CHAPTER VI

SUMMARY AND CONCLUSION
Lovastatin is one of the most important statins of biological origin and it is in
great demand because of its various clinical applications apart from lipid lowering ability.
Hence, in India, demand for lovastatin and its analogues currently exceed its production
capacity. The most important route of synthesis of lovastatin is by the way of
fermentation of microorganism rather than chemical synthesis. However from the
extensive literature it is evident that there are only few reports on the lovastatin
production through solid state fermentation. This survey of literature has stimulated the
exploration of lovastatin production through SSF, which is cost effective and promising
technology for the present day. The substrate i.e., carob pod (Ceratonia siliqua) used
under the present study is the first attempt to use as a novel substrate for the production
of lovastatin under SSF.

With the demand for cholesterol lowering drugs increasing significantly, efforts
are on the anvil to search for newer and cheaper substrates that are rich in fermentable
sugars. Hence the present study has been focused on the cost effective approaches for
lovastatin production, with the use of novel substrates like carob pods (Ceratonia
siliqua), though rich in fermentable sugars and yet generally not used except in cattle
feeding. The carob pod used under the present study is the first attempt to use as novel
substrate for the production of lovastatin under SSF. The most common microorganism
that has been employed in the fermentation of carob substrate is Aspergillus terreus,
which was described as the culture of choice for the production of lovastatin. Thus the
main aim of the present study is to produce lovastatin by solid state fermentation of carob
pods employing A. terreus strain as fermenting agent and hence making the process more
profitable.
Apart from this, lovastatin and its analogues hold a significant investment opportunity as the market shows immense demand for statins both in India and international market. This scenario is likely to intensify as more statins go off patent. Hence, this work would also benefit the prevailing demand for lovastatin in the market.

The summary of the results obtained and the conclusions arrived at on the basis of these results presented in the present thesis are briefly outlined as below:

1. The Chapter I include a brief introduction to the research undertaken, wherein the necessity of undertaking the work is justified and the aims and objectives of the study are specified.

2. In Chapter II, an exhaustive review of the relevant aspects of statins, chemistry and historical aspects, the characteristics of the lovastatin and substrates, uses, etc., is presented and also an attempt has been made to evaluate the different substrates used in fermentation for the production of lovastatin.

3. The Chapter III deals with the materials used and methodology adopted in the present study. The aspects covered are mainly – Isolation, screening and rapid confirmation of lovastatin producing *A. terreus* strains, the chemical analysis of agro-based waste carob pod, solid state fermentation procedure and methodology adopted to optimize the fermentation parameters, as well as process economization through nutrient supplementation and mutation of *A. terreus* KLVB 28 for maximum production of lovastatin, characterization of lovastatin through UV, IR, NMR spectral analysis and finally by HPLC.
4. In the Chapter IV, the results obtained during the study are presented. The results are indicated in brief as below:

i. The isolates of *A. terreus* were initially subjected for lovastatin production through agar plug culture-bioassay technique by evaluating their zone of inhibition. Out of 45 isolates, 6 isolates exhibited inhibition zone more than 12 mm. Of these 6 isolates, one isolate *A. terreus* KLVB 28 exhibited inhibition zone of 16 mm and hence the same was considered as the promising strain for lovastatin production.

ii. Screening of various agricultural substrates, like wheat bran, maize bran, rice bran, sunflower seed cake, ground nut seed cake as well as carob (*Ceratonia siliqua*) pod, were carried out. The results of suitability of substrate for solid state fermentation indicated that carob pod has been selected as a suitable substrate for the selective fermentation as it has very high concentrations of fermentable sugars.

iii. The success and direction of fermentation depends on obtaining a proper balance between the substrate, the process itself and the fermenting organisms. Hence, optimization of solid state fermentation parameters like initial moisture content, pH of the substrate, the ambient temperature and the inoculum size, particle size of the substrate, bed depth and role of fungal biomass in lovastatin production was carried out. Once a parameter was optimized, the optimum level of the parameter was continued in the next set of experiment.

   a. The studies reveal that the optimum fermentation period required to produce maximum lovastatin form carob substrate was 120 hr by employing *A. terreus* KLVB 28.

   b. The optimum initial moisture content for maximum production of lovastatin by the organism was observed at 65%, pH of 4.5, temperature of 35°C, inoculum size of $1 \times 10^8$ spores/ml, particle size of 2mm and bed depth of 2cm using deseeded carob pods as substrate.
iv. In the studies dealing with the process economization, attempts have been made to improve the production of lovastatin during solid state fermentation of the carob pod substrate through supplementation of various nutrients and also by mutation of the fermenting organism.

In studies involving nutrient supplementations, the substrate was amended with varying concentrations of different carbon sources, organic & inorganic nitrogen, phosphates, lower alcohols, metal ions individually and also in step-wise addition of each of these nutrients in their optimum levels as determined in previous experiment.

a. Amongst all the carbon sources tested for lovastatin production sucrose proved to be beneficial. The optimum concentration of sucrose needed to be supplemented to the carob pod substrate to effect maximum lovastatin production by *A. terreus* KLVB28 was observed to be 2%. At this level of sucrose supplementation, *A. terreus* KLVB28 produced 462.62 µg lovastatin/g substrate at 120 hr of fermentation.

b. Amongst all the organic nitrogen sources tested for lovastatin production yeast extract proved to be beneficial. The optimum concentration of yeast extract needed to be supplemented to the carob pod substrate to effect maximum lovastatin production by *A. terreus* KLVB28 was observed to be 1%. At this level of yeast extract supplementation, *A. terreus* KLVB28 produced 488.73 µg lovastatin/g substrate at 120 hr of fermentation.

c. The optimum concentration of inorganic nitrogen (ammonium chloride) needed to be supplemented to the carob pod substrate to cause maximum lovastatin production by *A. terreus* KLVB28 was observed to be 0.5%. At this level of ammonium chloride supplementation, *A. terreus* KLVB28 produced 560.11 µg lovastatin/g substrate at 120 hr of fermentation.

d. Amongst all the phosphate sources tested for lovastatin production K$_2$HPO$_4$ proved to be beneficial. The optimum concentration of K$_2$HPO$_4$ needed to be supplemented to the carob pod substrate to effect maximum lovastatin production by *A. terreus* KLVB28 was observed to be 0.5%. At this level of K$_2$HPO$_4$ supplementation,
*A. terreus* KLVB28 produced 512.32 µg lovastatin/g substrate at 120 hr of fermentation.

e. Amongst all the alcohol sources tested for lovastatin production glycerol proved to be beneficial. The optimum concentration of glycerol needed to be supplemented to the carob pod substrate to effect maximum lovastatin production by *A. terreus* KLVB28 was observed to be 2%. At this level of glycerol supplementation, *A. terreus* KLVB28 produced 440.13 µg lovastatin/g substrate at 120 hr of fermentation.

f. Amongst all the metal ions tested for lovastatin production Mg$^{2+}$ proved to be beneficial. The optimum concentration of Mg$^{2+}$ needed to be supplemented to the carob pod substrate to effect maximum lovastatin production by *A. terreus* KLVB28 was observed to be 0.2g/Kg. At this level of Mg$^{2+}$ supplementation, *A. terreus* KLVB28 produced 361.41 µg lovastatin/g substrate at 120 hr of fermentation.

g. In the studies involving step-wise addition of each nutrient (at optimum levels as observed previously) it was observed that a step-wise increase in the lovastatin production from the carob pod substrate by *A. terreus* KLVB28. Upon supplementation of optimum levels of the nutrients maximum production of lovastatin (842.42 µg lovastatin/g substrate) was effected by *A. terreus* KLVB28. The production of lovastatin by the organism increased as one nutrient after another nutrient was added in the order of steps VI > V > IV > III > II > I.

h. Induction of mutation was attempted by EMS treatment at concentration of 5mg/ml for 5 min, exhibited inhibition zone of 2.4 cm against *N. crassa* FGSC 4200. Based on the performance of the strain obtained on bio-assay plate, the strain *A. terreus* KLVB 28 mu21 was selected for further studies.

i. The results obtained after solid state fermentation revealed that the mutant strains yielded 504µg/g DW when compared to the parent strain *A. terreus* KLVB 28, which has yielded 289.63 µg/g DW on deseeded carob substrate for 120 hr fermentation.

j. The IR, NMR spectra and HPLC analysis unequivocally prove the identity of the isolated sample from the fermentation medium as lovastatin.
5. In Chapter V, a detailed discussion of the findings of the present work is made with reference to the works done by the earlier workers in this field. The present study reveals that the substrate, carob pod, is a suitable and alternate substrate as the process becomes economical; due to the inherent nature of the carob tree itself (its growth in barren lands and yet its pods being rich in fermentable sugars). The mutants *A. terreus* KLV B 28 and *A. terreus* KLV B 28mu21 are efficient in producing lovastatin from the carob pod substrate in good quantities, though the former is superior to the latter.

6. The bibliography referred in the present thesis is included in the References section at the end of the thesis.