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A Modified Spectrophotometric and Methodical Approach to Find Novel Inhibitors of Ornithine Decarboxylase Enzyme: A Path through the Maze

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Method Article

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Abstract

A precise method has been developed for measuring the activity of Ornithine decarboxylase \(ODC) enzyme, a promising target of cancer and infectious diseases using petite quantity of chemicals. The modified spectrophotometric assay has been used to find inhibitors of ODC enzyme. The methodology is based on the cognizance that enzyme transforms L-ornithine hydrochloride \(substrate) to yellow colored putrescine \(product) soluble in pentanol, the absorbance of which was measured spectrophotometrically. The technique described herein was optimized for evaluating efficacy of phytomolecules as ODC attenuator but could also be used for exploration of novel bioactive compounds as chemopreventive and/or chemotherapeutic agents. A reaction concoction was prepared and hydrophobic withdrawal of yellow colored TNP-Putrescine-TNP complex was recorded at 426nm. Advanced validation and understanding of the factors affecting the enzyme activity i.e. substrate concentration, inhibitor concentration, temperature, pH of the reaction medium and incubation time were also investigated.

Introduction

The ever increasing approach of finding novel natural product inhibitors of ODC enzyme has intrigued the wits of researchers for many years. In the lime light of newer gadgetry of information, we have developed a swift, responsive and lucrative method for measuring the ODC enzyme activity. The method is suitable for regular laboratory purposes and could be helpful for screening of molecules under in vitro drug discovery programme. The assay endowed a colorimetric end point that is non-destructive, indefinitely stable and perceptible to the naked eye. Based on the surveillance that picrylsulfonic acid \(2,4,6-trinitrobenzenesulfonic acid, TNBS) reacts at alkaline pH with amines and the amino groups give colored trinitrophenyl \(TNP) adducts.1-2, this assay is based on the observation that the TNP adduct of ornithine \(TNP-Ornithine-TNP) is soluble in water whereas TNP adduct of putrescine \(TNP-Putrescine-TNP) is soluble in 1-Pentanol and this can be differentially distinguished as upper layer, the structures and the reaction of which are hypothetically depicted in Figure 1. The sensitivity of this assay contrasted favorably with sensitivities of radiometric and HPLC based assays as reported earlier.1 Naturally occurring polyamines are ubiquitous, diminutive, evolutionarily ancient physiological cations, essential for cell growth, differentiation and proliferation. They play a key role in regulating ion channels/receptors, cell cycle involving internal ribosome entry site active only in mitosis in the human ODC mRNA, stabilizes cellular nucleic acids and/or membranes, activates protein kinases and affecting translation, transcription and chromatin structure.3-8 Being involved in cell motility and cell-cell interactions, the polyamine pathway is of immense interest because increased levels are associated with tumor formation. Hence, it is considered to be a promising target for chemotherapeutic intervention in cancer and parasitic diseases. 9-18\(Figure 2) The polyamine biosynthesis is tightly regulated by ODC \(EC 4.1.1.17), an enzyme first discovered in 1968, catalyzing the pyridoxal-5' phosphate \(PLP)-dependent decarboxylation of L-ornithine to generate putrescine. This enzyme is highly inducible specific to pH, temperature, time and substrate concentration. Mammalian ODC is a well known mediator of c-Myc-

induced apoptosis and is a direct target of c-Myc, thus recognized as a proto-oncogene.19-23 The plant ODC is very similar in sequence to animal ODCs, and its expression is correlated with cell proliferation.24 Since ODC is involved in cell growth and differentiation, it's constitutive over expression ensures a crucial role in numerous disease and disorders including cancer, autoimmune diseases.25 Account of its vital role in cell proliferation, numerous synthetic and natural products have been reported to inhibit the enzyme in polyamine biosynthesis.25-26 Subject to both positive and negative feedback regulation, ODC activity increases at low concentration of polyamine and vice versa. The regulation appears to be a mixture of post-transcriptional and induction of a unique ODC-specific inhibitor termed antizyme. 27-30 ODC expression regulated by vital factors such as Myc, Jun, Fos, and cAMP explicate the allocation of more than a single polyamine in nature. 31 A wide array of ODC inhibitors has been toiled out and the best-characterized was α-difluoromethylornithine \(DFMO; eflornithine), synthesized by Metcalf et al. \(1978). Investigated as a possible cancer chemotherapeutic agent, it had limited efficacy in early-phase clinical therapeutic trials. Since then unfortunately no other satisfactory inhibitor of ODC enzyme has been discovered in drug discovery and development program.23-26,32,33 Consequently, the urge to find out new ODC inhibitors is progressively increasing and the use of natural products e.g. phytomolecules is advantageous over synthetic counterparts due to fewer side effects and toxicity. Recently researchers have tried to find ODC inhibitors comparable to DFMO and thus there is a requisite for a simple spectrophotometric assay for easy and fast screening of inhibitors. Earlier, numerous methods have been reported for estimating ODC activity which can be categorized to radioactive and non-radioactive methods34-52\(Table 1). Radioactive methods are hazardous \(spurious CO2 release) while non-radioactive methods have many disadvantages involving sophisticated instruments, and cost-effectiveness. Hence, we tried to develop a simple, contemptible method which is not based on CO2 formation \(which give sometimes false result) but simple solubility factor. We evaluated samples53 to see the interaction of the enzyme with molecules to find out inhibitors of ODC enzyme. Mechanism of inhibition may be non-specific, irreversible, reversible, competitive and/or non-competitive. The assay is an improved, uncomplicated and cost- effective involving petite quantity of chemicals \(Figure 3). The capacity to verify and validate ODC as candidate biomarkers especially in cancer and parasitic disease is limited and for that reason biomarkers already discovered needs to be validated with simple bioassays. Therefore, in this paper, we have modified the assay to simplify the systematic validation of ODC enzyme activity with thorough testing of hefty sample sets.

Reagents

• 2.5mM 2-Mercaptoethanol, for electrophoresis \(Sigma Cat. No. M7154) • 1.5mM E.D.T.A. Disodium salt \(Himedia Cat. No. RM 119) • 75nM Pyridoxal 5- Phosphate hydrate \(Sigma Cat. No. P3657) • 3mM L-Ornithine monohydrochloride, approx. 99% \(Sigma Cat. No. O6503) • Putrescine Dihydrochloride \(Sigma Cat. No. P5780) • 4N Sodium Hydroxide extrapure AR \(SRL Cat. No. 1949181) • 1- Pentanol 99%, A.C.S reagent \(Sigma Cat. No. 398268) • 0.1M Sodium tetraborate decahydrate, ACS reagent, 99.5% \(Sigma Cat. No. S9640) • 10mM Picrylsulfonic acid solution \(Sigma Cat. No. 92823) • Dimethyl sulphoxide \(Merck Cat. No. K36760512728) • Di-potassium hydrogen phosphate, A.R., 99% \(Sigma Cat. No. P5379) •

Potassium phosphate monobasic, minimum 99.0% \(Sigma Catalogue No. P5379) • Perchloric acid, 70%, redistilled, 99.99% metal basis \(Sigma Cat. No. 311421) • Ornithine Decarboxylase Enzyme from Escherichia coli, 965mg solid,0.033units/mg solid, 0.06 units/mg protein \(Sigma Cat. No. O3001)

Equipment

• Eppendorff tubes from Genaxy Scientific Solan, Himanchal Pradesh, India. • Pipettes from ThermoFisher Scientific India Pvt. Ltd., Noida, India. • Vortex and Spinwin from M/s Tarsons Products Pvt. Ltd., New Delhi, India. • Centrifuge from Eppendorf, New York, USA. • Spectrophotometer from Molecular Devices Corporation, California, USA. • 96 well plates from Greiner Bio-One B.V. Alphen aan den Rijn, Netherlands • pH meter from Eutech Instruments, Navi Mumbai, India.

Procedure

Phosphate Buffer \(0.1M, pH 7.0) It is prepared by dissolving 1.74g of Dipotassium hydrogen phosphate \(K2HPO4) in 100mL Deionized water and 1.36g of Potassium phosphate monobasic \(KH2PO4) in 100mL deioinized water. Mix 61.5mL of K2HPO4 and 38.5 mL of KH2PO4 and raise the volume 1 Litre to get 0.1M Phosphate buffer \(pH 7.0). Phosphate Buffer \(150mM, pH 7.1) It is prepared by dissolving 2.61g of K2HPO4 in 100mL Deionized water and 2.04g of KH2PO4 in 100mL deioinized water. Mix 61.5mL of K2HPO4 and 38.5 mL of KH2PO4 and raise 1 Litre to get 150mM Phosphate buffer \(pH 7.1) Preparation of Standard Solution Ornithine-HCl: Dissolve 1.682mg of L-Ornithine HCl in 10mL of phosphate buffer \(pH 7.0). Dilute the stock solution to obtain working solution in the range of 0.2-100µM. Putrescine Dihydrochloride: Dissolve 1.6107mg of Putrescine Dihydrochloride in 10mL of phosphate buffer \(pH 7.0). Dilute the stock solution to obtain working solution in the range of 0.2-100µM. Reaction Mixture: Dissolve 17.57µL of β-mercaptoethanol \(2.5 mM), 55.84mg of Disodium EDTA \(1.5mM), 2µL of stock solution of Pyridoxal phosphate prepared in 150mM Phosphate buffer \(75nM) and 50.6mg of L-Ornithine HCl \(3mM) in 150mM Phosphate buffer \(pH 7.1). Picryl Sulfonic acid \(TNBS): 25.7µL of TNBS dissolved in 10mL of 1-Pentanol, freshly prepared and kept at 4ºC Caution\! β-mercaptoethanol is a toxic by inhalation, cause damage to skin and eyes by prolonged exposure, so this chemical bottle should be opened inside fume hood and for very short time. Wear masks and gloves while using it. 0.1M Sodium Borate: Dissolve 3.8137g in 100mL Deionized water, adjust pH \(8.0) by 1M either NaOH and/or 1M HCl. 4N sodium Hydroxide: Dissolve 16.0g in 100mL of Deionized water. 1M Perchloric acid: Commercial solution available is calculated as 12M. To use we have to make 1M solution. For this, take 86mL of stock pure solution and raise up to 1000mL with deionized water. Caution\! Heating may cause explosion, contact with combustible material should be avoided, use carefully as it causes eye irritation. Perchloric acid and 10% Trichloroacetic acid is highly corrosive and handling should be done in a chemical fume hood Ornithine Decarboxylase Enzyme solution: Stock solution is made as 10mg of enzyme dissolved in 1mL of phosphate buffer \(150mM). It means that 0.6 units/mg protein in 1mL The enzyme is kept at -20ºC and stock solution is prepared in 150mM Phosphate buffer immediately before starting experiment. The enzyme is highly labile and should be kept

at 4 ºC while performing the assay. 10mM Picrylsulfonic acid solution: 25.7µL of TNBS solution dissolved in 10mL of 1-Pentanol. Caution\! Store 1-Pentanol in cool place, containers opened must be carefully resealed and kept upright to prevent leakage as it is flammable. It is a harmful by inhalation, cause skin dryness, irritating to respiratory system and eyes. Wear masks and gloves while using it. PROCEDURE 1. For each sample there must be at least two sets of eppendorf tubes one with the enzyme and another without enzyme. The same case applies to control set also. 2. Add the test sample/standard to each set. 3. Add 5µL of enzyme from stock solution to one of the sets. The concentration of enzyme is optimized between 10-100µg/mL and ample activity is observed at 50µg/mL. 4. Add 400µL of substrate reaction mixture to every eppendorf tubes. 5. Incubate for 37ºC for 30 min. 6. Terminate the reaction by adding 400µL of perchloric acid \(1M) or 400µL of trichloro acetic acid \(10%). Precipitation must be done to stop the enzymatic reaction. 7. Centrifuge at 5000rpm for 5 min at room temperature. 8. Mix 100µL of standards \(Ornithine/Putrescine 0.2-100µM) and/or terminated reaction mixture with 200µL of 4N NaOH. At this moment supernatant should be pipetted very carefully and mixing should be done vigorously \[Critical Step]. 9. Add 400µL of 1-pentanol. 10. Mix vigourously for 1 min with vortex mixer \[Critical Step]. 11. Centrifuge for 5 min at 2000rpm. Two layers form, bottom hydrophilic and top hydrophobic layer. At this point demarcation line should be clear. 12. Transfer 200µL of upper \(organic) phase to a fresh tube containing 200µL of sodium borate \(0.1M, pH 8.0) and mix. \[Critical Step]: pH of Sodium borate is very crucial, it should be 8.0 and mixing should be done properly. 13. Add 200µL of TNBS \(picryl sulfonic acid, 10mM in 1-pentanol). \[Critical Step]: Prepare the TNBS dye reagent immediately before use. It is prepare in 1-Pentanol, so thawing of TNBS should be done beforehand as it is kept at -20◦C. After that mix for 1 min on vortex mixer. 14. Add 400µL of DMSO and mix for 1 min. DMSO should be added after proper mixing of supernatant and TNBS. 15. Centrifuge for 5 min at 3000 rpm. \[Critical Step]: A very fine two layers are observed with top layer contains TNP-Putrescine-TNP, while bottom layer contains TNP-Ornithine-TNP. Sensitivity of the assay depends on the precise pipetting of the upper fine layer. The volume of the layer varies depending on the enzymatic reaction \(30-100uL). 16. Take upper layer and read absorbance at 426 nm. 17. Calculate the percentage of enzyme inhibition using the formula below. For initial screening, we use a threshold of 50% enzyme inhibition as a cut off for compound behaving as ODC inhibitors. However the threshold can be increased or decreased according to inhibitory criteria selected by the investigators. For IC50 determination, plot a concentration responsive graph between compound dose and percent inhibition. IC50 values can be derived using curve fitting methods with statistical analysis. Control Difference = Mean OD Control with Enzyme-Mean OD Control without Enzyme Sample Difference = Mean OD Sample with Enzyme-Mean OD Sample without Enzyme Percent Inhibition = Control Difference- Sample Difference X 100 Control Difference It is also possible to calculate the amount of product formed by plotting a standard graph of Putrescine and calculating the absorbance of test sample in terms of µM of Putrescine produced.

Timing

Timeline \(Estimated for 5 Samples and 1 Control) Step1-4 : 1 h Step 5 : 30 min Step 6-16 : 1 h

Troubleshooting

PROBLEM	POSSIBLE REASON	WAY OUT
Inconsistent absorbance reading	pH of reaction mixture	pH should be checked appropriately
Enzyme degradation		Mixing gently of enzyme, sample and substrate reaction mixture
Sample degradation		Sample should be freshly prepared/a check should be there for degradation due to high temperature, light sensitive etc
Variation in temperature		Reaction should be carried out at an ideal temperature
Layers may get mixed during the separation step	Mixing is a critical step at steps 8, 10, 13 and 15	Mixing should be done by vortex for at least 1-2 min
pH of sodium borate solution		pH of Sodium Borate buffer should be 8.0
Enzyme may get functionally inactive	Cofactor PLP is essential for the activity of enzyme	PLP stock solution is made in phosphate buffer and it is kept at -20°C, therefore it should be quickly thawed, used and kept back to -20°C, otherwise it will degrade

Anticipated Results

Various concentrations of TNP-Putrescine-TNP and TNP-Ornithine-TNP extracted in pentanol give the standard curve shown in Figure 4. The curve is linear \(r > 0.9624 for putrescine and r > 0.062 for ornithine) in the range of 0.2 to 100μM concentration. The average molar extinction co-efficient \(1.91 ± 0.58 x 103 M-1 cm-1), absorption spectra \(λ max 426nm) of Putrescine and Ornithine with TNBS and linearity of enzyme concentration \(20-200μg/mL) have already been revealed.1 \(Figure 4). Effect of substrate concentration For the validation of the assay, changes were made in concentrations of L-Ornithine HCl. The modification is made in the substrate reaction mixture solution in which all the components are same except the concentration of L-Ornithine HCl varies from 0 to 5mM, i.e. different substrate solution is being made for changed concentration \(Figure 5). Effect of pH To study the effect of pH on enzyme activity, pH of the substrate solution was varied by using different amount of K2HPO4 and KH2PO4 used in phosphate buffer. The pH is varied from 5.0 to 9.0 \(Figure 6). Effect of incubation time The reaction mixture containing enzyme \(control with enzyme) and reaction mixture \(control without enzyme) was subjected to different incubation time at Step 5 ranging from 5 to 120 min \(Figure 7). Effect of temperature For evaluation of the effect of temperature on enzyme activity, tubes were incubated to different temperature from 25 to 61◦C with a difference of 12◦C \(Figure 8). Effect of

different concentration of DFMO For evaluation of the effect of known inhibitor of ODC activity, different concentration of DFMO mixed in reaction mixture along with the enzyme and same method of calculation was done as for other samples. Concentration used was from 1-5mM and very less activity was shown in 1mM while in other concentrations negligible activity was shown \(Figure 9).

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Figures

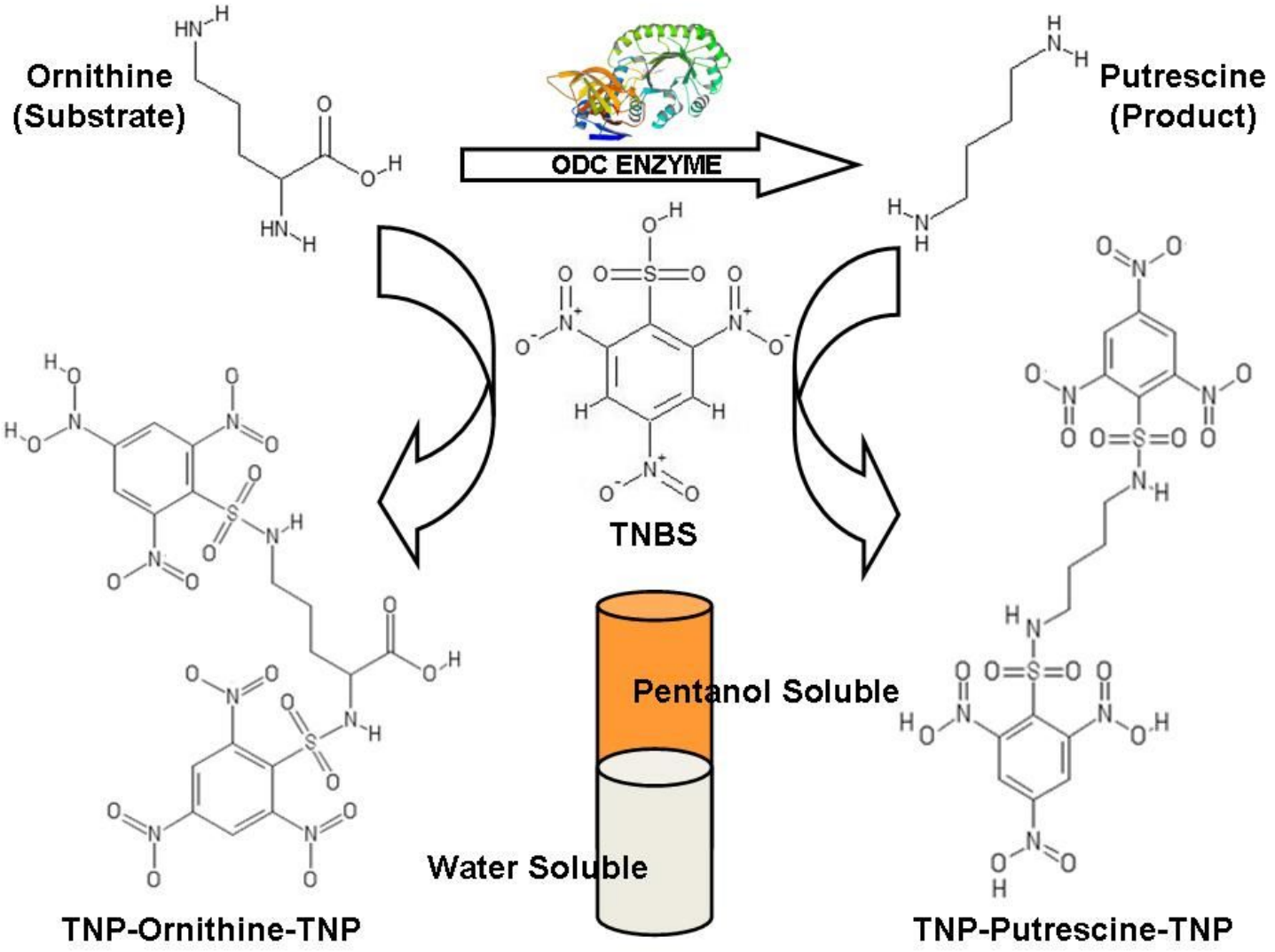


Figure 1

L-Ornithine HCl is converted to Putrescine by the enzyme ODC The adduct TNP-Putrescine-TNP and TNP-Ornithine-TNP are differentially extracted as hydrophobic and hydrophillic layer respectively i.e. TNP-Putrescine-TNP soluble in 1-Pentanol and insoluble in water and vice versa for the TNP-Ornithine-TNP. At alkaline pH, TNBS reacts with the amino group of the substrate and product formed which are having different solubility in 1-Pentanol and water.

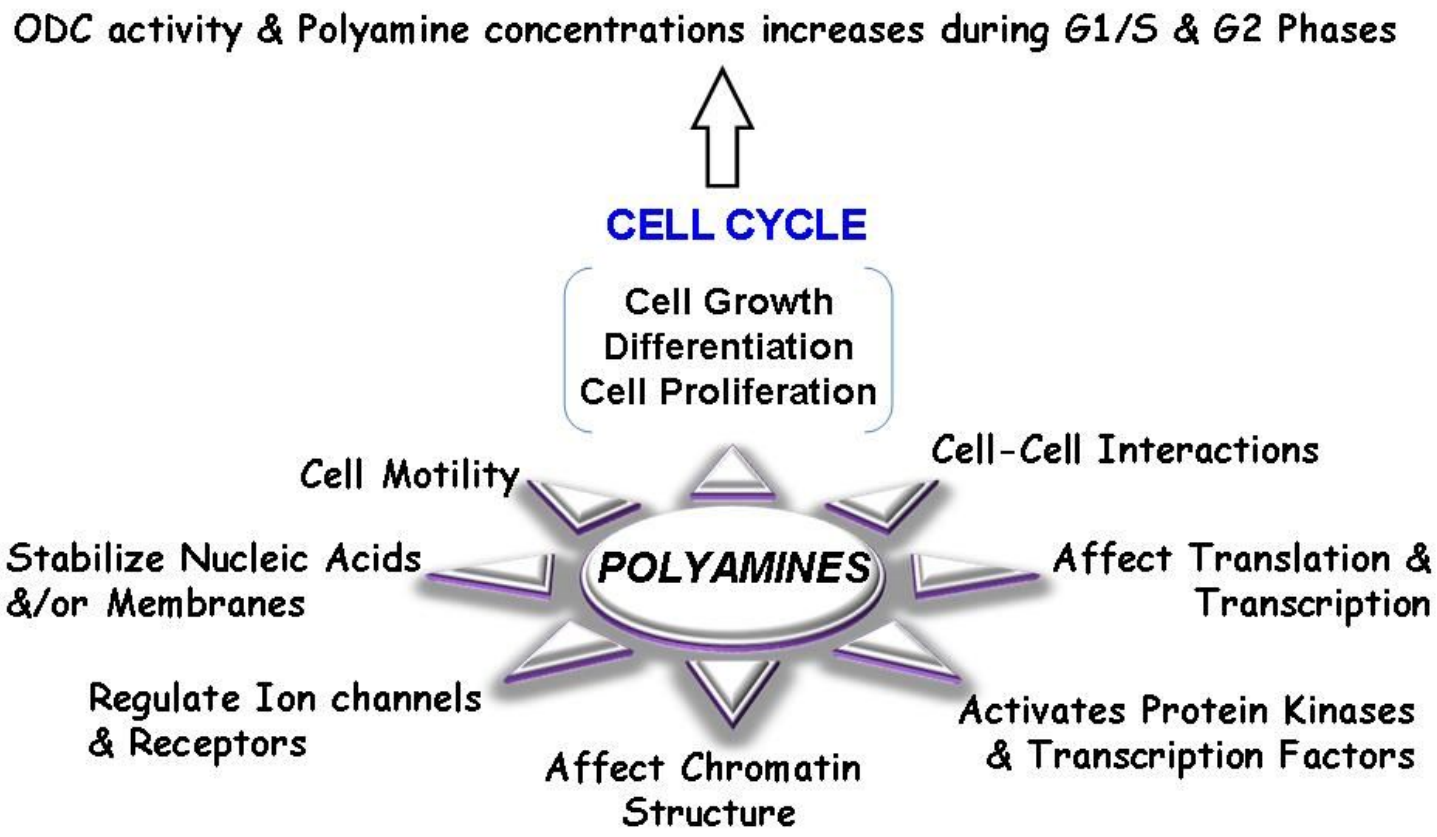


Figure 2

Polyamines and its vital role

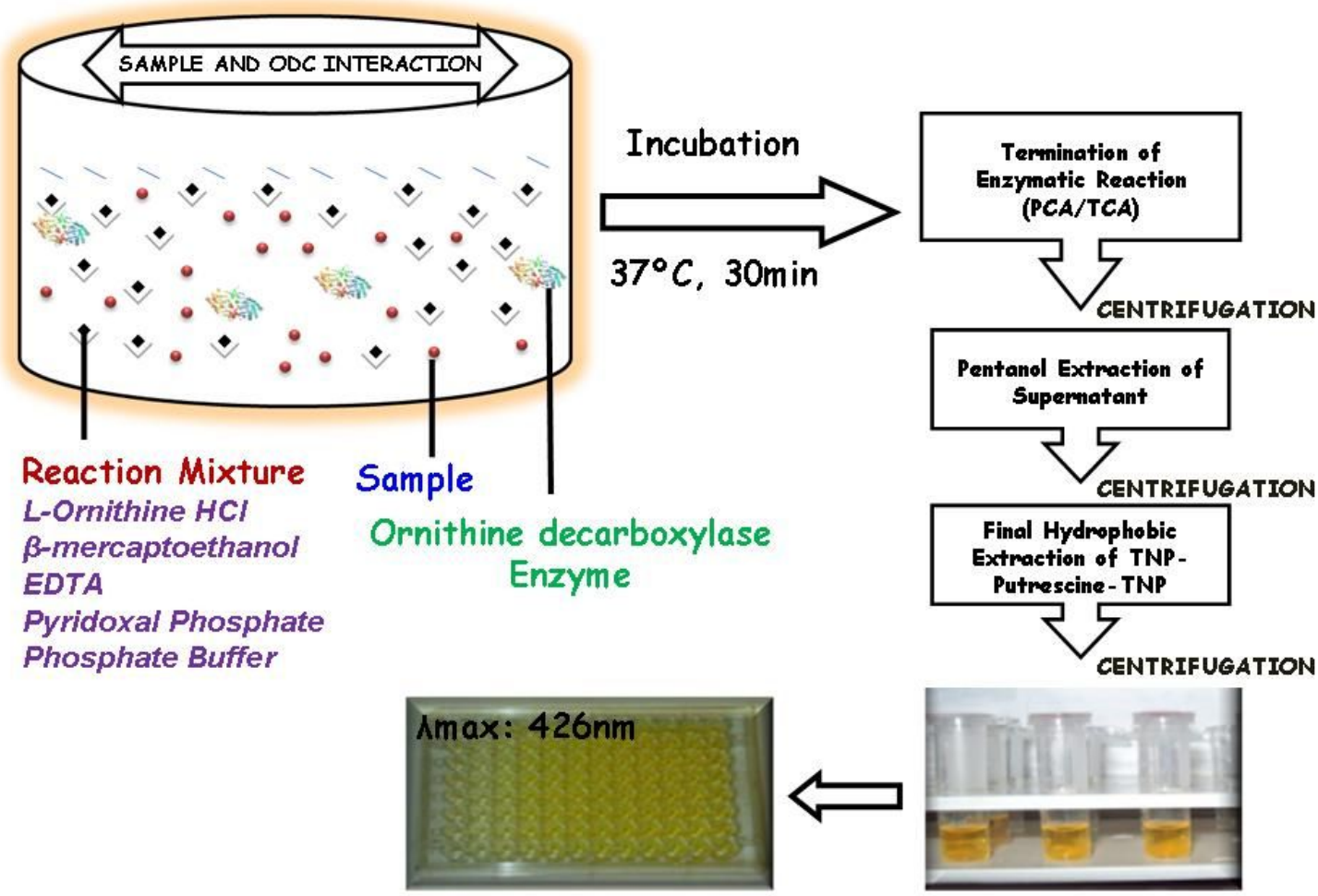


Figure 3

Diagrammatic representation of customized ODC assay

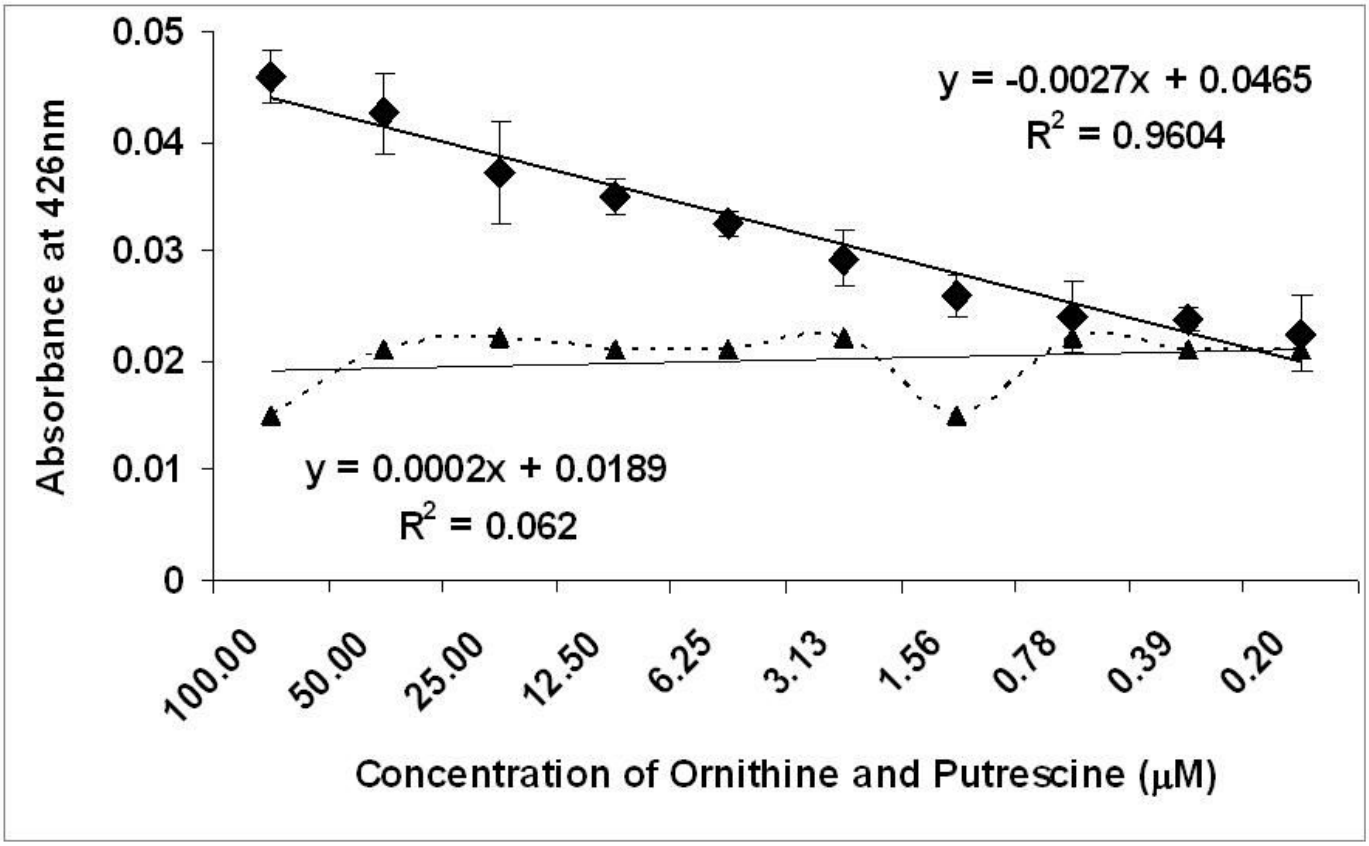


Figure 4

Standard plot of Putrescine and Ornithine

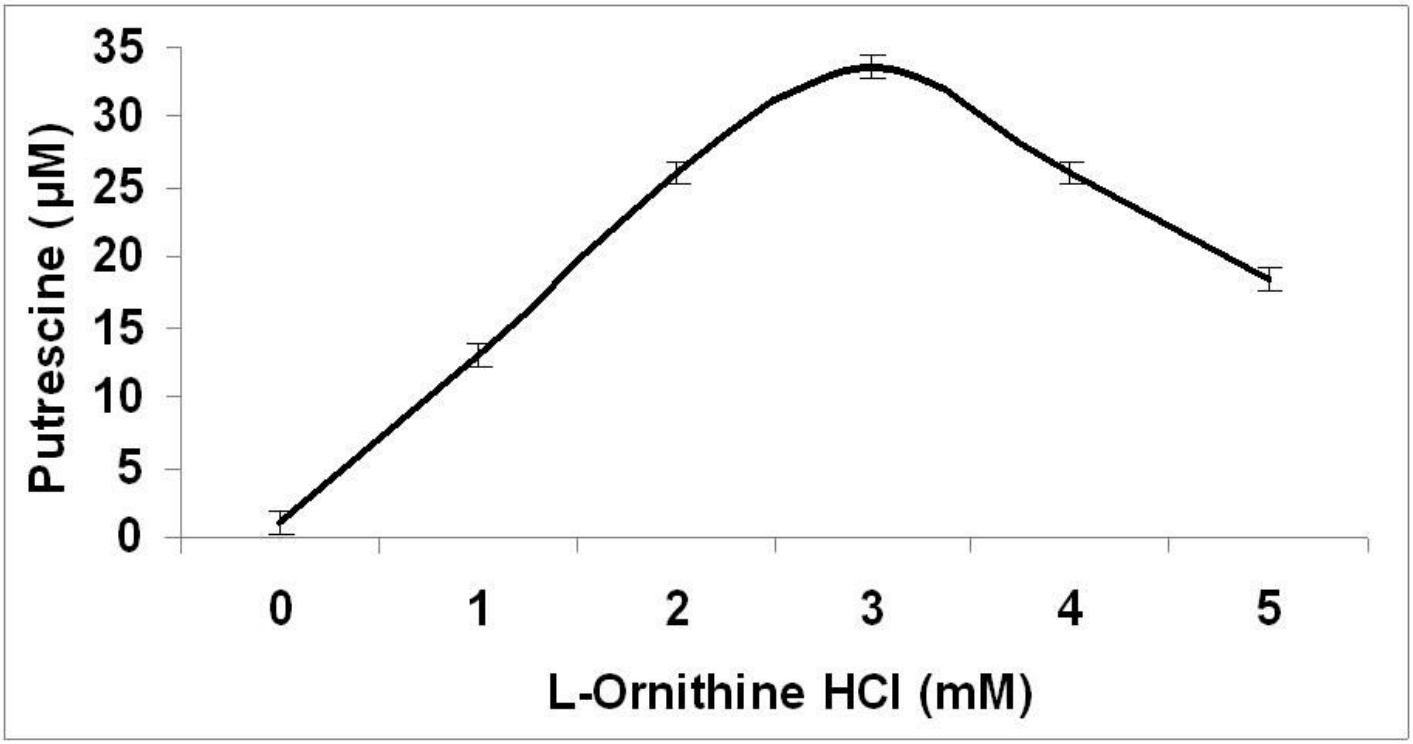


Figure 5

Substrate-responsive graph of Ornithine decarboxylase enzyme activity A gradual increase observed in the activity of the enzyme as the substrate concentration increases and the optimum activity is being found at 3mM L-Ornithine HCl. Further increase in the concentration of substrate results in ebbing of the enzyme action.

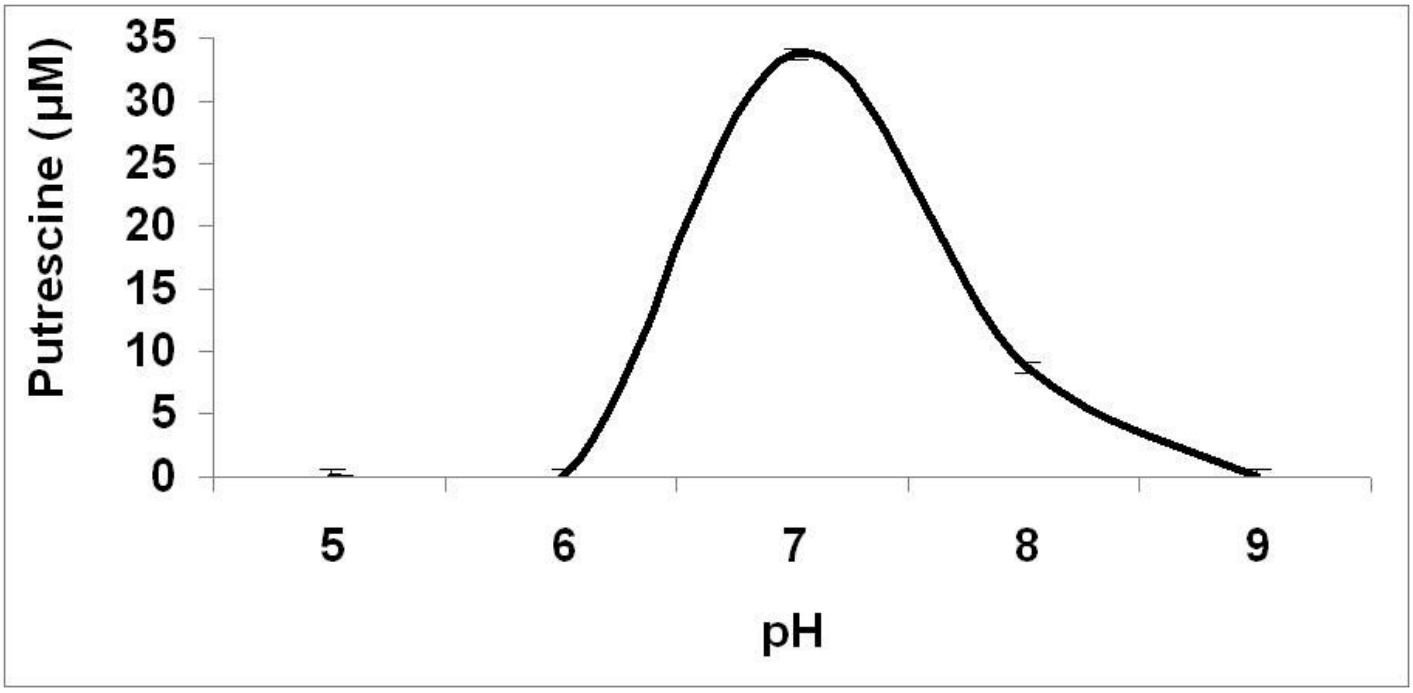


Figure 6

pH-dependent Ornithine decarboxylase activity Maximum activity is found at pH 7.0 while low and high pH values exaggerated the commotion of the assay.

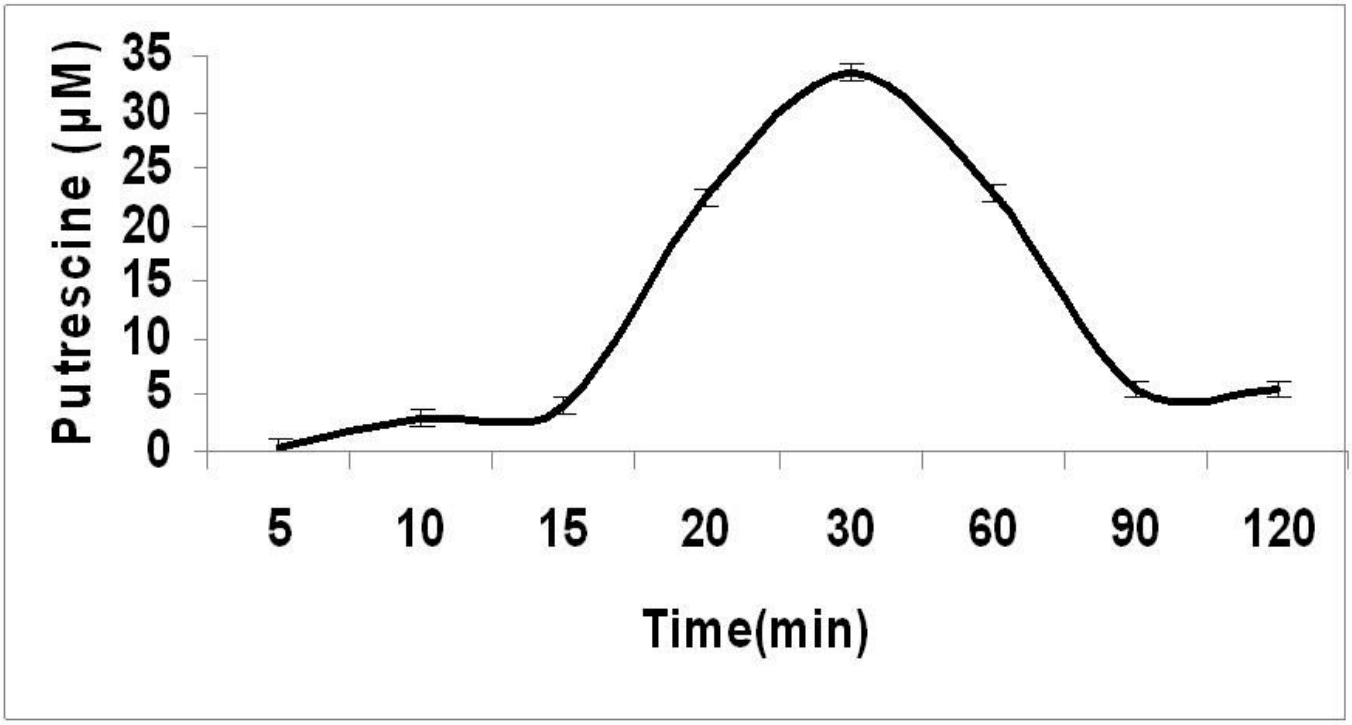


Figure 7

Time-dependent Ornithine decarboxylase enzyme activity The graph is plotted as a function of product formed in micromolar concentration. The maximum product formed is at 30 min incubation, further increase decreases the product formation which may perhaps be due to inhibition of the enzyme activity at higher concentration.

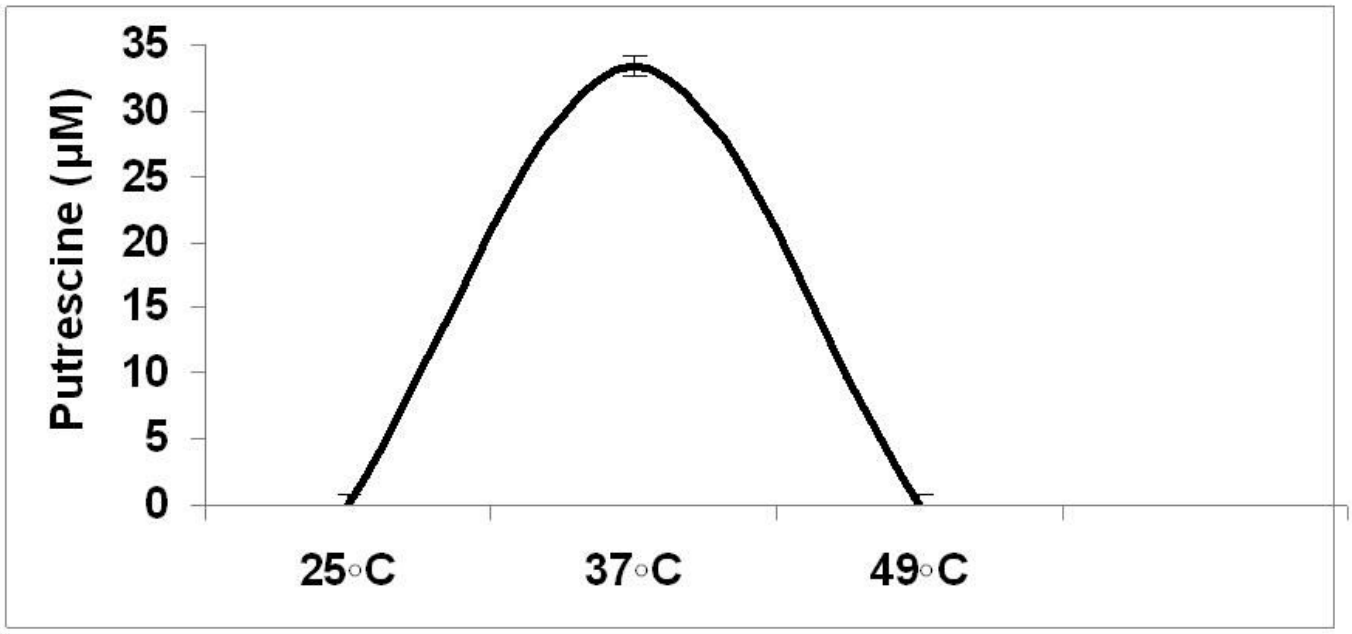


Figure 8

Temperature-dependent Ornithine decarboxylase enzyme activity Best optimum activity is seen at 37ºC while lower and higher temperature conceited the enzyme activity.

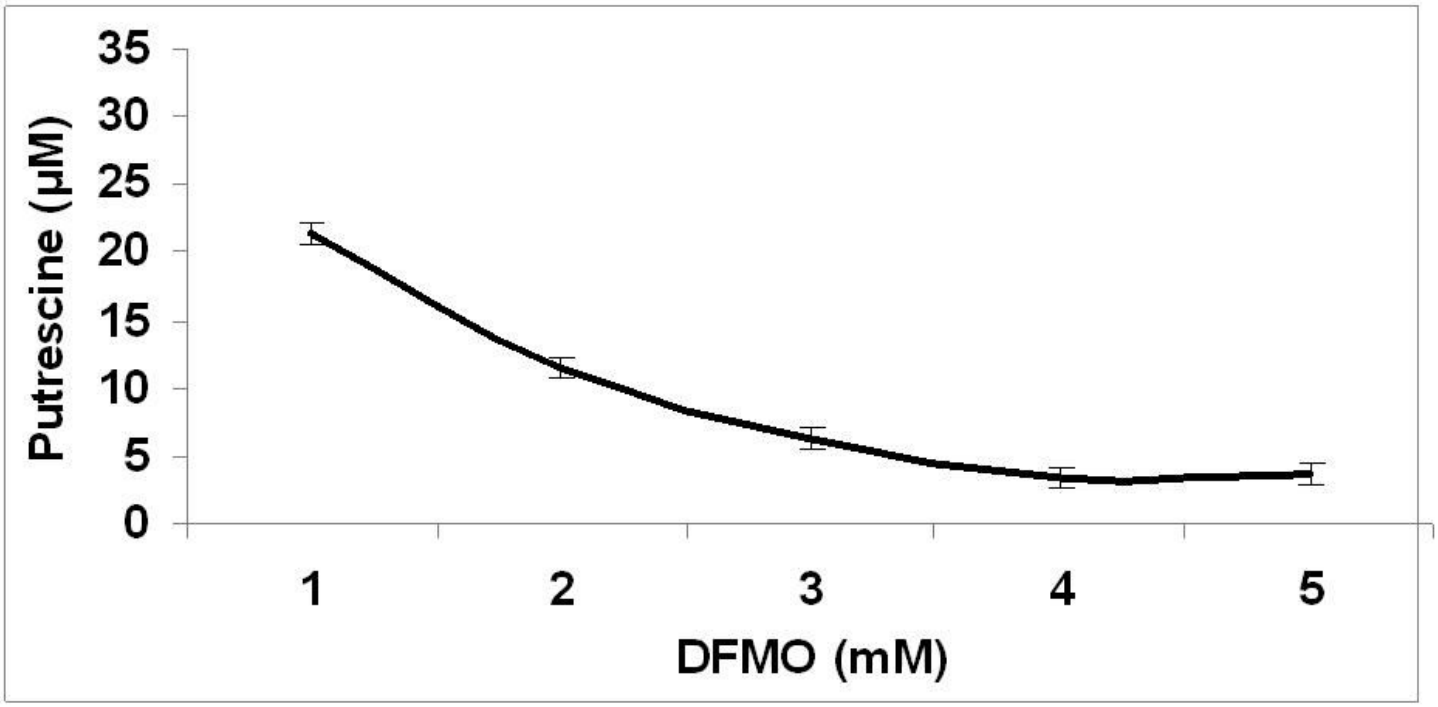


Figure 9

Concentration-dependent inhibition of Ornithine decarboxylase enzyme activity with DFMO Diminishing linear gradient is seen as we increase the concentration of DFMO, known inhibitor of ODC enzyme.