Drug discovery: mining microbes for bioactive compounds

A collaborative programme involving industry and academia was initiated in September 2007 by the Department of Biotechnology to screen the bacterial diversity from various ecological niches in the country to search for novel bioactive molecules for therapeutic applications. The programme was designed to collect resource samples from the less-explored ecological niches at regular intervals for three years, to ensure a steady supply of microbes for high-throughput screening (HTS). The project had two major components. The first was targeted to generate a repository of 250,000 bacteria in three years by the academic research groups. The other component was preparation of microbial extracts and their screening on HTS platforms in four therapeutic areas followed by chemical profiling of potent extracts. Figure 1 shows the overall project plan.

Microbial diversity and isolation strategy

Microbial communities survive in diverse habitats, according to the availability of nutrients and prevailing stress conditions. Therefore, each habitat is likely to be populated by a unique microbial community. To survive and proliferate in such habitats, microbes synthesize secondary metabolites which provide competitive advantage to them in such environments. These metabolites can be accessed by isolating and cultivating bacteria with desired phenotypic traits using specific growth conditions[1]. The broth cultures of these isolates can be then extracted and subjected to bioactivity-guided isolation procedures using advanced platforms of analytical chemistry[2]. The first objective of the project was to encompass as wide a microbial diversity as possible. To achieve this, a team of microbiologists was brought together from 10 different collaborating institutions. Each investigator was assigned a different habitat as summarized in Figure 2; and is reported earlier[3]. To bring some uniformity in the collection process with appropriate quality control, a meticulous standard operating protocol (SOP) was developed for sample collection, isolation of microbes and their purification. To accommodate for the variety of growth requirements, 30 different growth media were created. For isolation of bacteria from the samples collected from marine or brackish environment, extra sodium chloride was incorporated into the medium.

Outcome of the isolation strategy towards microbial diversity

As pointed out earlier, the first part of the project activity involved isolation of bacteria from different habitats and screening them for four therapeutic areas. As the result of this concerted activity, 247,964 pure bacterial species, were chosen, purified and confirmed for purity. These were then preserved in 15% glycerol at –70°C in duplicate cryovials, one copy of which was mailed to Microbial Culture Collection (MCC), Pune for long-time storage after cross-checking. A pilot study was carried out based on the growth characteristics and data generated through the screening assay, to evaluate the redundancy during isolation. Statistical analysis showed that almost 77–84% of the isolates was distinct. It was observed that on an average 25% of culture could not be revived. For bacteria isolated from marine and mangrove ecological niches, non-revivability was almost 65–70%. Eventually, about 175,000 pure isolates could be obtained, which were preserved at MCC for further studies.

Initially, all the 247,000 barcoded isolates (Figure 2) from different academic investigators were provided to the concerned scientists, where the extracts prepared from these were screened for their 'possible' anti-cancer, anti-diabetes, anti-inflammation and anti-infective activities. Assays were carried out using HTS; Freedom EVO200 workstation by M/s Tecan at Piramal Enterprises Limited (PEL), Mumbai. For identifying extracts with anti-cancer potential, screening was done for cytotoxicity assay using fluorescence-based propidium iodide method with tumour cell line model. Screening for the inhibitors of proinflammatory cytokine released by LPS-induced monocytic cell lines was evaluated by ELISA, and parallel cytotoxicity assays were performed as part of anti-inflammatory assay. The inhibition in glucose uptake assay using myotubes from rat skeletal muscle was used as the assay protocol for anti-diabetic activity. The primary screening for the anti-infective activity was carried out by spot diffusion assay on agar plate having the test organisms. These organisms included *Candida albicans*, *Aspergillus fumigatus*, *Staphylococcus aureus*, *Enterococci* sp., *Pseudomonas* and *Escherichia coli.*

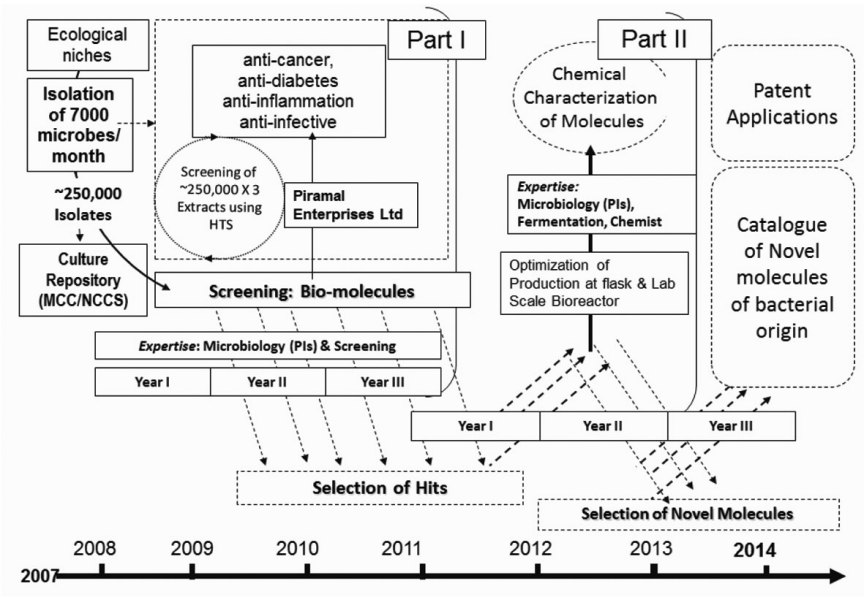

Figure 1. Overall project plan.

This screening activity led to the 'discovery' of 16,000 bioactive extracts (Figure 3).

Natural products research has adopted advanced strategies and hyphenated instrumentation that have helped surmount to a large extent, the existing technical barriers in HTS of molecular targets[4]. Strategies of pre-fractionation of extracts are employed by application of advance chromatographic techniques and robotic liquid handlers. Pre-fractionation followed by de-replication has improved the speed of obtaining novel scaffolds in natural products drug discovery programmes[5,6]. De-replication involves identification of the active fractions which contain previously reported molecules. This is achieved through the application of MS libraries and databases, which allow hit searching in terms of molecular weight, UV profile, chemical structure, bioactivity and taxonomy[5].

The second part of the project activity focused on chemical profiling of the bioactive extracts for mining out the active molecules present in them. It was decided that initially 300 extracts randomly selected to represent each ecological niche, that were active in all the therapeutic areas would be taken up for chemical characterization. In the second round, an additional 700 extracts would be selected following the same protocol. For chemical characterization, 2–4 mg of crude extracts was generated at CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nagpur, which were then tested, fractionated and characterized at PEL. Crude ethyl acetate extracts for each isolate were subjected to de-replication for novel molecules or molecules with novel activity. Taxonomic characterization of the strains was done at MCC, Pune.

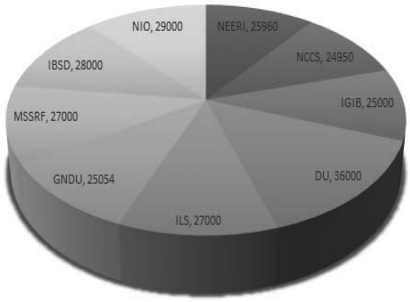

Figure 2. Spectrum of microbes from different ecological niches. CSIR-NEERI (National Environmental Engineering Research Institute, Nagpur): Effluent treatment plant and contaminate sites; NCCS (National Centre for Cell Sciences, India): Western Ghats and insect guts; CSIR-IGIB (Institute for Genomics and Integrative Biology, New Delhi): River sediment; DU (Delhi University, Delhi): Hot springs and contaminated soils (north India); ILS (Institute for Life Sciences, Bhubaneshwar): Extemophiles (Odhisha, Bihar and West Bengal); GNDU (Guru Nanak Dev University, Amritsar): Wetland ecosystems of northwest India; MSSRF (M.S. Swaminathan Research Foundation, Wardha): Eastern Ghats and mangrove areas; IBSD (Institute of Bioresources and Sustainable Development, Imphal): North East India; CSIR-NIO (National Institute of Oceanography, Goa): Marine isolates.

The 1000 bioactive microbial extracts thus obtained were subjected to phase-1 fractionation, which included automated small-scale HPLC fractionation with simultaneous detection of UV profile, MS and MS/MS fragment data. Fractions were collected in 96-well deep well plate and *in vitro* bioassays were carried out for all fractions HTS platforms. These active crude extracts simultaneously underwent UPLC–UV–ELS–MS fingerprinting to ensure omission of duplicates. Dendrograms were generated, and representatives of each group and unique ones were selected.

A detailed de-replication data analysis was carried out for the active fractions identified by HTS platform to find the novelty of compound/s, if any, on the basis of public databases and in-house LC-MS/MS database (called LC-MS library). Retention time, mass and MS/MS fragmentation were checked with LC-MS library and any 'hits' thus obtained were further evaluated for bioactivity and UV profile. In 'no hit' cases, accurate mass of compounds, obtained from Q-TOF MS analysis, was searched in various public domain databases such as Dictionary of Natural Products (DNP), AntiMarine (Marinlit + Antibase) and Scifinder. This analysis led to the identification of known compounds with novel activities (known as novel chemical activity (NCA)) or novel chemical entities (NCE). Fractions with known compounds and known reported activity were omitted from further studies.

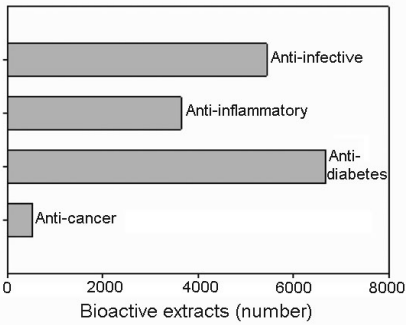

Figure 3. Therapeutic profile of 16,000 bioactive extracts.

Extracts which fulfiled the above criteria were considered for large-scale fractionation (phase-2 fractionation). As the phase-2 fractionation required 5–10 g of crude extracts, 'selected' cultures were first grown to 20 l level and then using automated liquid handlers, advanced drying techniques and HPLC, required amounts of extracts were obtained.

Pure and semi-pure fractions were tested for bioactivity as a check for enrichment. Characterization of a pure compound was done by NMR and mass spectrophotometry analysis. Further structural search for novelty was carried out with Scifinder and STN.

Overall, chemical characterization study led to the discovery of one NCE, confirmed by search on various databases, and Scientific and Technical Information Network (STN), four probable NCEs (Scifinder search, but to be confirmed by STN), and three known compounds with novel bioactivity. During this process, 235 compounds were dereplicated, and 51 bioactive compounds were purified and catalogued. Eighty-two postulated structures have been catalogued from phase-1 studies, which can be produced based on the requirement. The catalogue of bioactive compounds and related reports are available with DBT and MCC, Pune. Fifty-one pure compounds are being maintained at MCC, Pune.

For the NCA compound N2072, a US Provisional Patent Application entitled 'Use of a thiopeptide in the treatment of *Clostridium difficile* associated infections' was filed at the US Patent Office on 31 October 2013. The Provisional Patent Application No. is 61/898,237. This was followed by a PCT application (PCT/IB2014/065702) in October 2014.

Development programmes and future directions

A next logical step for the 'possible' utilization of these molecules would be to incorporate the NCEs into a development programme that would include the structure–activity relationships, drug metabolism and pharmacokinetics studies and toxicity studies. Studies will be also required to establish how many of these novel scaffolds would be druggable and,

if so, whether they could be amenable to modifications by chemistry approaches to define activity–toxicity profiles. It is to be noted that out of a total of 175,000 extracts, 16,000 showed activity in four therapeutic areas, among which only a 1000 were examined further. Even then, four NCEs and three NCAs could be detected. This encouraging result indicates that if properly exploited, the microbes collected through this programme could lead to the discovery of many more useful molecules.

Creation of a repository

This exercise also resulted in the creation of a repository of microbes collected under the project. All the 175,000 isolates are being characterized, maintained and preserved here, making it the largest collection in the world and the only one that is biotechnology linked. The microbial culture collection at National Centre for Cell Sciences, Pune set up by Department of Biotechnology, Govt of India has acquired the status of International Depositary Authority under the Budapest Treaty and is also designated as National Repository by the Ministry of Environment, Forest and Climate Change, GoI, under the Biodiversity Act, 2002. This is the single largest repository in the Asian region. It has recently been renamed as the 'National Centre for Microbial Resource'. The cultures collected under this project are available to any desirous researcher for large-scale screening under a Material Transfer Agreement. Besides this, the collection also offers a variety of microbial storage and identification services.

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C. N. R. Rao wins the Materials Research Society's Von Hippel Award

The Von Hippel Award, the Materials Research Society's (MRS) highest honour, recognizes brilliance and originality of intellect, combined with vision that transcends the boundaries of conventional scientific disciplines. The award that includes a cash prize, honorary membership in MRS, and a unique trophy was presented to Rao in Boston on 29 November 2017 during the Materials Research Society's Annual meeting. Rao who is the first Asian to receive this award is the National Research Professor at the Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India. He received this award 'for his immense interdisciplinary contributions to the development of novel functional materials, including magnetic and electronic properties of transition metal oxides, nanomaterials such as fullerenes, graphene and 2-D inorganic solids, superconductivity and colossal magnetoresistance in rare-earth cuprates and manganates.'

Rao started his independent research efforts in materials chemistry when the subject was in its nascent stage. With the meagre facilities available then, he investigated phase transformations of TiO_2 and CsCl, and also carried out defect calculations. While working on rare-earth oxides, he made TbO_2 and PrO_2 using a simple solution route – this is an early example of chimie douce. He started working on metal oxides by building simple instruments including a thermobalance and furnaces. In 1987 he was able to fully characterize the first N_2 superconductor ($YBa_2Cu_3O_7$) using a home-built AC suseptometer. He has worked on various aspects of transition-metal oxides including metal-insulator transitions, colossal magnetoresistance and multiferroics. In the last two decades, he has been engaged in the synthesis, characterization and measurements of properties of various nanomaterials, especially 2D nanosheets (graphene and its inorganic analogues). As part of his interest in designing new materials, he has covalently cross-linked 2D sheets and other nanomaterials to derive new materials with novel properties. Rao is actively working on water splitting and reduction of CO2, besides using aliovalent anion substitution to generate novel inorganic materials (Zn2NF in place of ZnO). He has authored more than 1500 research papers and 45 books.

Current Science had intended to publish the news of Rao winning the award under the news section in its 25 November 2017 edition. The misclassification of this news in the 25 November issue of the journal is deeply regretted by the Editor and Editorial staff.

1. https://www.mrs.org/fall-2017-von-hippel

Infosys Prize 2017

The Infosys Science Foundation announced the winners of the Infosys Prize 2017 on 15 November 2017. Every year, the foundation gives away awards for outstanding achievements to contemporary researchers and scientists in the areas of Engineering and Computer Sciences, Humanities, Life Sciences, Mathematical Sciences, Physical Sciences and Social Sciences.

The winners of 2017 were shortlisted from over 236 nominations by a scholarly