Chapter 5

Discussion

Nature is an exceptionally rich source of useful/beneficial microorganisms. Therefore, in the present study natural sources have been chosen for the isolation and screening of microorganisms capable of producing HMGR inhibitors. Of the different soil, water and biological fluids, eighty one microorganisms, irrespective of their type, were used in rapid screening for HMGR inhibitor production. Upon subjecting the isolates to rapid screening for production of Simvastatin (most common and potent semi-synthetic HMGR inhibitor) by thin layer chromatography and yeast inhibition bioassay technique (Kumar et al., 2000a). Only three isolates (i.e. about 4% of total isolates) have exhibited the corresponding Rf and inhibition zone on bioassay plate. Of these three isolates, two were bacterial isolates (BG 17 and BG 188) obtained from biological fluid and one was fungal isolate (FG 7) obtained from marine water. These exhibited inhibition zone of 16 mm and 14 mm (BG 17 and FG 7 respectively) and hence were considered as good HMGR inhibitor producers while BG 188 which showed a moderate zone was also included for further studies. After the preliminary bioassay guided screening, the extracts were subjected to thin layer chromatography. The compounds (SK-02, SK-03 and SK-04) produced by the strains (FG 7, BG 17 and BG 188) exhibited similar Rf values (0.71) as that of standard Simvastatin. These results are similar to the observations of Gunde-Cimerman *et al*., 1993 in the fungal systems. Further, in addition to evaluation of Simvastatin production by the isolates on TLC plates, quantitative estimation was also carried out using UV-VIS spectrophotometer. The spectral studies of standard Simvastatin revealed a maximum absorption peak λ_{max} at 238 nm. Therefore, 238 nm was also taken as the wavelength for extracts purified from all others cultures and optical densities were measured for

estimation of Simvastatin concentration. The results observed under these studies were in good agreement with characterization data for Simvastatin published in Merck Index (Windholz 1983). Therefore, on the basis of results observed in the preliminary screening based on yeast inhibition bioassay, thin layer chromatography, UV-Vis absorption spectra, it was found that the strains FG 7, BG 17 and BG 188 are potential HMGR producing candidates for further detailed studies. The three strains were purified and identified based on morphological as well as biochemical characters. Further all three were characterized in detail and identified based on 16S rRNA sequence identity. The gene sequences encoding the 16S rRNA are highly conserved across all bacterial species (Dhruva et. al., 2003). The 16S rRNA gene sequence has been widely used as a molecular clock to identify unknown bacteria to genus or species level (Sacchi et. al., 2002). The three strains were found to be *Aspergillus cervinus* (the fungi, FG 7) and the bacteria were found to be *Enterococcus faecalis* and *Bacillus anthracis* (BG 17 & BG 188 respectively).

A. terreus, is well described as the culture of choice for the production of natural Lovastatin and semi-synthetic Simvastatin (Alberts *et al.*, 1980; Gunde-Cimerman and Cimerman 1995; Samiee *et al.*, 2003). Apart from *Aspergillus terreus* few other species of *Aspergillus* have also been reported such as *A. flavus* (Shindia 1997; Samiee *et al.*,2003), *A. niger, A. repens, A. versicolor* (Gunde-Cimerman *et al.*,1993a), *A. oryzae* (Hajko *et al.*,1994; Shindia 1997), *A. fischeri, A. parasiticus, A. umbrosus* (Samiee *et al.*,2003). All these *Aspergillus* sp have been mainly isolated from terrestrial soil sources and *A. terreus* has been optimized for commercial production of statins. The present study evaluated *Aspergillus cervinus* (not reported before for any kind of statin

production) from a marine source for the enzyme mediated production of HMGR inhibitors. The demonstration of ability of this marine fungi to produce Simvastatin emphasizes the need for further investigation of fungal isolates from different/unexplored ecological niches for useful applications. Actinomycetes and filamentous fungi (most common producers of statins) have the drawback that their growth rate is much lower than that of bacteria and thus more time is required for obtaining enough cells for the fermentation reaction. Further, there is the problem of controlling the culturing of actinomycetes and filamentous fungi in a fermenter. As actinomycetes and filamentous fungi grow by elongating hyphae, the viscosity of the culture rises as they grow in a fermenter. This often causes shortage of oxygen and makes the culture heterogeneous, which lowers the efficiency of reaction with time. To solve this problem of oxygen shortage and keep the culture homogenous, the stirring rate of the fermenter must be raised; but hyphae are liable to be broken by stirring at a higher rate, which can again lower the production of statins due to damage to growing hyphae. Thus, culturing of actinomycetes and other filamentous fungi is bereft with many such problems. On the other hand, culturing of bacteria can be readily carried out because the viscosity of the culture hardly rises, and insufficiency of aeration and lack of homogeneity of the culture are not observed at all. Moreover the growth rate of bacteria is faster and allows for easier downstream processing. As of now heterologus expression of fungal genes in *E. coli* has been used for bacterial production of semi synthetic statins. For example, fungal acyltransferase coding LovD gene has been isolated from *Aspergillus terreus* and expressed in *E. coli* to be used in production of Simvastatin (Xie et al, 2007). Similarly Pravastatin is normally produced in a second stage by biotransformation (hydroxylation) of compactin by *Streptomyces carbophilus*.

Ykema, Streekstra, and Luiten 1999 developed a one-step biosynthesis process to produce pravastatin, by transforming the *P. citrinum* strain with a *S. carbophilus* hydroxylase gene that converts compactin to pravastatin. However, it is generally difficult to efficiently express genes of actinomycetes and filamentous fungi because their codon usage is widely different from those of bacteria such as *Escherichia coli*. Even otherwise the tools available for efficient expression of genes in actinomycetes, such as vectors and promoters are limited. Therefore, it is desirable to employ bacteria, in which various vectors, promoters, etc. can be used, in order to express genes at a high level and to carry out reactions more efficiently. Any genes from bacteria can be readily expressed in other bacteria at a high level. Finding a novel source of acyltransferase or related class of enzyme with a potential to synthesize HMGR inhibitor molecules is an important milestone in bio-catalysis of statin or their semi-synthetic analogs. This is the first attempt to work with novel bacterial strains isolated from natural source for Simvastatin production. This study first time reports the potential of two bacteria *Enterococcus faecalis* and *Bacillus anthracis*, isolated from human biological fluid for production of semi synthetic HMGR inhibitor, Simvastatin. So far, there are no reports known for a microorganism belonging to class of natural bacteria isolated from any source for bioconversion ability to produce Simvastatin, like those reported in the present study. The two bacteria were used for production of Simvastatin in optimum conditions and metabolite produced was characterized analytically and biologically. Temperature is an important parameter that governs the process of fermentation as well as growth of bacteria. It is widely accepted that the secondary metabolism of microorganisms represent an important pathway for survival and in turn depends on the incubation temperature (Lai *et al*., 2005). However, the

influence of temperature-shift varies from strain to strain. Since various temperature levels have been reported as optimum for bio-catalytic reactions, it seemed desirable to check the influence of incubation temperature upon the growth of microorganism and the product yield. The yield of Simvastatin increased significantly with the increase in fermentation temperature from 25^{o}C-37^{o}C and decreased above 37^{o}C for BG 17, while maximum yield was obtained at 30^{o}C for BG 188. The maximum Simvastatin yield obtained at 37^{o}C was 130µg/ml for BG 17 and 90µg/ml at 30^{o}C in case of BG 188. The two bacteria also showed the maximum growth at corresponding temperatures of maximum yield only. Similarly maximum Simvastatin yield was found to be 121 µg/ml from marine fungi, *A. cervinus*. Previous studies have also reported a similar yield of Simvastatin from wild strain of standard *A. terreus* strain i.e 120 µg/ml (Nasmetova et al; 2015) and 96 µg/ml (Xie et al; 2007). However, the yield was increased up to 250 µg/ml after strain enrichment.

It is noticed herein and also in most of the industrial fermentations that the control of pH of the medium at optimum level is essential for achieving maximum product formation. The response of the microorganism to pH variation differs in strains of same species. The data obtained in the present study on the effect of initial pH on product yield and growth of bacteria is shown in the results section. Simvastatin production increased with the increase in the initial pH of the substrates up to pH 7.5 and thereafter a decrease was observed. After completion of the conversion reaction at optimum conditions, the desired compound was directly isolated, separated or purified using solvent-solvent extraction as described in methodology section followed by characterization of the compound using analytical techniques along with biological potential to inhibit yeast and human HMGR. For the detection and determination of

compound obtained analytical characterisation was done using UV spectroscopy, Infra red spectroscopy, mass spectroscopy and high performance liquid chromatography. Biological activity of the compound was determined using yeast growth inhibition assay as well as effect on human HMGR gene expression in recombinant yeast. Further, the cytotoxicity and effect on triglyceride levels were estimated as HMGR inhibitors are reported to have anti-cancer properties and other pleiotropic clinical effects. The UV spectral studies on standard Simvastatin revealed a maximum absorption peak λ_{max} at 238 nm. The results thus obtained are in good agreement with the observations of Endo *et al*., (1976a) and Windholz (1983). The extracted compounds also showed the λ_{max} at 238 nm. Hence, these results indicated the presence of Simvastatin in culture filtrates. The calibration curve drawn from readings of standard Simvastatin dilutions were used to estimate the amount of Simvastatin produced in the culture medium. In other studies Simvastatin and its analogues were identified based on their retention time and UV absorption maxima (Vinci *et al*., 1991; Morovjan *et al*., 1997). Hence fermented sample was monitored at 238 nm. Identification of Simvastatin from the fermented extract was confirmed by HPLC using Hichrome C_{18} column with a mobile phase of acetonitrile (HPLC grade) 70% and acidified water (HPLC grade) 30% (70:30). The chromatogram reveals that the compound has retention time of 7.7 min at 238 nm. The results obtained herein are in good agreement with the findings of Vinci *et al*., (1991) and Morovjan *et al*., (1997), and confirmed the presence of Simvastatin in culture filtrates. The IR spectra of standard Simvastatin showed (Fig.4.17) sharp stretching vibrations at 1641.17, 1634.99 cm^{-1} respectively, indicating the presence of lactone (C=O) group and another weak stretching vibrations at 1220 cm^{-1} indicating presence of ether bridging (C-O-C).

A broad stretching vibration developed at 3524.33 and 3438 cm^{-1} in standard and isolated compounds respectively indicating the presence of phenolic –OH group. The indications of stretching vibrations obtained at 2999.66 cm^{-1} and 2925.56 cm^{-1} reveal the presence of methylene ($-CH_2$) groups. Thus, the observation made from the above analysis clearly confirms the identical functional groups as present in the standard Simvastatin and the extracts from culture medium. Further, the spectra thus obtained are similar with the findings of Endo (1979). Simvastatin has a number of pleiotropic effects. In the last few years, many studies have demonstrated that in addition to their lipid lowering effects, statins have anti-inflammatory and immunomodulatory properties. To test the biological efficacy of the extracted compound, it was tested against yeast growth. The three compounds SK-02, SK-03 and SK-04 inhibited the growth of yeast in YEPD culture broth by 62.8 %, 72% and 65% respectively and correspondingly clear zone of inhibition was also found on YEPD agar plates. The results obtained are in agreement with previous reports showing similar effect of statins on yeast growth. The compounds were also used to test their effect on human HMGR gene expressed in recombinant yeast strain. Using the RT-PCR technique for quantification of HMGR gene expression with and without treatment, the three extracts (SK-02, 03 & 04) were found to induce the HMGR expression by 1.16, 1.36 and 1.29 folds as compared to buffer treatment respectively. The results are in agreement as obtained by Beata et al, 2013 while studying the effect of various kinds of statins on HMGR expression in recombinant yeast.

The most known mechanism of statin production in fungi requires the Lovastatin biosynthetic operon. Lovastatin is derived from acetate via a polyketide pathway (Moore et al. 1985). Pioneering genetic research by Reeves, McAda, and workers at

MDS Panlabs Inc., identified a type I polyketide synthase (PKS) gene essential for Lovastatin biosynthesis by *A. terreus* which is now known as Lovastatin nonaketide synthase (LNKS) (Hendrickson et al. 1999). LNKS is a multi-domain enzyme that contains seven activities, and functions in a way similar to animal fatty acid synthases (FAS) and bacterial type I PKS, i.e., the ketosynthase (KS) which performs decarboxylative claisen condensation for chain elongation (Kennedy et al. 1999); the malonyl-CoA:ACP acyltransferase (MAT) selects and transfers the extender unit in the form of malonic esters, while the acyl carrier protein (ACP) serves as the tether (or bind) for the extender unit and the growing chain. In addition, tailoring enzymes such as ketoreductase (KR), dehydratase (DH), methyltransferase (MT) and enoylreductase modify the carbon backbone and introduce structural diversity. Kennedy et al, 1999 studied the complete Lovastatin gene cluster, from a genomic library. They reported the presence of 18 genes over a span of 64 kb. In this operon the gene lovD is particularly interesting because its product, an acyltransferase, catalyzes the last step that joins together the two polyketide components, Monacolin J and side chain to yield different statins. LovD has broad substrate specificity towards the acyl group. Most notably, LovD was able to catalyze the direct acylation of Monacolin J by acyl-CoA thioesters or membrane permeable thioesters acyl donors like N-acetylcysteamine (SNAC) thioesters. This work showed the feasibility of using LovD as a biocatalyst for a single-step synthesis of Simvastatin using α-dimethylbutyryl-S-NAC (or α-dimethylbutyryl-S-methylthioglycolate) as a substrate. Due to its broad substrate specificity, expression of LovD leads to production of various semi-synthetic statins which are not usually produced till different side chains are provided in the culture media. LovD gene is encoded by 1329 bp, and has been identified and isolated from

several statin producing fungal strains. The expression of LovD was investigated in house isolated three strains, which showed the production of Simvastatin in culture medium, via PCR amplification of total bacterial DNA with specific primers designed to amplify overlapping portions of Lovastatin biosynthetic operon of *A. terreus*. It was determined, that in the genome of two bacteria there were no genes that could be amplified with the chosen primers. Only for the fungal strain, identified as *A. cervinus* (FG 7) positive PCR products of 998 bp and 339 bp for lovD primer pairs were obtained. The PCR products were sequenced and the sequence showed 97% identity to that of LovD gene of *A. terreus* using BLAST which explains the presence of acyltransferase enzyme in this marine fungus capable of producing Simvastatin. None of the natural bacterial isolates appeared to possess genes similar to LovD gene described for fungi. The absence of PCR products (when primers used were designed using fungal LovD gene template in the investigated bacterial strains does not mean that these strains cannot produce Simvastatin, Further work is required for identifying the genes in the investigated strains which seem to be quite different from Lovastatin biosynthetic operon lovD of the fungal genome. The diversity of polyketide synthase genes is much greater and more complex and hence it is quite possible that many of these isolates do contain acyltransferase genes sequences but they are sufficiently different from the selected primer sequences, and hence were not detectable by the approach used. In this respect, it can be concluded that the arrangement of the acyltransferase gene in different bacterial and fungal genera varies. Therefore, further in depth study is needed to confirm the presence of homologs of LovD gene/s for Simvastatin production in bacteria. In-silico methods were used to test this hypothesis in the present study. Determining structural similarities between proteins is an

important aspect since it can help identify evolutionary and biological function similarities. One of the foundations of molecular biology is that a protein's sequence determines the structures, which in turn determines how the protein functions. These sequence-structure-function dependencies allow us to better deduce evolutionary relationship between proteins and between organisms and to better understand the function of thousands of genes and proteins. Since the structure of a protein determines what function it can perform, knowledge of structure is useful in predicting protein function. As many experimentally determined protein sequences are available and because a protein sequence will determine its structure, we can use this information to predict the structure and function of related proteins. Protein sequence-structure-function relationships have been investigated and quantified in various ways and many studies have observed and established sequence-structure relationship (Chothia and lesk 1986, Hubbard and Blundell 1987, Flores et al. 1983, Wilson et al. 2000, Yang and Honig 2000). The relationship between structural and functional similarity have also been studied (Thornton et al.1999, Wilson et al. 2000, Whisstock and Lesk 2003, Shakhnovich and Harvey 2004). A correlation has been observed between functional similarity and RMSD between pairs of proteins.

The functional convergence hypothesis would posit that sequence and structural similarities between proteins are observed because the shared features are strictly required for these proteins to perform their identical or similar functions. In several cases of low sequence similarity proteins retain the fold as well as retain the broad biochemical features and/or functional properties, suggesting an evolutionary connection (Murzin et al 1995; Russell and Sternberg 1996, 1997; Sowdamini et al 1998; Bray et al 2000). Level of similarity, between two divergently-evolved proteins,

increases as the proteins are viewed from "Sequences of bases in the genes" to "Amino acid sequences of the proteins" to "3-D structures of proteins". Modern similarity searching programs, such as BLAST (Altschul et al ., 1990, 1994, 1997), FASTA (Pearson & Lipman, 1988), and search3 (Pearson, 1996) do not use percent identity, or even raw similarity scores, to characterize protein sequence similarity; they use bit scores, probabilities, or expectation values that reflect the statistical significance of the alignment score. However, homologous sequences do not always share significant sequence similarity; there are thousands of homologous protein alignments that are not significant, but are clearly homologous based on statistically significant structural similarity or strong sequence similarity to an intermediate sequence. Thus, when a similarity search finds a statistically significant match, we can confidently infer that the two sequences are homologous; but if no statistically significant match is found in a database, we cannot be certain that no homologs are present. Although a common rule of thumb is that two sequences are homologous if they are more than 30% identical over their entire lengths (much higher identities are seen by chance in short alignments), the 30% criterion misses many easily detected homologs. While 30% identical alignments over more than 100 residues are almost always statistically significant. A 30% identity threshold for homology underestimates the number of homologs detected by sequence similarity between humans and yeast by 33% (this is a minimum estimate; even more homologs can be detected by more sensitive comparison methods). The bacterial BG 17 amino acid sequence is found to be 30% identical to the protein of LovD protein of *Aspergillus terreus* with significant E-value. This 202 amino acid bacterial protein was found to be belonging to transpeptidase family coding for beta-lactamase protein. To understand the structural

relationship between fungal LovD and bacterial protein PDB Id: 3CJM, structural alignment was performed and the RMSD between the aligned structures was calculated using CHIMERA online tool and was found to be 2A; the RMSD cutoff for similar structure is chosen to be up to 3A (Maiorov and Crippen, 1994). This shows that two proteins are sufficiently similar to have similar function. Further the bacteria BG 17 was found to be resistant to beta-lactam class of antibiotics including penicillin, ampilcillin and amoxycillin which confirms the strong expression of beta-lactamase enzyme in this bacterium so as to impart the high bio-catalytic activity to produce semisynthetic HMGR inhibitor.