



## Isolation, Screening, and Characterization of L-Arginase Producing Soil Fungi in Saudia Arabia

Nourah Hassan Alzahrani

Department of Biology Sciences, Faculty of Science, University of Jeddah, Jeddah, KSA.

### ABSTRACT

Therapeutic enzymes have important roles in cancer treatment. Thus, our research target was to isolate fungi from Saudi soil; so examined their ability to produce L-arginase and identified them by 18S rRNA. From among the 20 fungal isolates recovered from the rhizosphere in the north and south of the city of Jeddah Province in Saudi Arabia, eight isolates produced high quantities of L-arginase. Highly produced fungal isolates were identified in the 18S rRNA sequence using DNA sequences of ITS1 and ITS4 and deposited in GenBank under joint numbers such as *Aspergillus oryzae*XR\_002735719.1, *Penicillium oxalicum* MH156644.1, *Penicillium oxalicum* MH171483.1, *Aspergillus niger* MF093522.1, *Rhizopus oryzae* AB250174.1, *Aspergillus fumigatus* KJ746594.1, *Fusarium equiseti* KY945342.1, and *Rhizopus oryzae* AB250174. The highest producer of the enzyme was *Fusarium equiseti*KY945342.1 (2.492IU/mg & 1.952 IU/mg by *Penicillium oxalicum* MH156644.1).

**Keywords:** Fungi, L-arginase, 18S rRNA

**Corresponding author:** Nourah Hassan Alzahrani

**e-mail** ✉ [nhalzahrani@uj.edu.sa](mailto:nhalzahrani@uj.edu.sa)

**Received:** 20 May 2020

**Accepted:** 24 September 2020

### 1. INTRODUCTION

L-Arginase (argininetransaminaseEC3.5.3.1) is a manganese-contained enzyme catalyzing L-arginine deamination to L-ornithine and urea and can be an anti-cancer agent and against tumors argininosuccinate synthase (Unissa et al., 2015). High rates of microbial generators and thermal reduction stabilities for enzyme could be the main biochemical barriers (El-Sayed et al., 2014). L-arginase is a therapeutic enzyme for orthostatic carcinogenic carcinomas, while low anti-cancer activities, low protein tolerances, and shorter serum half-life are its shortcomings (Hussain et al., 2017). L-arginase has strong anti-cancer activity against many tumors, by depleting arginine, which stops the cell cycle in stage G1. Arginine deiminases purify heat-loving *Aspergillus fumigatus* KJ434941 to its electrode homogeneity by 5.1 times, with a 50KD molecular subunit, a new thermostatic, less antigen and a strong candidate for clinical trials (Elsayed et al., 2015). L-arginase has counteracted a wide range of arginine auxotrophic cancers as hepatocellular carcinomas (Yoon et al., 2007; Kim et al., 2009; Glazer et al., 2011; Yau et al., 2010).

Many microbial strains have potentially produced enzyme and could have isolated and characterized. Enzymes can be produced by several microbial strains differed in physiological, biochemical, catalytic, and immunological characterization which leads to the continuous screening programsto isolate the novel microbial strains which might produce effective enzymes with little limitations in the usage sector (Unissa et al., 2015).

### 2. MATERIALS AND METHODS

#### 2.1. Collection of Samples

Different soil samples were collected from some plant rhizosphere from Jeddah province(Khulais &Bahra) from the surface and depth of 10 to 20 cm and were serially diluted. Aliquots of 0.1ml of the suspension were evenly spread on Modified Czapek Dox Agar. The plates were incubated at 28C for 72h.

#### 2.2. Qualitative Screening of L-arginase Production of Isolated Bacteria:

Fungal isolates were appropriately screened for L-argininase production capacity by a rapid qualitative screening of the plates using (Czapek yeast extract agar medium) and phenol red (2g in 100ml ethanol) with last concentrations of 0.009% pre-poured plates as an indicator, yellow color zone around the colonies were reflected L-argininase enzyme and incubated in inverted position/ 28±2°C for 48 to 96 hours. Production colonies were purified and sub-cultured from each plate for further studies.

#### 2.3. L-Arginase Assay:

Arginase activity was measured in terms of the hydrolysis rate from L-arginine to l-ornithine and urea by measuring the amount of urea released in the reaction. Urea was measured spectrophotometrically by the Archibald method (Unissa et al., 2015). Urea gave a dark pink color when heated in alcohol with  $\alpha$ -isonitrosopropiophenone (0.2 ml glycine solution, 0.5 ml diluted enzyme & 0.1 ml manganese chloride). After incubation at 37°C for 10 minutes, check the enzyme started by adding 0.1 ml of arginine was a mixture incubated again at 37°C. After 30

minutes, the examination was stopped by adding 1 ml of a perchloric acid solution and mix.0.1 ml from 4% α-isonitrosopropiophenone added and tubes covered well and put in a boiling water bath. After 1 hour, the solution was removed and cooled at room temperature and was absorbed in 540 nm. The urea produced from the prepared urea curve was estimated by changing the urea concentration between 0.1 to 1microliter\mol and a visual density graph was drawn against the urea concentration to extrapolate the arginase activity. The expression of one unit of L-arginase through the amount of enzyme which releases 1 μM of Lornithine under examination standard, based on the original Lornithine (El-Sayed, 2014).

**2.4. Calculating Enzyme Activities Formula:**

Enzyme activity (μ/ml) = μmoles of urea released

time of enzyme action x volume of the enzyme (ml).

**2.4.1. Molecular Identification**

**2.4.1.1. Genomic DNA Extraction:**

DNA isolated from fungal isolates using DNeasy<sup>®</sup> fungi Mini Kit

**2.4.1.2. PCR**

The DNA-amplified product was used for ITS1 and ITS4 use in PCR technology. The primer is designed according to Leio et al. (2016).

**ITS1** (FORWARD) TCCGTAGGTGAACCTGCGG  
**ITS4** (REVERSE) TCCTCCGCTTATTGATATGC

**2.4.1.3. Database Research**

The obtained sequence results were subject to a BLAST search program to identify similarities with genes in the gene bank.

**3. RESULTS:**

**3.1. Isolation of Fungal Strains**

Based on serial dilution 22 fungal isolates were obtained.

**3.2. Screening and Qualitative Assay of L-enzymes Activity of Fungal Isolates by Rapid Plate Method:**

The primary screening of fungal isolates show their abilities for L-arginase activities carried out on modified MCD with arginine as nitrogen source using phenol red as the pH indicator.

Isolates show a yellow zone around colonies indicated decreasing in pH. The pink color turned to yellow in medium supplemented with L-Gln which means producing L-arginase, the yellow zone has appeared in plate number AAB1,AAD2,AAD4,AAB1,AAD3, AFA1, AFA3(Fig.1).

**3.3. Quantitative Assay of L-Arginase Activity Produced by the Selected Fungal Isolates by Nessler Reaction:**

The selected fungal isolates were assayed quantitatively for L-arginase production for 7 days of incubation. It was noticed that all the fungal isolates produced L-arginase after three days of incubation, these amounts increased by increasing the incubation period, but the maximum amount of the enzyme was accumulated after five days of incubation by all of the fungal isolates. Results in Fig. (2) show the highest producer AFA3 2.492IU/mg, followed by isolate AAB4 1.952 IU/mg.

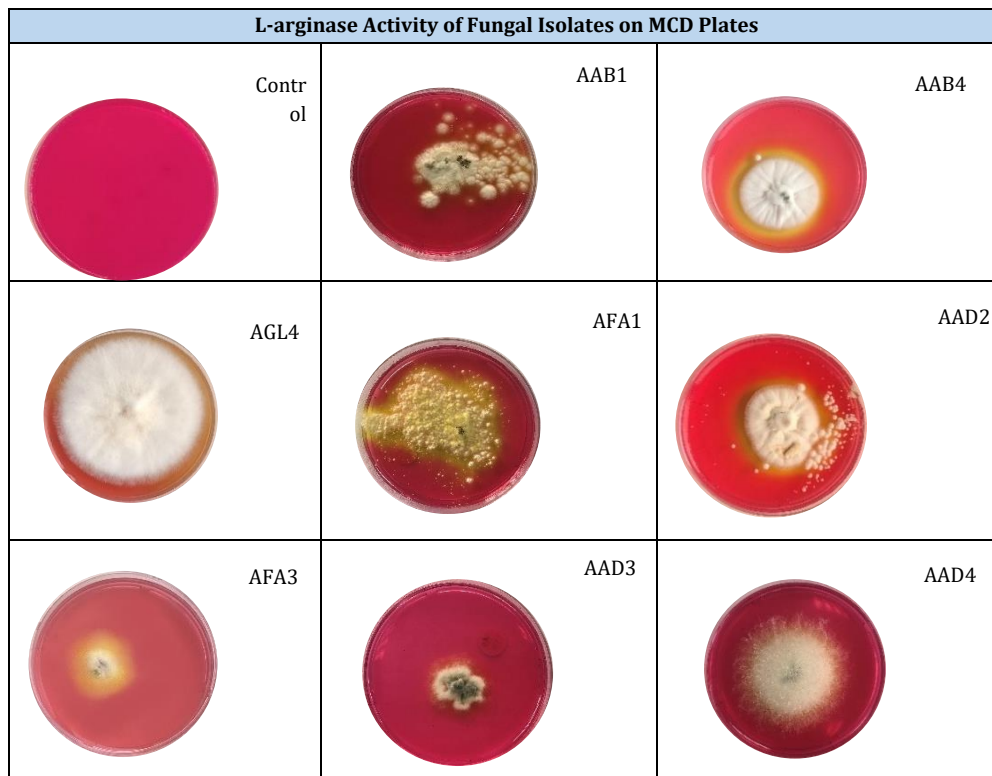


Figure 1: Qualitative Assay for L-arginase Activity of Fungal Isolates on MCD Plates

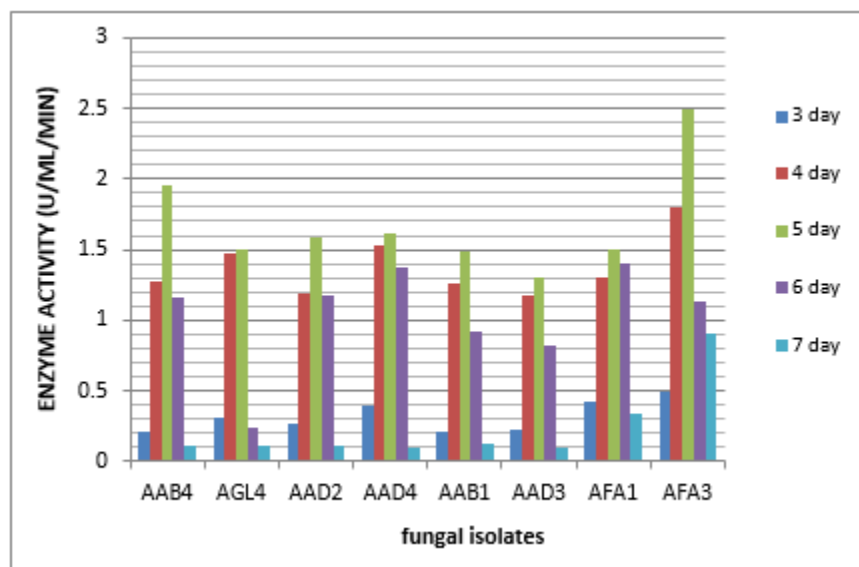


Figure 2: Quantitative Assay of L-Arginase Activity Produced by the Selected Fungal Isolates by Nessler Reaction

### 3.4. Molecular Identification of Fungal Isolates

The fungal isolates were subjected to molecular analysis and identification using DNA sequencing of ITS1 and ITS4 primers (Cheng et al., 2016). First, the 750 bp amplicons were

separated on 1% agarose gels to prove their accuracy and specificity. Then, the fragments with the molecular size representing the ITS4 and ITS1 primers were purified for sequencing (Table 1).

Fungal Isolate	Name and Accession of No. of the most related Strain in NCBI GenBank	Fungal Isolates	Identity(%)	Coverage (%)
AAB1	XR_002735719.1	<i>Aspergillus oryzae</i>	100%	100%
AAB4	MH156644.1	<i>Penicillium oxalicum</i>	100%	100%
AAD2	MH171483.1	<i>Penicillium oxalicum</i>	100%	100%
AAD3	MF093522.1	<i>Aspergillus niger</i>	100%	100%
AAD4	AB250174.1	<i>Rhizopus oryzae</i>	100%	100%
AFA1	KJ746594.1	<i>Aspergillus fumigates</i>	100%	100%
AFA3	KY945342.1	<i>Fusarium equiseti</i>	100%	100%
AGL4	AB250174.1	<i>Rhizopus oryzae</i>	100%	100%

Table 1: DNA Sequencing of 18s rRNA Using Universal Primer for Fungal Isolates

## 4. DISCUSSION:

Screening enzymatic activity by various methods such as measuring the color change region or the filtration region on agar with the completion of the appropriate substrate was used (Balagurunathan et al., 2010b, Nagaraju and Ram, 2019). Phenol red shows the basic pH change which is red in alkaline condition turning to yellow under acidic condition (Theantana et al., 2007). Isolates show yellow color surrounding colonies which indicate pH reduction; these results agree with the observation of Gulati et al. (1997) and Jyothi (2011). Nadaf et al. (2019) have screened newer strains able to fabricate new L-arginase that had bug yield and few adverse effects. Twenty isolates confirmed a red zone around the colonies, indicating that they produce L-arginase. There are only a few investigations for resources for the manufacturing of this enzyme. There are only a few investigations about sources of producing this enzyme. Pink

color surrounded colonies are due to the liberation of L-arginase (Zhang et al., 2013).

According to Nocker et al. (2004) amplification, ITS1, and ITS4 sequences of fungi can produce 750bp amplicon. The DNA sequences were analyzed using Blast alignment tools of GenBank and showed that the eight isolates identified as *Aspergillus oryzae*XR\_002735719.1 were, *Penicillium oxalicum* MH156644.1, *Penicillium oxalicum* MH171483.1, *Aspergillus niger* MF093522.1, *Rhizopus oryzae* AB250174.1, *Aspergillus fumigates* KJ746594.1, *Fusarium equiseti* KY945342.1, *Rhizopus oryzae* AB250174.1 with the similarity percentages of 100%. Using ITS1 and ITS4 as semi-nested PCR performed positively on samples by 18S rRNA PCR (Ferrer et al., 2001). Our results are consistent with those of Anand et al., (2001) who found PCR examination effectiveness compared to traditional methods. The PCR-based test is a fast, sensitive, and useful method for detecting fungal isolates.

Enzyme activities were determined in cultures both using Nessler's reagent (Dhevagi and Poorani, 2006). All the fungal isolates produced L-arginase after three days of incubation, these amounts increased by increasing the incubation periods, but the maximum amount of the enzyme was accumulated after five days of incubation by all of the fungal isolates; the highest producer of the enzyme was *Fusarium equiseti* KY945342.1 AFA3 (2.492 IU/mg and 1.952 IU/mg by *Penicillium oxalicum* MH156644.1 (AAB4). Previous studies have reported the highest L-arginase activity of *Bacillus thuringiensis* SK 20.001, and *Rummelii baciluspycnus* SK31.001 that were 8.4 U/ml at 24 h and 40.2 U/ml at 16 h respectively with an optimal temperature of 80°C (Nadaf et al., 2019). Very few reports on prokaryotic L-arginase have been characterized (Zolfaghari et al., 2019) such as *Bacillus brevis* (Kanda et al., 1997), *B. anthracis* (Viator et al., 2008), and *Helicobacter pylori* (Zhang et al., 2011). Studies of fungi enzyme such as *Neurospora crassa* (Borkovich and Weiss 1987), *Aspergillus nidulans* (Borsuk et al., 1999), and *Penicillium chrysogenum* (El-Sayed et al., 2014) have been reported. Microorganisms can be easily cultivated and manipulated, hence they are considered as good sources for enzymes production (Kumar and Sobha 2012).

## 5. CONCLUSION:

The current study refers to the result of testing the production of the enzyme L-arginase by twenty fungal isolates obtained from different soil samples collected from the rhizosphere of a number of plants in Jeddah, Saudi Arabia. The quantitative and qualitative results showed the high ability of the fungal isolates to produce the enzyme which is one of the most important candidate enzymes for use in treating types of cancer. Fungal isolates were molecularly defined by 18S rRNA. It can be concluded from this study that the rooting fungicide activity test has enzyme activity and the extract is expected to be used as therapeutic, industrial and anti-tumor agents.

## REFERENCES

- Anand, A., Madhavan, H., Neelam V., & Lily, T. (2001). Use of polymerase chain reaction in the diagnosis of fungal endophthalmitis. *Ophthalmology*, 108(2):326-330.
- Balagurunathan, R., Radhakrishnan, M. & Somasundaram, S. (2010). L-glutaminase producing Actinomycetes from marine sediments selective isolation, semi-quantitative assay, and characterization of potential strain. *Aust. J. Basic Appl. Sci.* 4, 698-705.
- Borkovich, K.A., & Weiss, R.L. (1987). Purification and characterization of arginase from *Neurospora crassa*. *Journal of Biological Chemistry*, 262: 7081-86.
- Borsuk, P., Dzikowska, A., Empel, J., Grzelak, A., Grzeskowiak, R., & Weglenski, P. (1999). Structure of the arginase coding gene and its transcript in *Aspergillus nidulans*. *Acta Biochimica Polonica*; 46: 391-03.
- Cheng, T.; Xu, C.; Lei, L.; Li, C.; & Yu Zhang, Y. (2016). Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. *Molecular Ecology Resources*. 16(1): 138-149.
- Dhevagi, P., & Poorani, E. (2006). Isolation and characterization of L-asparaginase from marine actinomycetes, *Ind J Biot*, 5:514-520.
- El-Sayed, A. S., Shindia, A. A., Diab, A. A., & Rady, A. M. (2014). Purification and immobilization of L-arginase from thermotolerant *Penicillium chrysogenum* KJ185377. 1; with unique kinetic properties as thermostable anticancer enzyme. *Archives of Pharmacal Research*, 37(10):1-10.
- El-Sayed, S. A. Hassan, M. N. & Nada, H.M.S., (2015). Purification, immobilization, and biochemical characterization of L-arginine deiminase from thermophilic *Aspergillus fumigatus* KJ434941: anticancer activity *in vitro* *Biotechnol.* (31)396-405.
- Ferrer, C., Colom, F., Frases, S., Mulet, E., Abad, J.L., & Alio, J.L. (2001). Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J Clin Microbiol.* 39(8):2873-2879.
- Glazer ES, Stone EM, Zhu C, Massey KL, Hamir AN, & Curley SA. (2011). Bioengineered human arginase I with enhanced activity and stability controls hepatocellular and pancreatic carcinoma xenografts. *Transl Oncol.*; 4(3): 138-146.
- Gulati, R. Saxena, R. K., & Gupta, R. (1997). A rapid plate assay method for screening L-asparaginase producing microorganisms, *Lett Appl Microbiol*, 24: 23-26.
- Husain, I., Bala, K., Abubakar Wani, A., Makhdoomi, U., Malik, F. & Sharma, A., (2017). Arginase purified from endophytic *Pseudomonas aeruginosa* IH2: Induce apoptosis through both cell cycle arrest and MMP loss in human leukemic HL-60 cells. *Chemico-Biological Interactions*, 274 (25): 35-49.
- Jyothi, H., Shivaveerakumar & Vandana, R. (2011). Production of L-Glutaminase by *Pseudomonas* VJ-6. *Research Journal of Biotechnology*, 6, 42-49.
- Kanda M, Ohgishi K, Hanawa T, & Saito Y. (1997). Arginase of *Bacillus brevis* Nagano: Purification, properties, and implication in gramicidin S biosynthesis. *Archives of Biochemistry and Biophysics* 344: 37-42.
- Kim, R. H., Coates, J. M., Bowles, T. L., McNerney, G. P., Sutcliffe, J., Jung, J. U., ... & Kung, H. J. (2009). Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. *Cancer research*, 69(2), 700-708.
- Kumar, D., & Sobha, K. (2012). L-Asparaginase from microbes: a comprehensive review. *Advances in BioResearch*, 3: 137-57.133:S68-S75.
- Liao, C. S., & Cheng, J. X. (2016). In situ and in vivo molecular analysis by coherent Raman scattering microscopy. *Annual Review of Analytical Chemistry*, 9, 69-93.
- Nadaf, P.D.; Kulkarni, A. G. & Vedamurthy, A.B. (2019). Isolation, Screening, And Characterization Of L-Arginase Producing Soil Bacteria. *IJPSR*, 10(7): 3440-3444.
- Nagaraju, K. & Ram, M. R.' (2019). *Bacillus cereus* Rn-6, Potential L-Glutaminase Producing Bacteria From Forest Soils. *Life Science Informatics Publications*. 5(4):134-142.
- Nocker, A., Lepo, J. E. & Snyder, R. A. (2004). Influence of an oyster reef on the development of the microbial

- heterotrophic community of an estuarine biofilm. *Appl. Environ. Microbiol.*, 70, 6834-6845.
21. Theantana, T., Hyde, K. & Lumyong, S. (2007). Asparaginase production by endophytic fungi isolated from some Thai medicinal plants. *Current Applied Science and Technology*, 7, 13-18.
  22. Unissa, R., Sudhakar, M., Reddy, A. S. K. J. W. J. o. P., & Sciences, P. (2015). Selective isolation and molecular identification of L-arginase producing bacteria from marine sediments. 4(06), 998-1006.
  23. Viator, R.J., Rest, R.F., Hildebrandt, E., & McGee, D.J. (2008). Characterization of *Bacillus anthracis* arginase: effects of pH, temperature, and cell viability on metal preference. *BMC Biochemistry*, 9: 1-14.
  24. Yau, C. C., Chan, P., Pang, R., Chan, W., Cheng, P. N., & Poon, R. (2010). A phase I study of recombinant human arginase I (rhArgI) for patients with advanced hepatocellular carcinoma. *Journal of Clinical Oncology*, 28(15\_suppl), e13503-e13503.
  25. Yoon, C. Y., Shim, Y. J., Kim, E. H., Lee, J. H., Won, N. H., Kim, J. H., ... & Min, B. H. (2007). Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase. *International journal of cancer*, 120(4), 897-905.
  26. Zhang T, Guo Y, Zhang H, Mu W, Miao M, & Jiang B. (2013). Arginase from *Bacillus thuringiensis* SK 20.001: Purification, characteristics, and implications for L-ornithine biosynthesis. *Process Biochemistry*, 48: 663-668.
  27. Zhang, J, Zhang, X, Wu, C., Lu, D., Guo, G., Mao, X., Zhang, Y., Wang, D.C., Li, D. & Zou, Q. (2011). Expression, purification, and characterization of arginase from *Helicobacter pylori* in its apo form. *PLoS One*, 6: 1-8.
  28. Zolfaghar, M., Amoozegar, M.A., Khajeh, K., Babavalian, H. & Tebyanian, H. (2019). Isolation and screening of extracellular anticancer enzymes from halophilic and halotolerant bacteria from different saline environments in Iran. *Molecular Biology Reports*, 1-12.