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Production of the Anti-Leukemic Therapeutic Enzyme, L-Asparaginase, by A Brackish Sediment Strain of *Aspergillus candidus*

Atim Asitok*, Maurice Ekpenyong
Environmental Microbiology and Biotechnology Unit, Department of Microbiology, Faculty of Biological Sciences, University of Calabar, P.M.B.1115 Calabar, Nigeria.

ABSTRACT

Aspergillus candidus strain IR-A4 was isolated from the brackish sediment of Itu River, Akwa Ibom State, Nigeria on Czapex-Dox yeast-extract agar and screened for L-asparaginase production by the rapid plate technique and in submerged fermentation. Protein was detected in cell-free fermentation broth by the Bradford method and confirmed as L-asparaginase by rapid development of pink colour on asparagine-minimal medium. The protein was partially purified by $(NH_4)_2SO_4$ precipitation and dialysis against Tris-HCl buffer. L-asparaginase activity, evaluated by the colorimetric Nesslerization method, was 1282 ± 70.5 U with a specific activity of 17.26 U/mg. The enzyme demonstrated very minimal glutaminase specific activity of 0.03 U/mg with a 352-fold lower specific activity relative to L-asparaginase activity suggesting near-free allergic reactions in the course of therapy. The anti-leukemic activity of the enzyme, demonstrated by *in vitro* cytotoxicity assay using HL-60 cell lines, showed that 87.98% of the dialyzed fraction of the enzyme could destroy 50% (IC50) of leukemic cells. There was 97% association, ω^2, between fermentor size and enzyme activity suggesting great potential for large-scale fermentative production of the therapeutic enzyme. Optimal conditions for enzyme activity were set at (i) Substrate concentration, 220-260 mM (ii) pH 8-9 and (iii) Temperature, 35-45ºC. The mould is recommended for large-scale production of low-glutaminase activity L-asparaginase for treatment of lymphoblastic leukemia patients.

Keywords: *Aspergillus candidus* strain IR-A4; L-asparaginase production; Glutaminase activity; Anti-leukemic activity.

*Corresponding Author Email: atimasitok@yahoo.com
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INTRODUCTION

Cancer is an all-encompassing terminology that describes diseases that ensue following the uncontrolled growth and division of cells driven by cellular changes that could be mediated, very frequently, by genetic and environmental cues like radiation and pollutants pesticides[1,2]. The affected cells could be parts of tissues and organs including blood, liver, kidney, cervix, ovary, thyroid, breast, prostate, colon and bladder; the target of which frequently describes the type of cancer under consideration[1]. A very significant difference between cancer and normal cells is the inability of cancer cells to obey the instruction to stop dividing and proceed to death, so they could be replaced by newer and more functional cells.

Leukemia refers to cancers of the bone marrow and the tissues derived therefrom, especially blood[2]. These neoplasms are the leading kinds of cancer in many countries, especially in developed countries, where incidence rate is reported as 40 cases per million[3]. Documented evidence reports 9-10 acute lymphoblastic leukemia (ALL) cases in every 100,000 patients with childhood (≤ 15 years) cases constituting 80% and adult cases just 20%[3]. Lymphoblasts are immature B- and T-lymphocytes which become cancerous when triggered by genetic and environmental factors especially exposure to ionizing radiations and pesticides[2,4].

For nearly three decades now, cytotoxic chemotherapy had been one of the treatment options for ALL. Other documented options include radiation therapy, stem cell transplantation and targeted drug therapy. L-asparaginase (EC 3.5.1.1) is an antineoplastic aminohydrolase which has been used as a component of the combination cytotoxic chemotherapy regimen for childhood ALL[5]. In its pharmacodynamics, the enzyme exploits the auxotrophic status of leukemic cells for L-asparagine; a biosynthesizing ability that they lost as a result of mutation in the gene that codes for asparagine synthetase[6,7]. The amino acid is therefore an essential amino acid in leukemic cells but a non-essential one in normal cells[8]. As a consequence, leukemic cells scavenge this amino acid from the blood and other sources. Starving leukemic cells of L-asparagine by L-asparaginase (ASPNase) hydrolysis is a major thrust in the regulation of progress and treatment of ALL in both children and adults. ASPNase hydrolyzes L-asparagine into L-aspartic acid and ammonia, thereby starving leukemic cells to death through shut-down of protein synthesis[9]. The enzyme is produced by all life forms including microorganisms, plants and animals except humans[10]. However, biotechnological exploitations have mostly been conducted on species of bacteria, yeast and mold genera[11] owing to their cost effectiveness and eco-friendliness[12]. Commercial production and clinical application of ASPNase for ALL treatment has focused on ASPNases from only a few bacteria especially *Escherichia coli* and *Erwinia chrysanthemi*[13,14].

A very significant drawback of ASPNase-dependent chemotherapy for ALL is side activity of glutaminase activity which is reported to be responsible for most of the allergies and hypersensitivity reactions from L-asparaginase therapy with resultant liver, circulatory and neurological complications[15,16.] Production of glutaminase-free ASPNase and/or those with minimal L-glutaminase activity from filamentous bacteria and fungi is being vigorously pursued by microbial biotechnologists primaritliy owing to the low to non-toxic nature of their enzymes[17,18,19,20,21].

This paper reports on the production of low glutaminase activity L-asparaginase from *Aspergillus candidus* strain IR-A4 with potential for treatment of acute lymphoblastic leukemia (ALL). Its propensity for pilot and industrial scale production is also reported. Although there have been reports on the production of L-asparaginase from *Aspergillus terreus, A. tamari* and *A. niger* [7,11], this, to the best of our knowledge, is the first report on the production of low-glutaminase activity L-asparaginase from a strain of *Aspergillus candidus*.

MATERIALS AND METHOD

Study area description, sampling and mycological analysis

Description of study area

Itu River is a narrow river lying 285.8 m between the settlements of the Itu people of Akwa Ibom State and Eniong people of Cross River State and located along the geographical coordinates of 5º 39' 0" North, 8º 39' 0" East. Itu, particularly Esuk Itu and Obot Itu and Eniong settlements are fisher folk communities which form part of the 12 clan structure of the Efik people of Nigeria. Small creeks branch off the river from the right flank into and out of Eniong settlements. Towards the head of the river is a beach, its market and an abattoir, which processing wastes are emptied chronically into the river. The river is tidal and receives domestic and agricultural wastes from the market and the abattoir, in addition to t industrial wastes of complex composition from the larger Cross River, and distributes them through its small creeks into neighboring streams. By reason of poor depth of the river, a large proportion of waste readily reaches the river bed and forms part of its sediment, thus meeting the nutritional needs of the micro- and macro-flora and fauna. The river sediment is therefore a sink for wastes from the settlements and a good source of biotechnologically-relevant microorganisms.

Sampling

A total of 60 intertidal sediment samples were collected from 12 different locations (5 per location) in the river by means of a Grab sampler. Sampling points were located by means of Global Positional System (GPS). Intra-flank sample distances were 200 m and the 5 samples

were collected 40 m from each other within the location. Samples were transported in sterile wide-mouth glass bottles to the laboratory for analyses.

Mycological analysis

The 5 samples from each location were mixed and sun-dried for 72 h and large sediment clumps broken and homogenized by means of a vortex mixer (XH-C, PEC MEDICAL, USA). The resulting 12 homogenous composite samples, labeled A to L, were diluted 1000-fold and triplicate dilutions plated in triplicates onto Czapek-Dox Yeast Agar (CYA). Plates were incubated at room temperature (28 ± 2ºC) for 72 h. All morphologically-distinct fungal colonies (evaluated in terms of colonial colour, texture and reverse plate colouration and pattern) were isolated and repeatedly sub-cultured onto CYA medium to obtain pure cultures. Purified cultures were maintained on agar plates at 4ºC until required for further studies.

Primary screening for L-asparaginase

All purified mold isolates were screened for ASPNase production on minimal medium without extraneous nitrogen and carbon sources using the rapid plate assay of Gulati *et al*.[22] The minimal medium contained (g/L): Na_2HPO_4 6; KH_2PO_4 3; $MgSO_4.7H_2O$ 0.2; $FeSO_4.7H_2O$ 0.05, NaCl 0.5; $CaCl_2$ 0.5; KCl 0.5 and was supplemented with 1 mL trace mineral solution with composition (g/L): $ZnSO_4.7H_2O$ 0.005; $MnSO_4.4H_2O$ 0.005; H_3BO_3 0.005; $CuSO_4.5H_2O$ 0.005; $CoCl_2.6H_2O$ 0.005; $MoNa_2O_4.2H_2O$ 0.005 and $NiCl_2.6H_2O$ 0.005 [23]. The minimal medium was supplemented with 1% (w/v) L-asparagine (Merck) as sole sources of carbon and nitrogen, 2% agar-agar, 0.35 mL 2.5% phenol red as indicator and pH adjusted to 5.5 using 1N HCl. The medium was sterilized by autoclaving at 121ºC for 15 min. Upon cooling at ambient laboratory conditions, agar plugs (6 mm) of each pure mold culture, retrieved from the refrigerator and allowed to equilibrate at room temperature, was aseptically transferred to the surface of sterile medium. Plates were incubated at room temperature (28 ± 2ºC) for 120 h. ASPNase-positive plates were selected on the basis of pink colouration that developed between 48 and 120 h of incubation while ASPNase-negative plates that remained amber coloured were discarded.

Secondary screening of L-asparaginase-positive molds in submerged culture

All molds that developed pink colonies in the primary screening were subjected to shake flask experiments. Medium composition was as described in section 2.2 minus agar-agar and phenol red. The medium was dispensed in 10 mL aliquots into 50 mL Erlenmeyer flasks and sterilized as described earlier. A 96-h old agar plug of each positive mold isolate was introduced into correspondingly labeled flasks and flasks plugged with sterile cotton wool and incubated in an orbital shaker agitating at 100 rpm for 120 h. After fermentation, flask contents were filtered by means of Whatman No.3 filter paper, followed by filtration of resultant culture filtrate with

0.22 µM (Millipore) membrane filters. The sterile broth was subjected to protein determination by the Bradford method of protein detection as described in Ekpenyong *et al*. [24]. The procedure is based on the principle of protein-dye binding. Development of blue colour from the light-green colour of the protein reagent (coomassie brilliant blue G-250) was indicative of the presence of protein in sterile test samples. Protein quantification was conducted using a protein calibration curve with bovine serum albumin as standard protein.

Confirmation of L-asparaginase presence by enzyme action

Sterile crude ASPNase fermentation broth, earlier used for protein detection in section 2.3, was tested for ASPNase action on the agar medium used in section 2.2 for the primary screening. Upon cooling and setting, 0.1 µL of each sterile ASPNase broth was dropped onto the surface of the L-asparagine-minimal agar medium and observed for development of pink colouration within 5 min. Time to pink colour development was monitored with a stop watch.

Production of L-asparaginase in submerged culture by selected mold

Most productive mold selected on the basis of protein quantity and time to development of pink colour in the confirmatory test, was employed in ASPNase production in different Pyrex® Erlenmeyer flask sizes of dimensions (base diameter x flask height): 125 mL (6.7 cm x 11.4 cm), 250 mL (8.2 cm x 13.2 cm), 500 mL (10.1cm x 17.6 cm), 1000 mL (12.9 cm x 21.6 cm), 2000 mL (16.0 cm x 26.8 cm) and 4000 mL (20.6 cm x 36.0 cm).

Preparation of spore suspension of selected mold

A 72-h old Potato Dextrose Agar (PDA) spores and mycelia of selected mold were transferred to an agar slant of similar medium in a Roux bottle. The bottle was incubated at room temperature for 96 h to obtain sufficient sporulation. Thereafter, spores were harvested with 5 mL sterile phosphate buffer (pH 7), washed twice with same buffer and suspension prepared, again, in the same buffer. Spore working concentration of 10^8 spore forming units per milliliter (sfu/mL) was prepared by dilution from the spore stock suspension.

L-asparaginase production in submerged culture

Medium composition was as described in section 2.2 except that agar-agar and phenol red were excluded. Medium was supplemented with L-asparagine at 1% (w/v), pH adjusted to 5.5 and medium dispensed into Erlenmeyer flasks of different sizes at 20% (v/v). Flasks were sterilized as described earlier. Upon cooling, each flask was inoculated with 2% (v/v) of 10^8 sfu/mL spore suspension and flasks incubated at room temperature on an orbital shaker agitating at 100 rpm for 72 h. ASPNase was harvested from the flasks by filtration with Whatman No.3 filter paper. The filtrate was centrifuged at 8,000 rpm for 10 min and supernatant subjected to membrane filtration using 0.22 µM Millipore membrane filter. Sterility was confirmed by spread plating 0.1 mL of presumed cell-free fermentation broth onto Czapek-Dox yeast agar

medium and incubating at room temperature for 120 h. Total protein, ASPNase activity and glutaminase activity were determined. All determinations were carried out in triplicates. Means of all triplicate determinations were compared using one-way analysis of variance (one-way ANOVA) in SPSS v 20 (IBM, USA). Significant means were separated by Bonferroni post hoc multiple comparison test.

L-asparaginase activity assay

L-asparaginase activity assay was conducted using the Nesslerization method [25]. Briefly, 0.1 mL of the concentrated supernatant from section 2.7 was added to 0.2 mL of 0.05M Tris-HCl buffer at pH 8.5. This was followed by the addition of 1.7 mL of 10 mM L-asparagine as substrate. The preparation was incubated in a water bath at 37ºC for 10 min. The reaction was stopped by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA) and the resultant suspension centrifuged at 10,000 rpm for 15 min to remove precipitated protein. A blank was prepared by adding enzyme solution after stopping reaction with TCA. An aliquot of 0.5 mL of supernatant was made up to 7.0 mL with sterile distilled water and thereafter treated with 1 mL Nessler's reagent; a 0.09 mol/L solution of potassium tetraiodomercurate (II) ($K_2[HgI_4]$) in 2.5 mol/L potassium hydroxide (KOH). The mixture was incubated at room temperature (28 ± 2ºC) for 15 min until yellowish-brown precipitate, indicative of ammonia presence, developed. The absorbance of the yellowish-brown coloration was read off a UV/Vis Spectrophotometer (Mettler Toledo, Ohio, USA) at a wavelength of 475 nm. Amount of ammonia liberated from L-asparagine hydrolysis by ASPNase was determined from a pre-plotted ammonium sulfate standard curve. One unit of ASPNase activity was defined as the amount of enzyme that liberated one micromole of ammonia per milliliter per minute at 37ºC.

Glutaminase activity assay

The assay protocol for ASPNase activity was repeated for glutaminase activity assay except that glutamine replaced asparagine as enzyme substrate for glutaminase activity according to the protocol by Imada *et al*.[25].

Mold characterization and identification

Mold was characterized based on macromorphological, micromorphological and molecular approaches by 18S rRNA sequencing using internal transcribed spacer (ITS) region universal primers (ITS1-F and ITS4) as described in Manter and Vivanco[26] and Asitok and Ekpenyong[27].

Partial purification of L-asparaginase

The sterile (cell-free) L-asparaginase broth was gradually brought to 40% saturation with ammonium sulfate (enzyme grade) and the precipitate removed by centrifugation at 12,000 rpm for 10 min. The resulting supernatant was then brought to 80% saturation with the salt and incubated overnight in the refrigerator at 4ºC. The proportion of protein precipitated between

40 and 80% saturation was recovered from the mixture by centrifugation, dissolved in 10 mL of 20 mM Tris-HCl buffer at pH 7.5. Total protein, ASPNase activity and glutaminase activity were quantified as described earlier. Furthermore, the precipitated protein was dialyzed against the Tris-HCl buffer for 36 h to remove residual ammonium sulfate. The dialyzed protein suspension was concentrated in a refrigerated centrifuge at 16,000 rpm for 10 min and particulate material removed. Total protein, ASPNase activity and glutaminase activity were determined from the concentrated dialyzed supernatant.

In-vitro anti-cancer activity assay

Here, we adopted the cytotoxicity assay of Skehan *et al*.[28] using the human leukemia cell line, HL-60. The lymphoma cells were first tested for *Mycoplasma* using MycoAlert assay (Lonza) before being cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 100U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum (FBS). The cell culture was maintained in humidified atmosphere of 5% CO_2 at 37ºC and sub-cultured every 72 h. For viability testing, cells were made to adhere to micro titer plates by seeding them in 96-well tissue culture plates at a density of 1 x 10^5 cells/mL and incubating plates in 5% CO_2 at 37ºC for 24 h. The cytotoxicity of the enzyme was tested against the cell line using the sulforhodamine B (SRB) assay. Cells in the exponential phase of growth were collected using 0.25% trypsin-EDTA and plated in 96-well plates at 100 µL/well. The cells were exposed to different concentrations of ASPNase for 72 h. The concentrated supernatant served as 100% concentration while lower concentrations were prepared by dilutions in sterile distilled water. Thereafter, cells were fixed with 10% TCA (100 µL/well) for 1 h at 4°C and subsequently exposed to a 0.4% SRB solution for 10 min in a dark chamber and then washed with 1% glacial acetic acid to remove unbound dye. The preparation was dried overnight and SRB dye solubilized with 10mM Tris-HCl (50µL/well, pH 7.4) for 5 min on a shaker at 1600 rpm. The optical density of each well was read off a microplate reader at 570 nm.

Effects of temperature, pH and substrate concentration on L-asparaginase activity

Optimum temperature for ASPNase activity was determined over a range of temperatures from 5ºC to 70ºC. Selection of optimum pH for ASPNase activity was determined by conducting ASPNase activity assay over a range of pH values from 3 to 12 using appropriate buffers [21,29]. Effect of substrate concentration on enzyme activity was conducted by assaying ASPNase activity over asparagine concentrations ranging from 10 mM to 260 mM. Data was subjected to regression analyses and models explained by adjusted goodness-of-fit, R^2 values.

RESULTS AND DISCUSSION

Results of mycological analyses of samples are presented in Table 1 and show that sample A, located at the head of the river and closest to waste release points, had the highest number of

morphologically-distinct mold species. This is suggestive of a great diversity of substrates released into the river from various domestic, agricultural and industrial activities. The sample with the least number of morphologically-distinct mold species of 3 was sample L, located the furthest from the river head. This suggests that readily metabolizable substrates disappear from the river with increasing distance from river head. The table also shows that sample A had the highest number of ASPNase-positive molds of 20 constituting 95% of all mold species isolated in the area. However, only 4 (19%) of the molds that tested positive to ASPNase in solid medium could do so in secondary screening. The mold isolates that could produce detectable amounts of ASPNase in submerged culture are presented in Figure 1. The figure, which also shows the time to pink colour development for the respective ASPNases, revealed that isolate A4 had a total protein amount of 142.67 mg and ASPNase activity was confirmed by pink colour development on asparagine-minimal agar medium within 47 s (Plate 1). This was followed by mold A2 with 93.25 mg and mold C2 with 83.82 mg of protein. Isolate J1 had the least protein concentration of 14.53 mg but with shortest time to pink colour development of 45 s suggesting that although the mold produced little amount of protein, its protein was most efficient in enzyme action. None of the 3 mold species in sample L could produce ASPNase in either solid or liquid media.

Table 1: Distribution of L-asparaginase producing mold isolates in Itu brackish sediment samples

Sample code	No. of mold colonies	ASPNase positive mold (SM)	% ASPNase positive mold (SM)	ASPNase positive mold (LM)	% ASPNase positive mold (LM)
A	21	20	95.2	4	19.0
B	17	15	88.2	1	5.9
C	16	13	81.2	2	12.5
D	14	11	78.6	1	7.1
E	14	10	71.4	1	7.1
F	13	10	76.9	1	7.7
G	12	8	66.7	1	8.3
H	10	6	60	1	10
I	10	4	40	0	0
J	8	3	37.5	1	12.5
K	6	2	33.3	0	0
L	3	0	0	0	0
TOTAL	144	102	70.8	13	9.0

LASNase – L-asparaginase; SM-Solid medium; LM-liquid medium

Two separate one-factor analysis of variance (one-way ANOVA) were conducted to elucidate the effect of fermentor (flask) size on total protein and enzyme activity as dependent variables. The spread of data met all six assumptions of the statistic. The test for independence of data was taken care of at the point of design while test of normality was conducted using Shapiro-Wilk's test at $\alpha = .001$. The results are presented in Figures 2a and b using Box plots. All the

groups (flask sizes) showed that $p > \alpha$ at .001, indicating that every single level of the independent variable (flask size) for the two dependent variables was normally distributed, thus meeting the normality assumption.

Next, we tested the homogeneity assumption that the variances of the six flask sizes were equal; that is, they were not significantly different. The result of Levene's test showed that the test was not significant; $F(5, 12) = .242$, $p = .936 > .05$ for total protein and $F(5, 12) = .600$, $p = .701 > .05$ for enzyme activity. The assumption of homogeneity of variance was accordingly, not violated, suggesting that one-way ANOVA could be conducted on the data.

Examination of the ANOVA table revealed that the overall *F* ratio for total protein was significant; $F(5, 12) = 50.37$, $p < .001$ and that for enzyme activity was also significant; $F(5, 12) = 119.04$, $p < .001$. These results indicate that at least one of the means of the six flask sizes for either dependent variable was significantly different from others and so the null hypothesis that all six group means were equal was rejected.

Since the *F* ratios were significant, we proceeded to measure the strength of association, ω^2, between the flask sizes (independent variable) and the dependent variables using the equation;

$$\omega^2 = SS_B - (K-1)MS_W \div SS_T + MS_W$$

where ω^2 is the strength of association; SS_B the sum of squares of between groups, *K* is the number of groups, MS_W the mean square for within groups and SS_T the total sum of squares. Solving for ω^2 for total protein gave strength of association of .932 suggesting that flask sizes accounted for 93.2% of the variance in total protein. The ω^2 between flask sizes and enzyme activity was calculated as .97 suggesting that the independent variable accounted for 97% of the variations observed in enzyme activity values.

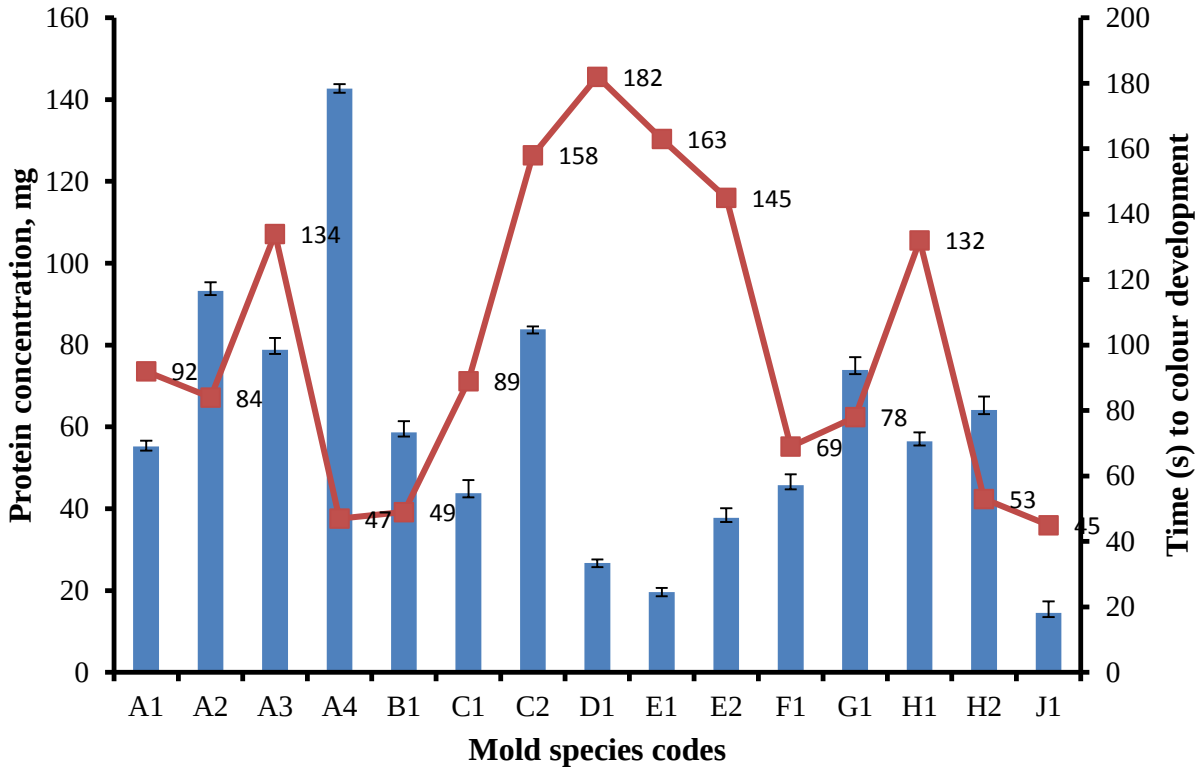
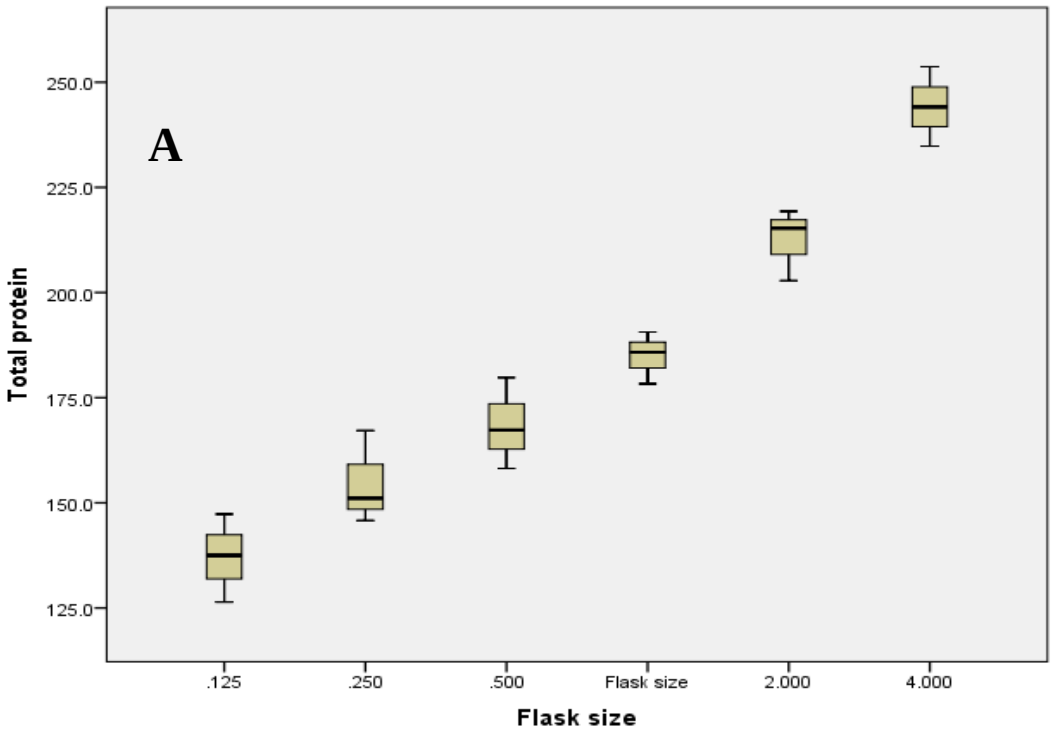


Figure 1: Total protein-dependent selection of ASPNase-positive mold species



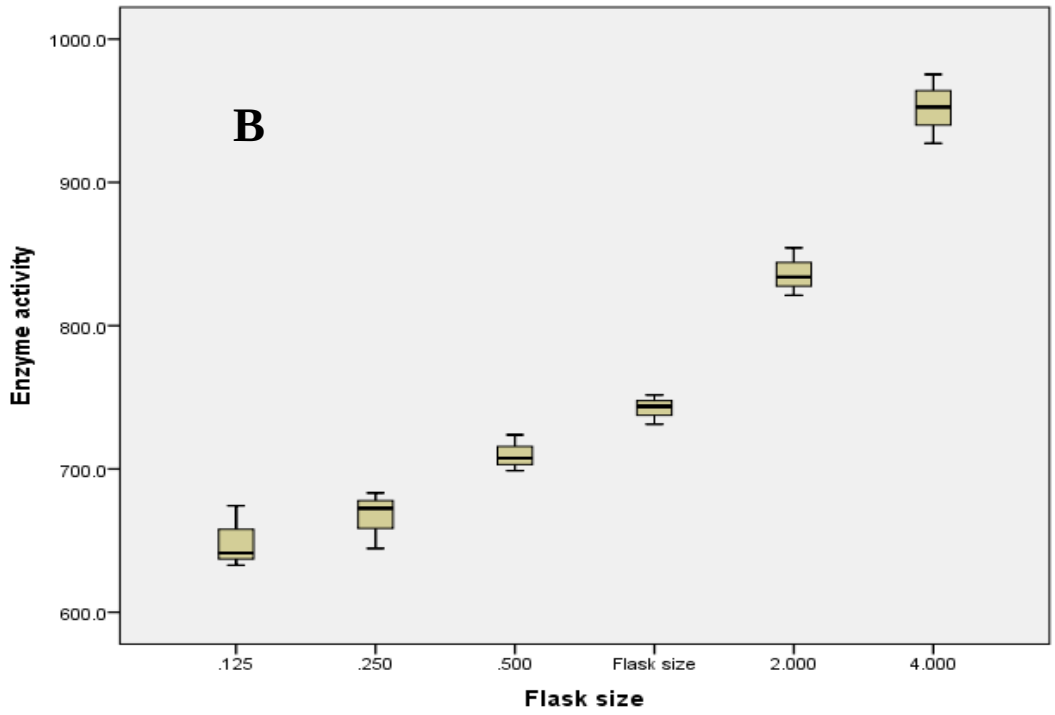


Figure 2: Box plots for test of normality assumption of one-way ANOVA of effect of flask size on amount of total protein (A) and enzyme activity (B).

*The flask size on the figure was set by software as default and represents 1 L

A post hoc test was conducted to separate significant mean differences. Since the sample size was small, we adopted the Bonferroni multiple comparison test which is more stringent and discriminating than the popular Turkey HSD test frequently adopted for researches in the social sciences. Results showed that mean total protein from 4 L Erlenmeyer flask (M = 244.2, SD = 9.45) was significantly different ($p < .05$) from those obtained from .125 L (M = 137.07, SD = 10.46), .250 L (M = 154.7, SD = 11.14), .5 L (M = 168.43, SD = 10.84), 1 L (M = 184.9, SD = 6.20), 2 L (M = 212.47, SD = 8.61). There were no statistically significant differences ($p > .05$) in total protein amounts produced in .125 L (M = 137.07, SD = 10.46) and .250 L (M = 154.7, SD = 11.14), .250 L (M = 154.7, SD = 11.14) and .5 L (M = 168.43, SD = 10.84) and 1 L (M = 184.9, SD = 6.20) as well as between 1 L (M = 184.9, SD = 6.20) and 2 L (M = 212.47, SD = 8.61).

Bonferroni post hoc tests also showed that mean enzyme activity from 4 L fermentor (M = 951.77, SD = 24.06) was significantly different ($p < .001$) from those obtained from .125 L (M = 649.5, SD = 21.90), .250 L (M = 666.8, SD = 20.04), .5 L (M = 710.03, SD = 12.69), 1 L (M = 742.17, SD = 10.29) and 2 L (M = 836.47, SD = 16.70). However, there were no statistically significant differences ($p > .05$) in enzyme activity produced in .125 L (M = 649.5, SD = 21.90) and .250 L (M = 666.8, SD = 20.04); .250 L (M = 666.8, SD = 20.04) and .5 L (M = 710.03, SD = 12.69); .5 L (M = 710.03, SD = 12.69) and 1 L (M = 742.17, SD = 10.29). It was interesting to note that although there was a statistically significant difference in enzyme

activity between 1 L and 2 L flasks, the difference in the amount of total protein obtained from these two flask sizes was not statistically significant. The higher sensitivity of enzyme activity to flask sizes is confirmed by the strength of association, ω^2, which variance of 97% could be attributed to variations in flask sizes.

Plates 2a and b are pictorials of the selected mold on Czapek-Dox yeast agar (CYA). Plate 2a depicts the mold as pure colonies after 120 h at 30ºC (below room and environmental temperatures). It shows the white cottony texture with granular conidia upon maturity. Plate 2b shows a pure culture of the mold showing the slow-growing, dull-white or creamy concentric mold with a pinkish red center after growth on CYA for 72 h. Microscopy revealed narrow phialide heads attached directly to conidiophores. The conidia were globose, smooth and thin-walled. The selected mold was identified after 18S rRNA sequencing using universal internal transcribed spacer (ITS) primer sequences (ITS1-F and ITS4) as a strain of *Aspergillus candidus* with 100% sequence similarity with *Aspergillus candidus* strain JN-YG-3-2 (GeneBank accession number: MG554228.1). The strain in this study was named *Aspergillus candidus* strain IR-A4 and was deposited in the University of Calabar Collection of Microorganisms (UCCM).

One of the major draw-backs in ASPNase treatment of acute lymphoblastic leukemia is simultaneous glutaminase activity which is responsible for the hypersensitivity problems of the treatment. Glutaminase activity-free ASPNase or at least those with low glutaminase activity are accordingly sought after during ASPNase bioprospecting and applications [21]. Results of the effect of purification levels on ASPNase activity, glutaminase activity and yield are presented in Table 2. The table shows that as purification levels increased, total protein, ASPNase and glutaminase activities decreased. However, specific activities of both enzymes increased with increasing purification of the target enzyme. Results showed that the concentrated dialyzed ASPNase had a specific activity of 10.57 U/mg but with glutaminase specific activity of 0.03 U/mg. This implies that at this stage of purification, the specific activity of ASPNase was about 350-fold higher than that of glutaminase. The dialyzed ASPNase fraction was 2.71-fold purer than the crude fraction while the $(NH_4)_2SO_4$ fraction was 1.94-fold purer. It is believed that at the final purification of the therapeutic enzyme, glutaminase activity will be completely negligible relative to ASPNase activity. The % yield of ASPNase which decreased marginally with increasing purification step is also shown in the table. *Per cent* yield of ASPNase in the dialyzed fraction was reported as 82.63%.

The anti-leukemic activity of the ASPNase obtained from *Aspergillus candudus* strain IR-A4 is presented in Figure 4 and showed that % viability of HL-60 cells decreased as concentration of the therapeutic enzyme increased. Regression analysis conducted to show to establish the

relationship between ASPNase concentration and cytotoxicity (% viability) confirmed a significant; $F(1,9) = 842.69$, $p < .001$; strong negative linear correlation, $r = -.995$; and a goodness-of-fit, $R^2 = .989$ with an adjusted R^2 of .988. This indicates that 98.8% of the loss of viability of human leukemia cell lines (HL-60) was due to the cytotoxicity of ASPNase.

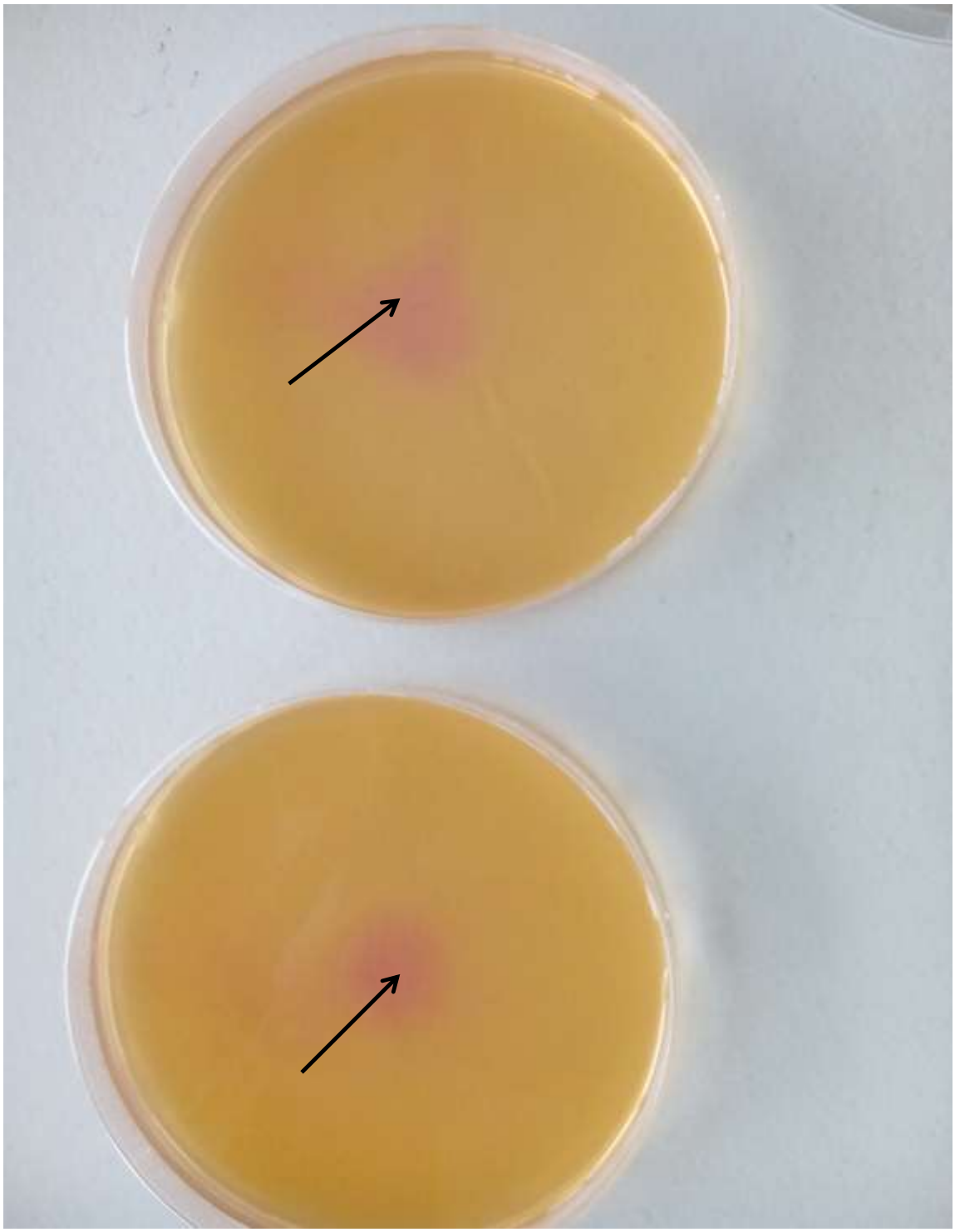


Plate 1: Confirmatory ASPNase activity of *Aspergillus candidus* strain IR-A4 on asparagine-minimal medium. Arrow shows pink colour development under 50 s of drop contact with medium

Plate 2: The white-spore colonies of *Aspergillus candidus* strain IR-A4 on potato dextrose agar (PDA) after 72 h (A) and its cottony pure culture (B) on same medium after 120 h

The therapeutic enzyme hydrolyzes the amino acid, asparagine; the specific growth factor required for viability of lymphoblastic cells, into aspartic acid and ammonia. This biochemical reaction results in the starvation of HL-60 cells leading to loss of viability and cell death (refs). Solving for IC50 from the regression equation revealed that 87.98% of the concentrated dialyzed L-asparaginase supernatant would bring about a 50% cytotoxicity of the HL-60 cells.

A more purified form of the enzyme is expected to reduce the concentration of the therapeutic enzyme required to achieve this level of treatment.

Results of the effects of substrate concentration, pH and temperature on ASPNase activity are presented in Figure 4a, b and c respectively. Figure 4a shows that ASPNase activity increased gradually with increasing concentrations of asparagine until it peaked at 220 mM of asparagine where enzyme activity was 1282 ± 70.5 U. A statistically non-significant ($p > .05$) rise to 1284 ± 59.2 U was recorded at substrate concentration of 240 mM and an equally non-significant drop to 1281 ± 42.8 U at 260 mM of asparagine. L-glutaminase activity did not significantly increase with increase in substrate concentration at 20 mM of glutamine. The non-significant increase in glutaminase activity with increasing substrate concentration is viewed as salutary since L-glutaminase side activity is not required for the cytotoxic chemotherapy of leukemic cells by ASPNase [30].

The overall effect of substrate concentration tested by one-way ANOVA of a regression model showed significant ($p < .001$) linearity ($R^2 = .904$) in the relationship with increasing activity as substrate concentration increased from 20 to 220 mM. Above that concentration the plot flattened out suggesting that substrate saturation point had been reached and that no further increase in enzyme activity would occur with further increases in substrate concentration. Similar substrate concentrations have earlier been reported by Moharib[31] for the ASPNase produced from *Vigna unguiculata* and Aghaiypour *et al*.[32] from *Erwinia chrysanthemi* ASPNase. To explain the curvature in the graph, a second-order polynomial regression was employed and it revealed an R^2 value of .9583, indicating that the effect of substrate concentration on ASPNase activity was not entirely linear because a 96%

Table 2: Effects of purification steps on total protein, enzyme activities and yield

Purification steps	**Total protein (mg)**	**L-asparaginase assay**		**Glutaminase assay**		**ASPNase Yield (%)**
		ASPNase activity (U)	**Specific activity (U/mg)**	**GLUNase activity (U)**	**Specific activity (U/mg)**	
Crude Extract	244.2	951.8	3.90	1.9	0.008	100
$(NH_4)_2SO_4$ precipitated fraction	116.8	894.3	7.66	2.1	0.018	93.96
Dialyzed fraction	74.4	786.5[a] 1284.5[b]	10.57[a] 17.26[b]	2.23[a] 2.56[b]	0.03 0.034	82.63

[a]- Activity at assay substrate concentration; [b]-Activity at maximum substrate concentration

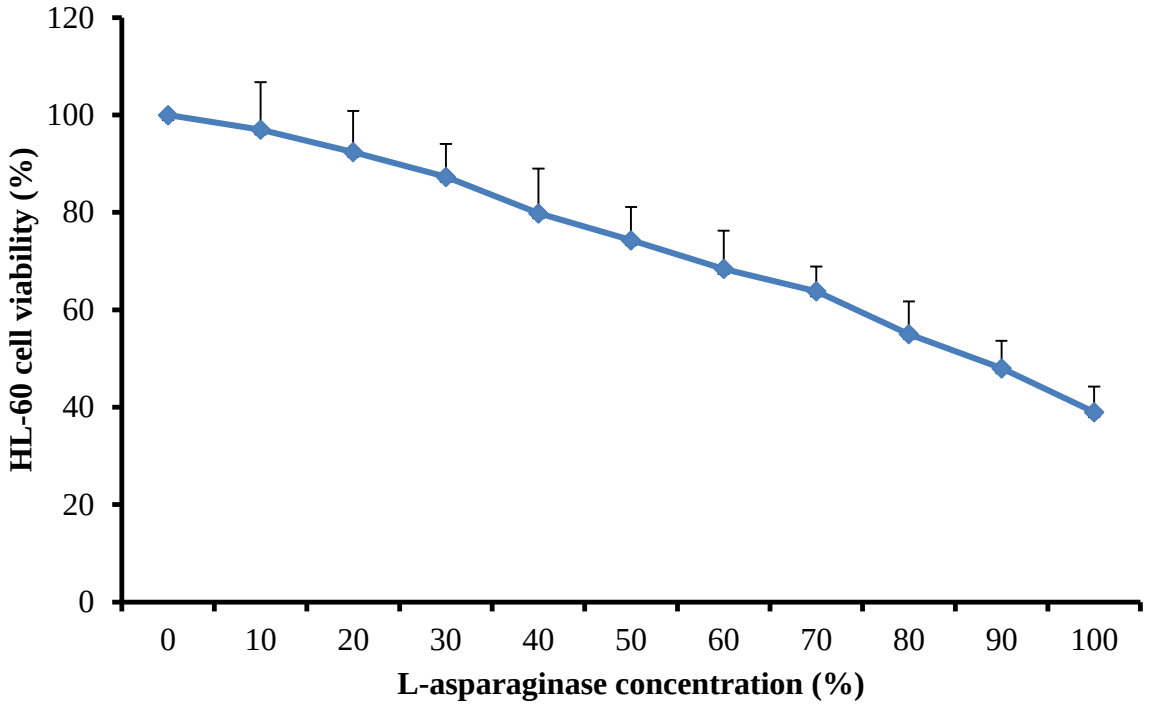


Figure 3: Anti-leukemic effect of L-asparaginase on HL-60 cells

accountability of a model is better than 90% as observed for polynomial and linear regressions respectively.

Figure 4b shows the effect of pH on ASPNase activity which revealed that optimum pH for *Aspergillus candidus* strain IR-A4 ASPNase was between 8 and 9. L-asparaginase activity, at maximum substrate concentration of 220 mM of asparagine, was found to increase as pH increased from 3 to 8 and started its decline upon further increase in pH. Similar pH effects have earlier been reported for ASPNase by *Pseudomonas aeruginosa* 50071 produced under solid state fermentation[33]. L-asparaginases active at physiological pH of humans had been reported as effective and efficient, with minimal side effects[34] owing to their compatibility with the physiological state of leukemia patients. Moreover, the very nature of ASPNases as amidases with optimum activity at alkaline pH is well established[35]. The activity response plot shows a perfect curvature best explained by a polynomial regression model as accounting for 89.4% of the observed variations in ASPNase activity among independent groups, in contrast to the linear model which was only responsible for 38.2% of those variations. Use of polynomial regression models to account for effects of curvature in response plots had earlier been reported in Ekpenyong et al.[36].

Figure 4c shows the effect of temperature on ASPNase activity which increased steadily at a significantly constant rate from 5ºC to 35ºC where activity showed no significant increase with further rise in temperature. Since ASPNase activity did not significantly beyond 45ºC, a temperature range between 35ºC and 45ºC as optimum for the ASPNase from this study is

proposed. A similar temperature optimum for *Bacillus subtilis* strain hswx88 ASPNase activity had earlier been reported by Pradhan *et al.*[37]. The linear regression model for the relationship between temperature and ASPNase activity was not significant ($p = .29 > .05$, $R^2 = .094$) and could explain only 6% of the variations observed in the dependent variable. A significant second-order polynomial regression that took care of the curvature in the response pattern could explain 97% of those variations.

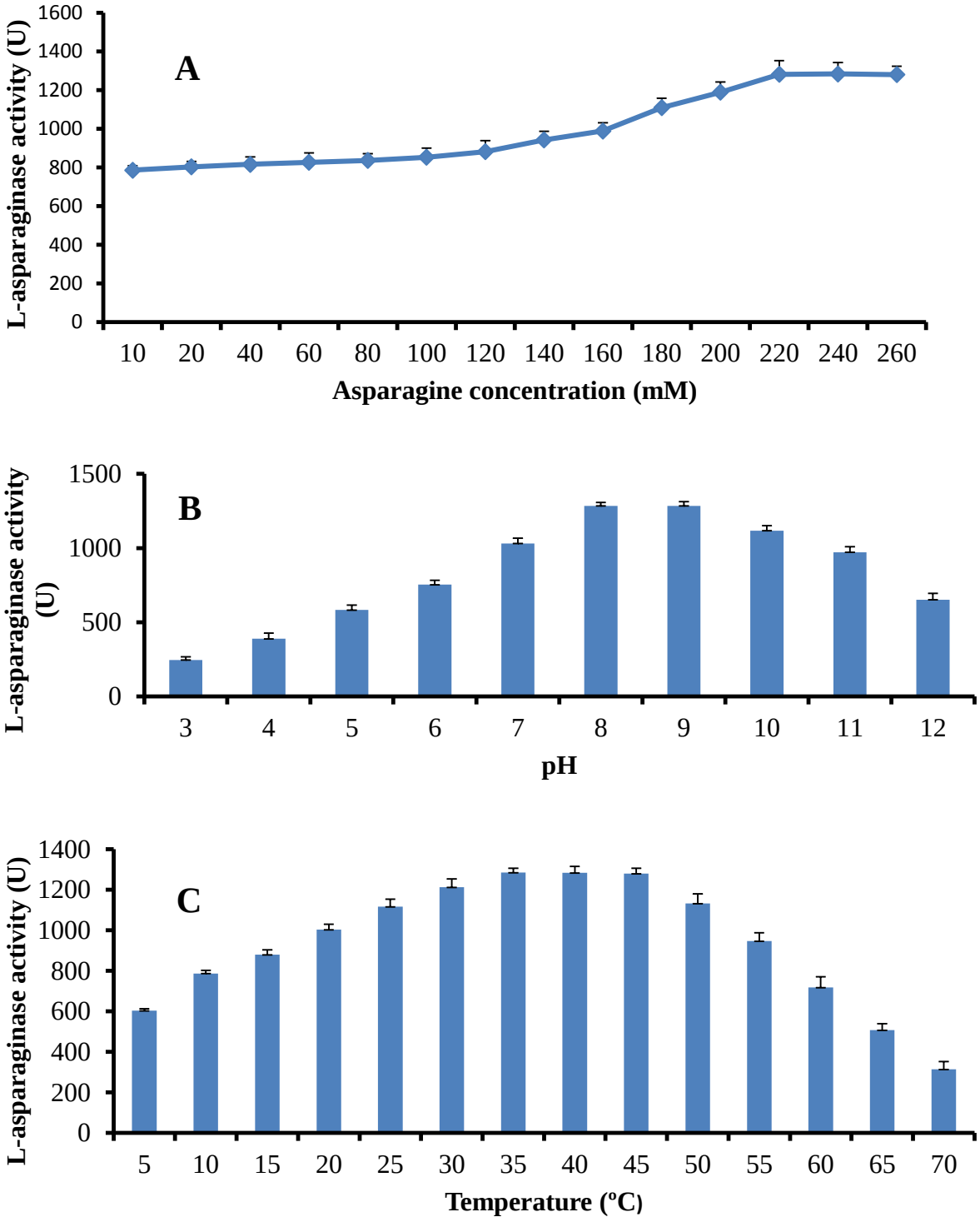


Figure 4: Effects of substrate concentration (A), pH (B) and temperature (C) on L-asparaginase activity.

CONCLUSION

Aspergillus candidus strain IR-A4, isolated in this study, demonstrated ability to produce glutaminase-near-free ASPNase in submerged fermentation. Enzyme activity of the dialyzed fraction was 786.5 U with a specific activity of 10.57 U/mg at assay conditions but 1284.5 U and 17.26 U/mg respectively, at optimum substrate concentration. Anti-leukemic activity of the enzyme was demonstrated by cytotoxic assay using HL-60 cell lines. IC50 of the partially purified enzyme was approximately 88% of total protein. The specific glutaminase activity of 0.03 U/mg was about 350-fold lower than target enzyme activity. ASPNase activity had a significantly strong association, ω^2, with fermentor size suggesting potential for large-scale fermentative production. Increasing concentrations of asparagine increased ASPNase activity to a maximum activity of 1282 ± 70.5 U at 220 mM substrate concentration. Effects of increasing pH and temperature on enzyme activity were non-linear, with pH and temperature optima, recorded at 8-9 and 35-45ºC respectively. The mold is recommended for large-scale production of the anti-leukemic enzyme.

Ethical Issues: None

Conflict of Interest: None declared

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