Covenant Journal of Physical & Life Sciences (CJPL) Vol. 8 No. 2, Dec 2020
ISSN: *print 2354 - 3574 electronics 2354 – 3485 DOI:*

An Open Access Journal Available Online

Arginase Production in Wild-Collected *Neurospora crassa* isolate from Lagos, Nigeria

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Received: 15.06.2020 Accepted:06.08.2020
Date of Publication: December, 2020

Abstract: There are inadequate records of wild-isolated *Neurospora crassa* from Nigeria. Most of the isolates reported are from air spores outdoors, and indoor environment of buildings including laboratories where it is a contaminant. A wild strain of *N. crassa* isolated from soil on a burned site was assayed for an important pharmaceutical metabolite, arginase. This isolate was identified by traditional cultivation methods and by its ribosomal DNA internal transcribed spacer (rDNA-ITS) sequence. Its average daily growth rate was 3.2 cm and as typical with *N. crassa*, it utilised different carbon sources. Among the sugars tested, sucrose, lactose, glucose and glycerol yielded the most biomass while ethanol supported the least growth. Arginase activity was determined by the amount of urea produced per unit time and its activity in the newly isolated *N. crassa* strain peaked on the second day and declined steadily thereafter in the course of five days.

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Keywords: *Neurospora crassa*, arginase, urea and carbon source

1.0 Introduction

Neurospora was first recorded in 1843 as *Monilila sitophila*, a contaminant in French bakeries [1, 2, 3]. Currently, the use of preservatives in bakery products has inhibited the presence of moulds except in artisan bread devoid of preservatives [4, 5]. It was in *N. crassa* that Beadle and Tatum [6] conducted their studies of the one-gene-one-enzyme hypothesis [7]. Its seven chromosomes were identified by Barbara McClintock [8] and the sequencing of its genome revealed that it has a size of 40 Mb and encodes 10,082 protein-coding genes [9]. *N. crassa* is a haploid, heterothallic ascomycete, exhibiting both asexual and sexual life cycles. It is used as a leading model organism in genetics/biochemical genetics [10], circadian clocks in eukaryotes [11, 12] and population studies [13]. It is easy to culture, reproduces quickly and has simple nutritional requirements. Mutant strains exist and are maintained at the Fungal Genetics Stock Center (FGSC) in Kansas, USA.

Morphologically, they can be identified by their distinctive orange mycelia, rapid growth, and profuse production of powdery

conidia [14, 15]. Orange-coloured masses of *Neurospora* have been seen in sugar factories and on other carbohydrate-rich foods [16]. It can be found in the tropical and subtropical regions of the world; [17] where it is observed to be the earliest colonizer after wildfires [18,19]. This explains why its spores require temperatures ranging from 60-65°C to germinate [17,20]. *N. crassa* has also been recorded on burnt trees and shrubs in the temperate forests of North-Western and Southern America [21], burned sites in African countries such as Ivory Coast, Gabon and Congo [18]. There are scarce records of other efforts to collect *N. crassa* from burned sites in other parts of Africa. In Nigeria, *N. crassa* has been isolated from the rhizosphere of a weed [22] and its spores recovered from air sampling in Victoria Island and Ikeja areas of Lagos State [23].

N. crassa has applications in metabolite production [24] especially the enzyme arginase [25], with ornithine and urea as the products of its action [26]. Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is a 105 kD homotrimeric enzyme that contains a binuclear manganese cluster and catalyzes the hydrolysis of L-arginine to form the non-protein amino acid L-ornithine, and urea [27,28]. It was first found in the mammalian liver, and ARG-1 is conserved in yeasts (*Saccharomyces cerevisiae*, *S. pombe*), moulds (*Eremothecium gosypii*, *Magnaporthe oryzae*, *N. crassa*) and mammals (cat, dog and humans) [26]. The structural gene for arginase is found between *wc-1* and *arg-10* genes on *N. crassa's* chromosome VIIR [29, 30]. Its two isoforms in *N. crassa* have been characterized [31]. In recent times, arginase has found use in the treatment of acute neurological disorders [32], allergic asthma [33], tumour inhibition and an extensive range of activity against cancer cells [34, 35]. Hence, the present investigation deals with the production of L-arginase from a strain of *N. crassa* isolated from nature and the determination of the best sole carbon source for its growth.

2.0 Materials and Methods

2.1 Sample Sites

Soil samples were collected from Ikorodu, Lagos State [36] and Atan, Ijebu-Ode, Ogun State in southwestern Nigeria. These sites were chosen during the dry season because the farmers set the land on fire after harvesting crops. The GPS coordinates for the sites were obtained with Google maps [37, 38]. Soil samples were collected up to a depth of 7.5 cm -10 cm [36] and five sites per location were randomly selected.

2.2 Strains and Culture Media

N. crassa strains, CECT 2727 and 2728 were ordered from the Spanish Culture Centre, Valencia and the freeze-dried pellets were cultured according to supplier's instructions. Rose Bengal Chloramphenicol Agar (RBCA) (Hi-Media, Mumbai India), Potato Dextrose Agar (PDA) (Hi-Media, Mumbai India) supplemented with chloramphenicol (250 µg/ml) and Vogel's minimal medium (1X Vogel's 2% glucose (V2G), 1.5 % agar) was used to isolate and cultivate the strains used in this study.

One litre (1 L) of 50X stock solution of Vogel's contained $Na_3C_6H_5O_7$. 5.5 H_2O (125 g), KH_2PO_4 anhydrous (250 g), NH_4NO_3 anhydrous (100 g), $MgSO_4$. 7 H_20 (10 g), $CaCl_2$. 2 H_20 (5 g), Trace Element (5 ml), Biotin Solution (2.5 ml), pH 6.0 and Chloroform (5 ml) was added as a preservative for storage at room temperature [39, 40]. Stock Trace element solution (100 ml) was composed of $C_6H_8.H_20$ (5 g), $ZnSO_4.7H_20$ (5 g), Fe $(NH_4)_2(SO_4)_2.6$ H_20 (1 g), $CuSO_4$. 5 H_20 (0. 25 g), $MnSO_4$. $1H_20$ (0.05 g), H_3BO_3 anhydrous (0. 05 g), $Na_2MoO_4.2H_20$ (0.05 g).

2.3 Soil Screening for *N. crassa*

One gram of soil from each of the ten samples was aseptically transferred into Erlenmeyer (250 ml) flasks containing 99-ml of sterile distilled water. The soil-water mixture was shaken for 5 min and hundred-fold serial dilutions were made to get dilutions of 10^{-2}, 10^{-4} and 10^{-6}. Aliquots (1 ml) from 10^{-2} and 10^{-4} flasks and (0.1 ml) from 10^{-4} and 10^{-6} were used to get 10^{-2}, 10^{-4} and 10^{-3}, 10^{-5}

dilutions respectively. Each soil dilution was aseptically transferred into the Petri plates using the pour plate technique. This was carried out in triplicates using Potato Dextrose Agar (PDA) and Rose Bengal Chloramphenicol Agar (RBCA). The plates were incubated at 30°C for 5 days and were observed daily for the presence of characteristic *N. crassa* orange hyphae.

To obtain pure cultures of *N. crassa*, using an inoculating needle, bright orange mycelia from the mixed culture plates were sub-cultured on RBCA, PDA and Vogel's 2% Glucose (V2G) plates. A second round of sub-culturing from the initial pure culture plates was done. Cultures were maintained on PDAC and Vogel's slants. The new isolate was assigned a laboratory stock number.

2.4 Genomic DNA Isolation, ITS Amplification and Sequencing

Genomic DNA was extracted from a 3-day old pure culture agar plate of *N. crassa* using Zymo Quick-DNA™ Fungal/Bacterial Miniprep Kit according to the manufacturer's instructions. PCR amplification was done using ITS4R and ITS5F primers and amplicon sequencing were done as previously described [36].

2.5 Determination of Growth Rate

Hyphal extension of the new isolate and *the other* two strains CECT 2728 and CECT 2727 were measured daily in 16 x 150 mm long growth tubes at 25°C $\pm$ 2. A loopful of conidia was inoculated on one end of V2G 2% agar medium. Growth fronts were marked over 4 days. Replicates were used for each *N. crassa* strain and an average growth rate of the six tubes was measured.

2.6 Dry weight mycelia biomass on different carbon sources

To determine if the new isolate was a nutritional variant, it was tested on different sugars commonly utilised by *N. crassa* and the mycelial weights were compared with the other strains CECT 2727 and CECT 2728. Ten millilitres of 1X Vogel's medium and each sugar were dispensed into 50 ml Erlenmeyer flasks. The monosaccharides tested included glucose, fructose, galactose and dextrose; the disaccharides were sucrose, maltose and lactose, glycerol (a polysaccharide) and ethanol. Each carbon source was added at a concentration of 0.2 % (w/v) and 2% (w/v). Mycelia mats cut out from plates with a sterilized 5 mm cork borer was inoculated into the flasks and incubated with shaking at 100 rpm (ZHP-100 PEC Medical USA) for 7 days at 30°C. The 2 % culture flasks were not agitated. Mycelia mass was harvested after 7 days and dried before weighing. Three replicates were set up for each treatment. As a control, flasks that contained sterile distilled water were inoculated with a mycelial plug of *N. crassa* [41].

2.7 Arginase Assay

2.7.1 Growth conditions

A 9 mm mycelium mat was picked from a 7-day old culture plate of *N. crassa* isolates IYN65 and CECT 2728 using a cork borer. The strains were sub-cultured in 20 ml Vogel's Minimal Medium with 2 % Glucose and 1mM of arginine to set the favourable growth conditions for the production of arginase. Flasks were shaken at 100 rpm at 25°C [42].

2.7.2 Cell Disruption

To breakdown the fungi and release its enzymic contents, the mycelial mass in the flasks of each *N. crassa* strain was centrifuged at 5000 rpm for 10 min at 4°C and the supernatant was discarded. Enzyme permeabilization was performed on the pellet as earlier described [43]. Three millilitres of permeabilization buffer (0.02 M Potassium phosphate, pH-7.5, 0.01 M EDTA) was added to the flask and mixed vigorously and then 0.2 ml of Toluene/ Ethanol (1:4 v/v) was added. The resulting mixture was then vortexed for 2 min and the supernatant discarded. The disrupted mycelia were then centrifuged and another permeabilization buffer without EDTA was used to re-suspend the permeabilized mycelia/pellet. The enzyme solution containing mycelia and permeabilization buffer (without EDTA) was used for arginase assay.

2.7.3 Arginase assay

To assay for arginase, a pre-incubation stage was set up. A mixture of 0.2 ml of glycine buffer, 0.5 ml of enzyme solution and 0.1 ml of manganese chloride was made and dispensed into test tubes. The solution was then placed in an incubator at 37 °C for 10 min to allow binding of manganese, its co-factor. To initiate the experiment, 0.1 ml of arginine was added to the incubated mixture and incubation was extended for another 30 minutes at 37 °C. The reaction was halted by the addition of 1 ml of perchloric acid [44]. An aliquot of this was used to assay for urea.

2.7.4 Urea colourimetric assay

To determine the ability of the enzyme solution to produce urea, an existing protocol by [45] was modified. Two millilitres of the final arginase assay mixture was dispensed into a test tube and sterile distilled water was added to bring the total volume up to 3.5 ml. To the solution, 1.4 ml trichloroacetic acid was added and mixed. Next, 1 ml of diacetylmonoxime (DAMO) and 1 ml of perchloric acid-sulfuric acid mixture were added, and the test-tube was placed in a boiling water bath for 30 min. Finally, the test-tube was removed and cooled in a water bath. The experiment was done in duplicates. Using a photoelectric colorimeter (Microprocessor photo colorimeter 1312, Electronics India), the absorbance of the final solution was measured at 480 nm. To avoid fading of the colour, absorbance was read immediately after the cooling step. The concentration of urea was determined from the calibration curve [46].

2.7.5 Urea standard curve

Arginase was indirectly assayed by measuring the amount of urea produced and its activity determined by extrapolation from the urea standard curve. The standard curve was constructed by making concentrations of urea standard solution and plotting them against their absorbance values at 480 nm. To get the concentrations, urea standard solution (30 mg/ml) was diluted 200 times and aliquots of the standard were dispensed into test tubes (13 x100 mm) in a final volume of 3.2 ml diluting with distilled water. This was necessary to get the corresponding concentrations of urea (mg/ml). Trichloroacetic acid (TCA) (2.8 ml) was added to each aliquot and mixed thoroughly. Thereafter, 1 ml of DAMO reagent and 1 ml of perchloric acid-sulfuric acid mixture were added and the solution placed in a boiling water bath for 30 min. The solution was allowed to cool to room temperature and the absorbance was measured at 480 nm. The control for this experiment contained the following constituents: glycine buffer, enzyme solution, $MnCl_2$ and perchloric acid, TCA, DAMO solution and perchloric acid-sulfuric acid mixture except for arginine [44]. Urea concentration used in the estimation of Arginase activity was calculated from the straight-line equation.

The formula for the calculation of enzyme activity is as follows:

$$\text{Enzyme activity U/ml} = \frac{\mu\text{moles of urea released}}{(\text{Time of enzyme action x Volume of enzyme (ml)})} \quad \ldots\ldots (1)$$

3.0 Results

3.1 Sampling Locations

Soil samples were collected from two locations - burnt vegetation farmland in Atan town, Ijebu-Ode with coordinates 4°00'59.5"E 6°52'31.8"N (**Figure 1A**), while the second was a freshly burnt harvested *Manihot esculenta* (cassava) plantation in Ikorodu, Lagos with coordinates 3°34'37.9"E 6°37'20.4"N) [36] (**Figure 1B**).

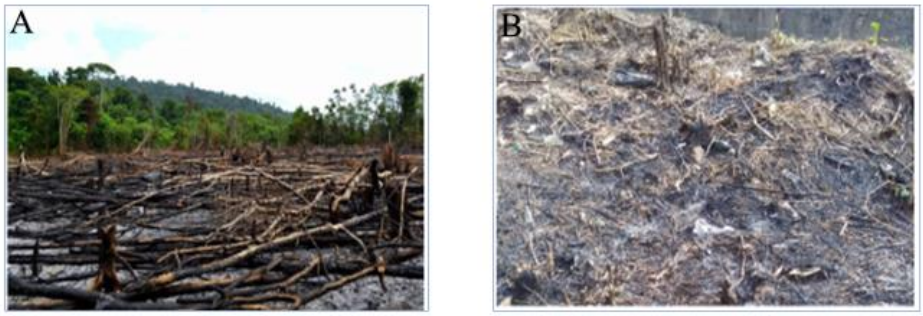

Figure 1. Burned sites of sample collection **A**. Atan, Ijebu-Ode Ogun State. **B.** Ikorodu, Lagos State.

3.2 Isolation and Cultural characteristics of *N. crassa*

Mixed cultures were obtained from all the ten soil samples (A-J) cultured on RBCA and PDA. However, only soil sample B had orange mycelia among its 9 fungal isolates on the plate. The patches of bright orange conidia on the RBCA and PDAC mixed culture plates that suggested the presence of *Neurospora* species were selected (**Figure 2**). Pure cultures of this isolate were obtained and stored as laboratory stock *N. crassa* IYN 65. Predictably, isolate IYN 65 grew more vigorously on PDA and Vogel's minimal medium than on RBCA (**Figure 3A**). A lactophenol wet mount of the strain revealed conidiophores bearing conidia (**Figure 3B**).

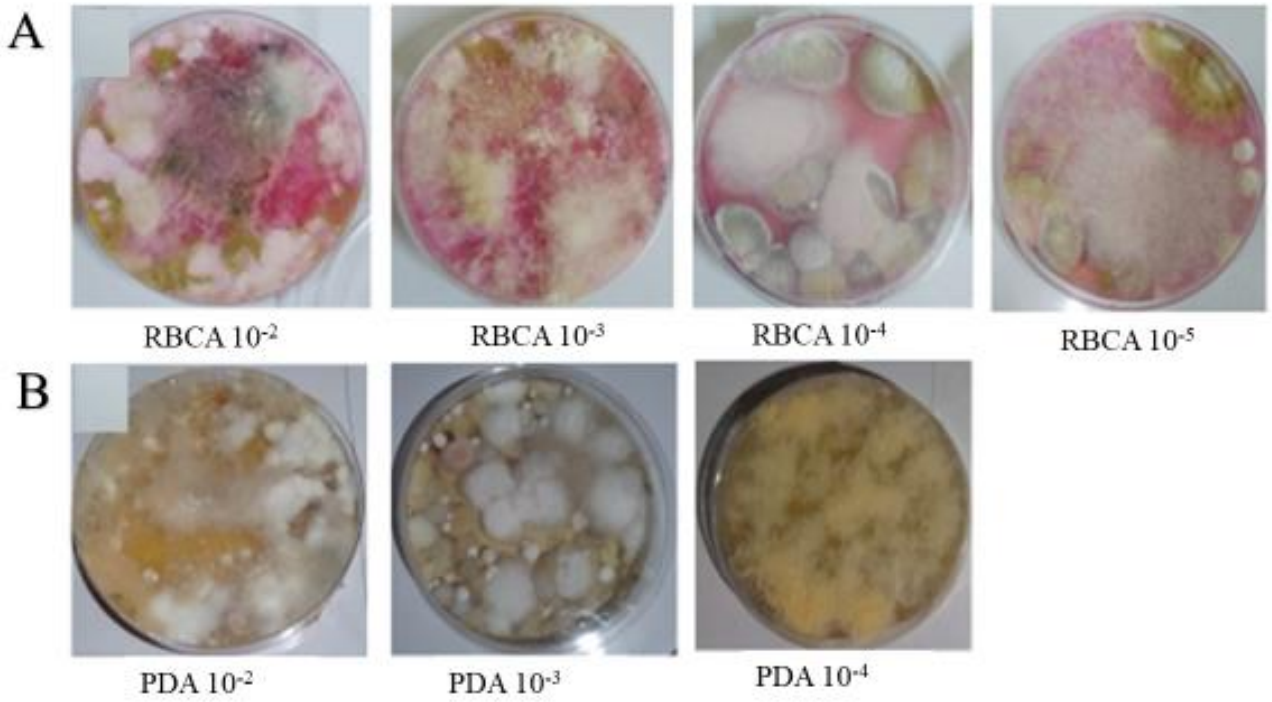

Figure 2. Mixed culture dilution plates of soil sample B with patches of *N. crassa* on **A.** Rose Bengal Chloramphenicol Agar **B.** Potato Dextrose Agar

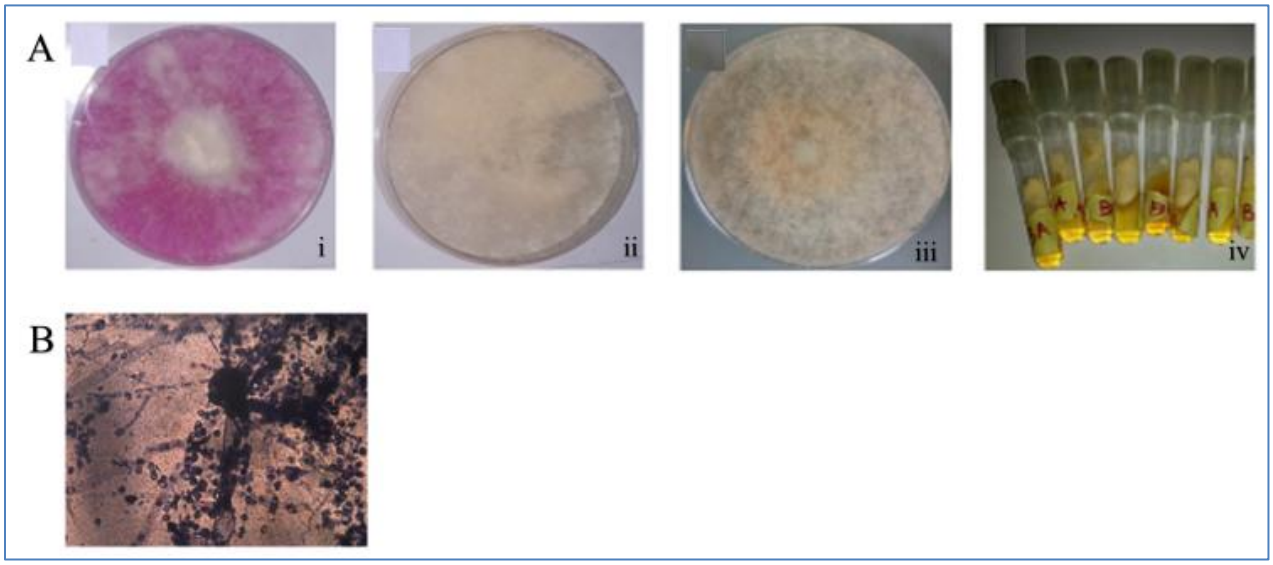

Figure 3. A. Pure culture of *N. crassa* grown on i) RBCA ii) PDA iii) V2G agar iv) PDA and Vogel's slants. **B.** Micrograph of *N. crassa* with conidiophores bearing conidia.

3.3 Molecular Identification of *N. crassa* Isolate IYN 65

To confirm the identity of the new *N. crassa* isolate, IYN 65, DNA was extracted and run on a 1% agarose gel (**Figure 4A**) and the expected amplicon size was confirmed (**Figure 4B**). Amplicon sequences were edited using Bioedit v 7.2.5 [47]. The ITS-1, partial sequence; 5.8S rRNA gene and ITS-2, complete sequence; and large subunit rRNA gene has been deposited in GenBank under accession number MT367687.

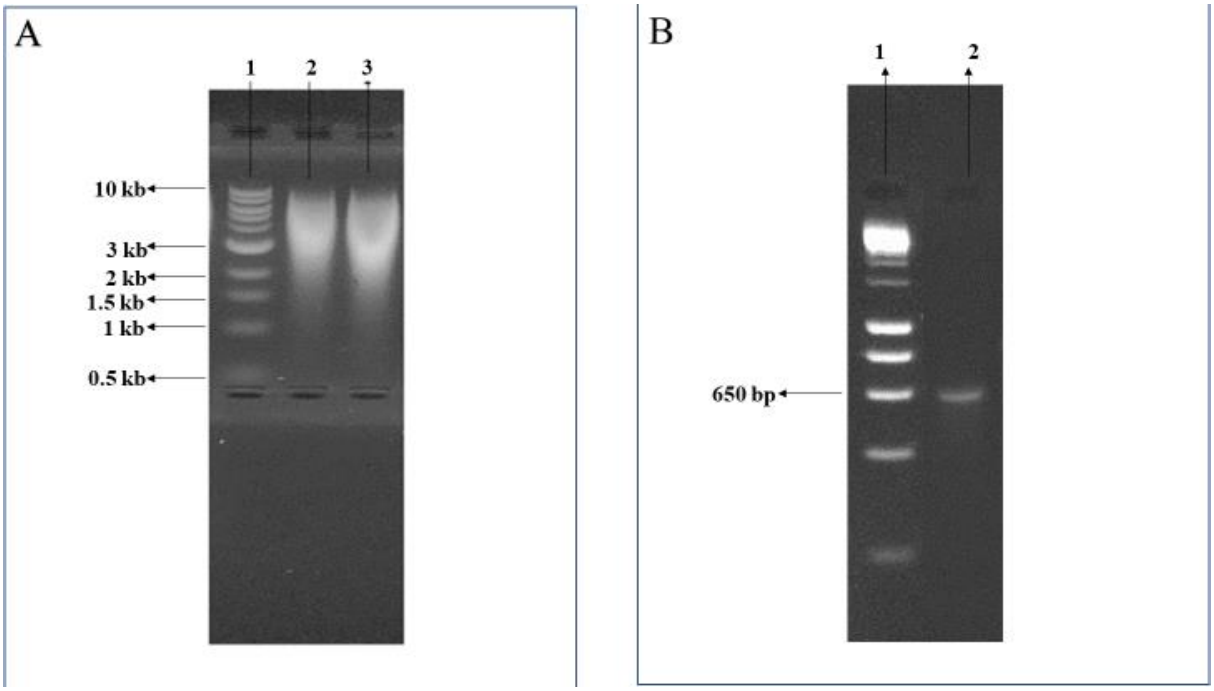

Figure 4. A. Gel electrophoresis of the extracted genomic DNA. Lane 1: 1kb NEB DNA Ladder, Lanes 2 and 3: IYN65. **B.** PCR amplicon Lane 1: 1kb Bioline DNA Ladder, Lane 2: *N. crassa* IYN65.

3.4 Growth Rate

The average daily growth rate of *N. crassa* strains used in this study as determined on V2G agar at room temperature was 3.2 ± 0.3 cm for IYN 65, 3.2 ± 0.15 cm for CECT 2727 and 3.1 ± 0.4 cm for CECT 2728 (**Table 1**).

Table 1. The growth rate of *N. crassa* strains

Strain	Growth Rate ±SD (cm)	Origin
IYN 65	3.2 ± 0.3	This study
CECT 2727	3.2 ± 0.12	Spanish Culture Collection Centre
CECT 2728	3.3 ± 0.3	Spanish Culture Collection Centre

3.5 Weights (g) of mycelial biomass on various carbon sources

The best-utilized carbon sources by IYN 65, CECT 2728 and CECT 2727 were sucrose (0.94 g± 0.06), lactose (0.92g ± 0.05) and glycerol (0.90 g ± 0.10) respectively (**Figure 5A**). In this same order, these strains yielded the least mycelial weights with ethanol at 0.23 g ± 0.06, 0.26 ± 0.04g, 0.29 g ± 0 (**Figure 5B**).

At a lower concentration with aeration, all three strains IYN 65, CECT 2728 and CECT 2727 grew best in lactose (**Figure 6A**). with the following weights 0.18 g ± 0.04, 0.17 g ± 0.02, 0.16 g ± 0.03 and least in ethanol (0.05 g ± 0.01, 0.04 g ± 0.02, 0.05 g ± 0.02 (**Figure 6B)**. Generally, the strains grew poorly in maltose. The best sole carbon source among the monosaccharides was glucose.

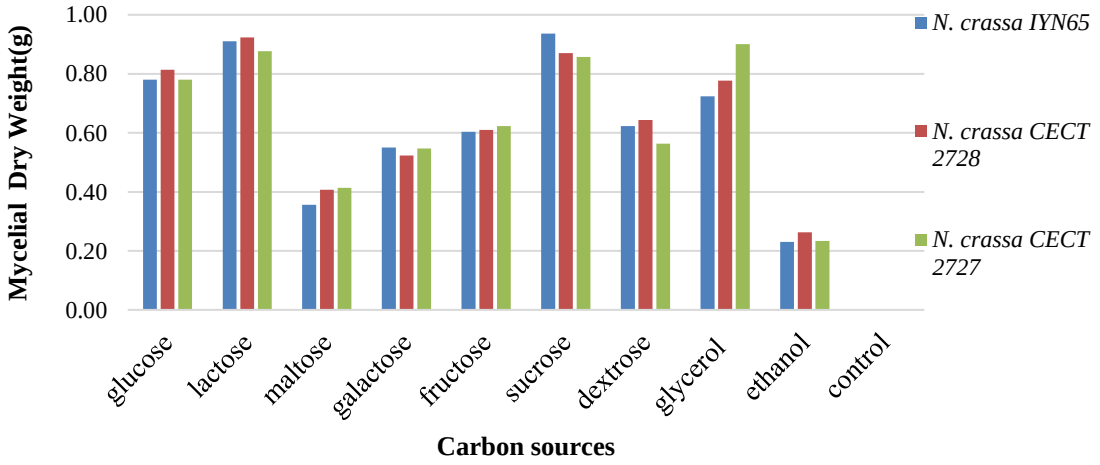

Figure 5A. Mycelial weights of *N crassa* strains on media containing 2% carbon sources.

Figure 5B. Mycelial biomass of *N. crassa* at 2 %

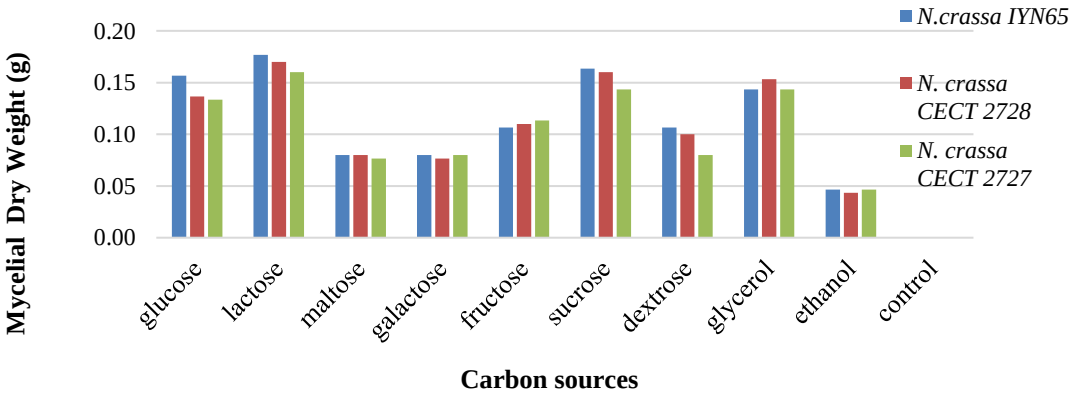

Figure 6A. Mycelial weights of *N. crassa* strains 0.2% carbon source media.

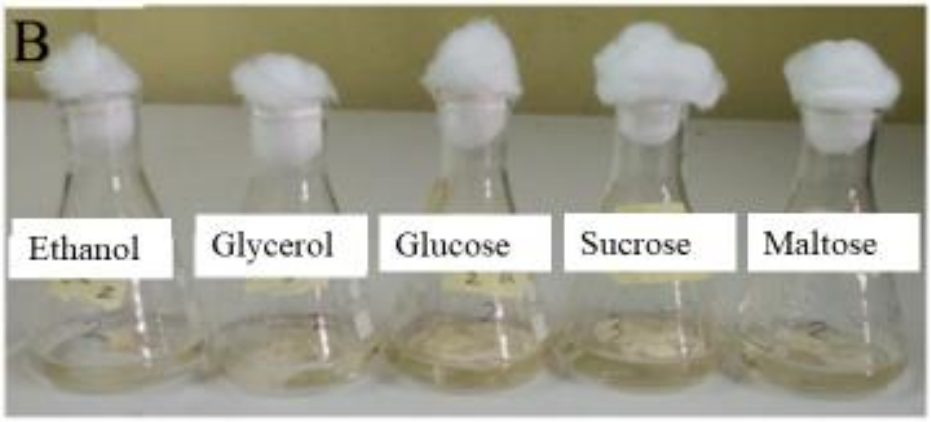

Figure 6B. Mycelial biomass of *N. crassa* at 0.2 %

3.6 Production of Arginase from *N. crassa strains*

This following urea concentrations of 7.5, 15, 22.5, 30, 45.0, 60, 75, 90, 105, 120, 135, 150, 165, 180, and 195 mg/ml were obtained from urea standard aliquots of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4, 1.6, 1.8, 2.0, 2.2, 2.4 and 2.6 ml (**Figure 7**). The equation obtained from the urea standard curve was y= 0.0008x+0.0104; where y represents the absorbance value and x was the urea concentration. Average optical density (OD) readings for each strain were substituted in the equation to give corresponding urea concentration values (**Table 2**). Enzyme activity was calculated for each time point from Equation (1) using the urea concentration values (**Table 2**) and the

enzyme volume was two millilitres. One unit of arginase activity was defined as the amount of urea (mg) released per ml of enzyme every 24 h under standard assay conditions.

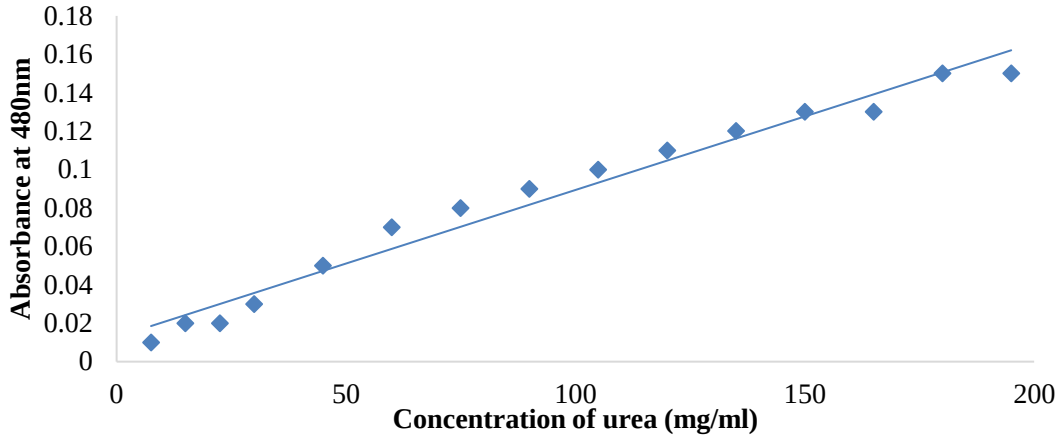

Figure 7. Urea standard chart.

Table 2. Optical density and Urea concentration levels in *N. crassa* strains

***N. crassa* Isolate IYN 65**					***N. crassa strain* CECT 2728**			
	Absorbance values (at 480 nm)				**Absorbance values (at 480 nm)**			
Hours	OD1	OD2	Avg. OD	Urea concentration (mg/ml)	OD1	OD2	Avg. OD	Urea concentration (mg/ml)
24	0.15	0.17	0.16	187	0.02	0.06	0.04	37
48	0.65	0.52	0.59	725	0.48	0.45	0.47	575
72	0.56	0.45	0.51	625	0.36	0.34	0.35	425
96	0.27	0.26	0.27	325	0.14	0.14	0.14	162
120	0.20	0.19	0.20	237	0.12	0.16	0.14	162

Enzyme activity monitored over the course of 5 days revealed that in strain IYN 65, the arginase activity (7.55 U/ml) peaked at 48 h with a urea concentration of 725 mg/ml; CECT 2728 also recorded its maximum enzyme activity (5.98 U/ml) at the same time from 575 mg/ml of urea. For both strains, the decline in enzyme activity (4.34 U/ml; 625 mg/ml of urea) and (2.95 U/ml; 425 mg/ml of urea) was observed at 72 h and continued downward until day 5 (1.98 U/ml; 237 mg/ml of urea) (1.35 U/ml; 162 mg/ml, of urea) (**Figure 8**). Colour intensity diminished from yellow (maximum activity) to clear (least activity) (**Figure 9**).

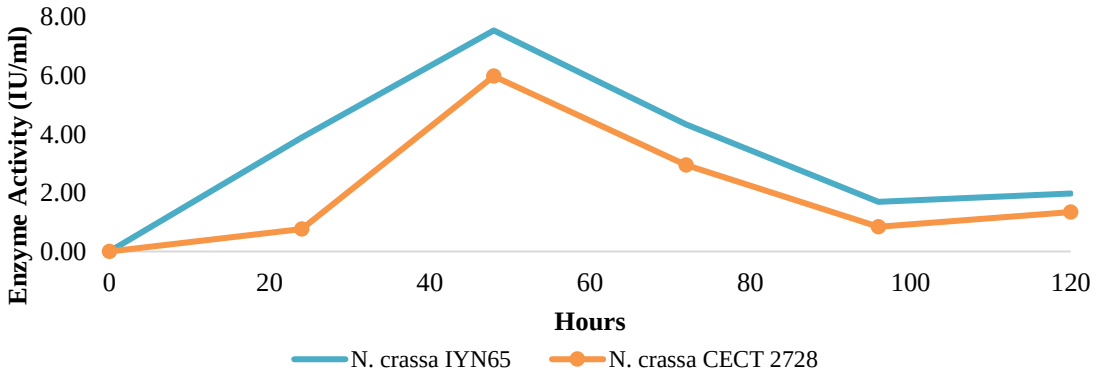

Figure 8. Time course of arginase activity of the *N. crassa* strains.

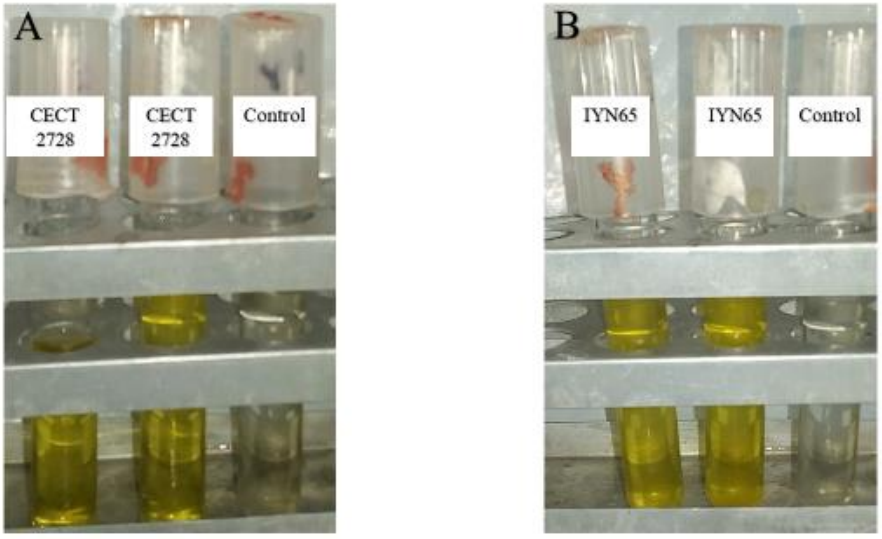

Figure 9. Test tubes for colorimetric arginase activity of *N. crassa* strains on Day 2. **A**. CECT 2728 and **B.** IYN 65.

4.0 Discussion

Here, the primary goal of isolating *N. crassa* strains from nature is to expand current information on their ecology in Nigeria. Historically, *N. crassa* has been isolated from burnt vegetation [22] and undergrowth of grass and *Mimosa* on a coconut plantation in India [48] and in association with *Pinus sylvestris* [49]. Laboratory stock cultures of *N. crassa* isolates previously obtained on a coal dump site from the rhizospheres of weed species *Urena lobata* [22] and grass *Brachiaria disticophylla* (unpublished data) had been lost during storage. Our subsequent attempts to isolate *N. crassa* from rhizospheres of plant species have not been successful.

Similar to a previous report in which two *N. crassa* isolates were obtained from the screening of thirty soil samples [48], only one of the ten soil samples screened in this study yielded *N. crassa*. This soil sample collected from a post-harvest burnt *Manihot esculenta* (cassava) plantation in Ikorodu, Lagos State was the same one from which another thermotolerant fungus, *Lichtheimia ramosa* had been obtained [36]. A BLAST search of the partial ITS1 and ITS2 sequence of *N. crassa* IYN 65 showed 100% identity to *Neurospora crassa* isolate CCMGE112 [50].

The average daily growth rate of 3.2 cm for the *N. crassa* isolate IYN 65 is consistent with the known range of 3 cm to 4 cm for *N. crassa* strains [51, 52]. As typical of *N. crassa* strains, IYN 65 was able to use a

variety of sugars. Its mycelial mass was significantly improved by glycerol, the disaccharides; sucrose and lactose, and glucose. In comparison with monosaccharides, disaccharides were better carbon sources for growth as seen in lactose and sucrose unlike fructose, galactose and dextrose. Overall, maltose and galactose were the least utilized sugars, while ethanol was the least growth-promoting carbon source. In a previous mycelia growth study [41], a similar trend was observed with sucrose and glucose being excellent sole carbon sources for *N. crassa* wild type strain 74A over ethanol. Based on the culture conditions in this study, there seemed to be no significant variation in the growth rate or mycelial mass of *N. crassa* IYN 65 and strains CECT 2727 and CECT 2728.

Of the many metabolites produced by *N. crassa*, arginase although discovered a long time ago, has gained renewed prominence because of its immense potential in clinical applications [53]. Furthermore, ornithine, a product of arginase metabolism is exploited in the treatments of diseases [54]. This preliminary study shows that the activity of arginase in *N. crassa* IYN 65 and the laboratory strain CECT 2728 capped between day 2 and 3. In a related study, the arginase activity obtained from four *N. crassa* strains by similar enzyme permeabilization procedures peaked around 60-66 hours [55].

In conclusion, arginase was produced from a new *N. crassa* isolate from the natural environment. Future work will entail whole-genome sequencing of the isolate and full characterization of its arginase. Efforts to isolate more *N. crassa* strains from different conditions in nature and other zones within Nigeria would be intensified to enable comparative studies.

5.0 References

[1] Payen, A. (1843). (Rapporteur) Extrait d'un rapport adresse a M. Le Marechal Duc de Dalmatie, Ministre de la Guerre, President du Conseil, sur une alteration extraordinaire du pain de munition. Ann. Chim. Phys. 9: 5–21.

[2] Shear, G.L and Dodge, B.O. 1927. Life histories and heterothallism of the red bread-mould fungi of the Monilia sitophila group. J. Agric. Res. 34(11):1019-1041.

[3] Perkins, D. D. (1992). *Neurospora*: The Organism behind the Molecular Revolution. Genetics 130: 687–701.

[4] Gioia, L., Ganancio, J. and Steel, C. (2017). Food additives and processing aids used in breadmaking. Intech Open. http://dx.doi.org/10.5772/intechopen.70087

[5] Dalmasso, J. (1985). Control of molds in breads, The Science Workbook: Student Research Projects in Food Agriculture-Natural Resources, College of Agriculture, Ohio State University.

[6] Beadle, G. W. and Tatum, E. L., (1941). Genetic Control of Chemical Reactions in *Neurospora*. PNAS USA 27(11): 499-506.

[7] Strauss B. S. (2016). Biochemical Genetics and Molecular Biology: The Contributions of George Beadle and Edward Tatum. Genetics 203(1): 13–20. https://doi.org/10.1534/genetics.116.188995

[8] McClintock, B. (1945). *Neurospora*. I. Preliminary observations of the chromosomes of *Neurospora crassa*. Am. J. Bot. 32(10): 671–678

[9] Galagan, J. E., Calvo, S. E., Borkovich, K. A., Selker, E. U., Read, N. D., Jaffe, D. and Rehman, B. (2003). The genome sequence of the filamentous fungus *Neurospora crassa*. Nature 422(6934): 859-865.

[10] Aramayo, R., & Selker, E. U. (2013). Neurospora crassa, a model system for epigenetics research. CSH Perspect. Biol. 5(10): a017921. https://doi.org/10.1101/cshperspect.a017921

[11] Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L. and Zoran, M. J. (2005). Circadian rhythm from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. 6(7): 544-560.

[12] Cockrell, A., Pirlo, R., Babson, D., Cusick, K., Soto, C., Petersen, E., Davis, M., Hong, C., Lee, K., Fitzgerald, L. and Biffinger, J. (2015). Suppressing the Neurospora crassa circadian clock while maintaining light responsiveness in continuous stirred tank reactors Sci. Rep. 5(10691). DOI: 10.1038/srep10691

[13] Gladieux, P., De Bellis, F., Hann-Soden, C., Svedberg, J., Johannesson, H. and Taylor, J. (2020). *Neurospora* from Natural Populations: Population Genomics Insights into the Life History of a Model Microbial Eukaryote. Methods Mol. Biol. 2090: 313-336. DOI: 10.1007/978-1-0716-0199-0_13.

[14] Jacobson, D.J. (1992). Control of Mating Type Heterokaryon Incompatibility by the *Tol* Gene in *Neurospora crassa* and *N. tetrasperma*. Genome 35(2): 347-353.

[15] Kanti, A and Sudiana I. (2016). Comparison of *Neurospora crassa* and *Neurospora sitophila* for phytase production at various fermentation temperatures Biodiversitas 17 (2): 769-775.

[16] Rashmi, K., Latha, J. N., Sowjanya, T. N., Kiranmayi, P., Rao, M. V., Menon, C. P. and Mohan, P. M. (2003). *Neurospora* in full bloom. Curr. Sci. 85: 1670-1672.

[17] Perkins, D. D. and Davis, R. H. (2000). Evidence for Safety of Neurospora Species for Academic and Commercial Uses. Appl. Environ. Microbiol. 66(12): 5107-5109.

[18] Turner, B. C., Perkins, D. D. and Fairfield, A. (2001). *Neurospora* from Natural Populations: A Global Study, Fung. Genet. Biol. 32(2): 67-92.

[19] Mukati, A., Vyas, A. and Vyas, H. (2012). A Study of Natural Population of *Neurospora* and Isolation of Novel Morphological Mutants. J. Environ. Res. Develop. 7: 923- 935.

[20] Moore, D., Robson, G. and Trinci, A. (2019). Spore germination and dormancy. In: 21st Century Guidebook to Fungi. 2nd edition. http://www.davidmoore.org.uk/21st_Century_Guidebook_to_Fungi_PLATINUM/Ch0402.htm

[21] Jacobson, D.J., Powell, A.J., Dettman, J.R., Saenz, G.S., Barton, M.M., Hiltz, M.D., Dvorachek, W.H., Glass, N.L., Taylor, J.W. and Natvig, D.O. (2004). *Neurospora* in Temperate Forests of Western North America. Mycology, 96(1): 66–74.

[22] Nsa, I., Igbinigie, E., Adeogun, O., Adekunle, A., Ilori, O., Adongbede, M. et al (2017). The potential of mycorrhizal associations of a coal dumpsite. J.I.R.T. 6(1):165-177.

[23] Odebode A., Adekunle, A., Stajich, J. and Adeonipekun, P. (2020). Airborne fungi spores distribution in various locations in Lagos, Nigeria. Environ. Monit. Assess. 192: 87-101.

[24] Mora, J., Tarrab, R. and Bojalil, L. F. (1966). On the structure and function

of different arginases. B.B.A. 118: 206-209.

[25] Turner, G.E. and Weiss, R.L. (2006). Developmental expression of two forms of arginase in *Neurospora* crassa. B.B.A. 1760: 848-857.

[26] Kossel, A. and Dakin, H.D. (1904). Über die Arginase. Physiol. Chem. 41: 321–331.

[27] Kuldeep K, Teena P and Shefali W. (2012). L-arginase Based Biosensor for Detection of L-arginine in Juice Samples. J. Nat. Prod. Plant Res. 2 (4): 494-499.

[28] Markus M. (2009). Arginase an Emerging Key Player in the Mammalian Immune System. Br. J. of Pharmacol. 158: 638–651.

[29] Davis, R. H., Lawless, M. B. and Port. L. A. (1970). Arginaseless *Neurospora*: genetics, physiology, and polyamine synthesis. J. Bacteriol. 102: 299-305.

[30] Morgan, D. H. 1970. Selection and characterisation of mutants lacking arginase in *Neurospora crassa*. Mol. Gen. Genet. 108: 291-302.

[31] Borkovich, K.A. and Weiss, R.L. (1987) Purification and Characterization of Arginase from *Neurospora crassa.* J. Biol. 262(15): 7081-7086.

[32] Esch, F., Lin, K.I., Hills, A., Zaman, K., Baraban, J.M., Chatterjee, S., Rubin, L., Ash, D.E. and Ratan, R.R. (1998). Purification of a Multipotent Antideath Activity from Bovine Liver and Its Identification as Arginase: Nitric Oxide-Independent Inhibition of Neuronal Apoptosis. J. Neurosci. 18(11): 4083-4095.

[33] Munder, M. (2010). Role of arginase in asthma: potential clinical applications. Expert Rev. Clin. Pharmacol. 3(1): 17-23.

[34] Cavanaugh, P.G. and Nicolson, G.L. (2000). Partial Purification of a Liver-Derived Tumor Cell Growth Inhibitor That Differentially Inhibits Poorly-Liver Metastasizing Cell Lines: Identification as an Active Subunit of Arginase. Clin. Exp. Metastas 18(6): 509-518.

[35] Wheatley, D.N., Philip, R. and Campbell, E. (2003). Arginine Deprivation and Tumor Cell Death: Arginase and Its Inhibition. Mol. Cell Biochem. 244: 177-185.

[36] Nsa, I.Y., Kareem, R.O. Aina, O.T., and Akinyemi, B.T (2020). Isolation of an Emerging Thermotolerant Medically Important Fungus, *Lichtheimia ramosa* from the soil. Afr. J. Microbiol. Res. 14(6): 237-241.

[37] Google. (n.d.). Atan/imuku. Retrieved Apr 6, 2019 from https://goo.gl/maps/zULhcsjSB9Wz3SDo7

[38] Google. (n.d.). Ikorodu. Retrieved Apr 11, 2019 from https://goo.gl/maps/AKDPSaAT52w65nvt6

[39] Vogel H. J., (1956). A convenient growth medium for *Neurospora* (medium N). Microb. Genet. Bull 13: 2–43.

[40] Metzenberg, R. L., (2003). Vogel's medium N salts: Avoiding the need for ammonium nitrate. Fungal Genet. Newsl. 50: 14.

[41] Anupama, M., Kumar, T. V. and Latha, J. N. L. (2014). Growth and metal removal by *Neurospora crassa* from marine water. Int. J. Biol. Pharmaceut. Res. 5: 22-26.

[42] Marathe, S., Yu, Y. G., Turner, G. E., Palmier, C., & Weiss, R. L. (1998). Multiple forms of arginase are differentially expressed from a single

locus in Neurospora crassa. J. Biol. 273(45): 29776-29785.

[43] Drainas, C. and Weiss, R.L. (1980). Effect of carbon source on enzymes and metabolites of arginine metabolism in *Neurospora*. J. Bacteriol. 141(1): 205-212.

[44] Davis, R.H. and Mora, J. J. (1968). Mutants of *Neurospora crassa* deficient in Ornithine-transaminase. J. Bacteriol. 96: 383-388.

[45] Kitamura, M. and Iuchi, I. (1959). An Improved Diacetylmonoxime Method for the Determination of Urea in Blood and Urine. Clin. Chim. Acta 4: 701-710.

[46] Keenan, Kelly A. "Enzyme and Protein Assay" n.d., Fungal Genetics Stock Center. Web (http://www.fgsc/teaching/Kennan). Date accessed: 08/07/2020.

[47] Hall, T. (1999). BioEdit: a user - friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

[48] Maheshwari, R. and Antony. A. (1974). A Selective Technique for the Isolation of *Neurospora crassa* from Soil. J. Gen. Microbiol. 81: 505-507.

[49] Kuo, H. C., Hui, S., Choi, J., Asiegbu, F.O., Valkonen, J.P. and Lee, Y. H. (2014). Secret lifestyles of *Neurospora crassa*. Sci. Rep. 4: 5135. Published 2014 May 30. DOI:10.1038/srep05135

[50] Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ (1990). Basic Local Alignment Search Tool. J. Mol. Biol. 215: 403-410.

[51] Ryan, F., Beadle, J. and Tatum, E. (1943). The Tube Method of Measuring the Growth Rate of *Neurospora*. Am. J. Bot. 30(10): 784-799.

[52] King, B. (2009). Race Tube Activity: Circadian Rhythms using Conidiation of *Neurospora crassa*. Conference Paper. Conference: Urban High School Development, At Louisville, Volume: 200

[53] Caldwell, R. B., Toque, H. A., Narayanan, S. P., & Caldwell, R. W. (2015). Arginase: an old enzyme with new tricks. Trends Pharmacol. Sci. 36(6): 395–405. https://doi.org/10.1016/j.tips.2015.03.006

[54] Corraliza, M. (2002). Increased expression of arginase II in patients with different forms of arthritis. Implications of the regulation of nitric oxide. J. Rheumatol. 29: 2261–2265.

[55] Kaur, G., Verma, N. and Wheatley, D. N. (2008). Comparison of Various Cell Disruption Techniques for the Extraction of Arginase from *Neurospora crassa*. Res. J. of Biotechnol. 3(1): 39-44.