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Original Article

Screening and Production of Fungal Arginine Degrading Enzymes and Testing Their Effects in Auxotrophic Cancers

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ABSTRACT

The aim of the work: Finding and evaluating fungal arginine-degrading enzymes from various Egyptian soil habitats as anticancer mediators against auxotrophic cancer cells was the goal.

Methods: Fungal cultures that produced enzymes degrading arginine were subcultured on malt agar medium. The enzymes were then readily isolated after expression by salting out with ammonium sulfate and purified using anion exchange resin chromatography. Additionally, the evaluation of their auxotrophic anticancer properties in male rabbit animal models weighing close to 2 kg and several cancer cell lines. In this work, fungal arginine degrading enzymes were generated at pH 6.5, 25 °C, and incubation for three days using mineral arginine agar [MAM] selective medium [developed to select only fungi capable of using arginine as a single metabolic source of carbon and nitrogen].

Results: DNA blotting hybridization analyses identified Aspergillus as the primary fungal genus isolating these enzymes in the current investigation. Aspergillus fumigatus produced arginine decarboxylase and arginine deiminase, while Aspergillus niger was the producer of the L-arginase enzyme. They proved to be powerful anticancer drugs when used against these auxotrophic cancer cells. L-arginine amino acid is converted by arginine deiminase into Citrulline and ammonia. Ornithine and urea are produced from arginine by L-arginase. Extracellular proteins were identified as the enzymes degrading arginine. Based on spectrophotometric measurement of the Citrulline concentration and the ammonia concentration resulting from the hydrolysis of L-arginine by arginine deiminase, arginine deiminase synthesis and activity were examined. Using mass spectrometry and western blot-gel electrophoresis, the molecular masses of fungal L-arginase, arginine deiminase and arginine decarboxylase were determined to be 51 kDa, 53 kDa, and 37 kDa, respectively. L-arginase's Km and Vmax values were 8.63 mmol/l and 7.41 µmol/min, respectively.

Conclusion: The study presented a unique fungal source of arginine-degrading enzymes from different soil conditions as anticancer mediators.

Keywords: Fungal; Arginine; Enzymes; Screening; Anticancer.

INTRODUCTION

Auxotrophic cancers for L-arginine such as hepatic carcinoma and melanoma represent a major cause of death worldwide which necessitate exploring new biological sources of management such as mycotic enzymes due to availability of few effective anticancer drugs in this regard. L-arginine is essential for the growth of auxotrophic cancers of L-arginine due to their inability to synthesize it; thus, the deprivation of L-arginine from the external surrounding media is a strategic technique to overcome this type of overwhelming lethal cancers [1].

Mold and yeast are the two kinds of mycoses [2]. Mold forms mats [mycelium] form of long threads called hyphae [3]. While non-septum hyphae do not generate lateral walls, certain hyphae [septum hyphae] form lateral walls [4]. Instead of growing by dividing into new cells, hyphae elongate their terminals [5]. Hyphae that are not septate are coenocytic [6].

No fungus is an obligate anaerobe; all are obligatory aerobes, with a small number being facultative aerobes [7].

Several significant medicinal mushrooms exhibit temperature dimorphism [8]. They can be found as mold in the environment at room temperature and as yeast in human tissues at body temperature [9]. Normal cells can synthesize arginine, but auxotrophic tumors require it for proliferation [10].

Liver cancer, melanoma, and colon cancer cells are examples of arginine-auxotrophic cancer cells that die when extracellular arginine is depleted by arginine-degrading enzymes. When used against these auxotrophic cancer cells, they proved to be potent anticancer drugs [11]. These include the enzymes arginine decarboxylase, arginine deiminase, and L-arginase [12].

L-arginine is converted to urea and Ornithine by the enzyme L-arginase. L-arginine amino acids are converted to ammonia and Citrulline amino acids by arginine deiminase [13].

Many adverse effects, including hyper-sensitivity responses [anaphylactic reactions and medication neutralization] are caused by bacterial arginine degrading enzymes, which are also thermolabile [14].

Because the fungal enzymes and human arginine-degrading enzymes are produced from eukaryotic cells, they are comparable and do not cause the same adverse effects of bacterial enzymes mentioned above [15].

In the current study, different soil samples were collected from various locations in Egypt to determine the physiological and environmental factors that affect the growth of fungal-producing arginine-degrading enzymes as anticancer agents. Additionally, the production of enzymes was investigated and identified.

METHODS

Ethical statement: In the present study, all applicable national, international, and/or institutional guidelines for the attention and utilization of humans and animals were preceded. All processes carried out in the study including animals were authorized by the local authorities, the Ethical committee for animal handling at Cairo university [ECAHCU], at the Faculty of Pharmacy, Cairo University, Egypt in agreement with the recommendations of the weather-all report with approval number P187/2021. All efforts were performed to ablate the number of animals utilized and their suffering during the study.

Collection of the samples: A total of one hundred and fifty grassland soil samples were randomly selected from various governorates in Egypt at a depth 0-10 cm, including acidic soils and the areas next to wheat mills. The samples were then preserved in sterile polythene bags at four ^{o}C until needed. The study procedure sought to gather samples of grassland soil from various soil settings across Egypt and to extract the enzymes responsible for degrading arginine, resulting in fungal isolates on metallic arginine agar media [MAA]. Additionally, the physiologic and environmental variables influencing the growth of certain fungal isolates that produce arginine-degrading enzymes were determined. Additionally, the synthesis and activity of these enzymes were characterized by specific fungal isolates.

Material source: The Egyptian business Algomhuria Pharmaceutical Chemical business provided all of the chemical and biological materials. The business Accegen-biotic sold us the cell lines. The present investigation employed only analytical grade chemical reagents.

Table [1]: List of instruments

Instrument	Model and manufacturer
Autoclaves	Tomy, japan
Aerobic incubator	Sanyo, Japan
Digital balance	Mettler Toledo, Switzerland
Oven	Binder, Germany
Deep freezer -80 ℃	Artikel
Refrigerator 5	Whir-pool
PH meter electrode	Mettler-toledo, UK
Deep freezer -20 °C	whirlpool
Gyrator shaker	Corning gyratory shaker, Japan
190-1100 nm Ultraviolet-visible spectrophotometer	UV1600PC, China
Light[optical] microscope	Amscope 120X-1200X,China

Type of study: This study was a screening experimental study.

Place and date of the study: This study was done in the faculty of pharmacy, at Cairo University, Egypt between August and December 2021.

Screening of positive fungal arginine degrading enzymes producing isolates

Mineral arginine plate [g/L]: This medium was used to screen isolates produced by fungal arginine-degrading enzymes. KH2PO4 [1.2], MgSO4 [0.6], FeSO4 [0.005], KCL [0.4], D-glucose [7], Agar [13], L-arginine [15], and Thiamphenicol antibiotic [0.15] were its constituent parts. The addition of Thiamphenicol stopped the development of bacteria. The medium had a PH of 6.5, and it was incubated for three days at 25 °C. In this selective medium, only the colonies that were able to use L-arginine as a source of carbon and nitrogen were allowed to develop. For future research, the positive isolates were kept refrigerated at 4 °C.

Malt extract agar medium: It is an all-purpose, acidic growth medium for isolating and cultivating fungus. NaOH was used to bring the pH down to 6.5. After three days of incubation at 25 ℃, subcultures of the positive isolates [produced on MAM] were cultured and isolated on malt extract agar medium at temperature 20 °C, PH 6.5. After being dissolved in 10% KOH, the positive fungal isolates were examined under a standard light microscope. The molecular identification of positive fungus isolates using hybridization between DNA blotting probes came next.

Determination of fungal arginine degrading enzymes production and activity

Direct nesslerization test: It was applied to evaluate the activity and synthesis of enzymes from positive fungal isolates cultivated on MAA plates. The hydrolysis of L-arginine amino acid to Citrulline amino acid and ammonium occurred by arginine deiminase. The ammonium that was emitted was detected and measured using a spectrophotometer at a wavelength of 425 nm. The ammonium content was closely correlated with light intensity. The enzyme activity was closely correlated with the amount of ammonium released.

Salicylate method: This approach is a variant of the widely recognized phenate approach. It contains no phenol or mercury salts. Low-range ammonium nitrogen determination can benefit from it. The hydrolysis of L-arginine amino acid into Citrulline amino acid and ammonium is catalyzed by arginine deiminase. At a UV wave-length of 425 nm, the released ammonium was detected and analyzed using a spectrophotometer. The ammonium content was closely correlated with light intensity. The enzyme activity was closely correlated with the amount of ammonium released.

The photometric measurement of the Citrulline concentration: Arginine deiminase hydrolyzes arginine, which is the cause of L-citrulline, based on the modified Diacetyl-monoxime Thiosemicarbazide at 520 nm. The concentration of Citrulline was closely correlated with the light's intensity. The amount of the amino acid Citrulline that was released was closely correlated with the activity of the enzyme. Because the L-arginase enzyme hydrolyzes L-arginine, the urea content may be measured spectrophotometrically using the Diacetyl Monoxime technique at 478 nm wavelength. The concentration of the released area was closely correlated with the light's intensity. Following centrifugation, these extracellular enzymes can be extracted using ion exchange resin chromatography or by salting out the supernatant in the centrifuge tube with ammonium sulfate. The mass spectrometer and western blot-gel electrophoresis method were used to estimate the molecular weights of the enzymes that degrade arginine. Using Nessler's experiment, the kinetics [Vmax and Km] of the enzymes that degrade arginine were graphically determined.

I**n vitro cell viability assay using MTT assay:** The physiologic, pharmacologic, and toxicological effects of the enzyme on the hepatic cancer cells were evaluated using the JHH4

hepatic carcinoma cell line. The physiologic, pharmacologic, and toxicological effects of the enzyme on human colorectal cancer cells were evaluated using the LIM1215 cell line. The physiologic, pharmacologic, and toxicological effects of the enzyme on melanoma cancer cells were evaluated using the UPMM3 melanoma cancer cell line. To evaluate the physiologic, pharmacologic, and toxicological effects of the enzyme on lung cancer cells, the Calu-3 lung adenocarcinoma cell line was employed. The physiologic, pharmacologic, and toxicological effects of the enzyme on the heart cancer cells were evaluated using the HL1 cardiac cancer cell line.

The Vero cell line was employed to evaluate the enzyme's pharmacologic, toxicological, and physiologic effects on normal mammalian cells. The in vitro cell viability test of arginine degrading enzymes was conducted using the MTT [dimethylthiazol-2-yl] diphenyl tetrazolium] technique. In order to assess and gauge the vitality of the cells, 5000 cells per well were seeded into 96-well microplate wells using a 10- to 50-µl cell solution. Following a 24-hour incubation period at 37 °C in a CO2 incubator, the plates were subjected to varying doses of the enzymes that degraded arginine. Each well-received 10 µl of 10% MTT [3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide] for the cell viability experiment, which was then incubated for three hours. After adding 100µl of dimethyl sulfoxide [DMSO] to each well, the optical absorbance was measured using a BioTek ELIZA reader at 570 nm. Rather than using the control [samples not subjected to arginine degrading enzyme treatment], the cell viability was determined as a percentage of the number of healthy cells in the samples. The concentration at which the growth of the cells is 50% inhibited was used to get the IC 50 values.

BrdU incorporation assay: An ELISA kit [50-Bromo-20-deoxyuridine [BrdU]] was used for colorimetric analysis of genomic DNA synthesis and cell proliferation rates [Roche, Germany]. Various cell lines were plated in a 96-well culture plate at a density of 5000 cells per well, with the appropriate concentration of generated arginine degrading enzymes, for 48 hours. The cells were then treated for 8 hours at 37 °C with the BrdU labelling solution. After that, the FixDenat solution was used to denature the DNA in the fixed cells. After fixed cells were exposed to the substrate tetramethylbenzidine, they were treated with anti-BrdU antibody coupled with peroxidase. Finally, a microplate reader examined the plates at 370 nm. The percentage of viable cells divided by the total amount of DNA synthesis yielded the amount of DNA synthesis per cell.

Caspase-3 activity assay: The Caspase enzyme, which is essential for cell death, was found using a caspase-3 test kit. The colorimetric caspase-3 activity test was performed in compliance with the guidelines provided by the manufacturer, Sigma-Aldrich Chemie Gmbh. After treating cell lines with arginine-degrading enzymes, 10 micrograms of the supernatant were incubated in a 96-well plate for four hours at 37 °C with 85 microliters of assay buffer and 10 microliters of caspase-3 substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide [Ac-DVD-pNA]. The quantities of p-ni-troanilide [p-NA] were used to measure fold changes in caspase-3 activity. Caspase 3 and their enzymatic activity resulted in the production of this molecule, as determined by the absorbance values of p-NA at 405 nm.

The activity of these enzymes in acidic, neutral, and alkaline PH media is then assayed, along with the impact of cobalt and manganese ions as cofactors and the ideal pH and temperature for their activity. Next figuring out the ideal temperature for this enzyme's functioning.

The design of a new primer for expression of fungal L-arginase enzyme

Forward primer for expression: The direction of a sequence was from 5^{-} to 3^{-} *GGGTTGACTGACTGGAGAGC*. T.annealing= Tm-5=60.04-5=55.04 °C

Reverse primer for expression: The direction of the sequence was from 5^{-} to 3^{-} *TGCATTTCCTGGCCAGATGTA*. T.annealing =Tm-5=59.72-5=54.72 °C.

The two restriction enzymes that were used and chosen according to the web cutter website were Hind III and EcoRI.

The design of novel primer for the recombinant expression of arginine deiminase from the potent ADI producing fungal isolate

Forward primer for expression: The direction of a sequence was from *5^{-} to 3^{-}* *GGCAAAGTGAAGCCAACCAG*. T.annealing= Tm-5=59.97-5=54.97 °C.

Reverse primer for expression: The direction of a sequence was from *5- to 3- TGGGGCCCTCGAGTTACATA*. T.annealing= Tm-5=60.03-5=55.03°C.

The two restriction enzymes that were used and chosen according to the web cutter website were Bam HI and Sphl.

Production of arginine degrading enzymes by fungal recombinant DNA technology: The use of *Saccharomyces cerevisiae BJ1824* as an expression host in the recombinant DNA technique to synthesis arginine degrading enzymes. Six times histidine made up the C-terminal, methanol served as the inducer, promoter AUG1, and the expression system vector was PYES2-DEST52. The arginine degrading enzymes' genes [cDNA] were cloned using PCR and subsequently sub cloned into PYES2-DEST52 utilizing restriction endonuclease II [Bam HI and Sph1 for arginine deiminase, but Hind III and EcoRI for the L-arginase gene] to aid in plasmid digestion and ligation by ligase enzyme. After being identified and propagated in *E. coli Top 10*, the recombinant plasmid was converted into *Saccharomyces cerevisiae BJ1824.* In order to produce arginine-degrading enzymes using galactose as an inducer, yeast transformants were grown at 30 degrees Celsius in YNBG selective medium [0.67% yeast nitrogen base without amino acids supplemented with appropriate nutrients and 2% galactose], which was then maintained in YPG-rich media [2% bacteriopeptone, 1% yeast extract, and 2% galactose].

Clarification and the purification of recombinant arginine degrading enzymes: The enzymes of interest were extracellular proteins that were isolated from the centrifuge tube supernatant by precipitation using 70% ammonium sulphate [every 100 ml of supernatant, 52 ml of 4.1 M saturated solutions of ammonium sulphate was added], centrifugation for three minutes at 4000 rpm, and ion exchange resin chromatography purification.

Formulation of fungal arginine degrading enzymes: The goal of the current study was to find the best way to provide fungal arginine-degrading enzymes as an anticancer treatment against auxotrophic tumors by experimenting with various pharmaceutical dose forms and administration routes. The preparation of injectable products was done in the presence of isotonic aqueous solutions, which have a pH of 7.4, which is similar to that of blood and bodily tissues. The antibacterial drug Thiamphenicol was added to the injections that were ready in containers. Through intra-muscular injection of aqueous solutions containing carboxymethylcellulose [CMC], the release of arginine-degrading enzymes was regulated by changing the viscosity of the vehicle. In order to extend the duration of effect to a once-daily dosage administration rather than multiple-day injections, ethylene glycol was added afterwards. Through the use of the wet granulation process, tablets containing micro-particles of arginine degrading enzymes 10 mcg/g were created. An excipient of 3% magnesium aluminum silicate was introduced. In addition to being a disintegrant, it was a glidant. 17% w/w starch used as diluent. The lubricating substance applied was 1% w/w magnesium stearate. When arginine-degrading enzyme medications were combined with excipients in various injectable or oral formulations, FTIR and DSC analyses were conducted to look for drug-drug interactions. Auxotrophic anticancer medicines, such as fungal arginine degrading enzyme medications, were tested on rabbit animal models that had previously been engineered to develop lung, heart, lung, and hepatic carcinoma. The study examined the anticancer effects of the enzyme test medications in comparison to two control groups: one that got a placebo and the other that received conventional doxorubicin, an anticancer treatment. There were one hundred bunny animal models in each group. The rabbits were males, around two kilograms in weight. The MTT test was used to measure the cytotoxic activity.

Experimental animals: The Nephrology and Urology Centre at Cairo University in Egypt provided 100 male rabbit animal models, each weighing around 2 kg. These animals were used to screen and evaluate the cytotoxic effects of arginine-degrading enzyme medicines that were refined from fungal isolate producing arginine degrading enzymes. The rabbits were kept in a conditioned environment with regular light and dark cycles lasting 12 hours with 55 ± 5% relative humidity and 25 ± 1 °C. They also had unrestricted access to normal laboratory food and water. Every intervention was carried out in accordance with the rules and ethical guidelines for laboratory animals that were approved by Cairo University's Faculty of Pharmacy Ethical Committee, Egypt.

Induction of tumerogenicity in mice models of xenografts: Below conditions devoid of pathogens, 100 male mice aged 6 to 8 weeks, weighing 180-190 grams, were kept for establishment counselling. Mice flanks were subcutaneously implanted with a 1.0 × 105 aliquot of cancer cell lines [hepatic, lung, colorectal, heart, and melanoma]

suspended in 225 µl of PBS containing 31% Growth Factor Ablated Matrigel [sourced from Dickinson of Becton, USA] in order to establish tumors for xenografts. Two weeks later, the mice were randomly divided into three groups, each consisting of five mice. Every four days, mice were administered 100 µl of PBS intraperitoneally along with either arginine degrading enzymes [5 U/animal model] or placebo [PBS] in addition to the usual anticancer medication doxorubicin [10 U/mice]. The tumor's volume [V] mm3 was measured every four days using the formula V = d2× D× 0.3, where d stood for longitudinal diameter and D for latitudinal diameter. After the mice were slaughtered 28 days later, the tumors were removed and weighed.

Assessment of IgG antibodies to fungal arginine degrading enzyme drugs: Detection of IgG antibodies was performed through ELISA.

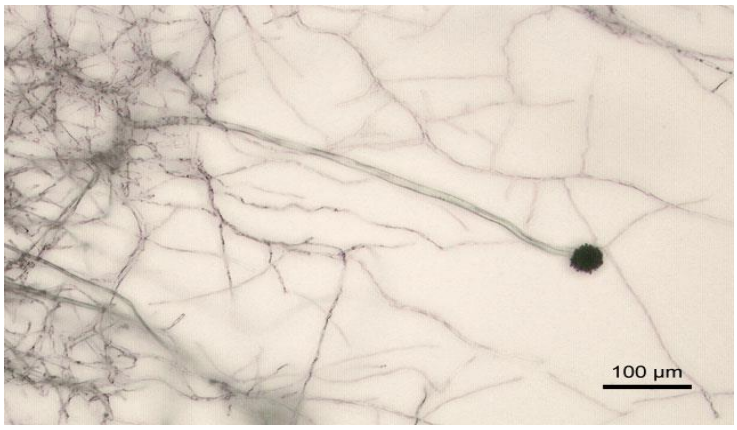

Figure [1]: Molds of *Aspergillus niger spp* with septate hyphae producing arginine degrading enzymes as anticancer agents

Figure [2]: *Aspergillus niger* growing on MAM

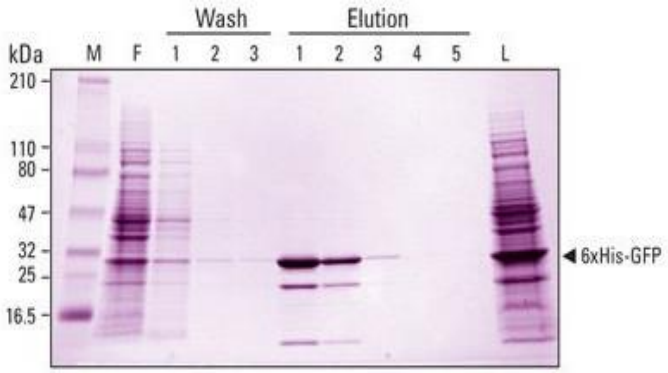

Figure [3]: Recombinant proteins of arginine degrading enzymes measured via the western blot technique. The maximum yield of recombinant proteins was 65 mg/l. The purity of recombinant Arginine degrading enzymes was approximately 86%

Statistical analysis: All cultures were conducted in triplets. Their presentation was by means and standard deviation. One-way analysis of variance [p value ≤ 0.05] was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software. F test was used during the present study.

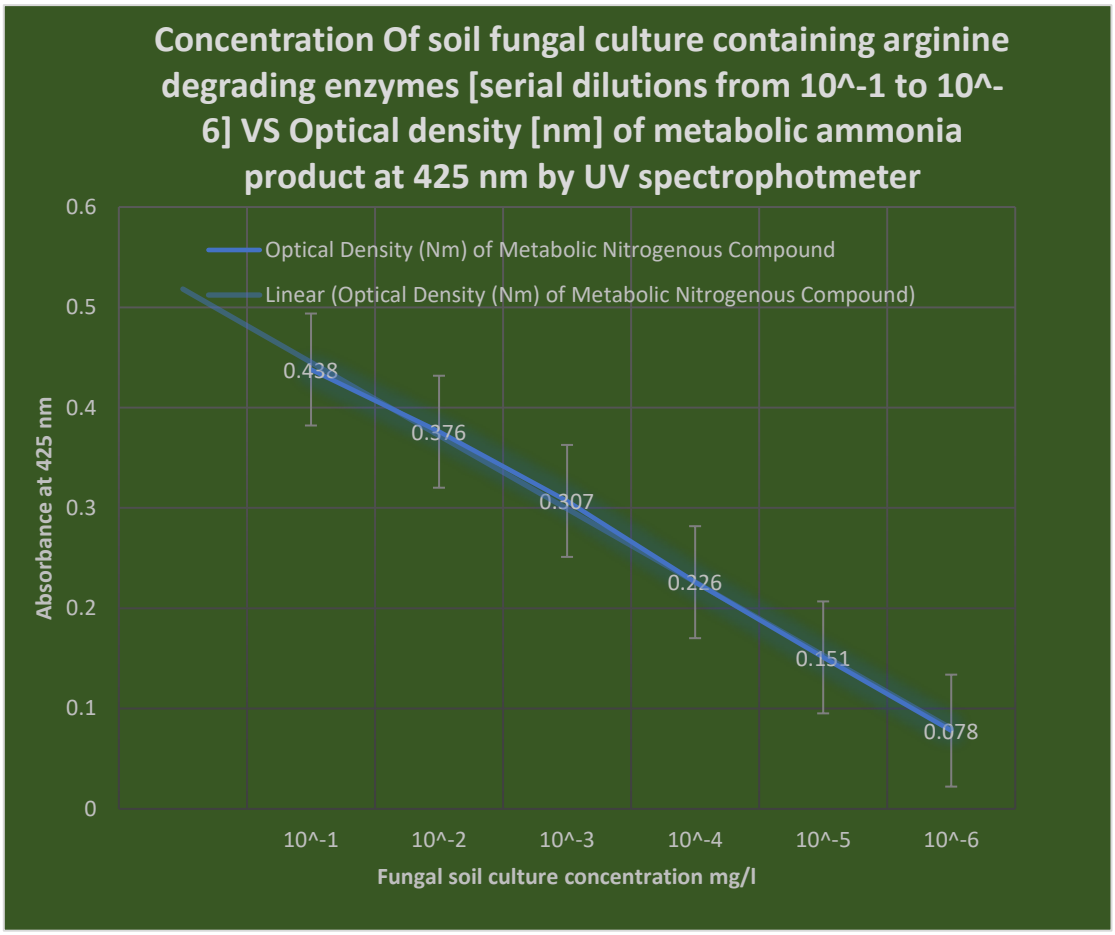

Figure [4]: A Nesslerization screening assay of different concentrations of soil fungal arginine degrading enzymes

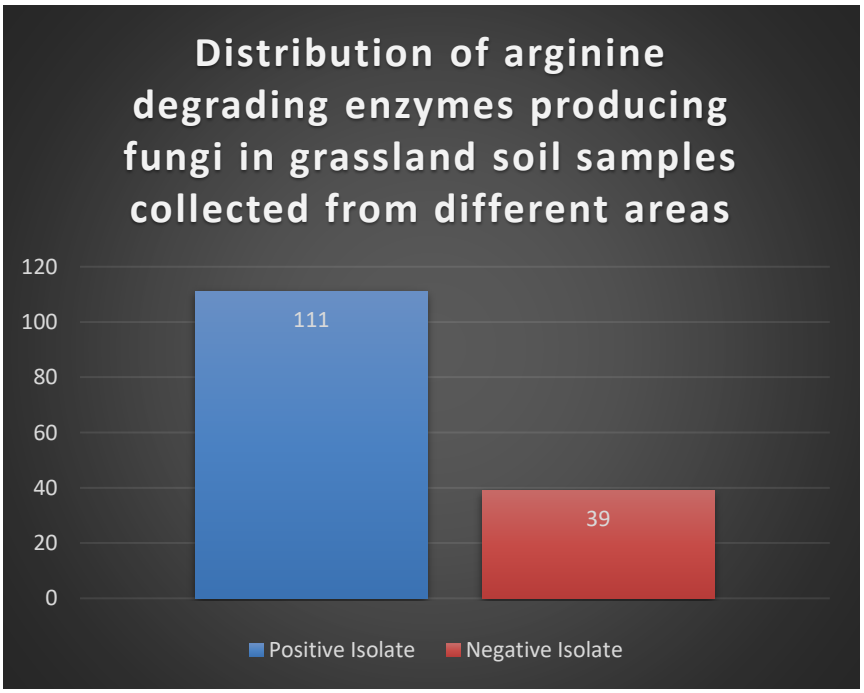

Figure [5]: A distribution of arginine degrading enzymes producing fungi

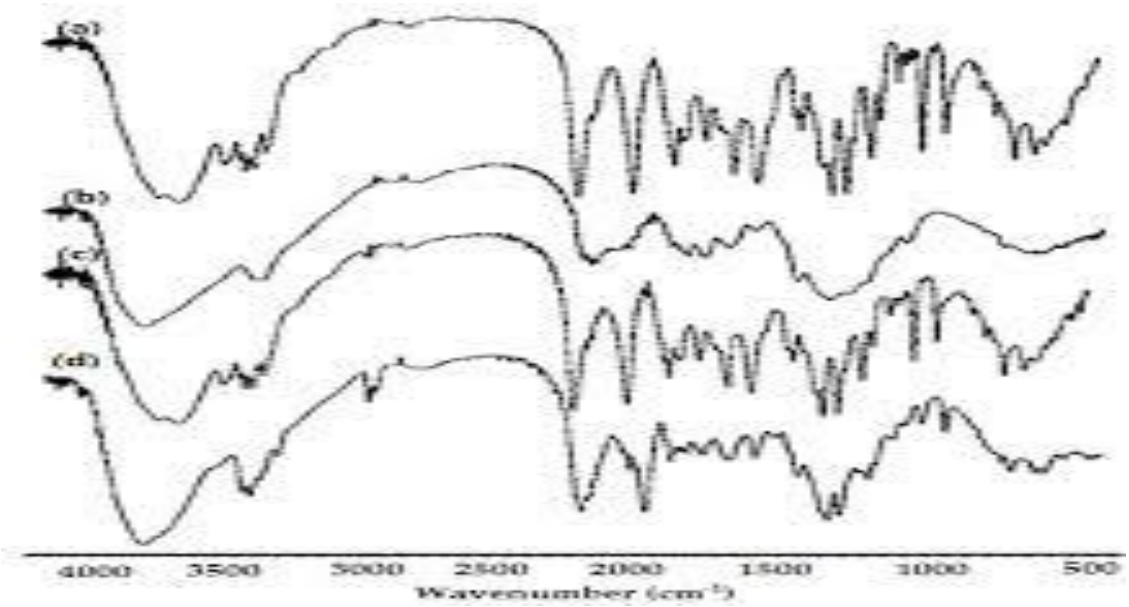

Figure [6]: FTIR spectroscopy shows no drug-drug interaction or incompatibility between arginine degrading enzymes and excipients

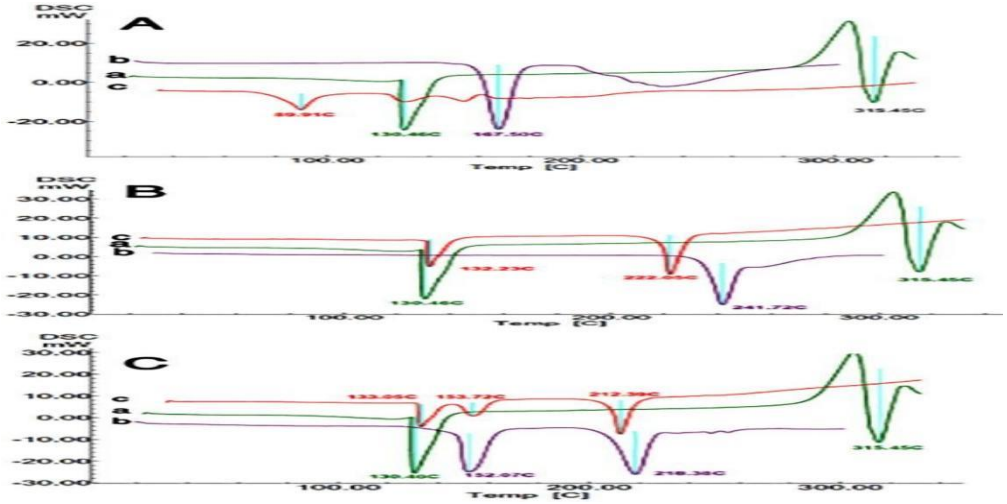

Figure [7]: DSC study shows no drug-drug interaction or incompatibility between arginine degrading enzymes and excipients

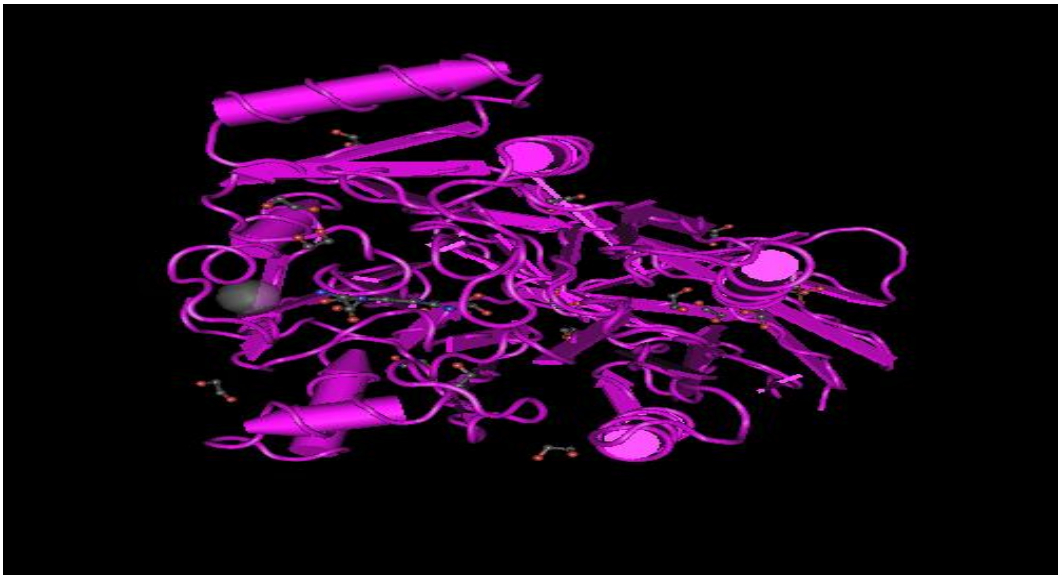

Figure [8]: 3D structure of Arginine deiminase. It is composed of 418 amino acids

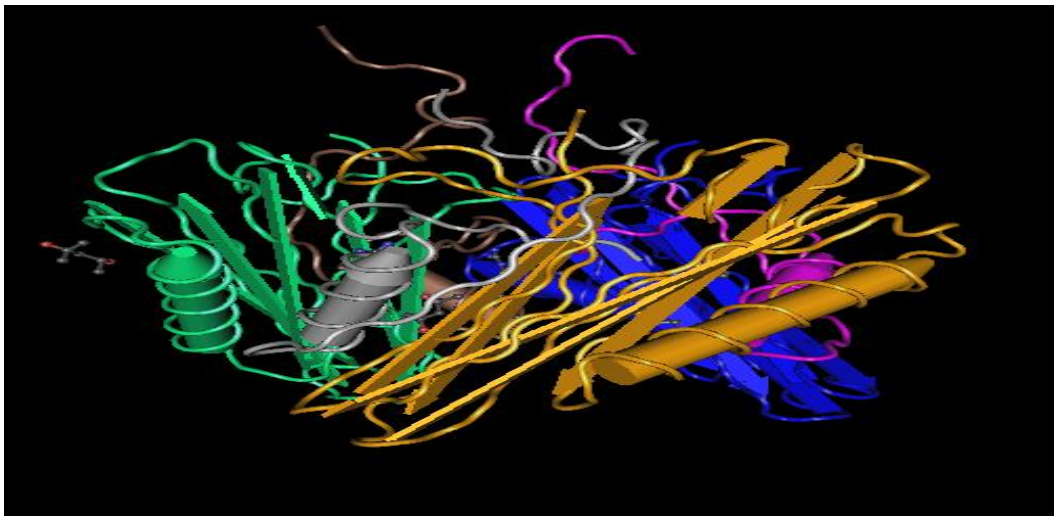

Figure [9]: 3D structure of Arginine decarboxylase. It is composed of 670 amino acids

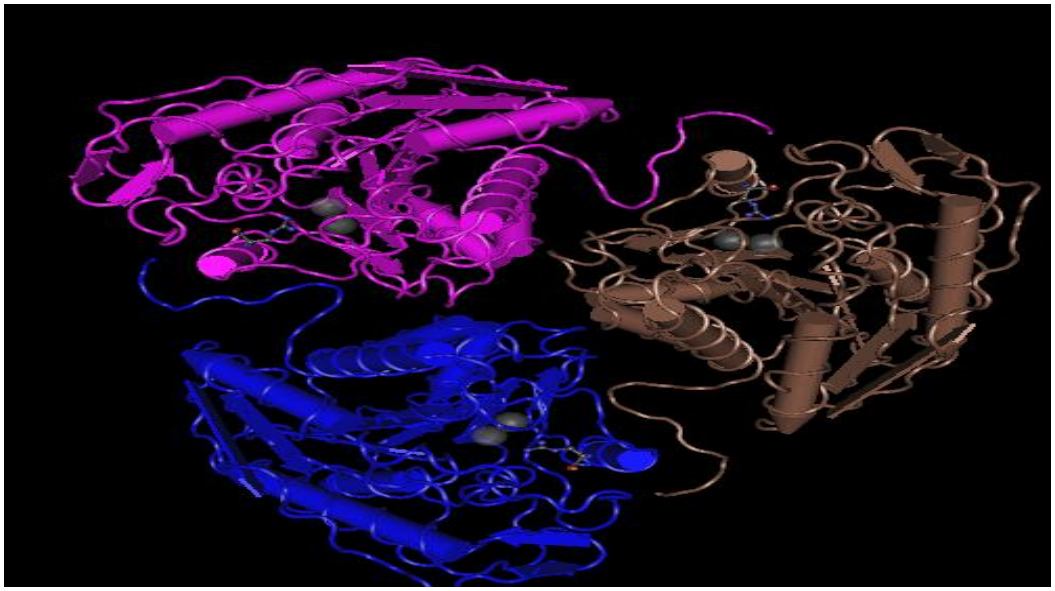

Figure [10]: 3D structure of L-arginase enzyme. L-arginase comprises 323 aminoacids structure

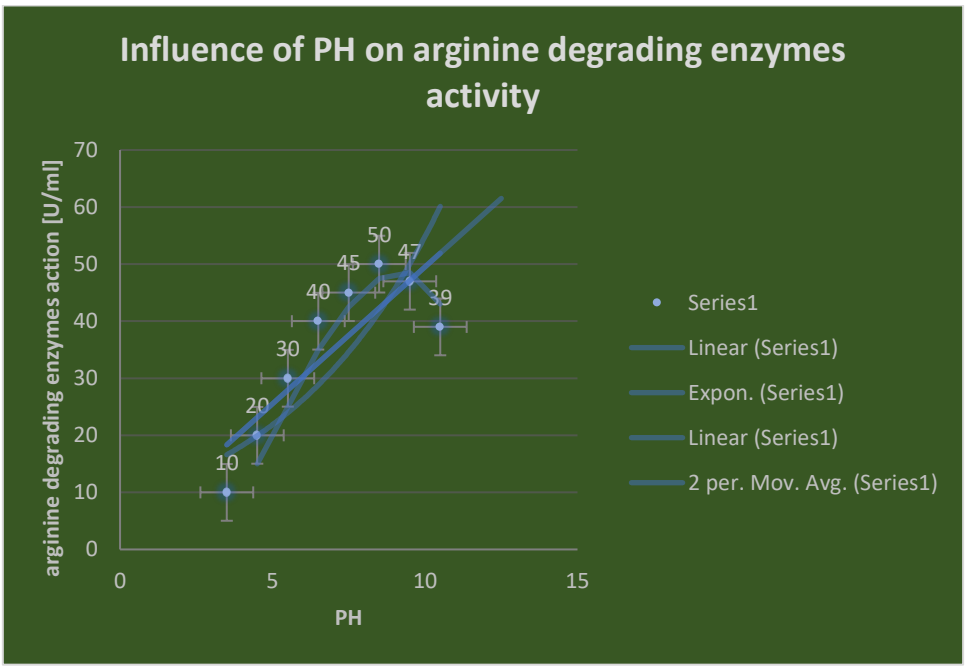

Figure [11]: The action of fungal arginine degrading enzymes as a function of the pH of the reaction

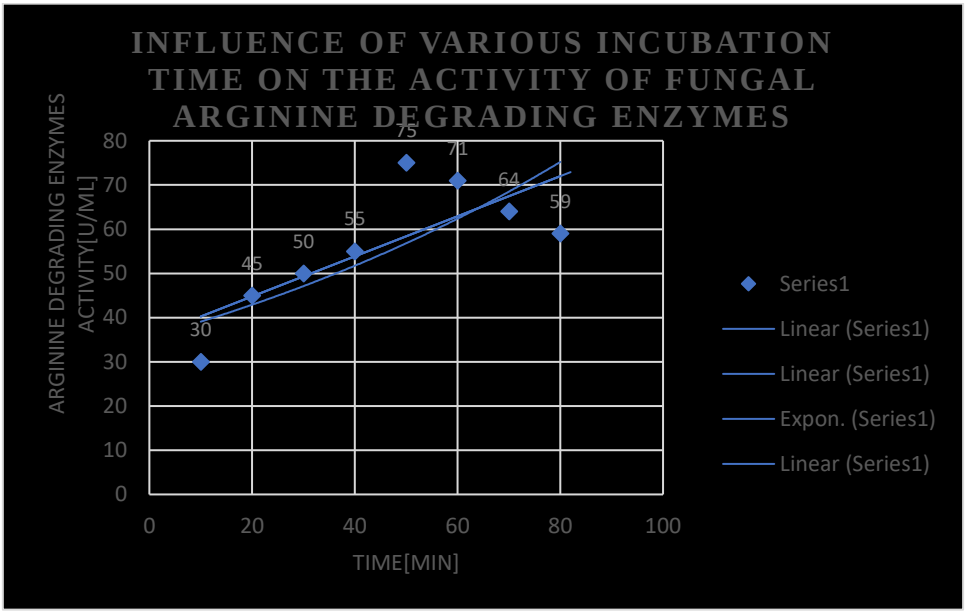

Figure [12]: The influence of various incubation times on arginine degrading enzymes activity

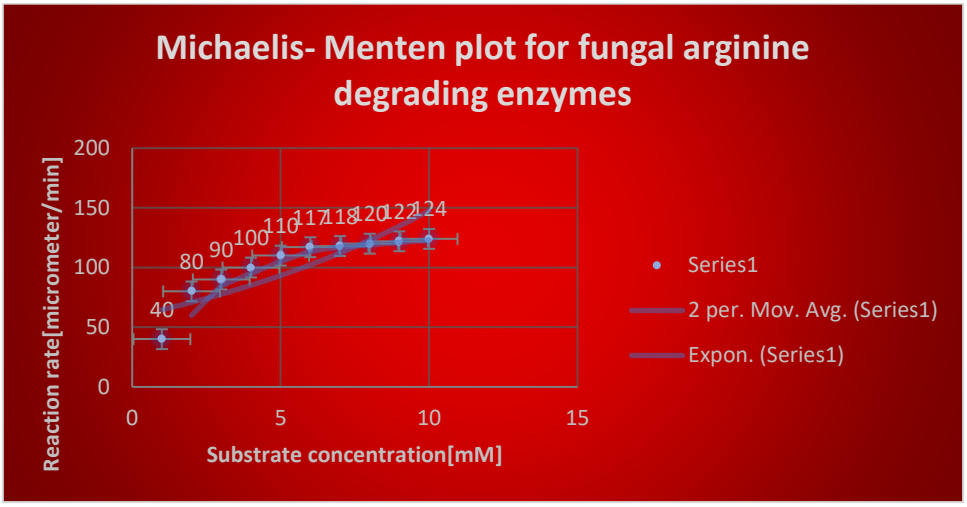

Figure [13]: The Michaelis-Menten plot for arginine degrading enzymes

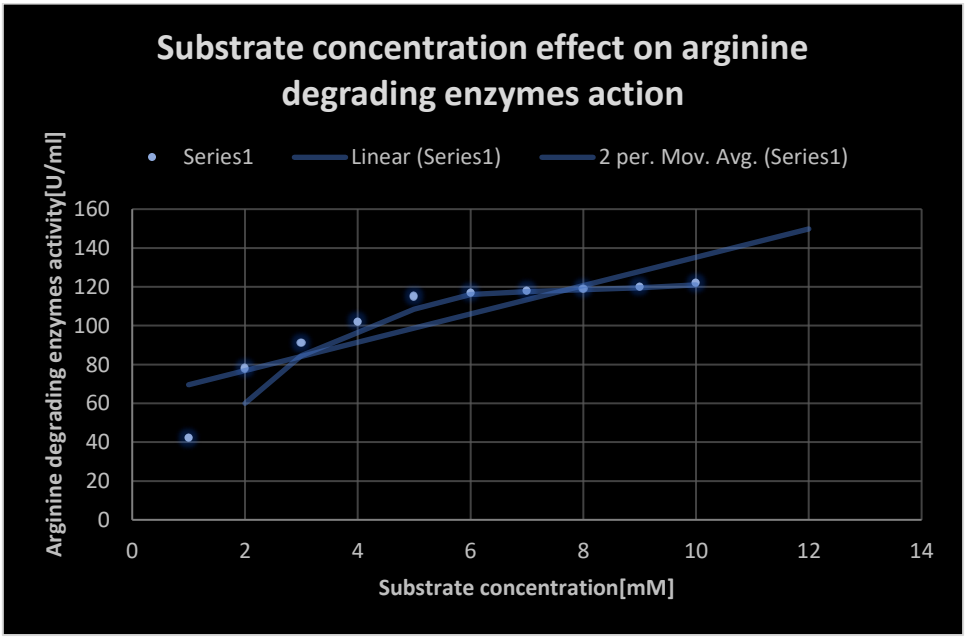

Figure [14]: The Influence of substrate concentration on the enzyme activity of arginine degrading enzymes

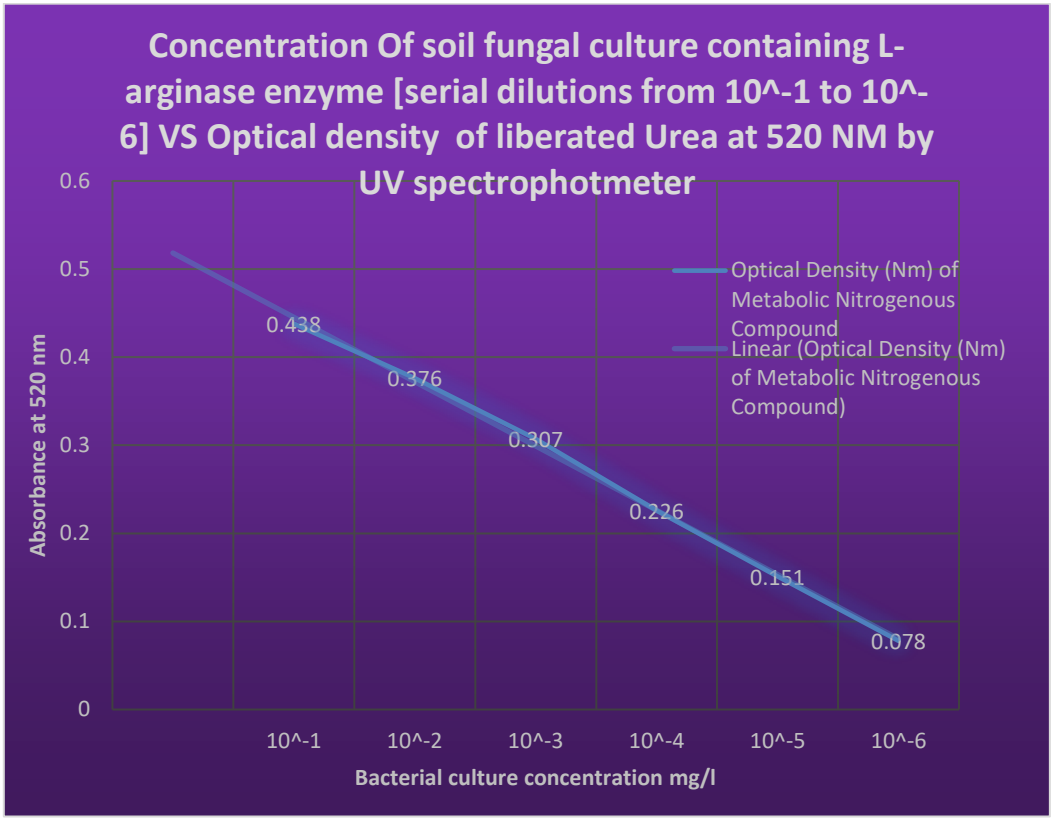

Figure [15]: Concentration of soil fungal culture containing L-arginase enzyme [serial dilutions from 10^{-1} to 10^{-6}] VS Optical density of liberated Urea at 520 NM by UV spectrophotometer

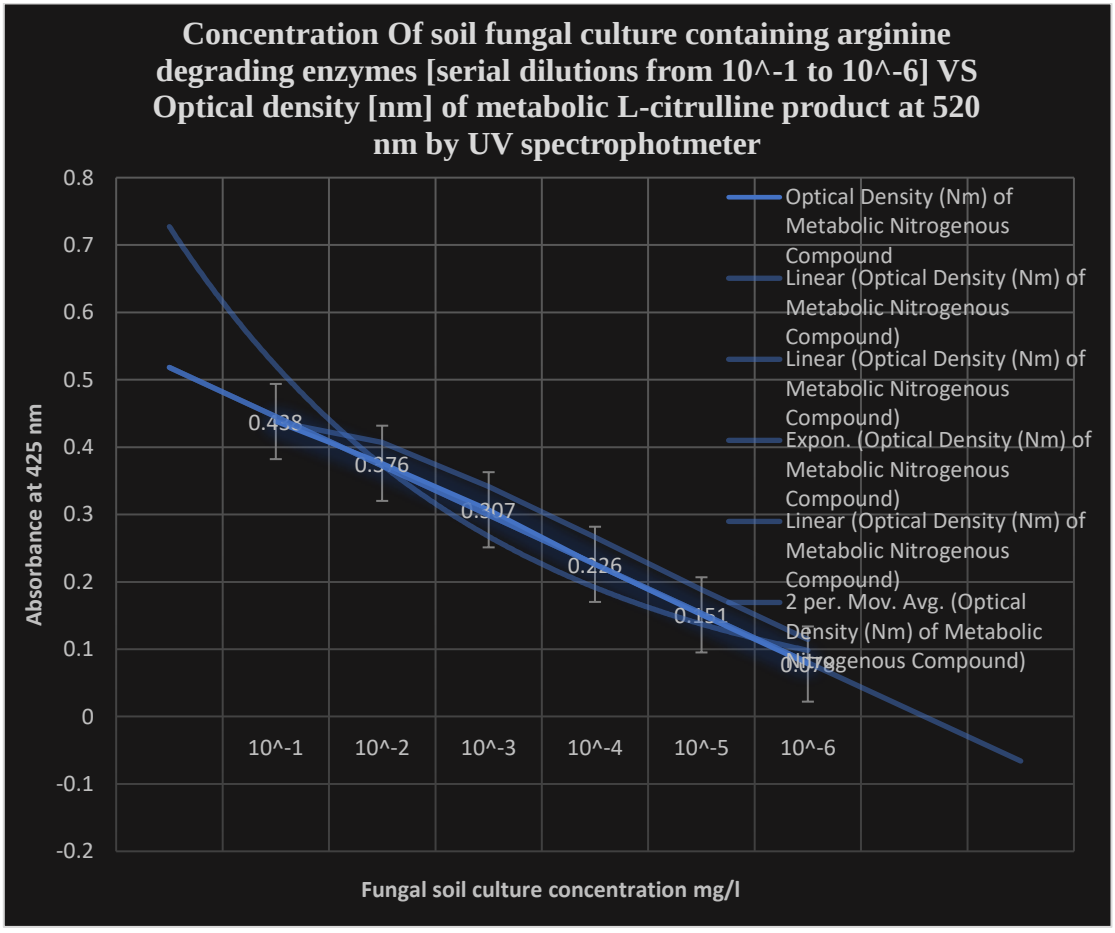

Figure [16]: Modified Diacetylmonoxime thiosemicarbazide screening assay of different concentrations of soil fungal arginine degrading enzymes

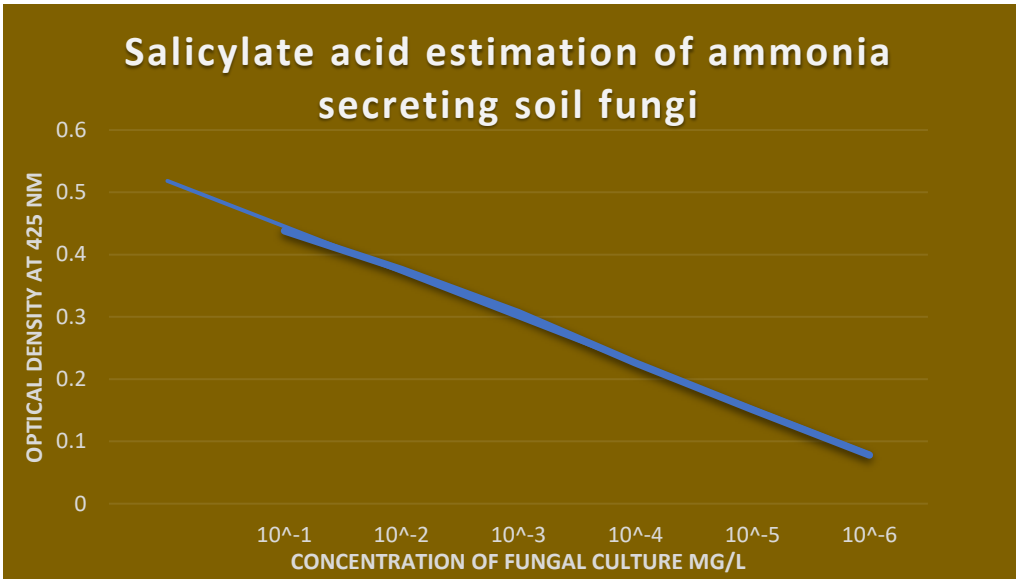

Figure [17]: Liberated ammonia at 425 nm by different Concentrations Of soil fungal culture containing arginine degrading enzymes

RESULTS

The study analyzed 150 soil samples gathered from various locations in Egypt and found that the primary source of arginine-degrading enzymes was the fungus *Aspergillus species*, which was cultivated on malt extract agar and MAA plates. It was identified by light microscopy as mold with septate hyphae. On MAA plates with malt extract agar, molds with green spores and conidia in radiating channels were cultivated. By using DNA probe hybridization as a molecular approach, this was verified. It was discovered that Aspergillus *fumigatus* generated arginine decarboxylase and arginine deiminase, two anticancer drugs, whereas Aspergillus niger produced the enzyme L-arginase. L-arginine amino acid was hydrolyzed into Ornithine amino acid and urea by L-arginase; while it was converted into ammonia and L-citrulline using arginine deiminase. The urea content was measured spectrophotometrically using the Diacetyl Monoxime technique at 478 nm wavelength, which was the consequence of the L-arginase enzyme hydrolyzing L-arginine. The concentration of the released urea was closely correlated with the light's intensity. The transformation of arginine into Citrulline and ammonia was catalyzed by arginine deiminase. Direct nesslerization and Salicylate assays were used to measure the ammonia content. The enzyme activity was closely correlated with the amount of ammonium released. Additionally, the modified Diacetylmonoxime thiosemicarbazide was used to detect the concentration of arginine deiminase using photometric measurement of the Citrulline concentration, which was the product of arginine hydrolyzed by arginine deiminase. The concentration of Citrulline was closely correlated with the light's intensity. The amount of the amino acid citrulline that was released was closely correlated with the activity of the enzyme. Using a mass spectrometer and the western blot-gel electrophoresis method, the molecular masses of fungal L-arginase, arginine deiminase, and arginine decarboxylase were discovered to be 51 kDa, 53 kDa, and 37 kDa, respectively. L-arginase's Km and Vmax values were 8.63 mmol/l and 7.41 µmol/min, respectively. The ideal pH and temperature for the synthesis of enzymes were 6.5 and 25 °C, respectively. The enzyme synthesis was activated by KH2PO4 [1.2 g], MgSO4 [0.6 g], FeSO4 [0.005 g], and KCL [0.4 g]. Using five different cancer cell lines, the pure fungal arginine degrading enzymes created by recombinant DNA technology were evaluated for their potential as in vitro anticancer agents using the MTT test. The tested human cancer cell lines [JHH4 hepatic carcinoma cell line, LIM1215 human colorectal cancer cell line, UPMM3 melanoma cancer cell line, Calu-3 lung adeno-carcinoma cell line, HL1 cardiac cancer cell line] and the normal Vero cell line were all subjected to varying degrees of inhibitory activity by fungal arginine degrading enzymes. The IC50 values for arginine degrading enzymes were found on several cancer cell lines, including the JHH4 hepatic carcinoma cell line, the LIM1215 human colorectal cancer cell line, the lung adenocarcinoma cell line [Calu-3], the cardiac cancer line [HL1], and the UPMM3 melanoma cancer cell line. The ICs50 values for fungal arginine deiminase enzyme were 19.1, 15.6, 17.3, 4.51, and 4.83 U/ml, respectively, and the IC50 values for fungal L-arginase enzyme were

21.4, 17.8, 14.5, 5.1, 4.91 respectively. Standard doxorubicin inhibitory concentration [IC50] against the hepatic cancer cell line [JHH4] was 5.2 U/ml; against the colorectal cancer cell line [IM1215], 4.19 U/ml; against the melanoma cancer cell line [UPMM3] was 3.88 U/ml; against the lung adenocarcinoma cell line [Calu-3] was 4.30 U/ml; and against the cardiac cancer cell line [HL1] was 5.03 U/ml.

After a full day of incubation, cancer cell lines treated with 10 U/ml of arginine-degrading enzymes showed reduced cell viability. While inhibiting the cell viability of the normal Vero cell line by 51.4%, the following values of arginine deiminase were found: 78.9% [JHH4], 74.3% [LIM1215], 67.2% [UPMM3], 70.3% [Calu-3], and 68.3% [HL1]. While inhibiting the cell viability of the normal Vero cell line by 50.7%, L-arginase was found in 79.4% [JHH4], 75.2% [LIM1215], 69.1% [UPMM3], 71.5% [Calu-3], and 66.7% [HL1] of the samples. Injections subcutaneous or intramuscular were used to provide arginine-degrading enzymes. These require administration often daily due to their short half-lives [3–4 hours]. The present investigation suggests that using 70% poly-ethylene glycol might prolong the duration of action and overcome this difficulty. Fungal arginine degrading enzymes were shown to have selectivity indices against cancer cell types. In terms of arginine deiminase, the values were 3.82 for the hepatic carcinoma cell line [JHH4], 3.35 for the colorectal cancer cell line [IM1215], 3.59 for the melanoma cancer cell line [UPMM3], 3.16 for the lung adenocarcinoma cancer cell line [Calu-3], and 3.71 for the cardiac cancer cell line [HL1]. In terms of L-arginase, the results were 3.94 for the hepatic carcinoma cell line [JHH4], 3.23 for the colorectal cancer cell line [IM1215], 3.67 for the melanoma cancer cell line [UPMM3], 3.19 for the lung adenocarcinoma cancer cell line [Calu-3], and 3.86 for the cardiac cancer cell line [HL1]. Utilizing a colorimetric BrdU proliferation test, it was investigated if arginine degrading enzyme therapy had an inhibitory effect on DNA synthesis in auxotrophic cancer cell lines. After treating auxotrophic cancer cells with arginine degrading enzymes for 48 hours, a dose-dependent decrease in their proliferation was seen. Enzymes that degrade arginine were shown to reduce cell proliferation at 0.3, 0.5, 0.8, 0.4, and 0.9 IU/ml, respectively, for JHH4, 11 and 54% for IM1215, 41 and 89% for UPMM3, 23 and 67% for Calu-3, and 29 and 76% for HL1.

The arginine degrading enzymes treated JHH4, IM1215, UPMM3, Calu-3, and HL1 cell lines showed a dose-dependent increase in the Caspase-3 apoptosis index, according to the Caspase-3 activity assay. The findings confirmed that the arginine-degrading enzymes generated cause apoptotic cell death by raising caspase-3's enzymatic activity. In JHH4, IM1215, UPMM3, Calu-3, and HL1 cells, respectively, arginine degrading enzymes at 0.5 IU/ml concentration increased caspase-3 activity to 14, 13, 15, 18, 11, and 19%. Additionally, this induction was raised to 23, 19, 31, 28, and 35% in JHH4, IM1215, UPMM3, Calu-3, and HL1 cells, respectively, at 0.9 IU/ml concentration. In rabbit models induced with liver carcinoma, melanoma, colorectal cancer, lung cancer, and heart cancer treated with test enzymes, tumor growth inhibitions were determined to be 79%, 82%, 64%, 75%, and 56%, respectively; in contrast, tumor growth inhibitions were 51%, 63%, 57%, 61%, and 71%, respectively, in rabbits challenged with a standard anticancer drug [doxorubicin]. In animal models of rabbits used as negative controls, no growth of inhibition was observed. When IgG antibodies were evaluated using ELISA against medications that degrade fungal arginine, no detectable IgG antibodies were found.

Table [2]: An estimation of L-arginase activity by the direct Diacetyl monoxime method and computation of fungal arginine degrading action via the direct Nesslerization technique

Concentration Of soil fungal culture containing L-arginase enzyme [serial dilutions from 10*-1 to 10*-6 mg/l]	The optical density of liberated metabolic nitrogenous compound [liberated urea] at 478 nm by UV spectrophotometer	The absorbance of liberated ammonia at 425 nm by UV spectrophotometer
0.00	0.00	0.00
1/10	0.438	0.410
1/100	0.376	0.382
1/1000	0.307	0.341
1/10000	0.226	0.283
1/100000	0.151	0.177
1/1000000	0.078	0.076

Table [3]: A computation of fungal arginine deiminase action via the modified acetyl monoxime thiosemicarbazide technique and computation of fungal arginine degrading enzymes action via the Salicylate technique

Concentration Of soil fungal culture containing arginine degrading enzyme [serial dilutions from 10^{-1} to 10^{-6} mg/l]	The absorbance of liberated L-citrulline at 520 nm by UV spectrophotometer	The absorbance of liberated ammonia at 425 nm by UV spectrophotometer
0.00	0.00	0.00
1/10	0.384	0.421
1/100	0.367	0.386
1/1000	0.299	0.372
1/10000	0.261	0.297
1/100000	0.249	0.188
1/1000000	0.015	0.082

Table [4]: The action of fungal arginine degrading enzymes as a function of the reaction pH and various incubation times

PH	Arginine degrading enzymes action	Incubation time [min]	Arginine degrading enzymes action[U/ml]
3.5	10	10	30
4.5	20	20	45
5.5	30	30	50
6.5	40	40	55
7.5	45	50	75
8.5	50	60	71
9.5	47	70	64
10.5	39	80	59

Table [5]: The Michaelis-Menten plot for arginine degrading enzymes

Substrate concentration[mM]	Rate of reaction[µM/min]
1	40
2	80
3	90
4	100
5	110
6	117
7	118
8	120
9	122
10	124

Table [6]: The Influence of substrate concentration and temperature on the enzyme activity of arginine degrading enzymes

Substrate concentration[mM]	Arginine degrading enzymes activity[U/ml]	Temperature ˚C	Arginine degrading enzymes activity[U/ml]
1	42	20	70
2	78	25	75
3	91	30	89.5
4	102	35	90
5	115	40	89
6	117	45	80
7	118	50	79
8	119	55	70
9	120	60	64
10	122	65	60

DISCUSSION

In addition to characterizing recombinant forms of fungal arginine degrading enzymes made using fungal recombinant DNA technology, the current study sought to screen for and identify physiological and environmental factors influencing the growth of fungal arginine degrading enzymes in various soil environments in Egypt. The impact of temperature on the activity of arginine-degrading enzymes is displayed in Table 6. The Michaelis-Menten plot for the enzymes that degrade arginine is displayed in Table 5. Fungal arginine-degrading enzyme activity was displayed in Figure 11 about the reaction's pH. The influence of substrate concentration on the arginine-degrading enzymes' activity is displayed in Table 6. An assessment of L-arginase activity using the direct Diacetyl monoxime approach is displayed in Table 2. A calculation of the fungal arginine degrading activity using the direct Nesslerization approach is displayed in Table 2. The effect of different incubation durations on the activity of arginine-degrading enzymes is displayed in Table 4. A calculation of the fungal arginine deiminase activity using the modified acetyl monoxime thiosemicarbazide method was displayed in Table 3. Fungal arginine-degrading enzyme activity was plotted against reaction pH in Table 5. A calculation of the fungal arginine-degrading enzymes' activity using the Salicylate approach is displayed in Table 3. Figure 17 shows how varied concentrations of soil fungal culture containing arginine-degrading enzymes released ammonia at 425 nm. A modified Diacetyl-monoxime thiosemicarbazide screening test of various soil fungus arginine-degrading enzyme concentrations was shown in Figure 16. The effect of different incubation durations on the activity of arginine-degrading enzymes is displayed in Figure 12. The concentration of a soil fungal culture containing L-arginase enzyme [serial dilutions from 10^{-1} to 10^{-6}] was shown in Figure 15, along with the UV spectrophotometer's optical density of released urea at 520 NM. The influence of substrate concentration on the enzyme activity of arginine-degrading enzymes was demonstrated in Figure 14. The Michaelis-Menten plot for enzymes that degrade arginine is shown in Figure 13. Aspergillus niger species molds with septate hyphae were depicted in Figure 1 as generating arginine-degrading enzymes as anticancer agents. Figure 10 shows the L-arginase enzyme's three-dimensional structure. The structure of L-arginase consists of 323 amino acids. Arginine deiminase's three-dimensional structure was shown in Figure 8. There are 418 amino acids in it. The 3D structure of arginine decarboxylase was shown in Figure 9. There are 670 amino acids in it. The location of fungi that produce enzymes that degrade arginine was shown in Figure 5. Figure 7 of the DSC analysis indicates that there are no drug-drug interactions or excipient incompatibilities between arginine degrading enzymes. No drug-drug interaction or incompatibility between arginine degrading enzymes and excipients is evident in Figure 6, which is alluded to in FTIR spectroscopy. A Nesslerization screening experiment with varying quantities of soil fungus arginine-degrading enzymes was shown in Figure 4. Aspergillus niger was shown growing on MAA plates in Figure 2. The western blot approach was used to quantify the recombinant proteins of arginine degrading enzymes, as shown in Figure 3. Recombinant protein yields reached a high of 65 mg/l.

The recombinant enzymes that degraded arginine had a purity of about 86%. When administered intravenously, medications that break down arginine have a relatively brief biological half-life of one hour. The short half-life of the injectable enzyme dosage form, administered intramuscularly or subcutaneously without the use of polyethylene glycol, was three hours. With the addition of polyethylene glycol, the duration of action was increased to eighteen hours. Because of their chemical instability in an acidic environment and their breakdown by proteolytic enzymes in the gastrointestinal tract, oral administration devices have demonstrated low effectiveness. After precipitating with ammonium sulphate, arginine-degrading enzymes were generated as extracellular proteins and isolated from the centrifuge tube supernatant using ion exchange resin chromatography.

When used as an anticancer mediator against auxotrophic tumors, recombinant arginine-degrading enzyme medicines show great yield, productivity, stability, specificity, and few side effects. Arginine degrading enzymes were found to have higher selectivity towards auxotrophic cancers for L-arginine, such as hepatic, lung, colorectal, melanoma, and heart cancers, when compared to earlier studies and standard doxorubicin as an anticancer agent against auxotrophic cancers for arginine. The disadvantages of the bacterial arginine-degrading enzymes could be resolved by the fungal enzymes. Compared to bacterial arginine degrading enzymes, they exhibited greater thermostability and fewer hypersensitivity responses [drug neutralization

and anaphylactic reactions]. The capacity to store fungal arginine enzymes at ambient temperature outside of the refrigerator for extended periods was proved by their great thermostability. It was observed that the resemblance between fungal and human enzymes—both are derived from eukaryotic cells, whereas bacterial enzymes are derived from prokaryotic cells—was the source of the superiority of fungal arginine-degrading enzymes over bacterial enzymes. Post-transcriptional and posttranslational alterations seen in eukaryotic cells, such as splicing and protein folding, are absent in prokaryotic cells. The in vitro cell viability MTT assay test on cell lines demonstrated these benefits.

This experiment demonstrated that the test fungal arginine degrading enzymes were more effective as an anticancer drug against auxotrophic cancer cell lines, such as tissue-culture-prepared hepatic carcinoma, melanoma, lung, and cardiac cancer cells. At an acidic pH, these enzymes exhibited no activity. They were more active at alkaline pH values, peaking at pH 8.7, although they did not surpass pH 10. Enzymes that break down arginine were equally efficient under alkaline cobalt or manganese ions as cofactors at a neutral pH of 7.3. In the physiologic medium, 37 °C was the ideal temperature for the fungal arginine-degrading enzymes to be biologically active. Fungal arginine degrading enzymes were found to have strong anti-proliferative effects and strong cytotoxic activity against hepatic carcinoma and melanoma, while it showed moderate cytotoxic activity and antiproliferative effects against colorectal cancer, lung adenocarcinoma, and heart cancer. These findings were compared with previous studies [16] and standard doxorubicin as an anticancer agent against eutrophic cancers for arginine. The enzymes that break down arginine in fungi showed minimal cytotoxic action and no anti-proliferative effects on human normal cells, indicating that they are good anticancer mediators against auxotrophic tumors for L-arginine with minimal negative effects on human normal cells.

The best way to provide fungal arginine-degrading enzymes in this investigation was by intramuscular or subcutaneous injection. They have to be administered many times a day due to their short half-lives [t1/2, around three hours]. In this investigation, the issue may be resolved by adding polyethylene glycol to prolong their duration of effect. Consequently, intramuscular or subcutaneous injection of arginine degrading enzymes was advised as a once-daily anticancer therapy against auxotrophic tumors for L-arginine. Due to the enzymes' chemical instability in the stomach's acidic environment and their destruction by proteolytic enzymes such pepsin and chymotrypsin pro-teases in the gastrointestinal tract, oral medication delivery solutions for arginine-degrading enzymes were not practical.

Drug-drug interactions between arginine-degrading enzymes and excipients of intra-muscular and subcutaneous injection drug delivery systems were not seen, according to FTIR and DSC tests. IgG antibodies were discovered in bacterial arginine degrading enzymes but were not detectable by ELISA. Apoptosis of the auxotrophic cancer cells for L-arginine, the cancer cytostatic effect, and preventing the auxotrophic cancer cells from obtaining L-arginine—the vital metabolic source for carbon and nitrogen for these cells—from the external surrounding media have all been observed to contribute to the fungal arginine degrading enzymes' promising anticancer activity against auxotrophic cancers.

The current study found that arginine degrading enzymes were very effective against auxotrophic cancers for L-arginine and solid tumors that form a mass of cells inside human organs like liver, lung, and colorectal cancer; however, they were ineffective against liquid tumors that circulate throughout the body, have high replication rates, rapidly dividing cells, and myeloma, lymphoma and leukemia. According to study by **Feun *et al.*** [17], arginine catabolizing enzymes including L-arginase and arginine deiminase demonstrated a critical function in the therapy of auxotrophic tumors for L-arginine. This led to arginine deprivation. Conversely, L-arginine had similar anticancer properties against auxotrophic tumors when degraded by enzymes that were isolated from fungi. A small sample size and poor methodology were the current study's limitations.

Conclusion: The finding of a novel fungus source for arginine-degrading enzymes made this work promising. Fermentations of Aspergillus fumigatus and Niger on metallic arginine agar media were used to manufacture medications containing enzymes that break down arginine. Compared to bacterial enzymes, they demonstrated a greater anticancer effect against auxotrophic tumors. In comparison to bacterial arginine degrading enzymes, they also showed less hypersensitive responses and more thermostable

reactions. It is advised that future research investigate the optimization of fungal arginine degrading enzymes as anticancer medicines against auxotrophic tumors employing L-arginine.

Conflict of interest: None

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