Summary

Cholesterol oxidase (EC 1.1.3.6) is a microbial enzyme that catalyzes the first step in cholesterol degradation pathway. Its mammalian homolog has not been reported so far. It oxidizes the cholesterol into cholest-4-en-3-one and hydrogen peroxide and releases hydrogen peroxide. It is used by clinical diagnostic industry for cholesterol estimation. Cholesterol oxidase is developing as potential enzyme which can be used in biotechnological industries. Till date it have been searched from many diverse habitats was reported from various microbial genera, *Brevibacterium, Nocardia*, and *Streptomyces, Rhodococcus*.

In order to explore new sources in this present study, total 1200 actinobacterial strains were isolated from mangrove sediments and screened for cholesterol oxidase activity. Among 1200 isolates 48 stains were positive for cholesterol oxidase activity on plate in which 21 strains shows equivalent cholesterol oxidase activity in comparison to positive control and 12 strains shows least activity as compared to control. These ChOx positive strains were further identified using 16S rRNA gene sequencing approach, 30 strains were identified as *Streptomyces* species, 7 strains were identified as *Rhodococcus* species, and one species from each genera of *Amycolatopsis, Brevibacterium, Nocardiopsis, Kitatospora* was also identified. Some potential novel strains was also identified such as BKS 15-14 was identified as *Rhodococcus triatomae* with 98.20%, BKS 10-45 *Streptomyces gramineus* 98.66%, BKS 12-139 *Streptomyces yunnanensis*98.74%, BKS 16-54 *Kitasatospora putterlickiae* 98.02% and BKS 16-23 *Streptomyces griseoruber* 98.72% on the basis of 16S rRNA gene sequence similarity.

To establish molecular phylogeny among the cholesterol oxidase producing actinobacterial strains, phylogenetic analysis was performed. This evolutionary history was inferred using Neighbor-Joining method, Maximum Likelihood method and Maximum Parsimony method. The NJ tree shows 5 clade, clade I represented by *Streptomyces badius,* clade II *S. grieseoruber,* clade III *S. lunalinharseii,* clade IV *Kitatospora putterlickiae,* and clade V *Nocardio psislucentensis*. Maximum Likelihood method was based on Kimura 2-parameter model shows 5 clade representing clade I *Streptomyces koyangensis*, clade II *S. setonii*, clade III *S. albulus*, clade IV *Kitatospora putterlickiae,* and clade V *Nocardiopsislucentensis*. The evolutionary history was also inferred using Maximum Parsimony method it has eight clade representing clade I *S. griseofuscus*, clade II *S. setonii*, clade III *Kitatospora putterlickiae,* clade

IV *S. albulus,* clade V *S. koyangensis*clade VI *Sacchropolyspora gloriosae,* and clade VIII *Brevibacterium epidermidis.* (NJ, ML, MP) Most of the isolates in clade V belongs to non-mycelium group of *actinobacteria*. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed.

In this study two major genera namely *Streptomyces* and *Rhodococcus* were found. Since the genus *Rhodococcus* are well reported for its survival in extreme environmental conditions such as organic solvent, detergent tolerant conditions three *Rhodococcus* strains were selected for further experiments are as follows i.e., *Rhodococcus ruber* BKS 20-38 strain, *Rhodococcus qinshengii* BKS 20-40 strain, *Rhodococcus tritomae* BKS 15-14 strain.

Using sequence details, primers were designed to amplify and sequence the cholesterol oxidase genes. The cholesterol oxidase nucleotide sequence was 1734bp, 1755bp, 1743bp for *Rhodococcus ruber* BKS 20-38, *Rhodococcus qingshengii* BKS 20-40 and *Rhodococcus triatomae* BKS 15-14 respectively which encodes single poypeptide of 577, 584, 580 amino acid length. Multiple sequence alignment of the deduced amino acid sequences of *R. ruber, R. qingshengii,*and *R. triatomae* cholesterol oxidases with *Rhodococcus* sp. CECT 3014, *Chromobacterium sp.* DS-1, *Brevibacterium sterolicum*, *Rhodococcus equi* ATCC 33707, was performed showing low similarities of 44, 26, 27, and 27%, respectively.

The genes of all the species were cloned in pET28a vector at *Nde*I and *hind*IIIsite which results in His-tag at the N-terminus of the recombinant protein. The pI of cholesterol oxidase of *R. ruber, R. qingshengii,*and *R. triatomae* deduced by protparam Expasy tool were found to be 7.2, 8.8, and 8.5 respectively. The estimated size of cholesterol oxidases on SDS-PAGE was found to be around 60 kDa.

All three cholesterol oxidase were characterized biochemically and compared with already reported in literature. In the literature Optimum pH of cholesterol oxidases from *Streptomyces* sp., *Streptomyces violascens* pH (7.5) and in *R. equi* to be 8.0 was reported. Cholesterol oxidases of this study exhibited optimum pH as 7.0 which are close to the biological pH (7.35-7.45). Optimum temperature of RrChOx, RqChOx and RtChOx was found to be 35°C similar to that of the results of cholesterol oxidases of *Brevibacterium sterolicum*, *Nocardia rhodochorus* now classified as *Rhodochorus rhodochorus.* Cholesterol oxidases of *Streptomyces fradie* optimum temperature of were found to be 70°C, whereas optimum temperature of

cholesterol oxidase of *Chromobacterium* sp. DS-1 was reported to be 65°C. The pH stability of all cholesterol oxidases examined in this study ranged from pH 5.0 – 11.0 and is similar to those of *Chromobacterium* sp. DS-1, *Pimelobacter simplex* the cholesterol oxidases examined in this study were found to be stable up to 55°C like those of *Bacillus* sp., As per the available literature, the cholesterol oxidase of *Streptomyces fradie* was stable till 70°C.

Kinetic studies showed that the cholesterol oxidase of RrChOx to be better among all the cholesterol oxidases in our experiments. RrChOx has higher affinity and catalytic efficiency than any other cholesterol oxidase used in our study. The value of k_{cat}/K_M gives the enzyme efficiency in the reaction. The higher the k_{cat}/K_M value higher is the enzyme efficiency in the reaction. The k_{cat}/K_M value was higher for the cholesterol oxidase of RrChOx 0.065, RqChOx 0.042, RtChOx 0.039.

We assembled and annotated all the three ChOx producing *Rhodococcus* genomes and used comparative genomics analysis to identify shared characteristics among the strains specifically related to secondary metabolism and xenobiotic degradation. In these four genome protein coding genes with function prediction assigned to *R. equi* 79.21%, *R. opacus* B4 58.86%, *R. ruber* 66.13%, *R. qingshengii* 59.70% of the genes with genome size ranging from 5.0 to 8.8 Mb majority of genes were related to cell metabolism.

Predicted proteins were also compared using the COGs protein database which is used to conclude orthologus groups and provide clues about functional characteristics of microorganism. In these four genomes Cogs distribution was similar except Carbohydrate transport and metabolism and energy production and conservation and Lipid transport and metabolism. For category of energy production and conservation percentage of gene is highest in *R. opacus* environmental isolate due to its vast metabolic degradation ability. In Lipid transport and metabolism percentage of genes is equal in both *R.opacus* and *R.ruber* which describes their ability to survive in harsh condition.

By using BLASTN analysis an online version of the Artemis Comparison Tool (WebACT) and Mauve software four genomes were compared *R. equi, R. opacus* B4, *R. ruber, R. qingshengii* and *R. triatomae.* Blast atlas, provides a quick overview of genomic regions of gene conservation across many genomes also supported the genome alignments results.

Syntenic dotplots of *R. equi* 103S (x-axis) and *R. opacus B4, R. ruber, R. qingshengii, R. triatomae* (y-axis) (Figure 6.6) shows that *R. opacus* is undergone many insertions, horizontal gene transfer events, deletions, and transposon activity when compared with *R.equi*. The genes that were conserved among the different *Rhodococcus* species have provided clues to the common characteristics of *Rhodococcus* genera, such as xenobiotic degradation and secondary metaboilsm (nutrient catabolism and transport, resistance to various environmental stresses, cell wall). The strain-specific genes differentiated each strain on the basis of its habitat, specific ecological adaptations, and ability to xenobiotic degradation. The strong adaptability of *Rhodococcus* genomes to their environment is associated to putative genes involved in catabolism and transport xenobiotic compounds and resistance to various environmental stresses (heavy metals, ROS, cold-, heat-, or osmotic-shock). These genes were very common in the genomes of *Rhodococcus*.

Comparative studies have yielded important insights into the catabolism and physiology of *Rhodococcus* genomes. So far reported *Rhodococcus* genomes such as *R. opacus* B4, *R. erythropolis* PR4, *R. erythropolis* SK121, and *R. equi* 103S, *R. jostii* all are predicted to encode cholesterol degradation pathway, suggesting that steroid degradation is a common, perhaps ubiquitous, characteristic of this genus this is also supported by biochemical characterization of RrChOx, RqChOx, RtChOx enzyme used in this study showing similar molecular weight and biochemical characteristics as earlier reports.

With recent availability of more *Rhodococcus* genomes and advanced tools for studying *Rhodococcus* physiology, our understanding of these organisms will improve, which in turn, accelerate the development of diverse biotechnological applications harboring the unique characteristics of this genus.