CHAPTER-V

DISCUSSION
5.1. ISOLATION AND SCREENING OF *A. TERREUS* STRAINS FOR LOVASTATIN PRODUCTION

Soil is the exceptionally rich sources for potential drug producing organisms especially for fungi. Therefore, in the present study soil has been chosen as a source and the soil samples were collected from various places of Gulbarga, Karnataka for the isolation of *A. terreus*, which is described as the culture of choice for the production of lovastatin (Alberts et al., 1980). Of the different soils tested for the presence of *A. terreus*, coconut soil has revealed the highest number of isolates. Thus from thirty five soil samples, irrespective of its type, totally forty five strains of *A. terreus* were isolated (Table 4.1).

Upon subjecting the *A. terreus* isolates to rapid screening for lovastatin production by the *Neurospora crassa* bioassay technique as described by Kumar et al., (2000a) only five isolates (i.e. only 11.1% of total isolates) have exhibited the inhibition zone on bioassay plate. Of these, three isolates exhibited inhibition zone of 12mm and these are considered as poor lovastatin producers (KLVB3, KLVB9 and KLVB40) and two isolates showed moderate (KLVB12 and KLVB29) while the remaining one isolate has exhibited inhibition zone of 16 mm and hence it was considered as good (KLVB28) lovastatin producer (Table 4.2).

Therefore, on the basis of results observed on the bioassay plates, it was considered that the strain *A. terreus* KLVB28 as a potential lovastatin producing candidate for further studies (Lingappa et al., 2004).
5.2. RAPID CONFIRMATION OF LOVASTATIN PRODUCING A.TERREUS BY THIN LAYER CHROMATOGRAPHY (TLC) AND ULTRA VOILET (UV) ANALYSIS

To ensure whether the zones of inhibition exhibited by the different isolates in bioassay studies were due to lovastatin activity, the agar plug extract were subjected to TLC for rapid confirmation. A TLC separation was developed for primary screening which effectively separated the lovastatin from other major co-metabolites (Fig. 3.6). In these studies, the compound produced by the strains (KLVB3, KLVB9, KLVB12, KLVB28, KLVB29 and KLVB40) exhibited similar Rf values (0.57) as that of standard lovastatin solution (Table.4.3). The results thus obtained were in close proximity with the observations of Gunde-Cimerman et al., (1993a).

Further, in addition to confirmation of lovastatin production by the isolates on TLC plates, quantitative estimation of lovastatin was also carried out for different isolates using UV-Vis spectrophotometer. The spectral studies of standard lovastatin and the culture extract from the strain A.terreus KLVB28 revealed the maximum absorption peak $\lambda_{\text{max}}$ at 238 nm (Fig 4.16). Therefore, 238 nm was taken as constant for all culture samples and measured the optical densities for lovastatin concentrations for further studies. The results observed under these studies were in good agreement with the data published in Merck Index (Windholz 1983).

5.3. SCREENING OF AGRO WASTE SUBSTRATES FOR LOVASTATIN PRODUCTION

Taking into account of economic profile of lovastatin production, attempts were made to use inexpensive agricultural wastes as substrates for the production of lovastatin under solid state fermentation employing A.terreus KLVB28 strain. In the present study
screening of various agricultural substrates like wheat bran, rice bran, maize bran, ground
nut seed cake, sunflower seed cake and deseeded carob pods were carried out for the
production of lovastatin. Amongst the 6 substrates employed for the production of
lovastatin, the carob pod was found to be most potential substrate for the production of
lovastatin. Since carob pod yielded maximum amount of lovastatin under the present
study it has been selected for further detailed study and **this is the first attempt to work
with this novel substrate for lovastatin production under SSF.**

### 5.4. COMPOSITION OF SUBSTRATE

It is always essential to analyse the chemical components of substrates before a
specific substrate is chosen for further fermentation studies, because such analyses reveal
the suitability of the substrate for required product synthesis. The biochemical
composition of the carob pod satisfies the conditions of fermentation process (Table 4.4).
It has good proportion of fermentable sugars (54%) and also has sufficient nutrients for
growth of the fermenting organisms as revealed by its biochemical composition. Further
it does not have any component toxic to the organisms as revealed by the seasonal growth
of the organisms (Fig. 3.13).

All solid substrates have a common feature i.e. their basic macromolecular
structure. In general, substrates for SSF are composite and heterogeneous products from
agriculture or by-products of agro-industry. The structural macromolecule may simply
provide an inert matrix within which the carbon and energy source are adsorbed
(Raimbault 1998). But generally, the macromolecular matrix represents the substrate and
it also provides the carbon and energy source to the fermenting organism in any solid
state fermentation process. The hypal mode of growth gives a major advantage to
filamentous fungi over unicellular microorganisms in the colonization of solid substrates and for the utilization of available nutrients. Further, the above kind of growth of filamentous fungi on solid substrate provides the higher penetrating power to the fermenting organism thereby increases its accessibility to all available nutrients within particles (Raimbault 1998; Pandey et al., 2000).

As such the carob pod satisfies many parameters of suitability. Hence, carob pod has been preferred and selected as the suitable substrate for production of lovastatin through solid state fermentation. The carob pod employed as substrate under the present study has served as best anchorage medium (Fig. 3.12 & Fig. 3.13) for the growth of mold A terreus KLVB28 strain. This in turn led to the proper utilization of water soluble sugars by the mold and produces good amount of lovastatin (Lingappa and Vivek Babu 2004).

5.5. OPTIMISATION OF FERMENTATION PARAMETERS

Bacteria, yeast and fungi can grow well on solid substrates and find application solids state fermentation processes. However, filamentous fungi are best adapted for solid state fermentation and dominate in research works, which was attributed due to their physiological, enzymological and biochemical properties (Raimbault 1998). The hyphal mode of fungal growth and their tolerance to low water activity and high osmotic pressure conditions make fungi efficient for bioconversion of solid substrates.

Generally, the outcome of any fermentation depends on the substrate, fermenting organisms and the process itself. As such a proper balance needs to be achieved amongst the three factors, in order to achieve maximum product recovery. The important parameters that govern the solid state fermentation process and product recovery are the
moisture content, initial pH, temperature, particle size and bed depth of the fermenting substrate, and the inoculum size of the fermenting organism; hence in order to obtain maximum lovastatin production, these parameters need to be optimized.

5.5.1. EFFECT OF MOISTURE CONTENT

The moisture content of the substrate is one important factor that affects the performance of solid state fermentation and it is governed by the nature of the substrate, the type of end product and requirement of the microorganism (Lonsane et al., 1985). Generally, the moisture level of substrates in solid state fermentation processes vary between 30% to 85% and have a marked effect on growth kinetics of fermenting organism (Lonsane et al., 1985; Oriol et al., 1988; Raimbault 1998). However, the optimum moisture content for the growth and substrate utilization is between 40% and 70% but it depends upon the organism and the substrate used for cultivation. The optimum moisture level for cultivation of Aspergillus niger on starchy substrate (rice) was 40%, whereas on coffee pulp the level was 80%, which clearly illustrates the unreliability of moisture level as parameter for predicting microbial growth (Raimbault 1998). High moisture level of substrates leads to decreased porosity, lower oxygen diffusion, increased risk of bacterial contaminations, enhanced aerial mycelium formation, reduction in gas volume, decreased gaseous exchange and change in the rate of degradation of lignin (Silman et al., 1979; Zadrazil and Burnnet 1981) while low moisture level cause suboptimal growth, a lower degree of substrate swelling and high water tension (Silman et al., 1979).

Lovastatin production through any type of fermentation (shallow pan, submerged or solid state) depends mainly on the carbon and nitrogen content of the substrate. Since
moisture content of the substrate is chiefly meant for making available of sugars and nutrients to the fermenting organism, the precise level of moisture requirement for the mold growth on carob substrate for the optimum lovastatin production was determined initially.

In the present study, the lovastatin production and sugar utilization enhanced with increase in the substrate moisture content up to 65% beyond which it declined (Fig. 4.2.1 and Fig. 4.2.2). The lovastatin yields were very low at lower initial moisture content of the substrate, whereas at the optimum levels, the yield was more even during the early duration of fermentation (i.e. 24, 48, 72 and 96 hr of fermentation). At moisture level higher than the optimum level, the lovastatin yield was significantly reduced. The highest yield of lovastatin was obtained at a moisture level of 65% and resulted in 218.88 μg/gDW of lovastatin and it declined sharply at lower levels of moisture content. The reduction in the production of lovastatin in reduced moisture content might be due to the reduction in solubility of nutrients of the solid substrate, lower degree of swelling and higher water tension. Likewise, the higher moisture levels decreased porosity, stickiness, and reduced air volume and diffusion that reduced oxygen transfer (Zadrazil and Burnnet 1981). The results obtained were are in close proximity with the results of Szakacs et al., (1998), reported the lovastatin production in sweet sorghum pulp and wheat bran with optimum of 75% moisture level and declined thereafter.

The substrate used in the present study (deseeded carob pod) was complex in nature with widely varying biochemical composition (Table 4.4) and it also differs with its water imbibing capacity. Hence, the lower and higher initial moisture levels than the
optimal in the substrate cause the differential swellings of the components (Moo-Young et al., 1983) leading to different degrees of fermentation. However, at optimum initial moisture content of substrate provides proper swelling assisting in good penetration to the mold and thereby helps in maximum mass transfer and higher product formation (Lonsane et al., 1985; Raimbault 1998; Pandey et al., 2001; Holker et al., 2004). In the present study, the strain A.terreus KLVB28 requires moisture level of 65% in the substrate indicating that the strain exhibits their normal metabolic activity at this level.

5.5.2. EFFECT OF pH

An important factor that affects the performance of carob pod fermentation is the initial pH of the substrate. Initial pH level of the substrate is one of the crucial factors for successful lovastatin production under solid state fermentation. It is noticed herein and also in most of the industrial fermentations the control of pH of the medium at optimum level is essential for achieving maximum product formation. The initial pH of the substrate mainly depends on the carbon sources used during the fermentation. However, the kinetics of pH variation depends highly on the microorganism. With Aspergillus sp., Penicillium sp., and Rhizopus sp., the pH can drop very quickly below 3.0; for other types of fungi, like Trichoderma, Sporotrichum and Pleurotus sp. the pH is more stable between 4 and 5. Besides this the nature of the substrate has a strong influence on pH kinetics, due to the buffering effect of lignocellulosic materials (Raimbault 1998). Most of the fungi are capable of growing in a wide range of pH 4-8 (Chahal 1983). 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 carob pod fermentation is shown in Fig. 4.3.1 and Fig. 4.3.2 which reveals that the yield of lovastatin
increased with the increase in the initial pH of the substrates up to pH 4.5 and thereafter the decrease of lovastatin was occurred. The maximum yield of lovastatin (220.18 µg/g DW) was obtained at pH 4.5 and the minimum yield of lovastatin (102 µg/g DW) was observed at pH 2.0. As in case of Szakacs et al., (1998), the maximum production of lovastatin was observed in wheat bran and sweet sorghum pulp is at 6.2. This variation in pH optimum for lovastatin production may be due to strain of the organism used, chemical composition of the substrate, fermentation system and finally, the conditions under which the fermentation takes place (Roukas, 1999). Thus from the above studies it clearly indicates that the yield of lovastatin mainly depends on the strains employed during the fermentation.

Buckland et al., (1989) reported the pH of fermentation medium play crucial role in the behaviors of secondary metabolite production as well as cell growth of fungi. The change in pH of fermentation medium might alter the cell metabolism, which inturn affect the lovastatin synthesis. Further, the unexpected reduction in the lovastatin synthesis caused by a large deviation of pH from its optimum was due to activation of unfavorable enzymes, which might decompose the molecular structure of lovastatin (Lai et al., 2005).

The above findings on the optimum initial pH values for maximum production of lovastatin from carob pod substrate are in good agreement with results of Szakacs et al., (1998) and Lai et al., (2005).
5.5.3. EFFECT OF TEMPERATURE

Temperature is an important parameter that governs the process of fermentation as well as the recovery of desired product. It is widely accepted that the secondary metabolism of microorganisms represent an important pathway for survival and inturn depends on the incubation temperature (Lai et al., 2005). However, the influence of temperature-shift varied from strain to strain employed during lovastatin production and it was also speculated that the temperature-shift might induce the associated gene expression and activate the key enzymes that dominated the lovastatin synthesis by the fungus. Since various temperature levels have been reported as optimum for the fermentation, it seemed desirable to check the influence of incubation temperature upon the fermentation.

The result on the effect of temperature on lovastatin production was shown in Fig. 4.4. The yield of lovastatin increased significantly with the increase in fermentation temperature from 20°C-35°C and decreased above 35°C. The maximum lovastatin yield obtained at 35°C was 243.33 µg/gDW and the least yield was observed at 45°C resulted in only 76 µg/gDW of lovastatin at 120 h of fermentation period. The decline in the yield of lovastatin at higher temperatures may have been due to decay in the enzyme system responsible for the production of lovastatin upon exhaustion of the fermentable sugars (Hajjaj 2001). Szakacs et al., (1998) have reported that the lovastatin production under solid state fermentation using wheat bran and sweet sorghum pulp was maximum at 25°C by employing *Aspergillus terreus* TUB-F 514 strain. Similarly Manzoni et al., (1998) have studied the production of statins using *Aspergillus terreus* in submerged fermentation systems and reported 25°C was the optimum temperature for lovastatin.
production. Whereas Hajjaj et al., (2001), reported 28° C is the optimum for maximum production of lovastatin in SmF. Hence the present observations were in accordance with the result of Hajjaj et al., (2001) and Lai et al., (2005).

5.5.4. EFFECT OF INOCULUM SIZE

Optimization of inoculum size (spores/ml) is necessary in solid state fermentation because low density of spores leads to insufficient biomass and end product synthesis as well as permit the growth of undesirable contaminants and too high densities of spores may cause a quick and too much biomass production thereby leading to fast nutrient depletion and ultimately reduction in the end product quality. As such attempts have been made in the present study to optimize the inoculum size for maximum lovastatin production from carob substrate.

The data obtained in the present study on the effect of inoculum size on carob pod fermentation is shown in Fig. 4.5. The data revealed that the inoculum size of 1x10^8 spores/ml of Aspergillus terreus KLVB28 yielded highest amount (280.66 μg/g DW) of lovastatin at 120 h fermentation period, while the lowest yield was observed at inoculum size of 1x10^5 spores/ml, producing 155 μg/g DW of lovastatin in carob pods at the same fermentation period. Thus, the present study reveals that an inoculum size of 1x10^8 spores/ml is suitable for maximum yield of lovastatin with A. terreus KLVB28 strain by employing carob pod as a substrate.

Gunde-Cimerman et al., (1995b), Novak et al., (1997), Szakacs et al., (1998), Manzoni et al., (1999), Hajjaj et al., (2001), Bradamante et al., (2002), Lopez et al., (2003; 2004), Lai et al., (2003; 2005) observed inoculum size of 5 x10^7, 5 x10^8, 1x10^7, 1x10^6, 1x10^7, 1x10^6, 5x10^6, 2x10^8, 9x10^7, 5x10^6 and 1x10^7 spore/ml were optimum for
the maximum yield of lovastatin from submerged fermentations by employing *A. terreus* strains respectively. Further, Szakacs et al., (1998), Lingappa and Vivek Babu (2004), Chi-Su et al., (2003) reported $1 \times 10^7$, $1 \times 10^8$ spores of *A. terreus* strains and 5% of *M. purpureus* spores were optimum for lovastatin production under solid state fermentation.

The present study revealed that an inoculum size of $1 \times 10^8$ spores/ml is suitable for maximum yield of lovastatin with *A. terreus* KLVB28 strain on deseeded carob substrate. Hence this optimal density of spore inoculum is close agreement with the findings of Novak *et al.*, (1997), Szakacs *et al.*, (1998), Lopez *et al.*, (2003), Lingappa and Vivek Babu (2004).

5.5.5. EFFECT OF PARTICLE SIZE

The particle size of the substrate greatly influences the production of lovastatin under solid state fermentation process (Hasseltine 1972). Smaller the particle size, larger is the surface area for heat and mass transfer leading to higher nutrient concentration and shorter nutrient diffusion pathways. On the other hand, small size of the particle also leads to closer packing density and reduction in the void space leading to reduced heat transfer area and gas exchange with the surrounding medium. Hence, agitation of the smaller particles becomes necessary to provide for a high degree of particle separation. Therefore, the particle size should not be too small that heat transfer or gaseous exchange is hindered and too large that nutrient transfer is limited (Mudgett 1986). Hence optimum particle size needs to be studied at which maximum productivity can be obtained.

The results on the optimization of particle size for lovastatin production with *A. terreus* KLVB28 strain on carob pods are presented in Fig. 4.6. Amongst different
particle sizes tested, the substrates with 2mm particle size has yielded maximum of 252.14 μg/g DW lovastatin at 120 h. of fermentation period, where as the lowest yield 162 μg/g DW was observed with particle size 4mm and above. The maximum production at 2mm particle size indicated that the substrate provides sufficient surface area for the fungus growth and adequate sugar diffusion (Roukas 1994).

Our results on the optimum particle size of 2 mm are in close agreement with the works of Szakacs et al., (1998) and this optimum values are also reported from our laboratory (Lingappa and Vivek Babu 2004).

5.5.6. EFFECT OF BED DEPTH

The thickness of substrate layer under any natural fermentation conditions play a key role in the desired end product formation. Further, it also affects the growth and enzyme activity of the organism involved during the fermentation. Maximum depth upto which uniform growth occurs during lovastatin fermentation (Koji dish) has been reported is 5 cm in thickness (Chi-Su et al., 2003). The more substrate layer depth, growth is obtained only upto 1 cm thick on the surface part, while the heat generated beneath the substrate layer inhibits the further mold growth. The increased bed depth also extends the fermentation period to achieve the maximum product formation. Hence it is necessary to optimize the bed depth during any solid state fermentation, with any new type of substrate employed for lovastatin production.

The result obtained in the present study is presented in Fig. 4.7 which indicates that the bed depth has a role in SSF of the carob pods to produce lovastatin. The yield of lovastatin enhanced as the bed depth increases from 1cm to 2cm and thereafter it decreases by further increase in bed depth of substrate layer. The maximum yield of
289.63 µg/g DW of lovastatin was obtained at 2cm bed depth, during 120 h of fermentation period. Since the studies were conducted under static conditions, proper aeration and gaseous exchanges could be obtained for optimum fermentation at this 2cm bed level or height. At higher bed depths lower yields of lovastatin were observed, that might be attributed due to lower metabolic activity of the organism coupled with improper aeration, gaseous exchanges as well as unsuitable temperature elevations have impaired the lovastatin yields. The results observed in the present study are in good agreement with the reports of Chi-Su et al., (2003) and this study was also reported in our laboratory (Lingappa and Vivek Babu 2004).

5.5.7. EFFECT OF FUNGAL BIOMASS

Biomass is a fundamental parameter in the characterization of microbial growth. In SSF, estimation of biomass, which is essential for kinetic studies, poses certain difficulties such as the microbial cells remain attached with the substrate and it is difficult to separate them. In case of fungal cultures, even it is more difficult because fungal mycelia penetrate into the substrate particles. Fig.3.12 shows scanning electron micrograph of carob pod particles for SSF and Fig 3.13 shows fungal mycelia growing on the particles embedding and degrading it. Thus, generally estimation of growth of microorganisms in SSF is carried out by indirect methods. Among the indirect or the other methods used for the estimation of microbial growth in SSF, assay of biomass components such as DNA, glucosamine, ergosterol and protein Kjeldal or assay of metabolic activity (respirometry) have been considered useful. However estimation of glucosamine for fungal biomass assay has been considered as the most suitable and frequently used method. Glucosamine is a useful component for the estimation of fungal
biomass, taking advantage of the presence of chitin, poly-N acetyl glucosamine, in the cell walls of many fungi (Raimbault 1998). Interference with this method may occur when using complex agricultural substrates containing glucosamine (Aidoo et al., 1982). However, the proportion of chitin in the mycelium will vary with age and the environmental conditions. Mycelium glucosamine contents ranged from 67 to 126 mg/g dry mycelium.

In the present study the biomass on carob substrate was estimated in terms of glucosamine content and the result on biomass formation during lovastatin production in solid state fermentation by employing A.terreus KLVB28 are presented in Fig. 4.8.

The data reveals that the fungal biomass formation increased with increase in fermentation duration up to 120 h and thereafter no significant increase in biomass is noticed up to 168 h of fermentation. The maximum biomass formation (118 µg/g substrate) was observed in 120 h of fermentation, which yielded 289.63 µg/g DW of lovastatin. Further, the above amount of lovastatin synthesized during the fermentation was due to consumption of 67.03 % sugar of carob substrate at 120 h of fermentation period.

Szakacs et al., (1998), Hajjaj et al., (2001), Lai et al., (2002, 2005) and Lopez et al., (2002, 2004) noticed the biomass content of 16g/L, 9.5g/L, 0.035g/L, 12g/L respectively during 7 days of lovastatin fermentation. Hence the results observed in the present study are in accordance with the findings of Lai et al., (2002, 2005).
5.6. PROCESS ECONOMIZATION

During the manufacture of any product, there is an always a trend towards economization of the process, i.e., to reduce the cost of and also time required for production of the product. So that the process of fermentation that is aimed at obtaining a specific product. In such fermentation processes, always importance was given to supplementation of nutrients and improvement methods.

The production of secondary metabolites is often coupled with the stationary growth phase of the microorganism used, and depends on N or P limitation together excess carbon and energy source (Holker et al., 2004).

5.6.1. SUPPLEMENTATION OF NUTRIENTS

In general, type, source and nature of carbon and nitrogen are the most important factors for any fermentation process. Generally it is believed that the ratio between the mass of carbon and nitrogen (C/N) ratio is most crucial for a particular process to obtain a specified product. This statement is valid when the metabolite to be produced is non-growth associated or even partially associated. In the case when the metabolite is associated to growth it is better from the beginning of the process to bring the balanced medium (Pandey et al., 2001).

5.6.1.1 Influence of carbon sources

It is a common practice to use carbohydrates as the carbon sources in microbial fermentation processes. Further most of the industrial micro-organisms are chemo-organotrophs; therefore the commonest source of energy will be the carbon sources such as carbohydrates. However some micro-organisms can also use hydrocarbons or
methanol as carbon and energy sources. Hence, energy for the growth of desired micro-
organism during industrial fermentation is derived either from the oxidation of medium 
components or from sun light (Stanbury et al., 1995). In general, the main product of a 
ermentation process will often determine the choice of carbon source, particularly if the 
product results from the direct dissimilation of it. Hence, it is now well recognized that 
the rate at which the carbon source is metabolized can often influence the formation of 
biomass or production of primary or secondary metabolites.

In general, carbon concentration had a positive effect on lovastatin production and 
high titers of lovastatin can obtained with medium rich in carbon source having limited 
reported the improvement in lovastatin yield with high sources of carbon energy. 
However, the carbon sources that produced slowest growth generally yields higher 
lovastatin in a fermentation medium (Lopez et al., 2003). In other words, a high 
productivity and final yield of lovastatin are generally obtained using a slowly 
metabolized carbon source under conditions of nitrogen limitation. Apparently, it was 
due to the metabolic pathways for the synthesis of lovastatin from carbon and much 
slower than the pathways that convert carbon to biomass. Therefore, growth suppression, 
through nitrogen limitation improves the synthesis of lovastatin by diverting more carbon 
to its synthesis (Lopez et al., 2003). In addition the carbon source may exert complex 
regulation on gene expression and enzyme activities for polyketide synthesis (Hajjaj et 
al., 2001).

Hence in the present study various carbon sources such as glucose, fructose, 
galactose, xylose, arabinose, lactose, maltose and sucrose at 0.5-2.5% level were
analyzed for lovastatin production and the results obtained are depicted in the Fig 4.9.1 to 4.9.9.

Various researchers have reported the supplementation of different carbon sources to fermentation medium for lovastatin production. The pioneering work on this aspects were started with Szakacs et al., (1998), presented the systematic and statistical approach for lovastatin production with different carbon sources such as glucose, lactose, sucrose, cellulose and starch at 2% concentration using A. terreus TUB-F 514 strain and they noticed lactose was the best and inexpensive carbon source, followed by glucose and sucrose yielded 400 µg/ml, 168 µg/ml, 160 µg/ml lovastatin respectively.

Hajjaj et al., (2001) observed the influence of carbon sources, glucose (4.5%) and lactose (7.0%) on A. terreus ATCC 74135 employed or lovastatin production yielded 35 mg/L, 25 mg/L lovastatin respectively and further they stated that a combination of both rapidly and slowly metabolized sugar may be beneficial to lovastatin production. Addition of glucose and lactose as the best carbon source to the fermentation medium was observed to be beneficial for lovastatin production employing A. terreus (Bradamante et al., 2002; Lai et al., 2003; Lopez et al., 2003; 2004) and M. ruber (Chang et al 2002 ab).

The present studies indicate that almost all carbon sources enhances the yield of lovastatin upto 2.0 % level thereafter no significant production in lovastatin is observed on all the days of fermentation period. Hexose monosaccharides like glucose, fructose, galactose increased lovastatin production upto 2% level for 120 h of fermentation and resulted in maximum lovastatin yields of 439.16, 430.42, 462.62 µg /g DW concurrent with 57.58, 60.01, 59.21% sugar conversion efficiency of A.terreus KLVB28
respectively. Pentose monosaccharides like xylose and arabinose has also revealed the
same pattern of hexose monosaccharides and resulted in higher lovastatin yields of
401.33, 404.12 µg/g DW concurrent with 59.33% and 57.99% sugar conversions
respectively. Similar kind of observations were also noticed even with disaccharides like
lactose, maltose and sucrose and yielded nearly 398.04, 362.12, 492.23 µg/g DW
lovastatin concurrent with 58.34, 63.58, 66.22 % sugar conversion efficiencies
respectively. Further addition of carbon sources after 2.5 levels to the substrate led to the
decrease in lovastatin yields, indicating beginning of catabolic repression of lovastatin
biosynthesis. Amongst various carbon sources tested, sucrose at 2% level enhanced
lovastatin production in better way (Fig. 4.9.9). The results obtained under the present
study are in good agreement with the findings of Szakacs et al., (1998).

5.6.1.2 Influence of nitrogen sources

Nitrogen is an important factor which plays a key role in lovastatin biosynthesis.
Most of the industrially used microorganisms, especially A.terreus can utilize nitrogen
sources either in organic and inorganic form, sometimes both. In many instances growth
will be faster with a supply of organic nitrogen and only a few micro organisms have an
absolute requirement for amino-acids. Generally, for lovastatin production organic
nitrogen were supplemented in the form of proteinaceous nitrogen compounds such as
beef extract, yeast extract, soyabean meal, corn steep liquor, peptonised milk and
peptone. In some instances inorganic nitrogen sources were supplied in the form of
ammonium salts or nitrates (Stanbury et al., 1995; Hajjaj et al., 2001). In general,
lovastatin biosynthesis proved dependent on the nitrogen sources and the influence of
nitrogen sources on secondary metabolism has been well illustrated for most of the
filamentous fungi (Hajjaj et al., 2001). Production of lovastatin is generally associated with the stationary phase of nitrogen limited growth with excess carbon source, during secondary metabolism and synthesized via the polyketide pathway. However, the biomass, and lovastatin production performance of \( A. \) terreus cultures depends on the type of nitrogen sources employed during fermentation (Lopez et al., 2003; 2004).

Hence, studies have been conducted with various organic and inorganic sources on lovastatin production and the results obtained in the present study context are depicted in the Fig 4.10.1 to Fig. 4.10.12. The results obtained in the present studies revealed that various organic nitrogen sources like beef extract, yeast extract, corn steep liquor, soyabean meal, peptonised milk and peptone at concentrations of 0.5, 0.75, 1.0, 1.25 and 1.50% was supplemented to the carob substrate. The results depicted in Fig. 4.10.1-4.10.6 indicated that the yield ofLovastatin increased with increase in nitrogen concentration up to 1.0% thereafter no significant increase in lovastatin was noticed on all the days of fermentation period with all nitrogen sources. Thus, nitrogen sources like beef extract (1%), yeast extract (1%), corn steep liquor (1%), soyabean meal (1.25%), peptonised milk (1%), peptone (1%) has yielded 468.46, 488.73, 370.66, 342.49, 352.8 and 296.03 µg/g DWLovastatin concurrent with 66.70, 74.05, 66.62, 59.27, 60.62 and 50.45% sugar conversion efficiencies respectively for \( A. \) terreus KLVB28 strain.

The results pertaining to inorganic nitrogen sources are presented in Fig. 4.10.7 to 4.10.12 which indicates that lovastatin yield increased with increase in inorganic nitrogen concentration up to 0.5% and further increase in nitrogen source has revealed no significant change in lovastatin concentration. The highest yield of lovastatin, 560.11, 458.26, 456.13, 467.51 and 469.33 µg/g DW concurrent with 57.57, 64.86, 62.67, 56.10
and 57.93% of sugar conversion efficiencies for *A. terreus* KLVB28 strain grown on deseeded carob substrate which was observed at 120 h of fermentation period. Thus amongst all inorganic nitrogen sources provided for lovastatin production ammonium chloride (0.5%) appears to be a good nitrogen source under SSF process.

Szakacs *et al.*, (1998) reported effect of three organic (0.3%) (soyabean meal, corn steep liquor and rapeseed meal) and three inorganic (0.1%) ((NH₄)₂SO₄, (NH₄)₂HPO₄, KNO₃) nitrogen sources on lovastatin production employing *A. terreus* TUBF 514 and further they observed rapeseed meal as the best organic nitrogen source, followed by corn-steep liquor and soyabean meal yielded 378 µg/ml, 22 µg/ml of lovastatin respectively in 7 day of fermentation. Amongst inorganic nitrogen sources, KNO₃ (0.2%) followed by (NH₄) HPO₄ (0.1%) (NH₄)₂SO₄, yielded 378 µg/ml and 22 µg/ml of lovastatin. The requirement of nitrogen sources mainly depends upon the strain and substrates employed during the fermentation. Various organic nitrogen sources such as soyabean meal, peptone, corn--steep liquor, yeast extract at 0.5% level were supplemented to the cultures of *A. terreus* (Manzoni *et al.*, 1999; Lai *et al.*, 2003; Lopez *et al.*, 2003) and *Monascus purpureus* (Chang *et al.*, 2002a; Chi-Su *et al.*, 2003). Cornsteep liquor and soyabean meal significantly enhances the lovastatin titers in the production medium (Lai *et al.*, 2003) as compared to peptone or yeast extract. However, yeast extract and soyabean meal are preferred as the best nitrogen sources when compared to corn-steep liquor at 0.1% level, supplemented to cultures of *A. terreus* ATCC 20542 (Lopez *et al.*, 2003). Hence the results obtained on the influence of organic nitrogen sources (Fig 4.28) are in good agreement with the observations of Lopez *et al.*, (2003).
Amongst inorganic nitrogen sources ammonium nitrate, urea, ammonium sulphate were supplemented (0.4-1%) level to the cultures of *A. terreus* and *M. purpureus* (Hajjaj *et al.*, 2001; Chi-Su *et al.*, 2002). Ammonia plays a vital role in nitrogen metabolism in filamentous fungi, which can be consumed by certain fungi during cell metabolism (Hajjaj *et al.*, 2003).

In the present study, results reveal that, amongst all inorganic nitrogen sources such as ammonium chloride, Ammonium sulphate, Ammonium carbonate, Ammonium nitrate and urea at 0.5% level, ammonium chloride proved to be beneficial to lovastatin synthesis, as it has yielded 560.11 µg/g DW of lovastatin at 120 h of carob fermentation by employing *A. terreus* KLV28. The above observations made in the present study are consistent with the findings of Hajjaj *et al.*, 2001 and Chi-Su *et al.*, 2003).

### 5.6.1.3 Influence of phosphate source

Phosphate is a key nutrient of regulatory importance during metabolism. All microorganisms require certain amount of phosphate for growth and metabolism. In certain secondary metabolic processes concentration of phosphates may be very critical. Some secondary metabolic processes have a lower tolerance range to inorganic phosphate than vegetative growth (Stanbury *et al.*, 1995). The reason for the effect of phosphate on growth is metabolic rather than genetic and may be related to the role of phosphate in the regulation of a number of enzyme systems or even in the overall energetic processes.

Generally potassium dihydrogen phosphate and sodium dihydrogen phosphates are supplemented to the substrate under solid state fermentation to enhance lovastatin production by the fermenting organisms and is usually added to give final concentration
in between 0.01-0.25% level in submerged fermentations (Szakacs et al., 1998; Chi-Su et al., 2003; Lopez et al., 2004).

Szakacs et al., (1998) supplemented the production medium with KH₂PO₄ in the range from 0.2-0.4% level and found 0.3% as optimum for lovastatin production under submerged process employing A. terreus TUBF-514 in 7 days of fermentation. Chi-Su et al., (2003) carried out studies employing M. purpureus to produce lovastatin from rice grains supplemented with 0.1% of KH₂PO₄, as basic nutrients during solid state fermentation. Lopez et al., (2004) supplemented KH₂PO₄ to A. terreus ATCC 20542, cultures in the range from 0.5-2.5 g/dm³ in 7-11 days of submerged fermentations and indicated 0.79 g/dm³ of phosphate as optimum for lovastatin production.

In the present study, K₂HPO₄, KH₂PO₄, Na₂HPO₄ and NaH₂PO₄ at 0.25-1.25% level have been supplemented to the carob substrate in order to evaluate their influence on lovastatin production by A. terreus KLVB28. Maximum production of lovastatin by the organism was obtained on supplementation of 0.5% of K₂HPO₄ during 120 h of carob fermentation and yielded 512.32 µg/g DW lovastatin, followed by NaH₂PO₄, Na₂HPO₄, and KH₂PO₄ (Fig 4.11.1 to Fig.4.11.5). When compared with the results obtained, amongst all phosphate sources K₂HPO₄ found to be beneficial for lovastatin production. Results on sugar conversion also indicate maximum (66.66%) utilization with K₂HPO₄.

Thus, supplementation of K₂HPO₄ to the substrate was found to be more beneficial than additions of any other phosphate source. The similar observations were also made by Szakacs et al., (1998) and Lopez et al., (2004).
5.6.1.4. Influence of alcohols

Addition of lower alcohols to the solid substrate medium enhances the lovastatin production from crude carbohydrates. The optimum amount of alcohols (glycerol, ethanol, methanol and mannitol) depends upon the strain and the composition of the fermentation medium. Generally, alcohols have been shown to act on membrane permeability in microorganisms by affecting their growth and sporulation through the action not only on the cell-permeability but also the spatial organization of the membrane and change the lipid composition of the cell wall (Pandey et al., 2001).

Generally for the lovastatin production, alcohols like glycerol, ethanol were supplemented to give final concentration between 2-13% in submerged and solid state fermentation (Hajjaj et al., 2001, Bradamante et al., 2002; Chang et al., 2002b; Lopez et al., 2003).

Manazoni et al., (1998) supplemented 5% glycerol to defatted soyabean flour medium for lovastatin production by employing A. terreus TUBF-514 strain. Hajjaj et al., (2001) observed the effect of ethanol and glycerol at 2% level on A. terreus in submerged fermentation and reported that ethanol and glycerol are reduced substrates that generate NADH, which is essential for the biosynthesis of polyketides and further described these two substrates are very poor additives for lovastatin production (Hajjaj et al., 2001).

In contrast to the above statement, Bradamante et al., (2002) reported that the significant lovastatin yields obtained in a medium supplemented with 7% glycerol along with 3% lactose, by employing A.terreus MIM BST strain (Manzoni et al., 1998). The stimulation of biosynthesis of secondary metabolites, such as lovastatin takes place in
presence of high source of energy and also by high level of glycolytic intermediate accumulation which influences reducing equivalent fluxes in the fermentation medium, thereby elicits lovastatin production (Bradamante et al., 2002).

Chang et al., (2002ab) also reported the efficiency of glycerol along with glucose as suitable media supplement for lovastatin production employing M. ruber. Lopez et al., (2003) and Lai et al., (2003) reported the interaction of glycerol with other carbon sources at 3% concentration, enhances the lovastatin production in the growing cultures of A. terreus ATCC 20542.

In the present study, glycerol, methanol and mannitol at concentration of 0.5-2.5% have been supplemented to deseeded carob substrate in order to evaluate their effect on lovastatin production by A. terreus KLVB28. Maximum yield of lovastatin (440.13μg/g DW) by the organism was obtained on supplementation of 2% of glycerol during 120 h carob fermentation, followed methanol, mannitol (Fig 4.12.1 to 4.12.4). Thus from the results amongst all lower alcohols tested glycerol at 2% concentration found to be beneficial for lovastatin production. The observations made herein are in consistent with the results of Lopez et al., (2003).

5.6.1.5 Influence of metal ions

All microorganisms require certain mineral elements for growth and metabolism. Certain trace elements like magnesium, manganese, copper, zinc, iron, cobalt and calcium are needed at appropriate concentrations and must be added as distinct components to fermentation processes. Further, few metal ions need to be supplemented
to a fermenting medium, as they are essential for cell mass formation and also acts as co-factors for several biosynthetic enzymes.

Several workers have reported the importance of trace elements/metal ions in lovastatin synthesis. Magnesium ions supplementation to medium as trace element solution to get final concentration between 200-500 mg/l during lovastatin production (Szakacs et al., 1998; Hajjaj et al., 2001, Chi-Su et al., 2003, Lopez et al., 2004).

Manganese ions were added to the growing cultures of A. terreus at 1.6-100 mg/l, as trace element solution during submerged fermentations (Szakacs et al., 1998; Hajjaj et al., 2001; Lopez et al., 2003; 2004).

Copper ions were generally supplemented as copper sulphate, at concentration ranges between 5-250 mg/l to the fermentation medium employing A. terreus (Hajjaj et al., 2001; Lopez et al., 2003; 2004).

Zinc source to the lovastatin biosynthesis from A. terreus cultures, was supplemented at the concentration ranges from 1mg to 200 mg (Szakacs et al., 1998; Hajjaj et al., 2001; Lopez et al., 2004).

The concentration of iron for the optimum production of lovastatin ranges from 2-200 mg (Szakacs et al., 1998; Hajjaj et al., 2001; Lopez et al., 2004). Only few reports are available on the incorporation of cobalt and calcium ions on lovastatin synthesis. Usually cobalt was supplemented at 2 mg/l concentration (Szakacs et al., 1998) whereas calcium ions, at the range of 20-50 mg/l, which is slightly higher than the former (Hajjaj et al., 2001 and Chi-Su et al., 2003).
In the present study, various metal ions such as Mg$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, and Fe$^{3+}$ were supplemented at 0.05g - 0.25 g concentration to the carob substrate in order to evaluate their influence on lovastatin production by *A. terreus* KLVB28.

The results obtained on the influence of metal ions are depicted in Fig 4.13.1 to 4.13.8. The data revealed that almost all metal ions enhance the lovastatin production; except calcium and cobalt, appears to have negative effect on lovastatin synthesis. Amongst all metal ions the beneficiality of the trace elements in enhancing the yield of lovastatin by *A. terreus* strain grown on carob substrate appears to be in the order of Mg$^{2+}$ > Mn$^{2+}$ > Cu$^{2+}$ > Zn$^{2+}$ > Fe$^{2+}$ > Co$^{2+}$ > Ca$^{2+}$. However, maximum production (361.41 µg/g DW) by the organism obtained for Mg$^{2+}$ followed by Mn$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ at 200 mg of each metal ion.

**5.6.1.6. Influence of combination of nutrients**

Apart from evaluating beneficiary effect of the supplementation of nutrients individually to the carob pod substrate, an effort has been made to evaluate the influence of addition of optimum levels of nutrients and minerals step by step to the fermentation medium, as described in the section 4.7.6.

The results obtained in the present study, as depicted in Fig. 4.14.1 and 4.14.2 revealed, lovastatin production by *A. terreus* KLVB28 strain continued to increase as step-wise addition of nutrients and minerals supplemented in the sets. Thus, at the finals set, highest levels of 842.42 µg lovastatin/g DW substrate was obtained, when all the nutrients and minerals (sucrose, yeast-extract, NH$_4$Cl, K$_2$HPO$_4$, glycerol and MgSO$_4$) were supplemented to the carob substrate, in their optimum concentrations. As a consequence of supplementation of all nutrients, additional yield of lovastatin enhanced
by 2.92 times, from the carob substrate. Hence it can be clearly inferred here that supplementation of nutrients and metal ions is beneficial for the yield of lovastatin through SSF. Further, the substrate utilization efficiency of *A. terreus* KLVB28 have revealed similar trend as above i.e. 1.03%, 1.01%, 1.03%, 1.1%, 1.21% with the stepwise additions of the nutrients and minerals, when considering the preceding set. Thus, it is clear from the above observations noticed herein, that all the nutrients and minerals are needed for maximum utilization of substrates and for the production of lovastatin.

**5.6.2 MUTATION**

Strain improvement is an essential part of process development for microbial fermentation products, as a means of reducing costs by developing strains with increased productivity and ability to use cheaper raw materials. Thus, strain improvement is regarded as a complex and multidisciplinary activity. However, amongst the newer emerging techniques such as rational screening and genetic engineering, the traditional method of strain improvement by mutagenesis, still plays a central role as it is most reliable and cost effective procedure (Thoma 1971; Rowlands 1984). Stanbury *et al.*, (1995) opinioned improvement of the microbial production of strain offers the greatest opportunity for cost reduction without significant capital investment.

Generally, mutation which is a permanent alteration of one or more nucleotides at a specific site along the DNA strand can be induced by both physical and chemical mutagens (Thoma 1971 and Parekh *et al.*, 2000). Further the efficiency of strain improvement also dependent on various factors such as type of organism to be used, the mutagen dose, exposure time and the condition of treatment and post-treatment.
The EMS employed in present study context belongs to the class of alkylating agent, induces alkylation of nitrogenous bases cytosine and adenine thereby causes GC→AT transition in the fermenting organism and produces higher relative effect (Parekh et al., 2000). Mutagenesis and screening has been the method of choice for improvement of industrial lovastatin production strains of A. terreus. Since the genus Aspergillus lacks a sexual reproductive cycle and is refractory to classical genetic approaches. The strains derived by this methodology exhibit improved lovastatin production and also have better utilization of less expensive raw materials, under adapted fermentation conditions. Hence in the present study of investigation, the mutants obtained as described in earlier section (3.14.2) was employed for lovastatin production under SSF in order to evaluate its product enhancement efficiency.

The perusal of the results (Fig 4.15) indicate that the mutant strain, A. terreus KLVB28 mu21 produced higher yield ofLovastatin (505 µg/g DW) where as the parent strain A. terreus KLVB28 yielded 289.63 µg/g DW on deseeded carob pod fermented at 120 h. It is observed herein that, there is an enhancement of lovastatin yield by 1.74 times than that of parent strain. Further, the sugar conversion efficiency of strains employed also enhanced in the present study context. A. terreus KLVB28, the parent strain revealed 67% of sugar conversion whereas the mutant strain, A. terreus KLVB28 mu21 has 72.6%. Thus sugar conversion efficiency too enhanced by 1.08 times than the parent strain.

Many investigators have reported, the increased yields of lovastatin by employing mutants of A. terreus Kumar et al., (2000ab) reported the increased lovastatin yields by the treatment with combined UV and EMS to the spores of A. terreus ATCC 20542.

Thus it is apparent that induction of mutation helps in getting better strains from the point of product synthesis and an increase in the yield of 1.5-1.7 fold during strain improvement helps a lot on process economization when subjected to large scale production systems.

5.7. CHARACTERIZATION OF LOVASTATIN THROUGH SPECTRAL STUDIES AND HIGH PERFORMANCE CHROMATOGRAPHY (HPLC)

5.7.1. Ultra violet (UV) spectral analysis

The UV spectral studies on standard lovastatin and the extract from A.terreus KLVB28 revealed the maximum absorption peak $\lambda_{\text{max}}$ at 238 nm. The results thus obtained (Fig. 4.16) are in good agreement with the observations of Endo et al., (1976a) and Windholz (1983). Hence, these results indicated that the presence of lovastatin in culture filtrate and this was reported from our laboratory (Lingappa et al., 2004).

5.7.2. Infra red (IR) spectral analysis

The IR spectra of both samples of lovastatin (standard and the one isolated from fermentation medium) showed (Fig.4.17.1 & Fig. 4.17.2) 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 indicates the presence of phenolic –OH group. The
indications of stretching vibrations obtained at 2999.66 cm\(^{-1}\) and 2925.56 cm\(^{-1}\) reveals 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 lovastatin and the one from fermented carob pod. Further, the spectra thus obtained are similar with the findings of Endo (1979).

5.7.3. Proton nuclear magnetic resonance (\(^1\)H NMR) spectral analysis

The proton NMR spectra of both reference lovastatin and the fermented extract were recorded on JEOL model GSX 270 MHz. The proton NMR spectral details (Fig.4.18.1 & Fig. 4.18.2) obtained in CDCl\(_3\) of both samples of lovastatin (standard and one isolated from fermentation medium) reveals proton signals 1-2.7 ppm indicating the presence of methylene (-CH\(_2\)) and methyl (-CH\(_3\)) groups. The proton signals obtained at 6-7.8 ppm as multiplets correspond to the aromatic ring protons. The peak multiplicity observed due to the presence of aromatic ring protons in different environments (Bradamante et al., 2002). Thus, from details of NMR data unequivocally prove the identity of the isolated sample from the fermentation medium as lovastatin.

5.6.4. High performance liquid chromatography (HPLC) analysis

Generally lovastatin 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 lovastatin from the fermented extract was carried out by HPLC using Hichrome C\(_{18}\) column with a mobile phase of methanol (HPLC grade) 90% and water (HPLC grade) 10% (90:10).

The results of HPLC chromatogram of lovastatin, isolated from the fermentation medium of A. terreus KLVB28 strain is depicted in (Fig. 4.19). The chromatogram reveals that the compound has retention time of 21.54 min and with UV absorption
maxima of 238 nm. The results obtained herein are in good agreement with the findings of Vinci et al., (1991); Morovjan et al., (1997) and indicated the presence of lovastatin in culture filtrates.