CLONING AND EXPRESSION OF ARGINASE FROM *LACTOBACILLUS Sp.*

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by

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CERTIFICATE

This is to certify that the work titled "**Cloning and Expression of L-arginase from *Lactobacillus sp.***" submitted by **Rohit Kumar** and **Madhur Bhardwaj** in partial fulfillment for the award of degree of **B. Tech Biotechnology** from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institute for the award of this or any other degree or diploma.

Bansal 25/05/13

Signature of Supervisor

Name of Supervisor : Dr. Saurabh Bansal

Date: 25.05.2013

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ABSTRACT

Arginase catalyzes the hydrolysis of L-arginine to urea and ornithine. It catalyzes the final step in the urea cycle, a process in mammals during which the body disposes of harmful ammonia. In most mammals, two isozymes of this enzyme exist: arginase I and arginase II. Arginase is also found in other organisms like worms, molluscs, fishes, bacteria, fungi, yeast, actinomycetes, algae and plants. The enzyme has been found to possess profound therapeutic benefits in treatment of various physiological disorders in the body. It has also been demonstrated that arginase is involved in the pathogenic bacteria's (such as Helicobacter pylori and Bacillus anthracis) evasion of the host's immune system. In the present study we are trying to clone and express L-arginase from Lactobacillus acidophilus in a suitable expression host through various techniques of rDNA Technology for its purification and further characterization.

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LIST OF ABBREVIATIONS

DNA : Deoxyribose Nucleic Acid

EDTA : Ethylene Diamine Tetra-acetic Acid

EtBr : Ethidium Bromide

PCR : Polymerase Chain Reaction

TE : Tris EDTA Buffer `

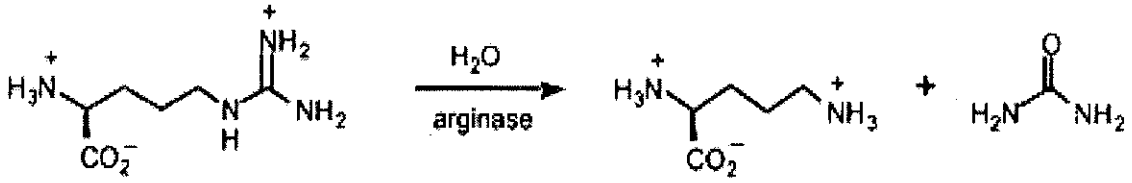
TAE : Tris Acetic acid EDTA

CTAB : Cetyltrimethyl ammonium Bromide

dNTP : Deoxyribonucleotide

CHAPTER-1
INTRODUCTION

Arginase, the arginine hydrolytic enzyme, was first discovered in the mammalian liver. It is a metalloenzyme that catalyzes the hydrolysis of L-arginine to urea and ornithine.



Arginine **Ornithine**

Urea

Arginase catalyzes the fifth and final step in the urea cycle, a series of biochemical reactions in mammals during which the body disposes of harmful ammonia. In most mammals, two isozymes of this enzyme exist; the first, Arginase I, functions in the urea cycle(1), and is located primarily in the cytoplasm of the liver. The second isozyme, Arginase II, regulates the arginine/ornithine concentrations in the cell. It is located in mitochondria of several tissues in the body, such as kidney, prostate, lactating mammary glands and brain(2). It may also be found at lower levels in macrophages. Important roles of Arginase II have been reported to be the biosynthesis of polyamines, amino acids ornithine, proline and glutamate and in the inflammatory process. Various experiments suggest that arginase II functions in L-arginine homeostasis by regulating L-arginine concentrations for cellular biosynthetic reactions such as nitric oxide biosynthesis. The human type I and type II arginases are related by 58% sequence identity and are immunologically distinct.

In both eukaryotes and prokaryotes, Mn^{2+} is the physiologic activator of arginase. But in some cases Co^{2+}, Ni^{2+}, Fe^{2+} and Cd^{2+} may also activate the enzyme(3).

Lactobacillus acidophilus is a homofermentative bacterium which ferments sugars into lactic acid, and grows readily at pH values 6.2 to 6.6. It has an optimum growth temperature of around 35 °C. *L. acidophilus* occurs naturally in the human and animal gastrointestinal tract, mouth, and vagina.

Though arginase is found in both eubacteria and eukaryotes, only a few eubacterial arginases have been characterized; most characterized arginases are from animals and

yeasts. *L.acidophilus* has LBA1022 gene which codes for this enzyme. Our aim here is to clone and express arginase from *L. acidophilus* in *E. coli*, to check for its activity and try to discover some new, interesting and unique properties among the arginase superfamily.

CHAPTER-2

REVIEW OF LITERATURE

2.1 HISTORY:

Arginase was discovered by Kossel & Dakin (1904) in mammalian liver, who showed that the products of its action are ornithine and urea. Since then, the enzyme has attracted a great deal of interest from many points of view. The enzyme has been found to exist in two forms that have evolved with differing tissue distributions. Crude preparations of arginase were reported as early as 1931. Hellerman and Perkins first showed activation by bivalent metal ions including cobalt, nickel, manganese, and iron. In 1940 an improved purification developed by Richards and Hellerman showed that the activity could be restored to pH-inactivated arginase by Mn^{2+} and (to a lesser extent) Fe^{2+}.

In 1956, the "partial" purification was further improved by Robbins and Shields. They demonstrated that the activity of arginase was dependent upon manganese, and found the optimal pH to be 9.2 (which has since been adjusted to 9.4).

Much work was done investigating inhibitors in the late 1970s and early 1980s. Bedino studied the effect of the product/inhibitor ornithine, and proposed an allosteric model for the regulation of the enzyme's activity.

Recent research has investigated the roles of arginases in vascular disease, pulmonary disease, infectious disease, and cancer. Varying levels of arginase have been found in the reproductive system of cattle, as well as in the immune system of mice and humans.

2.2 APPLICATIONS OF ARGINASE

2.2.1 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(4). Upregulation of endogenous arginase I causes the activation of neural regeneration pathways, the reaction being mediated by polyamines and leading to novel roles of arginase in cell survival, regeneration and translation in the central nervous system(5). It is known that arginase-I is constitutively expressed in human granulocytes and

participates in fungicidal activity by a novel antimicrobial effector pathway likely through arginine deprication in phagolysosome(6). A deficiency of the liver enzyme results in hyperargininemia- inherited in an autosomal recessive manner(7). Arginase I & II levels rise remarkably in asthma and lung infection thus, showing the involvement of arginase in the pathology of the disease. Measurements of plasma arginase activity provide a useful marker for underlying metabolic disorder and efficacy of treatment for asthma.

The use of Arginase as a potential chemotherapeutic agent has shown a lot of potential and promise. Remission of hepatocellular carcinoma was achieved by arginine depletion through endogenous human hepatic arginase released from transhepatic arterial embolizationng(8). The combination of the recombinant arginase with an antineoplastic agent 5 flourouracil (5FU) for treatment of human malignancies was tested in nude mice, and this treatment methodology was shown to be effective for arginine depletion(9). 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)(10).

The other major applications of L-arginase in medicine are described in the following sub-headings:

2.2.1.1 In cell growth

Arginase upregulates the synthesis of polyamines and proline via arginine hydrolysis thus, being necessary to provide compounds for cell proliferation and growth. This greatly accelerates wound healing(11).

2.2.1.2 In sexual arousal

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(12).

2.2.1.3 As a diagnostic tool

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 (13).

2.2.1.4 Maintaining semen quality

Increase in psychological stress results in raising nitric oxide level which results in poor sperm quality. Thus, arginase levels are important to keep NO levels (via arginine degradation) in control for maintenance of semen quality(14).

2.2.1.5 Ornithine production

Ornithine, produced by arginase is necessary for the production of collagen, is helpful in therapy of rheumatoid arthritis(15). The results suggested that L-ornithine has an antifatigue effect in increasing the efficiency of energy consumption and promoting the excretion of ammonia. L-Ornithine reduced stress in mice and made them more sociable toward other mice as well as the scientists conducting the experiment. L-Ornithine L-aspartate (LOLA), a stable salt of ornithine and aspartic acid, has been used in the treatment of cirrhosis. Amino acid supplements, including L-ornithine, are frequently used by bodybuilders and weightlifters under the belief that it will increase levels of human growth hormone (hGH). Ornithine is used for improving athletic performance, reducing glutamine poisoning in the treatment of a brain condition due to liver disease (hepatic encephalopathy).

2.2.1.6 As a biosensor

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.

A potentiometric sensor has been developed for monitoring arginine by coupling *Streptococcus faecium* cells with an ammonia gas-sensing membrane electrode(16).

Electrochemical biosensors for chiral analysis of amino acids including L-arginine by bienzymes L-amino acid oxidase and horseradish peroxidise have been described(17).

2.2.2 Arginase as an alternative to chemical pesticides

An Invention at Michigan State University provides an alternative to chemical pesticides by producing plants with an enhanced resistance to insect pests. Arginase, which produced in plant tissues, is an enzyme that degrades amino acid (arginine) essential for insect growth. This enzyme does not affect amino acid concentrations until the enzyme is activated in the insect gut. Overproduction of arginase in plants provides enhanced resistance to arthropod herbivores by acting as an anti-nutritive defense against phytophagous insects.

- Reduces need for chemical pesticides: The invention will reduce the amount of chemical pesticides needed to control plant herbivores.
- Alternative or complement to Bt: This gene uses an insect-control mechanism different from Bt, which is the current GMO standard for insect control. Therefore, this gene may be useful for control of insects that are resistant to or poorly controlled by Bt. This gene may also be used to improve the level of control provided by Bt genes(18).

2.3 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.

2.3.1 Bacterial Sources:

Among bacteria producing Arginase, the prominent ones include many bacilli, the *mycobacteria, Proteus* spp., *Lactobacillus*, *T. aquaticus*, *Agrobacterium-Rhizobium* group, *Streptomyces, Helicobacter pylori* and other *Cyanobacteria*. The enzyme produced from bacteria is said to be highly specific for L-arginine and inducible by the addition of L-arginine to the growth medium.

In mammals, L-arginine is used by macrophages to produce nitric oxide and other downstream reactive nitrogen species. The production of nitric oxide by activated host

macrophages is an effective antimicrobial agent and serves as an initial innate defense mechanism against pathogens. It has been proved that the availability of the intracellular arginine is a rate-limiting factor in nitric oxide synthesis, although extracellular arginine concentration has been shown to play a more important role in regulating NO synthesis compared to intracellular arginine. Arginase and nitric oxide synthase (NOS) use arginine as a common substrate and compete with each other for this substrate. Interestingly, many pathogenic bacteria such as *Helicobacter pylori* and *Bacillus anthracis* produce arginase which inhibits host nitric oxide production, allowing for survival of the organism when co-cultured with activated macrophages. Additionally, *H. pylori* arginase decreases T-lymphocyte proliferation and CD3ζ expression, arguing for the importance of this enzyme in multiple facets of host-pathogen interactions.

2.3.2 Protozoa:

Arginase has also been reported from various kind of protozoa. For example: Species of *Leptomonas*, *Leishmania*, *Crithidia, Blastocridhidia* and *Entamoeba histolytica*(19).

2.3.3 Plants:

Arginase activity has been reported to be present in parts (tissues and extracts) from several plants. There have been reports of presence of arginase in *Lathyrus sativus*(20) and pumpkin seeds. 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. Arginase has been extracted from embryonic axes of Soybean *Glycine max* and its properties have been studied. Jack bean (*Canavalia ensiformis*) leaf arginase can effectively hydrolyse both L– and D–arginine. Arginase activity was shown to be enhanced 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. The two genes *Le*ARG1 and *Le*ARG2 encoding arginase from *Lycopersicon esculatum* (tomato) were characterized and it was found that the genes were regulated by wounding. It was also reported that in Tomato plants-*Solanum lycopersicum* Arginase was induced along with threonine deaminase (TD) due to the activity of plant hormone- Jasmonic acid(21). 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. Xylem sap of the deciduous tree *Quercus ilex* was reported to possess arginase which degrades arginine. The enzyme activity increases before spring time to breaks down the L-arginine to ornithine. Arginase has also been found to be operative in cotyledons of somatic embryos of pine at late developmental stages.

2.3.4 Fungi:

Arginase has been purified and characterized from *Neurospora crassa*. *Aspergillus nidulans* produces arginase which enables the fungus to utilize arginine as the sole nitrogen source. Mycelial extracts of *Trichoderma sp.* were reported to be a source of arginase. It has been reported that the presence of arginase in members of family Agaricaceae including *Agaricus bisporus has* led to the accumulation of urea in its fruit bodies(22).

2.3.5 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. The purification and characterization of arginase from a plasmid-containing, enzyme-overproducing, protease deficient yeast strain (*S.cerevisiae*) was reported in 1989. Arginase has also been purified from *Schizosaccharomyces pombe* (23).

2.3.6 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, next to the liver in man, is also expressed by mature fetal and adult red blood cells and activated monocytic cells. Arginase II is expressed by kidney, nucleated red blood cells, brain, spinal cord, gastro-intestinal tract, mammary gland and prostrate.

2.4 METHODS OF L-ARGININE ESTIMATION

Many methods for arginine determination are available to monitor arginine. These include 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:

2.4.1 Chinard's ninhydrin reaction

Single reactions are prepared containing sample, 0.25 ml sample containing arginase and 0.5ml (100 μM) arginine to initiate the reaction. The samples are incubated for 60 minutes, followed by the addition of 1 ml of 0.72 M HCl to stop the reaction. After centrifugation, 2 ml of 6% ninhydrin is added to 1 ml of the filtered sample in a spectronic cuvette and the samples are boiled for 25 minutes, with a marble placed on top of each cuvette. Cooled samples are read using spectrophotometer at an absorbance of 505 nm. One unit of enzyme activity is defined as the amount of the enzyme that produces 1 μmol of ornithine/min at 37°C. Specific activity is expressed in enzyme units per mg of protein.

2.4.2 Flow-injection technique

L-arginine determination by flow-injection technique involves L-arginase immobilization on an epoxy matrix and the urea production by the reaction is measured spectrophotometrically through indophenols formation at an absorbance of 629 nm.

2.5 CATALYTIC MECHANISM

The catalytic mechanism for this reaction is illustrated in **Figure 2.1**. L-arginine contains a charged guanidinium group. In step one of the scheme, the charges guanidinium group binds to carboxylate anion side chain of Glu277. Then the μ-aquo ligand ionizes to the μ-

hydroxide. In step two the μ-hydroxide attacks the guanidine carbon and protons are transferred from the μ-hydroxide to the substrate N-omega hydroxy by the way of Asp128 thus creating a triply-bridging μ3-oxo adduct. In the third and final step protons are transferred to create L-ornithine and urea. In this non-toxic form, urea is excreted through the urine. Excess L-ornithine is recycled through the final step in the cycle to react with citrulline and eliminate ammonia from the animal's body.

Arginase's active site is extraordinarily specific. Modifying the substrate structure and/or stereochemistry severely lowers the kinetic activity of the enzyme. This specificity occurs due to the high number of hydrogen bonds between substrate and enzyme; direct or water-facilitated hydrogen bonds exist, saturating both the four acceptor positions on the alpha carboxylate group and all three positions on the alpha amino group. N-hydroxy-L-arginine, an intermediate of NO biosynthesis, is a moderate inhibitor of arginase. Crystal structure of its complex with the enzyme reveals that it displaces the metal-bridging hydroxide ion and bridges the binuclear manganese cluster.

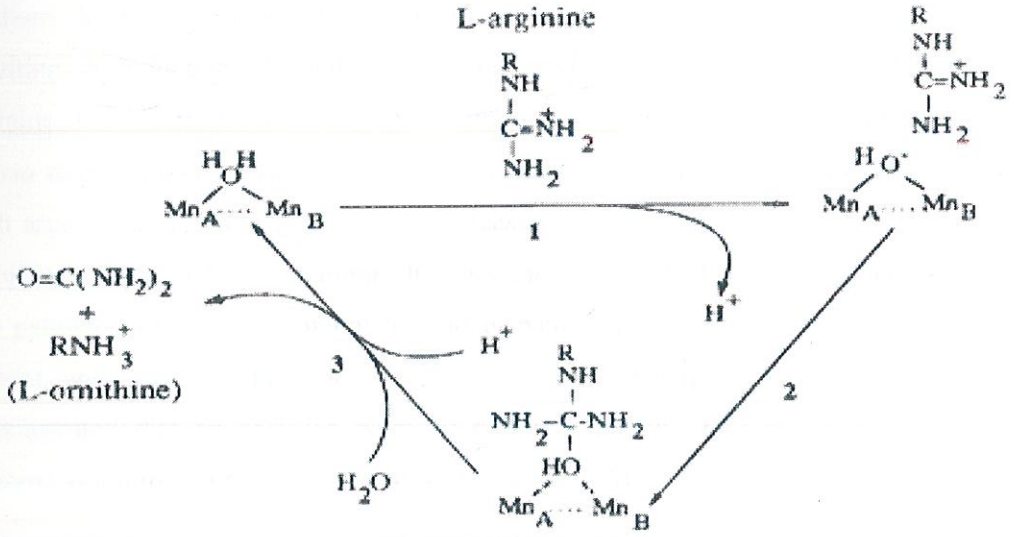


Figure 2.1. Proposed Mechanism for L-Arginine Hydrolysis by Arginase

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 available.

2.6.1 Production of arginase:

Arginine has been reported to be utilized as a nitrogen source in *Neurospora crassa*. *Neurospora crassa* was grown 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(24). *Saccharomyces cerevisiae* 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(25). 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. In arginine supplemented medium, the cytosolic pool dramatically increases resulting in induction of catabolic enzyme arginase as well as rapid catabolism of arginine. The effect of various nitrogen sources on arginase production was tested 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. It was 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. It was also demonstrated that an arginase pathway deficient mutant, BL196, failed to grow on arginine as a nitrogen source under these conditions(26) .

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. *Synechcoystis sp.* PCC 6803 were grown axenically in nitrate containing medium. Cultures were supplemented with 1mM filter

sterilized L-arginine. The effect of various carbon sources on arginase activity was studied in *Neurospora crassa* and it was 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. It was 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. *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 5 mM arginine, 1mM histidine or NH_4NO_3, later these were replaced with 5 mM arginine which acted as nitrogen source. Liquid cultures were inoculated with 106 conidia per ml of. Two forms of arginine with differing molecular weights i.e., 36 kDa and 41 kDa were produced in unsupplemented and arginine-supplemented media respectively.

2.6.2 Purification and kinetic characterization of arginase:

Rabbit liver arginase has been purified by chromatographic techniques of DEAE cellulose and Sephadex G-200. It was strongly inhibited by EDTA and lost activity in absence of Mn^{2+} ions. SDS-PAGE showed that enzyme had a subunit molecular weight of 36,500, kDa increasing probability of its tetrameric nature(27). Purification and properties of rat kidney arginase have been studied showing the enzyme was highly stable at high temperatures such as 60°C and had a K_m value of 18 mM and was strongly inhibited by borate and L-ornithine. Rat small intestinal arginase was purified and shown to have optimum pH of 10.0 and K_m of 19 mM. The enzyme was almost completely inactivated by treatment with EDTA(28). Arginase from human liver and erythrocytes has been isolated and purified. The human liver arginase had a molecular weight of was 107, 000 and its K_m for arginine was 10.5mM. The properties of human heart arginase studies revealed its K_m to be 5 mM and molecular weight of about 30, 000 kDa with the enzyme having strong dependence on Mn^{2+} ions for its activity(29).

Purification and physical properties of Arginase from *Xenopus laevis* liver were studied and its molecular subunit weight was determined and found to be 76,000 daltons(30). *Neurospora crassa* arginase has been reported to have a subunit weight of 38,300

determined by SDS-PAGE. The enzyme exhibited hyperbolic kinetics at pH 9.5 with an apparent K_m for arginine of 131 mM. The optimum pH and temperature for purified ox erythrocyte arginase have been reported to be 11.5 and 55°C respectively.

A thermostable arginase from the extreme thermophile *Bacillus caldovelox* has been purified by chromatographic techniques and its kinetic properties were studied. Its activity is optimal at pH 9 and temperature of 60°C(31). The K_m for arginine is 3.4 mM. Extrahepatic arginase (ArgII) was isolated from rat mammary gland and its properties were investigated. This enzyme had a pH optima of 10 and K_m of 12-14 mM. In situ characterization of *Helicobacter pylori* arginase employing NMR spectroscopy, spectrophotometry, radiotracer analysis and protein purification techniques was done. A K_m of 22+/- 33 mM was determined for the enzyme activity and differences of V_{max} were observed between strains. Divalent cations stimulated arginase activity & most potent activators were $Co^{+2} > Ni^{+2} > Mn^{+2}$(32).

Recombinant human arginase II has been expressed in *E. coli* and purified to homogeneity. The enzyme reportedly had a K_m 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. *Helicobacter pylori* arginase expressed in *E. coli* has been purified. 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. It was reported that arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity.

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 revealed that fully Mn-activated arginase contains two Mn2+ ions per subunit .The high resolution X-ray

crystal structure of rat arginase I was provided 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(33). The crystal structure of arginase structure of inactivated *Bacillus caldovelox* arginase-L-arginine complex was studied 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 α-carboxylase group and all three donor positions on α-amino group. It was noted the binding of Nω-hydroxy-Larginine (NOHA)-an intermediate of NO biosynthesis and modest inhibitor of the enzyme with rat arginase I which revealed that the Nω-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 Nω-hydroxyl group of the inhibitor displaces the metal bridging hydroxide ion.. The structure for human kidney II type arginase 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 α/β fold of rat arginase I and hexameric arginase from *Bacillus caldovelox.* The 2.15 A resolution crystal structure of arginase from the cerebral malarial parasite *Plasmodium falciparum* was reported 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. cDNA phARG6 for human liver arginase was used for expression of human liver arginase in *E. coli* strain KY1436 by. 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. 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. The *B. subtilis* arginase encoding rocF gene was cloned and expressed in *E. coli* K-12 for enhanced production of urea by arginase pathway. Modulation of mRNA levels of liver arginase by insulin and vanadate in experimental diabetes has been studied 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. The rocF gene encoding arginase in *Helicobacter pylori* was purified and expressed in *E. coli* and was found to confer arginase activity to *E. coli*. The transformed cells expressing arginase showed enhanced enzyme activity than the native *Helicobacter* enzyme. 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. The recombinant enzyme produced was used for treatment of human malignancies by subsequent pegylation. A highly active recombinant arginase was obtained by expressing arginase gene from *Leishmania amazonensis* in *E. coli* BL21 (DE3) cells. Antibody against the recombinant protein confirmed a glycosomal cellular localization of the enzyme in promastigotes.by means of disposable all solid-state potentiometric electronic tongue microsystem.

2.9 REGULATION OF ARGINASE LEVELS

Arginase levels in vascular smooth muscle cells may be influenced by interleukin-4 and interleukin-13, transforming growth factor- β, lysophosphatidycholine, and mechanical strain. Expression of arginase in endothelial cells may be controlled by lipopolysaccharides, tumor necrosis factor- α, or a combination of the two. Levels may

also be induced by thrombin, high glucose concentrations, oxidized low-density lipoprotein, or hydrogen peroxide. There are few suppressants of arginase in endothelial cells. A few plant compounds have been found to suppress arginase levels and prevent induction into the endothelial cells. These include genistein, cocoa flavanols, and simvastatin.

2.9.1 Consequences of High Concentration of Arginase in the Body

Imbalances of arginase levels in the body have been found to consequently induce vascular disease, pulmonary disease, infectious disease, immune cell function, and cancer. Over-expression of arginase has been found to affect proteins, nitric oxide, urea, and ornithine.

2.9.1.1 Effects on Proteins

If the level of arginase far exceeds that of arginine, nitric oxide synthesis could be reduced and thus nitric oxide synthase could be promoted. Nitric oxide synthase catalyzes the oxidation of the amidine nitrogen in arginine and serves as a vasodilator in the body. This could lead to pulmonary hypertension. In addition, this could trigger an increase or decrease in the expression of proteins such as the cationic amino acid transporter and the isoform of nitric oxide synthase known as Inos. If the level of arginase is too low in relation to the amount of arginase, the body is prone to pulmonary hypertension. iNOS can readily react with oxygen and superoxide and create peroxynitrite. Peroxynitrite reactions can often lead to cellular damage of cytotoxicity. If iNOS is absent in the body, the individual is often susceptible to infections.

2.9.1.2 Effects on Nitric Oxide

In endothelial cells, an over-expression of either arginase I or arginase II can reduce nitric oxide synthesis. Nitric oxide is physiologically important in the body. It serves as a vasodilator and is important in blood pressure regulation. Nitric oxide influences or serves a role in penile erection, neurotransmitters in the brain, the peripheral autonomic nervous system, long-term potentiation, neurotoxicity, muscle relaxation, and the primitive immune system. It has been found to inhibit adhesion, activation, and

aggregation of platelets. Low levels of nitric oxide can induce pylorospasm in infantile hypertrophic pyloric stenosis.

2.9.1.3 Effects on Urea

Urea can inhibit the amount of nitric oxide that can be produced by activated pulmonary artery endothelial cells. In addition, urea can reduce arginase's K_m for arginine and activate the arginase. High levels of urea in the increase the likelihood that urea-dependent activation of arginase can cause pulmonary hypertension. High concentrations of urea commonly lead to hyperammonemia, increased levels of ammonia in the body, and to often the consequences are fatal.

2.9.1.4 Effects on Ornithine (polyamines, proline, and glutamate)

Ornithine serves as a precursor for polyamines, proline, and glutamate synthesis. Therefore, arginase is thought to be the limiting factor in the synthesis of these products. An over-expression of arginase I in vascular smooth muscle cells will result in an increase in polyamine synthesis and cell production and there may initiate or progress intimal hyperplasia after a vascular injury. In endothelial cells, an over-expression of either arginase I or arginase II can promote polyamine synthesis, cell production, and proline synthesis.

2.9.1.5 Effects on K_m

Cysteine residues 168 and 303 may be found in arginase I. These residues may undergo S-nitrosylation. The S-nitrosylation of Cys303 can stabilize the trimer of arginase. This stabilization can decrease the K_m for arginine by a factor of six(34).

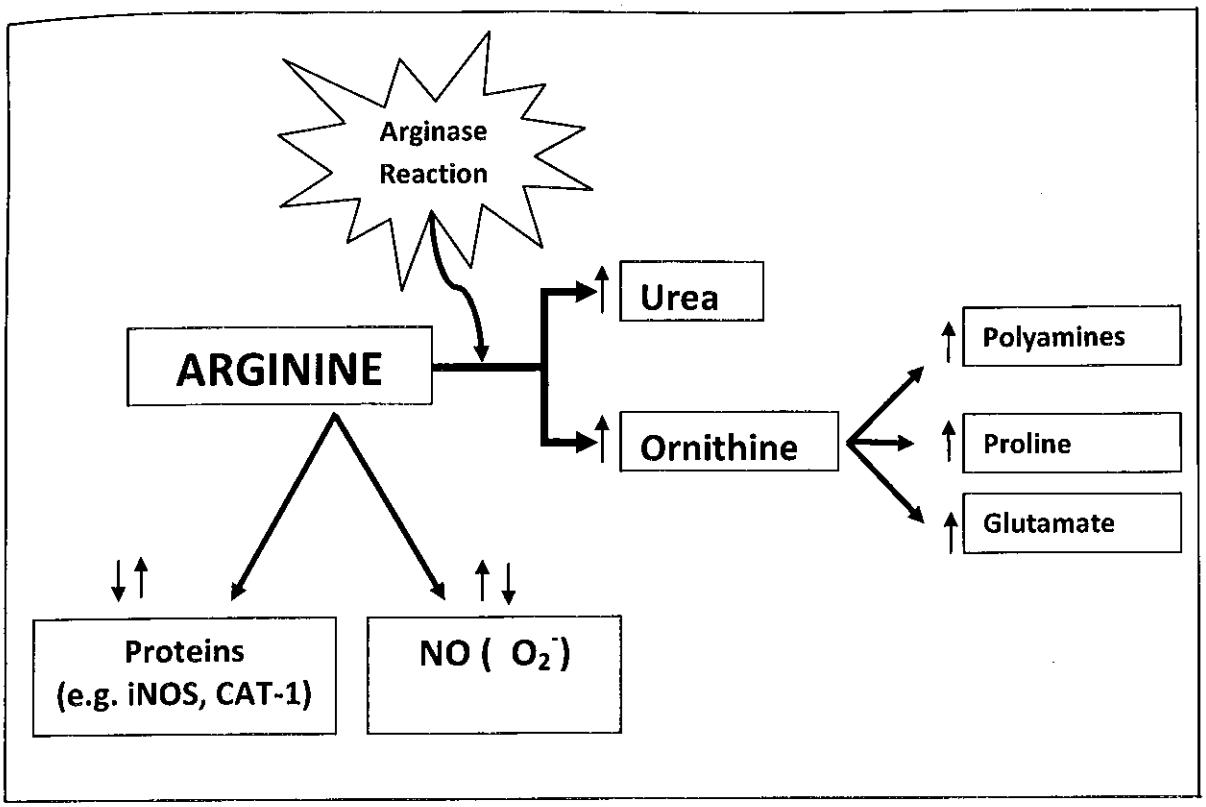


Fig 2.2: The effects of increased arginase activity

2.9.2 Consequences of Low Concentration of Arginase in the Body

Arginase deficiency is an inherited disorder that causes the amino acid arginine and ammonia to accumulate gradually in the blood. This deficiency is commonly referred to as hyperargininemia or arginemia. Ammonia, which is formed when proteins are broken down in the body, is toxic if levels become too high. The nervous system is especially sensitive to the effects of excess ammonia.

Arginase deficiency usually becomes evident by about the age of 3. It most often appears as stiffness, especially in the legs, caused by abnormal tensing of the muscles (spasticity). Other symptoms may include slower than normal growth, developmental delay and eventual loss of developmental milestones, intellectual disability, seizures, tremor, and

difficulty with balance and coordination (ataxia). Occasionally, high protein meals or stress caused by illness or periods without food (fasting) may cause ammonia to accumulate more quickly in the blood. This rapid increase in ammonia may lead to episodes of irritability, refusal to eat, and vomiting.

In some affected individuals, signs and symptoms of arginase deficiency may be less severe, and may not appear until later in life.

2.10 ROLE OF ARGINASE IN ASTHAMA

The enzyme, arginase, converts L-arginine into L-ornithine and urea, and has been implicated in the pathogenesis of lung diseases, related to dysregulation of L-arginine metabolism and remodeling. Allergic asthma is a chronic condition characterized by inflammation, lung remodeling and airways hyperresponsiveness (AHR). Increased expression of arginase may contribute to AHR in asthma by reducing L-arginine bioavailability for the nitric oxide synthase (NOS) isozymes, thus, limiting the production of the endogenous bronchodilator, nitric oxide (NO). Reduction of intracellular L-arginine concentrations as a consequence of augmented arginase expression and activity may also promote NOS uncoupling, resulting in increased formation of peroxynitrite, a powerful oxidant that promotes bronchoconstriction and inflammation. In chronic asthma, increased arginase expression may also contribute to airways remodeling, through increased synthesis of L-ornithine, and hence the production of polyamines and L-proline, which are involved in cell proliferation and collagen deposition, respectively. New drugs targeting the arginase pathway could have therapeutic benefits in asthma. The study focused on recent developments in the understanding of the role of arginase in AHR, inflammation and remodeling, highlighting studies that advance our knowledge of L-arginine dysregulation in human asthma and animal studies that explore the therapeutic potential of arginase inhibition(35).

2.11 ARGINASE AS A POTENTIAL TARGET IN THE TREATMENT OF CARDIOVASCULAR DISEASE

Functional integrity of the vascular endothelium is of fundamental importance for normal vascular function. A key factor regulating endothelial function is the bioavailability of nitric oxide (NO). Recently, the enzyme arginase has emerged as an important regulator of NO production by competing for l-arginine, which is a substrate for both arginase and NO synthase. Increased activity of arginase may reduce the availability of l-arginine for NO synthase, thus reducing NO production, increasing formation of reactive oxygen species, and leading ultimately to endothelial dysfunction. Increased activity and expression of arginase have been demonstrated in several pathological cardiovascular conditions, including hypertension, pulmonary arterial hypertension, atherosclerosis, myocardial ischaemia, congestive heart failure, and vascular dysfunction in diabetes mellitus. Experimental studies have demonstrated that inhibition of arginase under these conditions increases NO bioavailability, reduces oxidative stress, improves vascular function, and protects against ischaemia–reperfusion injury. Initial clinical interventional studies are also promising. The purpose of this study was to discuss the role of arginase in cardiovascular pathologies, its contribution to the development of several cardiovascular disease states and the feasibility of using arginase inhibition as a therapeutic strategy(36).

2.12 MODULATION OF THE ARGINASE PATHWAY IN THE CONTEXT OF MICROBIAL PATHOGENESIS

Recently, arginase was identified to show some immunosuppressant properties and is probably involved in the pathogenic bacteria's evasion of the host's immune system [11]. Firstly, arginase competes with host inducible nitric oxide synthase for the common substrate L-arginine, thus reduces the synthesis of NO, an important component of innate immunity and an effective antimicrobial agent that is able to kill the invading pathogens directly. Research indicates that arginase-deficient bacteria are more sensitive to NO-dependent killing by host macrophages, whereas the wild-type bacteria exhibited no loss

of survival. Secondly, arginase is involved in inhibiting human T cell proliferation and T cell CD3ζ expression, and thus efficiently reduces host cellular immune response by contributing to the inability of T cells. These clue that arginase could inhibit host innate defense and adaptive immune response simultaneously to facilitate the pathogenesis of pathogenic bacteria(37).

CHAPTER 3

MATERIALS AND METHOD

3.1 MATERIALS: All the chemicals prepared are listed in APPENDIX A.

3.1.1 *Lactobacillus acidophilus* strain:

Lyophilized *L. acidophilus*culture (MTCC No. *10708*) was obtained from *IMTECH CHANDIGARH.*

3.1.2 Chemicals:

The chemicals used in the present study were obtained from Merck Limited and Qualigens fine chemicals Limited. The various media used in the study namely Agerose gel, MRS Agar, MRS Broth, were manufactured by Merck Ltd. (Appendix-A).

3.1.3 Primers:

Forward primerwas designed using an initial stretch of 20 nucleotides from gene of interest and NheI restriction site was added with few additional bases (ATTT) at the 5' end

5' ATT GCT AGC ATG ATA GAA AAA ACA ATT CGG 3'

Reverse primer was designed using reverse compliment of last 20 nucleotides from gene of interest XhoI restriction site was added with few additional bases (ATTAA) at the 5' end.

5' ATT CTC GAG CTA AAA AAT ATT AGT ATT ATT 3'

3.2 METHODS:

3.2.1 Media preparation:

MRS medium were used as solid or liquid media for growth of *Lactobacillus acidophilus* which was prepared by mixing a desired concentration of media in distilled water and then followed by autoclaving at 121°C and 15 lb/in^2 pressure for 15-20 minutes.

3.2.2 Inoculation

After media sterilization, transferred Lyophilized *Lactobacillus acidophilus* to the media in Laminar Airflow Chamber. Then the media was incubated at 37 °C in incubator shaker for 48 hours.

3.2.3 Streaking On MRS Agar Plate

L. acidophilus was streaked on the MRS agar plate from the culture flask. Plates were incubated at 37 degree Celsius in incubator for 48 hours.

3.2.4 Gram's Staining

- A primary stain (crystal violet) was applied to a heat fixed smear of a *Lactobacillus* culture, kept it stand for 1 minute. The bacteria were affixed on the slide through heating not to rinse out during staining procedure.
- Gram's Iodine was added (which binds to crystal violet and traps it in the cell.)
- Rapid decolorization with alcohol was done
- Counterstaining with Safranin was done

3.2.5 Competent Cells Preparation

The standard method for making the bacteria permeable to DNA involves treatment with calcium ions. In $CaCl_2$ method, the competency can be obtained by creating pores in bacterial cells by suspending them in a solution containing high concentration of calcium. DNA can then transformed in to the Host cell by heat shock treatment at 42°C for the process of transformation.

Procedure

Primary culture: One colony of *E. coli* from LB plate was inoculated into 2 ml LB liquid medium and kept in a Shaker at 37 °C for overnight. 1ml of overnight cell culture was inoculated into 100 ml LB medium (in a 500 ml flask). It was then shake vigorously at 37 °C for about 2 hours to get an OD600 ~ 0.25-0.3. The culture was chilled on ice for 15 min and it was made sure that the 0.1M $CaCl_2$ solution and 0.1M $CaCl_2$ plus 15% glycerol were on ice. Now the cells were centrifuged for 10 min at 3300g at 4°C. The medium was discarded and the cell pellet was re-suspended in 30-40 ml cold 0.1M $CaCl_2$. Then the cells were kept on ice for 30 min. Centrifugation was done of the cells, as above. The supernatant was removed and the cell pellet re-suspended in 6 ml 0.1 M $CaCl_2$ solution plus 15% glycerol. 0.4-0.5ml of the cell suspension was pipette into sterile 1.5ml micro-centrifuge tubes. These tubes were frozen on dry ice and then transferred to -80°C freezer.

3.2.6 Plasmid Isolation: pET 28a DH5α (Appendix B)

5 ml LB medium containing proper antibiotics was inoculated with a single bacterial colony. The tube was incubated at 37°C overnight with vigorous shaking at 360 rpm. Bacteria were then centrifuged at 10,000g for 5 minutes at room temperature. The supernatant was discarded. Bacterial pellet was re-suspended in a total of 100 µl ice-cooled solution I (25mM). The bacteria were fully re-suspended by vortexing. 200 µl room temperature solution II was added to the suspension and it was mixed thoroughly by repeated gentle inversion. 150 µl ice-cold Solution III was added to the lysate. It was then mixed thoroughly by repeated gentle inversion. Vortexing was avoided. Lysate was centrifuged at 12,000g for 5 minutes at 4°C. Resulting supernatant was recovered. Equal volume of PCI was added to precipitate the plasmid DNA. It was mixed thoroughly by repeated gentle inversion. Vortexing was avoided. Upper layer was taken. It was then washed with isopropanol. Then again washed with 70% ethanol. TE was added to dissolve the pellet. After addition of 2µl RNAse A (10mg/ml), the mixture was incubated for 30 minutes at 37°C temperature to remove RNA.

3.2.7 DNA Extraction

Cells were centrifuged at 10000rpm for 5minutes. 700ul of CTAB was added. CTAB was incubated for 15min at 60°C in a water bath and then kept at water bath for 1hr at 60°C. To each tube 700µl of Chloroform was added: Isoamyl Alcohol (24:1) and the solution was mixed by inversion. After mixing, the tubes were centrifuged at 10000 rpm for 10 min. The upper aqueous phase was only transferred (contained the DNA) to a clean microfuge tube. To each tube 500µl of ice cold isopropanol was added. The tubes were then slowly inverted several times to precipitate the DNA. Following precipitation, the DNA was pipetted off by slowly rotating/spinning tip in the cold solution. Then the DNA was washed by transferring the precipitate into a microfuge tube containing 200µl of ice cold 70 % ethanol and the tube was slowly inverted. The tube was centrifuged at 10000 rpm for 2 minutes. After the wash, all the supernatant was removed and the DNA pellet was allowed to dry. The DNA was re-suspended in sterile DNAse free water (approximately 50-400µl H_2O). Then 10µl RNAse A in 10ml H2O was dissolved in 50-

100ul TE buffer for 1 hour. Agarose gel electrophoresis of the DNA showed the integrity of the DNA.

3.2.8 DNA quality confirmation

- 1 % solution of agarose was prepared by melting 1 g of agarose in 100 mL of 0.5x TAE buffer in a microwave for approximately 2 min.
- It was then allowed to cool for a couple of minutes and 2.5µl of Ethidium bromide was added. It was stirred to mix.
- A gel was casted using a supply tray and comb. It was then allowed to set for a minimum of 20min at room temperature on a flat surface
- The following were loaded into separate wells:

  3µL 1kb ladder

- 5µL sample + 2µL 6x Loading Buffer
- Run the gel for 30 min at 100 V
- Expose the gel to UV light and photograph (demonstration).

3.2.9 PCR Cycle (Table 3.1)

S. NO.	STAGE	STEP	TEMPERATURE	TIME
1.	Stage 1 *1 cycle		94° C	5 mins
2.	Stage 2 *30 cycles	**Step 1**	94° C	1 min
		STEP 2	47^{0}C	90 sec
		STEP 3	72° C	1 min
3.	Stage 3 *1 cycle		72° C	12 mins

Pcr Master Mix (Table 3.2)

MATERIALS	1X
Template DNA (50ng)	5µl
Primers (10µm)	1µl (each)
10X PCR Buffer(with Mgcl2)	5µl
10mM DNTPs	1µl
Autoclaved Water	36µl
Taq DNA Polymerase	1µl
TOTAL	**50µl**

To perform several parallel reactions, master mix was prepared containing autoclaved distilled water, buffer, dNTPs, primers and Taq DNA polymerase in a single tube, and aliquoted into individual tubes. Template DNA solutions was added. The solutions were gently vortexed and centrifuged after thawing before addition. Master-Mix was added, in a thin-walled PCR tube, on ice. Samples were vortexed again in order to collect all drops from walls of tube. Samples were the placed in a thermocycler PCR was started. Products were resolved using 1X TAE, 1.8% agarose gel.

3.2.10 Agarose gel electrophoresis of PCR Products

8 µl of the amplified PCR product was mixed with 2µl of gel loading dye and was loaded on 1.2% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding Ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

3.2.11 Restriction Digestion (plasmid vector)

Restriction digestion is basically done by restriction endonuclease in order to cleave the restriction sites in the vector plasmid and the gene of interest with the same enzyme in order to proper cloning within a suitable expression host.

3.2.11.1 Material and Method :

Restriction enzymes used:

- Forward Primer:- Nhe 1 (5' G/CTAGC 3')
- Reverse Primer:- Xho 1 (5' C/TCGAG 3')

Single Digestion (Table 3.3a)

Vector Plasmid	8µl
Forward Nhe 1 Primer	1µl
10X Assay Buffer(2.1)	1µl
TOTAL	**10µl**

Double Digestion(Table 3.3b)

Vector Plasmid	7µl
Forward Nhe 1 Primer	1µl
Reverse Xho 1 Primer	1µl
10X Assay Buffer(2.1)	1µl
TOTAL	**10µl**

3.2.11.2 Procedure

- The reaction mixture was made upto 10µl each.
- It was then kept at 37^{0}C for 4 hours.
- It was then loaded on Agarose Gel(2µl product+ 1µl Dye) with a ladder of 1Kb.
- The gel was exposed to UV light and photograph was taken.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 GRAM'S STAINING OF *LACTOBACILLUS ACIDOPHILUS*

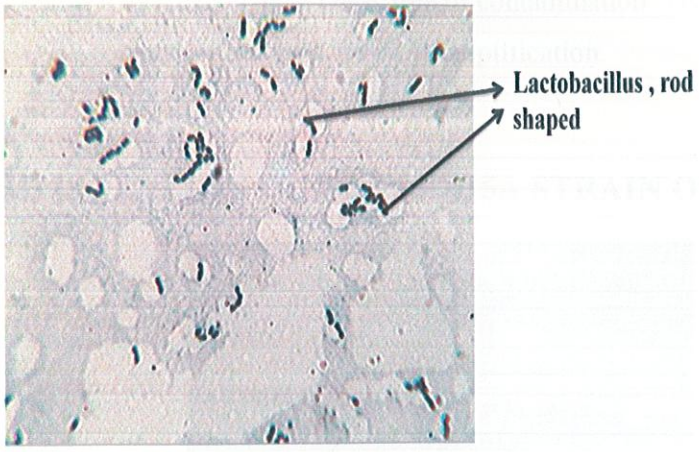


Fig 4.1 Gram's Staining of *Lactobacillus acidophilus*.

Gram's staining was performed to see the morphology of *Lactobacillus acidophilus.* The result showed that it was a gram positive bacteria as violet colour was retained because of hard cell wall. The colonies were round an opaque.

4.2 GENOMIC DNA ISOLATION

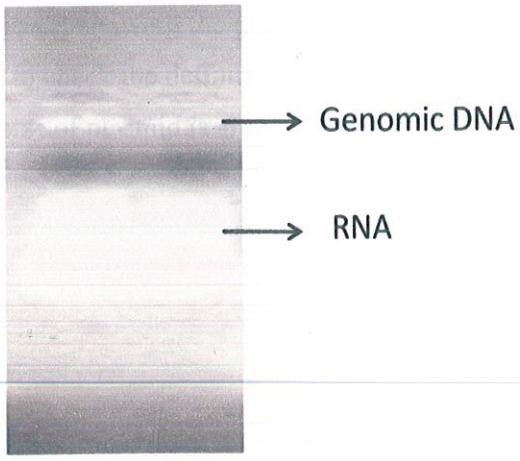


Fig 4.2 Genomic DNA isolated from *Lactobacillus acidophilus*.

The intact genomic DNA was successfully isolated from *L. acidophilus.* We confirmed it by seeing the bands in two lanes, the contamination of RNA was also seen so we gave RNAase treatment to it in order to have DNA free of contamination. This genomic DNA was further used for PCR amplification.

4.3 PLASMID ISOLATION OF PET 28DH5A STRAIN OF *E.COLI*

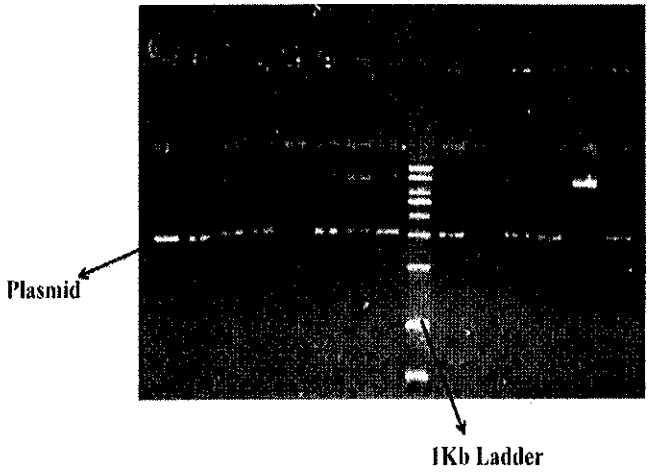


Fig 4.3 Plasmid Isolation of *pET 28DH5α strain of E.coli.*

The Plasmid was isolated from pET28 strain of *E.coli* by standard protocol all the samples were loaded on to the gel showing the presence of plasmid in all the wells. 1Kb ladder was also used in order to check the definite size of the plasmid, the result shown in the third lane shows that the size of plasmid is around 4-5Kb.

4.4 PCR AMPLIFICATIONS OF LBA1022 BY USING SPECIFIC DESIGNED PRIMERS

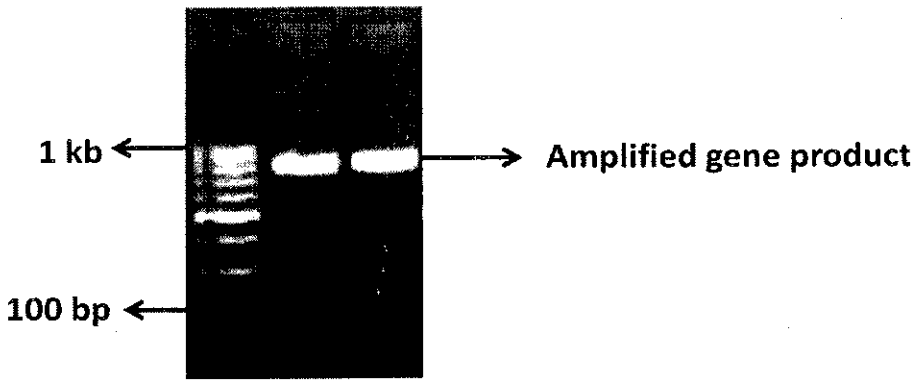


Fig. 4.4 PCR amplification of products

4.5 RESTRICTION DIGESTION OF PLASMID VECTOR

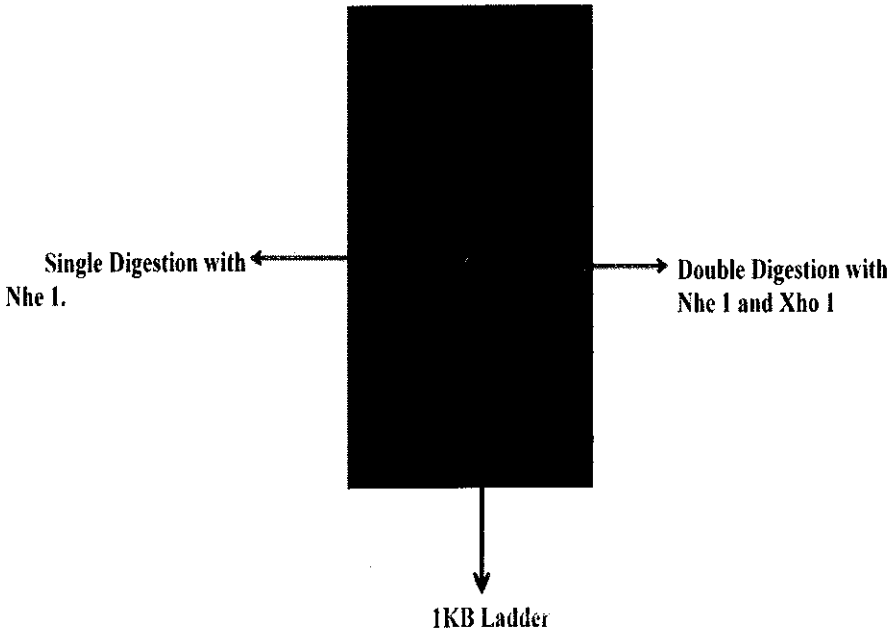


Fig. 4.5 restriction digestion of plasmid vector

The isolated Plasmid was treated with two restriction enzymes ie. Nhe1 and Xho1. The single digestion and double digestion results were seen. In both the digestion single bands were observed showing the restriction of plasmids around 5Kb.

4.6 CHARACTERISTICS OF ENZYME

Number of amino acids: 287

Molecular weight: 32645.0

Theoretical pI: 5.16

Total number of negatively charged residues (Asp + Glu): 38

Total number of positively charged residues (Arg + Lys): 29

Extinction coefficients:

Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.

Instability index:

The instability index (II) was computed to be 32.58

This classified the protein as stable.

CHAPTER 5

CONCLUSION

The arginine hydrolytic enzyme, arginase, is found ubiquitously in both eukaryotes and prokaryotes. This enzyme has got many applications in the field of therapeutic medicine (treatment of cancer, ornithine production, cell growth) and plant biotechnology (defense mechanism). We basically hypothesized that since *L. acidophilus* is a human friendly bacterium, arginase from this source will also be safe for usage in humans and can be used in various industries like pharmaceuticals for the treatment of various human disorders. This enzyme also has a pathogenicity factor in various pathogenic bacteria like *Helicobacter pylori* and *Bacillus anthraces.* So *L. acidophilus* arginase (being a bacterial arginase) can be used to carry out inhibition studies of this enzyme from pathogenic sources.

In this study we have isolated genomic DNA from *L. acidophilus*, and pET28a plasmid vector from *E. coli* DH5α. We were able to optimize PCR conditions for the amplification of LBA1022 gene from *L. acidophilus* using specific Primers and obtained an amplified ~0.87 kb gene fragment.

APPENDIX-A

LIST OF CHEMICALS:

Competent cells chemicals:-

- Single colony of E. coli cells to be transformed
- LB medium
- M CaCl2, ice cold
- LB amp plates
- 42 °C water bath
- 1M CaCl2 +15% glycerol, sterile

Plasmid Isolation chemicals:-

Lysis Buffer(solution 1):-

50mM Glucose	10ml
0.5M EDTA	1ml(pH-8.0)
1M Tris	12.5ml(pH-8.0)
Autoclaved water	85.5ml(store at 4^0C)

Solution II (Lysis buffer II): Freshly prepared 0.2 N NaOH, 1% SDS. Stored at room temperature (RT)

Isopropanol: Stored at -20 °C

Solution III(Lysis buffer II):

5M Potassium Acetate	30 ml
Glacial acetic acid	5.75 ml
Autoclaved water	14.25 ml

1M Tris:

30.28 g of Tris was added in 250 ml of dH2O and the final pH was adjusted to 8.

0.5M EDTA:

46.53 g of EDTA was dissolved in 250 ml of dH_2O and the final pH was adjusted to 8.

TE Buffer(1M)

Composition of TE Buffer (100ml)

0.2 M Tris base	50ml
0.1M EDTA	50ml

Chloroform – Isoamyl alcohol (24:1) – 5ml

Chloroform	4.8ml
Isoamyl alcohol	0.2ml

Extraction Buffer (100ml)

1% CTAB	1gm
50 mMTrisHcl	5ml of 1M
50mM EDTA	10ml of 0.5M
700Mm Nacl	70ml of 1 M
Water	Raise volume to 99ml and autoclave
1% β-Mercaptoethanol (added after autoclaving)	1ml

TAE Buffer

50X Stock solution of TAE was prepared by adding the following:

Composition of TAE Buffer

Tris base	24.2gm
Glacial acetic acid	5.71ml
EDTA(0.5M, pH 8.0)	10ml
Distilled water	Make up the vol. to 100ml

Gel loading dye(6X)

Composition of Gel loading dye

Bromophenol Blue	0.25%(w/v)
Xylene Cyanol	0.25%(w/v)
Glycerol in DW	30%(v/v)

APPENDIX B

DH5-Alpha *E. coli*

This strain of *E. coli* is not a pathogen and thus, was developed for laboratory cloning use.

lacZ Delta M15 mutation: Allowed for blue-white screening for recombinant cells.

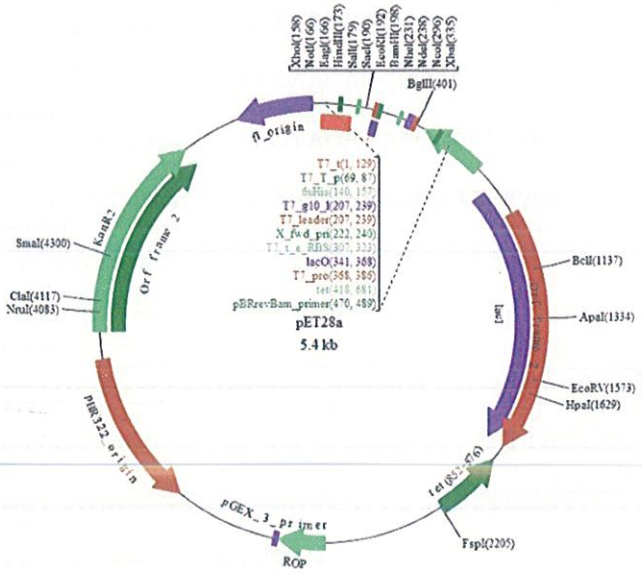
Genome structure

The genomic structure of this strain is a singular circular chromosome consisting of 4,686,137 nucleotides, 4359 genes, and 4128 protein encoding genes.

This strain also contains plasmids, and has the ability to accept plasmid insertion exceptionally well.

pET28a Vector

The pET28a-LIC vector was derived from expression plasmid pET28a. It is used for T7 promoter driven expression of recombinant proteins with the addition of a 19 amino acid N-terminal fusion tag containing a 6X His-tag followed by a thrombin protease cleavage site. Two stop codons are included in the vector at the C-terminal cloning site.



GENE CARD FOR LBA1022 FROM BACMAP

PID	58337317
Start	1014416
Stop	1015279
Strand	Direct
Synonym	LBA1022
Gene	Not Available
Product	arginase
Upstream 100 bases	>100 bases TGTGTGACAATATTCTGCCATCTTACCAATAGAAAACATATTTACTCCTT GGCTCTATAG TTACTATAGACTTTATCATAAATTCAAAGGAGTGAATATT
Gene Sequence	>864 bases ATGATAGAAAAAACAATTCGATTAGTTGTTCCAGATTGGCAAGCAGGTGA TAATCCCGTC TATAAATTAGGAGCAAAAGTTTTAAAAGCAATAGCACCGGAGAATAAAGA ACAAAAGACA ATTACGGTAAAAACTGCTGATAGTAACCAAACGTTAGAGAAAGAAAATAA CGTAACAGCT CAATCAGCTGTATTAAAAAATATTAGAAATACTAAAAAAGTCATCATTAC CGAACAACCA AATAAAATTATTACCTTCGGTGGTAATTGTTTAGTATCGCAGCAACCAAT TGACTATCTT AACGGAATCTATGGAGAAAAATTAGGTGTTATCTGGATTGATGCACATCC TGATATTTCT AATCCGGAAGTTTTTTATAATGAACACGCTATGGTAGTGGGAAATCTATT ACATCGTGGT GATCCAGCTATTCAAAAAGAGGTAGATAATCCGTTAAAATCTAATCAAAT TTTCTACGCT GGTTTACAAGAGCCGACTAACAATGAAAAGAAATTAATTGAACAAGCAGG TATCAAATAT AAGGTTCAAGATGAAAATCAAATAGATTCTGAAAGAGTAATAGATTGGAT CAAAGAAAAT AATTTTGAACATATTTATATTCACTTAGATGTGGATGTAATGAACTCTGA TTCAAAGAAT TTCTATGCCACATATTTTAACAATCCTGATCTAGGAGAAATACCTGACAA TGCAGCAGTA GGTAAAATGAGTAGAAAGAGCATCTGGCAATTTATTAGTAACTTCAGCCA AGAATATAAT CTTGTTGGTCTAACCTTAGCTGAGTATTTGCCTTGGAGTGCTAAAGAAAT GTTAGATTTA ATGAATAATACTAATATTTTTTAG
Downstre	>100 bases

am 100 bases	ATTTTCTTGAGCACTTATAAAAAGTAAGTTAAAATGTATTTAACTTAATT AAAAAATCAT TTATTAATTCAAGGAGGAGATAATCATGCAAATTTTTGTA
Protein Sequence	>287 residues MIEKTIRLVVPDWQAGDNPVYKLGAKVLKAIAPENKEQKTITVKTADSNQ TLEKENNVTA QSAVLKNIRNTKKVIITEQPNKIITFGGNCLVSQQPIDYLNGIYGEKLGV IWIDAHPDIS NPEVFYNEHAMVVGNLLHRGDPAIQKEVDNPLKSNQIFYAGLQEPTNNEK KLIEQAGIKY KVQDENQIDSERVIDWIKENNFEHIYIHLDVDVMNSDSKNFYATYFNNPD LGEIPDNAAV GKMSRKSIWQFISNFSQEYNLVGLTLAEYLPWSAKEMLDLMNNTNIF

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