SUMMARY

Statins are considered as the most widely used therapeutic agents for their role in lowering serum cholesterol level by inhibiting HMG-CoA reductase, a key enzyme of cholesterol biosynthetic pathway. Recent report suggests pleiotropic beneficial effects of statins in preventing or curing a disease like Alzheimer's, cancer, etc. It is no wonder that the worth of statins will be tens of billions of dollars in near future if they prove to be effective as anticipated.

Biotechnologically lovastatin is produced by Aspergillus terreus strains. A. terreus wild type strains are known to produce lovastatin in range of 1-240 mg/l and therefore isolation, screening and developing commercially viable lovastatin producing strains was taken up in this study. Studies have revealed that lovastatin producing fungi isolated from different geographical regions of the world differ in their statin producing capabilities. Therefore, isolation and screening of potential statin producer fungal strains including those from soil and certain plants as sources of lovastatin was envisaged. The potential statin producing strain selected after extensive screening can be subjected to optimization of process parameters that influence lovastatin production. In addition strain improvement approaches for obtaining hyper-producing strains based on rational selection procedures can be included. In recent years system biology approaches (proteomic/genomics) have also come to fore in understanding the complex pathways and evolving strategies for direct yield improvement of lovastatin. In light of these points the following study was planned with the following objectives.

- Isolation and screening of lovastatin producing soil and fungal isolates.
- Optimization of culture conditions for production of lovastatin by the selected strain.
- Improvement of lovastatin producing strain through cyclic mutagenesis and selection.
- Breeding of improved lovastatin producing strains by protoplast fusion and recombination.
Isolation and screening of fungal isolates for statin production

In this study, 219 fungal strains were isolated from different soil samples and plants parts. These soil isolates, 134 from soil and 85 endophytic fungi represented samples collected from different regions: Amritsar, Gurdaspur (Punjab, India) and Nanded (Maharashtra, India) in addition to endophytes from plant (Bambusa arundinacea, Terminalia chibula, Ocimum sanctum, and Aloe vera) parts collected from Guru Nanak Dev University campus, Amritsar.

The strains were grown on lactose based statin production medium and the culture extracts prepared in ethyl acetate were screened for statin production employing bioassay against Candia albicans and Rhodotorula rubra as test organisms. Out of total 134 soil isolates, 57.463 % were biologically active against both C. albicans and R. rubra, whereas 16.42 % were active against either C. albicans/R. rubra. Amongst soil isolates, maximal zone of inhibition against C. albicans was observed in an unidentified isolate GD7 (3.7 cm) whereas, A. flavus (GD11) produced largest zone of inhibition against R. rubra.

Out of 85 endophytic cultures, 24.7 % were bioactive against both the test organisms and 12.94 % showed zone of inhibition against either of the test organism. Isolate TL-b from O. sanctum leaf, showed largest zone of inhibition against C. albicans (3.7 cm) and R. rubra (2.0 cm). The bioassay positive culture extracts were resolved on TLC but no discernible correlation between bioassay and lovastatin production was observed. The quantitative and qualitative analysis of lovastatin was performed using HPLC fitted with PDA detector. The HPLC profile of 219 extracts showed that of 134 soil isolates, 38 (28.4 %) fungi were lovastatin (0.14-190 mg/l) producers. Out of 85 plant isolates, 9 endophytes (10.6%) produced lovastatin though at low levels ranging between 0.36-2.40 mg/l. Maximal lovastatin was produced by A. terreus (GD13 and GD12) strains isolated from the Maharashtra soil samples producing 190 mg/l and 153.94 mg/l of lovastatin, respectively. Melanocarpus sp. (15.98 mg/l), Stemphylium (0.42 mg/l) and Stachybotrys (2.8 mg/l) strains identified for lovastatin production are being reported for the first time.
Some of the isolates producing appreciable levels of lovastatin, in addition to morphological, microscopic features were also identified at molecular level using amplified rDNA (ITS I-5.8 S-ITS II) sequences for BLAST. The phylogenetic tree was constructed on the basis of aligned sequences using NJ plot. The analysis of dendrogram indicated that selected most potent lovastatin producing strain A. terreus GD\textsubscript{13} is phylogenetically distinct when compared to other A. terreus strains. The isolate 49 was identified as Aspergillus sp., G as Aspergillus fumigatus.

Anti-proliferative activity of the culture extracts

The culture extracts from lovastatin producing isolates, A.terreus (GD\textsubscript{13}) strain, Aspergillus sp. (49), an A.flavus (GD\textsubscript{11}) and A.nidulans (GD\textsubscript{6}) were screened for anti-proliferative activity against different cell lines derived from human HeLa (Hep-2), neuroblastoma (IMR-32), adenocarcinomic human alveolar basal epithelial cells (A-549) and human colorectal adenocarcinoma cells (HCT-15). The extract from A. terreus (GD\textsubscript{13}) that produced maximal lovastatin titres also showed high % cytotoxicity (81-83\%) against all cell lines tested in the experiment.

Optimization of process parameters for lovastatin production by A. terreus GD\textsubscript{13}

Aspergillus terreus GD\textsubscript{13} strain, chosen after extensive screening, was optimized for lovastatin production using one factor at a time (OFAT) approach followed by statistical Box-Behnken design of experiments. Of the different carbon sources used, lactose (@ 11 \%) supported maximal lovastatin (191.05 mg/l) production. Lovastatin yield was appreciably higher when either beef extract (310 mg/l) or soybean meal (290 mg/l) was used as a nitrogen source in the production medium. Soybean meal, being less expensive when compared to beef extract and of plant based origin, was preferred over beef extract in further experiments. Maximal level of lovastatin (470.70 mg/l) was obtained using 0.945 \% soybean meal, when pH of the medium was in the range of 5.0 to 6.0. For achieving these levels 96h old inoculum grown on PDA slants @ 6 \% (v/v) spore suspension (6.6x10\textsuperscript{7} spores/ml) was used. Medium without MgSO\textsubscript{4} resulted in drastic decrease in lovastatin (45.1 mg/l) production when compared to control (470.98 mg/l). The removal of NaCl, ZnSO\textsubscript{4} and TES also yielded lower lovastatin corresponding to 180.02, 260.68 and 280.7 mg/l, respectively.
Further optimization of lovastatin production was carried out using statistical approach employing Box Behnken design of experiments. Lactose level, soybean meal concentration, inoculum level (spore count/ml) and inoculum age, identified as significant process parameters for lovastatin production during OFAT were chosen as independent variables to study their interactive effect on lovastatin production using response surface methodology (RSM). The experimental model highlighted the positive effect of inoculum level and soybean meal for achieving maximal level of lovastatin (1280 mg/l). Further increase in the level of soybean meal in cultivation medium resulted in enhanced production to 1648mg/l of lovastatin. The optimum culture conditions improved the lovastatin titre by 8.7-fold when compared to the titres obtained under unoptimized conditions. The optimized process parameters were validated at fermentor level where 1500±98 mg/l of lovastatin could be achieved. The fed batch culture conducted at shake flask levels, produced maximal levels of the lovastatin (934.407 mg/l), when culture was fed once at 48 h with lactose (@ 6 %, w/v), the initial lactose to initiate batch experiment was 5 % (w/v).

Production of lovastatin using solidified culture medium

A cheap medium comprising of vegetable waste/lignocellulosic residues as carbon source devoid of any other component of culture medium was tested for lovastatin production using solid state fermentation. Maximal statin production (6 g/kg substrate) was observed when pea pod was used as a carbon source followed by sorghum straw, wheat bran, rice straw and orange peel. Pea pod, however, did not show reproducibility, whereas, sorghum straw supported constantly high levels of lovastatin (5.2 g/kg). Surprisingly, when basal medium components were added instead of water the lovastatin production declined.

Cyclic mutagenesis for developing lovastatin hyper-producing strains

A. terreus GD13 strain that produced highest levels of lovastatin was also taken up for strain improvement employing cyclic mutagenesis and rational selection approach. The screening/selection approach relied on identifying mutants that were resistant to end product (lovastatin) or anti-metabolite/analogue resistant compounds that are associated with modulation in the key enzymes of the metabolic pathways and changes in
morphological characters as phenotypic markers. Two key enzymes polyketide synthesis pathway i.e., a nonaketide synthase (LNKS) and a diketide synthase (LDKS) involved in the synthesis of lovastatin were targeted to increase the yield of lovastatin using mutagenesis and the selection of mutants showing resistance to the presence of lovastatin and polyketide synthase inhibitors (iodoacetamide and N-ethylmaleimide) in different stages of mutation programme.

Out of the seventeen mutants obtained in the first stage and selected as resistant to lovastatin (2000 mg/l), both positive (1.01-1.96 folds increase) and negative mutants (producing lower levels of lovastatin) were observed. One of the mutants, named as ‘2-P’, that was highly stable, produced 600 mg/l of lovastatin with beef extract as a nitrogen source. Further, in the second stage of mutagenesis, thirty-two mutants resistant to iodoacetamide that produced 1.12-2.85 folds higherlovastatin yields in comparison to the parent strain (2-P) were identified. The mutant IA_{169}, showed resistance to iodoacetamide (110 µg/ml) produced 736 mg/l of lovastatin. The third stage of mutagenesis gave rise to 36 mutants that were resistant to N-ethylmaleimide. Nineteen mutants manifested 1.003- 1.93 fold increment in the titres of lovastatin in comparison to the parent IA_{169}. The maximum lovastatin of 1424 mg/l was exhibited by the mutant EM_{19} that showed 7.5-fold higher lovastatin in comparison to the wild type isolate (190 mg/l).

**Breeding of lovastatin hyper producing strains using Protoplast fusion**

*A. terreus*, deutromycete fungus lacks sexual cycle and therefore breeding of lovastatin hyper producing strains through protoplast fusion was employed for inducing parasexual cycle. For this genetically well characterized strains were selected as parents with distinct and contrasting phenotypic markers. The selected mutants obtained at each stage of mutagenesis were characterized using potato dextrose agar medium containing different concentrations of acriflavine, caffeine and sodium selenate. *A. terreus* GD_{13} was characterized as (Caf^{res}/ Acri^{sen}) and EM_{34} as (Acri^{res}/ Caf^{sen}), one of the hyperproducing mutant were chosen for protoplast fusion on the basis of resistance/sensitivity to acriflavine (900 µg/ml) and caffeine (1250 µg/ml). The
intraspecific hybridization between these strains resulted in 19 heterokaryotic fusants of different morphologies and different lovastatin titres. Maximal lovastatin production was observed in fusant 2b (1819.924 mg/l) and 9b (1141.458 mg/l). The fusants 2b, 5b, 9b and 13 were further subjected to diploidization on PDA medium containing 0.2 % (w/v) camphor. One of the diploids 2bVIII derived from heterokaryon 2b showed high levels of lovastatin (2235 mg/l) corresponding to 2.47-fold increase in production as compared to one of the parents (EM$_{34}$) strain. Few other diploids also showed higher lovastatin production but they were not stable under SmF. These strains were also studied for lovastatin production underSSF. The haploid 2b VIII (o) produced highest lovastatin titre (8 g/kg substrate) when rice straw was used as a carbon source. The wild strain GD$_{13}$, EM$_{34}$ (mutant parent) and 2b (heterokaryon) yielded 5.3, 7.2 and 7.6 g/kg lovastatin, respectively, when sorghum straw was employed as carbon source. However, the optimal carbon source for the diploids 2b III and 2b VIII was wheat bran that supported production of 7.3 and 7.5 (g/ kg substrate) lovastatin, respectively. When sorghum straw was used as a carbon source and the water was replaced with basal medium during culturing, the lovastatin production decreased in case of GD$_{13}$, EM$_{34}$ and the heterokaryon 2b. However, it showed an increase of 1.6-folds in diploid 2b III (9.08 g/kg substrate), 3-folds in diploid 2b VIII (6.6 g/kg substrate), 1.83-folds in haploid 2b VIII (o) (9.1 g/kg substrate), respectively.

Proteome/transcriptome analysis of lovastatin producing strains

Peptide mass fingerprinting of the proteins resolved by SDS PAGE showed that hyper producing diploid 2b VIII expressed higher levels of FAD-synthase, GGPP synthase, short chain dehydrogenase and thioredoxin reductase when compared to wild type strain GD$_{13}$ and mutant EM$_{34}$ as well as heterokaryon 2b from which it was derived. The RNA profiling showed that the maximal expression of $lov$ $b$ $ks$ and $lov$ $b$ $mt$ genes was observed in diploid 2b VIII, expression of $lov$ $f$ $ks$ was the highest in heterokaryon 2b and $lov$ $f$ $mt$ was highest in the mutant parent EM$_{34}$. The results suggested up-regulation of nonaketide synthase gene in the diploid which possibly led to the higher lovastatin production.
Conclusions

*Stachybotrys*, *Stemphylium* and *Melanocarpus albomyces* were reported for the first time as lovastatin producers. An *A. terreus* strain GD13 was isolated from the soil samples of Deccan Plateau region that was the efficient producer of lovastatin. It showed 94% variability from known *A. terreus* strains when analyzed through nBLAST. The culture extracts of this potent lovastatin producing strain showed potential cytotoxic activity against all tested cell lines, namely, Hep-2, IMR-32, A-549 and HCT-15. Optimization of the production medium resulted in 7.0 folds increment in lovastatin production when compared to the lovastatin titres obtained under unoptimized condition. Strain improvement using cyclic mutagenesis resulted in a hyper-producing mutant (EM19) exhibiting 7.5-fold (1424 mg/l) higher levels of lovastatin when compared to wild type parent strain. A heterokaryon resulting from protoplast fusion showed 9.47-folds higher lovastatin as compared to the parental strain GD13 (190 mg/l). Non-sporulating heterokaryons produced very low levels of lovastatin. Morphologically similar recombinants to the parent strain were better producers of lovastatin. A diploid showing 11.26-folds higher lovastatin production was obtained by diploidization of the heterokaryon when compared to parental GD13 strain (190 mg/l). Further haploids derived from the diploid 2b VIII showed 1.06-1.93 folds higher lovastatin production than the respective diploid strain.

Future Prospects

- The results of the present study thus support the isolation of potential producers from diverse geographical regions
- The robustness of optimization and the strain improvement strategy adopted in this work is also evident from the many fold improvement in lovastatin production which can be helpful to develop commercial level strains