REVIEW OF LITERATURE

Overview

Keeping in view the various fields under which the present investigation was carried out, the following topics were chosen for literature survey:

• 2.1 Sources of L-arginase
  ➢ Bacterial sources
  ➢ Protozoan sources
  ➢ Fungal sources
  ➢ Algal/Lichen sources
  ➢ Yeast sources
  ➢ Plant sources
  ➢ Insects
  ➢ Sea-organisms
  ➢ Mammalian sources

• 2.2 Arginase- the multilateral medical therapeutic

• 2.3 L-arginine- the versatile amino acid with enormous health benefits

• 2.4 The need for L-arginine quantification in clinical and food samples

• 2.5 Methods of L-arginine estimation

• 2.6 Production, purification and characterization of arginase

• 2.7 Structure related studies

• 2.8 Recombinant arginase

• 2.9 Modification of arginase: immobilization and chemical modification

• 2.10 Assay procedures for arginase

• 2.11 L-arginine detection by Biosensors

• 2.12 Sol-gel immobilization technology

• 2.13 Fiber-optic Biosensors- a magnificent skill of sensing
2.1 Sources of Arginase:

Arginase apart from being ubiquitously present in mammalian tissues has also been characterized from various worms, molluscs, fishes, bacteria, fungi, yeast, actinomycetes, algae and plants.

**Bacteria**

Among bacteria producing Arginase, the prominent ones include many bacilli, the mycobacteria (Zellar et al., 1954), Proteus spp. (Prozesky et al., 1973), T.aquaticus (Degryse et al., 1976), Agrobacterium-Rhizobium group, (Dessaux et al., 1976; Vissers et al., 1981) and cyanobacterium Aphanocapsa 6308 reported by Weathers et al., (1978) and other cyanobacteria, (Gupta et al., 1981). Among the bacilli, Bacillus licheniformis arginase has been purified by Simon and Stalon (1976). Arginase production was studied closely in Bacillus subtilis 168 by Baumberg and Harwood (1979). Arginase activity was reported in Streptomyces spp. (Vargha et al., 1983) and Streptomyces calvuligerus by Bascaran et al., (1989). Arginase was also reported in cyanobacterium Anabaena cycadeae by Singh and Bisen, (1994). Arginase from extreme thermophile Bacillus caldovelox has been purified and characterized by Patchett et al., (1991). Moreno et al., 1992, have described arginase production and characterization in the phototrophic bacterium Rhodobacter capsulatus E1F1. Arginase, urease, L-ornithine 5-aminotransferase and L-ornithine cyclodeaminase activities have been detected in R. capsulatus and R. sphaeroides in the study of arginase pathway by Igeno et al., 1993. A thermostable arginase has been cloned and expressed from Bacillus caldovelox by Maria et al., (1996). Arginase activity in cephamycin producers Streptomyces clavuligerus and Nocardia lactamdurans was reported by Funte et al., (1996). Bacillus brevis Nagano - gramicidin S- producing bacteria was shown to be a source of arginase by Kanda et al., (1997). The enzyme produced from bacteria is said to be highly specific for L-arginine and inducible by the addition of L-arginine to the glutamate medium. Helicobacter pylori- the gastric human pathogen has been reported to have rocF gene that encodes arginase (David et al., 2004). In pathogens such as Helicobacter, by consumption and outcompeting the host for the limited arginine available, arginase pathway is a major escape mechanism
helping to evade the toxic antimicrobial effects of host nitric oxide. Arginine catabolism in the cyanobacterium *Synechocystis* sp. strain PCC 6803 involves urea and arginase pathway (Quintero et al., 2000). An arginase extracted from *Arthrobacter sp.* KUJ 8602 acted on D-arginine, 4-guanidinobutyrate and showed little activity with L-arginine and hence has been regarded as a kind of guanidinobutyrase and termed as D-arginase by Arakawa et al., (2002). In *Cyanobacteria* (Flores and Herrero, 2005) arginase is useful for the utilization of arginine into urea and subsequently into ammonia which is taken up by the bacteria as a nitrogen source. The spore-forming, gram positive bacterium *Bacillus anthracis*, the causative agent of anthrax has shown arginase activity. Mainly *B. anthracis* bacilli and endospores exhibit anthrax has shown arginase activity. (Raines et al., 2006). An engineered L-arginine sensor (arginine-regulated transgene, ART) of *Chlamydia pneumoniae* enables arginine-adjustable transcription control in mammalian cells and mice (Hartenbach et al., 2007).

(1) **Protozoa**

Species of *Leptomonas, Leishmania, Crithidia* and *Blastocridhidia* have been reported by Yoshida and Camargo (1978) to possess arginase. *Entamoeba histolytica* has been shown to have arginase called *Entamoeba histolytica* arginase (EhArg). Its cloning and expression has been discussed by Elnekave et al., (2003). Structural metal dependency of the arginase from the human malaria parasite *Plasmodium falciparum* has been described by Muller et al., (2005).

**Fungi**

Arginase has been reported to be purified and characterized from *Neurospora crassa* by Borkovich and Weiss, 1987. Arginine specific carbamyl phosphate metabolism in mitochondria of *Neurospora crassa* was studied by Rowland and Janet in 1987 where it was reported that arginine efficiently feedback-inhibits intramitochondrial ornithine synthesis. Two forms of Arginase expressed by *Neurospora crassa* as reported by Marathe et al., 1998 is the only reported example of multiple forms of arginase in a microbial organism. The larger form was produced by mycelia growing in arginine-supplemented medium. *Aspergillus nidulans* produces...
arginase which enables the fungus to utilize arginine as the sole nitrogen source (Dzikowska et al., 1994). Mycelial extracts of *Trichoderma sp.* were reported to be a source of arginase by El-Meleigy et al., (1998). An extensive survey of higher fungi carried out by Wagemaker et al., (2005) revealed that the presence of arginase in members of family Agaricaceae including *Agaricus bisporus* that led to the accumulation of urea in its fruit bodies.

(2) **Lichens:**

Planelles and Legaz (1987) reported the purification three forms of arginase from thallus of *Evernia prunastri* incubated at different time intervals. The third form was obtained by culture grown in presence of cycloheximide. Secreted, glycosylated arginase from *Xanthoria parietina* thallus was reported to induce loss of cytoplasmic material from *Xanthoria* photobionts (Molina et al., 1998). Secreted arginases from *Evernia prunastri* and *Xanthoria parietina* thalli have been reported by Legaz et al., (2004) to utilize arginine in a Mn$^{2+}$ dependent manner and show lectin function by binding to the cell wall of both homologous and heterologous algae thus helping in further algal-lichen associations by acting as cross-recognition factors ensuring specificity in the associations. Diaz et al., (2009) accounted the production and secretion of arginase to a medium containing arginine by *Peltigera canina*, a cyanolichen containing *Nostoc* as cyanobiont, The secreted arginase acts as a lectin by binding to the surface of *Nostoc* cells through a specific receptor which develops urease activity. *Leptogium corniculatum*, a cyanolichen containing *Nostoc* as photobiont, has been reported to produce and secrete arginase to culture medium containing arginine (Vivas et al., 2009).

**Yeast**

In *Saccharomyces cerevisiae*, arginase has been reported to form a multienzyme complex with ornithine transcarbamoylase in which arginase acts as a negative allosteric effector of ornithine transcarbamoylase (Hensley, 1988). The purification and characterization of arginase from a plasmid-containing, enzyme-
overproducing, protease deficient yeast strain (*S. cerevisiae*) was reported by Green *et al.*, (1989). Arginase was purified from *Schizosaccharomyces pombe* by Kang (1995).
Review of Literature

Plant sources
Arginase activity has been reported to be present in parts (tissues and extracts) from several plants. Reports regarding presence of arginase in *Lathyrus sativus* L. plants (Cheema *et al.*, 1969) and pumpkin seeds (Kollofel & Van Duke, 1975) and seedlings have been quite interesting. Evidence for arginase in apple trees was provided through studies in which $^{14}$C-labeled L-arginine was administered to trees and its subsequent metabolic products were determined by Hill-Cottingham *et al.*, (1973). **Roubelakis and Kliewer** in the year 1977 demonstrated the presence of arginase in grapevine- *Vitis vinifera*. Desai (1983) studied the properties of arginase obtained from *Arachis hypogea* L seedlings. Arginase obtained from cotyledons of *Canavalia lineata* was purified and characterized by Yu *et al.*, (1988). Arginase has been extracted by Kang and Cho (1990) from embryonic axes of Soybean *Glycine max* and its properties were studied. Jack bean (*Canavalia ensiformis*) leaf arginase can effectively hydrolyse both L– and D–arginine (Kavanaugh *et al.*, 1990). The activity of arginase from roots and leaves of kiwifruit vines- *Actinidia deliciosa* was investigated by Hale *et al.*, (1997). Isolation and study of properties of arginase from roots of a three-year-old shade plant, ginseng (*Panax ginseng C.A. Meyer*) was done by Hwang *et al.*, (2001). Regulation of loblolly pine (*Pinus taeda L.*) arginase in developing seedling tissue during germination and post-germinative growth was studied by Todd *et al.*, (2001). Arginase activity was shown to be enhanced (Millanes *et al.*, 2005) in sugarcane plants - *Saccharum officinarum* cv. Mayari in response to infection by teliospores of *Ustilago scitaminea*. The increase in enzyme activity increases putrescine which obstructs the polarization of teliospores at the cell walls. Papadakis *et al.*, (2005) reported that in totipotent tobacco protoplasts, extremely high levels of endogenous polyamine putrescine can be attributed to increased arginase activity. The characterization of two genes *LeARG1* and *LeARG2* that encode arginase from *Lycopersicon esculatum* (tomato) and the regulation of this arginase by wounding, jasmonate and phytoxin coronatine has been described where Arginase along with threonine deaminase (TD) has been reported to be induced by the plant hormone- Jasmonic acid in Tomato plants-*Solanum lycopersicum*. Often called a Jasmonic acid inducible protein (JIP), the enzyme has been shown to attack arginine in
the mid-gut causing intestinal issues in *Maduca sexta* larvae which infest the tomato plant hence conferring plant protection against herbivores (Chen et al., 2004). Xylem sap of the deciduous tree- *Quercus ilex* was reported to possess arginase by Nabais et al., (2005) which degrades arginine- a storage protein in the tree which breaks down to ornithine by the increased activity of arginase before spring time. Dabir et al., (2005) reported arginase extraction form cotyledons of *Vigna catjang*. Arginase has been found to be operative in cotyledons of somatic embryos of pine at late developmental stages (Rodriguez et al., 2006). *Arabidopsis* was reported to be a source of arginase by Palemeiri et al., (2006).

(3) **Insects**

Two arginases A and B were reportedly produced from developing embryos of the tick *Hyalomma dromedarii* (Fahmy et al., 1994). Samson (2000) reported a *Drosophila* gene encoding a 351-amino acid-long predicted arginase (40% identity with vertebrate arginases). Tanuja et al., (2005) reported that the levels of arginase in *Bombyx mori-* the silk worm decreased sharply during development of the pupa but rose during adult development. Arginase from the flatworm *Fasciola gigantica* has been described by Mohamed et al., (2005). Two arginases- Arginase I and Arginase II were reported to be present.

(4) **Sea-organisms**

Larva of *Phoronis pallida* was shown to possess arginase activity (Santagata, 2004). The giant African snail-*Achatina fulica* has been shown to possess 3.5 fold increased arginase activity after an injection of ammonium chloride (Hiong et al., 2005). Arginase has been reported in African lungfishes- *Protopterus aethiopicus* and *Protopterus annectens* in the cytosolic fractions of their livers (Loong et al., 2005). South American fish pacu (*Piaractus mesopotamicus*) when supplemented with arginine utilized the supplementation efficiently with normal hepatic arginase activity as reported by Tesser et al., (2005). Arginase activity was found to be highest in to juveniles of sea bream *Sparus aurata* fed with yeast diets (Olivia-Teles et al., 2006). Joerink et al., (2006) reported that basal arginase I expression was found in carp
Cyprinus carpio mid kidney while arginase II was found to be distributed in all organs examined with highest levels in liver. Pacific spiney dogfish shark Squalus acanthias showed enhanced activity of arginase with elevated nitrogen intake (Kajimura et al., 2006). The effect of protein in diet was directly related to increase in arginase activity in (Bibiano et al., 2006) liver of juveniles of Rhamdia quelen. L-arginine metabolism by arginase in mitochondria isolated from the liver of Antarctic fish Notothenia rossii and Notothenia neglecta was studied by Rodrigues et al., (2006).

(5) Mammals

In mammals, two types of arginases are known: Arginase I and II. The cytosolic enzyme found primarily in liver is Arginase I, a 35kD protein that circulates as a trimer. Arginase II is exclusively located in the mitochondrion. Arginase I is next to the liver in man also expressed by mature fetal and adult red blood cells and activated monocyctic cells. Arginase II is expressed by kidney, nucleated red blood cells, brain, spinal cord, gastro-intestinal tract, mammary gland and prostrate. Characteristics of Rat liver and chicken liver arginases have been described (Breitburd and Orth; 1971 Grazzi and Magri, 1971). Hepatic arginase of Salamandra salamandra was investigated at three different stages of intra- and extrauterine larval development and at fully metamorphosed juveniles (Schindelmeiser et al., 1983). The two human arginase isozymes have been studied in greater detail by Grody et al, (1987). There are abundant examples of isolation, characterization and purification of arginases from mammalian tissues. Other examples of mammalian arginases include those from the liver of Genypterus maculatus (Carvajal et al., 1987) and Mus booduga (Prasad et al., 1997) to name a few. Hepatic arginase from Pygoscellideae Penguins has been found to be comparable to liver arginase from laboratory mammals and may be considered as evidence of ureogenic activity displayed by them (Edson et al., 1999). Yu et al., (2001) studied arginase isoforms-Arg I and Arg II in mouse brain using in situ hybridization and immunohistochemistry. Arginase activity was reported in little skate Raja erinacea by Steele et al., (2005). AI and AII were detected only in neurons and not in glial cells. Arginase status in cattle reproductive system was studied by Razmi
et al., (2005) who showed that the maximum activity of the enzyme was present in the mucosal layer of vestibula. Dabir et al., (2005) reported arginase extraction from buffalo liver and studied its properties. Aminlari et al., (2007) have reported the distribution of arginase in a number of tissues of tissues of cat (*Felis catus*) besides its high activity in liver suggesting other roles of the enzyme in cat apart from its primary role in ammonia detoxification. Nikolaeva et al., (2008) studied arginase activity and its properties from the common frog *Rana temporaria* L. urinary bladder epithelial cells. The comparison of arginase activity in various frog tissues revealed the following pattern: liver > kidney > brain > urinary bladder (epithelium) > heart > testis.

### 2.2 Arginase: the multilateral medical therapeutic

The enzyme has been found to possess profound therapeutic benefits in treatment of various physiological disorders in the body. Measurement of circulating Arginase I i.e., serum arginase levels have been used experimentally as rapid marker for liver injury (Puri and Kaul, 1995). Arginase has been found to be essential for the treatment of acute neurological disorders (Esch et al., 1998). Ornithine, produced by arginase is necessary for the production of collagen, is helpful in therapy of rheumatoid arthritis (Corraliza et al., 2002). Arginase upregulates the synthesis of polyamines and proline via arginine hydrolysis thus, being necessary to provide compounds for cell proliferation and growth (Mori and Gotoh, 2004). Arginase competes with NOS for endogenous arginine pools, this way its levels acts as an indirect regulator of penile and vaginal flow thus playing an important role in male and female sexual arousal (Kim et al., 2004). Upregulation of endogenous arginase I causes the activation of neural regeneration pathways, (Lange et al., 2004) the reaction being mediated by polyamines and leading to novel roles of arginase in cell survival, regeneration and translation in the central nervous system. Munder et al., (2005) reported that arginase-I is constitutively expressed in human granulocytes and participates in fungicidal activity by a novel antimicrobial effector pathway likely through arginine deprecation in phagolysosome. A deficiency of the liver enzyme
results in hyperargininemia- inherited in an autosomal recessive manner (Scaglia and Lee, 2006). Raised arginase activity in serum of 85% patients suffering from colorectal cancer liver metastases (CRCLM) led to the conclusion that Arginase can be a useful marker for diagnosis of CRCLM (Meilczarek et al., 2006). Human granulocyte arginase has been reported to be a promising pharmacologic treatment to reverse unwanted immunosuppression (Munder et al., 2006). Increase in psychological stress results in raising NO level which results in poor sperm quality. Thus, arginase levels are important to keep NO levels (via arginine degradation) in control for maintainence of semen quality (Eskiocak et al., 2006). Arginase has been shown to be effective for treatment of Hepatitis- B (Cheng, 2006). A finding that Hepatitis C virus targets overexpression of arginase I in hepatocarcinogenesis (Cao et al., 2009) suggests that insights into this disease and its control may be through altering arginine metabolism by means of arginase activity alterations.

The arginase levels in NOS related processes prove to be important for pathogenesis of a host of diseases relevant to arginine-NO balance in the body. Due to arginase and nitric-oxid synthase (NOS) utilizing the same substrate arginine, a reciprocal regulation and metabolic balance exists between both of them for numerous processes that are dependent on these enzymes, occur inside the body. In rheumatoid arthritis patients a significant correlation between serum concentration of arginase protein and rheumatoid factor has been found (Huang et al., 2001) suggesting that increased arginase production may play an important role in pathogenesis of this disease. Arginase I & II levels rise remarkably in asthma and lung infection thus, showing the involvement of arginase in the pathology of the disease (King et al., 2004). Measurements of plasma arginase activity provide a useful marker for underlying metabolic disorder and efficacy of treatment for asthma (Morris et al., 2004). Increase in levels of arginase during Sickle cell disease (Morris et al., 2005) makes it a candidate for pathogenesis of this disease. The pathogenesis of cystic fibrosis has been reported to be arginase dependent since arginase levels are enhanced leading to depletion of arginine for NOS action to occur contributing to nitric oxide deficiency (Grasemann et al., 2005). Because of its role in epidermal
hyperproliferation by upregulating CAT-1 expression in psoriatic skin, arginase is important in pathophysiology of psoriasis (Schnorr et al., 2005). Similarly, since nitric oxide is a chief vasodilator, high arginase activities have often been associated with heart ailments such as atherosclerosis, mycordial infarction and ischemia. Arginase has been shown to modulate myocardial contractility by nitric oxide synthase 1-dependent mechanism (Steppan et al., 2006). Higher arginase activity in lower Mg$^{2+}$ ions have been observed in diabetic children (Bjelakovic et al., 2009) suggesting the pathophysiology of this disease could be understood to be related with increased arginine catabolism due to arginase action. The arginase-NO synthase competition for arginine has led to various effects on airway hyperresponsiveness, bronchial obstruction and lung inflammation in asthma due to increase in arginase activity. Munder (2009) have reported arginase modulation to have a noteworthy role in the mammalian immune system due to L-arginine metabolism being regulated by it leading to immunosuppression by suppression of T-cell immune responses. Maarsingh et al., (2009) and Munder (2010) have reported arginase to be a key enzyme in pathophysiology of allergic asthma opening novel therapeutic roles for asthma control through action of arginase inhibitors.

The use of Arginase as a potential chemotherapeutic agent has shown a lot of potential and promise. Arginase treatment of cultured HeLa, human diploid fibroblasts and L1210 cells proved to be as efficient as the use of AFM (Arginine Free Medium) by reducing arginine in the medium to micromolar levels within 5-30 minutes resulting in cell death in both the cultures (Wheatley et al., 2003). Remission of hepatocellular carcinoma was achieved by arginine depletion through endogenous human hepatic arginase released from transhepatic arterial embolization (Cheng et al., 2005). Using state-of-the-art DNA technology, researchers of the Hong Kong Polytechnic University (PolyU) produced a human recombinant arginase as a novel method in the treatment of liver cancer by arginine depletion (Cheng et al., US Patent no. 20050244398. 2005). The combination of the recombinant arginase with an anti-neoplastic agent 5 fluorouracil (5FU) for treatment of human malignancies was tested in nude mice bearing an ADI-resistant HCC xenograft and this treatment methodology
was shown to be effective for arginine depletion (Cheng et al., 2006, International Application No. PCT/CN2005/002001). BCT-100 pegylated recombinant human arginase manufactured by large scale fermentation of a recombinant *B. subtilis* strain LLC101 has been reported as a novel anti-melanoma agent for treatment of human melanoma cells as reported by Hsueh et al., (2006). Pegylation of recombinant human arginase (rhArg-peg 5000mw) produced in *B. subtilis* expression system was done and the pegylated enzyme has been shown to have *in vitro* and *in vivo* anti-proliferative potential and apoptotic activities in human hepatocellular carcinoma (HCC) (Cheng et al., 2007). While studies for conducting clinical trials of PEG BCT-100 are reported to be in process to assess its safety and efficacy in humans (http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00988195), recently further studies on pegylation of recombinant arginase conducted by the Cheng group (Tsui et al., 2009) by conjugation of rhARG with methoxypolyethylene glycol-succinimidyl propionate (mPEG-SPA 5,000) has been reported as having comparable anti-tumor efficacy to native rhArg1. That the inhibition of human hepatocellular carcinoma by recombinant arginase (rhARG1) is by the inducing cell cycle arrest at the G2/M or S phase, possibly mediated by transcriptional modulation of cyclins and/or cyclin dependent kinases (CDKs) has been observed by Lam et al., (2009). This group has further proposed the use of rhArg1 alone or in combination with chemotherapeutic drugs for treatment of liver cancer.

*In vitro* cytotoxicity of human arginase I (Stone et al., 2010) by replacing the two Mn$^{2+}$ ions normally present in the enzyme with Co$^{2+}$ significantly lowered the Km value of the enzyme, increased its serum stability and showed incredible ability to eliminate human hepatocellular carcinoma and melanoma cell lines proving it to be a capable new contender for treatment of L-Arg auxotrophic tumors. A study conducted by Hernandez et al., (2010) published just a few months back (June 2010) in the prestigious journal ‘Blood’ reports of the potential therapeutic role of pegylated Arginase I in the treatment of adult patients with acute lymphoblastic T cell leukemia (T-ALL) through arginine depletion.

2.3 L-arginine- the versatile amino acid with enormous health benefits
Arginine has multiple metabolic fates in human physiology with the enormous inherent versatility of this amino acid in various pathways involved with normal growth and maintenance of the body. Besides being a precursor of nitric oxide, creatine, polyamines, agmatine and urea it is metabolically interconvertible with amino acids glutamate and proline. It has been classified as a dibasic, cationic, semi essential amino acid since it becomes necessary under periods of growth as well as recovery after injury. The body needs high levels of arginine when it is under conditions of stress, illness, malnutrition or injury. There are also some genetic disorders which lower arginine levels and make the conditional supplementation of dietary arginine necessary. The food sources high in arginine include carob, chocolate, coconut, dairy products, gelatine, fish, poultry, meat, oats, peanuts, soybeans, walnuts, brown rice, wheat, wheat germ, sesame and sunflower seeds, popcorn and raisins.

Free arginine in the body is derived from diet, endogenous synthesis and turnover of proteins. Arginine is synthesised from citrulline in many cell types in body but a major part of endogenous synthesis is through the teamwork of epithelial cells of small intestine and proximal tubules of kidney. During conditions of arginine deficiency caused due to catabolic stress (inflammation and infection) or due to dysfunction of kidneys or small intestine levels of arginine is not sufficient and arginine needs to be supplied through diet (Morris, 2006). Arginine has a history of safe usage as a supplement. Being a common substrate for arginase and nitric oxide synthase its levels in the body are a result of competition and reciprocal regulation between these two key enzymes. Medical researchers have gathered enough clinical evidence to bring L-arginine to the forefront of modern medicine as an accepted treatment for a variety of human ailments.

For a more organised layout about the immense benefits of dietary arginine and its supplementation, the topic has been divided into the following sub-titles depending on numerous roles that arginine performs in the body.

2.3.1 In aging process: Studies in aging NMRI-mouse show that L-arginine reduces kidney collagen accumulation and N-epsilon-(carboxymethyl)lysine thus inhibiting
one of the primary mechanisms of the aging process by inhibiting the process of cross-linking (Radner et al., 1994). Arginine increases the release of the human growth hormone (HGH) (also known as the anti-aging hormone) from the pituitary gland (Gianotti et al., 2000).

2.3.2 In cardiovascular system: Most of the significant effects of arginine in therapy of the cardiovascular system arise from its being a metabolic precursor of nitric oxide—an endogenous neurotransmitter that helps to prevent vasoconstriction and which initiates vasodilation by relaxing the smooth muscle cells of the blood vessels. In patients of moderate congestive heart failure, arginine significantly increases stroke volume and cardiac output (without effect on heartbeat rate) as well as increase in vasodilation (leading to increased blood circulation) in congestive heart failure patients (Koifman et al., 1995). It improves blood circulation, improves exercise capability and facilitates vasodilation in angina Patients (Ceremuzynski et al., 1997). In studies conducted on young men with coronary artery disease, oral L-arginine has been shown to improve endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells thus helping to prevent atherosclerosis and reducing the severity of existing atherosclerosis (Adams et al., 1997). Cigarette smoking has been observed to be associated with increased human monocyte adhesion to endothelial cells (an underlying event in the course of atherosclerosis whose inhibition has been reported by use of arginine (Adams et al., 1997). L-arginine treatment directly influences the kinetics of nitric oxide and superoxide production thus improving blood circulation (Huk et al., 1997) thereby also helping in to prevent free radicals-induced damage to the lining of blood vessels. Reports recognise (Wallace et al., 1999) that L-arginine infusion dilates coronary vasculature in patients undergoing coronary bypass surgery. Khosh & Khosh (2001) have reported that Arginine lowers blood pressure in some hypertension patients (by facilitating the body’s production of nitric oxide (NO) and by inhibiting the angiotensin converting enzyme (ACE). The combination of arginine with cholesterol lowering statin namely Atorvastatin has been shown to hinder the spread of atherosclerotic plaques as compared with monotherapies thereby opening a new area of therapeutic strategy (Rasmusen et al., 2005). Miller (2006)
showed from an experimental study that a moderate dose of sustained-release L-arginine can improve endothelial function and blood pressure. Tripathi and Misra (2009) have reported that the free radical scavenging role of L-arginine is of immense therapeutic value to patients of myocardial infarctions and acute angina since it inhibits pro-oxidative enzymes and acts as an anti-oxidant by scavenging harmful free radicals that cause substantial damage to cardiomyocytes.

2.3.3 **In digestive system:** While its deficiency can cause constipation, the supplementation of L-arginine may decrease the incidence of gallstones. Arginine reduces intestinal permeability (due to arginine’s role in the production of nitric oxide) (Miller, 1997) and alleviates many cases of ulcerative colitis by promoting the healing of the ulcers that occur in the colon of ulcerative colitis patients (Segala, 2000) and helps prevent post surgical damage after intestinal manipulation (Thomas *et al.*, 2001).

2.3.4 **In excretory system:** Oral arginine lessens the pain and discomfort associated with interstitial cystitis (Smith *et al.*, 1997) and significantly improves the function of the kidneys and helps to prevent age-related degradation of the kidneys (Reckelhoff *et al.*, 1997).

2.3.5 **In immune system:** Arrest of liver cancer (Weisburger, 1969) and of mammary tumor growth by l-arginine was reported in 1980 by Cha-Chung where it was observed that arginine boosted the ability of the immune system to fight breast cancer. Later it was observed that supplemental arginine increases thymic cellularity in normal and murine sarcoma virus-inoculated mice and increases the resistance to murine sarcoma virus tumour (Rettura *et al.*, 1979). Arginine retards the growth of tumors and cancer by enhancing immune function. The process underlying this function is the ability of arginine to enhance the size and health of thymus gland- the “director” of immune system which produces enzyme thymosin which in turn stimulates the spleen and lymph nodes to manufacture high levels of active and effective T-lymphocytes (T-cells) (Moriguchi *et al.*, 1987). Kirk *et al.*, (1993) accounted that Arginine stimulates wound healing and immune function in elderly human beings as well as decreases inflammation in general (Efron *et al.*, 1998). Arginine helps to prevent bacterial &
viral diseases in persons with suppressed immune systems (Field et al., 2000). Patterns of arginine and nitric oxide in patients with sickle cell disease with vaso-occlusive crisis and acute chest syndrome suggested that arginine alleviates sickle-cell disease (Morris et al., 2000). Arginine enhances the ability of the immune system to recover from elective surgery and accidental injury (Wilmore, 2001).

2.3.6 In metabolism: Arginine deficiency can cause alkalosis in body however its presence scavenges super-oxide production and therefore exerts anti-oxidant effects in the body. Studies carried out on animals proved that L-arginine lowers serum LDL levels (Ryzenhov et al., 1984) and normalizes endothelial function (Rossitch et al., 1991) in cerebral vessels from hypercholesterolemic rabbits by reducing their total cholesterol levels. In diabetes, arginine has shown to reverse damages of diabetes (Giugliana et al., 1997) and in some cases prevent diabetes (Mohan and Cas, 1998). By stimulating the release of human growth hormone (HGH) from the pituitary gland, arginine alleviates obesity and facilitates weight loss Gianotti et al., (2000). Piatti et al., (2001) stated that arginine reduces insulin resistance and improves blood sugar disposal in diabetes type 2 patients. Due to its role in production of nitric oxide which in turn improves blood circulation via vasodilation, arginine has been shown to increases oxygen uptake in the lungs in persons with hypoxia and altitude sickness (Beall et al., 2001).

2.3.7 In muscoskeletal system: By producing nitric oxide, arginine causes the relaxation of smooth muscle. It facilitates muscle growth (by inhibiting muscle loss) and is required for the transport of the nitrogen used in muscle metabolism (Barbul, 1986). By stimulating the release of human growth hormone (HGH) which is an important mediator of bone formation and bone turnover and stimulation of nitric oxide synthesis which is a potent inhibitor of osteoclasts that cause the resorption of bone arginine may prevent and alleviate osteoporosis. (Visser & Hoekman, 1994). Stevens et al., (2000) reported that arginine enhances high intensity dynamic human muscle performance. The role of arginine in sepsis has been studied by Luiking et al., (2005)
who reported that arginine supplementation could reduce the effects of infection and sepsis through the NO pathway.

2.3.8 In nervous system: As stated by Cestaro (1994) arginine is essential for the regeneration of damaged axons of neurons by acting as an agent for degrading proteins that have been damaged through axon injury. By stimulating the production of nitric oxide (NO) - a neurotransmitter responsible for the potentiation (storage) of long term memory it serves as a memory enhancer (Pautler, 1994). In athletes, arginine supplementation improves pituitary responsiveness and modulates hormonal control (di Luigi et al., 1999). Due to its ability to repair damaged axons by increasing polyamine levels, arginine may be useful for the treatment of Alzheimer’s disease (Tarkowski et al., 2000).

2.3.9 In sexual system: Now known as the ‘Natural Viagra’ the role of arginine has been accounted since long in improving sperm count and sperm motility (Keller et al., 1975). Due to its involvement in the manufacture of endogenous spermidine resulting in improvement of sperm count and sperm motility, arginine alleviates male infertility. It enhances sexual performance in women too (Block, 1998). Chen et al., (1999) reported that arginine lessens male impotence by alleviating erectile dysfunction. In animal models, arginine resulted in endogenous nitric oxide-mediated relaxation and nitrinergic innervation which suggests that it may improve prostate function (Aikawa et al., 2001).

2.3.10 In Skin: Arginine is essential for and accelerates the healing of wounds by stimulating the release of human growth hormone (HGH), stimulating the production of collagen and by stimulating the proliferation of fibroblasts (Barbul et al., 1978). Arginine stimulates wound healing and immune function in elderly human beings by proliferation of fibroblasts (skin cells) (Kirk et al., 1993). Apart from accelerating the healing of burns (Yu et al., 1996) when applied topically it keeps the skin young by increasing the level of vascular endothelial growth factor in the skin (Block, 1998) besides improving scleroderma (Freedman et al., 1999). It has been shown to
dramatically speed up the healing of wounds in patients with high risk of infection after cardiac surgery (Tepaske et al., 2001).

2.3.11 In treating heatstroke: A study conducted in mice showed that heatstroke induced death in mice could be controlled by administration of arginine at appropriate concentration and time by increase in Th-2 cytokines and levels of splenic and hepatic arginase (Chatterjee et al., 2006).

2.4 The need for L-arginine quantification in clinical and food samples: As accounted above, being vital for an enormous number of processes in the body, the determination of arginine is of prime importance in physiological fluids. The levels of arginine have been found to vary in conditions of metabolic disorders and disease. Thus, arginine level in the body constitutes an important marker for diagnosis and treatment of various diseases. L-arginine levels in patients undergoing surgeries such as thoracoabdominal surgery (Nijveldt et al., 2000) and heart surgeries are particularly reduced and in such cases careful monitoring of arginine is necessary for speedy recovery. Being a precursor of the vasodilator NO, quantification of arginine levels is imperative in NO related processes such as atherosclerosis and strokes. L-Arginine levels in blood have proved to be a marker of nitric oxide–mediated brain damage in acute stroke (Armengou et al., 2003). In tumor cells undergoing therapy with arginine degrading enzymes (arginase, arginine deiminase and arginine decarboxylase), arginine quantification is particularly vital to assess the efficacy of the anti-cancer agent as well as the stage/phase of tumour removal.

Regarding foods, arginine is one of the most abundant amino acids present in grape juice and its monitoring in grape juices is essential for winemakers to realize hazard analysis critical control point (HACCP) since they are the raw material for making wines and must. Elevated arginine concentrations in grape juice and must can lead to an increased potential for Ethyl Carbamate (named as a potent carcinogen by FDA) concentrations in wine (Austin and Butzke, 2000). Arginine determination has been mainly carried out in fruit juices such as pineapple juice (Elkins et al., 1997), green tea (Lvova et al., 2003) and grape juice (Spayd and Anderson-Bagge, 1996; Hua
et al., 2008) by means of spectrophotometric (in pineapple & grape juice) and by biosensor (in green tea) methods.

Ough et al., (1988) reported that arginine concentration higher than 1000 mg/L could produce 15µg /L of ethyl carbamate which is currently the voluntary limit of EC in wines in the US while Canada has legalised this limit to 30 µg /L (Orduna et al., 2001). Arginine present in wines can be converted to ammonia and citrulline by heterofermentative malolactic bacteria during malolactic fermentation in grape must and wine. Ammonia produced increases the pH and hence the risk of growth by spoilage bacteria and citrulline is a precursor to the formation of potential carcinogenic ethyl carbamate (urethane) (Orduna et al., 2001). Also alcoholic fermentation by yeasts produces urea from arginine which can spontaneously react with ethanol to form ethyl carbamate (Uthurry et al, 2006). Ethyl carbamate content in wines with malolactic fermentation as determined at different points in vinification process in a recent study conducted by Masque et al., (June, 2010) was reported to be lower than the existing legal limit after malolactic fermentation but observed to increase with a storage time of 8 months.

Fruits such as watermelon have been found to be good and novel sources of arginine and their regular consumption has been shown to increase plasma levels of arginine (Mandel et al., 2005; Collins et al., 2007). A significant finding has been the potential that dietary sources of arginine (such as watermelon juice) may have in treating severe forms of malaria due to providing the body with the requisite precursor (arginine) for Nitric oxide (NO) which helps cure fever and other forms of complications in the disease (Lopansri et al., 2003; Hobbs et al., 2003).

The above facts make the quantification of arginine in biological and food samples absolutely necessary and indispensable.

2.5 Methods of L-arginine Estimation:

A host of methods for arginine determination are available to monitor arginine. These include (amongst others) the estimation of arginine through its metabolic
products such as urea and ornithine. Estimation of arginine in clinical samples is of supreme significance due to its versatility in participating in several important biochemical reactions in the body. Arginine is a precursor of several important intermediates so its determination is imperative as a marker for several diseases.

Some of the important reported methods for arginine determination are accounted as follows:

A number of amino acids including arginine were determined through microtititration method developed by Sobel et al., (1945). Amino acids were allowed to react with ninhydrin and the resulting ammonia was finally aerated over into boric acid and titrated with standard acid, with a capillary micro burette.

One of the earliest methods of arginine determination via ornithine formation is Chinard’s reaction (1945) whereby ornithine when treated with a mixture of glacial acidic acid and ninhydrin reagent forms a red color product which can be measured spectrophotometrically at a wavelength of 515 nm.

For determination of amino acids and related compounds, Moore and Stein (1945) prepared a modified ninhydrin reagent composed of 2 percent ninhydrin and 0.3 percent hydrindantin in 3: 1 methyl Cellosolve- N sodium acetate buffer (pH 5.5). Determination of arginine in plasma and urine by employing arginase has been accounted by using Chinard’s ninhydrin reaction by Gopalakrishna and Nagarajan (1980).

Another method reported by Rosenberg et al., (1956) describes the reaction of arginine with n-propanol and diacetyl in presence of increased concentration of $\alpha$-naphthol to give red color product whose absorbance can be measured at 535 nm.

Another widely popular method of colorimetric arginine determination is the Sakaguchi reaction. It comprises of the reaction of alkaline solution of arginine with $\alpha$-naphthol and hypochlorite giving a red color. Since its inception (Sakaguchi, 1925) many variations of the Sakaguchi reaction have been reported where the reaction was
suitably modified by the addition of hypobromite and oxine (8-hydroxyquinoline) by Weber (1930) and Sakaguchi (1950) respectively. A further modification for the reaction for quantitative determination of arginine was reported by Akamatsu and Watanabe (1961) using sulfosalicylic acid and oxine solution with hypobromite. Notenboom et al., (1967) made a fluorescent modification of the Sakaguchi reaction by using 2,4 dichloro α-naphthol for reaction with arginine. The reaction product was fluorescent and henceforth employed for fluorescent arginine determination.

Konings (1988) described another interesting procedure of arginine quantification in serum. It involved transformation of arginine and ATP into phospho-arginine and ADP by arginine kinase. The ADP formed is measured by two coupling reactions involving pyruvate kinase and lactate dehydrogenase with measurement of NADH consumption at 340 nm. L-arginine determination by flow-injection technique was reported by Alonso et al., (1995) whereby L-arginase was immobilized on an epoxy matrix and the urea produced by the reaction was measured spectrophotometrically through indophenols formation at an absorbance of 629 nm.

Orduna (2001) reported an enzymatic end –point arginine analysis method by using enzymes arginase, urease, and glutamate dehydrogenase in this simple assay. Other methods of arginine determination include the use of High performance liquid chromatography techniques (HPLC) such as those reported where amino acids are derivatized with 2-chlorobenzoxazole to yield highly fluorescent N-(2-benzoxazolyl)-amino acids (BOX-AAs) whose separation on a C18 reversed phase column is done for quantitative estimation (Anumula, 2000; United States Patent: 6,800, 486 B1) and recently discussed by Wu and Meininger (2008) involving precolumn derivatization with o-phthalaldehyde. Fluorescence in the latter report is monitored at excitation and emission wavelengths of 340 and 455 nm, respectively.

2.6 Production, Characterisation & Purification of arginase from various sources: Numerous reports regarding the characterization of arginase & its purification ranging from microbial sources to vertebrate and invertebrate sources are accessible in scientific literature.
2.6.1 Production of arginase: Arginine has been reported to be utilized as a nitrogen source in *Neurospora crassa* (Castaneda *et al.*, 1967; *Davis et al.*, 1970). Mora *et al.*, (1972) grew *Neurospora crassa* in minimal medium supplemented with 1.5% sucrose and reported that arginine synthesized from exogenous citrulline was not effectively utilized as exogenous arginine due to feedback inhibition of arginase *in vivo* and *in vitro* by arginine pool formed from citrulline. *Saccharomyces cerevisiae* ATCC 9763 was cultured aerobically at 30°C in defined media containing arginine as a sole source of nitrogen. After 5 hrs of growth, arginase was extracted by sonication and partially purified by gel filtration (Chan & Cossins, 1973). Most of the arginine pool in *Neurospora* is available inside the vesicle and lesser arginine concentration is found to be present in cytosol in cells growing in minimal medium (Weiss, 1976). In arginine supplemented medium, the cytosolic pool dramatically increases resulting in induction of catabolic enzyme arginase as well as rapid catabolism of arginine. Vaca and Mora (1977) tested the effect of various nitrogen sources on arginase production in a *N. crassa* mutant ure-1 lacking urease activity and found that arginase was hyperinduced with arginine as the nitrogen source whereas in the wild-type strain the induction was completely repressed by glutamine. In *Bacillus licheniformis* the arginase pathway enzymes are subject to strong catabolite repression during growth on glucose while glutamine antagonizes the induction of arginase pathway. This effect occurs only in a media containing a good carbon source. In this organism glutamine is a better nitrogen source than arginine, glutamate or ammonia. Broman *et al.*, (1978) demonstrated that *Bacillus licheniformis* has two pathways of arginine catabolism and in well aerated cultures the arginase route is present, and levels of catabolic ornithine carbamoyl transferase were low. They also demonstrated that an arginase pathway deficient mutant, BL196, failed to grow on arginine as a nitrogen source under these conditions. The intensity of carbon source (glucose, citrate) catabolite repression of arginase seems to be much weaker in *B. subtilis*. In the arginase pathway in the *B. subtilis* strain used by Baumberg and Harwood (1979) repression of the pathway was by glutamine and, in addition, ammonia while ornithine and citrulline were useful in
induction of the enzyme. In the genus *Agrobacterium*, arginine may be used as a nitrogen source by an inducible arginase and a constitutive urease. Many *Agrobacterium* strains are also able to use arginine and ornithine as carbon source. However, many *Agrobacterium* strains unable to grow on arginine or ornithine as a carbon source display this ability when they harbor a wild-type octopine or nopaline Ti plasmid (Ellis *et al*., 1979). *Synechocystis sp.* PCC 6803 were grown axenically in BG11 (nitrate containing) medium (Rippka *et al*., 1979). Cultures were grown 30°C in light with shaking (80-90 rpm) for liquid cultures and supplemented with 1mM filter sterilized L-arginine. Drainas and Weiss (1980) studied the effect of various carbon sources on arginase activity in *Neurospora crassa* and found that basal and induced levels of arginase were reduced in the following order sucrose, acetate, glycerol and ethanol with regard to carbon sources used. They also reported that arginine pools were similar regardless of carbon source in mycelia grown in arginine-supplemented medium and arginine degradation was proportional to level of arginase in both sucrose and glycerol grown mycelia suggesting a possible carbon metabolite effect on arginine metabolic enzyme genes in the fungus. Schreier *et al*., (1982) studied the regulation of inducible nitrogen catabolic enzyme arginase in *B. licheniformis* stating that although levels of enzyme were nitrogen source (Ammonia, Glutamine, Urea, Arginine, Ornithine) dependent its induction could still occur even in presence of preferred nitrogen sources. *Neurospora crassa* strain was grown at 30°C in Vogel’s minimal medium (VM) with 1.5% sucrose as carbon source. Initially it was supplied with 5mM arginine, 1mM histidine or NH₄NO₃, later these were replaced with 5mM arginine which acted as nitrogen source. Liquid cultures were inoculated with 10⁶ conidia per ml of media in baffled flasks and shaken at 250rpm (Marathe *et al*., 1998). Two forms of arginine with differing molecular weights i.e., 36 kDa and 41 kDa were produced in unsupplemented and arginine-supplemented media repectively.

### 2.6.2 Purification and kinetic characterization of arginase:

Rabbit liver arginase was purified by Breitburd and Orth (1971) by chromatographic techniques of DEAE cellulose and Sephadex G-200. It was strongly inhibited by EDTA and lost activity in absence of Mn²⁺ ions. SDS-PAGE showed that enzyme had a subunit molecular
weight of 36,500, kDa increasing probability of its tetrameric nature. Molecular characteristics of chicken liver arginase were accounted by Grazi and Magri (1971). Purification and properties of rat kidney arginase were studied by Kaysen and Strecker (1973) showing the enzyme was highly stable at high temperatures such as 60°C and had a Km value of 18 mM and was strongly inhibited by borate and L-ornithine. Tarrab et al., (1974) studied molecular isoforms of rabbit liver arginase by purifying them in a sequential manner by CM cellulose chromatography. Purification studies of arginases from human-leukemic lymphocytes and granulocytes were done by Reyero and Dorner (1975) employing purification procedure of acetone extraction, ammonium sulphate precipitation, DEAE-cellulose, CM-Sephadex chromatography and gel filtration on Bio-Gel A. Both the arginase proteins were strongly basic with pI values between 9.25 and 9.35. Rat small intestinal arginase was purified by Sephadex -100 filtration (Fujimoto et al., 1976) and shown to have optimum pH of 10.0 and Km of 19 mM. The enzyme was almost completely inactivated by treatment with EDTA. The isoforms differed in their kinetic and pH behavior. Arginase from human liver and erythrocytes was isolated (Beruter et al., 1978) and purified by DEAE-cellulose chromatography followed by gel filtration on Sephadex G-200. The human liver arginase had a molecular weight of was 107, 000 and its Km for arginine was 10.5 mM. The properties of human heart arginase studied by Baranczyk et al., (1980) revealed its Km to be 5 mM and molecular weight of about 30, 000 kDa with the enzyme having strong dependence on Mn²⁺ ions for its activity.

Gopalakrishna and Agarajan (1980) reported the purification of arginase from rat fibrosarcoma by heat treatment followed by DEAE-cellulose chromatography and gel filtration on Sephadex G-100 and accounted that the Km for arginine was 11 mM and had a pH optimum of 10. Purification and physical properties of Arginase from Xenopus laevis liver were studied by procedures of heat treatment, acetone fractionation, and isoelectric focusing (Peiser and Balinsky, 1982) and its molecular subunit weight was determined by gel filtration on Sephadex G200 and found to be 76,000 daltons. Arginase was obtained from Iris hollandica bulbs and purified approximately using DEAE-Sephadex chromatography, aminohexyl-Sepharose 4B
chromatography and gel filtration on Ultrogel AcA 34 by Boutin (1982). Constitutive arginase from *Evernia prunastri* thallus was purified 920-fold and is activated by endogenous L-arginine (Martin and Legaz, 1984). Borkovich and Weiss in 1987 reported that *Neurospora crassa* arginase has a subunit weight of 38,300 determined by SDS-PAGE. The enzyme exhibited hyperbolic kinetics at pH 9.5 with an apparent Km for arginine of 131mM. Antiserum was prepared against the purified enzyme and two proteins in the extracts of *S. cerevisiae* were detected that were weakly cross-reactive with the antiserum. Two forms of arginase were isolated from human erythrocytes and their immunological properties were studied by Kedra et al., (1988).

Later, Ikemoto et al., (1989) developed an efficient method involving hydrophobic chromatography and immunoaffinity chromatography for purification of human erythrocyte arginase. Patil et al., (1990) gave a high recovery protocol for purification of ox erythrocyte arginase involving heat treatment, CM and DEAE-Sepharose chromatography, arginine AH-Sepharose chromatography and molecular sieving through Biogel P-150. The optimum pH was 11.5 and temperature was 55°C for purified enzyme. Soyabean *Glycine max* axes arginase was purified by Kang and Cho (1990) and its properties were studied. The enzyme purification procedure followed was ammonium sulphate precipitation followed by chromatography on Sephadex G-200, DEAE Sephacel, Hydroxypatite and Arginine-Sepharose 4B affinity column. The enzyme had a Km of 83 mM and pH optimum of 9.5 with a molecular weight of 240,000 obtained by pore-gradient electrophoresis. Purification of recombinant human liver arginase expressed in *E. coli* was described by Ikemoto et al., (1990) by chromatographies on CM-Sephadex G-150, DEAE-cellulose and Sephadex G-150 followed by preparative gel electrophoresis. The purified enzyme was a monomer of molecular weight 35,000 kDa. Singh and Singh, (1990) purified liver arginase from teleostean fish-*Clarias batrachus* having Km of 15.38 mM for arginine and an optimum pH of 9.5. Ornithine and leucine act as competitive inhibitors whereas valine and isoleucine act as non-competitive inhibitors with respect to L-arginine as substrate. Purification of arginase from *S. cerevisiae* was done by Sepharose chromatography and TSK-G3000 gel filtration followed by its characterization (Green
et al., 1989) which revealed a Km of 15.7 mM for arginine and requirement of Mn$^{2+}$ ions for activity. CD spectra analysis showed significant spectral changes from removal of bound metal and dialysis against EDTA.

A thermostable arginase from the extreme thermophile *Bacillus caldovelox* was purified by chromatographic techniques and its kinetic properties were studied in 1991 by Patchett et al. Its activity is optimal at pH 9 and temperature of 60°C. The Km for arginine is 3.4 mM. Arginase obtained from the phototrophic bacterium-*Rhodobacter capsulatus* E1F1 was purified and studied for its properties. The molecular parameters and kinetic constants of *Rhodobacter* arginase resembled the *Saccharomyces cerevisiae* enzyme rather than those of bacterial arginases (Moreno et al., 1992). Extrahepatic arginase (ArgII) was isolated from rat mammary gland and its properties were investigated by Jenkinson and Grigor (1994). This enzyme had a pH optima of 10 and Km of 12-14mM. Purification of human hepatic arginase and its manganese (II) - dependent and pH-dependent interconversion between active and inactive forms was described by Kuhn et al., (1995) who showed that both the maximal velocity of catalysis and the Km toward arginine were markedly pH dependent in the physiological range. In situ characterization of *Helicobacter pylori* arginase employing NMR spectroscopy, spectrophotometry, radiotracer analysis and protein purification techniques was done by Mendz et al., (1998). A Km of 22+/33mM was determined for the enzyme activity and differences of Vmax were observed between strains. Divalent cations stimulated arginase activity & most potent activators were Co$^{2+}$ > Ni$^{2+}$ > Mn$^{2+}$.

Recombinant human arginase II was expressed in *E. coli* and purified to homogeneity by Colleluori et al., (2001). The enzyme reportedly had a Km of 4.8 mM at physiological pH and exists primarily as a trimer. Borate was a non-competitor inhibitor of the enzyme and ornithine which is an inhibitor of Arginase-I is not an inhibitor of the type-II enzyme showing that isozyme selectivity occurs between both forms with regard to substrate and product as well as inhibitor binding. *Pinus taeda* arginase (Todd et al., 2001) was purified by chromatographic separation on DE-52
cellulose, Matrex Green and arginine-linked Sepharose 4B and had a molecular mass of 140 kDa deduced by FPLC while the subunit size was shown to be 37 kDa observed by SDS-PAGE analysis. D-arginase obtained from \textit{Arthrobacter sp} KUJ 8602 was purified by anion exchange chromatography (Arakawa \textit{et al.}, 2003) using DEAE-Toyopearl 650M followed by hydroxyapatite chromatography and gel filtration chromatography with Superdex 200. The purified enzyme was proposed to be a homohexamer with each subunit of approximately 40,000 determined by SDS-PAGE. It had an optimum pH of 9.5 and needed Zn\textsuperscript{2+} for activation instead of Mn\textsuperscript{2+}. Bovine liver arginase as reported by Wheatley \textit{et al.}, (2003) is a remarkably heat resistant enzyme with a very long life on storage at 4°C in lyophilised form and is occasionally marginally more active at pH 7.2 than at pH 9.9. \textit{Helicobacter pylori} arginase expressed in \textit{E. coli} was loaded onto polypropylene columns containing nickel-nitrilotriacetic acid agarose resin for purification (McGee \textit{et al.}, 2004). The purified enzyme had significant activity with cobalt as cofactor and had acidic pH optima of 6.1. It was inhibited by low concentrations of reducing agents. Dabir \textit{et al.}, (2005) described the purification, properties and alternate substrate specificities of the enzyme from two different sources: \textit{Vigna catjang} cotyledon and buffalo liver. The Michealis-Menten constant for cotyledon arginase and hepatic arginase were found to be 42 mM & 2 mM respectively and their pH-optima were 10 & 9.2 respectively. Munder \textit{et al.}, (2005) have reported that arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. Two arginases Arg I and Arg II were separated from \textit{Fasciola gigantica} by purification on a DEAE-Sepharose column as reported by Mohamed \textit{et al.}, (2005). Further purification was carried out for Arg II and it was shown that the enzyme was activated by Mn\textsuperscript{2+} and inhibited by Fe\textsuperscript{2+}, Ca\textsuperscript{2+}, Hg\textsuperscript{2+}, Ni\textsuperscript{2+}, Co\textsuperscript{2+} and Mg\textsuperscript{2+} ions. Purification of recombinant \textit{Plasmodium falciparum} arginase (Muller \textit{et al.}, 2005) was done on Superdex S-200 column and its activity was found to be dependent on Mn\textsuperscript{2+}. Ni\textsuperscript{2+} when replaced with Mn\textsuperscript{2+} resulted in four-fold loss of activity. Viator \textit{et al.}, (2008) reported the purification of recombinant \textit{Bacillus anthracis} arginase expressed in \textit{E. coli} XL1-Blue MRF’ on arginase affinity columns.
2.7 Structure related studies

Most of the studies related with the structure of arginase are by means of studying the interaction of various inhibitor complexes with the substrate specific sites of the enzyme. Electron paramagnetic resonance (EPR) studies on rat liver arginase by Reczkowski and Ash (1992) revealed that fully Mn-activated arginase contains two Mn\(^{2+}\) ions per subunit and these form EPR spin-coupled binuclear centers. The high resolution X-ray crystal structure of rat arginase I was provided by Kanyo et al., (1996) which illustrated that the enzyme is a 105 kDa homotrimer with each subunit of 35 kDa containing a spin-coupled binuclear manganese cluster critical for its activity located at the bottom of a \(\sim 15 \text{ A}\) \(^\circ\)-deep active site cleft. Bewley et al., (1999) studied the crystal structure of arginase structure of inactivated \textit{Bacillus caldovelox} arginase-L-arginine complex which revealed that the structural basis for substrate and inhibitor specificity are an array of direct and water mediated hydrogen bonds saturating all four acceptor positions on \(\alpha\)-carboxylase group and all three donor positions on \(\alpha\)-amino group. Cox et al., (2001) noted the binding of N\(^\omega\)-hydroxy-L-arginine (NOHA)-an intermediate of NO biosynthesis and modest inhibitor of the enzyme with rat arginase I which revealed that the N\(^\omega\)-hydroxyl group displaces the metal-bridging hydroxide ion and bridges the binuclear manganese cluster and that in the binding of rat arginase I and nor-NOHA the N\(^\omega\)-hydroxyl group of the inhibitor displaces the metal bridging hydroxide ion. The structure for human kidney II type arginase (Cama et al., 2003) is nearly identical to that of rat arginase I due to conservation of all metal ligands between the two sequences and its polypeptide fold is topologically identical to \(\alpha/\beta\) fold of rat arginase I and hexameric arginase from \textit{Bacillus caldovelox}. Crystal structure of human arginase I at 1.29- angstrom resolution has been studied by Di-Costanzo et al., (2005). Inhibitor complexes of arginase I with inhibitors ABH and BEC were studied to know the inhibition of the enzyme in human and urine myeloid cells to study its effect on immune response. The role of the hyper-reactive histidine residue (H141) in rat liver arginase was studied by studying the structure of the enzyme (Colleluori et al., 2005) by chemical modulation, mutagenesis and X-ray diffraction. By the study, the H141 has been shown to possess
conformational mobility supported by its proton shuttling role and proposed to be an acid/base catalyst by deprotonating the metal-bridging water molecule to generate the metal-bridging hydroxide nucleophile, and by protonating the amino group of the product to facilitate its departure. Di Costanzo et al., (2007) determined the X-ray crystal structure of a perdeuterated human arginase I complexed with 2(S)-amino-6-boronohexanoic acid (ABH) at 1.90 Å resolution and found that the perdeuteration did not cause any structural and functional changes when compared with the unlabeled enzyme thus rendering the perdeuterated crystals suitable for neutron crystallographic study. Dowling et al., (2010) have reported the 2.15 Å resolution crystal structure of arginase from the cerebral malarial parasite *Plasmodium falciparum* in complex with the boronic acid inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) - the first report of crystal structure of a parasitic arginase. Inhibition studies with the enzyme structure lend important insights into the antimalarial therapy against liver-stage infection, and ABH may serve as a lead for the development of inhibitors.

### 2.8 Recombinant Arginase

Development of recombinant arginase has been an intriguing subject of research worldwide. Molecular cloning and nucleotide sequence of cDNA for human liver arginase facilitated the investigation of the enzyme and gene structures and helped in elucidating the nature of mutation in argininemia. Arginase activity was detected in *Escherichia coli* cells transformed with the plasmid carrying lambda hARG6 cDNA insert (Haraguchi et al., 1987). cDNA phARG6 for human liver arginase was used for expression of human liver arginase in *E. coli* strain KY1436 by Ikemoto et al., (1990). This *E. coli* expressed human liver arginase had chemical, immunological and most catalytic properties indistinct from purified human erythrocyte arginase. The cloning, expression and crystallization of a thermostable arginase from the thermophilic bacterium ‘*Bacillus caldovelox*’ has been discussed by Maria et al., (1996). In this study, the expression of recombinant arginase at high levels was achieved in *E. coli* using an inducible T7 RNA polymerase based system. Molecular cloning and nucleotide sequence of the arginase gene of *Bacillus brevis*
TT02-08 and its expression in *Escherichia coli* has been described by Shimotohno *et al.*, (1997). The *B. subtilis* arginase encoding rocF gene was cloned and expressed in *E. coli* K-12 for enhanced production of urea by arginase pathway (Tuchman *et al.*, 1997). Modulation of mRNA levels of liver arginase by insulin and vanadate in experimental diabetes has been studied by Salimuddin *et al.*, (1999) where it was shown that an increase in arginase activity and mRNA levels in diabetes and decrease in treated animals may be due to the transcriptional regulation of arginase gene. Kimura *et al.*, (2000) transformed *E. coli* strain KY1436 by pTAA12 expression plasmid vector containing human liver-type arginase cDNA and developed an ELISA system by using this recombinant enzyme as an antigen for the detection of anti-arginase antibodies in patients with Autoimmune Hepatitis (AIH). The expression, purification and characterization of human type II arginase involving genetic engineering experiments to develop a recombinant human type II arginase expressed in *Escherichia coli* was done by Colleluori *et al.*, 2001. The recombinant enzyme was studied for its kinetic properties and inhibitor effects on it. The findings indicated that isozyme selectivity exists between type I and type II arginases for binding of substrate and products, as well as inhibitors. The rocF gene encoding arginase in *Helicobacter pylori* was purified and expressed in *E. coli* (McGee *et al.*, 2004) and was found to confer arginase activity to *E. coli*. The transformed cells expressing arginase showed enhanced enzyme activity than the native *Helicobacter* enzyme. Recombinant arginase was developed by cloning DNA of *Plasmodium falciparum* (Pfarginase) into *E. coli* BL21. The recombinant enzyme had a Km of 13±2 mM, required Mn$^{2+}$ for activity and had high thermal stability (Muller *et al.*, 2005). The RocF gene of *Bacillus anthracis* was cloned in *E. coli* for development of a recombinant enzyme. The metal preference of the enzyme was shifted at pH 6 from Ni>Co>Mn to Ni>Mn>Co at pH 9. A novel recombinant *B. subtilis* prophage strain LLC101 was constructed for recombinant enzyme production by Cheng *et al.*, (2005). The recombinant enzyme produced was used for treatment of human malignancies by subsequent pegylation (Cheng *et al.*, 2005; Cheng *et al.*, 2007; Tsui *et al.*, 2009). A highly active recombinant arginase was obtained by expressing arginase gene from *Leishmania*
Review of Literature

(Leishmania) *amazonensis* (da Silva *et al.*, 2008) in *E. coli* BL21 (DE3) cells. Antibody against the recombinant protein confirmed a glycosomal cellular localization of the enzyme in promastigotes.

2.9 Modification of Arginase: Immobilization & Chemical Modification

Attempts to increase the activity and stability of the enzyme by various techniques of immobilization and chemical modification have been made. Vanillin polymethacrylates (vanacryls) were used for immobilization of arginase by Brown and Joyeau (1974). Carvajal *et al.*, (1977) reported the immobilization of human liver arginase by attachment to nylon with glytaraldehyde as a cross-linking agent. The immobilized enzyme tetrameric enzyme dissociated into monomers by treatment with EDTA. Influence of immobilization on solid matrix on rat liver arginase (Muszynska and Wojtczak, 1979) revealed that the immobilized enzyme was more resistant to effect of inhibitors, denaturing agents such as SDS and chelating agents such as EDTA than free enzyme. Bovine liver arginase was covalently immobilized by glutaraldehyde method to inner surface of Cuprophan hollow fibers of a conventional hemodialyzer (Rossi *et al.*, 1981). The immobilization method did not harmfully affect the physical and mechanical properties of neither hollow fibers nor their hemocompatibility. Aguirre and Kasche, (1983) by covalently coupling the enzyme to Sepharose beads and dissociating the resulting matrix-bound tetramer by acid or EDTA treatment, obtained catalytically active monomer forms of immobilized rat liver arginase. Savoca *et al.*, (1984) studied the therapeutic effectiveness of arginase in cancer therapy on a Taper liver tumor and the L5178Y murine leukemia by modifying it by covalent attachment of polyethylene glycol. The pegylation of the enzyme increased its stability. Beef liver arginase was modified by the covalent linking of monomethoxypolyethyleneglycol molecules (Visco *et al.*, 1987). The derivative enzyme had more convenient properties for a therapeutic use, such as increased structural stability, decreased digestion by proteolytic enzymes and an expanded clearance time in rats. Veronese *et al.*, (1988) discussed the immobilization of arginase by radiation induced polymerization of induced monomers. Properties of arginase
immobilized in a fibrin clot were studied by Diez et al., (1990). That chemical modification of rat liver arginase by N-bromosuccinamide leads to its inactivation was reported by Daghigh et al., (1992). Arginase isolated from beef liver was covalently attached to a polyacrylamide bead support bearing carboxylic groups activated by a water-soluble carbodiimide (Dala & Szajani, 1994). The pH optimum for the catalytic activity was pH 9.5 and apparent temperature maximum was 60°C. Immobilization markedly improved the conformational stability of the enzyme. An enzyme reactor for determination of L-arginine was described by Alonso et al., (1995) by immobilization on an epoxy resin matrix. Pegylation has been reported to greatly improve the stability of the enzyme (Wheatley et al., 2003). Pegylated recombinant human arginase has *in vitro* and *in vivo* anti-proliferative potential and apoptotic activities in human hepatocellular carcinoma (Cheng et al., 2005, 2007). For studying thermodynamics of nor NOHA/arginase binding and the role of the reactive histidine residue arginase was immobilized on a chromatographic support by Bagnost et al., (2007). Pegylation of rhARG with methoxypolyethylene glycol-succinimidy1 propionate (mPEG-SPA 5,000) proved to be the best for activity retention of the immobilized enzyme for use in arginine depletion as an anti-cancer therapy protocol (Tsui et al., 2009). Leishmania arginase was immobilized on a Ni²⁺ resin and its activation kinetics was studied by varying Mn²⁺ concentrations at a temperature of 23°C. Conformational changes were observed when enzyme interacted with Ni²⁺ present in the column (Silva and Winter, 2010). Konst et al., (2010) have reported the stabilization and immobilization of *B. subtilis* arginase on commercially available epoxy-activated supports out of which immobilization on Sepabeads EC-EP was most promising. The immobilized enzyme was employed in the biobased production of nitrogen-containing chemicals as an alternative to the petrochemical production.

### 2.10 Assay procedures for Arginase:

Numerous methods based on different principles for assaying arginase are available. Some of them are listed below:
2.10.1 Colorimetric Method: These methods are based on the colorimetric determination of urea or ornithine- the products of arginase reaction which can be assayed by the development of a colorimetric procedure. In the procedure of urea determination (Foster et al., 1971) - urea is determined using 2,3 – butanedione reagent (BUN reagent). This method has been reported for arginase assay (Basch, 1997). The colour is measured at 460 nm. In the method of Coulombe and Favreau, (1963) urea formed gives red colour on reaction with diacetyl monoxime and thiosemicarbazide in acid medium whose absorbance is measured at 535 nm. In the method of Davis and Mora, (1968), the urea formed is assayed by reaction with α-isonitrosopropiphenone. This procedure was originally devised for high accuracy urea determination in blood filtrates and urine by Archibald (1945). The absorbance change is read at 540 nm. A novel colorimetric assay was devised by Aminlari (1992) where determination of residual arginine, after its conversion with p-nitrophenyl glyoxal (PNPG) at pH 9.0 in the presence of sodium ascorbate was monitored. The decrease in absorbance in the presence of arginase correlates with the enzyme activity. A colorimetric microplate assay for arginase was recently developed by Iyamu et al., (2008) for high-throughput analysis of arginase activity in vitro. The method was a modified form of the Chinard reaction of ornithine-ninhydrin reaction.

2.10.2 Electrochemical Method

Booker and Haslam (1974) used the following reaction sequence for assay of arginase by means of a cation NH₄⁺ electrode that was used to sense the NH₃ liberated and immobilized urease was used. From 1.6 to 16 U of arginase could be assayed in 10 min with a CV of 3%.

\[ \text{L-arginine} \xrightarrow{\text{L-arginase}} \text{Urea} + \text{L-ornithine} \]

\[ \text{Urea} \xrightarrow{\text{Urease}} \text{NH}_4^+ + \text{CO}_2 \]
Larsen et al. (1975) have described a kinetic method for Arginase measuring the NH\(_3\) liberated with an air gap NH\(_3\) electrode. The above sequential reactions liberate NH\(_3\) whose determination as NH\(_4^+\) is done by the electrode. The determinations were based on monitoring the initial reaction rates of the selective release of ammoniacal nitrogen.

A tenfold excess of urease ensured direct proportionality between the rate of NH\(_3\) production and the arginase activity. The coefficient of variation (CV) was 2.8%.

**2.10.3 Spectrophotometric Method:** Ward and Srere (1967) reported a novel arginase spectrophotometric assay by based on the principle that the absorbancy of arginase below 2100 Å is larger than the combined absorbancies of ornithine and urea. A cleavage of arginine catalyzed by the enzyme thus results in a net decrease in absorbancy at these wavelengths, allowing a rapid and accurate assay for arginase activity. Ozer (1985) developed a new spectrophotometric assay for arginase in which the enzyme is coupled to urease and glutamate dehydrogenase and the decrease in absorbance at 340 nm due to the oxidation of NADPH is followed. Han and Viola (2001) developed an alternative assay for arginase by synthesising an alternative substrate of arginase has been synthesized in which the bridging guanidium nitrogen has been replaced with a sulphur. This thiol compound is a good substrate for arginase, leading to urea and 2-amino-5-mercaptovaleric acid which can be monitored with DTNB to produce a disulfide adduct and release 2-nitro-5-thiobenzoate as a chromophoric product.

**2.10.4 Flourimetric Method:** Orfanos et al., (1980) described a microfluorometric method for assay of arginase activity in dried bloodspots on filter paper. The urea formed from the arginase reaction is determined flurometrically by oxidation of NADH to NAD\(^+\) in a coupled kinetic reaction.

**2.10.5 Conductimetric method:** Macholan et al., (1989) have constructed a bioelectrode consisting of two parallel noble metal nets and a thin layer of gel-entrapped urease (for urea determination) and microbial cells or tissue slice (for arginase determination) for conductimetric measurements in tissue extracts.
2.10.6 **Radioisotopic Method:** This method was reported by Carulli *et al.*, (1967) with an underlying principle of hydrolysis of labeled substrate (arginineguanido-14C). The labeled urea produced is hydrolyzed by urease with the measurement of released 14CO2 in a liquid scintillation counter as a measure of arginase activity. Advantages of the method reported were sensitivity and accuracy derived from the absence of interference by nonradioactive urea in the incubation mixture.


2.10.7 **Other methods:** Bastone *et al.*, (1990) carried out measurement of arginase along with argininosuccinate lyase both in liver and erythrocytes, by using a commercial amino acid analyzer. The two different substrates used by both the enzymes give the similar product ornithine whose measurement is done by this method. Assay kits such as QuantiChrom™ Arginase assay kit for determining arginase activity are commercially available in market.

2.11 **L-arginine detection by Biosensors**

Biosensors are useful tools for clinical and medical analysis of compounds and their reactions and enable us to make practical analysis without time consumption. A number of studies have been carried out for development of Biosensor methods for monitoring L-arginine in physiological fluids and food samples.

Rechnitz *et al.*, (1977) developed a potentiometric sensor for monitoring arginine by coupling *Streptococcus faecium* cells with an ammonia gas-sensing membrane electrode. The development of a plug-flow reactor for determining arginine by immobilizing arginine decarboxylase on controlled pore glass beads and monitoring of the evolved CO2 by with CO2 sensing electrode was described by Vega *et al.*, (1980) and the process was used for monitoring arginine in peanuts to assess
their maturity. Grobler et al., in 1981 developed a bacterial electrode for arginine determination by means of immobilizing living Streptococcus lactis bacterial cells for conversion of arginine to ammonia by the bacterial enzyme machinery by using an ammonia gas-electrode as a detector.

Using microfabricated Clark oxygen electrodes, integrated amperometric amino acid sensors to detect L-glutamate, L-lysine, L-arginine, and L-histidin have been developed by Suzuki et al., (1994) where enzymes were immobilized on the gas-permeable membrane to detect L-lysine, L-arginine, and L-histidin, decarboxylases and autotrophic bacteria. Liu et al., (1995) determined arginine by development of a new sensor system called the surface acoustic wave (SAW)/conductance sensor system. The assay was based on two coupling reactions involving arginase and urease with measurement of frequency shift that resulted from the changes of conducting ions produced in the reactions. Koneki et al., (1996) described the construction of bienzymatic potentiometric electrodes for L-arginine determination by covalent binding of arginase and urease by using carbodiimide and glutaraldehyde on carboxylated poly(vinyl chloride) matrix of the ion-selective ammonium sensitive membrane. Biological determination of arginine by using electropolymerized polypyrrole (PPy) electrode in combination with a polyion complex of arginase and urease was performed by Komaba et al., (1998). Oungpipat and Lenghor (1999) developed an arginine biosensor based on immobilization of bovine liver arginase and Cajanus cajan tissue was used as source of urease. A stainless steel electrode was used for monitoring the pH change during the reaction. Sarkar et al., (1999) developed screen printed electrodes for monitoring L & D- amino acids by electrochemical biosensor preparation using enzymes D-amino acid oxidase (porcine kidney) &L-amino Acid Oxidase (Crotalus adamanteus). Both L- arginine and D-arginine were determined by these electrodes.

Electrochemical biosensors for chiral analysis of amino acids including L-arginine by bienzymes L-amino acid oxidase and horseradish peroxidise were described by Dominguez et al., (2001). L-arginine & other components giving unique
taste to Korean green tea samples with unknown manufacturer specifications have been determined by means of disposable all solid-state potentiometric electronic tongue microsystem (Lvova et al., 2003). Arginase selective biosensor based on arginase-urease coimmobilized in gelatin cross-linked with glutaraldehyde for arginine determination has been developed by Karacoaglu et al., (2003) for monitoring arginine in clinical samples such as blood serum.

2.12 Sol-gel immobilization technology:

By enzyme immobilization, enzymes are confined to selective supports/matrices for their multiple reuse, easy separation, retention of catalytic activity, control of reaction time, minimising enzyme loss and improved stability for biological, industrial and commercial processes.

Sol-gel immobilization technique has emerged as a widely acceptable, useful and interesting technique for immobilization of biomolecules and more particularly enzymes. The sol-gel technique offers advantages such as (a) simplicity, (b) the process occurs at room temperature and reduces protein denaturation, (c) biocompatibility, (d) resistance to microbial attack, (e) negligible swelling behaviour, (f) optical transparency, (g) the sol-gel system retains a large quantity of water making the enzyme catalytic centres long-term stable.

2.12.1 Fundamentals of Sol-Gel Process

The overall sol-gel process, as the name implies, usually involves two stages. First, the precursors (usually inorganic salts or organic metal alkoxides) initially undergo hydrolysis and polycondensation to form high molecular weight, yet soluble poly-intermediates called the sol. These intermediates then link together to form a three-dimensional network called a gel.

The sequential reactions that take place are can be shown as follows:

\[(RO)_3 SiOR + H_2O \rightarrow (RO)_3 SiOH + ROH\]
\[2(RO)_3SiOH \rightarrow (RO)_3Si-O-Si(OR)_3 + H_2O\]

\[(RO)_3SiOH + ROSi(OR)_3 \rightarrow (RO)_3Si-o-Si(RO)_3 + ROH\]

The resulted sol gel is a unified rigid network with pores of sub-micrometer dimensions and polymeric chains whose average length is greater than a micrometer. HCl and ammonia are the most generally used catalysts for the hydrolysis; however, other catalysts such as acetic acid, KOH, amines, KF, and HF are also used. Removal of liquid in the pore by thermal evaporation at or near ambient pressure results in drying and shrinkage of the gel, the resulting monolith being termed as “xerogel”. If the liquid is primarily alcohol, the monolith is termed as an “alcogel.”

**Figure 2.1: Sol-gel immobilization processes**

(http://sariyusriati.files.wordpress.com/2008/10/flowchart-sol-gel1.jpg)

The design flexibility of sol-gel matrices is enormous with the gels being able to form monoliths such as hydrogels and xerogels or their thin films can be easily deposited by dip-coating or spin-coating. The films can be modified as per need by incorporation of various dyes and sensing materials (eg. flourophores) for development of sensing devices (biosensors). Moreover, fibers can be drawn from
viscous gels and nanopores can be synthesised from colloidal dispersions. While fabricating sol-gels a very crucial parameter to be kept in mind and controlled suitably is the pore size of the gel. The pore-size is a vital characteristic of any sol-gel since it plays a major role in utility and success of encapsulation, it should be large enough to permit the entry and transport of molecules such as buffer ions, substrates & products of the reaction and analytes and simultaneously small enough to prevent leakage of immobilized macromolecules. The sol-gel method and its different variations have shown to be adequate for stability of biomolecules entrapped in them. Sol-gel matrix “cages” have been found to be biocompatible and satisfactory for preserving protein structure and functionality.

The foremost application of sol-gel chemistry in immobilization of enzymes lies in the development of sol-gel encapsulated enzyme-based Biosensors. An ample range of electrochemical and optical biosensors have been developed by sol-gel immobilized enzymes for the determination of various analytes. Sol-gel film based biosensors have been reported by a number of workers such as Doong et al., (2001) for immobilization of acetylcholinesterase and FITC-dextran (Paraoxan monitoring), cholinesterase and bromocresol purple in TMOS+PTMOS for pesticides-carbaryl dichlorvos (Andreou and Clonis, 2002), carbonic anhydrase coimmobilized with cresol red in TMOS for acetazolamide (Jeronimo et al., 2005). An inherently labile lactate oxidase was immobilized over Prussian blue modified electrode by sol-gel method using water–organic mixtures with the high content of organic solvent as a reaction medium for development of an advanced lactate biosensor was reported by Yashina et al., (2010).

Encapsulation of living cells is another splendid forte of sol-gel technology. These cells are infact miniaturized natural bioreactors having well-developed enzyme production machinery. These can be used for applications ranging from the development of biosensors to processes in food industry, waste treatments and
production of drugs or chemicals. Recombinant luminous bacteria (Premkumar et al., 2002) immobilized into TEOS sol-gel showed almost similar luminescence as free cells and remarkable stability of 4 weeks at 4°C. Ferrer et al., (2003) studied the expression of green fluorescent protein (GFP) of genetically engineered E. coli TG1/pPBG11 immobilized by sol-gel process in response to presence of alkanes and ketones. Alvarez et al., (2007) studied the immobilization of inorganic sol-gel formed by inclusion of citric acid in sol-gel matrices for use in immobilization of bacteria E. coli and Staphylococcus aureus and found that citric acid neutralized the alkalinity of silica precursors and hence improved the biocompatibility of the procedure. Kim et al., (2009) have reported the immobilization of E. coli in silica sol-gel matrix using alkoxy silane monomers, this being the first report to account the intact macromolecular protein synthesis machinery of E. coli in the sol-gel immobilization system.

2.13 Fiber-optic biosensors: a magnificent skill of sensing

Since 1950, when fiber-imaging bundles were first put together till now fiber optic research has evolved on just one pathway- expansion Optical fiber based biosensors are analytical devices formed as a result of combination of optical fibers and biorecognition molecules. The underlying principle of analysis is the transmission of light signals through optical fibers to sensing layer coupled to the fiber-end where different phenomena such as absorbance or luminescence are used to measure interactions between analyte and sensing layer.
Figure 2.2: The scheme of measurement of a fiber-optic enzyme based biosensor
The analyte (substrate) enters the enzyme layer where it is converted into products. The indicator (sensing) layer consists of an indicator dye to record the formation of reaction product or consumption of reactant. Exc: excitation light pathway; Em: Emission light pathway (Borisov & Wolfbeis, 2008)

The main components of a fiber-optic based analytical system (optrode) include (a) a light source; (b) an optical fiber that can act for both transmissions of light as well as a substrate; (c) the sensing material immobilized to the surface of the end face of the fiber; and (d) a detector to measure the output light signal. Computers and microprocessors control optrode instrumentation and analyse output signals. The working comprises the interaction of the sensing element with the analyte resulting in change in physicochemical properties which change its optical properties generating optical signals that can be correlated to the analyte concentration, optical signals can be measured from the light source through optical fiber to the fiber end, where the sensing element is immobilized. The same fiber or a different fiber, is used to guide the output light to the detector (eg., spectrophotometer, fluorometer) where the reflected, emitted or absorbed light is measured (Biran et al., 2008).

Fiber-optic biosensors offer advantages of (a) having a wide analytical sphere since almost every chemical analyte can be determined by using its spectroscopic properties; (b) capable of transmission of light over long distances since the bioreceptor need not be in intimate contact with the optical fiber; (c) having multiplex capability by guiding light of different wavelengths simultaneously in different directions for multiple analyte determinations; (d) can be used in harsh environments being resistant to electric and magnetic interference so are safer than electrochemical
biosensors; (e) do not need reference electrode and temperature dependence is lower than that of electrodes; and (f) can be easily miniaturized at low cost for application in *in-vivo* measurements.

Due to the large number of reactions they catalyse, the specificity of analytes (substrates, inhibitors and products) they detect and the variety of transducers that can be coupled to them, enzymes are the most extensively used biological components of fiber-optic biosensors. A particularly interesting field of Fiber-optic sensors and biosensors is their development by immobilizing various color reagents (dyes) and fluorescent dyes along with biocomponent machinery in sol-gel matrices for detection of a wide range of analytes. Bright et al., (1988) reported the development of a new fluorimetric ion sensor based on the measurement of quenching or enhancement of the Rhodamine 6G (entrapped hydrophobically and electrostatically on a Nafion film) fluorescence by various ions. Gao et al., (1995) discussed the fabrication of tapered tips for fiber-optic biosensor development by dipping the fiber tips into sol gel containing rhodamine 6G solutions resulting in side surface and distal end coating of the tip. Preininger et al., (1996) reported the development of a novel optical sensor for ammonia utilizing rhodamine dyes immobilized in thin membranes made from ethylcellulose, poly(vinyl chloride) or poly(vinyl acetate). A fiber-optic sensor for pH monitoring based on immobilization of phenol red, cresol red and bromophenol dyes in sol-gel has been developed by Gupta et al., (1997). Fluorosensors for ammonia based on fluorescence energy transfer were developed by Preininger and Mohr (1998) where donor-acceptor complexes such as ion pairs consisting of a fluorophore (rhodamine B or tetramethylrhodamine ethyl ester) and an absorber (bromophenol blue) were immobilized in PVC and in sol-gel. Excitation energy transfer between acriflavine (donor) to rhodamine 6G (acceptor) molecules in water with varying pH was used as a pH sensor by Misra et al., (2000). Reagentless fluorescence-based sensing films made by immobilization of urease containing fluorescein or carboxy-seminaphtharhodafuor-1 (SNARF-1), either free or conjugated to a dextran polymer backbone in sol-gel were reported by Gulcev et al., (2002) for urea detection. Fabrication of optical sensors for pH detection has been discussed by Wang et al., (2003) by spin coating silica sol obtained by acidic hydrolysis of tetraethoxysilane.
Goldfinch and Lowe (2004) discussed the development of solid-phase optoelectronic sensors for penicillin, urea, and glucose by derivatization of triphenylmethane dyes such as bromocresol green and bromthymol blue with glutathione and co-immobilized with penicillinase, urease, and glucose oxidase. Narang et al., (2004) discussed the entrapment of fluorescein and rhodamine 6G in TEOS sol-gel derived optical fibers. Tsai and Doong (2005) accounted an array-based optical biosensor for the simultaneous determination of pH, urea, acetylcholine and heavy metals by coentrainment of urease and acetylcholinesterase (AChE) with the sensing probe and FITC-dextran in a sol-gel matrix. Encapsulation of bromothymol blue and phenol red pH indicators into TEOS sol-gel matrix has been discussed by Zaggout (2006) where it has been reported that the indicators keep their structure and pH behaviour intact as their solution counterparts. Beltran-Perez et al., (2006) discussed the development of an optical fiber pH sensor using sol–gel deposited TiO$_2$ film doped with different organic dyes: brilliant green, rhodamine 6G, rhodamine B and coumarin. A urea biosensor from stacked sol-gel films with immobilized nile blue chromoionophore and urease enzyme was described by Alqasaimeh et al., (2007). Chen et al., (2007) described the construction of a gold nanoparticle and rhodamine 6G based fluorescent sensor for high sensitive and selective detection of mercury (II) in environmental water samples. TEOS based sol–gel films doped with fluorescein (FL) and phenol red (PR) dyes were fabricated by Persad et al., (2008) for efficient sensing of ammonia gas. The surface-enhanced Raman excitation profiles (REPs) of single molecule of rhodamine 6G (R6G) on Ag surfaces were studied by Dieringer et al., (2009) highlighting the role of excitation energy in determining the resonance Raman intensities for R6G on surface-enhancing nanostructures.

Though there are many conventional methods of arginine analysis available still there is an inevitable need for rapid, reliable, sensitive, specific and selective arginine monitoring. The present work besides developing sol-gel modified potentiometric methods, for the first time investigates the area of fiber-optic biosensors to scrutinize arginine for its fast, efficient and reliable monitoring.