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Microbial L-asparaginase as a Potential Therapeutic Agent for the Treatment of Acute Lymphoblastic Leukemia: The Pros and Cons

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Abstract: L-asparaginase has been used as an effective antineoplastic agent. Asparaginases are the cornerstones of treatment protocols for acute lymphoblastic leukemia (ALL), it has been an integral part of combination chemotherapy protocols of pediatric acute lymphoblastic leukemia for almost 3 decades and in the majority of adult treatment protocols. The clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumor cells unable to synthesize this amino acid are selectively killed by L-asparagine deprivation. However, L-asparaginase can cause hypersensitivity in the long-term used, leading to allergic reactions and anaphylaxis. Hence, studies are continued and focused on abatement of immune reactivity either by modifying the L-asparaginase using pegylation or by the screening of soil samples from various sources for isolation of potential microbes which have the ability to produce the desired enzyme. The search for other L-asparaginase sources, can lead to an enzyme with less adverse effects. The discovery of new L-asparaginase serologically different but having similar therapeutic effects is highly desirable.

Key words: Microbial L-asparaginase, antineoplastic agent, acute lymphoblastic leukemia

INTRODUCTION

L-asparaginase (L-asparagine aminohydrolase, E.C. 3.5.1.1) belongs to an amidase group that hydrolyses the amide bond in L-asparagine to L-aspartic acid and ammonia (Kumar and Verma, 2012). L-asparaginase is the first enzyme with anti-leukemic activity which has been thoroughly researched by many researchers throughout the world (Savitri and Azmi, 2003). It is an effective antineoplastic agent, used in the acute lymphoblastic leukemia (ALL) chemotherapy (Narta *et al*., 2007). The pioneer observation that turned out to be important for the development of L-asparaginase as a potential antineoplastic agent was made by Clementi (1922), revealing that the serum of guinea pig is a rich source of L-asparaginase. Kidd (1953) demonstrated the ability of guinea pig serum to inhibit the growth of transplantable lymphoid tumours in mice and rats as well as certain spontaneous and radiation-induced leukemias in mice. At the same time, several other observations were made. First, the inhibition was produced by the guinea pig sera alone and the sera from other animal species did not show this effect. Second, only certain tumor types were susceptible to guinea pig serum mediated growth suppression. Third, guinea pig serum inhibited the *in vivo* growth of certain lymphoma tumor cell lines but it failed to inhibit the *in vitro* growth of the same tumor cell lines. Tsuji (1957) reported the deamidation of L-asparagine by extracts of *E. coli* to L-aspartic acid and ammonia. Mashburn and Wriston (1964) successfully purified L-asparaginase from cell extract of *E. coli* and demonstrated its tumour-inhibitory activity similar to that of guinea pig serum. Broome (1961) discovered that the regression of lymphosarcoma transplants in mice treated with guinea-pig serum was due to the nutritional dependence of the malignant cells on exogenous L-asparagine and provided evidence that L-asparaginase in the serum is the antitumor factor (Pritsa *et al*., 2001).

The demand for L-asparaginase will increase several fold in coming years due to its potential industrial applications as food processing, aid in addition to its clinical applications (Pedreschi *et al*., 2008). Use of L-asparaginase has revolutionised the anti-leukemia therapy in acute lymphoblastic leukemia (Patil *et al*., 2011; Pieters *et al*., 2011; Jain *et al*., 2012). Its antitumor effect results from the depletion of asparagine, an amino acid essential to leukemia cells and subsequent inhibition of protein synthesis leading to cytotoxicity. However, its use has been limited by a high rate of hypersensitivity in the long-term used (Reynolds and Taylor, 1993) and

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development of anti-asparaginase antibodies which causes an anaphylactic shock or neutralization of the drug effect. To overcome these limitations, modified versions of L-asparaginase (such as L-asparaginase from other new sources, pegylated formulations and L-asparaginase loaded into erythrocytes) have been recently proposed (Thomas *et al*., 2010). The L-asparaginases of *Erwinia chrysanthemi* and *E. coli* have been employed for many years as effective drugs in the treatment of acute lymphoblastic leukemia and leukemia lymphosarcoma (Kozak *et al*., 2002; Graham, 2003) but their therapeutic response rarely occurs without some evidence of toxicity (Duval *et al*., 2002).

Literature reports indicated that the enzyme's biochemical and kinetic properties vary with the genetic nature of the microbial strain used (Eden *et al*., 1990). For example, *Erwinia* L-asparaginase exhibited less allergic reactions compared to the *E. coli* enzyme. However, *Erwinia* L-asparaginase had a shorter half-life than *E. coli* (Asselin *et al*., 1993), suggesting the need to discover new L-asparaginases that are serologically different but have similar therapeutic effects. Therefore, there is a continuing need to screen newer organisms in order to obtain strains capable of producing new and high yield of L-asparaginase with less adverse effects.

ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Leukemia is a malignant cancer of the bone marrow and blood, characterized by an uncontrolled accumulation of abnormal blood cells leading to the inhibition of normal blood cell functions and in many instances, death. Leukemia causes more deaths than any other cancer among children aged around twenty (Kwan *et al.,* 2009). Acute lymphoblastic leukemia is cancer of white blood cells (WBC), the cells that normally fight infections characterized by the excessive multiplication of malignant and immature WBC (lymphoblast) in bone marrow. Treatment of acute leukemia includes chemotherapy, steroids, radiation therapy and intensive combined treatments including bone marrow or stem cell transplants. Among these, chemotherapy is most preferred. The drugs being employed for treatment includes prednisolone, dexamethasone, vincristine, L-asparaginase, daunorubicin, cyclophosphamide, cytarabine, etoposide, thioguanine, mercaptopurine, hydrocortisone, methotrexate etc. Although, variety of drugs are available todays but their efficacy in treatment of cancers at third and fourth stage is doubtful. Also, the side effects caused by these chemotherapeutic agents are many such as infertility, secondary neoplasm, nausea and vomiting, immunosuppression etc (Jain *et al*., 2012). Among the antitumour drugs, bacterial enzyme L-asparaginase has been employed as the most effective chemotherapeutic agent in pediatric oncotherapy especially for acute lymphoblastic leukemia. The most common therapeutic indications of L-asparaginase are: Treatment of Hodgkin disease, treatment of acute lymphocytic leukemia (mainly in children), acute myelocytic leukemia, acute myelomonocytic leukemia and chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma (Stecher *et al*., 1999).

L-ASPARAGINE AND ITS ROLE IN NORMAL AND MALIGNANT CELLS

L-asparagine is an amino acid required by cells for the synthesis of protein molecules and survival of the cells. L-asparagine found in many vegetables, with higher concentrations in some varieties of potatoes. L-asparagine is not an essential amino acid in normal cells. L-asparagine can be synthesised by body itself, within the cell by an enzyme called L-asparagine synthetase from aspartic acid and glutamine (Fig. 1) or can be absorbed from the outside (consumed in the diet, absorbed into the body and made available to the body's cells). However, neoplastic cells cannot produce L-asparagine due to the absence of L-asparagine synthetase (Savitri and Azmi, 2003). Therefore, they obtain the required L-asparagine from circulating pools for their growth. Thus, depletion of L-asparagine in the circulating pools starves the tumor cells, leads to the destruction of the tumour cells, since they are unable to complete protein synthesis.

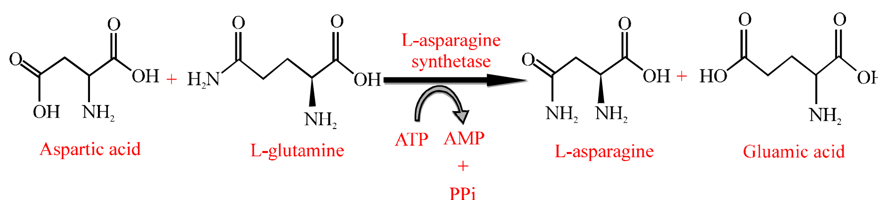

Fig. 1: Biosynthesis of L-asparagine. Aspartate is transaminated to asparagine in an ATP-dependent reaction catalyzed by asparagine synthetase and glutamine is the amino group donor

Unlike normal cells, malignant cells can only slowly synthesize L-asparagine and are dependent on an exogenous source of L-asparagine for survival (Lee *et al*., 1989). Prager and Bachynsky (1968) reported that leukemic lymphoblasts and certain other tumor cells lack or have very low levels of L-asparagine synthetase, do not synthesize L-asparagine de novo and thus rely on asparagine present in serum for their proliferation and survival. Neoplastic cells cannot synthesize L-asparagine due the absence of L-asparagine synthetase (Keating *et al*., 1993). Tumor cells, more specifically lymphatic cells, require huge amount of L-asparagine to keep up with their rapid malignant growth. This means they use both asparagine from the diet (blood serum) as well as what they can make themselves (which is limited) to satisfy their large L-asparagine demand. Therefore, L-asparagine is an essential amino acid for the growth of tumor cells, whereas the growth of normal cells is not dependent on its requirement as it can be synthesized in amounts sufficient for their metabolic needs with their own enzyme L-asparagine synthetase. The presence of L-asparaginase results in depletion of serum L-asparagine and kills tumor cells by depriving them of an essential factor required for protein synthesis. In contrast, normal cells are protected from L-asparagine-starvation due to their ability to produce this amino acid (Duval *et al*., 2002), with the help of the enzyme L-asparagine synthetase, which is present in sufficient amounts and thus are less affected by its rapid depletion produced by treatment with the enzyme L-asparaginase. L-asparaginase also inhibits protein synthesis by L-asparagine hydrolysis. The general medical approach to leukemia therapy is therefore based on a metabolic defect in L-asparagine synthesis of some malignant cells (Ravindranath *et al*., 1992).

L-ASPARAGINASE TYPES AND STRUCTURE

Two related families of L-asparaginase are designated type I and type II according to the terminology in *E. coli*. L-asparaginase I (*AnsA*) is a low-affinity enzyme found in the cytoplasm, synthesis of cytoplasmic L-asparaginase I is constitutive whereas L-asparaginase II (*AnsB*) is a high-affinity periplasmic enzyme, is activated during anaerobiosis. *AnsB* is changed by aeration, carbon source and variation of available amino acids (Jennings and Beacham, 1990). Further, only the type II enzyme has shown substantial anti-tumor activity (Usha *et al*., 2011).

The functional form of *E. coli* L-asparaginase II exists as a tetramer of identical subunits denoted A-D (Kozak *et al*., 2002) bound mainly by non-covalent forces with molecular mass in the range of 140-160 kDa (Aung *et al*., 2000; Aghaiypour *et al*., 2001a, b; Kozak *et al*., 2002). Each monomer consists of about 330 amino acid residues that form 14 β-strands and 8 α-helices, arranged into two easily identifiable domains, the larger N-terminal domain and the smaller C terminal domain, connected by a linker consisting of~20 residues (Pourhossein and Korbekandi, 2014). Each of the four active sites is located between the N- and C-terminal domains of two adjacent monomers. Thus, the L-asparaginase tetramer can be treated as a dimer of dimers. Although, the dimers contain all the structural elements and functional groups to create a complete active site environment, the active enzyme is always a tetramer (Khushoo *et al*., 2004) with one active center each and specifically catalyses the hydrolysis of L-asparagine producing L-aspartic acid and ammonia. Despite this fact, the active enzyme is always a tetramer created by subunits A and C or subunits B and D (Khushoo *et al*., 2004). The active site is formed by strictly conserved residues. Part of the active site is a flexible loop (between residues 10 and 40) that contains the two important residues Threonine-12 and Tyrosine-25 (Aghaiypour *et al*., 2001a, b). Threonine-12 is the nucleophile involved in the acylation reaction (Aung *et al*., 2000). Access to the active site cavity is controlled by this flexible loop that opens and closes in a ligand dependent fashion (Aung *et al*., 2000; Kozak *et al*., 2002). The amino acid sequence of several different asparaginases has been reported, including that of *E. coli* enzyme (Wriston, 1985).

L-ASPARAGINASE BRAND NAMES

Some of the commercially available L-asparaginase is existed under the brand name A-ase, ASN-ase, Colapase, Crasnitin, Elspar, Crisantas, Pasum, PEG-asparaginase and Pegasparagasum.

INTRACELLULAR AND EXTRACELLULAR L-ASPARAGINASE

Most of the microbial L-asparaginase is intracellular in nature except few which are secreted outside the cell (Narayana *et al*., 2008). Extracellular L-asparaginase is more advantageous than intracellular type because of higher accumulation of enzyme in culture broth under normal conditions, easy extraction and downstream processing (Amena *et al*., 2010), the extracellular L-asparaginase in bacteria is protease deficient and the liberated protein exported to the medium is mostly soluble, biologically active and has an authentic N-terminus, relatively free from endotoxins those results in minimization of adverse effects. Secretion also facilitates proper folding of proteins specially that requiring disulfide bridge formation, as it passes through a more favorable redox potential in the periplasmic space.

BIOCHEMICAL AND ENZYME KINETIC PROPERTIES

Several research groups have studied L-asparaginase production and purification in attempt to minimize impurities that produce allergenic reations (Campbell *et al*., 1967; Gallagher *et al*., 1988). In general, it is noticed that biochemical and enzyme kinetic properties vary with the microbial source. A source-dependent variation of physicochemical and biochemical properties, like optimum pH, temperature, substrate specificity, inhibition pattern, etc., of microbial enzymes is well documented (Eden *et al*., 1990). For example, *Erwinia* asparaginase is considered less toxic and is frequently employed compared with allergic reactions to *E. coli* asparaginase. However, *Erwinia* asparaginase had a shorter half life than *E. coli* (Asselin *et al*., 1993). Biochemical properties of *Yersinia pseudotuberculosis* L-asparaginase are similar with those of *E. coli* type II L-asparaginase (Pokrovskaya *et al*., 2012).

STABILITY OF L-ASPARAGINASE

The anti-tumour activity of L-asparaginase is a function of its half-life in the blood (Fernandes and Gregoriadis, 1997). Therefore, 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 mono methoxypolyethylene glycol (mPEG) (Kamisaki *et al*., 1982) which is on 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.

COMPARATIVE EVALUATION OF *E. COLI* AND *ERWINIA* L-ASPARAGINASE

Comparative evaluation of L-asparaginase for its potential activity from different microbial sources revealed that biochemical and therapeutic properties differ with source of strain in addition to enzyme properties. L-asparaginases from *Erwinia chrysanthemi* and *E. coli* are currently in clinical use as effective drugs in the treatment of ALL. They are also used in the treatment of Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma (Stecher *et al*., 1999; Duval *et al*., 2002). Because the L-asparaginases from *E. coli* and *Erwinia* possess different immunological specificities they offer an important alternative therapy if a patient becomes hypersensitive to one of the enzymes (Lee *et al*., 1989). Comparison of the two enzymes, lead to the conclusion that *E. coli* L-asparaginase can be recommended for first-line therapy, reserving *Erwinia* asparaginase for allergic patients, because most patients allergic to the *E. coli* asparaginase are not immediately allergic to the *Erwinia* asparaginase, which is reported to be less toxic (Duval *et al*., 2002). The toxicity is partially attributable to the glutaminase activity of these enzymes (Howard and Carpenter, 1972). *Erwinia* asparaginase is considered to be comparably less toxic and is frequently employed in the event of allergic reactions to *E. coli* asparaginase although *Erwinia* asparaginase has a shorter half life than *E. coli* asparaginase (Konecna *et al*., 2004).

APPLICATION OF L-ASPARAGINASE

The demand for this enzyme is expected to increase several fold in coming years owing to its potential industrial applications in food industries besides its clinical applications.

In food industry as a food processing aid: During heating the amino acid L-asparagine, naturally present in starchy foods, is the precursor of the Maillard browning reaction (Mottram *et al*., 2002) which is responsible for giving baked or fried foods their brown color, crust and toasted flavor. Unfortunately, suspected potential human carcinogens such as acrylamide (Gokmen and Palazoglu, 2008) and some heterocyclic amines are also formed in Maillard reaction. Acrylamide is formed from L-asparagine and reducing sugars in starch-based foods that are heated above 120°C (Fig. 2) and prepared by frying or baking or broiling, including potato chips, French fries, cookies, reaction flavors etc. (Tareke *et al*., 2002). L-asparaginase promises to be a potential way to reduce the amount of free L-asparagine in the starting materials of food production, thus reducing the imminent risk of generating acrylamide, a potentially carcinogenic and neurotoxic compound (Friedman, 2003). L-asparaginase is also used commercially to reduce the formation of acrylamide in fried foods (Pedreschi *et al*., 2008). By adding L-asparaginase before baking or frying the food, asparagine is converted into another common amino acid, aspartic acid and

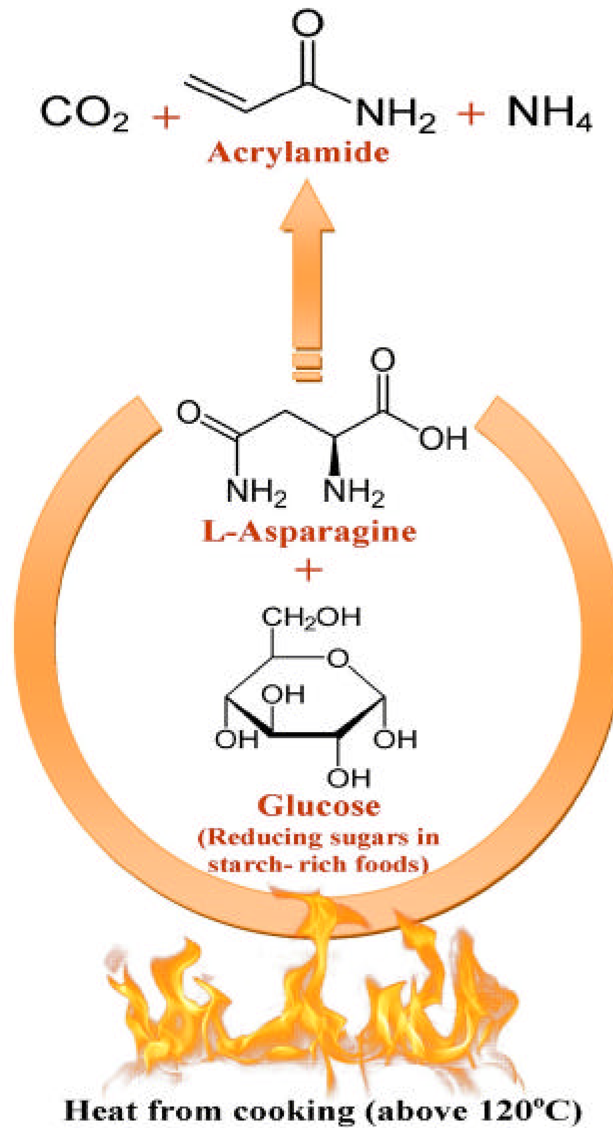

Fig. 2: Illustration of the formation of acrylamide: Potentially toxic carcinogen acrylamide is mainly formed through the heat-induced Maillard reaction between the amino group of the L-asparagine and carbonyl groups of reducing sugars in carbohydrate-rich foods (such as potatoes or cereals forms) at temperatures above 120°C. Less L-asparagine and reducing sugars means less acrylamide

ammonium ions. As a result, L-asparagine cannot take part in the Maillard reaction and therefore the formation of acrylamide is significantly reduced. L-asparaginase cleaves the L-asparagine in fresh starchy foods and ultimately reduces the levels of acrylamide formation in fried potato chips and french fries (JECFA, 2001; Pedreschi *et al*., 2008). Complete acrylamide removal is probably not possible due to other, minor L-asparagine independent formation pathways (Kornbrust *et al*., 2010). As a food processing aid, L-asparaginases can effectively reduce the level of acrylamide up to 90% in a range of starchy foods without changing the taste and appearance of the end product (Hendriksen *et al*., 2009). Recombinant L-asparaginase of *Aspergillus niger* and *Aspergillus*

oryzae used in processing of starchy food products. It converts the amino acid asparagine to aspartic acid then reduces acrylamide formation during processing of high starch food products (Pedreschi *et al*., 2008) (Fig. 2).

In biosensors: L-asparaginase is used for monitoring asparagine levels in mammalian and hybridoma cells (Taeymans *et al*., 2005).

L-asparaginase as a therapeutic agent: L-asparaginase constitutes one of the most biotechnologically and biomedically important group of therapeutic enzymes accounting for about 40% of the total worldwide enzyme sales. Global requirements of antileukemic and antilymhoma agents are far greater than those of other therapeutic enzymes of which L-asparaginase contributes one third (Hosamani, 2012). L-asparaginase is an important enzyme as therapeutic agents used in combination with other drugs in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma (Stecher *et al*., 1999; Verma *et al*., 2007). L-asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings (Savitri and Azmi, 2003). L-asparaginase used in the treatment of acute lymphoblastic leukemia was the major breakthrough in modern oncology as it induces complete remissions in over 90% children within four weeks (Gallagher *et al*., 1988). This enzyme is the drug of choice used in combination therapy with other drugs in the treatment of acute lymphoblastic leukemia in children (Verma *et al*., 2007). The role of L-asparaginase in lymphocytic leukemia cells treatment is based on the fact that these cells cannot synthesize L-asparagine due the absence of L-asparagine synthetase and are rely on the exogenous sources to get hold of L-asparagine (Lee *et al*., 1989; Keating *et al*., 1993). On the contrary, normal cells are protected from L-asparagine starvation due to their ability to generate this essential amino acid (Duval *et al*., 2002). For this reason the commonest therapeutic practice is to inject intravenously free enzyme in order to decrease the blood concentration of L-asparagine (the depletion of asparagine) affecting selectively the neoplastic cells (Mitchell *et al*., 1994) through inhibition of protein synthesis, cell cycle arrest in the G1 phase and apoptosis in susceptible leukemic cell populations which leads to cells death.

L-asparaginase as antioxidant: L-asparaginase has an anti-oxidant property (Moharam *et al*., 2010).

MECHANISM OF ACTION OF L-ASPARAGINASE

The mechanism of action of L-asparaginase is based on the fact that lymphocytic leukemic cells are deffcient in L-asparagine synthase and hence are dependent on an exogenous supply of L-asparagine. On treatment with L-asparaginase which catalyzes the conversion of L-asparagine to L-aspartate and ammonia (Fig. 3), the growth of these cells is critically affected with the rapid depletion of L-asparagine from the blood supply and the surrounding tissue (Muller and Boos, 1998).

Cells require a steady supply of the amino acid L-asparagine to build proteins. Most cells use the enzyme asparagine synthetase to make their own asparagine. The enzyme takes L-aspartate and adds an amine, forming the characteristic amide group of asparagine. Thus, most cells can make their own supplies of asparagine and do not need to obtain it in their diet. L-asparaginase performs the opposite reaction, it takes L-asparagine and pulls off its amine, releasing L-aspartate and ammonia (Hosamani, 2012). As a result, of the hydrolysis of L-asparagine, accumulation of large amount of L-aspartic acid and ammonia have been reported (Miller *et al*., 1969). However, in human body, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxaloacetate in the gluconeogenic pathway leading to glucose formation (Sieciechowicz *et al*., 1989). However, if a large dose of this enzyme is introduced into the blood, it will circulate and continually break down all the asparagine that it finds, ultimately starving the cells that rely on the blood-borne supply. The enzyme cuts off the supply of L-asparagine in the blood and the cancer cells die as they become unable to build their proteins (Graham, 2003). The enzyme also prevents free diffusion of L-asparagine from the surrounding tissues to the cancerous cells. Because of high pressure in the blood stream, the enzyme molecules can pass into the intercellular spaces from fine

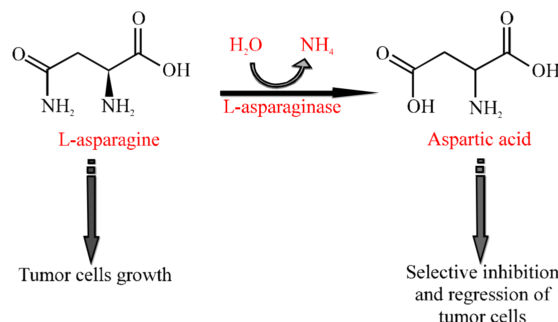

Fig. 3: Schematic illustration of mechanism of action of L-asparaginase

capillaries and catalyze the hydrolysis of L-asparagine (Putter, 1970). Reports have appeared demonstrating that L-asparaginase sequentially inhibits protein, RNA and DNA synthesis (Ellem *et al*., 1969) and L-asparaginase inhibition of glycoprotein synthesis in leukemic cells *in vitro* (Bosmann and Kessel, 1970) has been described. The reaction mechanism is represented in Fig. 3.

MICROBIAL SOURCES OF L-ASPARAGINASE

L-asparaginase is present in a wide range of organisms including animals, microbes, plants and in the serum of certain rodents but not in human beings (El-Bessoumy *et al*., 2004). Although, L-asparaginase has been found in various plant and animal species, due to the difficulty in extraction procedure of this enzyme, other potential sources like microorganisms were searched. Microorganisms like bacteria, fungi, yeast, actinomycetes and algae (Table 1) are very efficient producers and the better source of L-asparaginase, because they can be cultured easily and the extraction and the purification of L-asparaginase from them are also convenient, facilitating the large scale production (Patro *et al*., 2011). The enzyme properties vary from organism to organism, literature reports indicated that the enzyme's biochemical and kinetic properties vary with the genetic nature of the microbial strain used (Eden *et al*., 1990). The importance of microorganisms as L-asparaginase sources has been focused since the time it was obtained from *E. coli* and its antineoplastic activity demonstrated in guinea pig serum (Boyse *et al*., 1967). The *E. coli* and *Erwinia* enzymes were isolated, purified and experimentally used as an anti-leukaemic agent in human patients (Story *et al*., 1993).

Bacteria: The major bacterial species that produce L-asparaginase include *E. coli* (Cedar and Schwartz, 1968; Khushoo *et al*., 2004), *Erwinia cartovora* (Maladkar *et al*., 1993; Kotzia and Labrou, 2005), *Pseudomonas stutzeri* (Manna *et al*., 1995), *Pseudomonas acidovoras* (Davidson *et al*., 1977a), *Erwinia aroideae* (Peterson and Ciegler, 1969), *Thermus thermophilus* (Pritsa *et al*., 2001), *Thermus aquaticus* (Curran *et al*., 1985), *Staphylococcus aureus* (Rozalska and Mikucki, 1992), *Staphylococcus* sp.-6A (Prakasham *et al*., 2007a), *Vibrio succinogenes* (Kafkewitz and Goodman, 1974), *Citrobacter freundi* (Davidson *et al*., 1977b), *Proteus vulgaris* (Tosa **et al.**, 1972), *Zymomonas mobilis* (Pinheiro *et al*., 2001), *Bacillus subtilis* (Fisher and Wray, 2002), *Bacillus licheniformis* (Golden and Bernlohr, 1985),

Table 1: Microbial sources of L-asparaginase

Microorganisms	References
Bacteria	
E. coli	Cedar and Schwartz (1968)
Erwinia cartovora	Maladkar *et al.* (1993)
Pseudomonas acidovoras	Davidson *et al.* (1977a)
Erwinia aroideae	Peterson and Ciegler (1969)
Thermus thermophilus	Pritsa *et al.* (2001)
Thermus aquaticus	Curran *et al.* (1985)
Vibrio succinogenes	Kafkewitz and Goodman (1974)
Staphylococcus aureus	Rozalska and Mikucki (1992)
Citrobacter freundi	Davidson *et al.* (1977b)
Proteus vulgaris	Tosa *et al.* (1972)
Zymomonas mobilis	Pinheiro *et al.* (2001)
Bacillus subtilis	Fisher and Wray (2002)
Bacillus licheniformis	Golden and Bernlohr (1985)
Bacillus circulans MTCC 8574	Hymavathi *et al.* (2009)
Enterobacter aerogenes	Mukherjee *et al.* (2002)
Serratia marcescens	Khan *et al.* (1970)
Corynebacterium glutamicum	Mesas *et al.* (1990)
Actinomycetes	
Streptomyces griseus	Dejong (1972)
Streptomyces sp. PDK2 and PDK7	Dhevagi and Poorani (2006)
Streptomyces noursei MTCC 10469	Dharmaraj (2011)
Streptomyces karnatakensis, Streptomyces venezuelae	Mostafa (1979)
Streptomyces tendae	Kavitha and Vijayalakshmi (2010)
Streptomyces gulbargensis	Amena *et al.* (2010)
Streptomyces albidoflavus	Narayana *et al.* (2008)
Streptomyces plicatus	Koshy *et al.* (1997)
Streptomyces aurantiacus	Gupta *et al.* (2007)
Nocardia levis MK-VL_113	Kavitha and Vijayalakshmi (2012)
Fungi	
Cylindrocarpon obtusisporum	Raha *et al.* (1990)
Fusarium roseum, F. saloni	Nakahama *et al.* (1973)
Aspergillus terreus	Baskar and Renganathan (2009)
Aspergillus niger	Mishra (2006)
Aspergillus tamari	Sarquis *et al.* (2004)
Mucor species	Mohapatra *et al.* (1997)
Aspergillus nidulans	Saxena and Sinha (1981)
Yeast	
Saccharomyces cerevisae	Oliveira *et al.* (1999)
Candida utilis	Kil *et al.* (1995)
Rhodosporodium toruloids	Ramakrishnan and Joseph (1996)
Candida guilliermondii	Stepnyan and Davtyan (1988)
Algae	
Chlamydomonas	Paul (1982)

Bacillus circulans MTCC 8574 (Hymavathi *et al*., 2009), *Enterobacter aerogenes* (Mukherjee *et al*., 2000), *Serratia marcescens* (Khan *et al*., 1970) have been found to produce L-asparaginase. *E. coli* and *Erwinia chrysanthemi* asparaginases have been used successfully in the treatment of leukemia for the last 40 years (Fu and Sakamoto, 2007) and L-asparaginase from *E. coli* and *E. carotovora* is currently in clinical use for the treatment of ALL (Savitri and Azmi, 2003). Production of this enzyme has also been reported from *Corynebacterium glutamicum* (Mesas *et al*., 1990).

Actinomycetes: Actinomycetes are also a good source for the production of L-asparaginase (Dhevendaran and

Annie 1999; Narayana *et al*., 2008). Among the actinomycetes, *Streptomyces* species are distributed widely in marine and terrestrial habitats (Pathom-Aree *et al*., 2006) and are of commercial interest due to their unique capacity to produce novel metabolites. They are responsible for the production of about a half of the discovered bioactive secondary metabolites, notably antibiotics, anticancer compounds and enzymes (Manivasagan *et al*., 2013). Several *Streptomyces* species such as *Streptomyces griseus* (DeJong, 1972), *S. karnatakensis, S. venezualae,* (Mostafa, 1979), *Streptomyces* sp. PDK2 and PDK7 (Dhevagi and Poorani, 2006) and *Streptomyces* sp. S3, S4 and K8 (Basha *et al*., 2009), *Streptomyces gulbargensis* (Amena *et al*., 2010) have been explored for L-asparaginase production. There are also some reports of L-asparaginase production from *Streptomyces plicatus* isolated from the alimentary canal of the fish, Gerres filamentosus (Koshy *et al*., 1997), some *Streptomyces* sp. associated with *Theraponjarbua* and *Villorita cyprinoides* (Dhevendaran and Anithakumari, 2002), *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongia diffusa* (Dharmaraj, 2011) and *Streptomyces aurantiacus* isolated from mangroves of Bhitarkanika (Gupta *et al.,* 2007). However, very little information is available on the production of L-asparaginase by the genus *Nocardia*, *Nocardia levis* MK-VL-113, (Kavitha and Vijayalakshmi, 2012).

Fungi: Certain L-asparaginase producing fungal species also isolated and studied include *Aspergillus tamari* (Sarquis *et al*., 2004), *Aspergillus terreus* (Baskar and Renganathan, 2009), *Aspergillus awamori* (Prakasham *et al*., 2007a), *Cylindrocarpon obtusisporum* (Raha *et al*., 1990) and *Fusarium* species like *Fusarium roseum*, *F. saloni* (Nakahama *et al*., 1973).

Yeast: L-asparaginase production is also reported by a yeast species *Saccharomyces cerevisae* (Oliveira *et al*., 1999), *Candida utilis* (Kil *et al*., 1995), *Candida guilliermondii* (Stepnyan and Davtyan, 1988), *Rhodosporodium toruloids* (Ramakrishnan and Joseph, 1996).

Algae: An algal species named *Chlamydomonas* (Paul, 1982).

OPTIMIZATION OF L-ASPARAGINASE PRODUCTION

The growth and natural product production of an organism are strongly influenced by medium composition, thus the optimization of media components and cultural conditions is an important step for bioprocess development (Suresh and Raju, 2013). Production of L-asparaginase is greatly influenced by fermentation media composition and culture condition factors such as temperature, pH, inoculum size, agitation rate and incubation time (Hymavathi *et al*., 2009).

No defined medium has been established for the optimum production of L-asparaginase from different microorganisms. Each organism has its own particular conditions for maximum enzyme production. Screening and evaluation of the nutritional and environmental requirements of a microorganism are important stages to develop and determine the overall economic feasibility of bioprocess (Venil *et al*., 2009). It is essential to screen and evaluate various nutritional and environmental requirements for microbial growth and subsequent biocatalyst production (Prakasham *et al*., 2005), as culture conditions that promote optimum enzyme production differ significantly with the nature of the micro-organism (Prakasham *et al*., 2007b).

The traditional one-factor at a time technique used for optimizing a multivariable system is not only time consuming but also overlook the effects of interactions among factors, leading to misinterpretations of results. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by using statistical experimental designs. Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, the first step is to screen the important parameters and the second step is to optimize those parameters (Nawani and Kapadnis, 2005). These have several advantages that included less experiment numbers, suitability for multiple factor experiments, search for relativity between factors and finding of the most suitable condition and forecast response (Chang *et al*., 2006). Response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system can be determined. The factorial design and regression analysis in the RSM helps to evaluate the effective factors and to study the interaction between these factors. This method use mathematical models to analyze the experimental data and to predict the relationship between the response and the variables (Rajendran and Thangavelu, 2007). Several researchers in biotechnology have applied these techniques for optimization of different parameters (El-Naggar *et al*., 2013a, b; El-Naggar and Abdelwahed, 2014; Rajendran and Thangavelu, 2007; El-Naggar *et al*., 2014).

L-asparaginase production throughout the world is carried out mainly by submerged fermentation (SF). This technique however, has many disadvantages. It is cost intensive and has low product concentration. In addition,

it generates excess of effluents and consequently needs handling and disposal of large volumes of waste water during downstream processing (El-Bessoumy *et al*., 2004). Since microorganisms growing in submerged culture utilize oxygen dissolved in the fermentation medium, the supply in vessels used for shaken cultures may become critical for microbial biosynthesis of specific end products. In addition, oxidation reduction mechanisms existing in the fermentation mixture may exert a chemical influence on biosynthetic products. Heinemann *et al*. (1970) observed that agitation in shaken culture was essential for optimal growth and that the tumor inhibitory enzyme, L-asparaginase, was produced during a period of zero dissolved oxygen concentration in the fermentation medium.

In recent years, the production of enzymes by solid state fermentation (SSF) has emerged. SSF is a very effective technique as the yield of the product is many times higher than in submerged fermentation (Arima, 1964). It also offers many other advantages including high product concentration, less risk of bacterial contamination, ease of product extraction (Lonsane *et al*., 1985), simple cultivation equipment, low energy requirements and less waste water generation and less environmental concerns regarding the disposal of solid waste (Pandey *et al*., 2001). L-asparaginase production in SSF has been reported earlier on soy bean meal (El-Bessoumy *et al*., 2004) and wastes from three leguminous crops-bran of *Cajanus cajan, Phaseolus mungo* and Glycine max (Mishra, 2006).

TOXICITY OF L-ASPARAGINASE TO NORMAL CELLS

Notwithstanding its high therapeutic efficacy, the therapeutic use of L-asparaginase by the patients exerts toxicity to normal cells which in turn causes the unpleasant side effects to the patients (Table 2). L-asparaginase administration has been limited by problems of hypersensitivity and the rapid clearance of the enzyme from the blood stream associated with long-term therapy. The L-asparaginases of *Erwinia* and *E. coli* have been employed for many years as effective drugs in the treatment of acute lymphoblastic leukemia and leukemia lymphosarcoma (Graham, 2003) but their therapeutic response rarely occurs without some evidence of toxicity (Duval *et al*., 2002). In case of toxicities arising out of inhibited protein synthesis, normal tissues with high rates of protein synthesis (e.g., liver, pancreas and coagulation systems) are most frequently affected by L-asparaginase therapy. The majority of patients experience evidence of hepatotoxicity (Ohnuma *et al*., 1970), L-asparaginase causes a wide spectrum of side effects, such as fever, skin rashes, hepatic dysfunction (may raise liver enzyme blood tests and may cause liver disease in some patients), pancreatitis, diabetes (may raise blood sugar levels), leucopoenia, neurological seizures and abnormal coagulation tests (may interfere with blood clotting) that may lead to intracranial thrombosis or haemorrhage (Duval *et al*., 2002).

Another limiting factor of L-asparaginase treatment is the development of hypersensitivity, which ranges from mild allergic reactions to anaphylactic shock (Moola *et al*., 1994). A number of other problems also occurred, including azotemia and central nervous system dysfunction (Oettgen *et al*., 1967). Many of the side effects of L-asparaginase are due to the fact that is a foreign protein.

Pancreatitis is a well-documented complication of L-asparaginase, occurs in 10-16% of patients with a mortality rate between 1.8 and 4.6% (Ohnuma *et al*., 1970; Nguyen *et al*., 1987). This toxicity resembles other drug-induced pancreatitis and is characterized by abdominal and/or back pain, anorexia, nausea and vomiting (Chabner *et al*., 1990). Some patients develop signs and symptoms of diabetes due to damage in islet cells and subsequent decrease in synthesis of insulin. Hyperglycemia may be more severe when L-asparaginase is administered in combination with prednisone but the risk can be reduced if L-asparaginase is administered after prednisone (Ortega *et al*., 1977).

Table 2: Toxicity profile of L-asparaginase therapy

System	Complications
Liver	Hepatic dysfunction. Elevation in transaminase and bilirubin levels, abnormal alkaline phosphatase levels. A decrease in serum albumin, fibrinogen and serum lipoprotein levels may raise liver enzyme blood tests
Central nervous system	Central nervous system dysfunction. Neurotoxicity (depression, lethargy, fatigue, somnolence, confusion, irritability, agitation, dizziness) occurs in up to 25% of adult patients treated with L-asparaginase but rarely occur in children
Pancreas	Pancreatitis, acute pancreatitis, hemorrhagic, diabetes due to damage in islet cells and subsequent decrease in synthesis of insulin
Coagulation system	Imbalances in the formation of clotting factors, thromboembolism in adults may lead to intracranial thrombosis or haemorrhage
Hypersensitivity	Ranges from mild allergic reactions to anaphylactic shock
Skin	Skin rashes

Neurotoxicity (depression, lethargy, fatigue, somnolence, confusion, irritability, agitation, dizziness) occurs in up to 25% of adult patients treated with L-asparaginase (Pochedly, 1977) but rarely occur in children (Table 1). Neurotoxity may also result from lack of L-asparagine and L-glutamine in the brain. While many patients had elevation in blood ammonia levels but a correlation between ammonia levels and degree of toxicity has not been firmly established. Ohnuma *et al.* (1969) reported three patients with severe central nervous disorders who had a marked improvement of symptoms after administration of asparagines. Another study reproducibly established a relationship between marked neurological symptoms and a pronounced increase of the ammonia blood level (Leonard and Kay, 1986) but this was not confirmed by others.

The toxicity of L-asparaginases is partially attributable to the glutaminase activity of these enzymes (Howard and Carpenter, 1972). The low rate of L-glutamine hydrolysis by the *E. coli* L-asparaginase EC-2 has been reported (Campbell *et al.*, 1967) and shown to be a characteristic of this enzyme. In contrast, the L-asparaginase from guinea pig serum does not catalyze the hydrolysis of L-glutamine (Meister *et al.*, 1955). L-glutamine is required for several metabolic pathways including the formation of L-asparagine by the enzyme L-asparagine synthetase (Prager and Bachynsky, 1968). The L-glutaminase activity may cause such a reduction in glutamine in the body that it limits the tolerable therapeutic dose. Now a day's most of the research is focused on production of glutaminase free L-asparaginase by using microbial systems. L-asparaginases with high specificity for L- asparagine and low-to-negligible activity against L-glutamine are reported to be less troublesome during the course of anti-cancer therapy (Hawkins *et al.*, 2004). The interest in L-asparaginase from *Erwinia carotovora* arose from the fact that it shows decreased glutaminase activity and therefore it is expected to exhibit fewer side effects when used in the anti-cancer therapy (Krasotkina *et al.*, 2004).

However, due to the prolonged administration of L-asparaginase, the corresponding antibodies are produced in patients, it results in the development of high titers of serum IgG antibodies which in the majority of cases interfere with the therapeutic effect of the enzyme, resulting in anaphylactic shock and may also cause neutralization of drug effect.

As with most protein drugs, L-asparaginase is susceptible to degradation by serum proteases and elimination by the reticuloendothelial system. The plasma half-life of L-asparaginase is estimated to be in the range of 8-30 h (AHFS, 2001). This rapid clearance necessitates frequent injection of large doses, further elevating the possibility of inducing immunological responses.

The route of administration may play a role. Hypersensitivity was lower when native asparaginase was administered intramuscularly compared with intravenous administration (Capizzi *et al.*, 1970). In order to overcome its usual shortcomings and maintain a therapeutic level of L-asparaginase in the blood of a patient, abrogation or modifications of antigenicity and immunogenicity of the protein must be achieved without substantial loss of the biological activity necessary for therapeutic purposes. Methods such as physical entrapment and conjugation of the enzyme with water-soluble polymers have been developed to replace frequent-injection therapy (Capizzi *et al.*, 1971).

IMMOBILIZATION AND PEGYLATION

L-asparaginase circulates in the blood system for only a short time before being taken up and broken down by native proteases which restrictedly limit its effectiveness only to a certain degree. In order to reduce the immunological response caused by this enzyme, prolong its action time and enhance drug effects in blood, the native L-asparaginase was often chemically modified and physically embedded with various kinds of soluble and insoluble biocompatible polymers to produce various immobilized L-asparaginase. It has been reported that the immobilized enzyme was not only reduced the immunity and toxicity but also greatly improved the resistance to proteolysis with respect to native L-asparaginase. The use of immobilized enzymes lowers production costs as these can be readily separated from reaction mixture and hence can be used repeatedly and continuously. Several different methods have been employed for enzyme immobilization which includes adsorption onto insoluble materials, entrapment in polymeric gels, encapsulation in membranes, cross linking with bifunctional or multifunctional reagents and linking to an insoluble carrier (Klibanov, 1983).

L-asparaginase polymerized to dextran (Wileman *et al.*, 1986), albumin (Poznansky *et al.*, 1982), polyethylene glycol (Teske *et al.*, 1990), polyacryalmide gel (Shoemaker *et al.*, 1987) and water soluble carboxymethyl cellulose (Karsakevich *et al.*, 1988) have proved to be less or non immunogenic and increase circulatory persistence, resist inactivation by antibodies, lack side effects and have a greater therapeutic efficiency than native enzyme.

Pegylation: The term pegylation describes the modification of biological molecules by covalent conjugation with polyethylene glycol (PEG) and is used as a strategy to overcome disadvantages associated with some biopharmaceuticals such as L-asparaginase. A polyethylene glycol modified version of the enzyme was developed in the 1970s and 1980s and was first used in clinical trial since the 1980s (Hosamani, 2012). Pegylation changes the physical and chemical properties of the biomedical molecule. In general, pegylation improves drug solubility and decreases immunogenicity. Pegylation also increases drug stability and the retention time of the conjugates in blood, as well as shields the enzyme from proteolytic degradation, improving pharmacokinetics of the drug (Harris and Chess, 2003), decreases renal excretion, thereby allowing a reduced dosing frequency and shields antigenic determinants from immune detection without obstructing the substrate-interaction site (Molineux, 2003). Pegaspargase (Oncaspar) is a modified form of native *E. coli* L-asparaginase in which the enzyme is covalently linked to polyethylene glycol. The binding preserves the enzymatic function of the drug but decreases the immunogenicity of the protein, thus potentially reducing the risk of hypersensitivity reactions (Capizzi, 1993; Keating *et al*., 1993). The PEG conjugated L-asparaginase (pegaspargase) from *E. coli* is a safe and effective alternative to native enzyme for clinical antileukemia therapy, although, pegaspargase should not be routinely substituted for the native enzyme (Holle, 1997). Pegaspargase can be given safely to children with a history of allergic reaction to prior administration of *E coli* asparaginase (Ettinger *et al*., 1995) and is indicated for patients who require L-asparaginase but have developed hypersensitivity to the native form or as part of front-line treatment of ALL. Another advantage of pegaspargase is its prolonged half-life of elimination compared with the *E. coli* or *Erwinia* forms which may be important in improving the pharmacokinetic profile of the drug (Asselin *et al*., 1993). In addition, a single injection of pegaspargase can be given instead of the inconvenient administration of multiple doses of native L-asparaginase. Pegaspargase has shown antileukemic activity similar to that of *E. coli* asparaginase in children with recurring ALL (Hawkins *et al*., 2004) who have already been exposed to the native form, as well as in newly diagnosed patients (Silverman *et al*., 2001). In contrast to children, pharmacokinetic and toxicity data on pegaspargase in adults is very limited (Wetzler *et al*., 2007).

However, pegylation also has disadvantages. The attachment of PEG molecules to a protein can modify the interaction capabilities or active sites of the protein (L-asparaginase) that are responsible for its biological function (Gaberc-Porekar *et al*., 2008). In addition, the non-immunogenicity of PEG became questionable since specific anti-PEG antibodies were found in some clinical trials. Some reports indicate the loss of long circulating properties when the PEG-asparaginase conjugate is administered multiple times due to a strong anti-PEG immune response (Ishida *et al*., 2007). Although, widely used, pegylation is a rather complicated and expensive approach. In particular when the therapeutic protein is an enzyme, there is a serious risk of a decrease or a complete loss of enzyme activity. Furthermore, since pegylation includes a chemical modification, the whole developing process from bench to market needs to pass numerous trials, requiring a lot of time and money. Nevertheless, pegaspargase has not yet been proven to be superior to *E. coli* L-asparaginase for the first remission of ALL. Most critically, pegaspargase failed to completely abort the anaphylactic responses in patients who are hypersensitive to L-asparaginase therapy (Chung, 2010).

RED BLOOD CELLS AS A DRUG CARRIER FOR L-ASPARAGINASE

Among all carriers employed for L-asparaginase encapsulation, the use of RBCs (red blood cells) as the drug carrier appears to be most appealing, simply because the RBCs would not only protect the loaded protein drug from proteolytic degradation but also prevent detection of the drug by the host immune system (Chung, 2010). Furthermore, RBCs are completely biodegradable without generation of toxic products and they are also biocompatible, particularly when autologous RBCs are used. In addition, RBCs are the most abundant cells of the human body, therefore giving an affordable source of supply for use in drug encapsulation. Moreover, the biconcave disk shape of RBCs endows them with the highest surface to volume ratio (1.9×10^4 cm g^{-1}) usable for drug encapsulation. Most critically, RBCs possess a life span in circulation of approximately 120 days which is significantly longer than any of the currently existing drug carriers (Kwon *et al*., 2009). Encapsulation of L-asparaginase into the erythrocytes prevents the removal of the enzyme by reticuloendothelial cells, increases the serum half life of the enzyme and greatly decreases the immune response to the enzyme and is therapeutically more effective (Kravtzoff *et al*., 1990).

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