JJBS

Volume 14, Number 3, September 2021
ISSN 1995-6673
Pages 403 - 412

Jordan Journal of Biological Sciences

Production of Chemotherapeutic agent L-asparaginase from Gamma-Irradiated *Pseudomonas aeruginosa* WCHPA075019.

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Received: June 24, 2020; Revised: October 28, 2020; Accepted: November 10, 2020

Abstract

Because of the dangers and painful effects of chemotherapeutic drugs, the need for therapeutic agents with less adverse effects will increase several times in the coming years. L-asparaginase enzyme is an effective antitumor agent, especially acute lymphoblastic leukemia, with no side effects compared to other chemotherapeutic agents. Microorganisms are emerging as a safer source of L-asparaginase. Therefore, the findings of new L-asparaginase-producing bacterial strains with high yield for therapeutic applications become necessary. From twenty bacterial isolates tested for their L-asparaginase activity, 16S rRNA sequencing for the most potent isolate showed that the selected isolate had 100% identity to *pseudomonas aeruginosa* strain (accession number: WCHPA075019). In the presence of L-asparagine (1%) and glucose (1%) as nitrogen and carbon sources at a low dose of gamma radiation (0.75 kGy), the maximum productivity of L-asparaginase was reached after 2 days at 35 ° C, pH 7.6 under shaking at 200 rpm. Purification of L-asparaginase with 70% ammonium sulphate, followed by Sephadex G100 increases enzyme purity by 1.5-fold after gel filtration. Pure L-asparaginase had a molecular weight of 123 kDa by SDS- PAGE. The maximum activity of the enzyme against L-asparagine was detected at 35°C and pH 9.0 after 30 min and 200 mM substrate. L-asparaginase activated in the presence of metal ions such as K+, and Na+, not affected when exposed to EDTA and strongly inhibited in the presence of Ba2+, and Cd2+. The anticancer activity of the purified enzyme was tested in vitro against three types of cell line carcinoma. The growth inhibition of L-asparaginase for HEPG2 carcinoma cell line (IC50 value of 3.5 µg/ml) was greater than the inhibition of HCT and MCF-7 carcinoma cell lines with IC50 value of 3.8 and 12.5 µg/ml, respectively relative to the growth of the untreated control cells.

Keywords: *pseudomonas aeruginosa*, 16s rDNA analysis, L-asparaginase, Optimization, Gamma radiation, Purification, Enzyme activity, Anti-cancer.

1. Introduction

Microbial-source enzymes are potential biocatalysts used in various reactions and are part of the most essential products required to meet human needs in many fields (Olukunle and Ajayi, 2018). Approximately, 40% of global enzyme sales are L-asparaginase, which considered as one of the major important biomedical and biotechnological groups of therapeutic enzymes (Qeshmi *et al*., 2018). L-asparagine is an essential amino acid used in normal and cancer cell nutritional requirements. The enzyme L-asparaginase converts L-asparagine to ammonia and aspartic acid (Chand *et al*., 2020). The major medical use of L-asparaginase is L-asparagine elimination from the blood of acute lymphoblastic leukemia (ALL) treated patients in order to prevent a recurrence (Gutierrez *et al*., 2006).

In the 19th WHO list of specific medicinal products, L-asparaginase enzyme is listed as a cytostatic adjuvant to acute lymphoblastic leukemia, as well as in the WHO model list of essential medicinal products for children (WHO, 2015).

The enzyme is commonly used as an anticancer agent because it is non-toxic biodegradable, cheap, and can be easily supplied at the local site. Recent clinical trials have shown that this enzyme is also a promising agent in the treatment of certain forms of human's neoplastic cells (Alrumman *et al*., 2019).

Because of immeasurably useful medical applications, L- asparaginase biotechnological production has become the subject of extensive research by many researchers worldwide. L-asparaginase activity was frequently reported in plants, micro-organisms (bacteria, fungi, and actinomycetes), animals, and in the serum of certain rodents but was not isolated from a human source (Lalitha and Ramanjaneyulu, 2016). Many genera of bacteria, *Bacillus circuans* (Prakasham *et al*., 2010), *Bacilus brevis* (Narta *et al*., 2011), *Pseudomonas flurescens* (Sinha *et al*., 2015), *Pseudomonas aeruginosa* (Saeed *et al*., 2018), and *Escherichia coli* (Kante *et al*., 2019) are reported as L-asparaginase producers.

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Optimizing research conditions aiming to repeatedly increase L-asparaginase yield is the goal of many studies (Pallem, 2019). Among all the fermentation parameters tested, Prakasham *et al*. (2007) found that the inoculum volume incubation temperature and medium pH are the main effective parameters at a single level. These factors account for more than 60% of enzyme total yield. Furthermore, Arumugam and Senthil (2017) tested the effect of the nitrogen source on enzyme production under various conditions using the one factor at a time method (OFAT). Different studies indicated that low doses of gamma radiation can improve the growth and metabolism of microorganisms. Abdelrazek *et al*. (2019) use gamma rays to increase the productivity of L-asparaginase.

Specific purification steps were applied to the crude culture filtrate to obtain a pure enzymatic preparation. Various purifying steps, including ammonium sulfate fractionation, were followed by separation on Sephadex G-100 and CM-Sephadex C50 (El-Bessoumy *et al*., 2004) or partial purification of the ammonium sulfate precipitation and dialysis (Arumugam and Senthil, 2017). Obtained results demonstrated an increase in treated L-asparaginase activity relative to the crude enzyme.

Some L-asparaginase preparations are currently approved for ALL treatment (Horvath *et al*., 2019). Asparaginase from *Escherichia coli* and *E. chrysanthemi* was considered for therapeutic purposes. Due to their serious side effects, such as liver dysfunction, allergies, and central nervous system disorders, it did not achieve complete remission, (Egler *et al*., 2016). To overcome these defects, further studies are required to find new bacterial strains that produce L-asparaginase without these effects (Fatima *et al*., 2019).

The aim of this research is to optimize the culture conditions for l-asparaginase production by a selected local bacterial isolate and to investigate the effect of various gamma irradiation low-doses on the production. Extraction and characterization of the purified enzyme and determination for the purified enzyme therapeutic efficacy as an anti-cancer in vitro against standard cancer cell lines were also evaluated.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and media.

Analytical grade reagents and other chemicals were obtained from El-Gomhoria Company, Cairo, Egypt. From Sigma Aldrich (St. Louis, Missouri, USA), media and L-asparagine were bought.

2.1.2. Samples

Samples were collected from various asparagine rich sources and screened for isolation of bacteria-producing L-asparaginase. Bacterial samples have been isolated from fish, meat, cheese, rice, yellow lentils, asparagus, black lentils, potatoes, soybeans, eggs, milk, and beans. All sources were refined and exposed to the air for a day to activate bacterial growth.

2.2. Methods

2.2.1. Isolation, purification, and screening of bacterial isolates for L- asparaginase production

For each sample taken, 10g sample was inoculated in 90 ml nutrient broth. The samples were serially diluted (10^{-1}: 10^{-8}) and from each dilution, 0.1 ml was streaked over solid modified M9 medium (Gulati *et al*., 1997). After 24h of incubation at 37°C, the pink color of the plate indicated L-asparaginase production. A single colony of each isolate was collected and streaked on nutrient agar medium several times until single pure colonies were obtained. Pure cultures were reserved at 4°C on slants of nutrient agar medium for further studies (Atlas and Parks, 1993).

2.2.2. Screening of bacterial isolates for L- asparaginase production A. Qualitative assay of L –asparaginase

Screening procedure based on the principle that the pH indicator Phenol red was incorporated. It is yellow at pH below 7 (acidic pH), and above pH 7 it turns pink (alkaline pH).

Primary screening using agar well diffusion (Magaldi et al., 2004).

Fifty ml of modified M9 broth medium was taken in conical flasks, inoculated with 1 ml of 24 h aged bacterial culture suspension, and incubated for 24 h at 37 °C. After that, 30 min centrifugation for the culture broth was carried out at 4 °C and 5000 rpm. For each isolate, cell-free supernatant (100 µl) was poured into a well (8 mm diameter) in a modified M9 agar plate. At 4 °C for 12 h, the inoculated plate was left to diffuse the filtrate into the medium and then incubated for 24 h at 37 °C. Diameters measuring of the pink area with a yellow background around the hole (mm) stating L-asparaginase activity. The cultures with high enzyme production were selected for further studies.

Disc diffusion technique (Balouiri et al., 2016***).***

The isolated bacteria were inoculated in Erlenmeyer flasks of modified M9 broth medium and incubated for 24 h at 37 °C. On sterilized filter paper discs (6mm) 25µl from cell-free supernatant was suspended. Then saturated discs were placed on the solid modified M9 medium surface and kept for 12 h at 4°C to allow diffusion of the filtrate and then the plates incubated for 24h at 37°C. Diameters of the pink zone around discs were measured, and the more L- asparaginase producer was selected for further studies.

B. Quantitative assay of L –asparaginase (Imada et al., 1973***).***

The modified M9 medium (50 ml) has been inoculated with a 24-h old bacterial cell suspension (2 ml), and the un-inoculated medium has been used as a control. The flask was incubated at 37°C with shaking (250 rpm) for 48 h. Centrifugation of the bacterial culture was carried out at 6000 rpm for 20 min.

2.2.3. Estimation of L-asparaginase enzyme activity in culture filtrates

Nesslerization determined the culture filtrate enzyme activity. From the cell-free supernatant or enzyme solution, 0.1 ml of the sample was combined with 0.9 ml 0.1 M Tris-HCl buffer (pH 8.5), and then 1 ml 0.04 M L-asparagine substrate was added. After incubating at 37°C

for 30 minutes, the reaction was terminated with 0.5 ml of tri-chloro-acetic acid (TCA) 1.5 M.

Dilution of 0.1 ml of supernatant to 8 ml using distilled water after centrifugation for protein precipitation occurred before treatment with Nessler's reagent (1.0 ml). For 15 minutes, the brown reaction was allowed to proceed, and the ammonia release was estimated at 500 nm. A typical ammonium-sulfate graph at different concentrations (1.5-11.8 µg / ml) was used for evaluating the liberated ammonia.

2.2.4. Determination of enzyme activity

The released quantity of NH_3 from asparagine is used to calculate the activity of the L-asparaginase enzyme (Peterson and Ciegler, 1969).

The International Unit (IU) identified the activity of the L-asparaginase enzyme as the quantity of enzyme needed to release one micromole of ammonia from L-asparagine per ml per minute (µmole / ml/min) at pH 8.5 and 37 ° C (Manna *et al*., 1995).

Bovine serum albumin (BSA) was used for the determination of protein contents (Lowery *et al*., 1951).

The amount of enzyme required for releasing 1µ mole of the product/min /mg of protein was considered as specific activity (Lalitha and Ramanjaneyulu, 2016).

2.2.5. Characterization and identification of the most potent L-asparaginase producing strain.

According to the standard biochemical and physiological identification test described in Bergey's Manual for Systematic Bacteriology, the most active isolate was identified (Brenner *et al*., 2005), and 16S rRNA gene sequencing was used for confirmation of the identification.

Extraction of DNA

On a rotary shaker (120 rpm), the selected bacterial strain was cultivated (on nutrient broth) overnight at 30°C. Bacterial DNA has been extracted using the Bacterial Genomic DNA Mini-Prep Kit (Axygen cat. No. V110440-05).

Polymerase chain reaction (PCR)

The specificity of primers is revised by the ribosome database (PROBE CHECK function) and BLAST search tool. In the Perkin Elmer 2400 (Nowalk, CT) thermo cycler, DNA amplification is performed on a pure 2 µl to 3µl sample, each 1µl sample contains approximately 150 ng DNA. The final volume of PCR amplification reaction was 100µl; 0.2µM from each primer (F1 and R1), 200µM dNTPs, 2.0 mM MgCl2 and 2.5 units of Maxima® Hot start Taq DNA polymerase (Fermentas, www. fermentas.com) mixed by PCR buffer (1X). The thermal cycle (PCR) steps were applied as follows; 5 min initial denaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min at 55°C, 2min extension at 72°C. DNA was extended for 10 min at 72 °C after the last cycle (Khan *et al*., 2018).

DNA Sequencing

The amplification has been confirmed by analysis of 5 µl of the PCR product on 1% agarose gel (Promega) by electrophoresis. The size of the resulting PCR product ranged from 1450 to 1500 bp (Yamamoto and Harayama, 1998).

The PCR purification kit (Fermentas, Germany) was used for purification of the PCR products. Using the same PCR primers, the 16S rDNA amplicon was sequenced using an ABI377 DNA automatic sequencer (Perkin Elmer, Applied Bio-system Div., Waltham, USA).

2.2.6. One variable at a time method for Optimization of L-asparaginase production

Optimization of the experiments was carried out using a one factor at a time (OFAT) strategy. The effect of different nutritional and physiological parameters was evaluated by changing just one factor at a time and leaving the other factors stable. The physiological parameters that were investigated included initial pH (4.0-9.0), incubation time (18, 24, 48, 60, 72, 84, and 96 h), and incubation temperature (from 25 to 60 °C), with temperatures increasing by 5 °C each time. In order to test the impact of different sources of carbon and nitrogen (nutritional parameters), maltose, starch, fructose, lactose, xylose, sucrose, and mannitol (1% w / v) were added separately to the M9 fermentation medium by replacing the glucose, and various sources of nitrogen (L-arginine, yeast, peptone, NH_4Cl, $NaNO_3$, Glutamine, and NH_4SO_4) were added separately at final concentration equimolecular to locate in 5 g of L- asparagine. Under static and shaking conditions at various speeds of 100, 150, and 200 rpm, M9 basal liquid media were inoculated and incubated for 48 h at 35 ° C to study the effect of static and shaking conditions on the enzyme production.

2.2.7. Influence of different gamma radiation doses on L-asparaginase production

At the National Center for Radiation Research and Technology in Nasr City, Cairo, Egypt, using an experimental ^{60}Co Russian gamma chamber, the M9 broth medium from the 24h test bacteria was exposed to various low doses of gamma radiation (0.25, 0.5, 0.75 and 1.5 kGy). At the time of the experiment, the average dose rate was 1 kGy / 50 min. The irradiated samples were grown on flasks containing M9 medium at pH 7.6 under shaking conditions at 200 rpm and 35 ° C for 48 h (Abdelrazek *et al*., 2019).

The cell free filtrate was used at the end of each test period for measuring protein (mg / ml) and the activity of the enzyme (U / ml) as previously mentioned.

2.2.8. Purification of L-Asparaginase Crude enzyme preparation

The experimental strain was grown in the modified production medium (M9 medium) under optimal condition. The cell-free filtrate obtained after the culture fermentation was harvested, centrifuged (10,000 rpm) for 30 min and considered as the crude enzyme (Gulati *et al*., 1997).

Ammonium sulphate precipitation

A slow addition of ammonium sulphate to the crude enzyme by stirring was submitted at 4 °C until the desired saturation (70%) of ammonium sulphate was achieved (Bollag *et al*., 1996). The mixture was kept at 4 ° C overnight, and then the protein precipitation was carried out by centrifugation (10,000 rpm) for 15 min at 4°C. The

precipitate protein pellet was immediately dissolved at a minimal volume of 0.1 M buffer (citrate phosphate: pH 7). The protein content and enzyme activity of the dissolved fractional precipitate were tested.

Dialysis

Precipitated pellets were introduced into cellulose bag for dialysis against distilled water and then were dialyzed against phosphate buffer pH7.0 (Bhargavi and Jayamadhuri, 2016). The enzyme preparation was concentrated against polyethylene glycol crystals (PEG).

Sephadex gel filtration

The concentrated elution fractions were combined and applied to a Sephadex G-100 column (2.5x45cm) pre-equilibrated with the same buffer at a flow rate of 20 ml/h. Combine the active fractions, concentrate, and examined for protein (mg/ml) content and enzyme activity (U/ml). The fraction with a sharp peak was pooled and concentrated by the dialysis membrane and used for further study (Bhargavi and Jayamadhuri, 2016).

SDS- PAGE protein electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at the Regional Center for Mycology and Biotechnology Azhar University Cairo, Egypt. SDS-PAGE was made in accordance to the method of Laemmli (1970), using a 10% separating gel and 5% stacking gel containing 0.1% SDS. The gel was strained with coomassie brilliant blue R-250. Then distained with methanol, acetic acid and water in the ratio of 4:1:5.

Determination of molecular weight

The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers phosphorylase b (97.4 kDa), Bovine serum albumin (66.2kDa), Ovalbumin (45 kDa), carbonic anhydrase (25 kDa) and lactoglobulin (18.4 kDa). Standard curve for protein marker was drawn based on the electrophoretic mobility (Rf) of proteins against their log10 molecular weights.

2.2.9. Biochemical Properties of the purified L-asparaginase enzyme

Effect of pH: The purified enzyme and asparagine reaction mixture were adjusted to different pH values (4.0-9.0) at 35°C for 30 min.

Effect of different incubation temperature: The purified enzyme and asparagine reaction mixture has been incubated for 30 min at various temperatures (25, 30, 35, 40, 45, 50, 55 and 60 °C).

Effect of reaction time: The reaction mixture incubated for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min.

Effect of substrate different concentrations (L-asparagine): Different concentrations of L-asparagine (50, 100, 150, 200, 250 and 300 mM) were used.

Effect of Metal ions (activator / inhibitor): Purified enzymes were separately pre-incubated with different metal ions (Cu^{+2}, Fe^{+2}, Zn^{+2}, $EDTA^{+2}$, Co^{+2}, Ba^{+2}, Ca^{+2}, Mn^{+2}, Mg^{+2}, K^{+}, Na^{+} & Cd^{+2}) for 30 minutes prior to the addition of asparagine (40 mM). At the end of the incubation period, the enzyme activity was measured by using the cell-free filtrate, as previously mentioned.

2.2.10. Anticancer activity

Cell Viability Assay for three cell lines: human hepatocarcinoma hepG2 cell line, colon cancer HCT, and human breast adenocarcinoma (MCF-7), which were from the American Model Culture Collection (ATC Collection, Minisota, United States) have been performed by the Cairo National Cancer Institute in Egypt using MTT (3-(4, 5-dimethyl-2)-2, 5-diphenyltetrazolym bromide. The viability of MTT cells was determined according to Vichai and Kirtikara (2006).

Percentage of cell viability = Optical Density for the treated cells / control cells Optical Density * 100.

Sigmoidal dose-response curve-fitting models (Graphpad Prizm Software, version 3) were used to detect L- asparaginase as an anticancer against three human cell lines.

2.2.11. Statistics and calculations

For each analysis, results have been expressed as a mean ± SD (standard deviation). All tests have been conducted in triplicates, n=3.

3. Results and Discussion

3.1. Screening of isolated bacteria for production of L-asparaginase.

Twenty bacterial isolates were randomly isolated from fish, meat, cheese, rice, yellow lentils, asparagus, black lentils, potatoes, soybeans, eggs, milk, and beans. The data showed that the majority of the isolates are gram-positive, rod-shaped and spore-forming. Modified M9 with a sole source of nitrogen (asparagine, 1%) was the medium used for screening the activity of all isolated bacteria for L-asparaginase production. The change of yellow color of media to pink is a positive indicator for the enzyme production. The plate culture assay indicated that all bacterial isolates exhibited positive production for L-asparaginase with different zone diameter, which provides an assay for L-asparaginase activity. L-asparaginase efficacy was tested spectrophotometrically. Table 1 results show the activity of L-asparaginase in U / ml, and the pink zone (mm) diameter. L-asparaginase activity of the isolates was observed to range from 12.0 to 44 U / ml and the diameter to range from 12 to 37 mm using both agar-well and disk diffusion methods. It is in agreement with Gulati *et al*. (1997), who proved that the transformation of medium color to pinkish was triggered by the production of L-asparaginase.

Table 1. Screening of isolated bacteria for production of L-asparaginase

Isolate no	Sample source	Agar well diffusion (mm) Pink zone diameter	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg)
1	Fish	17	0.160	15.0	93.16
2	Fish	16	0.173	18.0	104.0
3	Fish	15	0.158	15.0	94.90
4	Meat	13	0.251	14.7	58.48
5	Cheese	12	0.160	12.0	75.00
6	Rice	18	0.190	10.5	55.63
7	Yellow lentil	29	0.240	32.6	135.0
*8	Asparagus	36	0.320	44.0	137.7
9	Black lentil	28	0.280	34.4	122.8
10	Black lentil	26	0.252	31.8	126.3
11	Potato	18	0.280	15.53	55.40
12	Potato	19	0.220	14.5	65.90
13	Potato	19	0.250	16.6	66.40
14	Soybeans	30	0.260	32.0	123.0
15	Soybeans	28	0.270	30.0	111.1
16	Egg	18	0.216	21.21	69.09
17	Milk	23	0.200	27.20	105.7
18	Milk	20	0.231	26.0	112.1
19	Beans	22	0.190	30.0	120.8
20	Beans	21	0.232	29.2	125.3

*Out of twenty isolates, isolate no 8 was selected as the most L-asparaginase producer.

3.2. Identification and characterization of the most potent isolate

The isolate number (8) was identified using the tests of systematic bacteriology guided by Bergey`s Manual, and the results indicated that it belonged to the genus pseudomonas (Paul and Sinha, 2014). For confirming the identification, isolate No 8 DNA was extracted directly from the organism. Based on the alignment of 16s rDNA available in the gene bank, two primer set was used. 1kb DNA product was obtained in confirmation genus identification. On the other hand, BLAST searches were performed to investigate whether high homology of tested strain exits to other Pseudomonas. The genomic DNA for the bacterial isolates was used as a template for the amplification of rRNA using the forward and reverse primers for 16S rDNA (Figure 1). After running of PCR and agarose gel (Figure 2), the purified PCR products from *P. aeruginosa* were sequenced and the sequence obtained was deposited with the accession number WCHPA075019 in the bank of gene (Figure 3). BLAST studies have shown that the strain tested has a 100% identity with *pseudomonas aeruginosa* and it was identified as *pseudomonas aeruginosa* WCHPA075019.

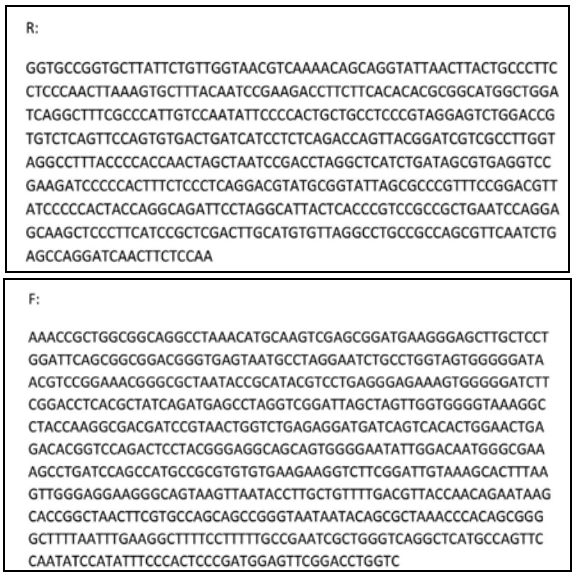


Figure 1. DNA sequences of *pseudomonas aeruginosa.*

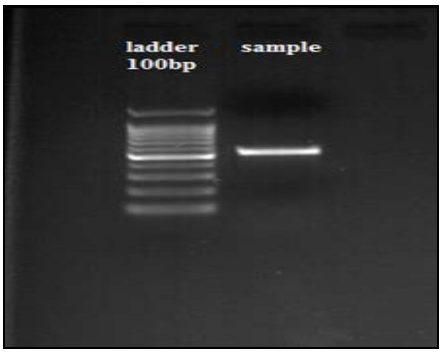


Figure 2. PCR product of 16S *rRNA* of *pseudomonas aeruginosa*.

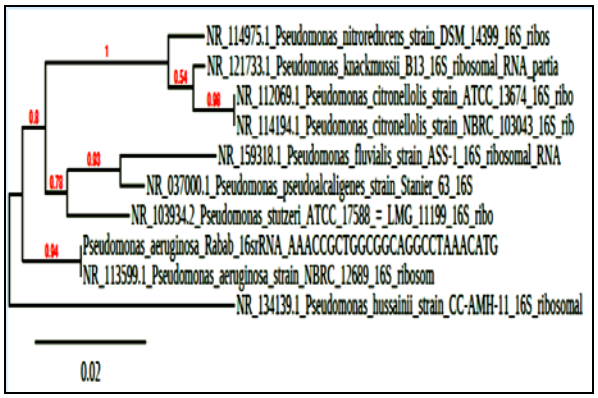


Figure 3. Phylogenetic tree analysis of *pseudomonas aeruginosa* WCHPA075019 obtained after performing 16S *rRNA* sequencing

This is in accordance with Badoei-Dalfard (2015) and Jois *et al*. (2013) who reported that *P. aeruginosa* is a good L-asparaginase producer.

3.3. Optimization of P. aeruginosa WCHPA075019 L-asparaginase production

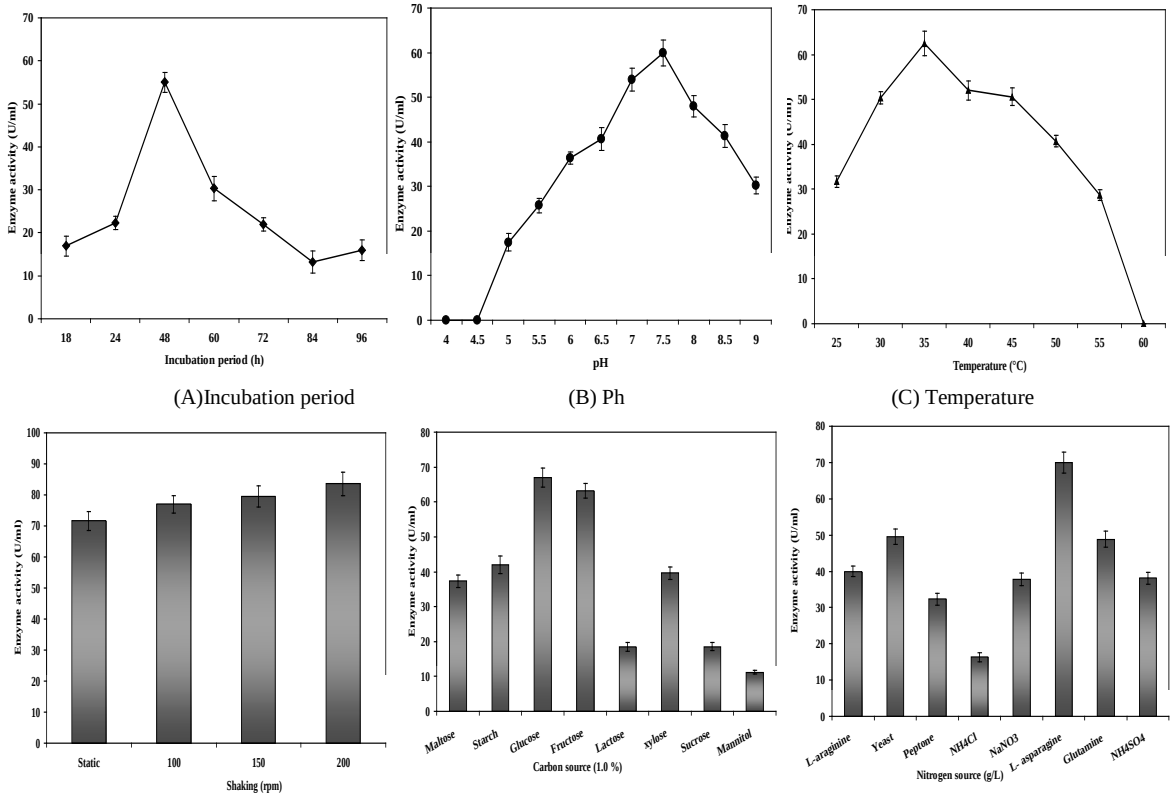
To optimize *Pseudomonas aeruginosa* L-asparaginase production, many cultural and nutritional parameters were examined. The maximum enzyme production was exhibited at 35°C for 2 days and pH 7.6 in presence of 1% glucose and L-asparagine under shaking condition at 200 rpm (Figure 4). Such findings are in accordance with Komathi *et al*. (2013) who stated that the maximum

enzyme production by *P. aeruginosa* was after 48 h of incubation at 35°C and pH 7.6.

Badoei-Dalfard (2016) revealed that with L-asparaginase and glucose as nitrogen and carbon sources, the largest amount of L-asparaginase by *P. pseudoalcaligens* JHS-71 was obtained at pH 7.0 and 37°C after 48 h. This result was consistent with the data recorded by Badoei-Dalfard (2015), which showed that *P. aeruginosa* strain SN004 maximum production was achieved when glucose was used as carbon source. Various sources of organic and inorganic nitrogen have been tested. The present data showed that *P. aeruginosa* WCHPA075019 was capable of using both organic and inorganic nitrogen sources. L-asparagine was the ideal nitrogen source for the L-asparaginase production (170.7 Umg-1), which indicates that L-asparagine is an L-asparaginase inducer. This results in accordance with Badoei-Dalfard (2015), who confirmed that (0.5%) L-asparagine is the best source of nitrogen for *P. aeruginosa* strain SN004 L-asparaginase maximum production (785 U / ml). Shukla and Mandal (2013) reported that the use of L-asparagine followed by peptone and yeast extract can achieve *Bacillus subtilis* L-asparaginase maximum yield. The maximum enzyme production by *P. aeruginosa* WCHPA075019 occurred at 200 rpm. Also, Kuwabara *et al*. (2015) reported that at 200 rpm *P. aeruginosa* PAO1 L-asparaginase maximum production took place.

3.4. Influence of different gamma radiation doses on L-asparaginase production

Numerous studies have shown that low gamma irradiation doses can improve metabolic activities and microbial development. *P. aeruginosa* was exposed to gamma rays at doses from 0.25 to 1.5 kGy using an experimental ^{60}Co Russian gamma chamber, (dose rate 1kGy/50min). Results showed that enzyme activity increased gradually from 0.25 to 0.75 kGy and maximum activity at 0.75 kGy and decreased sharply at 1.0 kGy dose and had no activity at 1.5 kGy (Figure 5). This result is in agreement with Abd EI-Aziz and Hassan (2010) who showed that radiation dose level 0.75kGy resulted in an increase in the elastase yield of *Bacillus subtilis* by 7.94% and in the final dry weight when compared with non-irradiated control. The inhibitory effect of radiation on a microbial enzyme may be due to the action of ionizing radiation on either of the two components of which the enzymes are made up, i.e. the protein or the prosthetic group. When acting on protein moiety they may oxidize reactive groups, amino groups or double bonds or may act by or precipitation, when acting on the prosthetic group they may produce chemical changes that alter the biological activity of the enzyme (Reisz *et al*., 2014). On the other hand, the improvement by gamma radiation may either be due to an increase in gene copy or gene expression or both (Rajoka *et al*., 1998) and by inducing mutagenesis in the microbial cell to enhance its activity for enzyme production (Awan *et al*., 2011).



(A)Incubation period (B) Ph (C) Temperature

(D) Static and Shaking (E) Carbon source (F) Nitrogen source

Figure 4. Optimization of the production of *P. aeruginosa* L-asparaginase

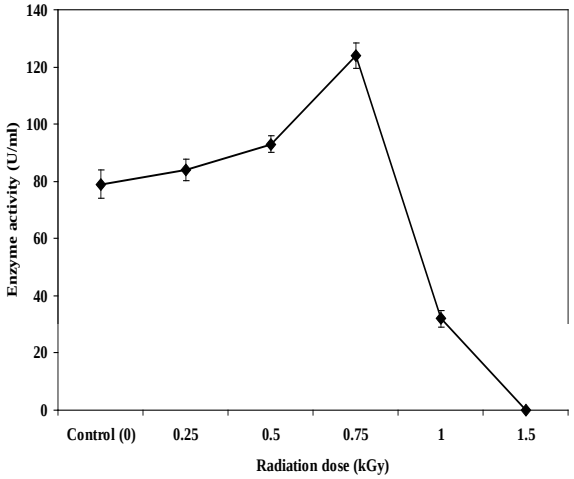


Figure 5. Effect of gamma radiation on *P. aeruginosa* L-asparaginase activity.

3.5. Enzyme Purification

L-asparaginase produced by *P. aeruginosa* in liquid media was purified by Ultra-filtration and combination of gel filtration and ion-exchange chromatography to obvious homogeneity with varying recovery and purification yield.

Results in Table 2 revealed that the final specific enzyme activity was 366 Um/g with 1.5 fold and 6.6% yield. When El-Bessoumy *et al*. (2004) grown *P. aeruginosa* 50071 on solid-state fermentation, the purified enzyme final specific activity was 1900 IU / mg, the purification rate was 106 -fold and the yield was 43 %.

L- asparaginase fractional purification by Sephadex G-100 column chromatography. As a result of gel filtration chromatography on Sephadex, G-100 the specific activity increased to 1.5 fold with a 6.6% yield. Fractions with the highest activity were pooled and dialyzed at 4.0 °C against distilled water (Figure 6).

Table 2. L-asparaginase purification profile.

Purification Steps	Volume (ml)	Enzyme activity (U/ml)	Protein content (mg/ml)	Total activity (U)	Total protein (mg)	Specific activity U/mg	Yield %	Purification fold
Culture filtrate (crude extract)	2730	124	0.48	338520	1310	245.8	100	1
Precipitation by amm.Sulphate (70%)	901	129	0.45	111711	405	287	33	1.17
Sephadex G100	160	139	0.38	22240	60.8	366	6.6	1.5

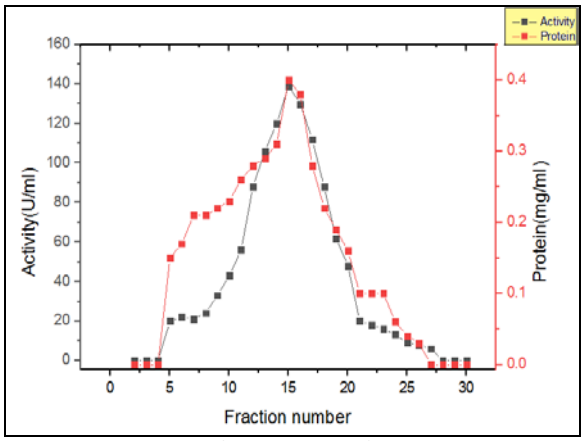
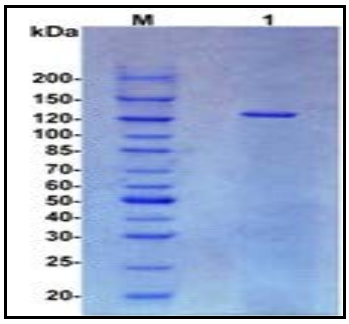


Figure 6. Fractional purification pattern of the L- asparaginase produced by *P. aeruginosa* applying Sephadex G-100 column chromatography.

In addition to demonstrating the purity of the enzyme, the molecular weight was measured. *Pseudomonas aeruginosa* L-asparaginase purified enzyme molecular weight was 123 KDa **(**Figure 7).

The L-asparaginase sizes vary from one organism to another in terms of genus and species. The Pseudomonas L-asparaginase molecular size and subunits vary from a single subunit of 34–33 kDa under non-denaturing and denaturing conditions respectively (Shakambari *et al*., 2019). The molecular weight of *Pseudomonas aeruginosa* pure and crude enzyme samples are found to be 75KDa by Jois *et al*. (2013). Also, *Pseudomonas aeruginosa* L-asparaginase exists as a monomer with a size of 160 kDa as reported by (El-Bessoumy *et al*., 2004). Thus, L-asparaginase shows a wide structural variation in the subunits of the above-mentioned bacteria.



* Lane M =marker protein, Lane 1= purified enzyme.

Figure 7. SDS- PAGE of *P. aeruginosa* purified L-asparaginase.

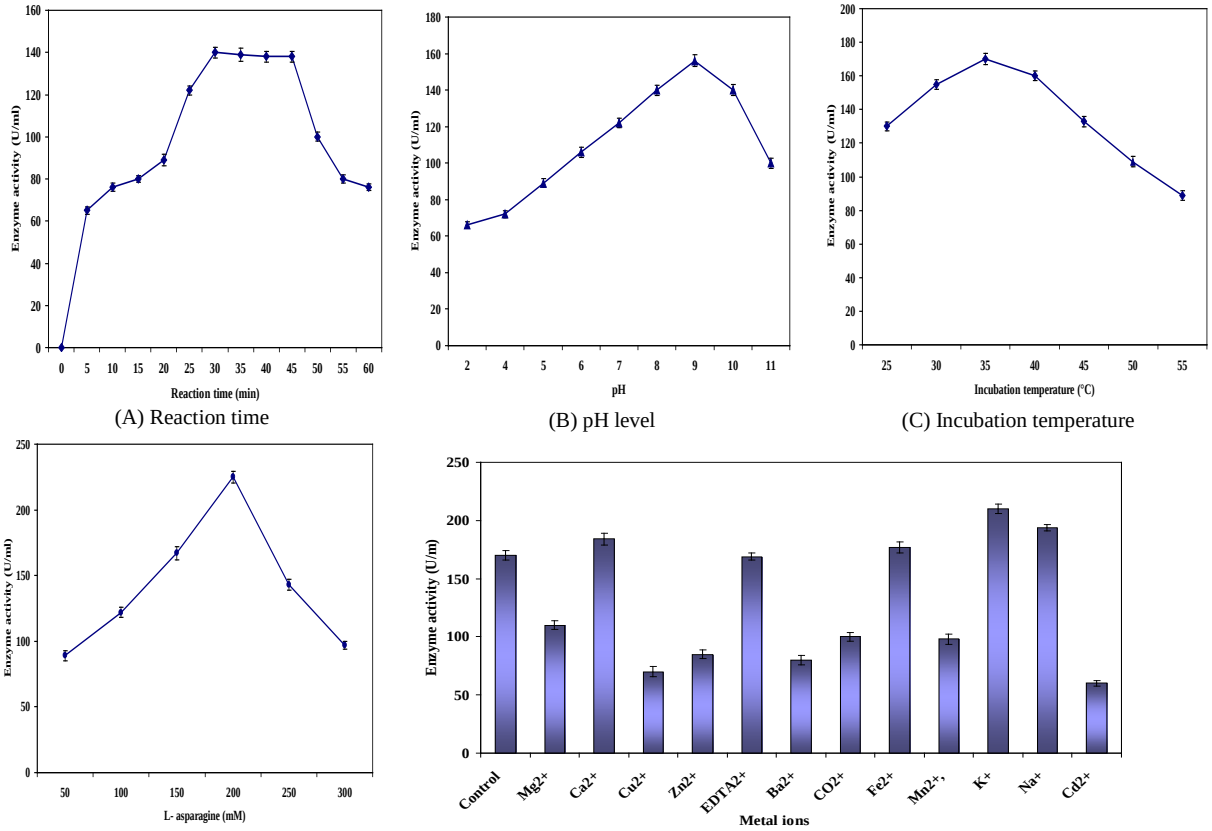
3.6. Properties of P. aeruginosa L- asparaginase purified enzyme

Clearly, after a 30 min incubation period, the maximum L-asparaginase enzyme activity was achieved (Figure 8A). This finding is consistent with (Komathi *et al*., 2013) who observed the maximum *P. aeruginosa* enzyme activity after 30 min. The maximum recorded activity was also 30 min for *Streptomyces noursei* asparaginase enzyme (Kumar *et al*., 2011). The optimal pH value for *P. aeruginosa* purified L-asparaginase was pH 9 (Figure 8B). El-Bessoumy *et al*. (2004) recorded comparable results for *P. aeruginosa* 50071. Moreover, Shukla and Mandal (2013) recorded maximum activity of *Bacillus subtilis* purified L-asparaginase at pH 9. For every enzyme, there is a specific optimal temperature beyond which there has been a decrease in activity (Kumar *et al*., 2011). In the current study, the optimum incubation temperature was reported at 35 °C (Figure 8C) for maximum activity of *P. aeruginosa* L-asparaginase. Also, the optimal temperature

for the maximum activity of *P. aeruginosa* asparaginase enzyme was observed at 35ºC by Komathi *et al*. (2013).

The findings in (Figure 8D) indicate that by increasing the L-asparagine concentration, the activity of L-asparaginase gradually increases. L-asparaginase maximum activity was determined at asparagine concentration of 200 mM (167 U/ml). *P. aeruginosa* SN004 L-asparaginase maximum production (785 U / ml) was achieved with 0.5% L-asparagine on an optimized medium as defined by Badoei-Dalfard (2015).

The activity of the enzyme decreased when of Mg^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, CO^{2+}, $Mn^{2+}$and Cd^{2} were present by 110, 70, 85, 80, 100, 98 and 60 U/ml, respectively (Figure 8E). However, K^{+} exerted a highly stimulatory effect to occupy the first rank among all tested compounds followed by Na^{+}, Ca^{2+}, and Fe^{2+} with increase in activity by 210, 194, 184, and 177 U/ml, respectively. L-asparaginase activity was not affected by the EDTA chelator agent which indicated that the enzyme was not a metalloprotein. The enzyme reached its maximum activity in optimizing media containing magnesium ions (Shukla and Mandal, 2013).



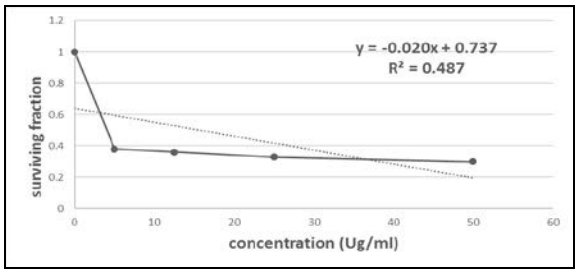
(A) Reaction time (B) pH level (C) Incubation temperature

(D) substrate concentration (E) Metal ions

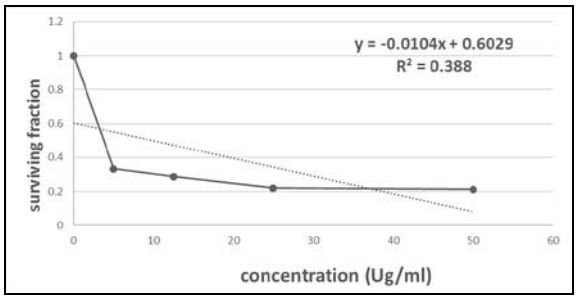
Figure 8. Characteristics of *P. aeruginosa* L- asparaginase purified enzyme.

3.7. Anticancer activity

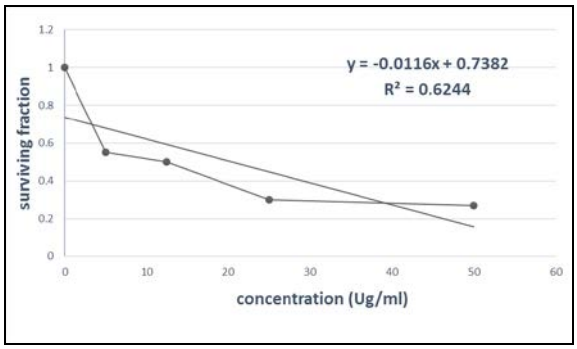
In the present study, three tumour cell lines were used to investigate the in vitro antitumor activity of *P. aeruginosa* L-asparaginase enzyme (Figure 9). Incubation of HEPG2-116 with progressive doses of the *P. aeruginosa* asparaginase enzyme causes progressive cell growth inhibition, as indicated by its IC_{50} value of 3.5 µg/ml. The enzyme anti-tumour activity against the breast adenocarcinoma MCF-7 was IC_{50} 12.5 µg /ml. The tested enzyme was found to have activity against HCT-116 cells (IC_{50}, 3.8 µg/ml) compared to the growth of the control (untreated cells). The in vitro cytotoxicity of *Helicobacter pylori* CCUG 17874 new L-asparaginase against a variety of cells has been studied by Cappelletti *et al*. (2008), they stated that gastric epithelial cells AGS and MKN 28 are most affected. When Moharam *et al*. (2010) examined the antioxidant and antitumor activities of *Bacillus sp* R36 asparaginase; they found two human cell lines were inhibited by the enzyme, including colon carcinoma (HCT-116) and hepatocellular carcinoma (HEPG2-116) with IC_{50} value of 218.7 µg/ml and 112.19 µg/ml, respectively.



(A) HCT-116 IC_{50}=3.8µg/ml



(B) HEPG2-116 IC50=3.5 µg/ml



(C) MCF-7 IC_{50}=12.5 µg/ml

The diagram represents the relation between the concentration (horizontal axis) and surviving fraction (vertical axis).

Figure 9. *P. aeruginosa* L-asparaginase toxic effect on cancer cells.

4. Conclusion

L-asparaginase is considered one of the therapeutic enzymes used in the treatment of blood cancer (ALL) in children. Using enzymes developed by these commercial strains causes adverse side effects for patients in the long run. So, finding new bacterial strains that can be used for L-asparaginase commercial production is essential. In this study, the most potent local bacterial isolate *P. aeruginosa* WCHPA075019 isolated from asparagus was selected for the production of L-asparaginase. The culture conditions, nutritional requirements, and low doses of gamma radiation were optimized to reach maximum l-asparaginase productivity. The study was, moreover, extended to purify L- asparaginase and investigate its physicochemical properties. The purified enzyme preparation showed anti-cancer activity against 3 human cell lines. From the results, *P. aeruginosa* WCHPA075019 L-asparaginase may be evaluated clinically as an anticancer pharmaceutical agent for the tested cancer cell lines.

Declaration of competing interests:

None

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