Discussion
5. DISCUSSION

5.1 ISOLATION AND SCREENING OF *ASPERGILLUS TERREUS* STRAINS FOR L-ASPARAGINASE PRODUCTION

Soil is the exceptionally rich sources for potential enzyme 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, India for the isolation of *Aspergillus terreus* strains. Totally thirty five strains of *A. terreus* were isolated (Table - 4.1). Of the different soils tested for the presence of *A. terreus*, coconut field soil has revealed the highest number of isolates.

After the isolation of *A. terreus* strains, they were subjected to rapid screening for the production of L-asparaginase by rapid plate assay method as described by Gulati et al. (1997). All the thirty five isolates exhibited the zone of diameter (Plate - 1) on rapid plate assay method. For the convenience, the groupings of strains of *A. terreus* isolated from the soils have been done on the basis of zone diameter they exhibited. Therefore, it is proposed that the strains exhibiting zone diameter above 0.9 cm are referred as good L-asparaginase producer, those strains with zone diameter 0.6 - 0.9 cm and those having below 0.6 cm zone diameter may be referred to as moderate and poor L-asparaginase producers. As per the groupings the strain *A. terreus* KLS2 exhibited higher zone diameter and was considered as potential strain for L-asparaginase production. As such, strain KLS3 KLS5 and KLS7 can be treated as moderate L-asparaginase producers and remaining isolates were treated as poor L-asparaginase producers.
Therefore, on the basis of results observed on the rapid plate assay method, it was considered that the strain *A. terreus* KLS2 as a potential L-asparaginase producing candidate for further studies.

**5.2 SCREENING OF AGRO BASED WASTE SUBSTRATES FOR L-ASPARAGINASE PRODUCTION THROUGH SOLID STATE FERMENTATION**

Taking into account of economic profile of L-asparaginase production, attempts were made to use inexpensive agricultural wastes as substrates for the production of L-asparaginase under solid state fermentation employing *A. terreus* KLS2 strain (Fig. 4.1). In the present study screening of various agro based substrates like rice chaff (husk), banana peel, groundnut seed cake, pigeon pea husk and deseeded carob pods were carried out for the production of L-asparaginase. Amongst the five substrates employed for the production of L-asparaginase, the carob pod was found to be the most potential substrate for the production of L-asparaginase. It is generally accepted that sugars act as catabolite repressors for the production of L-asparaginase at higher concentration (Jeffries 1976; Liu and Zajic, 1972). But, in our studies though the carob pod has large amounts of sugars in it served as best substrate for the production of L-asparaginase, this may be due to the presence of sufficient amounts of proteins present in the substrate has overcome the higher concentration of sugars (Table - 4.2). The highest production of L-asparaginase in presence of sugars in carob pod observed in the present study are in agreement with the observations of Tyulpanova et al. (1972) and Pastuszak and Szymona (1976).
Since carob pod produced maximum amount of L-asparaginase 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 L-asparaginase production under SSF.

5.3 BIOCHEMICAL ANALYSIS OF CAROB POD

It is always essential to analyse the chemical components of substrates before a specific substrate is chosen for further fermentation studies, because such analysis reveal the suitability of the substrate for required product synthesis. The biochemical composition of the carob pod satisfies the conditions of fermentation process (Table - 4.2). 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 (Plate - 5).

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 hyphal 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 there by increases its accessability to all available nutrients within the particles (Raimbault, 1998).

As such the carob pod satisfies many parameters of suitability. Hence, carob pod has been preferred and selected as the suitable substrate for the production of L-asparaginase through solid state fermentation. The carob pod employed as substrate under the present study has served as the best anchorage medium for the growth of mold A. terreus KLS2 strain. This inturn led to the proper utilization of water soluble sugars and proteins by the mold and produces good amount of L-asparaginase.

5.4 OPTIMISATION OF FERMENTATION PARAMETERS

Bacteria, yeast and fungi can grow well on solid substrates and find application in solid state fermentation processes. However, filamentous fungi are the 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 L-asparaginase production, these parameters are need to be optimized.

### 5.4.1 Effect of initial moisture content on L-asparaginase production

The moisture content of the substrate is one of the important factors that affect 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 in 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 *A. 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 (Schenel and Rypacek, 1955; 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).

L-asparaginase production through any type of fermentation (shallow pan, submerged) 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 L-asparaginase production was determined initially.

In the present study, the L-asparaginase production enhanced with increase in the substrate moisture content up to 65% beyond which it declined (Fig. 4.2). The L-asparaginase production was very low at lower initial moisture content of the substrate, whereas at the optimum levels, the production was more even during the early duration of fermentation (i.e. 24, 48, 72 hrs of fermentation). At moisture level higher than the optimum level, the L-asparaginase production was significantly reduced. The highest production of L-asparaginase was obtained at a moisture level of 65% and the maximum L-asparaginase activity of 4.96 IU was observed and it declined sharply at lower levels of moisture content. The reduction in the production of L-asparaginase 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 et al., 1981).

The substrate used in the present study (deseeded carob pod) was complex in nature with widely varying biochemical composition (Table – 4.2) 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-Yong 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 \textit{A. terreus} KLS2 requires
moisture level of 65% in the substrate indicating that the strain exhibits their normal
metabolic activity at this level. Gervais and Molin (1988) and Lingappa et al. (2004)
reported 75% and 65% moisture content were optimal for xylanase and lovastatin
production in solid state fermentation respectively, by \textit{A. terreus}. As such, in the
present study \textit{A. terreus} KLS2 has given high amount of L-asparaginase at 65% of
moisture content. Therefore, our findings were in good agreement with the findings
of Lingappa et al. (2004).

\textbf{5.4.2 Effect of initial pH on L-asparaginase production}

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 L-asparaginase 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
(Shankaranand and Lonsane, 1994).

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 \textit{Aspergillus} sp., \textit{Penicillium} sp., and \textit{Rhizopus} sp., the pH can
drop very quickly below 3.0; for other types of fungi, like \textit{Trichoderma},
\textit{Sporotrichum} and \textit{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) which reveals that the production of L-asparaginase increased with the increase in the initial pH of the substrates up to pH 4.5 and thereafter the decrease of L-asparaginase was observed. The maximum production of L-asparaginase 5.210 IU was obtained at pH 4.5 and the minimum production of L-asparaginase 3.83 IU was observed at pH 3.0.

De Angeli et al., 1970 and Ali et al. (1994) have reported pH 7 and 4.5 were optimum for the maximum production of L-asparaginase under SmF process respectively. Pushalkar et al. (1995) have reported the maximum (2.18 U/ml) production of beta-glucosidase by A. terreus in liquid shake cultures on the 7th day of growth with the initial pH of the medium in the range of 4.0 - 5.5. Gulati et al. (1997) have reported 6.2 was the optimum pH for L-asparaginase producing A. terreus strains. Similarly, Sarquis et al. (2004) have reported highest L-asparaginase production of 58 U/L when A. terreus strains cultivated in medium having pH of 6.2. In our study the data revealed that the pH of 4.5 was found as suitable for maximum production of L-asparaginase with A. terreus KLS2 strain on deseeded carob substrate under solid state fermentation. These variations in pH optima for L-asparaginase production may be due to the strain of the organism used, chemical composition of the substrate, fermentation system and finally the
conditions under which fermentation takes place (Roukas, 1994). Thus from the above studies it clearly indicates that the production of L-asparaginase mainly depends on the strains employed during the fermentation. As such our findings are in close agreement with the findings of Ali et al. (1994).

5.4.3 Effect of initial temperature on L-asparaginase production

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 in turn depends on the incubation temperature (Lai et al., 2005). However, the influence of temperature-shift varied from strain to strain employed during L-asparaginase production and it was also speculated that the temperature-shift might induce the associated gene expression and activate the key enzymes that dominated the L-asparaginase 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 L-asparaginase production was shown in Fig. 4.4. The production of L-asparaginase increased significantly with the increase in fermentation temperature from 25 - 35°C and decreased above 35°C. The maximum L-asparaginase production obtained at 35°C was 5.28 IU and the least production was observed at 40°C resulted only 3.026 IU of L-asparaginase at 72 hrs of fermentation period. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms and it is also
reported by various scientists that the metabolic activities of the microbes become slow at lower or higher temperature (Tunga et al., 1999 and Pandey et al., 2001).

Sarquis et al. (2004) reported 30°C is the suitable for L-asparaginase production through submerged fermentation by using *A. terreus* and *A. tamarii*. The present study revealed that the 35°C is suitable for maximum production of L-asparaginase with *A. terreus* KLS2 strain on deseeded carob substrate. This variation in temperature optima for L-asparaginase production may be due to different fermentation system and strain used for L-asparaginase production (Roukas, 1994).

### 5.4.4 Effect of initial bed depth on L-asparaginase production

The thickness of the substrate layer under any natural fermentation conditions plays 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. 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 L-asparaginase production.

The results obtained in the present study are presented in Fig. 4.5. The data indicates that the bed depth has a role in SSF of the carob pods to produce L-asparaginase. The production of L-asparaginase enhanced as the bed depth increases from 10 mm to 30 mm and thereafter it decreases by further increase in the
bed depth of the substrate layer. The maximum production of 5.57 IU of L-asparaginase was obtained at 30 cm bed depth at 72 hrs of fermentation period. Since the studies were conducted under static conditions, proper aeration and gaseous exchanges could be obtained for optimum fermentation at this 30 mm bed level or height. At higher bed depths lower productions of L-asparaginase 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 productions (Chandershekar Naik, 2002).

5.4.5 Effect of initial particle size on L-asparaginase production

The particle size of the substrate greatly influences the production under solid state fermentation process (Hesseltine, 1972; Kumar and Lonsane, 1989). 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. It was reported that substrates with finer particles showed improved degradation due to increase in surface area and greater growth of the fungal cultures was stimulated by smaller particle size of substrate (Pandey, 1992; Pandey et al., 2001)
The results on the optimization of particle size for L-asparaginase production with *A. terreus* KLS2 strain on carob pods are presented in Fig. 4.6. Amongst different particle sizes tested, the substrates with 2 mm particle size has produced maximum of 5.63 IU of L-asparaginase at 72 hrs of fermentation, where as the lowest production of 3.14 IU of L-asparaginase was observed with particle size of 6 mm. The maximum production of L-asparaginase at 2 mm particle size indicated that the substrate provided sufficient surface area for fungus growth (Roukas, 1994).

### 5.4.6 Effect of initial inoculum size on L-asparaginase production

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 L-asparaginase production from carob substrate.

Importance of inoculum size on microbial fermentation process is widely accepted. Out of six inoculum size tested (1 X 10^5 to1 X 10^10) a 1 x 10^7 spores/ml inoculum was found to be the most suitable for high production of L-asparaginase by *A. terreus* in SSF at 72 hrs of fermentation. From Fig. 4.7 it is clear that the L-asparaginase production steadily increased with the increasing in the size of the inoculum until it reaches to the magnitude when enzyme productivity became maximum, thereafter no appreciable change in production of L-asparaginase with high inoculum size could be observed. This indicates that the inoculum density does
not have unlimited effect on fermentation processes. It has some optimum value depending upon the microbial species and fermentation processes (Tunga et al., 1999). Similar reports are available on xylanase production (Archana and Satayanarayan, 1997). Sun et al. (1997) shown an inoculum size of 5% is suitable for maximum fibrinolytic enzymes by Fusarium oxysporium beyond this range of enzyme activity decreased.

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

5.6 SUPPLEMENTATION OF NUTRIENTS

In general the 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 in 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 Influence of carbon sources on L-asparaginase production

It is a common practice to use carbohydrates as the carbon sources in microbial fermentation processes. Further most of the industrial microorganisms are chemo-organotrophs; therefore the commonest source of energy will be the carbon sources such as carbohydrates. However some microorganisms can also use hydrocarbons or methanol as carbon and energy sources. Hence, energy for the growth of desired microorganism during industrial fermentation derived either from the oxidation of medium components or from light (Stanbury et al., 1995). In general, the main product of a fermentation 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 L-asparaginase production and high titers of L-asparaginase can be obtained in a medium rich in carbon source. Various researchers have reported the supplementation of different carbon sources to fermentation medium for L-asparaginase production. *Azotobacter* is capable of utilizing a large number of carbon sources (Burris et al., 1942). Some of the carbon sources, either specifically or non-specifically are known to influence the activity of L-asparaginase in other microorganisms). Results of Robinson and Berk (1969) indicated that glucose (0.25%) is required for maximal L-asparaginase synthesis by *E. coli*. Gaffar (1976) presented the systematical approach for L-asparaginase production with different carbon sources such as glucose, lactose,
sucrose, mannitol at 1% concentration using *Azotobacter vinelandii* and they noticed sucrose was the best and inexpensive carbon source, followed by glucose and the L-asparaginase obtained was 2.24 U/mg and 2.12 U/mg respectively. The role of glucose in the synthesis of L-asparaginase is controversial. It is generally accepted as a catabolic repressor in case of *E. coli* and *Erwinia aerioideae* at higher concentrations (Liu and Zajic, 1972; Jeffries, 1976). Repression of L-asparaginase by glucose has been showed even in other bacteria such as *Serratia marcescens* (Heinemann and Howard, 1969; Khan et al., 1970), *P. vulgaris* (Tosa et al., 1971). Sukumaran et al. (1979) observed the influence of different carbon sources on the production of L-asparaginase using colour less mutants of *Serratia marcescens* on medium having glucose (0.2% and 0.3%) and sucrose (0.3% and 0.4%). The results revealed that the glucose medium and sucrose medium produced 15.1 IU/10 ml and 18.9 IU/10 ml and 15.6 IU/10ml and 16.5 IU/10ml of L-asparaginase respectively. Borkotaky and Bezbaruah (2002) studied different carbon sources for L-asparaginase production. Amongst the various carbon sources tested, mannitol was shown to be the best for the production of L-asparaginase.

In the present study various carbon sources such as glucose, fructose, sucrose at 0.05 M - 2.0 M concentration level were analyzed for L-asparaginase production and the results obtained are depicted in the Fig. 4.8.1 - 4.8.3.

The present study indicate that the carbon sources enhanced the production of L-asparaginase upto 0.15 M and 0.10 M by supplementation of glucose, fructose and sucrose respectively at 72 hrs of fermentation, thereafter no significant production of L-asparaginase was observed on all the days of fermentation period. In case of
glucose and fructose the maximum L-asparaginase production of 7.45 IU and 6.88 IU was observed respectively, whereas sucrose produced 6.74 IU of L-asparaginase at concentration of 0.10 M. Further addition of carbon sources to the substrate led to the decrease in L-asparaginase productions, indicating beginning of catabolic repression of L-asparaginase biosynthesis. Amongst various carbon sources tested, the amendment of glucose at 0.015 M concentration level enhanced the L-asparaginase production in better way (Fig. 4.8.1 - 4.8.3).

The results obtained under the present study coincides with the findings of Robinson and Berk (1969) and Sukumaran et al. (1979).

5.6.2 Influence of nitrogen source on L-asparaginase production

Nitrogen is an important factor which plays a key role in L-asparaginase production. Most of the industrially used microorganisms, especially *A. terreus* can utilize nitrogen source either in organic form or inorganic form sometimes both. In many instances growth will be faster with supply of organic and inorganic nitrogen source. It is clear that the organism show slight differences in the growth pattern in the presence of nitrogen source than in the case of nitrogen free medium (Chandershekar Naik, 2002). Generally, for L-asparaginase production inorganic nitrogen will be supplemented in the form of ammonium salts those were ammonium sulphate, ammonium chloride and urea. In some instances, the organic nitrogen source in the form of proteinaceous nitrogen compounds such as peptone, yeast extract and tryptone. Among these nitrogen sources, some were capable of influencing the growth of the organism and production of enzyme.
The results pertaining to inorganic nitrogen sources are represented in Fig. 4.8.4 - 4.8.6, which indicates the L-asparaginase production increased with the increase in inorganic nitrogen concentration up to 0.13% only in ammonium sulphate, but in case of ammonium chloride and urea, the regulation of L-asparaginase production was observed. The maximum L-asparaginase production was 6.64 IU at 72 hrs fermentation period by supplementation of ammonium sulphate. The L-asparaginase production in case of ammonium chloride and urea was found to be 4.06 IU and 3.14 IU at 0.50% and 0.60% concentration at 72 hrs of fermentation period respectively. Amongst all inorganic nitrogen sources provided for L-asparaginase production, ammonium sulphate (0.13%) appears to be a good nitrogen source.

Gaffar (1976) showed positive effect of supplementation of ammonium sulphate (1%), ammonium chloride (1%) and urea (1%) in the production of L-asparaginase. Krishna Reddy and Reddy (1990) have reported ammonium sulphate for the maximum (986 IU/ml) production of L-asparaginase by K. pneumoniae. Similarly Mukherjee et al. (2000) have optimized the nitrogen source for L-asparaginase production using Enterobacter aerogenes under shake–flask culture method and found that ammonium hydrogen phosphate at 0.16% is suitable to achieve higher amount of L-asparaginase. Sarquis et al., (2004) have conducted experiments on biomass and L-asparaginase production using different nitrogen sources on A. terreus IOC 217 strain. Amongst the different nitrogen sources tested proline 2% found to be best nitrogen source for the production of L-asparaginase. Ferrara et al. (2004) have evaluated the use of proline for L-asparaginase production
and found urea as suitable substitute for proline during the L-asparaginase production. Under the present study, amongst all inorganic nitrogen sources tested for L-asparaginase production, ammonium sulphate (0.13%) appears to be good nitrogen source and the results are consistent with the findings of Krishna Reddy and Reddy (1990).

The studies were also conducted with various organic nitrogen sources on L-asparaginase production and the result obtained in the present study context are depicted in Fig 4.8.7 - 4.8.9. The results obtained in the present studies revealed that various organic nitrogen source like peptone, yeast extract and tryptone at concentrations of 0.25, 0.50, 0.75, 1.00 and 1.25% was supplemented to the carob pod substrate. The results indicated that the production of L-asparaginase increased with increase in organic nitrogen concentration upto 1.0% thereafter no significant increase in L-asparaginase production was noticed on all the days of fermentation period with all organic nitrogen sources. Thus, organic nitrogen sources like peptone (1%), yeast extract (1%) and tryptone (1%) have produced 5.287, 6.117 and 4.012 IU of enzyme respectively by A. terreus KLS2 strain.

Peterson and Cigeler (1972) have reported increased production of L-asparaginase by the addition of yeast extract (1.5%) to the growth medium by employing E. aroideae. Liu and Zajic (1972) studied the effect of organic nitrogen source like yeast extract (0.5%) and tryptone (0.5%) on the production of L-asparaginase by using E. aroideae. Amongst two nitrogen sources used yeast extract (2.76 IU) was found as the best organic nitrogen source. Mikucki (1977) has obtained maximum production of L-asparaginase when yeast extract was used as
nitrogen source during the production of L-asparaginase. Singh and Sukumaran (1986) have reported the effect of organic nitrogen source like peptone (2%) and meat extract (2%) for the production of L-asparaginase by employing *E. coli*. It is also reported that peptone was the good inducer for the production of L-asparaginase.

In the present study, results revealed that amongst all organic nitrogen sources tested for L-asparaginase production yeast extract has proved to be beneficial for supplementation of L-asparaginase production, as it has produced 6.177IU at 72 hrs fermentation periods. The above observations made in the present study are consistent with the findings of Peterson and Cigeler (1972) and Liu and Zajic (1972).

### 5.6.3 Influence of phosphate source on L-asparaginase production

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. 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 enzymes systems or even in an overall energetic process.

In the present study disodium hydrogen phosphate, dipotassium hydrogen phosphate, dihydrogen potassium phosphate at 0.05 M, 0.10 M, 0.15 M and 0.20 M levels have been supplemented to the carob pod substrate in order to evaluate their influence on L-asparaginase production by *A. terreus* KLS2. The results pertaining to this study are presented in Fig. 4.8.10 - 4.8.12.
The results reveal that maximum production (6.20 IU) of L-asparaginase by *A. terreus* KLS2 was obtained on supplementation of 0.10 M of diammonium hydrogen phosphate at 72 hrs fermentation followed by dipotassium hydrogen phosphate (4.91 IU) and potassium dihydrogen phosphate (4.14 IU). Amongst all the phosphate sources tested for maximum enzyme production disodium hydrogen phosphate was found to be beneficial.

Mukherjee et al. (2000) have studied the effect of dipotassium hydrogen phosphate (0.1%) and diammonium hydrogen phosphate (0.16%) under submerged conditions using *Enterobacter aerogenes* for L-asparaginase production. The study indicated that diammonium hydrogen phosphate has found best source for the production of L-asparaginase.

In the present study, results revealed that amongst all phosphate sources tested for L-asparaginase production, ammonium hydrogen phosphate produces maximum (6.20 IU) enzyme at 72 hrs fermentation period. The present study is consistent with the findings of Mukarjee et al. (2000).

5.6.4 **Influence of alcohol source on L-asparaginase production**

Addition of lower alcohols to the solid substrate medium enhances the production of microbial metabolite (Chandrashekar Naik, 2002). The optimum amount of alcohols depend upon the composition of the fermentation medium. Generally, alcohols have been shown to act on membrane permeability in microorganisms by affecting their growth and sporulation (Pandey et al., 2001).

Under the present study the alcohol, i.e., glycerol was supplemented at the concentration 0.10, 0.25, 0.50 and 1.0% on deseeded carob pod in order to evaluate
their effect on L-asparaginase production. The results on the effect of glycerol on L-asparaginase production with *A. terreus* KLS2 strain on carob pods are presented in Fig 4.8.13. The results revealed that maximum L-asparaginase production of 7.11 IU was obtained on supplementation of 0.50% of glycerol at 72 hrs of fermentation period.

Robinson and Berk (1969) supplemented alcohols like glycerol in the concentration between 0.05 - 1.00% in (submerged fermentation) H82 medium. In all the cases the enhancement of L-asparaginase production was observed but the maximum production was observed at 0.50%. Singh and Sukumaran (1986) have supplemented 0.4% of glycerol into the medium for the production of L-asparaginase by using wild and mutant *E. coli* strains and obtained 2 IU/ml and 9.00 IU/ml of L-asparaginase respectively.

Under the present study the data revealed that maximum L-asparaginase production of 7.11 IU was obtained on supplementation of 0.50% of glycerol at 72 hrs of fermentation period. These observations are similar to the observations of Robinson and Berk (1969).

**5.6.5 Influence of trace metal ions on L-asparaginase production**

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 the fermentation medium. Further, few metal ions need to be supplemented to a fermenting medium, as they are essential for cell mass formation and also acts as a co-factor for several biosynthetic enzymes.
The results on the effect of metal ions on L-asparaginase production with *A. terreus* KLS2 strain on carob pods are presented in Fig. 4.8.14 - 4.8.17.

In the present study various metal ions such as Mn$^{2+}$, Cu$^{2+}$, Zn$^{2+}$ and Fe$^{2+}$ were supplemented at 0.01 - 0.03% concentration to the carob pod substrate in order to evaluate their influence on L-asparaginase production by *A. terreus* KLS2. The data revealed that manganese and zinc showed beneficiary effect on the production of L-asparaginase whereas copper and iron did not increase the synthesis of L-asparaginase. The maximum production of L-asparaginase obtained was 6.64 IU at 0.01% concentrations for both manganese and zinc.

Only few reports are available on the importance of trace metal ions on the L-asparaginase production. Robinson and Berk (1969) have reported the supplementation of metal ions like copper, zinc, and manganese to the H82 medium in the concentration ranging 0.001-0.002% and obtained maximum L-asparaginase production of 1.73, 1.74 and 1.71 IU/ml by supplementation of 0.001 concentration of copper, zinc, and manganese respectively. As such our result coincides to the results of Robinson and Berk (1969).

**5.6.6 Influence of combination of nutrients on L-asparaginase production**

Studies were carried out to evaluate the influence of combination of nutrients on L-asparaginase production by using *A. terreus* KLS2 on carob pod substrate and the results are presented in Fig. 4.8.18.

The nutrients obtained at optimum levels in the previous study were added step by step to the substrate. The data of this study reveals that the gradual increase in
the production of L-asparaginase. When all the nutrients are added to the substrate, this (KLS2) strain produces 7.95 IU of L-asparaginase at 72 hrs fermentation period.

5.7 MUTATIONAL STUDY

Mutagenesis is achieved by different methods like irradiation, chemicals, fast neutrons etc. (Bradley, 1966). The use of fast neutrons is a costly affair. The chemical mutagens are inefficient, less potent and cause side effects to the researchers (Lawley, 1966). Therefore, irradiation mutagens were used for mutagenic study when mold are the target organisms. Generally X-rays, gamma rays and UV rays are frequently used. Of these X-rays and gamma rays have high penetrating power and cause, greater nuclear damages as well as lethal effect in the organisms. Hence, the powerful mutagens causing nuclear damages are generally avoided. On the other hand, UV rays with shorter wave length than the visible light are mild mutagens. Hence they are considered to be ideal for induction of mutations (Elander, 1969; Hopwood, 1970; Thoma, 1971).

The mutants were obtained from the strain of A. terreus KLS2 by UV irradiation, and used for L-asparaginase production (Fig. 4.9). The mutant strain A. terreus KLS2 mu showed maximum production of L-asparaginase of 8.15 IU at 72 hrs of fermentation. It is also apparent that induction of mutation helps in getting better strains from the point of product synthesis.

Sukumaran et al. (1979) showed highest L-asparaginase production in glycerol-peptone medium by two colorless mutants Serratia marcescens WF and 933 these organisms produced about 24 IU/10ml and 20 IU/ml of enzyme respectively. Similarly, Singh and Sukumaran (1986) reported that the L-asparaginase production
of 9.8 IU/ml by mutant strain of *E. coli*. Under the present study the data revealed that the mutant strain *A. terreus* KLS2 mu also showed increased production of L-asparaginase upto 8.15IU at 72 hrs fermentation period when compared to wild strain *A. terreus* KLS2 (6.05 IU) on carob pod substrates. Therefore, our results agree very close to the results of Singh and Sukumaran (1986).

### 5.8 PURIFICATION OF L-ASPARAGINASE

Purification of proteins is vital in the study of their function and expression. Separation involves removing any contaminants that are present in the mixture, these may be other proteins or completely different molecules altogether. For an enzyme to be employed as a reagent in any field, be it clinical chemistry or organic synthesis, it must first be purified to a degree that removes any other enzyme capable of catalyzing undesirable side-reactions. Enzymes are unstable molecules with a definite physico-chemical organization. Even a slight change in this organization reduces the activity of enzyme and some times the enzyme is totally inactivated. Therefore the enzymes have been isolated under controlled condition of pH, ionic strength and temperature, since they are proteinacious in nature. Purification of enzymes has been done due to separation of different types of enzymes, first at separation of the enzymes from its producer cells and secondly to removal of excess of water and most of the non protein materials have been removed. This may or may not mean purification to homogeneity. For the enzyme to be commercially viable, purification must yield tens or hundreds of grams of protein. Many standard procedures for the purification of proteins in the laboratory do not readily lend themselves to scaling up,
whereas, on the other hand, some techniques relatively unsatisfactory in the laboratory are much more effective on a large scale.

Generally ammonium sulphate precipitation method has been employed first for purification of enzymes followed by different chromatographic techniques like ion-exchange chromatography, affinity chromatography, gel filtration chromatography and HPLC were employed. In the present study we have used ion-exchange and gel filtration chromatography for purification of enzymes. After the crude extract purified by ammonium sulphate precipitation method.

The studies on the purification of enzymes are presented in Fig. 4.10.1 and 4.10.2. The results revealed that the crude enzyme extract showed highest specific enzyme activity of 2.6 U/mg proteins, whereas ammonium sulphate precipitation, DEAE- cellulose (Ion-exchange) and Sephacryl S-200 (gel filtration) chromatography showed highest specific enzyme activity of 3.4 U/mg proteins, 10.4 U/mg proteins and 11.5 U/mg proteins respectively. In the ammonium sulphate precipitation method about 1.27 fold increases in the specific enzyme activity was observed with 20.24% recovery of proteins. The DEAE- cellulose (Ion-exchange) and Sephacryl S-200 gel filtration chromatography showed 4.0 and 4.42 folds increase in the specific enzyme activity with 4.29% and 3.80% recovery of proteins respectively.

Gaffar (1976) has reported purification of L-asparaginase by ammonium sulphate precipitation, Sephedex-G-150 and DEAE-Cellulose methods. It has been observed that 6.5 U/mg of protein purification by ammonium sulphate precipitation method. Whereas in Sephedex G-150 (Gel filtration) chromatography and DEAE-Cellulose chromatography about 18 U/mg of protein and 50 U/mg protein purification
was observed respectively by using these steps. Similarly he has also reported that 1.6, 4.5 and 12.5 folds increase in the specific enzyme activity with 63%, 21% and 5.0% recovery of proteins respectively. Mesas et al. (1990) have reported that specific enzyme activity of 24.7 U/mg, 136 U/mg and 2020 U/mg of protein through protamine sulphate, DEAE-Sephacel and seapryl S-200 methods respectively. 

The increase in the folds of specific enzyme activity of 1.2, 6.6 and 98 was observed for each step of purifications with 105%, 35% and 12.5% recovery of proteins was reported respectively. Khushoo et al. (2005) have purified recombinant protein from the culture supernatant in a single step using Ni-NTA affinity chromatography which gave an overall yield of 95 mg/L of purified protein, with a recovery of 86%. This is approximately 8-fold higher in the enzyme activity. Krasotkina et al. (2004) have purified E. coli enzyme with a single chromatographic purification step more than 90% purity, complemented with 72% active enzyme recovery, was achieved. The activity of purified L-asparaginase was 630 IU/mg. This variation in the specific enzymatic activity from various workers may be due to choice of the column material used which plays critical role in the purification process Mukherjee (2000).

5.9 GENERAL PROPERTIES OF L-ASPARAGINASE

5.9.1 Effect of pH on enzyme activity

Fig. 4.11.1 shows that partially purified L-asparaginase was active over broad pH ranges (4.0 - 11.0) with an optimum at pH 9.0. The pH optimum reported from this study is comparatively high than the results reported by De-Angeli (1970) employing A. terreus under the submerged fermentation. This property of the enzyme makes clear that enzyme produced by A. terreus KLS2 under the present study has
effective carcinostatic property, because the physiological pH is one of the perquisites for anti tumour activity (Manna et al., 1995). The L-asparaginase activity below pH 8 would not be expected to be very effective for the treatment of tumour patients (Scheetz et al., 1971). Triantafilloss et al. (1988) have studied that membrane bound L-asparaginase from Tetrahymena pyriformis acts optimally at pH 9.6. Mesas et al. (1990) have found the optimal L-asparaginase activity at pH 7.0. The enzyme activity was slightly lowered at pH values of 7.5 or 8.0. Similarly, the pH 9.2 is optimum was reported by Pritsa and Pyrikidis (2001). Thus our results coincides with the results of Triantafilloss et al. (1988) and Pritsa and Kyriakidis (2001).

5.9.2 Effect of temperature on enzyme activity

The temperature optimum of L-asparaginase from A. terreus KLS2 is given in Fig. 4.11.2. The optimum temperature for L-asparaginase activity was found to be 37^\circ\text{C}. It is active at a wide range of temperature condition from 30^\circ\text{C} to 75^\circ\text{C}. Beyond this temperature the enzyme becomes unstable. This property of enzyme makes most suitable for complete elimination of asparagine from the body when tumour patient treated with L-asparaginase \textit{in-vivo}. Mannan et al. (1995) have found 37^\circ\text{C} to be the optimum temperature for the enzyme activity.

5.9.3 Effect of pH on stability of enzyme activity

The enzyme was stable at alkaline pH 8.0 and retains 100% activity even after incubation for 30 and 60 min at 37^\circ\text{C} (Fig. 4.11.3). The enzyme retains 67% and 46% activity at pH 11 for 30 and 60 min respectively. It is observed that the enzyme was more stable at alkaline pH than the acidic. The similar findings were reported by Mannan et al. (1995) using \textit{Pseudomonas stutzeri} MB–405. The enzyme obtained by

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P. stutzeri MB 405 was more stable at alkaline pH than at the acidic one. The enzyme was maximally stable at pH range from 7.5 to 9.5.

5.9.4 Effect of temperature on stability of enzyme activity

The results on the effect temperature on enzyme stability are presented in Fig. 4.11.4. The data indicated that no significant enzyme activity was lost when it is pre incubated at 70°C for 30 and 60 min. The residual activity is 100% at this temperature. At 80°C it retains 69% and 60% activity for 30 min and 60 min respectively. Similar results were reported by Pritsa and Kyriakidis (2001), they found 100% activity of enzyme at 77°C.

5.9.5 Substrate specificity of enzyme

The substrate specificity of the enzyme is presented in Table - 4.4. The results revealed that the enzyme was 100%, 5% and 4% active towards L-asparagine, D-asparagine and L-glutamine respectively. The data indicated that the enzyme extracted from A. terreus KLS2 is very much specific to its natural substrate asparagine. This property of the enzyme is very essential on the treatment of patients where incomplete removal of asparagine is required. The observations reported under the present study are in good agreement with the findings of Campbel and Mashburn (1969) and Mannan et al. (1995).