3. REVIEW OF LITERATURE

L-asparaginase (L-asparagine aminohydrolase, EC 3. 5. 1. 1) constitutes one of the most biotechnologically and biomedically important group of therapeutic enzymes accounting for about 40 per cent of the total worldwide enzyme sales (Warangkar and Khobragade, 2010). The discovery and development of L-asparaginases as anti-cancer drug began in 1953, when scientists first observed that lymphomas in rat and mice reversed after treatment with guinea pig serum (Kidd, 1953). Later it was found out that it is not the serum itself which provoke the tumour regression, but rather the enzyme L-asparaginase (Broome, 1963).

The enzyme catalyzes the deamidation of L-asparagine to L-aspartate and ammonia (El-Bessoumy et al., 2001). It plays an important role both in the metabolism of all living organisms as well as in pharmacology (Borek, 2001). L-asparaginase enzyme acts to maintain nitrogen balance and the level of amino acids within cells (Yun, 2007).

Schematic illustration of the reaction mechanism of L-asparaginase

![Schematic illustration of the reaction mechanism of L-asparaginase]

(Source: Elshafei et al., 2012)

Therefore, the commonest therapeutic practice to treat cancer, the technique is to intravenously administer L-asparaginase in order to deplete the blood. L-asparagine level and exhaust its supply to selectively affect the neoplastic cells (Theantana et al., 2009; Deokar et al., 2010; Warangkar and Khobragade, 2010). In addition, L-asparaginase plays a central role in the amino acid metabolism and utilization, whereas, in human body, it acts as a precursor for ornithine in the urea cycle and in transamination reactions.
forming oxalo acetate in the gluconeogenic pathway leading to glucose formation (Hosamani and Kaliwal, 2011). L-asparaginase is also being used in food industry to reduce the formation of carcinogenic acryl amides in deep fried potato recipes (Friedman, 2003).

**Structure and types of L-asparaginase**

All L-asparaginases are homotetramers with 222 - symmetry and a molecular mass in the range 130 - 180 kDa, with a highly conserved overall fold (Wikman et al., 2005). They are composed of four identical subunits denoted A, B, C and D (Kozak et al., 2002). Each monomer consists of about 330 amino acid residues that form 14 beta - strands and eight alpha - helices, which are arranged in two easily identifiable domains: the larger N - terminal domain and the smaller C - terminal domain. These are connected by a linker consisting of 20 residues. Each of the four active sites is located between the N and C - terminal domains of two adjacent monomers. Thus the asparaginase tetramer can be treated as a dimer of dimers, as the active site is derived from subunits A and C or from subunits B and D (Swain et al., 1993).

Crystallographic studies (Lubkowski et al., 1996) and sequence homology analyses (Bonthron and Jaskolski, 1997) have demonstrated that all known type II asparaginases possess two highly conserved amino acid motifs. L-asparaginases of this type are homotetramers with four active sites. Each active site is created by amino acids from two monomers; including the amino acids form the conserved motifs (Swain et al., 1993; Bonthron and Jaskolski, 1997). In the nomenclature of EcAII, the four subunits are labeled ABCD, and the active - site - competent intimate dimers are composed of subunits AC or BD. It was postulated (Swain et al., 1993) that the mechanism of the L-asparaginase reaction could be a variant of the reaction catalyzed by serine proteases, but with a threonine (12 or 90 in EcAII sequence) in the role of the nucleophilic serine.

Crystallographic studies of glutaminase - asparaginase (Lubkowski et al., 1994) have revealed that they share the same tertiary and quaternary structure with type II L-asparaginase. The *E. coli* genome also encodes a related, cytoplasmic (type I) L-asparaginase (EcAI). It has the conserved aminoacid motifs found in EcAII, but the quaternary structure of these two enzymes is probably different. EcAI has been reported
to function as a homodimer (Jerlstrom et al., 1989) and is characterized by a much lower substrate affinity ($K_m = 10^{-3} \text{M}$).

**General Classification of L-asparaginases**

Based on sequence homology analysis (Borek and Jaskólski, 2001) as well as on biochemical (Campbell et al., 1968; Ho et al., 1970; Dunlop et al., 1980) and crystallographic data (Aghaiypour et al., 2001; Jaskólski et al., 2001; Kolyani et al., 2001; Lubkowsky et al., 2003; Sanches et al., 2003; Yao et al., 2005) available L-asparaginase sequences can be divided into three families. The first family corresponds to the bacterial type L-asparaginase, the second to plant type L-asparaginase and the third to enzymes similar to *Rhizobium etli* asparaginase (Bonthron and Jaskólski, 1997). L-asparaginases are widely distributed in nature, in animal organs such as liver of guinea pig, placenta, kidney and intestine of beef and horse (Prista and Kyridio, 2001) but not in mankind.

Bacterial type L-asparaginases are classified into subtypes I and II, which is defined by their intra or extra cellular localization (Michalska and Jaskolski, 2006). Type I (cytosolic) has a lower affinity for L-asparagine, while type II (periplasmic) has a high substrate affinity. Plant type L-asparaginases differ structurally and have a different evolutionary origin than bacterial L-asparaginases (Michalska et al., 2006).

(Source: Michalska et al., 2006).
Occurrence of L-asparaginase

L-asparaginase is produced by a large number of microorganisms that include *E. coli* (Verma et al., 2007; Derst et al., 1994; Mercado and Arenas, 1999), *Er. caratovora* (Kotzia and Labrou, 2005; Kotzia and Labrou, 2007). *Enterobacter aerogenes* (Mukherjee et al., 2000), *Corynebacterium glutamicum* (Mesas et al., 1990), *Candida utilis* (Kil et al., 1995), *Staphylococcus aureus* (Muley et al., 1998), *Serratia marcescens* (Bernard and Howard, 1969) and also in plants such as soy beans, *Oryza sativa*, *Hordenum vulgare* and *Lupinus* species (Borek and Jaskolski, 2001). The therapeutic effect of L-asparaginase from these two bacterial species is accompanied by side effects that might include anaphylaxis, diabetes, leucopoenia pancreatitis, neurological seizures and coagulation abnormalities which may further lead to intracranial thrombosis or haemorrhage. These side effects are partially attributed to the presence of L-glutaminase activity obtained from these sources (Kotzia and Labrou, 2005). Therefore, it is desirable to search for other L-asparaginase producing microorganisms with novel properties that can produce an enzyme with less adverse effects.

Marine microorganisms continue to provide pharmacologically important secondary metabolites which are unique and novel chemical compounds. Marine microbes are continuously explored for drug discovery. Apart from microbes all other marine sources have also provided valuable chemical diversity (Trevan, 2004). *Streptomyces* is the largest prokaryotic genus with 562 valid species currently (Euzeby, 2009). Among marine microorganism, actinobacteria have gained special attention as the most potent source of antibiotics and other bioactive secondary metabolites (Newman et al., 2000). Actinobacteria represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria, including five subclasses and 14 suborders (Stackebrandt, 2000). Among the five subclasses, actinobacteria (bacteria belonging to the order Actinomycetales; commonly called actinomycetes) / actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005), notably antibiotics (Strohl, 2004) antitumor agents (Cragg et al., 2005), immunosuppressive agents (Mann, 2001) and enzymes (Pecznska - Czoch and Mordarski, 1998). Because of the excellent track
record of actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs in the past fifty years. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Fenical et al., 1999). Thus, it is crucial that new groups of actinomycetes from unexplored or underexploited habitats be pursued as sources of novel bioactive secondary metabolites.

**Actinomycete studies**

Actinomycetes, characterized by a complex life cycle, are filamentous Gram-positive bacteria belonging to the phylum *Actinobacteria* that represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria (Ventura, 2007). *Actinobacteria* are widely distributed in terrestrial and aquatic ecosystems, especially in soil, where they play a crucial role in the recycling of refractory biomaterials by decomposing complex mixtures of polymers in dead plant, animal and fungal materials. Although the diversity of life in the terrestrial environment is extraordinary, the greatest biodiversity is in the oceans (Donia and Hamann, 2003). More than 70 per cent of our planet’s surface is covered by oceans and life on Earth originated from the sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests (Haefner, 2003). As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds.

Early evidence supporting the existence of marine actinomycetes came from the description of *Rhodococcus marinonascene*, the first marine actinomycete species to be characterized (Helmke and Weyland, 1984). Further support has come from the discovery that some strains display specific marine adaptations (Jensen, 1991), whereas others appear to be metabolically active in marine sediments (Moran, 1995). However, these early findings did not generate enough excitement to stimulate the search for novel actinomycetes in the marine environment. Recent data from culture-dependent studies have shown that indigenous marine actinomycetes indeed exist in the oceans.
Microbial sources like actinomycetes are well recognized to produce a variety of chemical structures, several which are most valuable pharmaceuticals, agrochemicals and industrial products like enzymes (Okami, 1986). Actinomycetes are aerobic gram positive filamentous bacteria with high G + C (Guanine + Cytosine) content which form asexual spores and which are widely distributed in both terrestrial and aquatic habitats including extreme habitats like deep sea, desert, mountain etc. Actinomycetes are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry and which are ecofriendly filamentous bacteria. The value of actinomycetes to society in terms of providing useful drugs especially antibiotics and anticancer agent and to the pharmaceutical industry for revenue generating discovery platform, is indisputable (Baltz, 2007). Recent literature has revealed that at least 4,607 patents have been issued on actinomycetes related products and processes (Berdy, 2005). Out of 22,500 total bioactive secondary metabolites 10,100 (45 per cent) are reported to be produced from actinomycetes in which 7630 from Streptomyces and 2470 from rare actinomycetes (Berdy, 2005).

Traditionally, Actinomycetes have been isolated from terrestrial sources although, the first report of mycelium forming Actinomycetes being recovered from marine sediments appeared several decades ago (Weyland, 1969). Recently, the marine derived Actinomycetes have become recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties (Jensen et al., 2005). Actinomycetes represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world and especially significant for their role on the recycling of organic matter (Srinivasan et al., 1991).

The literatures suggested that, marine sediment sources are valuable for the isolation of novel Actinomycetes with the potential to yield useful new products (Goodfellow and Haynes, 1984). However, it has been resolved that actinomycetes form part of the autochthonous marine microbial community of sediment samples originated from terrestrial habitats and were simply carried out to sea in the form of resistant spores (Weyland, 1981; Goodfellow and Williams, 1983; Weyland and Helmke, 1988; Takizawa et al., 1993; Ravel et al., 1998).
Habitats for Microbes

Few Studies have investigated the vertical distribution of actinomycetes in soil (Hegedorn, 1976). The largest population of bacteria $(10^8/g$ of dried soil sample), actinomycetes $(10^7/g$) and fungi $(10^5/g$) were observed in soil from the top layer $(0 - 0.3m$) of black loam. Fungi were observed only in the upper layer. Reduced number of bacteria and actinomycetes were detected in soil of second layer $(0.5 - 5m)$. Bacteria $(10^6/g)$ were also found in silty and sandy soils at 40 m. The vertical distribution of actinomycetes was investigated in details in soils taken at 10 cm interval between 0 and 1 m. A comparative survey of all isolates demonstrated that the actinomycetes population was the largest in soil of the surface layer and although the number decreased gradually as depth increased, individual actinomycetes strains were present in all soil layers (Takahashi and Omura, 2003).

Strategies for the isolation of L-asparaginase producing microbes

Enzyme screening technology has undergone massive developments in recent years. In the 1990s, methods for high - throughput screening of enzyme activities were perceived as a critical bottleneck. Today, large repertoires of efficient screening strategies are available that allow testing of almost any reaction with high - throughput. Many investigators have evolved in screening of L-asparaginase producing microbes using different strategies. Novel bacterial isolation procedures and screening of their natural products, bioactive compounds and therapeutic enzymes in medicines are important in this direction. Among the bacteria, streptomyces gain a special importance because belong to the group actinomycetes which are known to produce a wide variety of enzymes and metabolites (Annie et al., 1997; Balagurunathan, 2004).

Screening of L-asparaginase producing actinomycetes using a rapid plate assay

Microbial strains producing L-asparaginase were identified by a pink colored colony on modified M9 agar medium with phenol red as an indicator for detection of L-asparaginase producing colonies (Prakasham et al., 2010).

During the production of L-asparaginase, the substrate L-asparagine was cleaved into L-aspartic acid and ammonia, an alkaline by product which increase pH of the medium there by color of the medium was changed from yellow to pink. The intensity of pink color is directly proportional to the quantity of L-asparaginase produced. Gulati et
al., 1997 developed a rapid plate assay method for detection of L-asparaginase producing actinomycetes using modified Czapek Dox’s medium supplemented with phenol red 0.009 per cent (Sabu et al., 2003). Among marine microorganism, actinobacteria have gained special attention as the most potent source of antibiotics and other bioactive secondary metabolites (Newman et al., 2000). Actinobacteria represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria, including five subclasses and 14 suborders (Stackebrandt, 2000).

**Isolation and identification of L-asparaginase producing actinomycetes**

Microorganisms found in marine environments have attracted a great deal of attention, due to the production of various natural compounds and their specialized mechanisms for adaptation to extreme environment (Solingen et al., 2001). The pre - treatment including enrichment, physical and selective media may be used to study the ecology of actinomycetes in natural habitats such as soil or water samples (Jensen et al., 2005).

Since marine sediments represent an environment which is markedly different from that associated with soil samples, it is not clear how effective the pre - treatment of such sediments would be for the recovery of bioactive actinomycetes. Marine sediment is an inexhaustible resource that has not been properly exploited. Reports from the East coast of India, suggests that soil is a major source of Actinomycetes (Sivakumar et al., 2005; Vijayakumar et al., 2007; Dhanasekaran et al., 2008; Vijayakumar et al., 2009).

Actinomycetes were isolated by soil dilution plate technique on starch casein agar medium, starch nitrate agar medium, glycerol glycine agar medium, and chitin agar medium (Haefner et al., 2003). The plates were incubated at 28°C and the numbers of colonies were determined after 15 days. All the medium containing 50.0 per cent sea water was supplemented with Nystatin 50.0 μg/ml and Nalidixidic acid 20.0 μg/ml to inhibit the bacterial and fungal contamination respectively (Haque et al., 1992). Serial dilutions were prepared from each sample and one ml of the forth dilution was spread on Starch nitrate agar medium (SNA) described by Shirling and Gottlieb (1966).

The morphological identification method consists of macroscopic and microscopic characterization. Macroscopically the actinomycetes isolates were differentiated by their colony characters, e.g. size, shape, color, consistency etc. For the
microscopy, the isolates were grown by cover slip culture method (Kawato and Sinobu 1959). They were then observed for their mycelial structure, and conidiospore and arthrospore arrangements on the mycelia under microscope (1000X). The observed morphology of the isolates was compared with the Actinomycetes morphology provided in Bergey’s Manual for the presumptive identification of the isolates (Buchanan and Gibbons, 1974)

*Actinomyces* is a genus of bacteria. The bacteria that were grouped in this genus share several characteristics. The bacteria are rod-like in shape. Under the light microscope, *Actinomyces* appear fungus-like. They are thin and joined together to form branching networks. Bacteria of this genus retain the primary stain in the Gram stain reaction and so are classified as being Gram positive (Hopp et al., 2008).

Biochemical tests were done for identification of the isolates (Howell and Pine, 1956 and Howell et al., 1959), except that the basal medium consisted of Heart Infusion Broth, 25 g; pancreatic digest of casein, 4.0 g and yeast extract, 5.0 g (per liter of distilled water at pH 7.0). Inoculum for all biochemical tests, except for the carbohydrate fermentations, was taken from 3-day-old cultures in AM broth. Inoculum for carbohydrate fermentations was taken from cultures made in sugar-free Heart Infusion Broth. The following biochemical tests were performed: catalase test, starch hydrolysis, nitrate reduction, litmus milk reactions, H₂S production and acid from carbohydrates (dextrose, xylose, mannitol, raffinose, and mannose). In the fermentation tests, the carbohydrates were added to the basal medium at a concentration of 0.5 per cent. A drop in pH of less than 0.49 pH unit (as compared with control tubes) was considered to be a negative reaction; a drop of 0.5 to 0.9 units, a plus-minus reaction and a drop of one pH unit or more was considered positive. Starch hydrolysis was determined by growing the strains on slants of basal medium to which had been added 5.0 g of soluble starch and 15.0 g of agar per litre. Hydrolysis was determined by flooding the slants with Gram’s iodine solution. Litmus milk reactions were studied in litmus milk reinforced with 0.5 per cent yeast extract and 0.3 per cent glucose. Nitrate reduction was tested by growing the strains on the basal medium containing 0.1 per cent KNO₃. H₂S production was demonstrated by growing the organisms on BHI slants in cotton-plugged tubes with strips of lead acetate paper suspended above the medium, and incubating at 37°C in an
anaerobic jar (95 per cent N₂ plus 5 per cent CO₂). (False reactions may be obtained with lead acetate paper, if tubes are sealed with pyrogallol - carbonate seals.) Catalase tests were performed according to standard procedures. All cultures made for the biochemical tests (except for H₂S production) were incubated at 37°C under pyrogallol - carbonate seals and read at 3 and 10 days (Howell et al., 1959).

The actinomycetes are a group of bacteria which possess many important and interesting features. They are of considerable value as producers of antibiotics and of other therapeutically useful compounds, they exhibit a range of life cycles which are unique among the procaryotes, and they appear to play a major role in the cycling of organic matter in the soil ecosystem (Lacey, 1978; Williams, 1978). The isolation of actinomycetes from the mixed microflora present in nature is complicated by their characteristic slow growth relative to that of other soil bacteria. This has resulted in the development of selective isolation procedures based primarily on one or both of the following approaches: (i) nutritional selection, in which media are formulated with nutrients which are preferentially utilized by actinomycetes (El-Nakeeb, 1963; Kuster, 1964; Tan 2006), (ii) selective inhibition, in which compounds such as antibiotics are incorporated into media to selectively inhibit non-actinomycete bacteria (Ara et al., 2007; Huang et al., 2008). The major difficulties with each of these approaches are that neither is strictly selective for actinomycetes and that each have the inherent potential to actually inhibit the growth of some actinomycetes.

**Molecular taxonomy determined by sequences and phylogenetic analysis.**

Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The nearly complete 16S rRNA gene sequences (averaging 1,445 nucleotides) were used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions. Sequences then were aligned by using ClustalX software (version 1. 8. 1) with representative actinomycete 16S rRNA gene sequences and the complete 16S rRNA gene sequence of *E. coli* K - 12 (Thompson et al., 1997). The genus level signature nucleotides of the emended family *Streptomycetaceae* were obtained from alignments as described by Koch et al., 1996. Phylogenetic trees were constructed by first generating a complete alignment of 16S rRNA gene sequences of selected members of each genus within the family *Streptomycetaceae* by using ClustalX (Thompson et al.,
The aligned sequences then were incorporated into Phylo - Win, and neighbor - joining and maximum - parsimony methods were used to generate bootstrap values (from 1,000 resamplings) and consensus trees (Galtier et al., 1996). Manipulation and tree editing were done by using Tree View (Page, 1996).

**Optimization of culture conditions for L-asparaginase production**

L-asparaginase formation has been shown to have a firm link to active cell growth. Savitri and Azmi, (2003) have found maximum L-asparaginase activity of *Streptomyces plicatus* at pH 7.0, while Narayana et al., (2007) has reported maximum L-asparaginase production of *S. albidoflavus* at pH 7.5. Gulati et al. (1967) have reported that 6.2 was the optimum pH for L-asparaginase producing *Aspergillus nidulans* strain. The maximum L-asparaginase production by *Emericella nidulans* of 0.96 IU was observed at 30°C, whereas lowest enzyme production of 0.56 IU was observed at 40°C after 48 hrs of fermentation period. Any temperature beyond the optimum range was 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 Pandy et al., 2001).

Sarquis et al., (2004) reported that 30°C is the suitable temperature for L-asparaginase production through submerged fermentation by using *A. terreus* and *A. tamarii*. Sutthinan Khamna et al., (2009) reported that *Amycolatopsis* CMU - H002 has showed that 30°C is optimum for L-asparaginase synthesis. *S. albidoflavus* produced high amount of L-asparaginase at 28 - 30°C when grown at 35°C (Narayana et al., 2007). The inoculum size of 1 ml showed maximum production of L-asparaginase as 1.11 IU at 48 hrs fermentation period.

The optimal pH determined for L-asparaginase activity of *Streptomyces gulbargensis* was 9. 0. Similar observations have been reported for asparaginase from *Pseudomonas stutzeri* MB - 405 (Manna et al., 1995). Dhevagi and Poorani, (2006) reported the maximum L-asparaginase activity of *Streptomyces sp. PDK7* between pH 8.0 and 8.5. Regarding the pH stability, the enzyme retained more than 80 per cent of the activity in the pH range of 7.0 - 10.0. Results are in good agreement with those of Manna et al., (1995). The enzyme was found to be maximally active at 40°C. This optimum L-
asparaginase activity at 40°C is similar to that of *Corynebacterium glutamicum*, reported by Mesas *et al.* (1990).

Production of L-asparaginase began after 24 hrs of cultivation and reached to maximum levels after 72 hrs of incubation. Maximum biomass production obtained with 72 hrs old culture of *Streptomyces albidoflavus* could also be correlated with high levels of L-asparaginase production. L-asparaginase formation has been shown to have a firm link to the active cell growth (Savitri *et al.*, 2003).

The acidic nature of fermentation medium of *Streptomyces albidoflavus* could inhibit L-asparaginase biosynthesis. Glucose is reported to be a repressor of asparaginase synthesis due to the acid production (Geckil *et al.*, 2006). Verma *et al.* (2007) also stated that yeast extract is important for cell growth and L-asparaginase synthesis, but in high concentrations L-asparaginase production was inhibited. Yeast extract (2 per cent) was found to be the best organic nitrogen source for L-asparaginase synthesis from *S. albidoflavus* when compared to peptone (3 per cent). Liu and Zajic (1972) reported yeast extract as the best nitrogen source for maximum yield of L-asparaginase. Production levels of L-asparaginase by *Streptomyces griseus* ATCC 10137 was 0.36 IU per mg of cell dry weight as reported by Jong (1971).

Mostafa and Salma (1979) reported that the 6 - day old culture of *Streptomyces collinus* produced high amount of L-asparaginase when grown at pH 8.5 and temperature 28 - 30°C. But Narayana *et al.* (2008) study reported that 3 - day old culture of *S. albidoflavus* was found to produce maximum amount of enzyme when cultured at pH 7.5 and temperature 35°C.

Maximum L-asparaginase activity occurred when *E. coli* was incubated with an optimum substrate concentration at pH 9.0 (Castaman and Rodeghiero, 1993 and Liboshi *et al.*, 1999), for *Pseudomonas aeruginosa* 10145 (Roberts *et al.*, 1968), and many other microbial asparaginase activities (Balcao *et al.*, 2001). A temperature profile showed that the enzyme had maximum activity at 37°C, and more than 52 per cent activity was attained at 50°C for asparaginases from *Pseudomonas stutzeri* MB - 405 (Manna *et al.*, 1995), *E. carotovora* (Maladkar *et al.*, 1993), and *Staphylococcus* (Sobis and Mikucki, 1991). Also, Qian *et al.* (1996) proved that *E. coli* L-asparaginase lost its activity more rapidly at higher temperatures. On the other hand, L-asparaginase from
Chrombacteriaceae had maximum activity at 20°C (Roberts et al., 1972). Incubation of L-asparaginase at 37°C for different times showed that the activity reached its maximum at 30 minutes.

The maximum L-asparaginase activity was reported as 10.69 IU/ml (22.45 IU/mg of protein) using L-asparaginase as sole source of carbon from P. carotovorum MTCC 1428. The most favorable substrate for the production of L-asparaginase from P. carotovorum MTCC 1428 was found to be L-asparagine or the combination of L-asparagine and glucose. The wide variation of L-asparaginase production from 0 to 23.93 IU/mg of protein (11.54 IU/ml) reflected the importance of the screening of nitrogen source. These results suggested that all variables (L-asparagine, yeast extract and peptone) significantly influence the production of L-asparaginase. At high levels L-asparaginase concentration (3.0 g/l) favored the increase in the production of L-asparaginase than at lower level (0.00 g/l) (SanjayKumar, 2010).

**L-asparaginase purification and properties**

Recent data from the supernatant fraction exhibited 70 per cent of Actinomycetes L-asparaginase activity compared to only 0.5 percent of activity by particulate fraction (Dhevagi and Poorani, 2006). It can be observed that the ammonium sulphate purified sample showed a specific activity of 0.83 IU/mg, approximately 1.09 - fold pure. In the final step, the enzyme showed a specific activity of 63.07 IU/mg, being approximately 83 - fold. The final recovery of protein was 2.18 percent. The final purification step in Sephadex G 200 showed a specific activity of 63.07 IU/mg of protein being approximately 85 - fold pure and the final recovery of protein was 2.18 per cent. The marine Vibrio sp., showed an enzyme specific activity of 14 IU/mg of protein, approximately 19 - fold pure and the final recovery of protein was 1.6 per cent (Selvakumar et al., 1991). A species of marine micro algae, Chlamydomonas was isolated with L-asparaginase enzyme activity of 78 IU/mg of protein and had been purified over - 600 fold (Paul, 1982). The large-scale production of L-aparaginase enzyme from strain Citrobacter sp C6 with an overall purification of 180 - fold and yield of 4.3 per cent was carried out successfully (Bascomb et al., 1975).

A strain of Pectobacterium carotovorum MTCC 1428 glutaminase - free L-asparaginase was purified to homogeneity after Sephadex G - 100 chromatography.
with 42.02 per cent of yield. The enzyme was purified approximately 72 - fold with a specific activity of 2020.91 IU /mg (Sanjay Kumar et al., 2011).

L-asparaginase from *Pseudomonas aeruginosa* 50071 has been purified in CM - Sephadex C - 50 column up to 106 - fold with 43 per cent yield (El-Bessoumy et al., 2004). L-asparaginase from a marine *Streptomyces* sp. PDK2 has been purified 85 - fold with 2.18 per cent recovery in the final Sephadex G - 200 purification step (Dhevagi and Poorani, 2006). SDS - PAGE analysis of purified L-asparaginase from CM - Sephadex C - 50 gel filtration exhibited a distinct protein band above 97 kDa which might be L-asparaginase with electrophoretic mobility of 0.33.

The partial purified L-asparaginase was obtained from *Penicillium brevicompactum* NRC 829 on Sephadex G - 100 column. The most active fractions for enzyme activity with specific activity 132.4 IU/ mg, and purification fold of about 35 and 63 per cent yield were pooled together, dialyzed against 0.01 M Tris - HCl buffer (pH 8.0), and concentrated by lyophilization (-50°C). The elution profile of the most active fractions collected from Sephadex G - 100 and loaded on Sephadex G - 200 column. A sharp distinctive peak of L-asparaginase activity, which fits with only one protein peak, was noticed. The most active fractions (F7 - F9) with specific activity 574.24 IU/ mg and about 151 - fold purification and 40 per cent enzyme recovery were pooled together, concentrated with lyophilizer and stored at -20°C (Elshafei et al., 2012).

Quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC - PAGE) is based on Bio-Rad’s Model 491 Prep Cell, an instrument for preparative gel electrophoresis of all kinds. The procedure is done in an unmodified Prep Cell. Through use of a high - pH buffer, most proteins are negatively charged and migrate from the cathode to the anode during electrophoresis (Kastenholz, 2004). Although the pH value (pH 10) of the electrophoresis buffer is not physiological, protein isomers are continuously eluted at physiological pH 8) (Kastenholz, 2006).

Dhevagi and Poorani (2006) who reported the maximal L-asparaginase activity of *Streptomyces* sp. PDK7 which was between pH 8.0 and 8.5, and the optimal L-asparaginase activity extracted from *Streptomyces gulbargensis* was 9.0 (Amena et al., 2010). L-asparaginase is one of the amidases that are generally active and stable at neutral and alkaline pH, whereas, pH 5.0 to 9.0 were reported earlier to be optimum for
amidase activity (Ohshima et al., 1976). L-asparaginase, purified from marine actinomycete, exhibited maximum activity between pH 7.0 and 8.0 (Basha et al., 2009), membrane bound L-asparaginase from Tetrahymena pyriformis acts optimally at pH 9.6 (Triantafillons et al., 1988) and the optimal L-asparaginase activity from Corynebacterium glutamicum was reported at pH 7.0 (Mesas et al., 1990). L-asparaginase extracted from Pseudomonas stutzeri MB - 405 reported to be maximally stable at pH range from 7.5 to 9.5 (Manna et al., 1995). The results also demonstrated that, L-asparaginase retained about 64 per cent of its activity after storing at pH 4.0 for 24 hrs. This means that, L-asparaginase of P. brevicompactum had higher pH stability over a wide range of pH values.

A previous study which reported that the optimum temperature for L-asparaginase activity obtained from Pseudomonas stutzeri MB - 405 was 37°C. L-asparaginase from Erwinia sp. showed maximum activity at 35°C (Borkotaky and Bezbaruaah, 2002), and maximum activity of L-asparaginase purified from Streptomyces gulbargensis was at 40°C (Amena et al., 2010). Similar results were also reported by Mesas et al., (1990) for L-asparaginase purified from Corynebacterium glutamicum. This property of L-asparagine enzyme makes it most suitable for complete elimination from the body when patient is treated with L-asparaginase in - vivo.

The anti - tumour activity of L-asparaginase is also a function of its half - life in the blood (Fernandes and Gregoriadis, 1997), attempts have been made to increase the half - life, for example by entrapment of the enzyme in liposomes (Neerunjun and Gregoriadis, 1976) or microcapsules (Chang, 1984), and by covalent coupling to macromolecules such as dextran (Wileman et al., 1986), albumin (Poznansky et al., 1982) or monomethoxypolyethylene glycol (mPEG) (Kamisaki et al., 1982) which is available in the market. Unfortunately, none of these approaches have managed to eliminate the disadvantages of L-asparaginase treatment, leaving scientists with the need to identify and characterize new enzymes with better properties.

Inhibition of P. carotovorum L-asparaginase activity with different metal ions and lack of effect with the metal chelator - EDTA possibly indicated that the enzyme was not a metalloprotein. The inhibition of activity in the presence of Hg²⁺, Cd²⁺ and Zn²⁺ might be indicative of the presence of essential vicinal sulphhydryl groups. The potential
presence of such a group(s) is also indicated by the stimulation of activity by the reducing agents, 2 - mercaptoethanol and glutathione, and inhibition by thiol group blocking reagents, p - CMBA and iodoactamide. Thiol reactivity was also observed with the purified L-asparaginase from *Erwinia carotovora* (Warangkar and Khobragade, 2010). L-asparaginase from *P. carotovorum* completely lost its activity in 2 M urea, and only 21 per cent of activity was retained in the presence of 2 mM of SDS. Only L-asparaginase and no glutaminase activity were observed. This property makes the *P. carotovorum* L-asparaginase potentially very useful in medical applications.

*Er. carotovora* L-asparaginase has decreased thermodynamic stability as compared to the *E. coli* L-asparaginase and get rapidly inactivated in the presence of urea (Papageorgiou *et al.*, 2008).

The study results revealed that L-asparaginase affinity towards acrylamide was quite close to that for L-asparagine and the report indicated that the enzyme extracted from *P. brevicaulis* NRC 829 is very specific to its natural substrate L-asparagine (Ali Mohamed Elshafei *et al.*, 2012). The Kₘ of L-asparaginase for L-asparaginase was found to be 1.05 mM. This result indicates the high affinity of L-asparaginase towards its natural substrate, which might relate to its degree of effectiveness against tumors. Higher Kₘ values 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively, has been reported (Chang and Franden, 1981). On the other hand, a lower Kₘ value (0.058 mM) was obtained for L-asparaginase from *Er. chrysanthemi* 3937 (Kotzia and Labrou, 2007).

In case of the purified L-asparaginase from *P. carotovorum* MTCC 1428 substrate affinity in terms of Kₘ was very low (0.657 mM) which was 5 - 9 times lower than the reported cytosolic L-asparaginase (Avramis and Tiwari, 2006). The affinity of L-asparaginase for its natural substrate was related to its degree of effectiveness against sensitive tumors. It is known that the L-asparaginase with Kₘ of 5.0 mM or less is used as a drug (El - Bessoumy *et al.*, 2004 and Prista and Kyriakidis, 2001).

Based on the Lineweaver-Burk analysis, the Kₘ and Vₘₐₓ values of L-asparaginase from *Pseudomonas aeruginosa* 50071 were 0.147 mM and 35.7 IU, respectively. This indicates the high affinity of the enzyme to the substrate. L-asparaginase of different microorganisms which has different substrate affinities and
probably plays different physiological roles in the enzyme activity (El-Bessoumy et al., 2004). Higher $K_m$ values (2.5 mM and 3.5 mM) for L-asparaginase from *C. glueamicum* and *E. coli*, respectively, have been reported (Willis and Woolfolk, 1974). On the other hand, a lower $K_m$ value (0.074 mM) was obtained for L-asparaginase from *Vibrio succinogenes* (Willis and Woolfolk, 1974).

The affinity of L-asparaginase to its substrate is related to its degree of effectiveness against tumors (James et al., 1966). The substrate affinity of L-asparaginase as measured by $K_m$ value was found to be $2.4 \times 10^{-5}$ M for L-asparagine. This compares favorably with $K_m$ values reported for antineoplastic L-asparaginases from other sources such as *E. coli* (1.25 x $10^{-5}$ M) (Scheetz et al., 1971), *Proteus vulgaris* (2.6 x $10^{-5}$ M) (Tosa et al., 1972) and *Erwinia aroideae* NRRL Q - 138 (3.0 x $10^{-5}$ M) (Tosa et al., 1972).

**Molecular Weight of L - asparaginase**

L-asparaginases purified from *Pseudomonas stutzeri* MB - 405, *Thermus thermophilus* and *Escherichia coli* were with smaller molecular values ranging from 33 - 34 kDa, (Manna et al., 1995, Prista and Kyridio, 2001; Soares et al., 2002). Purified L-asparaginase from *Bacillus* sp. (Moorthy et al., 2010), *Streptomyces gulbargensis* (Amena et al., 2010), *Streptomyces albido flavus* (Narayana et al., 2007) and *Streptomyces* PDK2 (Dhevagi and Poorani, 2006) exhibited a molecular weight of 45, 85, 112 and 140 kDa, respectively. Reports on production and purification of L-asparaginase from *Pseudomonas aeruginosa* by SDS PAGE revealed a peptide chain with molecular weight of 160 kDa (El-Bessoumy et al., 2004) and *Corynebacterium glutamicum* (Mesas et al., 1990) with Molecular weight of 80 kDa.

The molecular weight of the subunits of *Pectobacterium carotovorum* L-asparaginase was found approximately 36.5 kDa by SDS - PAGE analysis. The purified L-asparaginase has an approximate molecular weight of 146 ± 2.4 kDa as assessed by Native PAGE (Sanjay Kumar et al., 2011). The previous observation, which bacterial L-asparaginase exists as a tetramer of identical subunits with molecular mass in the range of 140-180 kDa (Aghaiypour et al., 2001; Aung et al., 2000; Kozak and Jurga, 2002; Prakasham et al., 2010).
The purified L-asparaginase from *V. Succinogens* had a molecular weight of 146.0 kDa and subunit molecular weight of approximately 37.0 kDa in 10 per cent SDS - PAGE (Distasio *et al.*, 1976). The purified L-asparaginase from *Chalmydomonas* sp. showed a higher molecular weight of 275.0 kDa in Disc gel electrophoresis (Paul, 1982).

The L-asparaginase from *E. carotovora* is active as homotetramer and its crystal structure (PDB ID 1ZCF) and its functions have been thoroughly characterized with the approximate molecular mass of 150.0 kDa as determined by native PAGE (Papageorgiou *et al.*, 2008). The molecular mass of enzyme subunit is 36.5 kDa as observed from SDS - PAGE separation and gel filtration. The SDS - PAGE of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of purification. It revealed only one distinctive band that was indicated by the pure preparation of L-asparaginase. Native PAGE has exhibited an approximate molecular weight of 150.0 kDa, which indicated that L-asparaginase purified from *E. carotovora* was homogeneous. SDS - PAGE and gel filtration chromatography indicated that enzyme subunit is made of one band with approximate molecular weight of 36.5 kDa and 37.0 kDa, respectively. This value was equal to tumor inhibitory L-asparaginase (Wriston and Yellin, 1973).

Molecular weight of L-asparaginase from *Streptomyces albidoflavus* was 112.0 kDa (Narayana *et al.*, 2007). Molecular weight of asparaginases produced by microorganisms may vary as shown below; molecular weights of L-asparaginase from *C. glutamicum, Streptomyces* sp - PDK2 and *P. aeruginosa* 50079 have been determined as 80.0 kDa, 140.0 kDa and 160.0 kDa respectively by SDS - PAGE (Savitri *et al.*, 2003; Dhevagi and Poorani, 2006 and El -Bessoumy *et al.*, 2004).

Differences in the half - life of unmodified L-asparaginases from *Escherichia coli* have been described and attributed to small differences in iso - electric points (Mashburn and Landin, 1970), and Putter and Gehrman, 1969, have reported that a chemically modified *E. coli* L-asparaginase with a reduced iso - electric point has been used clinically and found to have a half - life of about 24 hrs, compared with about 11 hrs for the unmodified enzyme.
The isoelectric focusing showed that the pI of purified L-asparaginase from *P. carotovorum* MTCC 1428 was close to 8.4, which is similar to that of the enzymes from *Erwinia sp.* (8.7) and different for the *E. coli* enzyme (5.0) (Muller and Boos, 1998). Recently, Abakumova et al. (2009) reported that the pI of L-asparaginase isolated from *Yersinia pseudotuberculosis* was 5.4.

The alignment of the complete amino acid sequence of the peptides from spots showed a good alignment with tyrosinases from other Streptomyces species. *De novo* determined peptide sequences were deduced manually and used for similarity searches using the FASTA, MS - BLAST, and the MS - Homology algorithm. Several amino acid sequences of the peptides were identified and showed very high similarity with database sequences for other tyrosinases from *Streptomyces* species. PMF is a technique used to identify proteins by matching their constituent fragment masses (peptide masses) to the theoretical peptide masses generated from a protein or DNA database of the peptides. (Dolashki et al., 2009)

The targeted protein spots were identified by MALDI - TOF - TOF followed by peptide sequencing using PEAKS Studio 4.0 *de novo* sequencing software. The generalized schematic of the methodology used to compile a database for *Macaca mulatta*. The precursor mass, m/z error (ppm), PEAKS and SPIDER score for confidence interval (per cent) for the PEAKS *de novo* generated peptide sequences and their corresponding homology searches (Nilesh and Scott, 2006).

**Recombinant Extracellular L-asparaginase II**

The characterization of new recombinant L-asparaginase and the development of rapid, simple and effective production methods are not only of academic interest but also of practical importance (Avramis and Panosyan, 2005).

There are very few reports on the efficient release of recombinant proteins into the culture medium of *E. coli*. Although extracellular secretion of recombinant proteins has been achieved by co-expression of permeabilizing proteins, it leads to non-specific secretion and also requires controlled low level expression of the co-expressing gene to avoid problems of cell lysis and lethality (Makrides, 1996; Jonasson, 2002).

Recombinant L-asparaginase high expression levels were showed in high specific growth rates (Shokr et al., 2002). However stationary phase protein over expression is a
fundamental characteristic of *E. coli*, and fairly high-level expression of the target protein has been obtained previously with induction in the stationary phase (Ou *et al.*, 2004). Similarly higher solubility of the recombinant protein and lower proteolytic rates were reported with late log phase induction which resulted in enhanced protein yields (Galloway *et al.*, 2003). The extra cellular environment is the preferred location for the accumulation of recombinant proteins (Mukherjee, 2004).

The promoter region of an *Er. chrysantherni* pectate lyase gene has also been shown to be efficiently utilized in *E. coli* (Keen *et al.*, 1984). Secretion of L-asparaginase by *E. coli* was also demonstrated, as the mature enzyme was predominantly located in the periplasmic space. It follows that the L-asparaginase structural gene should contain an N-terminal signal peptide sequence.

The level of expression of the asparaginase gene when under lac promoter control (pASN42) was lower than when enzyme synthesis was regulated by the natural asparaginase promoter (pASN30). This was surprising as the lac promoter is widely recognized as one of the more powerful *E. coli* promoters (Rosenberg *et al.*, 1982). Two possible explanations are that: (i) the mRNA produced from the lac promoter is inefficiently translated, possibly due to secondary structure (e.g. Schoner *et al.*, 1984) or (ii) fusion of the N-terminal portion of the lad’ gene with the asparaginase structural gene results in a decreased enzyme activity.

*Pichia pastoris* strain was harboring the *S. cerevisiae* ASP3 gene produces L-asparaginase with specific yields of 800 Ug/L (Antonieta *et al.*, 2006). This value is seven-fold higher in comparison to that obtained using a nitrogen de-repressed strain of *S. cerevisiae* ure2 dal80 whose specific yield was of 115 Ug/L (Ferrara *et al.*, 2004).

Cloning and expression studies of L-asparaginase in *E. coli* has been reported. Cloning was done as a DNA fragment generated by PCR. The recombinant plasmid PASN, containing L-asparaginase gene using expression vector PBV 220, was transformed in *E. coli* host strains. Higher activity was found in recombinant enzymes. Recombinant L-asparaginase from *Er. carotovora* expressed in *E. coli* and purified was reported by Borisova *et al.*, 2003. Large quantity of L-asparaginase mRNA was measured by RQ-PR as described by Irino *et al.*, 2004. The L-asparaginase mRNA level paralleled the L-asparaginase enzyme activity and as protein level. Krasotkina *et al.* (2004) has used
chromatography technique for purification of Recombinant L-asparaginase from *Er. carotovora*. The kinetic properties show that recombinant L-asparaginase combined the main advantages of *Er. chrysanthemi* and *E. coli* L-asparaginase - II. Kotozia and Labrou, 2005 reported that recombinant L-asparaginase was produced by cloning L-asparaginase form *Er. carotovora* NCYC 1526 (Er A) and expression was done in *E. coli*, purification was carried out by anion exchange chromatography and affinity chromatography.

The secretory production of recombinant proteins has several advantages, such as simplicity of purification, avoidance of protease attack and better chance of correct protein folding. Generally, secretory proteins are synthesized in the cytoplasm as premature proteins containing a short (15 - 30 amino acid residues) signal sequence that allows proteins to be exported outside the cytoplasm. A number of signal sequences have been used for efficient secretory production of recombinant proteins in *E. coli*, including PelB, OmpA, PhoA, endoxylanase and StII (Choi and Lee, 2004). The efficiency of protein secretion also depends on the genetic properties of the host strain, the expression vector, the nature of the protein being secreted, the co-expression of facilitator proteins and physical factors such as temperature, medium pH, specific growth rate and percentage of dissolved oxygen (Khushoo *et al.*, 2005). Existence of C-terminal His-tag lead to the decrease of extracellular export (Khushoo *et al.*, 2005), although Khushoo and colleagues reported that asparaginase activity in culture medium is lower than periplasmic. Researchers demonstrated that induction of recombinant *E. coli* cells during late log phase has led to increasing rate of recombinant L-asparaginase expression yield (Galloway *et al.*, 2003).

**Applications of L-asparaginase**

Human Hepatoma Cell Lines is the sixth most common type of cancer in the World, with rates increasing from year to year and its incidence is mainly correlated with chronic hepatitis B and C virus infection and liver cirrhosis (Parkin *et al.*, 2005, Bosch *et al.*, 2004 and McGlynn and London, 2005). HCC treatment with conventional chemotherapeutic agents is inefficient, due to several side effects linked to impaired organ function typical of liver disease; in fact these therapies offer a probability of 5-year survival of 50 - 70 per cent (Bruix, and Sherman, 2005). Therefore, the limited
effectiveness of chemotherapy and high recurrence rate highlights the urgent need to identify new molecular targets and to develop new treatments.

**Molecular Pathways in Hepato Cellular Cancer tumorigenesis**

(Source: Cornelia Braicu et al., 2009)

Cappelletti *et al.*, 2008 have studied the in vitro cytotoxicity of a novel L-asparaginase from the pathogenic strain *Helicobacter pylori* CCUG 17874 against different cell lines. Pritsa *et al.*, 2001, also studied the anticancer activity of L-asparaginase from *Thermus thermophilus* against different tumour cell line include K-562 (chronic myelogenous leukemia), Burkitt’s lymphoma, SK-N-MC (primitive neuroectodermal tumour), HeLa (cervical cancer), BT20 and MCF-7 (breast cancers), HT-29 (human colon cancer) and OAW-42 (ovarian cancer).

Iwamoto *et al.*, 2007, recently described the protective effect of mesenchymal cells in L-asparaginase cytotoxicity. They postulated that the interaction between ALL cells and the micro environment in which these cells reside protected the leukemic cells from asparagines depletion by a high expression of asparaginase synthetase in these mesenchymalcells. Surviving leukemic cells may therefore be rescued with asparagines produced by mesenchymal cells.

Since cancer cells are usually very heterogenous, different cancer express different markers and even cells from the same tumor may not be identical (Pelosi *et al.*, 2003). Thus, single - marker RT - PCR has its limitations in sensitivity and specificity. In recent years, several different multi marker assays have been developed for the detection
of tumor cells in the peripheral blood of patients with various malignancies (Kuffer et al., 2002).

The assay is carried out using \((3 - (4, 5 - \text{dimethyl thiazol} - 2\text{yl}) - 2, 5 - \text{diphenyl tetrazolium bromide (MTT)})\). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity (Mantani, 2001). L-asparaginase were tested for cytotoxicity against leukemia cancer cell line, U937 and compared with two potent chemotherapeutics drug (melphalan and 5 - FU) by using MTT assay as described by Prayong et al., (2008) and Mossman (1983). IC\(_{50}\) value is common to demonstrate the cytotoxic activity in previous studies (Irino et al., 2004). Using MTT assay, the in vitro cytotoxicity effect of Bacillus sp R36 L-asparaginase enzyme on the growth of two tumor cell lines were studied. The IC\(_{50}\) values were calculated from the linear equation of the dose effect of the enzyme against hepatocellular carcinoma Hep G2 cells \((y = -0.4069x + 95.648)\) and against colon carcinoma HCT - 116 cells \((y = - 0.2233x + 98.838)\) (E-Moharam et al., 2010).

Analysis of CCRF - CEM cells indicated a consistent increase in the sub - G1 cell population in flow cytometry and suggesting that the growth phase of the cell cycle was affected by this novel L-asparaginase (Prakasham et al., 2010). The purified L-asparaginase enzyme from \(E.\ carotovora\) MTCC 1428 and the commercial preparation \((E.\ coli)\) have been subjected to cytotoxic activity \textit{in vitro} on the Hep - 2C cell line. The cytotoxic activity of the purified L-asparaginase from \(E.\ carotovora\) MTCC 1428 and commercial preparation from \(E.\ coli\) were found to be similar at higher concentrations. However, the L-asparaginase purified from \(E.\ carotovora\) MTCC 1428 showed better toxicity on Hep - 2C cell line (84 per cent survival) in comparison to commercial L-asparaginase preparation (90 per cent survival) obtained from \(E.\ coli\). The sensitivity of the cell line to both L-asparaginases (purified and commercial preparation) appeared to be dose dependent, resulting in the significant decrease in viable cells. The treatment of cancerous cell line with increasing concentration of L-asparaginase (upto 0.25 IU) resulted in appreciable inhibition of cell growth. (Wmikazmi, 2012)
Cell cycle arrest and apoptosis of leukaemia cells induced by L-asparaginase from E. coli was observed by Ueno et al. (1997). Deprivation of L-asparagine from the culture of L5178Y cells by L-asparaginase caused the fragmentation of chromosomal DNA of the leukemia cells within 24 hrs. Prior to the degradation of DNA, cell cycle of L5178Y cells were found to be arrested in G1 phase, and evidence of the DNA strand breaks was initially observed in G1 phase cells as early as 8 hrs after the L-asparaginase treatment. Therefore, apoptosis of leukemia cells induced by L-asparaginase was found to be an event that has been associated with the cell cycle arrest in G1 phase.

Ando et al. (2005) have reported the selective apoptosis of natural killer cell tumors by L-asparaginase. Cappelletti et al. (2008) studied in vitro cytotoxicity of a novel L-asparaginase from the pathogenic strain H. pylori CCUG 17874 against different cell lines. They reported that AGS and MKN 28 gastric epithelial cells being the most affected. The antitumor activity of L-asparaginase from chicken liver was investigated by EI-Sayed et al. (2011).

The enzyme inhibited the growth of the two human cell lines including hepatocellular carcinoma (HepG2) and colon carcinoma (Het - 116) with IC$_{50}$ value of 8.38μg/mL and 4.67 μg/mL, respectively. However, IC$_{50}$ was greater than 10 μg/mL/well for MCF7 (breast carcinoma) cell line. L-asparaginase was identified as the factor responsible for the cellcycle inhibition of fibroblasts and gastric cell line [Scotti et al., 2010]. Its effect on cell - cycle was confirmed by inhibitors, a knockout strain and the action of recombinant L-asparaginase on cell lines. Interference with cell - cycle in vitro depended on cell genotype and was related to the expression levels of the concurrent enzyme asparagine synthatase. L-asparaginase from pathogenic bacteria E. coli showed cytotoxicity to U937 cell line with IC$_{50}$ 0.5 ± 0.19 IU/ml, and selectivity index (SI = 7.6) about 8 time higher selectivity over the lymphocyte cells (Aljewari et al., 2010).

One component of the immune system that molds the host response to foreign pathogens is cytokines, which are secreted or membrane - bound proteins that regulate the growth, differentiation, and activation of immune cells (Wiltrout, 2000; Lalla et al., 2004). Cytokines are released in response to a diverse range of cellular stresses including infection, inflammation, and carcinogen - induced injury. These proteins are generally grouped into two categories, pro - inflammatory or anti - inflammatory, although several
cytokines do not fit specifically into either category (Lin et al., 2003; kitoka et al., 2003; song et al., 2003). In fact, many cytokines have pleiotrophic functions, and some can act in a synergistic manner. Several cytokines, particularly those produced by CD4 - T_H cells, are defined as T_H1 or T_H2 and are comprised mainly of ILs. T_H1 cytokines (e.g., IL-1, IL-2, IL-12p35, IL-12p40, IL-15, and non - ILs, e.g., TNF-α and IFN-γ) are generally referred to as pro - inflammatory, and the T_H2 cytokines (e.g., IL-4, IL-8, IL-10, and IL-5) induce anti - inflammatory responses (Akiba et al, 2001, wagner et al., 2001, Wang et al., 2003). Cytokines normally function to stimulate a host response aimed at controlling cellular stress and minimizing cellular damage. The failure to resolve an injury can provoke excessive immune cell infiltration and would lead to persistent cytokine production. Therefore, the host response to stress provokes changes in cytokine expression, which can impact several stages of cancer formation and progression. The liver hosts many cell types that are susceptible to the actions of cytokines. The effect of cytokine function on tumorigenesis and progression is complex as a result of the pleiotropy and apparent redundancy of cytokine action.


The opposing roles of pro - and anti - inflammatory cytokines were involving in HCC metastasis. T_H1 and T_H2 cytokines generally function as pro - or
anti-inflammatory factors, respectively. Some TH2 cytokines, such as IL-10, can negatively regulate TH1 cytokines, such as IFNc, and vice versa. In the pro-inflammatory process, an insult will signal TH1 cytokines to activate immune cells such as macrophages and NK cells, which generate a pro-inflammatory response that is anti-metastatic. Conversely, certain conditions, such as the presence of a tumor or genetic predisposition, may cause immune cell populations to become “alternatively activated” and contribute to carcinogenesis by functioning to promote anti-inflammatory cytokines and the humoral response (e.g., mast cells, B cells, and eosinophils), thus contributing to metastasis. The causative factor associated with switching the cytokine balance is unknown, but factors produced by the tumor or by the microenvironment might play a role in tumorigenesis by polarizing cytokine production toward a TH2 phenotype (Wang and Budhu, 2006).

Mesenchymal stem cells (MSCs) may contribute to the tumor growth and progression through several mechanisms among which their immunomodulatory effects have great and special importance (Uccelli et al., 2008). It has been proposed that they may inhibit both innate and adaptive immune cells through the expressions of prostaglandin E2 (PGE2), TGF-b1, indoleamine 2, 3 - dioxygenase (IDO), hepatocyte growth factor (HGF) and inducible nitric-oxide synthase (iNOS) (Rasmusson et al., 2003). Also, it has been reported that MSCs induce downregulation of MHC class II molecules and IL-12 production which may lead to the defective CD4+ T cell activation and function (Rasmusson et al., 2003). They inhibit the proliferation of T cells and cause decreased IFNc and increased IL-4 productions by T helper 2 (TH2) cells, which leads a shift in immune responses from a pro-inflammatory to an anti-inflammatory response (Aggarwal et al., 2005). It has been demonstrated that MSCs have the ability to downregulate CTL-mediated cytotoxicity and to inhibit B-cell proliferation and differentiation (Corcione et al., 2006). Interestingly, they induce the generation and proliferation of regulatory T cells through the stimulation of IL-10 productions and the release of HLA-G5 (Selmani et al., 2008).

VEGF is considered one of the most important factors involved in tumorassociated angiogenesis, involved in neovascularization (Mas et al., 2007), development and/or progression of HCC, being associated with a poor survival (Zhu and
Raymond, 2009). HCC patients with level of serum and tissue VEGF over expression have a lower survival rate (Zhou et al., 2006). Another study was demonstrated for the first time that retinoblastoma protein (pRb2/p130) is inversely correlated with VEGF expression and tumor aggressiveness. VEGF together with pRb2/p130 may act a diagnostic or prognostic indicator in HCC (Claudio et al., 2004 and Spinizi and paggi, 2008).

In the cancer cells, Szajnik et al. demonstrated that activation of NF-κB induced by lipopolysaccharide (LPS), was responsible for the production of cytokines such as IL-6, IL-8 and VEGF in SKOV3 cells (Szajnik, 2009). Triggering of Toll-like receptor 4 (TLR4) by LPS induced tumor promotion by the induction of proliferation, activation of NF-κB, p65 binding to DNA, and resistance to NK cell-mediated cytotoxicity accompanied by the increased production of proinflammatory cytokines (IL-6 and IL-8), VEGF (Szczepanski et al., 2009). Abbas Ghaderi et al., 2011 has studied about high amounts of anti-inflammatory cytokines such as IL-4 and IL-10 and angiogenic mediators such as VEGF, MMP2, IL-8 and SDF-1 in normal Adipose derived stem cells ASCs supplemented with the supernatant of ASCs isolated from stage III breast cancer patients compared to those cultured with stage II and MCF7 supernatants.