MATERIALS AND METHODS

The present study was carried out in search of fungi which can produce the L-Arginase enzyme. The work involved selection of the potential fungi, optimization of the enzyme production, bulk crude enzyme preparation, purification of L-Arginase and determination of its biochemical characteristics and anticancer properties. Fungi from two genera i.e., Aspergillus and Penicillium were screened for their capacity to produce the enzyme L-Arginase in cultural conditions.
I. SCREENING OF THE FUNGI FOR THEIR L-ARGINASE ACTIVITY:

Pure cultures of the species of *Aspergillus* and *Penicillium*, maintained in the culture collection of our laboratory have been tested for L-arginase production. The fungi to be screened were grown in petriplates containing the Czapek-Dox medium of the following composition to prepare a fresh inoculum:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>20.0 g/l</td>
</tr>
<tr>
<td>KNO₃</td>
<td>2.0 g/l</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0 g/l</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g/l</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g/l</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 g/l</td>
</tr>
<tr>
<td>Distilled water</td>
<td>- make up to 1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.8</td>
</tr>
</tbody>
</table>

The culture plates were incubated at 28 ± 2°C till the sporulation began. This culture was used as inoculum. Five mm disc was inoculated to a broth medium of above composition except for KNO₃ which was replaced by L-arginine.
2.0 g/l as the nitrogen source which would induce L-arginase synthesis in the potential fungi.

The cultures were grown in 100 ml flasks containing 20 ml of the culture medium. The organisms were incubated at 28 ± 2°C for 4-6 days.

PREPARATION OF CELL FREE EXTRACT (CFE):

(Enzyme preparation)

The cultures were harvested during the late log phase of growth, after 4 days of incubation, and the mycelial mats were washed with chilled normal saline. The fungal mat was thoroughly homogenized in chilled normal saline (10 ml for each g of fresh weight of mycelium) with sterilized sand in pestle mortar at 4°C. The mycelial extract was centrifuged at 4000 rpm for 15 min at 4°C. The resulting supernatant was used as the source of crude enzyme for assay.

Enzyme Assay:

L-ARGINASE:

L-Arginase, which catalyzes the hydrolysis of arginine to ornithine and urea, was assayed by a modification of the
colorimetric assay of Green et al. (1990). The first step in assay involves heat activation of arginase in the presence of MnCl₂. Subsequent to the heat activation, arginine is added to start the reaction. A chromophoric derivative of the hydrolytic product, urea, is then measured spectrophotometrically. In heat activation step, equal volume of activation buffer containing 10 mM MnCl₂, 20 mM Hepes pH 7.0, 200 mM NaCl and 2.5 mg ml⁻¹ bovine serum albumin was mixed with enzyme. This solution was heated to 40°C for 15 min. In 400 ul of activated enzyme, 400 ul of ice cold 0.1M glycine buffer (pH 9.0) was added. Then it was equilibrated to 37°C by placing the tube in a water bath for approximately 2 min. The assay was initiated by the addition of 200 ul of 0.3 M arginine in 0.1 M glycine buffer pH 9.0. The reaction was stopped after 30 min by the addition of 2 ml of 30% TCA. Then it was centrifuged at 3000 rpm for 10 min. To 1 ml of supernatant 1 ml distilled water, 1.6 ml sulfuric acid + phosphoric acid + water (1:3:7) mixture was added. Then 0.4 ml of diacetyl mono oxime (2% in 2% acetic acid) was added, mixed well and the tubes were placed in boiling water bath for 30 min to allow color formation. The absorb-
ance was measured at 480 nm. One unit of arginase is defined as that amount which produces 1 umole urea min⁻¹ at 37°C.

Protein Estimation: Protein concentration was estimated by the method of Lowry et al. (1951).

II. CULTURAL AMENDMENTS:

(i) Effect of L-arginine concentration on production of L-arginase by P. citrinum:

To study the effect of L-arginine on the production of L-arginase by P. citrinum, varying concentration of L-arginine was supplemented in Czapek-Dox medium substituting the KNO₃. The concentration of L-arginine used was 0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% w/v. Cultures were incubated at 28 ± 2°C for 4 days.

(ii) Effect of Mn (II) and Fe (II) on the production of L-arginase by P. citrinum:

To study the effect of Mn (II) and Fe (II) on production of L-arginase by P. citrinum, 0.05%, 0.1% and 0.2% w/v of MnCl₂ and FeSO₄ were supplemented in Czapek-Dox
medium containing 0.2% L-arginine as nitrogen source. All the possible combinations of Mn(II) and Fe(II) were used to study the effect on enzyme production. The cultures were grown on 28 ± 2°C for 4 days.

III. PURIFICATION OF L-ARGINASE FROM Penicillium citrinum:

After the screening of fungi for L-arginase capacity, Penicillium citrinum was selected for detailed investigation.

The fungus was grown in 3000 ml Haffkine flask containing 400 ml culture medium. The culture was incubated at 28 ± 2°C and harvested on 6th or 7th day during late log phase of growth for extraction and purification of L-arginase.

The purification was done by two methods:

A. METHOD 1 : Multi step purification:

(a) Preparation of initial extract: The mycelium was separated and washed with chilled normal saline, 40 g of fungal mass was homogenized in 80 ml extraction buffer of following composition:
20 mM Hepes buffer (pH 7.0)
5 mM MnCl₂
2 mM β-mercaptoethanol
0.85% NaCl

The resulting homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was taken as the starting material for purification of the enzyme.

(b) Ammonium Sulphate precipitation / fractionation:

Ammonium sulphate was added to the initial extract to bring its concentration to 40%. It was then stirred at 4°C for 60 min. It was centrifuged at 10,000 rpm for 30 min at 4°C. The precipitate was discarded.

Ammonium sulphate was added in the supernatant to increase its concentration to 70% and stirred for 60 min at 4°C. It was again centrifuged at 10,000 rpm for 30 min at 4°C. Supernatant was discarded. The precipitate was resuspended in 10 ml Tris buffer pH 7.2, containing 5 mM MnCl₂ and 2 mM β-mercaptoethanol.

(c) Gel Filtration - Sephadex G-100:

The protein solution was subjected to next step of
purification by passing through Sephadex G-100 column with bead size of 40-120 μ (Sigma).

Method:

i. **Column**: A chromatographic column made of glass tubing having a diameter of 2.5 cm and a height of 45 cm was used.

ii. **Preparation of eluent and Gel slurry**: Tris buffer 100 mM (pH 7.2) with 5 mM MnCl₂ and 2 mM β-mercaptoethanol was used as eluent.

For the preparation of gel slurry 10.0 g dry gel was suspended in 500 ml of 100 mM Tris buffer pH 7.2 and kept in boiling water bath for 4 hr for swelling and degassing. Then it was cooled to room temperature. The gel with buffer was allowed to settle down and the fine particles remaining in the suspension were removed by decantation.

iii. **Packing of column**: The concentration of hydrated gel slurry was adjusted to permit convenient pouring. Column was mounted on a vertical stand. Dead space below the support (glass wool) was filled with liquid from the outlet end to ensure no bubbles are trapped below the glass wool, then outlet tubing was closed. The column was tilted at an angle and the slurry was poured directly down the inside wall of the
column and the column was returned to a vertical position immediately after pouring. Flow was applied to the column as soon as possible to achieve even sedimentation. Two to three column volumes of buffer was passed to stabilize the gel bed.

iv. Application of sample and elution: Buffer was drained from the surface of the bed without allowing the bed to run dry. The sample was layered on the top of the bed with a pipette. Then the column was refilled with eluent i.e., 100 mM Tris-HCl pH 7.2, 5 mM MnCl₂ and 2 mM β-mercaptoethanol and then was reconnected to the buffer reservoir.

v. Collection and analysis of fractions: Three ml fractions were collected using fraction collector (CYGNET, ISCO). Absorbance at 280 nm was taken on JASCO UV-VIS Spectrophotometer for each fraction to detect the protein fraction. The fractions with protein were analyzed for enzyme activity as mentioned earlier.

(d) Ion Exchange Chromatography:

This was performed on DEAE CELLULOSE column.

i. Column: A chromatographic column made of glass tubing having a diameter of 1 cm and a height of 20 cm was used.
ii. Method: Prior to setting up the column, DEAE-Cellulose matrix was washed with 0.5 N HCl and then 0.5 N NaOH. This was done by gentle centrifugation. Next the matrix was washed with 20 volumes of 100 mM Tris- HCl (pH 7.6) with 5 mM MnCl₂ and 2 mM β-mercaptoethanol. pH was maintained at 7.6. The whole matrix was then transferred to a column.

Pooled fractions containing maximum enzyme activity obtained from Sephadex G-100 gel filtration was loaded on the DEAE-Cellulose column of size 20 cm x 1 cm. Protein was eluted by developing the gradient forming by drop wise addition of 0.3 M KCl in to the buffer reservoir. Two ml fractions were collected with the help of fraction collector and the absorbance was monitored at 280 nm using JASCO UV-VIS spectrophotometer. The fractions with protein were analyzed for enzyme activity.

METHOD 2: Single step purification of L-arginase by substrate specific elution from DEAE Cellulose column.

(a) Preparation of Initial Extract:

The mycelial mat was separated and washed with chilled
normal saline and homogenized in extraction buffer of the following composition:

- 100 mM Tris HCl buffer pH 7.6
- 5 mM MnCl₂
- 2 mM β-mercaptoethanol

The resulting homogenate was clarified by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was used as initial material for purification.

(b) **Affinity chromatography**:

DEAE Cellulose column was regenerated by washing sequentially with 0.5 N HCl and then 0.5 N NaOH. Column was equilibrated with 100 mM Tris buffer (pH 7.6) containing 5 mM MnCl₂ and 2 mM β-mercaptoethanol. The enzyme extract was passed through the column. The column was washed with 3 bed volumes of the same buffer to remove unbound proteins.

Then the buffer containing substrate i.e., 0.3 M L-arginine in above mentioned buffer was passed through the column. Three ml fractions were collected with the help of fraction collector and protein was monitored at 280 nm using JASCO UV-VIS Spectrophotometer. The fractions with protein were analyzed for L-arginase activity. The fractions with enzyme activity were dialyzed against same buffer to remove
L-arginine and urea. Then again analyzed for the enzyme activity. Peak fractions were pooled together and stored in deep freeze.

IV. CONFIRMATION OF HOMOGENEITY OF L-ARGINASE & DETERMINATION OF MOLECULAR WEIGHT:

The purity of L-arginase and sub unit molecular weight was confirmed by polyacrylamide gel electrophoresis in denatured conditions. MW-SDS-70 kit (Sigma) was used for molecular weight marker.

(1) SDS Polyacrylamide Gel Electrophoresis:

(a) Preparation of Gels:

The gel tubes had an inner diameter of 0.5 cm and were long enough to hold a 10 cm gel. To prepare 12 gels 15 ml gel buffer (Reagent B) and 13.5 ml of acrylamide gel (Reagent C) were mixed. Into this 1.5 ml freshly prepared ammonium persulphate solution (Reagent E) and 0.05 ml TMEDA (Reagent D) were added. Two ml of solution was dispensed carefully into each tube and carefully layered a few drops of water on the top of gel solution, before the gel hardened.
(b) Electrophoresis:

500 ml gel buffer (Reagent B) was diluted with 1000 ml of distilled water and the compartments of electrophoresis apparatus were filled with diluted gel buffer. 50 ul of L-arginase sample and 10 ul of marker samples were loaded. Electrophoresis was carried out at constant current of 8 milliamps/gel with positive electrode in lower chamber until the marker dye (Bromophenol Blue) was 1 cm from the anodic end of gel. Gels were removed from tubes by squirting water from a syringe between the gel and glass wall, then used a pipette ball to exert pressure.

(c) Staining and Destaining:

Gels were stained in staining reagent (Reagent F) for overnight and then destained by diffusion against several changes of reagent G. The migration distance of tracking dye and of blue protein zones were recorded from the top of gel.

To determine the relative mobility \( (R_f) \) of a protein, the following formula was applied.

\[
R_f = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}
\]
The $R_f$ values (abscissa) were plotted against the known molecular weights (ordinate) on semi-logarithmic paper and molecular weight of L-arginase was estimated from the calibration curve.

(2) Determination of Molecular weight by Gel Filtration:

Molecular weight of L-arginase of *P. citrinum* was determined by Sephadex G-100. The column was prepared as mentioned earlier. This column was equilibrated with 0.05 M phosphate buffer pH 7.5, flow rate was maintained at 36 ml/hour. In the first run Blue Dextran 2000 was passed through the column to determine the void volume of the column. In the second run albumin (M. Wt. 66,000 Da) and trypsinogen (M. Wt. 24,000 Da) were passed. In the third run ovalbumin (M. Wt. 45,000 Da) and $\beta$-lactoglobulin (M. Wt. 18,400) were passed. Three ml fractions were collected and O.D. at 280 nm was monitored. The elution volume ($V_e$) for each protein was measured from the start of the sample application to the center of the elution peak. When the sample is Blue Dextran 2000, $V_e$ is then the void volume.
Preparation of Calibration Curve:

A molecular weight calibration curve defines the relationship between the elution volumes of a set of standard proteins and the logarithm of their respective molecular weights. Elution parameter $K_{av}$ was used for the preparation of calibration curve. The $K_{av}$ values for each protein was calculated using the equation

$$\frac{Ve - Vo}{Vt - Vo} = K_{av}$$

where

$Ve = \text{elution volume for the protein}$

$Vo = \text{Column void volume} = \text{elution volume for Blue Dextran 2000.}$

$Vt = \text{Total bed volume.}$

Using semilogarithmic graph paper, $K_{av}$ value for each (on the linear scale) was plotted against the corresponding molecular weight (on the logarithmic scale), and a straight line was drawn which best fits the points on the graph. From the $K_{av}$ of L-arginase of \emph{P. citrinum} the molecular weight was determined from the calibration curve.
V. Determination of Enzyme Kinetics and Their Characterization in Respect of Factors and Cofactors Including the Analogs:

(a) Substrate Specificity:

Substrate specificity of L-arginase was determined by substituting L-arginine by 6 different substrates i.e., L-asparagine, L-glutamine, L-lysine, L-phenylalanine, L-canavanine and Homoarginine in the enzyme assay. The concentration of these substrates was 0.3 M in the system.

(b) Effect of Substrate Concentration:

For determining the effect of substrate concentration on L-arginase activity, keeping the enzyme concentration fixed, L-arginine concentrations were varied from 0.02 M to 0.1 M in standard assay system described earlier. A graph of substrate concentration was plotted against the reaction velocity for determining the Michaelis Menten pattern. Km and Vmax were determined by the Lineweaver Burk
(c) Effect of manganese concentration on the activation of L-arginase:

In order to determine the effect of manganese concentration on the heat activation of L-arginase, manganese was removed by dialysis of the enzyme against 20 mM Hepes buffer pH 7.0, 2 mM β-mercaptoethanol. The manganese was then added to L-arginase at various concentrations for the heat activation.

The effect of manganese concentration on the heat activation of arginase was analyzed in terms of the following equation:

\[ V' = \frac{V_m [Mn]}{K_{0.5} + [Mn]} \]

where \( V' \) is the rate of urea formation at a particular manganese concentration, \( V_m \) the maximal rate of urea formation, \([Mn] \) the total manganese concentration in the heat activation step and \( K_{0.5} \), the concentration of manganese at which the velocity is half maximal.

(d) Effect of incubation time on the activation of L-arginase:

During the heat activation of L-arginase, the
equal volume of enzyme was added to solution containing 20 mM Hepes buffer pH 7.0, 10 mM MnCl₂ and 2 mM β-mercaptoethanol. This solution was heated to 40°C for different time intervals i.e., 5, 10, 15, 20, 25 and 30 min. Then further assay was carried out as mentioned earlier.

(e) **Effect of pH on the activity of L-arginase**:

To determine the effect of hydrogen ion concentration on the activity of L-arginase, the reaction was carried out at different pH from 2.5 to 11.0. The pH was maintained in the reaction mixture by 0.1 M glycine buffer of respective pH. L-arginine (0.3 M) was also prepared in respective buffer to avoid the change in the pH of the reaction mixture.

(f) **Effect of Temperature on the activity of L-arginase**:

To determine the effect of temperature on the activity of L-arginase, the reaction was carried out at different temperature i.e., 10, 20, 25, 30, 35, 40, 45, 50 and 60°C. For this, reaction mixture with substrate was kept at particular temperature for 15 min and then L-arginase was added to the reaction system and incubated for 30 min. Urea
formation was estimated as mentioned earlier.

(h) **Effect of substrate analogs on the activity of L-arginase:**

The three analogs of L-arginine namely L-lysine, L-canavanine and homoarginine were used to understand their effect on enzyme activity. Analogs were added to reaction system with varying concentration of L-arginine from 0.02 M to 0.1 M. L-lysine and homoarginine 10 mM and 100 mM, canavanine 5 mM and 50 mM were added to reaction system. The Km and Vmax were determined by Lineweaver Burk plot.

(i) **Effect of metal ion on the activity of L-arginase:**

To study the effect of metal ions on the activity of L-arginase of *Penicillium citrinum*, the metal ions considered were Cu(II), Co(II), Mg(II), Fe(II) and Zn(II). Each of these ions were taken in concentrations of 0.1 ppm, 1.0 ppm, 10 ppm, 100 ppm and 1000 ppm concentration in the reaction system and the activity was assayed as mentioned earlier.

Since the Fe(II) showed the increase in the activity of L-arginase it was of interest to determine the combined effect of Mn(II) and Fe(II) on the enzyme activity.
For this enzyme was dialyzed against 20 mM Hepes buffer pH 7.0 and 2 mM β-mercaptoethanol to remove manganese. MnCl₂ and FeSO₄, each of 100, 500 & 1000 ppm were then added to the reaction system with different combinations and assayed as earlier mentioned.

V. TESTING OF L-ARGINASE FOR ANTICANCER ACTIVITY:

The growth inhibitory potential of the enzyme was tested against Chang liver (Human), Mouse myeloma P3X.Ag8.653 and SP2/0.Ag14 cell lines.

Cell Lines:

The cell lines one from human origin i.e. Chang liver and two from Mouse myeloma P3X.Ag8.653 and SP2/0.Ag14 were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. These cells were cultured at atmosphere of 5% CO₂ and 95% air at 37°C.

Growth Medium:

Chang Liver cell line was grown on Basal medium (Eagle) with Earl’s balance salt solution (GIBCO, Grand
Island, NY) supplemented with 10% calf serum (Flow Laboratories).

Mouse myeloma SP2/0.Ag14 cell line was grown on Dulbecco's modified Eagle's medium with 4.5 g/l glucose (GIBCO) supplemented with 10% fetal calf serum (Flow Laboratories).

Mouse myeloma P3X.Ag8.653 cell line was grown on RPMI medium 1640 (GIBCO) supplemented with 20% fetal bovine serum (Flow Laboratories).

Subculturing of Chang Liver Cell Line: Chang liver cell line culture was grown to confluency in 50 ml plastic flask with 10 ml Basal medium (Eagle's) with Earl's balance salt solution supplemented with 10% calf serum. Medium was poured off and cells were rinsed with PBS. Four ml of TPVG (prewarmed to 37°C) was poured over the cells from the opposite side of monolayer. After 2-3 min TPVG was poured off and the bottle was shaken to remove cells from the surface of the bottle. Five ml of growth medium was added to resuspend the cells. An aliquot was taken for a viable cell count which was carried out as follows:

Viable count: 0.5 ml of 0.4% Trypan blue stain (Sigma) was
taken in a test tube and 0.3 ml PBS was added along with a 0.2 ml suspension of the test cell line (dilution factor of 5). Allowed it to stand for 5 to 15 min. With the cover slip in place, a small amount of Trypan blue - cell suspension was transferred to both the chamber of the haemocytometer by carefully touching the edge of the cover slip with the pipette tip and allowed each chamber to fill by capillary action. Starting with one chamber of the haemocytometer, counted unstained cells (viable cells) in all four 1 mm corner squares.

Each square of the haemocytometer, with cover slip in place, represents a total volume of 0.1 mm$^3$ or 10$^{-4}$ cm$^2$. Since 1 cm$^3$ is equivalent to approximately 1 ml, the cell concentration per ml was determined using following calculation:

\[
\text{Cells/ml} = \text{the average count per square} \times \text{dilution factor} \times 10^4
\]

Cell concentration was adjusted to 0.5 x 10$^5$ cells/ml by addition of basal medium (Eagle) with Earls balance salt solution supplemented with 10% calf serum and 10 ml of this was seeded in to 50 ml plastic culture bottle and incubated at 37°C in 5% CO$_2$ and 95% air atmosphere.
Tests on Human Liver Cell line:

(A) Chang liver cell line culture was grown to two third confluency in 50 ml plastic flask with 10 ml Basal medium (Eagle's) with Earl's balance salt solution supplemented with 10% calf serum. Culture medium was replaced with fresh medium along with 122.2 IU of the test L-arginase. The control cultures were taken with heat inactivated enzyme. Culture was again incubated for 18 hrs at 37°C in 5% CO₂ and 95% air atmosphere.

Staining of culture:

After completion of incubation, culture medium was removed and cells were fixed with Bouins fixative for 2 hrs. It was washed 3-4 times to remove yellow tinge from the cells and stained with Haematoxyline (1:5 dilution with distilled water) for 5-10 min and then washed in distilled water. Culture was passed through series of alcohol 50%, 70% and 90% for 2 min each and stained with Eosin for 10 min. Briefly rinsed in absolute alcohol and mounted in Euparol.

Microscopic examination was made to observe the effects of enzyme upon the Chang liver cells to record data of its activity in both the test and control sets.
(B). Two ml of this cell suspension i.e., $10^5$ cells was seeded in four replicates using 24 well tissue culture plates (Laxbro). The enzyme L-arginase was sterilized by passing through 0.2 u bacteriological filter before use. 25 IU and 50 IU L-arginase were added to the wells containing cells. Heat inactivated L-arginase was taken as control. The test cultures were incubated at 37°C in 5% CO₂ and 95% air atmosphere for 4 days. After due incubation medium was removed and cells were trypsinized for viable count as described earlier. The ratio (x100) of number of cells in a test culture to average number of cells in control culture was expressed as "relative cell number".

Subculturing of Mouse Myeloma Cell Lines (SP2/01, Ag14 & P3X, Ag8.653):

Both the cell lines grow in suspension. The cells were harvested by centrifugation at 2000 rpm for 5-10 min. The SP2/01. Ag14 cells were resuspended in 2 ml of Dulbeco's modified Eagles medium and P3X. Ag 8. 653 cells were resuspended in RPMI 1640 medium. 0.5 ml cell suspension was used for viable cell count as mentioned earlier. The cell concentration was adjusted to $1x10^5$ viable cells/ml with
their respective medium and 10 ml of the cell suspension was seeded in 50 ml culture flasks. The cultures were incubated in atmosphere of 5% CO₂ and 95% air at 37°C. After 3-4 days fluid renewal was done. After seven days of growth, a maximum of 1x10⁶ cells/ml may be is obtained.

Test on Mouse Myeloma:

Cell suspension of both the mouse myeloma cell lines was diluted to 0.5 x 10⁴ cells/ml in their respective media. Two ml of this cell suspension was seeded in four replicates using 24 well tissue culture plates (Laxbro).

The enzyme L-arginase was sterilized by passing through 0.2 u bacteriological filter before use. 50 IU and 100 IU L-arginase were added to the wells containing cells. Heat inactivated L-arginase was taken as control.

The test cultures were incubated at 37°C in 5% CO₂ and 95% air atmosphere for 4 days.

Viable cell count:

After 4 days, medium containing cells were removed from the wells of the plate with the help of Pasteur pipette and centrifuged at 1000 rpm for 2 min. Cells were resuspended in
phosphate buffer saline (pH 7.0). Viable cells were counted using Trypan blue as described earlier.

The ratio (x 100) of the number of cells in a test culture to average number of cells in control culture was expressed as "relative cell number".

Estimation of L-arginine concentration: After harvesting the cells by centrifugation, L-arginine concentration was determined in the culture medium at the end of experiment. For this proteins were precipitated by addition of 30% TCA and the supernatant was taken for estimation whose pH was adjusted to 7 with the help of 0.1 N NaOH. Its arginine concentration was measured with the help of Bovine arginase (Sigma). 100 ul medium was incubated with 200 ul glycine buffer (pH 9.4), 200 ul of activation buffer (20 mM Hepes buffer pH 7.0 with 5 mM MnCl$_2$) and 100 ul bovine arginase (25 IU) at 37°C for 30 min. Reaction was stopped by addition of 30% TCA. Urea produced was estimated with diacetyl mono oxime. The concentration of urea is equimolar to arginine present in medium.