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# EXTRACTION AND QUANTIFICATION OF L-ARGINASE PRODUCED BY *ALCALIGENES FAECALIS*

*AbdulGaniy B. Agbaje*[*1], Ganiyu P. Oyeyiola[2] and Ismaila O. Sule[2]*

***Address(es):***
[1]Department of Biological Sciences, Al-Hikmah University, Ilorin, Nigeria.
[2]Department of Microbiology, University of Ilorin, Ilorin, Nigeria.

*Corresponding author: tundeag@gmail.com

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**ABSTRACT**

L-arginase is one of the enzymes that have been used as therapy for cancer treatment. L-arginase catabolise L-arginine and reduce tumour growth by making them susceptible to other anti-cancer drugs. Previous works have focused on the use of radiotherapy and chemotherapy for the treatment of metastatic cells. However, both radiotherapy and chemotherapy have been reported to have severe side effects. This necessitates the development of other agents such as enzymes with minimal side effects. This study therefore examined the production of L-arginase and determination of the optimum fermentation conditions. Isolated bacteria were screened using rapid plate assay in order to determine their ability to produce L-arginase. Medium component includes L-arginine and relevant salts. Enzyme activity was determined using calorimetric assay. The promising isolate was selected and primed for identification using molecular technique. The highest zone of colour change was produced by *Alcaligenes faecalis*. The optimum incubation period was 60 hours. Optimum agitation rate was 150 revolution per minute. Enzyme yield was 163±0.78 U/mL when optimum fermentation conditions were used for enzyme production. The molecular weight of L-arginase determined was 120 kilodalton. *Alcaligenes faecalis* isolated during this work can be taken as a promising isolate for the large-scale production of L-arginase. There should be further search for other microorganisms with the potential to produce other industrially important enzymes.

**Keywords:** production; L-arginase; *Alcaligenes faecalis*, fermentation; temperature; L-arginine, enzymes

## INTRODUCTION

L-arginase contains manganese and it is the final enzyme in the urea cycle of the liver. L-arginase plays a very important role in the removal of ammonium ions from the body system **(Morris, 2002)**. L-arginine is produced through L-citrulline by the actions of argininosuccinate lyase and argininosuccinate synthase. The amino acid L-arginine is converted by L-arginase into urea and L-ornithine **(Ruth *et al.*, 2015)**.

Two isoforms of L-arginase are distributed in different tissues and subcellular locations that exist in mammals. L-arginase I is called the cytosolic form and it has been detected in erythrocytes and hepatic cells. It plays an important role in the process of ureogenesis. L-arginase II is an enzyme that is associated with the mitochondria. It has been found in kidney tissues, macrophages, and skeletal muscles. L-arginase II play significant roles in the synthesis of polyamines and ornithine **(Das and Prasad, 2010)**.

Certain cancer cells require L-arginine for proliferation and metastasis **(Cavanaugh and Nicolson, 2000)**. However, studies have proved that L-arginine deprivation therapy has been shown to be an effective means for the treatment of some forms of cancer. L-arginase has been used successfully in arginine-deprivation cancer treatment method. This type of cancer treatment also makes tumour cells susceptible to other anti-cancer drugs **(Patil *et al.*, 2016)**. Clinical trials have been used to demonstrate that human hepatocellular carcinomas (HCC) cells that are surviving due to the presence of arginine reduced in size when L-arginase was used as therapy. Selective removal of L-arginine using L-arginase enzyme may be used for treating patients affected with metastatic melanoma **(Conners, 2016)**.

It had been pointed that, amino acids contribute significantly to the regulation of cellular metabolism in normal and malignant cells. Amino acids also play key roles in the synthesis of hormones and peptides, and they also function in gene modulatory and gene expression **(Wu, 2013)**. Amino acids regulate RNA synthesis via mechanisms such as regulation of transcription factors **(Luo *et al.*, 2013; Loreni *et al.*, 2014; Proud, 2014)**. Most tumour cells alter their metabolic pathway. This necessitated additional nutrients requirement for survival and maintenance of biosynthesis and ATP production **(Locasale and Cantley, 2011; Cantor and Sabatini 2012; Ferreira *et al.*, 2012)**. The supply of nutrients within the cell is usually not adequate during high growth rate. Some tumour cells therefore need nutrient supply from outside the cells in order to meet the nutrients and energy requirements. The cancer cells then become auxotrophic for nutrient and energy. Depriving the tumour cells of amino acids may results into growth inhibition and death of the tumour cells **(Yamamoto *et al.*, 2014; Gelb *et al.*, 2015)**. Incorporating enzymes that can deprive tumour cells of amino acids exogenously may be adopted as an effective strategy for cancer treatment **(Graham, 2003; Nadaf *et al.*, 2019)**. This current study examined the potential of *Alcaligenes faecalis* isolated from the rhizosphere of maize plant to produce L-arginase that can be used exogenously for the depletion of L-arginine. The various fermentation conditions were also optimised.

## MATERIALS AND METHODS

### Isolation of bacteria

Bacteria were isolated from the rhizosphere of *Zea mays*. During the isolation process a series of five test tubes was prepared for the isolation of bacteria and fungi. Nine millilitres (9 mL) of sterile distilled water was put into each of the test tubes. To the first test tube, one gram of the soil sample was added to give a dilution of $10^{-1}$. The content was shaken properly and 1 ml of the solution was added to the next test tube containing 9 mL of sterile distilled water to make a concentration of $10^{-2}$. This process of serial dilution was repeated up to $10^{-5}$ dilution. Zero point one millilitre (0.1 mL) of the $10^{-4}$ dilution was cultured on the nutrient agar plates using the spread plate technique. A sterile spreader was used to spread the inoculum over each plate. The plates were incubated upside down at 37°C for 24 hours. These plates were examined after 24 hours for the isolation of bacteria **(Nadaf *et al.*, 2019)**.

### Screening of isolated bacteria for enzymes production by rapid plate assay

Twenty-nine bacterial (29) isolates were screened for the production of L-arginase using the rapid plate assay as described by **Umayaparvathi *et al.* (2013)**. Agar medium containing the L-arginine as nitrogen and carbon source was used. Chromogenic change from yellow to pink on the agar plates was used as an indication of enzyme production **(Sabu *et al.*, 2005)**. The agar medium contained 4% (w/v) of L-arginine, $KH_2PO4$ (0.2% w/v), $CaCO_3$ (0.002% w/v), $FeSO_4.7H_2O$ (0.001% w/v), $KNO_3$ (0.2% w/v), NaCl (w/v 0.5%), $MgSO_4.7H_2O$ (0.005% w/v), and 15g/L agar-agar with pH adjusted to 7.4. About 0.012 (g/L) of 2.5 % of phenol red was added as pH indicator. The plates were inoculated and incubated for 24 hours at 37°C. Colonies that showed highest chromogenic zones were selected and maintained on the agar slants at 4°C **(Hymavathi *et al.*, 2009; Abdallah *et al.*, 2012; Wakil and Adelegan, 2015)**.

**Molecular characterisation of the bacterial isolate**

Promising bacterial isolate was identified using the polymerase chain reaction (PCR) technique. The isolate (B26) was identified using 16S ribosomal RNA (rRNA) sequencing method. AxyPrep Multisource Genomic DNA Miniprep Kit was used for isolating DNA in accordance with the manufacturer's instructions. The DNA was subjected to cocktail mix containing forward and reverse primers. After amplification and denaturation of the DNA annealing of primers was carried out at 56°C for 30 second and extension at 72°C for 45 seconds. Agarose gel (1.5%) was used to load the amplicon. The ladder used was 50bp from NEB. The base pair of the amplicon was about 1500bp. The PCR product was then purified. The purified product was loaded on 3130xl genetic analyzer from Applied Biosystems to produce the sequences. The 16S rRNA sequences were checked for comparison at the National Center for Bioinformatics (NCBI) GenBank.

**Phylogenetic analysis of the promising Isolate (Project Isolate)**

Phylogenetic tree construction and molecular evolutionary analyses were conducted using MEGA version X following the procedure described by **Tamura and Nei (1993)** and **Kumar *et al.* (2018)**. Sequence of the promising isolate and its closest relatives were used to construct a dendrogram tree.

**Enzyme production**

Enzyme production was the submerged fermentation method using a medium containing L-arginine (4% w/v), NaCl (0.5% w/v), $MgSO_4$.7H2O (0.005% w/v), $KH_2PO4$ (0.2% w/v), $KNO_3$ (0.2%w/v), $FeSO_4$.7H2O (0.001% w/v) and $CaCO_3$ (0.002% w/v), pH was adjusted to 7.4. Culture was dispensed in sterile deionised water and to obtain cell density equal to 0.5 McFarland standard **(Veerapagu *et al.*, 2013)**. The fermentation was sustained for 72 hours at 37°C using 200 rpm in a shaking incubator. After fermentation, the culture was centrifuged for 20 minutes at 10,000 rpm under at 4°C. The supernatant was cell free and was recorded as crude enzyme **(Das and Prasad, 2010; Umayaparvathi *et al.*, 2013)**.

**Purification of the L-arginase produced**

The supernatant which contained the crude enzyme was precipitated using ammonium sulphate. Precipitated enzyme was dispensed into 20 mM potassium phosphate buffer and dialyzed against same buffer. Protein extraction and dialysis was carried out using dialysis cassette. Extracted enzyme was centrifuged at 16,000 xg for 5 minutes to pellet and the pellet was repeatedly washed with 1x Phosphate-buffered saline (PBS) until the supernatant becomes clear.

**Determination of molecular weight of L-arginase produced**

Molecular weight determination was carried out using the SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Polyacrylamide gel was arranged using 5% stacking and 12% separating gel (0.75 mm). The protein extract was centrifuged for 5 minutes at 16,000 xg. The supernatant was dispensed into a sterile tube. Adequate 5x SDS buffer was added and the incubation was carried out for 5 minutes at 95 °C. Initially stained protein ladder (4 µl and 8 µl) of the enzyme extract was placed on slots of the gel in an electrophoresis chamber. The chamber is filled with electrophoresis buffer. The gel was maintained for 1 hour at 200 V. Immediately after the process of electrophoresis, the resultant gel was stained using Coomassie Brilliant Blue R-250. Molecular weight was measured by comparing the migration distances of standard proteins (markers) along the length of ladder **(Aley *et al.*, 1986; Ansorge *et al.*, 1996; Das and Prasad, 2010; Umayaparvathi *et al.*, 2013; Reddy *et al.*, 2016)**.

**Determination of L-arginase activity**

About 2.0% w/v of L-arginine was used during the determination of enzyme activity. One milliliter (1 mL) of the purified enzyme was added to 1 mL of L-arginine in the presence of 50 mM Tris-HCl. The pH was moved to 8.5 and the set-up was incubated for 10 minutes at 30°C. The incubation process was halt by adding 2 mL of 0.4 M trichloroacetic acid. The precipitate was then removed through centrifugation for 10 minutes at 10,000 rpm. One millilitre (1 mL) of the resultant supernatant was neutralized using 0.4 M sodium carbonate (5 mL) and the content was incubated with 1 mL of 1 N Folin Ciocalteu's reagent for 20 minutes at 40°C. Absorbance was determined using spectrophotometer. The amount of enzyme that caused absorbance (340 nm) to move at 0.1 per minute per mL was designated as enzyme activity under the assay conditions **(Das and Prasad, 2010; Geetha *et al.*, 2012; Umayaparvathi *et al.*, 2013; Reddy *et al.*, 2016)**.

**Determination of optimum fermentation parameters**

Optimum fermentation conditions were determined using monothetic approach. Enzyme activity was determined at different incubation periods, substrate concentrations, temperatures, agitation rates, pH, carbon source and nitrogen sources, sodium chloride concentrations and inoculum sizes. Experiments were carried out in triplicate for each of the fermentation parameters **(Das and Prasad, 2010; Umayaparvathi *et al.*, 2013; Wakil and Adelegan, 2015; Reddy *et al.*, 2016)**.

**Study of enzyme production using optimum fermentation parameters**

L-arginase was produced using the optimum fermentation parameters. The production was done in triplicate and the average enzyme activity was recorded **(Das and Prasad, 2010; Umayaparvathi *et al.*, 2013; Wakil and Adelegan, 2015; Reddy *et al.*, 2016)**.

**Statistical analysis**

Statistical analysis was done using ANOVA and Duncan's test ($p \leq 0.05$).

**RESULTS**

Some of the bacterial isolates showed chromogenic change (yellow to pink) around their colonies, indicating positive results. The highest chromogenic zone (60±0.05 mm) recorded was produced by isolate B26 which was eventually identified as *Alcaligenes faecalis*.

One bacterial isolate that produced significant zone of colour change during the rapid plate was selected for further studies. The enzyme was then produced using the submerged fermentation process. The results obtained during the optimization process showed that fermentation conditions such as pH, temperature, inoculum concentration, incubation period, substrate concentration, and sodium chloride concentration significantly affected production and activity of the enzyme as enzyme yield was 163±0.78 U/mL when optimum fermentation conditions were used for enzyme production

The selected bacterial isolate showed highest enzyme activity at temperature ranged between 27ºC and 37ºC. The highest L-arginase activity of 157 U/mL was recorded when lactose (4% w/v) was added to the fermentation medium.

The various conditions for the fermentation process were optimised in order to determine the best parameters for enzyme production. The optimum quantity of L-arginine for enzyme production was 6%. The results were as shown in table 2. The optimum incubation period was 60 hours as shown in figure 2 and the optimum temperature for the production of the L-arginase was 35ºC. The optimum agitation rate was 150 rpm as shown in figure 5 and the optimum concentration of NaCl concentration was 6% as shown in figure 9.

The enzyme was produced using optimum parameters determined. The yield of L-arginase was 163±0.78 U/mL. The molecular weight of L-arginase recorded was 120 kDa as shown in table 3.

**Table 1** Screening of different bacterial isolates for L-arginase production

| Bacterial Isolates | L-arginase (mm) |
|---|---|
| B1 | 20.00 ± 0.09 $^{d}$ |
| B2 | 0.00 ± 0.00 |
| B3 | 21.00 ± 0.02 $^{e}$ |
| B4 | 25.00 ± 0.04 |
| B5 | 20.00 ± 0.04 $^{d}$ |
| B6 | 25.00 ± 0.03 $^{g}$ |
| B7 | 0.00 ± 0.00 |
| B8 | 48.00 ± 0.03 $^{n}$ |
| B9 | 15.00 ± 0.01 |
| B10 | 35.00 ± 0.04 |
| B11 | 13.00 ± 0.01 $^{b}$ |
| B12 | 0.00 ± 0.00 |
| B13 | 0.00 ± 0.00 |
| B14 | 27.00 ± 0.01 $^{i}$ |
| B15 | 0.00 ± 0.00 |
| B16 | 0.00 ± 0.00 |
| B17 | 25.00 ± 0.01 $^{g}$ |
| B18 | 20.00 ± 0.04 $^{d}$ |
| B19 | 47.00 ± 0.03 $^{m}$ |
| B20 | 0.00 ± 0.00 |
| B21 | 30.00 ± 0.03$^{j}$ |
| B22 | 0.00 ± 0.00 |
| B23 | 15.00 ± 0.02 $^{c}$ |
| B24 | 10.00 ± 0.01$^{a}$ |
| B25 | 0.00 ± 0.00 |
| B26 | 60 ± 0.05$^{p}$ |
| B27 | 45.00 ± 0.04$^{l}$ |
| B28 | 50.00 ± 0.02 $^{o}$ |
| B29 | 0.00 ± 0.00 |

**Key:** 0.00 ±0.00 = No change observed

Same letter on mean values indicate that the values are not significantly different ($p \leq 0.05$) at $\alpha = 5\%$

**Identification of the bacterial isolate**

Isolate B26
*Alcaligenes faecalis* 16S ribosomal RNA sequence
CGGATTTGACGCTCGCGGGATGCTTTACACATGCAAGTCGAACGGCA
GCGCGAGAGAGCTTGCTCTCTTGGCGGCGAGTGGCGGACGGGTGAGT
AATATATCGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAG
TGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATCGCAAG
ACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGG
TAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGAC
CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCC
GCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAA
GAAAAGGTATCCCCTAATACGGGATACTGCTGACGGTATCTGCAGAA
TAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTT
CGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATT
TTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGT
GTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGG
CAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAA
CTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAA
GTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGC
AACGCGAAAACCTTACCTACTCTTGACATGTCTGGAAAGCCGAAGAG
ATTTGGCCGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGTCATTAGTTGCTACGCAAGAGCACTCTAATGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCC
CTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGT
CGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCCGATCGTAGTCC
GGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAA
TCGCGGATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACA
CCGCCCGTCACACCATGGGAGTGGGTTTCACCAGAAGTAGGTAGCCT
AACCGTAAGGAGGGCGCTTACCACGGTGGGATTCATGACTGGGGTGA
AGTCGTAACAAGGTAGCCGTATCGAGGTGCTC

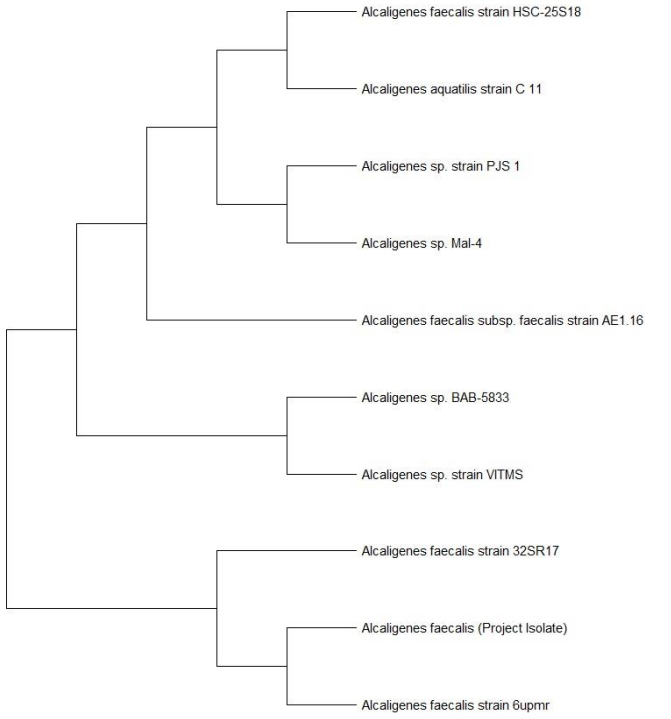


**Figure 1** Phylogenetic Tree (Project Isolate)

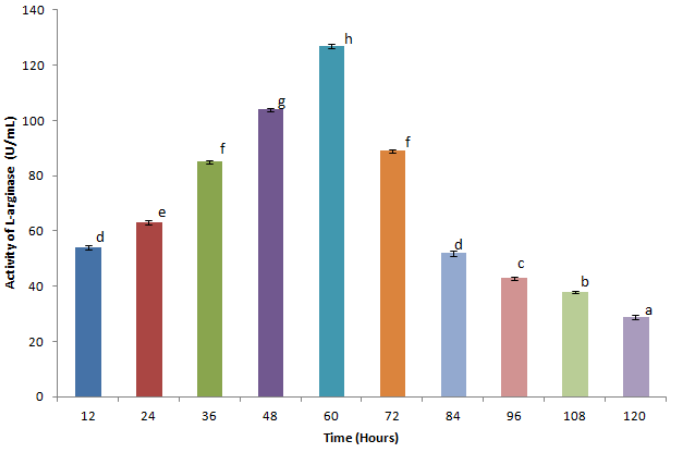


**Figure 2** Effect of Incubation Period on L-arginase Production
Same letter on mean values indicate that the values are not significantly different ($p \leq 0.05$) at $\alpha = 5\%$. Error bars stand for standard error of mean (SEM)

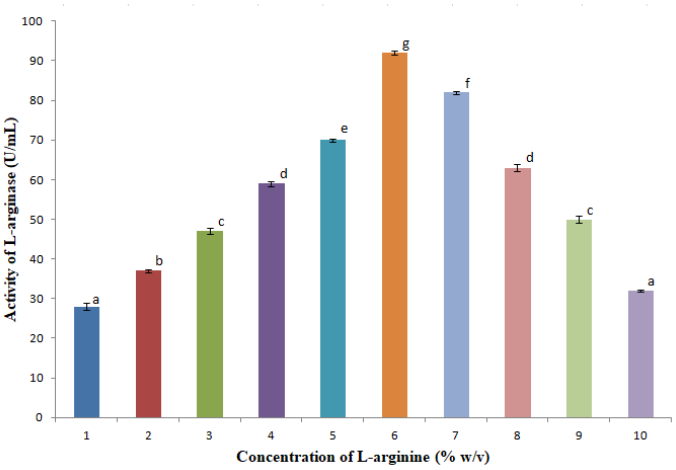


**Figure 3** Effect of L-arginine Concentration on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different ($p \leq 0.05$) at $\alpha = 5\%$. Error bars stand for standard error of mean (SEM)

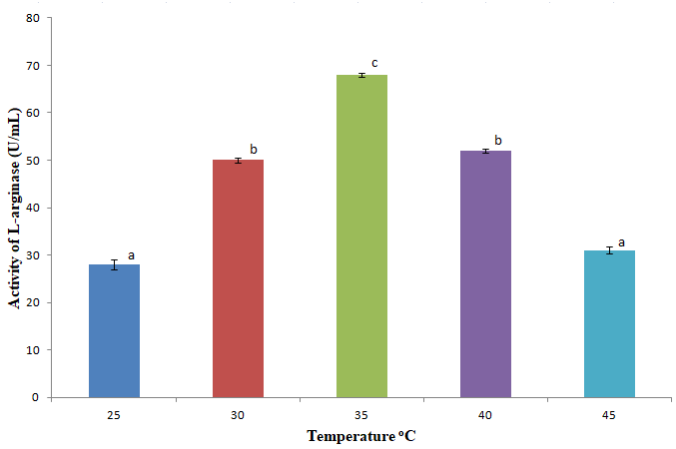


**Figure 4** Effect of Temperature on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different ($p \leq 0.05$) at $\alpha = 5\%$. Error bars stand for standard error of mean (SEM)

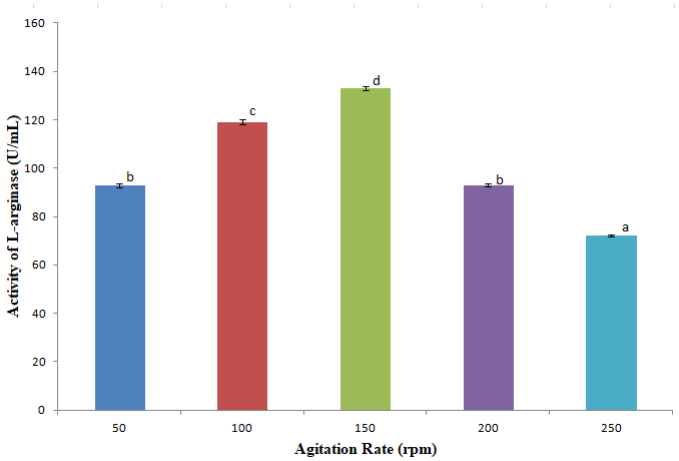


**Figure 5** Effect of Agitation Rate on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different (p≤0.05) at α = 5%. Error bars stand for standard error of mean (SEM)

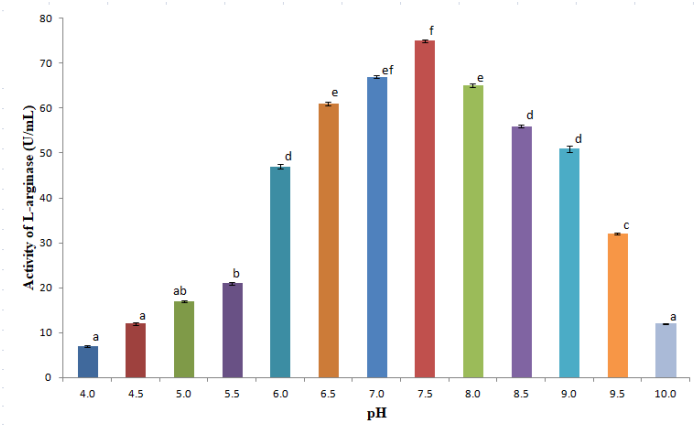


**Figure 6** Effect of pH on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different (p≤0.05) at α = 5%. Error bars stand for standard error of mean (SEM)

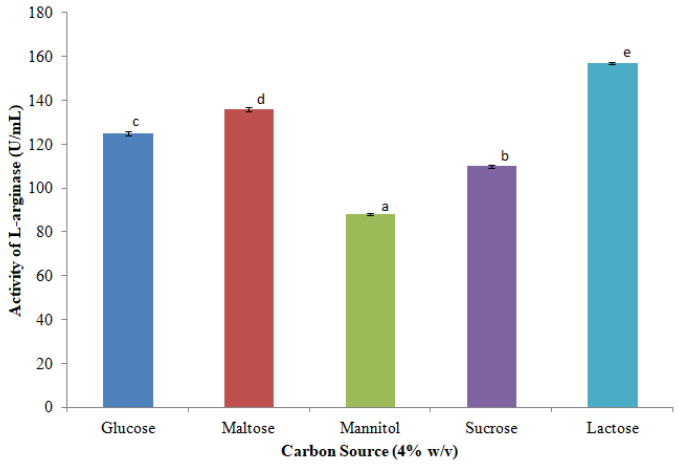


**Figure 7** Effect of Additional Carbon Source on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different (p≤0.05) at α = 5%. Error bars stand for standard error of mean (SEM)

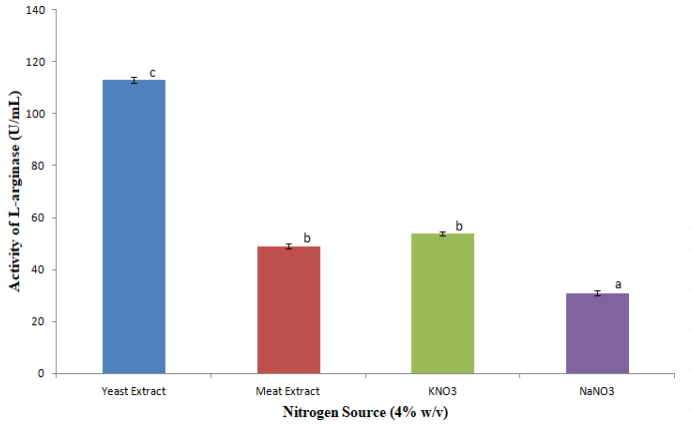


**Figure 8** Effect of Additional Nitrogen Source on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different (p≤0.05) at α = 5%. Error bars stand for standard error of mean (SEM)

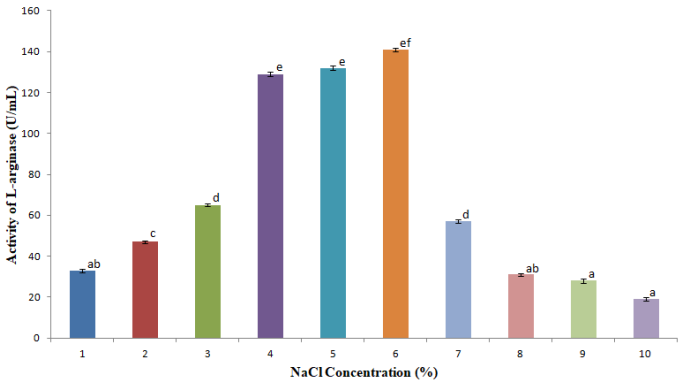


**Figure 9** Effect of NaCl Concentration on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different (p≤0.05) at α = 5%. Error bars stand for standard error of mean (SEM)

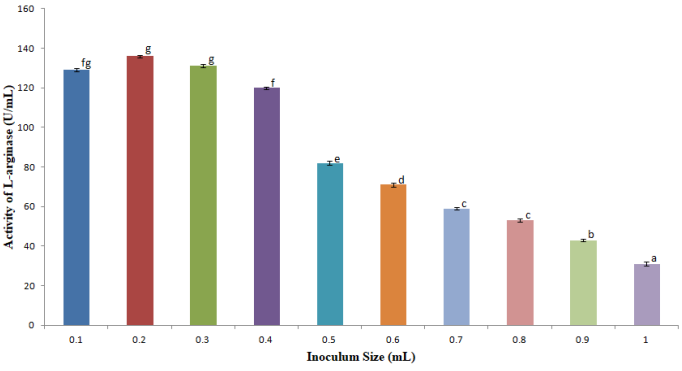


**Figure 10** Effect of Inoculum Size on the Production of L-arginase
Same letter on mean values indicate that the values are not significantly different (p≤0.05) at α = 5%. Error bars stand for standard error of mean (SEM)

**Table 2** Optimum conditions for the production of L-arginase

| S/N | Fermentation Condition | Optimum Condition |
|---|---|---|
| 1 | Incubation period (Hours) | 60 |
| 2 | Substrate Concentration (%) | 6 (L-arginine) |
| 3 | Temperature (°C) | 35 |
| 4 | Agitation rate (rpm) | 150 |
| 5 | pH | 7.5 |
| 6 | Additional carbon (4% w/v) | Lactose |
| 7 | Additional nitrogen (4% w/v) | Yeast extract |
| 8 | NaCl Concentration (%) | 6 |
| 9 | Inoculum size (mL) | 0.2 |

**Table 3** Enzyme yield using optimum fermentation parameters

| S/N | Enzyme | Production Isolate | Enzyme yield (U/mL) |
|---|---|---|---|
| 1 | L-arginase | *Alcaligenes faecalis* (Isolate B26) | 163±0.78 |

Values are Mean±SEM

## DISCUSSION

The selected bacterial isolate showed highest enzyme activity at temperature ranged between 27ºC and 37ºC which agreed with the findings of **Wakil and Adelegan (2015)**. The additional nitrogen source that enhanced the production of enzyme was yeast extract for L-arginase produced by *Alcaligenes faecalis*. It was observed that the additional carbon source had significant influence on enzyme production.

The highest L-arginase yield of 157 U/mL was recorded when lactose (4% w/v) was added to the fermentation medium. This agreed with the findings of **Umayaparvathi *et al.* (2013)** who posited that the influx of additional carbon will provide a platform for the enhancement of enzyme yield.

In a similar study, **Nadaf *et al.* (2019)** identified *Pseudomonas* sp. strain PV1 as the most potent L-arginase producing strain while screening L-arginase producing soil bacteria. The bacteria were tested on minimal media with phenol red as pH indicator. Fluconazole was added to the medium during that study in order to prevent fungal growth.

It is very important that the production media for microbial enzymes should be cheap and free from substances that can be poisonous to humans. This is particularly imperative if the enzymes will be used as biopharmaceuticals. The additional carbon and nitrogen sources including the salts added during the production of the enzyme were all cheap and considered safe for human consumption.

Among the various microorganisms that have been found to produce enzymes bacteria are preferred due to high yield, ease of cultivation and genetic manipulation. The species of bacteria reported in the present work was able to produce significant quantity of the enzyme.

## CONCLUSION

L-arginase has been considered as a biocatalyst that will be highly priced in future due to its properties and therapeutic roles. Rhizosphere environment has been shown in this study as a source of biopharmaceutically important microorganisms. In this study bacteria that are capable of producing L-arginase were isolated from rhizosphere soil. The use of phenol red as pH indicator enhanced the detection of chromogenic zones. There is therefore a need to conduct further research in the rhizosphere of other plants in order to isolate other microbes that can be of medical and industrial importance.

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Ansorge, I., Benting, J., Bhakdi, S., & Lingelbach, K. (1996). Protein sorting in Plasmodium falciparum-infected red blood cells permeabilized with the pore-forming protein streptolysin O. *Biochemistry Journal*, 315(1), 307-314. doi: https://doi.org/10.1042/bj3150307

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