.

314 **Data availability**

315 All data relevant to the article is included in the article and its supplementary information.

316

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442

443 **FIGURES AND TABLES CAPTIONS:**

444

445 **Figure 1** Rarefaction plot of the phylogenetic diversity, using [A] observed [B] chao1 and [C]
446 Shannon curve as alpha diversity metric. The curves reached a plateau indicating the
447 high depth sequencing of the sample.

448 **Figure 2** Stacked bar and dough nut chart representing [A] dominant prokaryotic phylum and
449 [B] the list of all Actinobacterial genus and [C] species.

450 **Figure 3** Summary of the top 10 genus predominant in the pond sediment.

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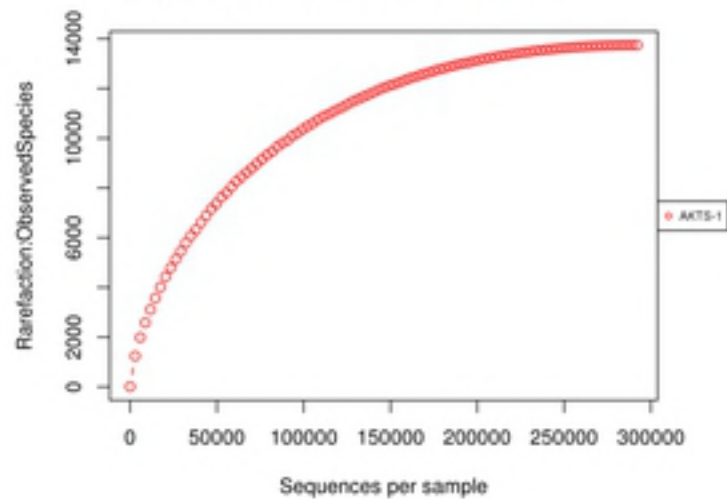
473 **Table 1. Physico-chemical characteristics of sediment sample AKTS**

| Test parameter | Unit | Result |
|--------------------------------|------------------|---------------|
| <i>Color</i> | | Red |
| <i>pH</i> | | 7.13 |
| <i>Salinity</i> | Mmhos/cm | 0.960 |
| <i>Acidity</i> | Mg/kg | 5.82 |
| <i>Humidity</i> | g/m ³ | 24.10 |
| <i>Water activity</i> | | Medium |
| <i>Temperature</i> | °C | 25.32 |
| <i>Soil moisture</i> | % | 24.55 |
| <i>Silt</i> | % | 55.6 |
| <i>Clay</i> | % | 25.2 |
| <i>Sand</i> | % | 18.4 |
| <i>Electrical conductivity</i> | Mmhos/cm | 0.952 |
| <i>Permeability</i> | Mm/hr | 26.34 |
| <i>Organic matter</i> | % | 10.7 |
| <i>Nitrogen</i> | % | 0.9 |
| <i>Phosphate</i> | % | 2.1 |
| <i>Potassium</i> | % | 0.86 |
| <i>Lime status</i> | % | 5.8 |
| <i>Magnesium</i> | mg/lit | 220 |
| <i>Sodium</i> | mg/lit | 145 |
| <i>Calcium</i> | mg/lit | 280 |
| <i>Zinc</i> | mg/lit | 264 |
| <i>Copper</i> | mg/lit | 22 |
| <i>Boron</i> | mg/lit | 12 |
| <i>Aluminum</i> | mg/lit | 14 |
| <i>Chromium</i> | mg/lit | 12 |
| <i>Fluoride</i> | mg/lit | 8 |
| <i>Silver</i> | mg/lit | 2 |
| <i>Mercury</i> | mg/lit | 2 |
| <i>Selenium</i> | mg/lit | 4 |

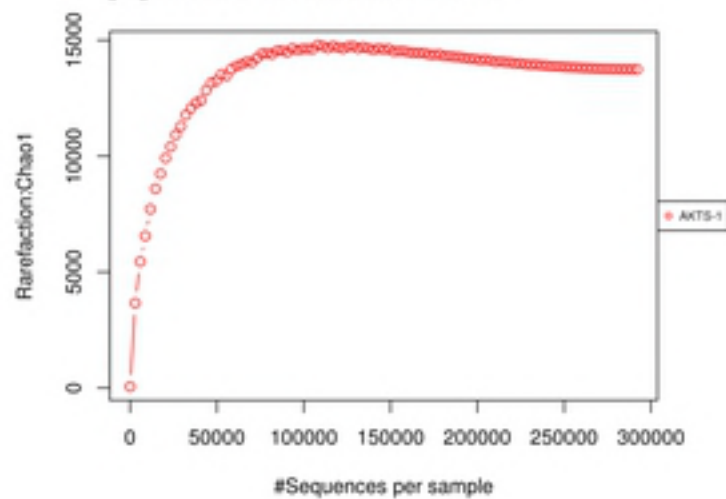
Table 2. Details of Actinobacterial strains isolated from sediment sample and their antimicrobial activity

| Strain | Cultural characteristics | | | | | | | Antimicrobial activity (mm) | | | | |
|---------|--------------------------|-------------|-------------|---------------|----|----|----|-----------------------------|-----------------|------------------|---------------------|----------------|
| | Growth | Consistency | AMC | RSP | SP | AM | SM | <i>E. coil</i> | <i>Bacillus</i> | <i>S. aureus</i> | <i>K. pneumonia</i> | <i>Candida</i> |
| AKTS-1 | Good | Powdery | White | Brown | + | + | + | | 14 | | | |
| AKTS-2 | Good | Powdery | Orange | Pale yellow | - | + | + | | | | | 9 |
| AKTS-3 | Good | Powdery | Pink | Pale red | - | + | + | 19 | 25 | 15 | 12 | 20 |
| AKTS-4 | Good | Powdery | Grey | Brown | - | + | + | | | | | |
| AKTS-5 | Good | Powdery | Dirty white | Red | - | + | + | | | | | |
| AKTS-6 | Good | Rough | Orange | Reddish brown | - | + | + | | | | | |
| AKTS-7 | Good | Powdery | White | pink | + | + | + | | | | | |
| AKTS-8 | Good | Rough | White | - | - | + | + | | | | | |
| AKTS-9 | Good | Rough | Pale yellow | Pale yellow | - | + | + | | | 28 | | |
| AKTS-10 | Good | Powdery | Pale yellow | Pale yellow | - | + | + | | | | | |
| AKTS-11 | Good | Leathery | Orange | Pale yellow | - | + | + | 16 | 17 | 30 | 11 | 14 |
| AKTS-12 | Good | Powdery | Pale yellow | Pale red | - | + | + | | | | | |
| AKTS-13 | Good | Leathery | Grey | Pale red | - | + | + | | | | | |
| AKTS-14 | Good | Leathery | Grey | Pale yellow | - | + | + | | | | | |
| AKTS-15 | Good | Rough | Grey | Pale yellow | - | + | + | | 23 | | | |
| AKTS-16 | Good | Powdery | White | Brown | + | + | + | | 23 | 18 | | |
| AKTS-17 | Good | Powdery | Dirty white | Pale red | - | + | + | | 16 | 11 | | 18 |
| AKTS-18 | Good | Rough | Dirty white | Light green | - | + | + | | 18 | | | 11 |

[A] Observed rarefaction curve



[A] Chao1 rarefaction curve



[A] Shannon rarefaction curve

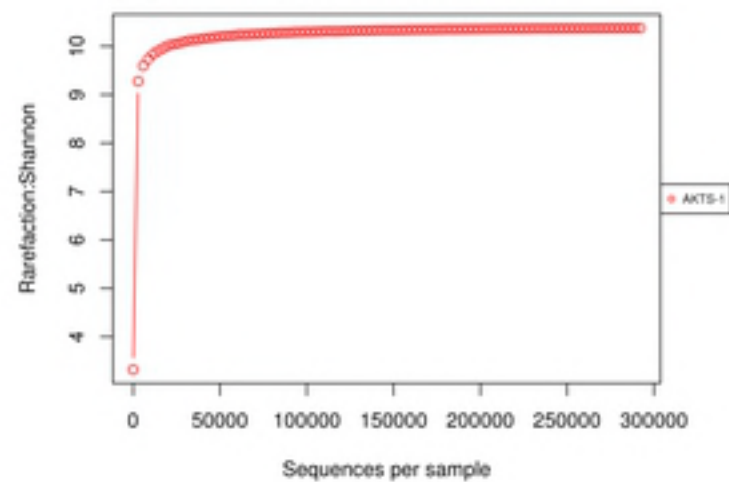


Figure 1

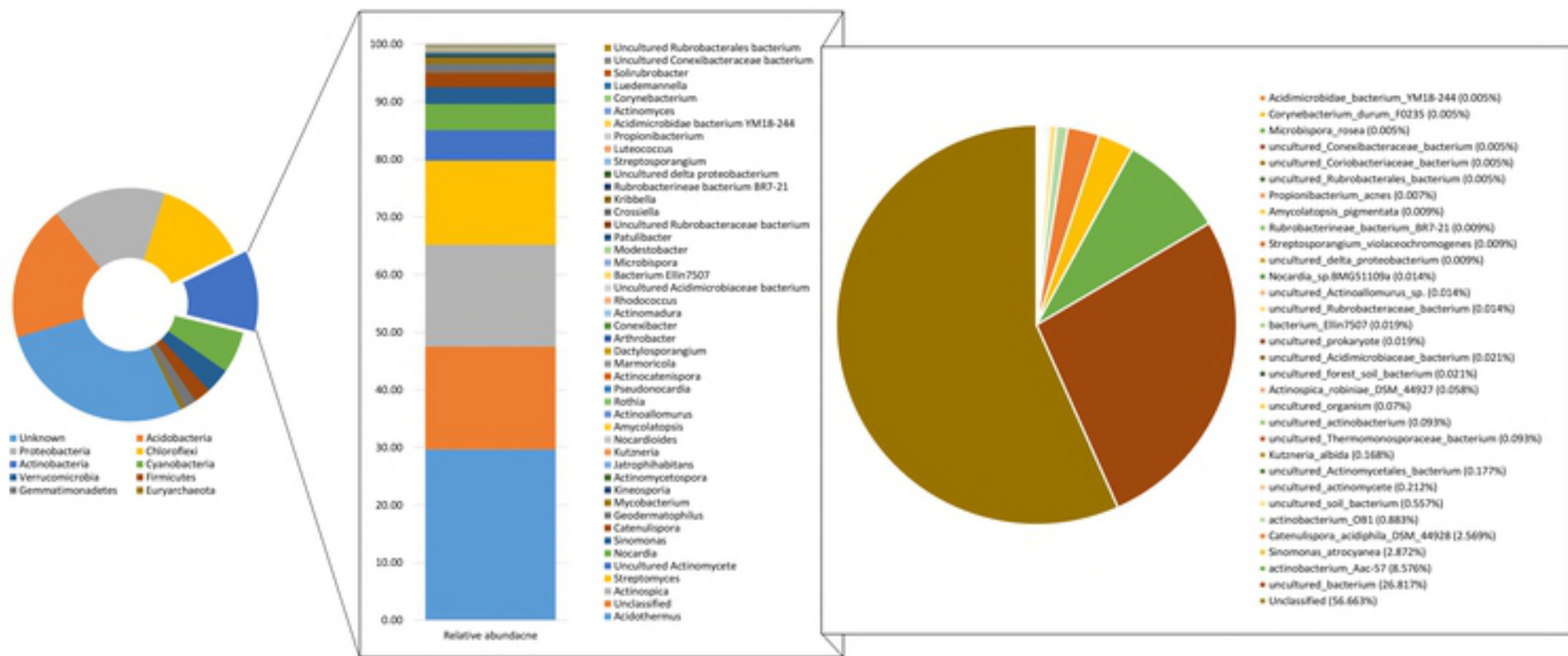


Figure 2

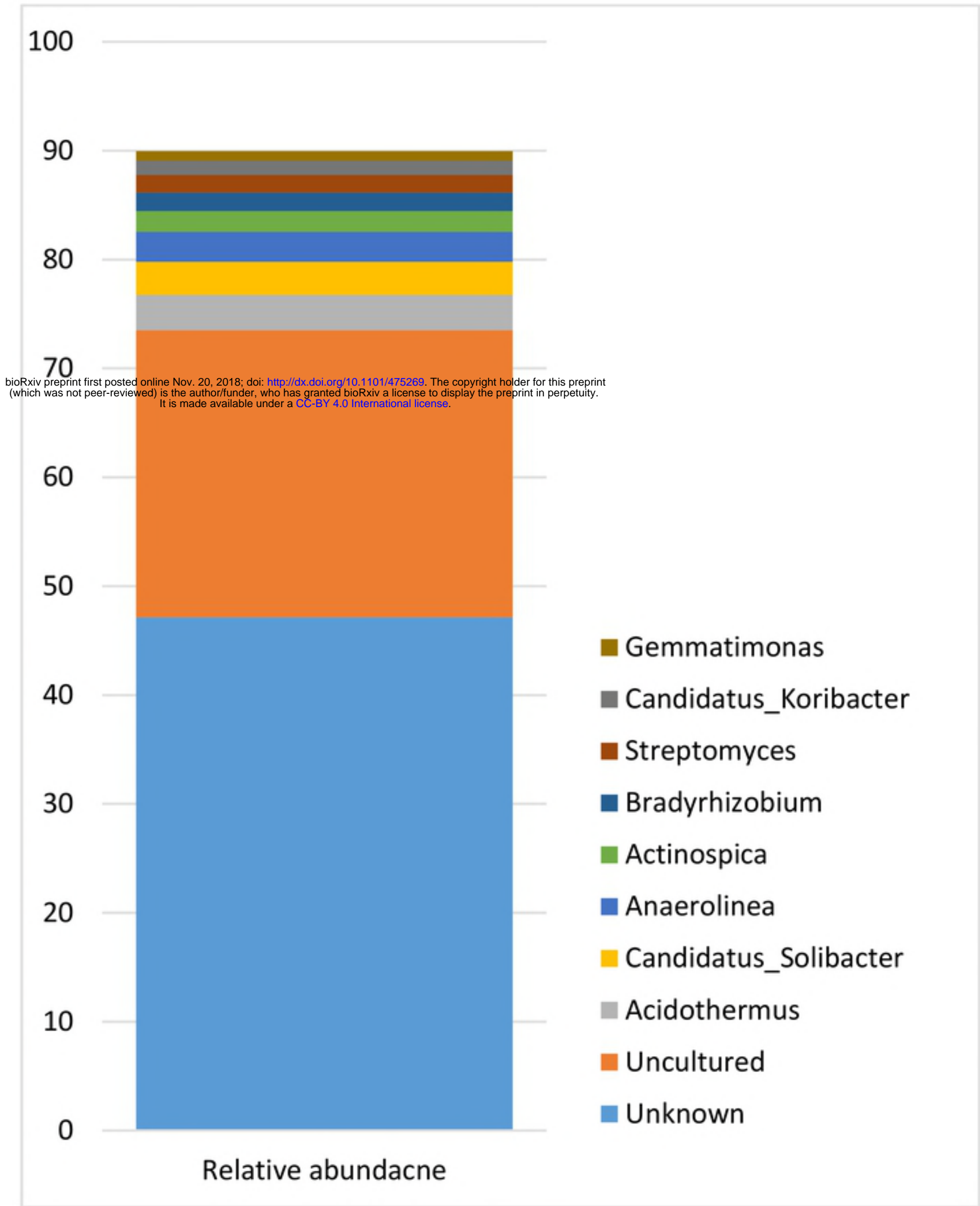


Figure 3