Article

Not peer-reviewed version

Isolation of an Arginase-Producing *Aspergillus niger* AUMC16187 from Sandy Soil at Al-Gharbia Governorate, Egypt and Characterization of the Produced Enzyme

Hossam H. H El-Wahsh , Nageh F. Abo-Dahab , Fareed Shawky El-Shenawy , Amro Abd Al Fattah Amara * , Nawal Abd El-Baky *

Posted Date: 11 March 2025

doi: 10.20944/preprints202503.0811.v1

Keywords: Arginase; 18S rRNA; Fungal enzymes; Carbon and nitrogen sources; Additional elements; Amino acids; Gel filtration

 doi:10.20944/preprints202503.0811.v1

Article

Isolation of an Arginase-Producing *Aspergillus niger* AUMC16187 from Sandy Soil at Al-Gharbia Governorate, Egypt and Characterization of the Produced Enzyme

Hossam H. H El-Wahsh [1], Nageh F. Abo-Dahab [1], Fareed Shawky El-Shenawy [1], Amro Abd Al Fattah Amara [2,*] and Nawal Abd El-Baky [2,*]

[1] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut Branch, Assiut, Egypt.
[2] Protein Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab City, P.O. Box 21934, Alexandria, Egypt
* Correspondence: aamara@srtacity.sci.eg (A.A.A.F.A.); nelbaky@srtacity.sci.eg (N.A.E.-B.)

Abstract: This study aimed to isolate arginase-producing fungi from 24 soil samples collected from four Egyptian governorates, then to identify the most potent fungal isolate both morphologically and genetically via detecting ITS regions of 18S rRNA sequence. Optimal conditions for enzyme production and activity including incubation or reaction time, temperature, pH, carbon and nitrogen sources, additional elements, different amino acids, and EDTA were also investigated. Arginase was purified from concentrated clear culture filtrate with gel filtration using Sephadex G-50 column. *Aspergillus niger* AUMC16187 was identified as the most efficient arginase-producer among 68 fungal isolates isolated from 24 soil samples and screened for arginase production. Maximum arginase production was achieved at 7th day of incubation, 27°C, and pH 7, using 1% dextrose and 2% ammonium chloride as carbon and nitrogen sources, respectively, 0.2% magnesium chloride as additional element, and 0.15% glutamine. SDS-PAGE analysis of purified arginase showed two bands at molecular weights of approximately 127 and 70 kDa. Purified arginase displayed maximum activity at 40°C, pH 7, after reaction time of 10 and 15 min, and was most stable at 30-50°C. All EDTA concentrations completely inhibited enzyme activity revealing that it is a metalloenzyme. The enzyme demonstrated significant cytotoxic effects against human melanoma cells (A431), indicating its potential as an anticancer agent.

Keywords: arginase; 18S rRNA; fungal enzymes; carbon and nitrogen sources; additional elements; amino acids; gel filtration

1. Introduction

A wide variety of microbes, including fungi, yeasts, and bacteria can produce therapeutic enzymes like arginase, asparaginase, and glutaminase [1–4]. Enzymes are currently being employed and researched for a variety of pathologies, including cancer and cardiovascular disorders, in addition to the therapy of metabolic deficits [5,6]. Even though the enzymes used in the clinical trials originated from bacteria. It was believed that eukaryotic enzymes would be more suitable for human therapy than bacterial ones [7].

Arginase, also known as arginine amidinohydrolase (EC.3.5.3.1), is a manganese metalloenzyme in the urea cycle that converts arginine into urea and ornithine. It is present in all five kingdoms of organisms, believed to originate in bacteria, and found in plants, yeasts, invertebrates, and vertebrates [8]. There are a few reports on arginase production from fungi, including *Neurospora crassa* [9], *Agaricus bisporus* [10], *Aspergillus nidulans* [11], *Aspergillus niger* [12], *Aspergillus versicolor* [13], and

 doi:10.20944/preprints202503.0811.v1

Penicillium chrysogenum KJ185377.1 [7]. *Neurospora crassa* expresses two forms of arginase [9,14]. Arginase in *Agaricus bisporus* causes urea accumulation in its fruit bodies [10].

Initially classified as a non-essential amino acid, arginine plays roles in antibacterial and anticancer activity, immunity, hormone release, and wound healing [15]. Medically, arginase is used in treating cancer, rheumatoid arthritis, neurological disorders, and asthma. Arginase deficiency causes hyperargininemia, a genetic disorder of the urea cycle [16].

It is interesting to conclude that arginase has served as an "accomplice" molecule in a number of cancers, such as digestive system cancer (including colorectal cancer [17], and liver cancer [18,19]), prostate cancer [20], central and peripheral nervous system (including glioblastomas [21]), endocrine and neuroendocrine system cancer (including thyroid cancer [22], neuroblastoma [23], melanoma [24], head and neck squamous cell carcinomas (HNSCC) [25]), lung cancer [26], breast cancer [27,28], and acute myeloid leukemia (AML) [29], promoting tumor growth or metastasis in several ways. Therefore, by inhibiting arginase activity or expression, arginase is a powerful target for the treatment of several types of cancer [30–33].

In contrast to the above-mentioned approach, arginine also significantly regulates T cells and nutritional metabolism, thus it may lower the risk of cancer. The role of arginine in carcinogenesis has recently gained more attention due to its stimulation of the growth of cancerous tissue cells [34]. Arginine pathway activation and availability of arginine have important effects on the microenvironments of cancer. Particularly, immunosuppression, initiation and development of tumors, adhesion and differentiation of tumor cells, and angiogenesis may all be affected by NO and polyamines, which are only produced from arginine. Furthermore, clinical trials using arginine depletion in cancer therapy have shown promising results [34].

The present work aimed to isolate arginase-producing fungi from 24 soil samples collected from four Egyptian governorates, then to identify the most potent fungal isolate both morphologically and genetically. The physical and chemical conditions for enzyme production and activity were also optimized. Additionally, the produced enzyme was purified and assayed for its in vitro properties as an anticancer agent against tumor cell line.

2. Materials and Methods

2.1. Collection of Soil Samples

In sterile containers, several soil samples were taken from the surface and at a depth of 10 to 20 cm at various sites in the Egyptian governorates of Alexandria, Al-Gharbia, Asyut, and Suhag. All samples were brought to the lab, either for immediate use or to be preserved at 4 °C until needed.

2.2. Isolation and Identification of Fungi

All collected soil samples were serially diluted. On modified Czapek Dox's agar [35], aliquots of the suspension containing 0.1 ml were equally distributed. The plates were incubated for 5-7 days at 28±2°C. By repeatedly sub-culturing, produced fungal colonies were separated and purified. The morphology of fungal colonies was investigated, along with microscopic features, and further screening was performed.

2.3. Maintenance of Fungal Cultures

Single colonies were grown on potato dextrose agar (PDA) slants supplemented with 0.01% l-arginine, stored at 4°C under sterilized paraffin oil, and sub-cultured annually [36]. Working culture was sub-cultured every other month and stored at 4°C without the addition of mineral oil [37].

2.4. Qualitative Screening of Arginase-Producing Fungi

Using the rapid plate assay, sixty-eight fungal isolates were properly examined for their ability to produce arginase. L-arginine served as the sole source of nitrogen in the Czapek Dox minimal agar medium. The synthesis of enzyme was determined by the chromogenic shift from pink to yellow on the agar plates. As a pH indicator, phenol red (2.5g in 100 ml ethanol) was utilized. The medium

 doi:10.20944/preprints202503.0811.v1

comprised L-arginine (1% w/v), glucose (1% w/v), KCl (0.05% w/v), $MgSO_4.7H_2O$ (0.05% w/v), KH_2PO_4 (0.1% w/v), $FeSO_4.7H_2O$ (0.01% w/v), and 22g/L agar-agar at a pH of 7.4. Following inoculation, the plates were incubated for 5-7 days at 28±2°C [2,38]. The colonies with the most chromogenic zones were chosen and kept on the agar slants at 4°C.

2.5. Arginase Assay

The ninhydrin test was used to assess the arginase activity spectrophotometrically based on the released ornithine as formerly described [39] with minimal modification. Briefly, the procedure involves 500 µL of L-arginine (100 mM) in potassium phosphate buffer (pH 7.0) with 1 mM manganese chloride ($MnCl_2$) and 500 µL of crude enzyme. After incubating the reaction mixture for 10 min at 40°C, 100 µl of 10% trichloroacetic acid (TCA) was added as a reaction stopper, followed by 25 µl of ninhydrin reagent. After boiling for 5 min, the liquid was centrifuged to remove precipitated proteins, and the produced color was assessed at 575 nm as previously described [40]. Based on authentic L-ornithine, one unit of arginase was defined as the quantity of enzyme that released one micromole of L-ornithine during a standard assay. The specific activity of arginase (U/mg protein) was calculated using the following formula: Arginase activity (U/mL)/protein content (mg/mL).

Bovine serum albumin (BSA) was used as the standard to quantify the enzyme protein concentration using Folin's reagent [41]. The protein concentration was indicated as mg of protein per ml of sample utilized.

2.6. Molecular Identification of the Hyperactive Producer Strain by ITS Analysis

The fungal isolate was cultured on Czapek's yeast extract agar (CYA) medium and incubated at 28°C for 5 days [35]. DNA extraction was performed at the Molecular Biology Research Unit, Assiut University using Patho-gene-spin DNA/RNA extraction kit (Intron Biotechnology Company, Korea). PCR and sequencing were done by SolGent Company (Daejeon, South Korea). The ITS regions of 18S rRNA sequence were amplified using the universal primers ITS1 (forward) and ITS4 (reverse) which were incorporated in the PCR reaction mixture. Primers have the following sequence: ITS1 (5' - TCCGTAGGTGAA CCTGCGG - 3'), and ITS4 (5'- TCCTCCGCTTATTGATATGC -3'). The purified PCR product was sequenced with the same primers [42].

The obtained sequences were analyzed using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) from the National Center of Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) website. Analysis of sequences and establishment of phylogenetic trees were done using MegAlign (DNA Star) software version 5.05.

2.7. Optimization of Arginase Productivity

The initial optimization stage was to determine whether characteristics of a modified Czapek Dox medium have a significant effect on the synthesis of arginase by *Aspergillus niger* AUMC16187.

2.7.1. Screening of Arginase Production Under Submerged Fermentation, Static and Shaking Incubation Conditions

For arginase synthesis at varied time intervals (3, 5, 7, 9, 11, and 13 days), *Aspergillus niger* AUMC16187 was cultivated in modified Czapek Dox's broth media under shaking (120 rpm) and static incubation. Arginase activity was evaluated at each time interval.

2.7.2. Effect of Different Temperatures on Arginase Production

Aspergillus niger AUMC16187 was grown in modified Czapek Dox's broth media with shaking incubation at 120 rpm at various incubation temperatures ranging from 4 to 47°C to determine the effect of incubation temperature on arginase production. At each temperature, arginase activity was assessed.

 doi:10.20944/preprints202503.0811.v1

2.7.3. Effect of Initial pH Values on Arginase Production

Modified Czapek Dox's broth media were initially adjusted to a range of pH values (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12) using 0.1 M of NaOH or HCl. The optimum initial pH for producing arginase by *Aspergillus niger* AUMC16187 was determined and used in subsequent tests.

2.7.4. Effect of Different Carbon Sources on Arginase Production

It was investigated what would happen if different carbon sources were added separately at 1% w/v in a modified Czapek Dox's broth medium. These carbon sources include xylose, dextrose, fructose, glucose, sorbitol, lactose, sucrose, raffinose, cellulose, apple pectin, and starch. In the subsequent studies, *Aspergillus niger* AUMC16187 was grown on the optimal carbon source that stimulated maximum enzyme production.

The ability of *Aspergillus niger* AUMC16187 to increase the production of arginase at different concentrations of the best carbon source (0.25, 0.5, 1, 1.5, 2, and 2.5% w/v) was also studied.

2.7.5. Effect of Different Nitrogen Sources on Arginase Production

The ability of approximately 11 organic and inorganic nitrogen sources to promote the development of enzyme was investigated. The final concentration of nitrogen in the medium, expressed as N-base, remained unaltered despite their separate additions to the modified Czapek Dox's broth medium. L-arginine, beef extract, peptone, urea, and yeast extract were the sources of organic nitrogen, whereas ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate, sodium nitrate, and sodium nitrite were the sources of inorganic nitrogen. The best nitrogen source for stimulating the maximum level of arginase production by *A. niger* AUMC16187 were determined by this investigation. The following investigations made use of the best nitrogen source.

The ability of *Aspergillus niger* AUMC16187 to improve arginase production was determined at different concentrations of the best nitrogen source (0.25, 0.5, 1, 1.5, 2, and 2.5% w/v).

2.7.6. Effect of Different Additional Elements on Arginase Production

Barium chloride, calcium chloride, cobalt chloride, di-potassium hydrogen phosphate, magnesium chloride, potassium di-hydrogen phosphate, and sodium chloride were added separately at 0.1% concentration to the modified Czapek Dox's broth media to investigate their effect on arginase production by *Aspergillus niger* AUMC16187. The following experiments used an element that stimulates the highest levels of enzyme synthesis.

A. niger AUMC16187 was examined for its capacity to increase arginase synthesisat different concentrations of the most suitable additional element (0.025, 0.05, 0.1, 0.15, 0.2, and 0.25% w/v).

2.7.7. Effect of Different Amino Acids on Arginase Production

At a concentration of 0.1% in a modified Czapek Dox's broth medium, and along with the substrate, thirteen amino acids were tested, including alanine, arginine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, and serine for arginase production by *Aspergillus niger* AUMC16187.

Aspergillus niger AUMC16187 was examined for its capacity to improve arginase production at different concentrations of the most appropriate amino acid (0.025, 0.05, 0.1, 0.15, 0.2, and 0.25% w/v).

2.8. Purification of Arginase

After fermentation periods at determined optimum physical and chemical parameters, crude arginase was obtained. The clear culture filtrate was collected by centrifugation at 4000 rpm for 10 min. Arginase was concentrated using Amicon® ultra centrifugal filter, 10 kDa molecular weight cut-off (MWCO), followed by purification using gel filtration on Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer . Each purification step was performed at 4°C [43]. The active fractions were then dialyzed.

 doi:10.20944/preprints202503.0811.v1

2.9. SDS-PAGE Analysis of Purified Arginase

Purified arginase was mixed with sample buffer and heated at 100°C for 10 min, then loaded on 12% SDS-PAGE [44].

2.10. Determination of Protein Content

By BSA as a standard, Folin's reagent was used to assess the protein concentration of purified enzyme spectrophotometrically at 550 nm [41].

2.11. Characterization of Purified Arginase Activity

2.11.1. Optimum Reaction Time of Purified Arginase

The optimum reaction time of the purified arginase was determined at different time intervals (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min) and then assayed [39].

2.11.2. Effect of pH and pH Stability on the Activity of Purified Arginase

By assaying the activity of purified arginase using 0.1 M of different buffer solutions at several pH values as follows: citrate buffer (pH 2, 3, 4, and 5), potassium phosphate buffer (pH 6-7), Tris-HCl buffer (pH 8-9), and glycine-NaOH buffer (pH 10, 11, and 12), the optimum pH level was determined. Purified arginase was incubated at different pH values (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12) overnight to determine pH stability. Enzyme activity was then measured [39].

2.11.3. Effect of Temperature and Thermos-Stability on the Activity of Purified Arginase

In order to determine the optimum temperature, the activity of purified arginase was measured using 100 mM potassium phosphate buffer (pH 7) at different temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ºC). The purified enzyme was incubated at different temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ºC) overnight in order to evaluate the thermal stability of arginase. The activity of arginase was then determined in all conditions [39].

2.11.4. Effect of Substrate Concentration on the Activity of Purified Arginase

In the reaction mixture, different concentrations of L-arginine were incubated separately with purified arginase. Arginine concentrations were 25, 50, 75, 100, 125, and 100 mM to determine the optimal substrate concentration for the enzyme assay under the optimized assay conditions. The main concentration of arginase in the reaction mixture was determined at 0.1 M of L-arginine.

2.11.5. Effect of Different Metallic Cations on the Activity of Purified Arginase

Different metal cations, such as barium, calcium, cobalt, copper, magnesium, manganese and nikal, at a concentration of 1.0 mM were separately incubated with the enzyme to study the effect of these chemicals on purified arginase. The assayed residual activities were compared to the control (which was defined as 100% activity in the absence of any additives) for each chemical included.

2.11.6. Effect of Different Concentrations of EDTA on the Activity of Purified Arginase

EDTA at different concentrations of 0.2, 0.4, 0.6, 0.8 and 1 M was individually incubated with the purified arginase to study the effect of this chemical on enzyme. For each concentration of EDTA included, the residual activity measured was compared to the control (without additives).

2.11.7. Effect of Enzyme Concentration on the Activity of Purified Arginase

The best concentration of substrate solution in the reaction mixture was individually incubated with different concentrations of purified arginase (0.019, 0.048, 0.095, 0.19, 0.285, and 0.39 mg) to determine the best enzyme concentration under optimal assay conditions.

2.12. In Vitro Anticancer Assay of Arginase

 doi:10.20944/preprints202503.0811.v1

2.12.1. Cell Culture

From Nawah Scientific Inc., (Mokatam, Cairo, Egypt), human melanoma A431 (skin/epidermis) and human skin fibroblast (HSF) cells were obtained. The cells were kept in Dulbecco's Modified Eagle's medium (DMEM) provided with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of 5% (v/v) CO_2 at 37°C [45,46].

2.12.2. Cytotoxicity Assay

Viability of cells was measured using the assay of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). About 100 µl of the cell suspension aliquot ($5x10^3$) was seeded into 96-well plates, and the cells were cultured in complete medium at 37 °C for 24 h with 5% CO_2. Another 100 µl aliquot of medium containing the enzyme at different concentrations was added to treat the cells. After an enzyme exposure time of 48 h, the medium was discarded, and 20 µl of 1 mg/ml stock MTT solution was mixed with 100 µl of PBS in each well. The mixture was then incubated for 4 h at 37 °C. Next, 100 µl of absolute DMSO was used to dissolve the formed formazan crystals. Formazan solutions' (purple product) absorbance was determined at λ_{max} 570 nm using a multi-well plate reader (BMGLABTECH® FLUOstar Omega, Germany) [45,46].

2.12.3. Detection of the Apoptotic Effect of Arginase on Tested Human Cancer Cells Using a Flow Cytometry Assay

Apoptosis and necrosis cell populations are determined using the Annexin V-FITC apoptosis detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK) coupled with flow cytometry using two fluorescent channels. After treatment with test enzyme (purified arginase at concentration of 660 µg/ml) for 24 h, 10^5 cells were collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Cells were then incubated in the dark with 0.5 ml of Annexin V-FITC/PI solution for 30 min at room temperature, according to the manufacturer's protocol. After staining, cells were injected using an ACEA Novocyte™ flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA) and analyzed for FITC and PI fluorescent signals using FL1 and FL2 signal detectors, respectively (λex/em 488/530 nm for FITC and λex/em 535/617 nm for PI). For each sample, 12,000 events were acquired, and positive FITC and/or PI cells were quantified by quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA) [47–50].

3. Results

3.1. Collection of Soil Samples, Isolation and Identification of Arginase-Producing Fungi

68 fungal isolates were isolated from 24 soil samples and characterized. **Figure S1** describes colonies of *Aspergillus niger* AUMC16187 (the most potent fungal isolate for arginase production among 68 obtained isolates) isolated from sandy soil at Al-Gharbia Governorate in Egypt after growth on CYA and PDA at 28±2°C. Meanwhile, their microscopic images are shown in **Figure 1**.

Figure 1. Light microscopic images at different magnifications showing *A. niger* AUMC16187. (a) *A. niger* AUMC16187 at 100 X; (b) *A. niger* AUMC16187 at 400 X; (c) *A. niger* AUMC16187 at 1000 X.

3.2. Qualitative Screening of Arginase-Producing Fungi

Qualitative screening of fungal isolates on Czapek Dox minimal agar medium with minor modifications showed their arginase activity. L-Arginine was used as the sole nitrogen source in the medium, with phenol red as an indicator that turned from pink to a yellow zone around the colonies due to low pH as a positive result (**Figure 2**). Sixteen fungal strains out of 68 isolates showed yellow zones around the colonies; these strains were screened quantitatively for the most potent isolate for arginase production under submerged fermentation.

Figure 2. Arginase activity assay for *A. niger* AUMC16187 on Czapek-Dox minimal agar plates using arginine as the sole nitrogen source. (a) negative control; (b) *A. niger* AUMC16187 after 3 days; (c) *A. niger* AUMC16187 after

 doi:10.20944/preprints202503.0811.v1

5 days. The yellow zone around the fungal colonies indicates arginase production, while a pink color indicates a negative result.

3.3. Quantitative Screening of Arginase Producing Fungi

Sixteen fungal isolates were selected based on their ability to produce arginase under constant conditions. These isolates were quantitatively screened for arginase production over 7 days of shaking incubation. HOS. 1 (*Aspergillus niger* AUMC16187) was the most potent arginase producer, showing activities of 0.43 U/ml and 1.94 U/mg. This fungal isolate that showed the highest arginase activity was selected for further studies. Based on morphology, microscopic, and molecular identification, HOS. 1 was identified as *Aspergillus niger* AUMC16187 (**Figures S1, 1** and **3**).

Figure 3. Phylogenetic tree based on ITS sequence of rDNA of *Aspergillus niger* AUMC16187 (arrowed) aligned with closely related strains in the GenBank. This strain showed 99.13% - 100% identity and 96% - 100% coverage with several strains of the same species including the type material *A. niger* ATCC16888 (gb: AY373852). *Penicillium chrysogenum* represents an outgroup strain. P. = *Penicillium*, A. = *Aspergillus*.

3.4. Molecular Identification of the Hyperactive Producer Strain by ITS Analysis

ITS sequence of HOS. 1 (*Aspergillus niger* AUMC16187) was as follows:

CGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTG
TACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCG
GGCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTG
ATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGA
ACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA
ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTC
AAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGGACGGGCCCGAAAGGCAG
CGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGC
CGGCGCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATAC
CCGCTGAACTTAAGCATATCAATAAGCCGGAG

3.5. Optimization of Arginase Productivity

3.5.1. Screening of the Most Potent Fungal Isolates for Arginase Production Under Submerged Fermentation, Static and Shaking Incubation Conditions

The best incubation period for *Aspergillus niger* AUMC16187 to produce more arginase was evaluated. Arginase production was also examined under static and shaking incubation conditions. The results showed that arginase production increased with increasing incubation time till the 7th day. On the other hand, static incubation conditions resulted in very poor productivity compared to the production under shaking conditions, so we chose shaking conditions for enzyme production.

The maximum specific arginase activity under shaking conditions [6.8 U/mg] was observed on the 7th day [168 h] of incubation, and then the arginase level gradually decreased to 0.63 U/mg after 13 days (**Table 1**). Therefore, we chose the optimal incubation period under shaking conditions for the production of arginase after 7 days.

Table 1. Screening of the most potent fungal isolate (*Aspergillus niger* AUMC16187) for arginase production under shaking incubation conditions.

Time	Arginase absorbance (mean±SD)	Arginase activity (U/ml/min)	Arginase specific activity (U/mg)	Protein content (mg/ml)
72 hours	1.15±0.008	0.75	4.14	0.18

 doi:10.20944/preprints202503.0811.v1

120 hours	1.41±0.003	0.92	3.52	0.26
168 hours	**2.3±0.005**	**1.5**	**6.8**	**0.22**
216 hours	1.88±0.02	1.22	3.06	0.4
264 hours	0.35±0.008	0.22	0.93	0.24
312 hours	0.21±0.029	0.13	0.63	0.21

3.5.2. Effect of Different Temperatures and Initial pH Values on Arginase Production

For seven days, *Aspergillus niger* AUMC16187 was incubated under shaking incubation conditions at different temperatures (4, 17, 27, 37, and 47 °C) and pH values (2-12) in order to determine the optimum temperature and pH for arginase production. According to the data in **Table 2**, the maximum arginase production levels (3.43 U/mg) were achieved by shaking incubation for 7 days at 27°C, while pH 7 achieved 3.27 U/mg under the same conditions (**Figure 4**). The levels of arginase decreased gradually with increasing temperatures and pH values.

Figure 4. Effect of different pH values on arginase production.

Table 2. Effect of different temperature values on arginase production.

Temperature	Arginase Abs (mean±SD)	Arginase activity (U/ml/min)	Arginase specific activity (U/mg)	Protein content (mg/ml)
4 °C	0.12±0.005	0.07	0.08	0.93
17 °C	0.85±0.002	0.55	2.75	0.2
27 °C	**2.11±0.003**	**1.37**	**3.43**	**0.4**
37 °C	0.49±0.027	0.31	2.86	0.11
47 °C	0.29±0.023	0.18	0.77	0.24

3.5.3. Effect of Different Carbon Sources on Arginase Production

Aspergillus niger AUMC16187 was grown in modified Czapek Dox broth medium supplemented with different individually added carbon sources, including xylose, dextrose, fructose, glucose, sorbitol, lactose, sucrose, raffinose, cellulose, apple pectin, and starch for arginase production. In contrast to the results of other carbon sources, dextrose was found to be the optimal carbon source for arginase production (5.56 U/mg) as demonstrated in **Figure 5**. *Aspergillus niger* AUMC16187 can produce arginase without a carbon source, even at a small level, as shown by the lower arginase production activity of the control (0.76 U/mg), i.e. in this case arginine was used as a carbon and nitrogen source.

Figure 5. Effect of different carbon sources on arginase production.

3.5.4. Effect of Concentration of the Best Carbon Source on Arginase Production

The results in **Table S1** illustrated the effect of different concentrations of dextrose (0.25, 0.5, 1, 1.5, 2, and 2.5% w/v) as the best carbon source for arginase production by *Aspergillus niger* AUMC16187. The maximum arginase level (6.28 U/mg) was obtained using 1% dextrose, which subsequently decreased to 0.82 U/mg at 2.5% concentration. As a result, the sole carbon source for the production of arginase was dextrose at 1% w/v concentration.

 doi:10.20944/preprints202503.0811.v1

3.5.5. Effect of Different Nitrogen Sources on Arginase Production

In order to produce arginase from *Aspergillus niger* AUMC16187 strain, we used different nitrogen sources, including organic and inorganic sources. The arginase results in **Figure 6** showed that ammonium chloride was the best nitrogen source (enzyme specific activity of 40.36 U/mg) for *Aspergillus niger* strain AUMC16187 to produce arginase in contrast to other nitrogen sources. Interestingly, the control sample showed activity of 3.07 U/mg for arginase. Therefore, this result verified that this strain can produce arginase in the absence of L-arginine (without substrate).

Figure 6. Effect of different nitrogen sources on arginase production.

3.5.6. Effect of Concentration of the Best Nitrogen Source on Arginase Production

The best nitrogen source at different individually added concentrations (0.25, 0.5, 1, 1.5, 2, and 2.5% w/v) was used to determine the most suitable concentration for arginase production by *Aspergillus niger* AUMC16187. From the data shown in **Table S2**, we recorded that the best nitrogen source, ammonium chloride, for maximum arginase production (9.99 U/mg), was at a concentration of 2% and subsequently decreased to 2.95 U/mg at 2.5%.

3.5.7. Effect of Different Additional Elements on Arginase Production

Barium, calcium, cobalt, magnesium, and sodium chlorides, as well as di-potassium hydrogen phosphate and potassium di-hydrogen phosphate, were each added separately at a 0.1% concentration to the modified Czapek Dox's broth media. This was done to determine their effect on arginase production by *Aspergillus niger* AUMC16187. Among all the mineral supplements added to the medium, even the results of the control sample (without additional elements), magnesium chloride showed the highest production of arginase, 6.22 U/mg (**Figure 7**).

Figure 7. Effect of different additional elements on arginase production.

3.5.8. Effect of Different Concentrations of the Most Suitable Additional Element on Arginase Production

Strain of *Aspergillus niger* AUMC16187 was examined for their capacity to increase arginase production at different concentrations of the most suitable additional element (0.025, 0.05, 0.1, 0.15, 0.2, and 0.25% w/v). The results in **Figure S2** illustrated that the maximum productivity (8.83 U/mg) of *Aspergillus niger* AUMC16187 for arginase was at 0.2% with the most suitable additive, magnesium chloride.

3.5.9. Effect of Different Amino Acids on Arginase Production

At a concentration of 0.1% in a modified Czapek Dox's broth medium, and along with the substrate, thirteen amino acids were tested for arginase production. The results in **Figure 8** showed that among all other amino acids and the control sample, glutamine was the most favorable for arginase production (8.51 U/mg) by *Aspergillus niger* AUMC16187.

Figure 8. Effect of different amino acids on arginase production.

3.5.10. Effect of Different Concentrations of the Most Suitable Amino Acid on Arginase Production

Different concentrations of glutamine (0.025, 0.05, 0.1, 0.15, 0.2, and 0.25% w/v) were added separately to the culture medium to determine the optimum levels of arginase production. The addition of 0.15% glutamine separately in the medium is most suitable for the production of arginase (2.97 U/mg) as shown in **Figure S3** by *Aspergillus niger* AUMC16187. Subsequently, at 0.25%, arginase production decreased to 2.23 U/mg.

3.6. Purification of Arginase

 doi:10.20944/preprints202503.0811.v1

Arginase was concentrated, then purified using gel filtration with a Sephadex G-50 column. **Table 3** showed the results of purification steps of arginase produced from *Aspergillus niger* AUMC16187. Protein concentration and specific activity of purified enzyme are showed in **Table 4**.

Table 3. Scheme of arginase purification from *Aspergillus niger* AUMC16187 culture filtrate.

Purification stage	Total volume (ml)	Protein content (mg/ml)	Activity (U/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude arginase after concentration step	300	0.29	0.7	87	210	2.5	100	1
Gel filtration using sephadex G 50-150 column chromatography	190	0.39	1.69	74.1	321.1	4.33	79.33	1.73

Table 4. Protein concentration and specific activity of purified enzyme.

Purified enzyme	Protein concentration (mg/ml)	Specific activity (U/mg)
Crude arginase	0.29	2.5
Purified arginase	0.39	4.33

3.7. SDS-PAGE Analysis of Purified Enzyme

On SDS-PAGE, the purified arginase produced by *Aspergillus niger* AUMC16187 was found to have two subunits with a molecular weight of about 127 and 70 kDa (**Figure 9**).

Figure 9. SDS-PAGE analysis of purified arginase. lane M: protein molecular weight marker; lane 1: purified arginase.

3.8. Characterization of Purified Arginase Activity

3.8.1. Optimum Reaction Time of Purified Arginase Activity

The activity of purified arginase was measured at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min, with the optimum reaction time found to be 10 min.

 doi:10.20944/preprints202503.0811.v1

The highest activity of purified arginase was found to be 100 and 98.9% at 40°C after 10 and 15 min of incubation, respectively, with a slight decrease at other incubation periods (**Figure S4**).

3.8.2. Effect of pH and pH Stability on the Activity of Purified Arginase

By assaying the activity of purified arginase using 0.1 M different buffer solutions and several pH values (2-12), the optimum pH level was determined. Purified arginase showed the highest activity of 100% at pH 7, according to results shown in **Figure 10a**. The activity increased continuously with increasing pH values of 2, 3, 4, 5, and 6, with activity of 1.35, 9.38, 16.27, 41.5, and 56.41%, respectively, while it dropped to 61, 34.62, 25.44, 10.53 and 0.21% at pH values 8, 9, 10, 11, and 12, respectively.

Figure 10. Effect of different pH values (a) and pH stability (b) on the activity of purified arginase.

The pH stability of purified arginase was also tested overnight at pH values of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. We observed that arginase was most stable at pH 7, with an activity of 95.41% (**Figure 10b**).

3.8.3. Effect of Temperature and Thermo-Stability on the Activity of Purified Arginase

In order to determine the optimum temperature, the activity of purified arginase was measured using 100 mM potassium phosphate buffer (pH 7) at different temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ºC). The results are shown below.

A wide range of arginase activity was demonstrated by the results shown in the **Figure S5**, from 4 to 80 °C. The activity was 91.19, 100, and 90.21% at 30, 40, and 50°C, respectively. Raising the temperature above this range decreased the enzyme activity to 68.68, 33.45 and 0.0% at 80, 90, and 100°C, respectively. Arginase activities changed little over a wide range of temperatures, meaning it is thermostable.

The purified enzyme was incubated at different temperatures (10, 20, 30, 40, 50,60, 70, 80, 90, and 100 ºC) overnight in order to evaluate the thermo-stability of arginase. As shown in **Figure S6**, the thermo-stability of purified arginase showed that the enzyme was most stable at 30, 40, and 50 °C with activity of 91.19, 100, and 90.21%, while its activity decreased to 68.68, 33.45, and 0% at 80, 90, and 100 °C, respectively.

3.8.4. Effect of Substrate Concentration on the Activity of Purified Arginase

In the reaction mixture, different concentrations of L-arginine were incubated separately with purified arginase. L-arginine concentrations were (25, 50, 75, 100, 125, and 100 mM) to determine the optimal substrate concentration for the assay of enzyme under the optimized assay conditions.

As shown in **Table 5**, the best activity of purified arginase was 102.15, 110.95, 104.29, and 100% at concentrations of 25, 50, 75, and 100 mM, respectively. While above these concentrations, the activity decreased to 91.41 and 76.39% at 125 and 150 mM, respectively.

Table 5. Effect of substrate concentration on the activity of purified arginase.

L-arginine conc. (mM)	Relative activity (%)
25	102.15
50	110.95
75	104.29

 doi:10.20944/preprints202503.0811.v1

100	**100**
125	91.41
150	76.39

3.8.5. Effect of Different Metallic Cations on the Activity of Purified Arginase

Different metallic cations, such as barium, calcium, cobalt, copper, magnesium, manganese and nikal, at a concentration of 1.0 mM were separately incubated with the enzyme to study the effects of these chemicals on purified arginase.

Manganese cations increased arginase activity by 12.79% compared to control. However, barium, calcium, cobalt, copper, magnesium, nickel, and sodium cations inhibited enzyme activity by 8.95, 98.49, 95.93, 99.77, 97.21, 95.93, and 93.37%, respectively (**Figure S7**).

3.8.6. Effect of Different Concentrations of EDTA on the Activity of Purified Arginase

EDTA at different concentrations of (0.2, 0.4, 0.6, 0.8 and 1 M) was incubated with the purified arginase to study the effects of this chemical on enzyme. For each concentration of EDTA included, the residual activity measured was compared to the control (without additives). The results in **Figure S8**, indicated that all EDTA concentrations completely inhibited the activity of purified arginase, suggesting that the enzyme is a metalloenzyme.

3.8.7. Effect of Enzyme Concentration on the Activity of Purified Arginase

Different concentrations of purified arginase (0.02, 0.05, 0.1, 0.19, 0.29, and 0.39 mg) were individually incubated with the best concentration of substrate solution in the reaction mixture to determine the best enzyme concentration under the optimal assay conditions. The highest activity of purified arginase (100%) was achieved at appropriate enzyme concentrations in the reaction mixture, which were found to be 0.19 mg (**Table 6**).

Table 6. Effect of enzyme concentration on the activity of purified arginase.

Enzyme concentration (mg)	Relative activity (%)
0.02	87.26
0.05	89.38
0.1	93.63
0.19	**100**
0.29	76.64
0.39	2.31

3.9. In Vitro Anticancer Effect of Arginase

3.9.1. Cytotoxicity Assay of Arginase Using MTT Method

The cytotoxic activity of arginase against human melanoma (A431) and human skin fibroblast (HSF) cells was determined by incubating the cells with different arginase concentrations (2.58, 5.16, 10.31, 20.63, 41.25, 82.5, 165, 330, and 660 µg/ml) for 24 h. The MTT colorimetric protocol was then used to determine cell viability. As shown in the **Table S3** & **Figure 11a**, arginase exhibited a concentration-dependent cytotoxic effect on the tested cancer cell line. It effectively inhibited the

 doi:10.20944/preprints202503.0811.v1

proliferation of A431 cancer cells with lesser effect on the viability of normal HSF cells (**Table S4 and Figure 11b**).

Figure 11. Cytotoxicity % of fungal arginase against A431 cancer cells (a) and normal HSF cells (b).

The IC_{50} value of arginase for the tested cancer cells was determined to be 2394 µg/ml for human melanoma (A431), while it was 2907 µg/ml for healthy cells (HSF). These results showed that the enzyme is more toxic to cancer cells than to healthy cells.

3.9.2. Flow Cytometry Analysis of Fungal Arginase Against A431 Cancer Cells

Apoptosis and necrosis cell populations were determined using annexin V-FITC coupled with 2 fluorescent channels flowcytometry following treatment of A431 cells with purified arginase at concentration of 660 µg/ml for 24 hours. **Figure 12** demonstrated the powerful effect of fungal arginase on A431 in comparison to control via inducing apoptosis of these cells.

Figure 12. Flow cytometry analysis of fungal arginase against A431 cancer cells.

4. Discussion

Arginase, which is crucial in the urea cycle, has potential as an anticancer agent by modulating polyamine levels, which are essential for cell proliferation [51]. This study aimed to isolate arginase-producing fungi from different sources and identify the most potent isolate morphologically and genetically. Additionally, the study sought to optimize the physico-chemical production conditions. Finally, the enzyme was purified, and its properties and applications were investigated.

In this study, we successfully produced arginase from soil fungi. Sixty-eight fungal strains were isolated from 24 soil samples and screened for arginase production through qualitative and quantitative assays. Quantitative screening allowed for the selection of the most potent fungal strain exhibiting superior enzyme production. *Aspergillus niger* AUMC16187 was identified as the most potent strain for arginase production. This strain was genetically identified. The use of soil fungi for arginase production aligns with recent studies. Alzahrani (2020) demonstrated the potential of soil fungi in producing arginase [52].

Subsequent optimization involved adjusting various culture conditions to enhance enzyme yield from *Aspergillus niger* AUMC16187. Numerous nutritional, physical, and chemical factors significantly influence the production of arginase through submerged fermentation. In our study, we incubated strain at varied time intervals (3, 5, 7, 9, 11, and 13 days) under both shaking and static conditions. The optimum incubation period for arginase production from *Aspergillus niger* AUMC16187 was determined to be 7 days under shaking conditions then enzyme levels decreased. This method resulted in a significant increase in enzyme production compared to static conditions. Maximum arginase activity (6.8 U/mg) was observed on day 7. The incubation period for arginase closely aligns with El-Sayed et al. (2014), who reported a specific arginase activity of 2.44 U/mg from thermostable *Penicillium chrysogenum* KJ185377.1 after a 6-day incubation [7], while it slightly differs from Alzahrani (2020), who observed the best fungal arginase production after 5 days [52].

We found that the highest arginase production level (3.43 U/mg) from *Aspergillus niger* AUMC16187 were achieved by shaking incubation for 7 days at 27°C, while pH 7 achieved 3.27 U/mg under the same conditions. These results agree with El-Sayed et al. (2014) regarding optimum pH of 7, who found that *Penicillium chrysogenum* KJ185377.1 had optimal arginase activity (2.44 U/mg) at pH 7 and 45°C (which noticeably differs from optimum temperature in the present work, 27°C) [7]. However, our results differ from those of bacterial arginase, which showed the highest arginase productivity from *Bacillus licheniformis* OF2 at pH 8 and 35°C, and from *Lactobacillus acidophilus* at 40°C and pH 6 [21,53].

A key parameter in the increased production of arginase is the nature and amount of carbon sources present in the fermentation medium. In the present study, dextrose (1% w/v) was identified as the optimal carbon source for arginase production from *Aspergillus niger* AUMC16187, with a

 doi:10.20944/preprints202503.0811.v1

specific activity of 6.28 U/mg. While this strain can produce arginase without a carbon source, the production level was much lower (0.76 U/mg) using arginine as the carbon and nitrogen source. These results differ from those of studies that reported maltose to be the best carbon source for arginase production from bacteria [21,54].

We observed that ammonium chloride was the best nitrogen source for maximum arginase production (9.99 U/mg) at a concentration of 2%. These results differ from that, which found peptone to be the best nitrogen source for arginase production from *Bacillus licheniformis* OF2 [21]. Similarly, yeast extract was found to be optimal for *Pseudomonas sp.*, and *Lactobacillus acidophilus* for arginase production [53,54].

Among all the mineral supplements added to the medium, magnesium (from magnesium chloride) at a concentration of 0.2% showed the highest production of arginase (8.83 U/mg) from *Aspergillus niger* AUMC16187. These results align with those obtained by Ibrahim et al. (2018), who used magnesium as an additive element (0.005% $MgSO_4.7H_2O$) for arginase production from *Alcaligenes faecalis*, although the concentration differs [55]. However, 0.02% $MnSO_4$ was found to be optimum for *Pseudomonas sp.* Strain PV1 arginase production, and 10 mg/L calcium chloride and 0.5 mg/L ferrous sulphate were optimum for *Lactobacillus acidophilus* arginase production [53,54].

At a concentration of 0.1%, thirteen amino acids were tested in the medium along with substrate, for arginase production by *Aspergillus niger* AUMC16187. In contrast to most previous studies, which did not use amino acids as additional substances with the substrate for arginase production, Borkovich and Weiss (1987) and Gumashta et al. (2021) supplemented their medium with arginine (0.1% w/v) for producing arginase from *Neurospora crassa* and *Streptomyces plicatus* KAR73, respectively [14,56]. In our study on *Aspergillus niger* AUMC16187, glutamine proved to be the most effective additional amino acid when combined with the substrate (arginine), achieving a specific activity of 2.97 U/mg at a 0.15% (w/v) concentration. Notably, this strain also produced arginase even in the absence of the substrate.

In the present work, arginase was concentrated from culture filtrate then purified using gel filtration with a Sephadex G-50 column. The purified arginase from *Aspergillus niger* AUMC16187 had a protein concentration of 0.39mg/ml, an activity of 1.69 U/ml, and a specific activity of 4.33U/mg, with a 1.73-fold purification. In contrast, El-Sayed et al. (2014) reported a total protein concentration of 412 mg and a specific activity of 2.44 U/mg for thermotolerant *Penicillium chrysogenum* KJ185377.1 [7]. In this work, protein concentration was higher than that reported by El-Sayed et al. (2014) (0.39 vs. 0.297 mg/ml) who used Sephadex G100 column gel filtration. However, the specific activity in this work (4.33 U/mg) was lower than their value (24.45 U/mg), as was the fold purification (1.73 vs. 10.02) [7]. Dzikowska et al. (1994) purified arginase from *Aspergillus nidulans* using Bio-Gel P-60 gel filtration chromatography, achieving a specific activity of 14.1 U/mg, 4.7-fold purification, and a 21.7% yield [57].

In this study, using SDS-PAGE, the purified arginase produced by *Aspergillus niger* AUMC16187 was found to have two subunits with molecular weights of approximately 127 kDa (very faint) and 70 kDa (very clear), indicating that the enzyme is dimeric. These results differ from previous studies on arginase from other sources. For instance, Borkovich and Weiss (1987) reported that the arginase from *Neurospora crassa* had a molecular weight of 38.3 kDa [14]. Dzikowska et al. (1994) found the molecular mass of the purified arginase from *Aspergillus nidulans* to be 40 kDa [57]. Zhang et al. (2013) noted that arginase from *Bacillus thuringiensis* SK 20.001 had a molecular weight of around 33 kDa using SDS-PAGE and 191 kDa according to gel filtration [38]. Yu et al. (2013) determined the molecular weight of arginase from *Bacillus subtilis* 168 to be 49 kDa using SDS-PAGE [58]. El-Sayed et al. (2014) reported that the molecular structure of arginase subunit from thermostable *Penicillium chrysogenum* KJ185377.1 was 37.0 kDa [7]. Gumashta et al. (2021) mentioned that arginase from *Streptomyces plicatus* KAR73 had a molecular weight of 23 kDa [56]. According to Hassabo et al. (2023), the molecular mass of the purified arginase from *Streptomyces diastaticus* MAM5 was estimated to be 39 kDa [27].

 doi:10.20944/preprints202503.0811.v1

Arginases have been studied for their molecular weights, with values ranging from 98 kDa [59] to 352 kDa [60]. Additionally, the number of subunits reported varies from three to ten. For example, there is a tetramer from mouse liver [61], a trimer from *Saccharomyces cerevisiae* [62], a hexamer with a molecular weight of 180 kDa from *Bacillus brevis* Nagano [63], a hexamer from Iris bulbs [64], and a decamer with a molecular weight of 352 kDa from ginseng root arginase [60]. These variations in molecular weights highlight the diversity in arginase structure across different species and strains, suggesting potential differences in enzyme functionality, stability, and regulatory mechanisms.

The present study demonstrated that the purified arginase from *Aspergillus niger* AUMC16187 shows high stability around 40°C at various incubation periods. The arginase activity peaks at 100% after 10 min and slightly decreases to 98.87% at 15 min. The activity remains stable between 96.6% and 97.73% throughout most of the incubation periods, with minor fluctuations observed. This consistent activity indicates that the enzyme is highly active and stable at this temperature, which is crucial for its potential applications, such as in cancer treatments. Similarly, it was reported that the optimal incubation time for arginase activity produced from *Bacillus thuringiensis* SK 20.001 and *Penicillium chrysogenum* KJ185377.1was 10 min at 40°C [7,38]. In contrast, it was found that the optimal incubation time for arginase activity from *Streptomyces diastaticus* MAM5 was 30 min at 40°C [27].

This work showed that the purified arginase is most efficient and stable at a neutral pH of 7, achieving peak activity (100%) and maintaining high stability (95.41%). The enzyme activity increased steadily from pH 2 to 6, indicating effectiveness under slightly acidic conditions. However, beyond pH 7, activity decreased significantly, particularly in more alkaline environments. These findings suggest arginase's suitability for medical applications, such as cancer treatment, where maintaining a neutral pH is crucial for optimal enzyme performance. In contrast, bacterial arginase from *Streptomyces plicatus* KAR73, *Bacillus licheniformis* OF2, *Streptomyces diastaticus* MAM5, *Pseudomonas aeruginosa* IH2, and *Bacillus thuringiensis* SK 20.001 had optimal activity at pH 7.5-10 (at alkaline pH with reduced activity in acidic conditions) and was stable between pH 8 and 12 [27,28,38,56,65].

The present study found that purified arginase is most effective at around 40°C and is thermostable over a wide range of temperatures. The enzyme was most stable at 30°C, 40°C, and 50°C, with activities of 91.19%, 100%, and 90.21%, respectively, but its activity decreased significantly at higher temperatures. These results are consistent with other studies on arginase purified from *Streptomyces plicatus* KAR73 [56], and from *Bacillus thuringiensis* SK 20.001 [38]. In contrast, it was observed that purified arginase from *Glaciozyma antarctica* PI12 had maximum activity and stability at 20°C, retaining 70% residual activity at 10-30°C [66], and arginase from *Streptomyces diastaticus* MAM5 had the highest activity at 50°C and remained stable up to 50°C [27].

In this study, purified arginase showed the highest activities at L-arginine concentrations of 25, 50, 75, and 100 mM, with activities of 102.15%, 110.95%, 104.29%, and 100%, respectively. Activity decreased to 91.41% and 76.39% at concentrations of 125 and 150 mM. The highest activity of purified arginase (100%) was achieved at an enzyme concentration of 0.19 mg in the reaction mixture. These results align with those reported by El-Sayed et al. (2014) and Hassabo et al. (2023), who used similar concentrations in the reaction mixture for arginase assays from *Penicillium chrysogenum* KJ185377.1 and *Streptomyces diastaticus* MAM5 [7,20]. Comparatively, Zhang et al. (2013) found similar results with arginase assays from *Bacillus thuringiensis* SK 20.001 at duplicated reaction mixture concentrations [38].

Among the tested metallic cations, manganese increased arginase activity by 12.79%, while barium, calcium, cobalt, copper, magnesium, nickel, and sodium inhibited it by 8.95%, 98.49%, 95.93%, 99.77%, 97.21%, 95.93%, and 93.37%, respectively. Our findings showed that all tested EDTA concentrations completely inhibited the activity of purified arginase, suggesting that the arginase is a metalloenzyme. According to Hassabo et al. (2023), the activity of *Streptomyces diastaticus* MAM5 arginase was enhanced by 59% with Co^{2+} and 14% with Mn^{2+} ions [27]. It was strongly inhibited by Cu^{2+}, Li^{2+}, and Hg^{2+} ions. EDTA, SDS, sodium azide, 8-hydroxyquinoline, and hydroxylamine also significantly reduced arginase activity. Similarly, *Pseudomonas aeruginosa* IH2 arginase was most

 doi:10.20944/preprints202503.0811.v1

active with Mn^{2+} and Mg^{2+}, while Al^{3+}, Cu^{2+}, and Li^{+} ions decreased its activity [65]. *Bacillus thuringiensis* SK 20.001 arginase showed maximum activity with Mn^{2+} and Ni^{2+} ions, while Zn^{2+}, Fe^{2+}, and Fe^{3+} slightly inhibited its activity [38]. Similar findings were reported for marine *Bacillus licheniformis* OF2 arginase [21], *Bacillus brevis* Nagano arginase [63], *Saccharomyces cerevisiae* arginase [62], and *Bacillus subtilis* arginase [67]. In contrast**,** it was found that *Helicobacter pylori* arginase preferred Co^{2+}, Ni^{2+}, and Mn^{2+}, respectively [39].

Since it is the precursor of multiple cell-signaling molecules that are crucial for tumor growth, arginine deprivation is a viable treatment strategy for a variety of cancers [68]. Normal cells can generate arginine from citrulline using arginosuccinate synthase (AS) and arginosuccinate lyase (ASL), so they are unaffected by arginase's conversion of arginine to ornithine and urea. Cancer cells, however, do not express these enzymes [69].

Our results, demonstrating the concentration-dependent cytotoxic effect of arginase on melanoma (A431) and human skin fibroblast (HSF) cells, which align with previous findings [7,21]. Selim et al. (2024) reported promising activity of arginase on breast and colon cell lines with minimal cytotoxicity on normal skin cells (BJ-1) [21], which is similar to our results of significant inhibition of melanoma cells and lesser impact on HSF cells. Both studies highlight arginase's potential as an anticancer agent with higher toxicity to cancer cells than to normal cells, reinforcing the role of arginase in selective cancer therapies. Additionally, our study results showed significant apoptosis in A431 cells treated with 660 µg/ml of purified arginase in flow cytometry analysis, which aligns with previous studies [27,28,65].

5. Conclusion

This study successfully isolated and identified *Aspergillus niger* AUMC16187 as the most efficient arginase-producer among 68 fungal isolates isolated from 24 soil samples and screened for arginase production. Maximum arginase production from this strain was achieved at 7th day of incubation, 27°C, and pH 7, using 1% dextrose and 2% ammonium chloride as carbon and nitrogen sources, respectively, 0.2% magnesium chloride as additional element, and 0.15% glutamine. SDS-PAGE analysis of purified arginase showed two bands at molecular weights of approximately 127 and 70 kDa. Purified arginase displayed maximum activity at 40°C, pH 7, after reaction time of 10 and 15 min, and was most stable at 30-50°C. The enzyme is a metalloenzyme that can be inhibited by all EDTA concentrations. Importantly, the purified arginase exhibited significant cytotoxic effects against human melanoma (A431) cancer cells. These findings suggest that arginase from *Aspergillus niger* AUMC16187 holds potential as an anticancer agent.

References

1. Shirazian, P.; Asad, S.; Amoozegar, M. A. The potential of halophilic and halotolerant bacteria for the production of antineoplastic enzymes: L-asparaginase and L-glutaminase. *EXCLI J.* **2016**, *18*(15), 268–279.
2. Nadaf, P.D.; Kulkarni, A.G.; Vedamurthy, A.B. Isolation, screening and characterization of L-arginase producing soil bacteria. *Int. J. Pharm. Sci. Res.* **2019**, *10*(7), 3440–3444. doi: 10.13040/IJPSR.0975-8232.10(7).3440-44.
3. Sidkey, N. M.; Abo Elsoud, M. M.; Elnemr, W.; Elhateir, M. M. Anticancer Glutaminase Production and Optimization Using Halotolerant *Aspergillus flavus* CZCU-9, F1H. *Al-Azhar Bulletin of Sci.* **2019**, *30*(1-C), 1–9.
4. Awad, M. F.; El-Shenawy, F. S.; El-Gendy, M. M. A. A.; El-Bondkly, E. A. M. Purification, characterization, and anticancer and antioxidant activities of L-glutaminase from *Aspergillus versicolor* Faesay4. *Int. Microbiol.* **2021**, *24*(2), 169–181. doi: 10.1007/s10123-020-00156-8.
5. Chen, H.; McGowan, E. M.; Ren, N.; Lal, S.; Nassif, N.; Shad-Kaneez, F.; Qu, X.; Lin, Y. Nattokinase: A promising alternative in prevention and treatment of cardiovascular diseases. *Biomark. Insigh.* **2018**, *13*, 1177271918785130.
6. Radadiya, A.; Zhu, W.; Coricello, A.; Alcaro, S.; Richards, N. G. J. Improving the treatment of acute lymphoblastic leukemia. *Biochem.* **2020**, *59*(35), 3193–3200.

 doi:10.20944/preprints202503.0811.v1

7. El-Sayed, A.S.; Shindia, A.A.; Diab, A.A.; Rady, A.M. Purification and immobilization of L-arginase from thermotolerant *Penicillium chrysogenum* KJ185377.1; with unique kinetic properties as thermostable anticancer enzyme. *Arch. Pharm. Res.* **2014**. doi: 10.1007/s12272-014-0498-y.
8. Dzik, J.M. Evolutionary roots of arginase expression and regulation. *Front Immunol.* **2014**, *5*, 544. doi: 10.3389/fimmu.2014.00544.
9. Marathe, S.; Yu, Y.G.; Turner, G.E.; Palmier, C.; Weiss, R.L. Multiple forms of arginase are differentially expressed from a single locus in *Neurospora crassa*. *J. Biol. Chem.* **1998**, *273*(45), 29776–29785. doi: 10.1074/jbc.273.45.29776.
10. Wagemaker, M.J.; Welboren, W.; van der Drift, C.; Jetten, M.S.; Van Griensven, L.J.; Op den Camp, H.J. The ornithine cycle enzyme arginase from *Agaricus bisporus* and its role in urea accumulation in fruit bodies. *Biochim. Biophys. Acta.* **2005**, *1681*(2-3), 107–115. doi: 10.1016/j.bbaexp.2004.10.007.
11. Olszewska, A.; Król, K.; Weglenski, P.; Dzikowska, A. Arginine catabolism in *Aspergillus nidulans* is regulated by the rrmA gene coding for the RNA-binding protein. *Fungal Genet Biol.* **2007**, *44*(12), 1285–1297. doi: 10.1016/j.fgb.2007.07.001.
12. Keni, S.; Punekar, N.S. Contribution of arginase to manganese metabolism of *Aspergillus niger*. *Biometals.* **2016**, *29*(1), 95–106. doi: 10.1007/s10534-015-9900-6.
13. Parmar, F.A.; Patel, J.N.; Upasani, V.N. Screening of microorganisms for production of therapeutic enzymes. *Screening* **2021**, *3*(6), 39.
14. Borkovich, K.A.; Weiss, R.L. Relationship between two major immunoreactive forms of arginase in *Neurospora crassa*. *J. Bacteriol.* **1987**, *169*(12), 5510–5517. doi: 10.1128/jb.169.12.5510-5517.1987.
15. Potenza, M.A.; Nacci, C.; Mitolo-Chieppa, D. Immunoregulatory effects of L-arginine and therapeutical implications. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* **2001**, *1*(1), 67–77. doi: 10.2174/1568008013341811.
16. Henriques C.F.; Fernandes, R.; Barreto, F.; Miranda, R.; Aguiar, T.C. Hyperargininemia: A Rare Diagnosis in Adulthood. *Eur. J. Case Rep. Int. Med.* **2024**, *11*(4), 004379. doi: 10.12890/2024_004379.
17. Maisonneuve, C.; Tsang, D.K.L.; Foerster, E.G.; Robert, L.M.; Mukherjee, T.; Prescott, D.; Tattoli, I.; Lemire, P.; Winer, D.A.; Winer, S.; Streutker, C.J.; Geddes, K.; Cadwell, K.; Ferrero, R.L.; Martin, A.; Girardin, S.E.; Philpott, D.J. Nod1 promotes colorectal carcinogenesis by regulating the immunosuppressive functions of tumor-infiltrating myeloid cells. *Cell Rep.* **2021**, *34*(4), 108677. doi: 10.1016/j.celrep.2020.108677.
18. Atta, I. S. Efficacy of expressions of Arg-1, Hep Par-1, and CK19 in the diagnosis of the primary hepatocellular carcinoma subtypes and exclusion of the metastases. Histol. Histopathol. **2021**, *36*(9), 981–993. doi: 10.14670/HH-18-361.
19. You, J.; Chen, W.; Chen, J.; Zheng, Q.; Dong, J.; Zhu, Y. The Oncogenic Role of ARG1 in Progression and Metastasis of Hepatocellular Carcinoma. *Biomed. Res. Int.* **2018**, *2018*, 2109865. doi: 10.1155/2018/2109865. Erratum in: *Biomed. Res. Int.* **2019**, *2019*, 6212386. doi: 10.1155/2019/6212386.
20. Matos, A.; Carvalho, M.; Bicho, M.; Ribeiro, R. Arginine and Arginases Modulate Metabolism, Tumor Microenvironment and Prostate Cancer Progression. *Nutrients.* **2021**, *13*(12), 4503. doi: 10.3390/nu13124503.
21. Azambuja, J.H.; Ludwig, N.; Yerneni, S.S.; Braganhol, E.; Whiteside, T.L. Arginase-1+ Exosomes from Reprogrammed Macrophages Promote Glioblastoma Progression. *Int. J. Mol. Sci.* **2020**, *21*(11), 3990. doi: 10.3390/ijms21113990.
22. Li, J.H.; Zhang, S.Q.; Qiu, X.G.; Zhang, S.J.; Zheng, S.H.; Zhang, D.H. Long non-coding RNA NEAT1 promotes malignant progression of thyroid carcinoma by regulating miRNA-214. *Int. J. Oncol.* **2017**, *50*(2), 708–716. doi: 10.3892/ijo.2016.3803.
23. Hackett, C.S.; Quigley, D.A.; Wong, R.A.; Chen, J.; Cheng, C.; Song, Y.K.; Wei, J.S.; Pawlikowska, L.; Bao, Y.; Goldenberg, D.D.; Nguyen, K.; Gustafson, W.C.; Rallapalli, S.K.; Cho, Y.J.; Cook, J.M.; Kozlov, S.; Mao, J.H.; Van Dyke, T.; Kwok, P.Y.; Khan, J.; Balmain, A.; Fan, Q.; Weiss, W.A. Expression quantitative trait loci and receptor pharmacology implicate Arg1 and the GABA-A receptor as therapeutic targets in neuroblastoma. *Cell Rep.* **2014**, *9*(3), 1034–1046. doi: 10.1016/j.celrep.2014.09.046.
24. Yu, Y.; Ladeiras, D.; Xiong, Y.; Boligan, K.F.; Liang, X.; von Gunten, S.; Hunger, R.E.; Ming, X.F.; Yang, Z. Arginase-II promotes melanoma migration and adhesion through enhancing hydrogen peroxide production and STAT3 signaling. *J. Cell Physiol.* **2020**, *235*(12), 9997–10011. doi: 10.1002/jcp.29814.

 doi:10.20944/preprints202503.0811.v1

25. Srivastava, S.; Ghosh, S.K. Modulation of L-Arginine-Arginase Metabolic Pathway Enzymes: Immunocytochemistry and mRNA Expression in Peripheral Blood and Tissue Levels in Head and Neck Squamous Cell Carcinomas in North-East India. *Asian Pac. J. Cancer Prev.* **2015**, *16*(16), 7031–7038. doi: 10.7314/apjcp.2015.16.16.7031.
26. Giatromanolaki, A.; Harris, A.L.; Koukourakis, M.I. The prognostic and therapeutic implications of distinct patterns of argininosuccinate synthase 1 (ASS1) and arginase-2 (ARG2) expression by cancer cells and tumor stroma in non-small-cell lung cancer. *Cancer Metab.* **2021**, *9*(1), 28. doi: 10.1186/s40170-021-00264-7.
27. Hassabo, A.A.; Abdelraof, M.; Allam, R.M. L-arginase from *Streptomyces diastaticus* MAM5 as a potential therapeutic agent in breast cancer: Purification, characterization, G1 phase arrest and autophagy induction. *Int. J. Biol. Macromol.* **2023**, *224*, 634–645. doi: 10.1016/j.ijbiomac.2022.10.152.
28. Selim, M.S.; Mounier, M.M.; Abdelhamid, S.A.; Hamed, A.A.; Abo Elsoud, M.M.; Mohamed, S.S. Characterization, modeling, and anticancer activity of L-arginase production from marine *Bacillus licheniformis* OF2. *BMC Biotechnol.* **2024**, *24*(1), 6. doi: 10.1186/s12896-024-00829-6.
29. Mussai, F.; De Santo, C.; Abu-Dayyeh, I.; Booth, S.; Quek, L.; McEwen-Smith, R.M.; Qureshi, A.; Dazzi, F.; Vyas, P.; Cerundolo, V. Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment. *Blood.* **2013**, *122*(5), 749–758. doi: 10.1182/blood-2013-01-480129.
30. Abdel Wahed, A. S. A. H.; Amer, M. A. M.; Abou Mohamed, N. M.; Mobasher, M. I. Serum arginase-2 level in patients with vasculogenic erectile dysfunction. *Al-Azhar Med. J.* **2016**, *45*(4), 805–812.
31. Abdel Wahed, A. S. A. H.; Amer, M. A. M.; Abou Mohamed, N. M.; Mobasher, M. I.; Mamdouh, H.; GamalEl Din, S. F.; ElSheemy, M. S. Serum Arginase II level can be a novel indicator for erectile dysfunction in patients with vasculogenic erectile dysfunction: a comparative study. *Int. Urol. Nephrol.* **2018**, *50*(8),1389–1395. doi: 10.1007/s11255-018-1921-y.
32. Mohamed, S. A. A.; Eldowik, Y. M. The role of glypican 3, arginase 1, and CD34 in differentiation between benign and malignant primary hepatic lesions. *Al-Azhar Assiut Med. J.* **2022**, *20*(3), 239–244.
33. Niu, F.; Yu, Y.; Li, Z.; Ren, Y.; Li, Z.; Ye, Q.; Liu, P.; Ji, C.; Qian, L.; Xiong, Y. Arginase: An emerging and promising therapeutic target for cancer treatment. *Biomed. Pharmacother.* **2022**, *149*, 112840. doi: 10.1016/j.biopha.2022.112840.
34. Zhang, Y.; Chung, S.F.; Tam, S.Y.; Leung, Y.C.; Guan, X. Arginine deprivation as a strategy for cancer therapy: An insight into drug design and drug combination. *Cancer Lett.* **2021**, *502*, 58–70. doi: 10.1016/j.canlet.2020.12.041.
35. Pitt J.I; Hocking A.D. In Fungi and Food Spoilage. *Springer Nature Switzerland AG. Part of Springer Nature* (524 pages). **2009**. doi: 10.1007/978-0-387-92207-2_1.
36. Stanbury, P.; Whitaker, A.; Hall S.J. Principles of Fermentation Technology, 2nd ed. *Elsevier.* **2013**, *42*, 97 & 101.
37. Bajpai, B.; Patil, S. Induction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of fungi imperfecti. *Enz. Microb. Technol.* **1997**, *20*(8), 612–614. doi: 10.1016/S0141-0229(96)00206-2.
38. Zhang, T.; Guo, Y.; Zhang, H.; Mu, W.; Miao, M.; Jiang, B. Arginase from *Bacillus thuringiensis* SK 20.001: Purification, characteristics, and implications for l-ornithine biosynthesis. *Process Biochem.* **2013**, *48*(4), 663–668. doi: 10.1016/j.procbio.2013.02.023.
39. Zhang, J.; Zhang, X.; Wu, C.; Lu, D.; Guo, G.; Mao, X.; Zhang, Y.; Wang, D.C.; Li, D.; Zou, Q. Expression, purification and characterization of arginase from *Helicobacter pylori* in its apo form. *PLoS One.* **2011**, *6*(10), e26205. doi: 10.1371/journal.pone.0026205.
40. Chinard, F.P. Photometric estimation of proline and ornithine. *J. Biol. Chem.* **1952**, *199*(1), 91–95.
41. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*(1), 265–275.
42. White, T.J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A guide to methods and applications/Academic Press, Inc*. **1990**.
43. Amarowicz, R.; Naczk, M. Gel filtration of condensed tannins and phenolic acids of canola hulls on Sephadex G-25 and G-50. *Polish J. Food Nutr. Sci.* **2006**, *15(4)*, 415–418.
44. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **1970**, *227(5259)*, 680–685. doi: 10.1038/227680a0.

45. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **1983**, *65(1-2),* 55–63. doi: 10.1016/0022-1759(83)90303-4.
46. Ahmed, A.H.H.; Mohamed, M.F.A.; Allam, R.M.; Nafady, A.; Mohamed, S.K.; Gouda, A.E.; Beshr, E.A.M. Design, synthesis, and molecular docking of novel pyrazole-chalcone analogs of lonazolac as 5-LOX, iNOS and tubulin polymerization inhibitors with potential anticancer and anti-inflammatory activities. *Bioorg. Chem.* **2022**, *129,* 106171. doi: 10.1016/j.bioorg.2022.106171.
47. Alaufi, O.M.; Noorwali, A.; Zahran, F.; Al-Abd, A.M.; Al-Attas, S. Cytotoxicity of thymoquinone alone or in combination with cisplatin (CDDP) against oral squamous cell carcinoma in vitro. *Sci. Rep.* **2017**, *7(1),* 13131. doi: 10.1038/s41598-017-13357-5.
48. Mohamed, G.A.; Al-Abd, A.M.; El-Halawany, A.M.; Abdallah, H.M.; Ibrahim, S.R.M. New xanthones and cytotoxic constituents from *Garcinia mangostana* fruit hulls against human hepatocellular, breast, and colorectal cancer cell lines. *J. Ethnopharmacol.* **2017**, *198,* 302–312. doi: 10.1016/j.jep.2017.01.030.
49. Bashmail, H.A.; Alamoudi, A.A.; Noorwali, A.; Hegazy, G.A.; AJabnoor, G.; Choudhry, H.; Al-Abd, A.M. Thymoquinone synergizes gemcitabine anti-breast cancer activity via modulating its apoptotic and autophagic activities. *Sci. Rep.* **2018**, *8(1),* 11674. doi: 10.1038/s41598-018-30046-z.
50. Fekry, M.I.; Ezzat, S.M.; Salama, M.M.; Alshehri, O.Y.; Al-Abd, A.M. Bioactive glycoalkaloides isolated from *Solanum melongena* fruit peels with potential anticancer properties against hepatocellular carcinoma cells. *Sci. Rep.* **2019**, *9(1),* 1746. doi: 10.1038/s41598-018-36089-6.
51. Hernández, V.M.; Arteaga, A.; Dunn, M.F. Diversity, properties and functions of bacterial arginases. *FEMS Microbiol. Rev.* **2021**, *45*(6), fuab034. doi: 10.1093/femsre/fuab034.
52. Alzahrani, N.H. Isolation, screening, and characterization of l-arginase producing soil fungi in Saudia Arabia. *World J. Environ. Biosci*. **2020**, *9*(3), 45-49.
53. Gautam, H.; Kumari, N.; Bansal, S. Enhancing the Production of Therapeutic Enzyme Arginase from *Lactobacillus acidophilus* Using Response Surface Methodology. *Braz. Arc. Biol. Technol.* **2022**, *65*, e22210041.
54. Nadaf, P.; Vedamurthy, A.B. Optimization of l-arginase production by *Pseudomonas sp.* Strain PV1 under submerged fermentation. *Int. J. Sci. Technol. Res.* **2020**, *9*(1), 4390–4394.
55. Ibrahim, H.O.; Agbaje, A.B.; Afolabi, H.A.; Usman, H. Effects of temperature, pH, and agitation rate on the production of microbial L-arginase. *J. Pharm. Biol. Sci.* **2018**, *13*(5), 01–12.
56. Gumashta, R.; Jain, R.; Pandey, A.; Tiwari, P.; Jain, A. Applicability of Native L-Arginase produced by *Streptomyces plicatus* KAR73 as Antineoplastic Agent. *J. Sci. Industrial Res.* **2021**, *80*(10), 841–849.
57. Dzikowska, A.; Le Caer, J.P.; Jonczyk, P.; Węgleński, P. Purification of arginase from *Aspergillus nidulans*. *Acta Biochim. Pol.* **1994**, *41*(4), 467–471.
58. Yu, J.J.; Park, K.B.; Kim, S.G.; Oh, S.H. Expression, purification, and biochemical properties of arginase from *Bacillus subtilis* 168. *J. Microbiol.* **2013**, *51(2),* 222–228. doi: 10.1007/s12275-013-2669-9.
59. Brusdeilins, M.; Hapke, C.; Huberts, H.H.; Schumacher, K. Identification of the apparently lymphocyte-specific human liver-derived inhibitory protein (LIP) as cytoplasmic liver L-arginase. *J. Immunol.* **1983**, *131*(5), 2427–2431.
60. Hwang, H.J.; Kim, E.H.; Cho, Y.D. Isolation and properties of arginase from a shade plant, ginseng (*Panax ginseng* C.A. Meyer) roots. *Phytochem.* **2001**, *58*(7), 1015–1024. doi: 10.1016/s0031-9422(01)00392-2.
61. Spolarics, Z.; Bond, J.S. Multiple molecular forms of mouse liver arginase. *Arch. Biochem. Biophys.* **1988**, *260(1),* 469–479. doi: 10.1016/0003-9861(88)90471-7.
62. Green, S.M.; Eisenstein, E.; McPhie, P.; Hensley, P. The purification and characterization of arginase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1990**, *265(3),* 1601–1607.
63. Kanda, M.; Ohgishi, K.; Hanawa, T.; Saito, Y. Arginase of *Bacillus brevis* Nagano: purification, properties, and implication in gramicidin S biosynthesis. *Arch. Biochem. Biophys.* **1997**, *344(1),* 37–42. doi: 10.1006/abbi.1997.0174.
64. Boutin, J.P. Purification, properties and subunit structure of arginase from Iris bulbs. *Eur. J. Biochem.* **1982**, *127(2),* 237–243. doi: 10.1111/j.1432-1033.1982.tb06861.x.
65. Husain, I.; Bala, K.; Wani, A.; Makhdoomi, U.; Malik, F.; Sharma, A. Arginase purified from endophytic *Pseudomonas aeruginosa* IH2: Induce apoptosis through both cell cycle arrest and MMP loss in human leukemic HL-60 cells. *Chem. Biol. Interact.* **2017**, *274,* 35–49. doi: 10.1016/j.cbi.2017.07.001.

 doi:10.20944/preprints202503.0811.v1

66. Yusof, N.Y.; Quay, D.H.X.; Kamaruddin, S.; Jonet, M.A.; Md Illias, R.; Mahadi, N.M.; Firdaus-Raih, M.; Abu Bakar, F.D.; Abdul Murad, A.M. Biochemical and in silico structural characterization of a cold-active arginase from the psychrophilic yeast, *Glaciozyma antarctica* PI12. *Extremophiles.* **2024**, *28(1)*, 15. doi: 10.1007/s00792-024-01333-7.
67. Nakamura, N.; Fujita, M.; Kimura, K. Purification and Properties of L-Arginase from *Bacillus subtilis*. *Agric. Biol. Chem.* **1973**, *37(12)*, 2827–2833. doi: 10.1080/00021369.1973.10861080.
68. Chen, C.L.; Hsu, S.C.; Ann, D.K.; Yen, Y.; Kung, H.J. Arginine Signaling and Cancer Metabolism. *Cancers (Basel).* **2021**, *13(14)*, 3541. doi: 10.3390/cancers13143541.
69. Prajapati, B.; Supriya, N.R. Review on anticancer enzymes and their targeted amino acids. *World J. Pharm. Res.* **2017**, *6(12)*, 268–284. doi: 10.20959/wjpr201712-9676.