

DEVELOPMENT OF BIOPROCESSES FOR PRODUCTION AND PURIFICATION OF L-ASPARAGINASE FROM *STAPHYLOCOCCUS AUREUS*, AND *IN VITRO* EFFECACY AGAINST HUMAN BREAST CNCER CELL LINE

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ABSTRACT

The present study was aimed to isolate the abundant *Staphylococcus aureus* produce asparaginase and evaluate the efficacy of purified L-ASNase against a breast cancer cell line. From one hundred local isolates of *S. aureus* which subjected to the primary screening processes for asparaginase production, thirty-seven isolates with maximum ability of hydrolysis zone in primary screening (the ratio of Z more than 10 mm) were selected for secondary screening. It has been indicated that *S. aureus* TG98 had the highest productivity of the enzyme (255.8 U/mg protein). The highest producing-isolate was identified according to vitek test. The optimal conditions for ASNase production by the selected isolate was performed using submerged fermentation, where the medium (5) was utilized as the best medium for production, lactose as the best carbon source, NH₄Cl as the best nitrogen source, pH 8 at 37 °C after 24 hr of incubation period, the specific activity was reached to 496.99 U/mg. The enzyme was purified by gel filtration chromatography utilizing Sephadex G-150. The results indicated that there is an elevating in final purification folds 2 time with 103% yield of enzyme. Also, it was exhibited maximal activity and stability at pH 8.0. Also it was active and stable at 37°C. The highest rate of enzyme specificity found with asparagine. The breast tumor cells proliferation was significantly suppressed by the cytotoxic effect of L-ASNase comparison with normal cell line (WRL-68).

Keywords: bacteria, enzyme, isolation, vitek test, optimization

حسن وحسين

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تطوير العمليات الحيوية في انتاج وتنقية الاسباراجينيز من المكورات العنقودية الذهبية وقياس كفاءته ضد خط خلايا سرطان

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باحث

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المستخلص

هدفت الدراسة الحالية الى عزل بكتريا *Staphylococcus aureus* الاغزر انتاجا لانزيم الاسباراجينيز ثم تقييم فعالية الانزيم المنقى ضد خط خلايا سرطان الثدي. من بين 100 عزله من بكتريا *S. aureus* والتي خضعت لعملية الغرله الاولى لتحديد العزله الاكفا في انتاج الانزيم ، شخصت 37 عزله كفاءة اعتمادا على نسبة (النمو/ الهاله الشفافه) على الوسط الصلب. ادخلت هذا العزلات الى الغرله الثانويه باستخدام الوسط السائل ، انتخبت العزله *S. aureus* TG98 يكونها الاكفا في انتاج الانزيم اذا بلغت الفعاليه النوعيه 255.8 وحدة / ملغم بروتين وشخصت العزله الاكثر انتاجا اعتمادا على اختبار vitek test ، حددت الظروف المثلى لانتاج الانزيم اذا بلغت 496.99 وحدة / ملغم بروتين باستخدام الوسط (5) كوسط امثل للانتاج المجهز بـ لاكتوز كمصدر كريوني امثل و كلوريد الامونيوم كمصدر نيتروجيني امثل برقم هيدروجيني 8 ودرجة حرارة 37م بعد 24ساعة من الحضانه. نقي انزيم الاسباراجينيز باستخدام كروماتوغرافيا الترشيح الهلامي بوساطه هلام السيفاكريل ج-150 بعدد مرات تنقيه 2 ويحصي له انزيمييه 103%. اظهر الانزيم المنقى اعلى فعاليه وثبات انزيمي عند رقم هيدروجيني 8 ودرجه حرارة 37 م باستخدام الاسباراجين كافضل مادة اساس. ثبت انتشار خلايا سرطان الثدي بشكل كبير من خلال التأثير السام L-ASNase على الخلايا مقارنة بخط الخلايا الطبيعي (WRL-68) .

كلمات مفتاحية: بكتريا، انزيم، عزل، اختبار الفايتهك، ظروف مثاليه

INTRODUCTION

Staphylococcus aureus (*S. aureus*) considered a fundamental microbiome of skin, nose and mucous membranes in human and animals. However, Staphylococci are the causative agent of many clinically important infections (12). *S. aureus* are gram positive bacteria that grow in the form of clusters with 0.5 to 1.5µm in diameter portrayed by individual cocci since division of cell happens in more than one plane, these bacteria are non-motile, non-spore forming and highlighting a complex nutritional requirement for development, characterized by resistance to heat and tolerance to high concentration of salt, although it grow best in aerobic condition they are facultative anaerobic (15, 33). A special property when studying the repertoire of *S. aureus* released virulence factors are the significant functional redundancy displayed by the most of toxins and many of these toxins have enzymatic characteristics. Little is known about staphylococcal asparaginase (36). L-asparaginase (L-ASNase) is a common enzyme found in bacteria, yeast, fungus, and plants. It catalyzed the hydrolysis of asparagine's amide group, resulting in aspartate and ammonia (24). L-ASNase is an amidohydrolase that is broadly utilized as an important anti-tumor agents. The enzyme acts via the L-asparagine catalysis, locating on the majority of tumor cells surface, to ammonia and L-aspartate, resulting in death of these cells because of the exhaustion of L-asparagine on their surfaces, since it is important for their proliferation and activation (30). L-ASNase contributes a significant portion of the overall medicinal enzymes used as anti-leukemia and anti-lymphoma agents worldwide (21). In the L-ASNase existence, the cancer cells not have an essential growth factor, so they cannot survive. Hence, this enzyme can be utilized as anti-leukemetic, potent anticancer drug for treat all (especially, children) (2). L-ASNase is an essential enzyme can be utilized as chemotherapeutic agents for treat several diseases, such as melanomasarcoma, reticlesarcoma, lymphosarcoma, lymphocytic leukemia, acute myelomonocytic leukemia, acute myelocytic leukemia and the Hodgkin disease (39). Production of L-asparaginase is greatly

influenced by fermentation medium composition and culturing conditions such as incubation time, agitation rate, inoculum size, pH, and temperature (23). Normal cells make their own L-asparagine via use L-asparagine synthetase. *E. coli*, *Bacillus* sp., *Streptomyces albidoflavus*, and *actinomycetes* from the rhizosphere of medicinal plants were the most widely employed microorganisms to manufacture L-asparaginase (19). Therefore; there is a urgent required to screen novel organisms for obtaining strain have ability to produce new and high productivity of L-asparaginase. Because of there is a fewer reports about L-asparaginase production and purification by *S. aureus*. The aim of this study was isolating L-asparaginase producing *S. aureus* bacteria from different pathogenic sources followed by the optimization and purification to reach the maximum enzyme activity to study its properties and antitumor effect for medical applications in future.

MATERIALS AND METHODS

Chemical materials used in this study:

Nutrient agar, nutrient broth, mannitol salt agar, phenol red, meat extract, yeast extract, coomasse blue 250 and all other reagent grand chemicals were purchased from Hi-Media and Sigma Aldrich, India, Switzerland.

Sample Collection and bacterial isolation

One hundred and seventy samples were isolated from the medical site, such as Medical City Hospital, Al-Kadhimiya Hospital, Al-Yarmouk Hospital / Burn Center, and Al-Hariri Hospital, during the periods (September 15 - December 15, 2021). These samples were proven from the skin, burns, wounds, urine, blood, tonsillitis, and ear infections. These samples were cultivated on brain heart broth then brain heart agar thereafter on mannitol salt agar medium as a selective medium for *Staphylococcus* sp. In order to identify *Staphylococcus aureus*, some biochemical tests and a Vitek 2 test were performed. The identified isolates of *S. aureus* were prepared for the screening experiment. Thereafter evaluated for the production of L-asparaginase.

Screening *S. aureus* isolates for L-asparaginase production

Primary screening (qualitative screening): Qualitative screening for asparaginase

production was done according to Sharma and Husain (34) with some modification. One hundred *S. aureus* isolates were screened using a plate assay with asparagine solid medium to find the best ones that produced asparaginase, this medium contained per g/L (10g asparagine, 2g glucose, 1.52 g KH_2PO_4 , 0.52 g KCl, 0.52 g MgSO_4 , 0.03 g FeSO_4 , 0.03 g Zn SO_4 , 0.05 g CuSO_4 and 15 g agar-agar) dissolve in 1 L of distilled water, it was mixed properly, pH was regulated at 7.0. Autoclaved then adding 0.09 g/l of phenol red after filtrating it in sterilized asparagine medium thereafter pour in the dishes to find the best ones that produced asparaginase. Following this, a well is aseptically poked with a sterile cork borer on agar surface plate. One hundred microliter of activated bacterial isolate was put in the well of asparagine agar plate and placed in incubator at 37°C for 24 hrs. The hydrolysis of clear zone around the colony wells was considered as an indicator for secretion of asparaginase.

Secondary screening (Quantitative screening): Thirty-seven isolates were selected with maximum yield on the basis of the initial screening and cultured on a solid asparagine medium. A 250 mL flask contains 50 mL of submerged medium with modification composing of per g/L (10 g asparagine, 2g glucose, 1.52 g KH_2PO_4 , 0.52 g KCl, 0.52 g MgSO_4 , 0.03 g FeSO_4 , 0.03 g Zn SO_4 and 0.05 g CuSO_4) versus 1 L distilled water with pH 7.0. Autocleave then inoculated with 1.0 mL of overnight culturing isolates containing 3×10^{11} cells/mL. The flasks were placed in rotary shaker incubator at 37 °C at 150rpm. After 24 hr. of incubation, the culture of each flask was centrifuged at 10,000 rpm for 10 minutes. The pure supernatant was used as a crude enzyme and evaluated for concentration of protein and asparaginase activity. Concentration of protein and activity of enzyme and were repeatedly turned on (34).

L-asparaginase assay

Asparaginase activity was tested using Nesslerization process, which is dependent on the conversion of L-asparagine into ammonia and L-aspartate and has an absorption limit at 625 nm, according to Al-Dulimi (4) with some modification. One milliliter of crude L-ASNase was added in test tube, then 1 mL of

200mM of L-asparagine prepared in potassium phosphate buffer (0.05 M, pH 8.0) was then added with gentle mixing, thereafter placed in incubator at 37C for 30 min. After incubation, 1mL of trichloroacetic acid (1.5 M) was applied to the solution mixture to stop reaction. The mixture was placed in centrifuge 8000 rpm for 10 minutes. L-ASNase activity was measured within the supernatant using the direct Nesslerization process for each sample by mixing 1mL of Nessler reagent and 1mL of enzyme supernatant with well shaking. Then the mixture was placed in incubator at 37°C for 30 min. The optical density was measured at 625nm. For preparation of blank, 1 mL of Nessler reagent and 1 mL of purified water were mixed. One L-ASNase unit was defined as the enzyme amount that releases 1 μ mol of ammonia per minute at the experimental conditions. Concentration of protein calculated based on the Bradford method (13).

Optimization of crude L-asparaginase production from selected *S. aureus*

L-asparaginase production was studied in relation to impact of different culture requirements. Numerous governing factors, such as fermentation media, carbon content, nitrogen content, pH value, temperature and incubation period, were investigated.

Effect of fermentation media

Eight different types of media were utilized to study the optimal medium for L-ASNase production. Including (g/L): (1) (asparagine 10 g, glucose 2g, KH_2PO_4 1.52 g, KCl 0.52 g, MgSO_4 0.52 g, FeSO_4 0.03 g, Zn SO_4 0.03 g and CuSO_4 0.05 g) (32). (2) (glucose 2g, KH_2PO_4 1.52 g, KCl 0.52 g, MgSO_4 0.52 g, FeSO_4 0.03 g, Zn SO_4 0.03 g and CuSO_4 0.05 g) (34) with modification. (3) Asparagine 1g/L, meat extract 0.3 g/L, yeast extract 0.3 g/L, peptone 1 g/L and glucose 1 g/L (16). (4) Meat extract 0.3 g/L, yeast extract 0.3 g/L, peptone 0.5 g/L, glucose 1 g/L (16) with modification. (5) Asparagine 1 g/L, glucose 0.1 g/L, K_2HPO_4 0.1 g/L, yeast extract 0.05 g/L, trypton 0.05 g/L (9). (6) Glucose 0.1 g/L, K_2HPO_4 0.1 g/L, yeast extract 0.05 g/L, trypton 0.05 g/L (9) with modification. (7) Asparagine 1g/L, yeast extract 0.06 g/L, trypton 0.02 g/L, Na_2HPO_4 1.075 g/L, KH_2PO_4 0.36 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0025 g/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.00027 g/L, MnCl_2 0.00025

g/L, CaCl₂ 0.0015 g/L (5). (8) Yeast extract 0.06 g/L, trypton 0.02 g/L, Na₂HPO₄ 1.075 g/L, KH₂PO₄ 0.36 g/L, MgSO₄·7H₂O 0.0025 g/L, FeSO₄·7H₂O 0.00027 g/L, MnCl₂·4H₂O 0.00025 g/L, CaCl₂·H₂O 0.0015 g/L (5) with modification. Erlenmeyer flasks (250) mL containing 50 mL of each tested medium in duplicate were autoclaved, then placed in inoculator with 1.0 mL of overnight culture consisting of 3×10^{11} cell/mL of selected isolate and incubated at 37°C for 24 hrs. in shaker incubator at 150 rpm. After incubation, the culture of each flask was filtrated by centrifuge and concentration of protein, the activity of enzyme and specific activity of the filtrate were all measured (4,13).

Optimal carbon sources

For determine the best carbon source, A 50 ml of optimal medium was placed in 250mL flasks, and the pH was changed to 7.0. After autoclaving for 15 minutes at 121°C, 0.1 g/L of each carbon source solution (lactose, glucose, sucrose, cellulose, maltose, fructose and starch) was separately placed to each flask after sterilized by filter unite with 0.22 µm, (in duplicate). The flasks were then placed in shaker incubator at 37°C (150 rpm) after inoculated with 1% (3×10^{11} cells/mL) overnight culture of *S. aureus* isolate for 24 hr. Thereafter, the cultures were placed in centrifuge (10,000 rpm for 10 minutes) and the supernatants were recovered for evaluating the activity of enzyme, concentration of protein and specific activity (4, 13).

Effect of nitrogen source

A 0.05 g/L of several nitrogen sources, including yeast extract, trypton, peptone, NH₄Cl, yeast extract with trypton, yeast extract with peptone, peptone with trypton and peptone with NH₄Cl, were added to optimal asparaginase production medium, the pH was set to 7.0. The flasks were inoculated with 1% (3×10^{11} cells/mL) of the highest productivity isolate and the medium was shaken in a shaker incubator (150 rpm) for 24 hr at 37°C. Enzyme activity, protein concentrations, and specific activities were all determined using the supernatant obtained after centrifuging each flask's culture following incubation (4,13).

Effect of temperature

Temperatures of 25, 30, 37, 40, 45, and 50°C were utilized for determination of the optimal

temperature degrees of L-asparaginase production. The selected medium was at optimum carbon and nitrogen sources, inoculated with 1% of overnight culture from bacterial isolate (3×10^{11}) cell/mL and incubated at different temperature for 24 hrs using shaker incubator for 150 rpm. After enzyme extraction with centrifugation, the activity of enzyme, concentration of protein and specific activity were measured (4,13).

The pH effect

In order to evaluate the enzyme production, the initial pH value of the medium was examined after selected the optimal temperature that gives the maximum productivity of asparaginase. A 250 mL Erlenmeyer flasks containing 50 mL of optimized medium were set to different pH values (4, 5, 6, 7, 8, 9 and 10), then autoclaved. Thereafter, the medium was inoculated with culture bacterial isolate at a concentration of 3×10^{11} cell/mL and placed in a shaker incubator at 150 rpm and 37°C for and 24 hrs. After incubation, the activity of enzyme, concentration of protein, and specific activity were determined (4,13).

Optimal incubation period

Asparaginase production efficiency was evaluated by experimenting with various incubation periods. The medium chosen at the optimal pH was inoculated with 1% of overnight selected isolate (3×10^{11} cells/mL) and incubated at 37 °C in different incubation time include 24, 48, 72, 96 hours. Activity of enzyme, concentration of protein and specific activity were measured (4,13).

Purification of L-asparaginase

The asparaginase purification from *S. aureus* isolate (TG98) was done by gel filtration chromatography utilizing sephadex G-150. The column was attended to and packed based on the directions of manufacturing company's (Pharmacia-Sweden). The crude enzyme was passed over a sephadex G-150 column (1.6 x 21) cm, and the elution step was performed with sodium phosphate buffer solution 0.2 M, pH 7.0, with flow rate 20 mL/h, 3mL for every fraction. The enzyme activity of these fractions was determined after the protein fractions were assessed at 280nm in each fraction. The volume of effective fractions was estimated, and subsequently activity and

concentration of protein were calculated. The volume was condensed and placed in separated tubes before being frozen for a period of time (7).

Characterization of partial purified L-asparaginase

Effect of pH on L-asparaginase activity: The effect of pH on activity of the partial purified L-ASNase was estimated by preparing asparagine as a substrate in different buffer solutions include 0.05 M sodium acetate buffer (pH 5, 6). 0.05M tris-base buffer (pH 9) and 0.05M potassium phosphate buffer (pH 7, 8). The activity was estimated and the relation between an enzyme activities towered values of pH were plotted to evaluate the optimal pH of L-ASNase activity (20).

Impact of different pH on the stability of partial purified L-ASNase

Partially purified enzyme and buffers were placed together in equal volumes (1:1 ratio) at different range of pH (5-9) with mixing. The solution was placed in a water bath at 37°C for 15min. After that, the samples were transferred immediately to an ice bath. The activity of enzyme was assayed then estimate the remaining enzymatic activity for each pH value and plotted to assay the optimal pH of L-asparaginase stability.

Impact of temperature on L-ASNase activity: Activity of partial purified L-ASNase was estimated at various range of temperature include (25, 37, 45, 50 and 55) °C. Then the association between enzyme activates and temperature was evaluated for assay the optimal temperature for L-asparaginase activity (20).

Impact of temperature on L-ASNase stability: Partially purified L-ASNase was placed in water bath at variable temperatures (25, 37, 45, 50 and 55°C) for 15 min. and then it transferred immediately to an ice bath. Thereafter, the enzyme activity was assayed at optimal temperature of enzyme activity. Then remaining activity (%) was estimated.

L-Asparaginase specificity

The impact of variable substrates on L-asparaginase activity was studied *via* utilizing different substrates, including (asparagine, collagen, gelatin, casein). For preparing these substrate solutions, 200mM from each substrate was placed into 100mL of 0.05M

potassium phosphate buffer at pH 8.0. The association between enzyme activities and type's substrates was evaluated for determination of the optimum substrates of L-asparaginase activity.

Effect of some chemical compounds on L-asparaginase activity: The impact of chemical compounds includes (ZnSO₄, CuSO₄, CaCl₂, HgCl₂, KCl, EDTA, and cysteine) on partial purified L-asparaginase activity was study. Solution for each one was prepared at concentration 1mM and 5mM by dissolving in 0.05 M of phosphate buffer. Enzyme solution was incubated with these metal ions solution at 1:1 (v/v) ratio for 15 min at 37 °C, then the enzyme activity was estimated and compared with control (the enzyme before treatment), thereafter remaining activity % was assayed.

Cytotoxic effect of partial purified L-ASNase : The possible cytotoxic effects of L-ASNase were investigated using *in vitro* approach. This was prepared in various concentrations and their cytotoxicity was tested on tumor cell lines (MCF7), WRL 68 cell line using as a control (37). MTT cytotoxic effect assay: based on the instructions of manufacturer agreed to Ascar *et al.*, (6). From (1x10⁴ to 1x10⁶ cells mL⁻¹) were cultivated in 96-well microplates to 200 µL well⁻¹. The microplates were sealed with a sterilized parafilm, with gentle stirring and placed in incubator for 24hrs at 37 °C with 5% CO₂. After incubation, the medium was removed, and 200 µL of a 2-fold serial dilution of the purified L-Asparaginase (25, 50, 100, 200, 400 mg mL⁻¹) was placed into the wells. Triplicate was carried out at every concentration and control. The microplates were placed in incubator for 48hrs at 37 °C with 5% CO₂. After exposing to L-ASNase, 10 mL of MTT solution was placed to every well. The microplates were then placed in incubator for 4hrs at 37 °C, 5% CO₂. Carefully, the medium was eliminated and 100µL of dissolution was placed to every well and then the microplates were placed in incubator for 5min. Optical density was estimated utilizing an ELISA reader (Bio-rad, Germany) at 575nm. Statistically, the data analysis was carried out on the optical densities that obtained to calculate the IC₅₀, based on this equation:

$$\text{Viability (\%)} = \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

RESULTS AND DISCUSSION

Screening *S. aureus* isolates for L-asparaginase production

Primary screening (qualitative screening)



Figure 1. L- asparaginase activity of *S. aureus* on solid medium at 37⁰C for 24 hr.

One hundred bacterial isolates were subjected for qualitative screening method using asparaginase plate agar medium and clear hydrolysis zone assay was done. By calculating the ratio of diameter of clear zone around the wells containing bacterial culture (Z), the efficiency of bacterial isolates was compared. Among all *S. aureus* isolates, thirty-seven isolates were asparaginase producer depending upon the intensity of produced yellow color (Figure 1). The formation of yellow color reveal the bacteria ability to alter pH of culture from acidic to alkaline because of asparagine degradation in solid medium containing phenol red existence as pH indicator, this can be interpreted by the breakdown of amide bonds in L-asparagine by L-asparaginase with accumulation of ammonia in the medium. L- ASNase activity was correlated with the formation of yellow zones around wells and their diameters. The diameter of yellow zone was ranged from (20-45) mm. These isolates were selected for more secondary screening. The activity L-ASNase zone was ranged from 0.8 to 13mm for soil bacterial isolates (17). In this context, Wakil and Adelegan (41) reported that the hydrolysis zone of L-ASNase that ranged from 3 to 5cm.

Secondary screening (Quantitative screening): For further revealing and screening, the 37 isolates of *S. aureus* with maximum production yellow color in primary screening, were screening again for their enzymatic activity using submerged

fermentation method by utilizing the Nesslerization procedure, this is based upon the transformation of L-asparagine to ammonia and L-aspartate with an absorption limit of 625nm (4). Among 37 isolates, *S. aureus* TG98 gave the highest activity of enzyme, specific activity of L- ASNase in crude supernatant was 255.8U/mg protein. While the asparaginase specific activity for the rest isolates was varied from 0.144 to 185U/mg, the *S. aureus* TG98 isolate which had the higher specific activity was chosen for further experiments. The difference between members in the same species for their ability of asparaginase production may be because of the source, type and genetic variation of isolates and condition of culture such as aeration, stirring, pH, temperature and component of media, which help to elevate the (TG98) isolate ability to producing the enzyme in a liquid media (22).

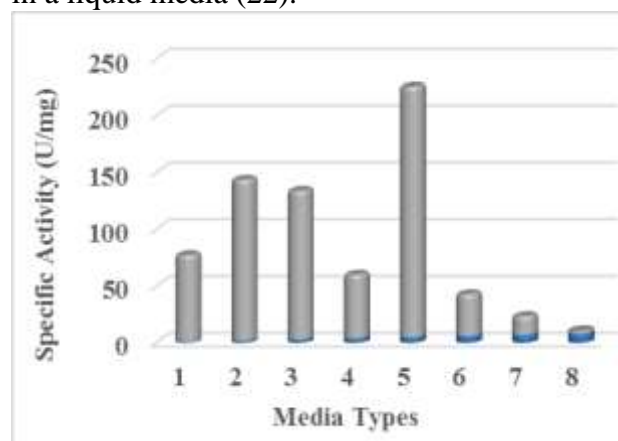


Figure 2. Production of asparaginase in different liquid media by *S. aureus* TG98 using shaker incubator 150 rpm at 37⁰C for 24 hr

Optimum condition for asparaginase production: Effect of fermentation medium: The effect of media on asparaginase synthesis was investigated using *S. aureus* TG98, which was grown in eight different media. According to particular activities, medium (5) was shown to be the best medium for asparaginase production among the eight media studied. The greatest specific activity of asparaginase detected was 220.71 U/mg, while the specific activity of asparaginase utilizing medium 1, 2, 3, 4, 6, 7 and 8 was reduced to (76.33, 141.71, 131.04, 56.17, 37.01, 16.77 and 2.85) U/mg protein, respectively (Figure 2) As a result, this medium was chosen to maximize the

activity of the other fermentation parameters. The medium composition and conditions of culture (such as pH, temperature and nutrients) have a critical role in production of L-ASNase. Among variable microorganisms, there is no specified medium for maximize the production of L-ASNase. Hence, there are specific requirements for each microorganism to achieve its maximal ability for formation of L-ASNase, the improvement of appropriate medium components and conditions of environment is obligatory. Different conditions of environment and components of medium needed for growth of microorganisms and production of enzyme should be adjusted as the culture conditions which allow the enzyme formation differs highly with the nature of microorganism (19).

Optimum carbon source

The production of asparaginase was investigated in the existence of various carbon sources added into the medium at a concentration of 0.1g/L. Lactose was found to enable good L- asparaginase production among seven distinct carbon sources (Figure 3). The specific activity of asparaginase was increased to 370 U/mg, compared to 22.72 U/mg with the sucrose-containing medium. . Carbon is an essential element required for organism growth, and most microbes prefer it as an energy source. As a result, it is largely used as a significant carbon source for microbial growth and metabolite production (11).

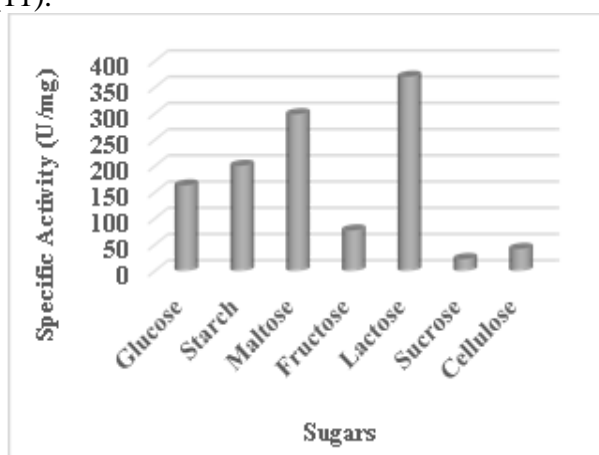


Figure 3. Effect of carbon sources on asparaginase produced by *S. aureus* TG98 utilizing submerged culture pH 7.0 at 37°C for 24hrs

Optimum nitrogen source: At the optimum carbon source, the impact of several nitrogen

sources was assessed. When compared to alternative nitrogen sources, NH_4Cl was the greatest source of nitrogen for supporting *S. aureus* TG98 enzyme production, increasing asparaginase specific activity to 419.3 U/mg (Figure 4). The concentration and nature of nitrogen in the cultivation medium used to grow the organism are critical for asparaginase synthesis. The source of nitrogen has a profound influence on the metabolism of microorganisms. Vi with his coworkers (40), indicated the microbial fermentation contained seeds of *Moringa oleifera* showed the highest activity of enzyme (4.073IU/mL) after using ammonium sulfate as source of nitrogen. Whereas the activity become 1.36 IU/mL after using ammonium chloride in cooked chicken bone. From five variable examined sources of nitrogen, sodium nitrate indicated to be the worst source of nitrogen for both substrates. Moreover, ammonium chloride reported giving (1.36 IU/mL) followed by ammonium sulphate (1.248 IU/mL) indicated to be the best source of nitrogen for production of L-ASNase by *E. coli* cultured in cooked chicken bone.

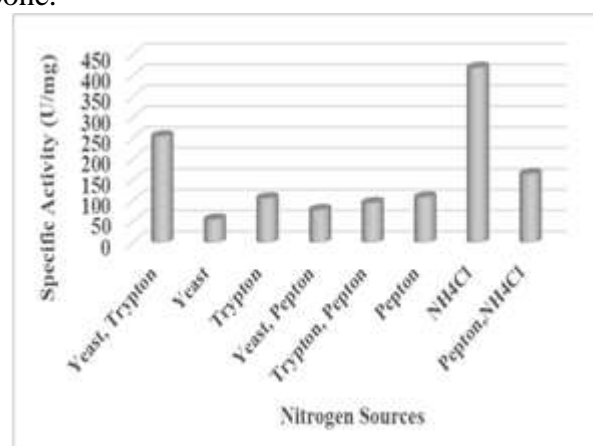


Figure 4. Effect of nitrogen sources on asparaginase production from *S. aureus* TG98 at 37°C in shaker incubator 150 rpm pH 7.0 for 24 hrs

Influence of temperatures on L-asparaginase formation: The findings in (Figure 5) investigate the ability of *S. aureus* TG 98 isolate to proliferate and release L-ASNase at broad temperatures ranges (25, 30, 37, 40, 45 and 50°C). The maximum production of asparaginase was achieved at 37 °C with specific activity of 464.28 U/mg. Lower yields obtained at lower and higher temperature 25, 50 which was 99.74 and 75.71

U/mg protein respectively. Temperature plays a vital role in the growth and metabolism of any microbe. The maximal specific activity of asparaginase for majority of species was reported at 37°C. Whereas the decrease or increase in the temperature of incubation less or more the optimal temperature result in a decline in activity of enzyme (1). The production of enzyme effected by temperature through effecting the oxygen solubility in the media, on speed of enzymatic reactions, on the vibration energy of molecules, and in the cell that affected negatively or positively on synthesis of enzyme (14).

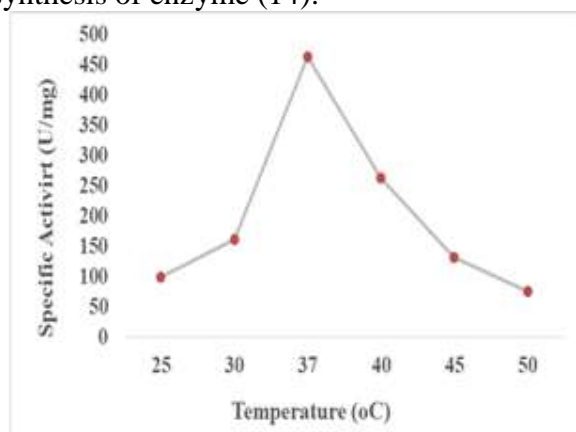


Figure 5. Impact of temperature of ASNase production from *S. aureus* TG98 using shaker incubator 150 rpm for 24hr

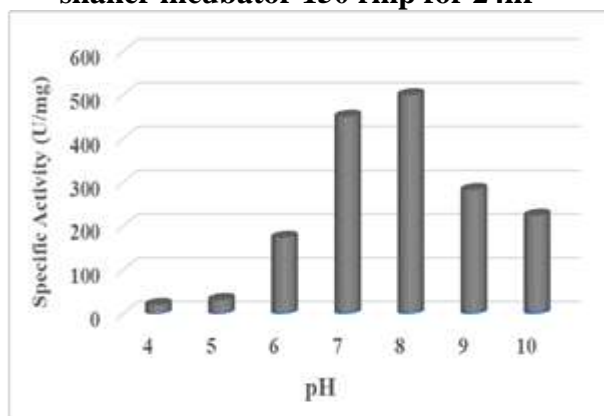


Figure 6. Effect of pH on asparaginase production from *S. aureus* TG98 in shaker incubator 150rpm for 24hr at 37°C

Optimum pH: *Staphylococcus aureus* TG98 was cultured in the production medium with various values of pH to investigate the influence of the initial pH for asparaginase synthesis (4, 5, 6, 7, 8, 9 and 10). The maximum asparaginase specific activity (492.5) U/mg was found at pH 8.0, as shown in (Figure 6), however, increasing or decreasing the pH value above or below 8.0

resulted in a drop in enzyme activity. The Asparaginase produced by *S. aureus* TG98 reported to have the highest activity at alkaline pH. It was reported that asparaginase produced from *E. coli* have its maximum activity in alkaline pH that probably due to the balance between L-aspartic acid and L-aspartate. L-aspartic acid in acidic pH has greater affinity to the active site of the enzyme (42). Any decline or elevate in concentration of hydrogen ions (H^+) lead to changes of pH in the reaction mixture which may causes drastic alteration in three-dimensional structure of protein, resulting in the denaturation of enzyme (38). pH of the culture media often influence the fermentation course and enzyme production rate, since pH of the medium drastically affects the conformation of the plasma membrane, and subsequently affects the membrane bound ribosomes involved in protein synthesis (3).

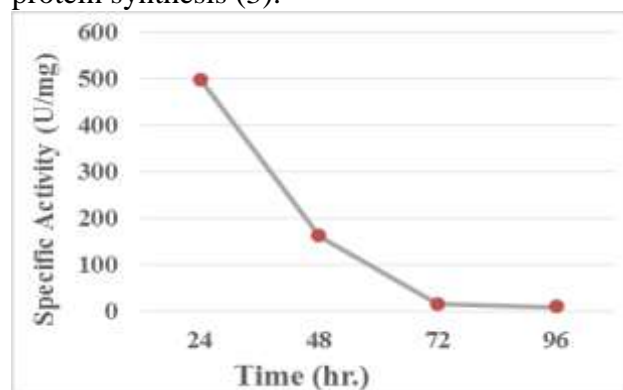


Figure 7. Effect of incubation period on asparaginase production from *S. aureus* TG98 in shaker incubator 150 rpm at 37°C

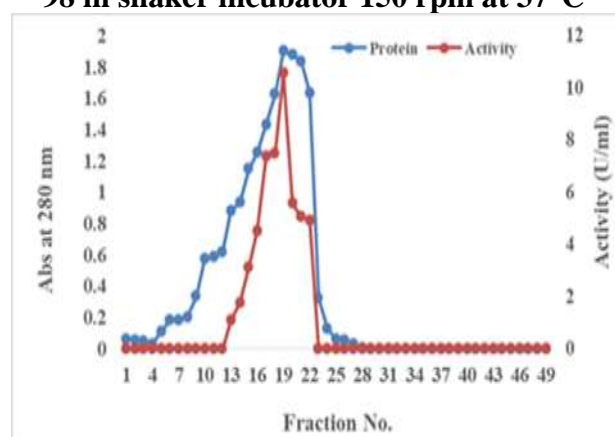


Figure 8. Gel Filtration chromatography for L-ASNase purification from *S. aureus* HN77 utilizing Sephadex G150 column (1.6 x 21) cm equilibrated and eluted with phosphate buffer (pH 7, 0.2 M), in rate of flow 20ml/hr. 3ml for every Fraction

Impact the incubation periods on production of L-ASNase : The optimal incubation time was tested to reveal the periods of growth and enzyme synthesis. The finding in (Figure 7), show that 24 hr. was better incubation period for asparaginase production from *S. aureus* TG 98, the specific activity was reached to 496.99 U/mg protein. The specific activity was declined with elevating the period of incubation, that because of the altering in the culture conditions during this periods, including accumulating the toxic metabolites, oxygen depletion, nutrients consumptions and which suppress the growth of bacteria (26).

Purification of L- asparaginase

Staphylococcus aureus TG 98 was cultivated under the optimum conditions for optimal asparaginase production, enzyme extracted by centrifuge at 10000 rpm for 10 min. The crude asparaginase was purified from the undesired proteins and other ingredients utilizing gel filtration by (Sephadex – G150) as follows:

Gel filtration chromatography

The crude enzyme solution was undergone cross a Sephadex - G150 gel filtration column with diameters of 1.6×21cm, and equilibrium with 0.05M of Tris-HCL pH 8. The results revealed that separated one protein peak in the eluted fractions from the column, also one peak of asparaginase activity in fractions (12-24), (Figure 8). The specific activity reached to 929.4 U/mg protein with 2 fold of purification and yield reached to 103% as in (table 1). Narayana *et al*, (31) were purified L-ASNase from *Streptomyces albidoflavus* and found that the specific activity of L-ASNase elevated and reached 101 and 437U/mg after purification utilizing the Sephadex G-100 and CM-Sephadex C-50, respectively. Purity of L-asparaginase was elevated achieved 99.3-fold with 40% recovery in CM-Sephadex C-50 step of purification. Dhevagi and Poorani (18) reported that the specific activity was 63.07U/mg protein with 85 fold of purification and with final recovery of protein 2.18% when purification L-asparaginase from marine actinomycetes using Sephadex G-200 gel filtration.

Table 1. Purification table of L- asparaginase from *S. aureus* TG 98

Sample	Volume (ml)	Activity (U/ml)	Protein cons (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crude Enzyme	50	4.6	0.01	460	230	1	100
Gel Filtration using Sephadex G-150	30	7.9	0.0085	929.4	237	2	103

Characterization of L-asparaginase

Influence of pH on the activity of L-ASNase: The pH influence on partial purified L-ASNase of *S. aureus* TG98 was assessed in a range of pH 5.0-9.0 as presented in (Figure 9). The best activity was in pH between (7-8) with maximal activity of enzyme at pH 8. It was reached to 10 U/ml, whereas in acidic pH (5 and 6) were 9.38 U/mL and 9.5 U/mL respectively, also the activity was decrease in alkaline pH (9) and reached to 7 U/mL. The changing in optimal pH because of the electrostatic interactions effected by the carrier microenvironment.

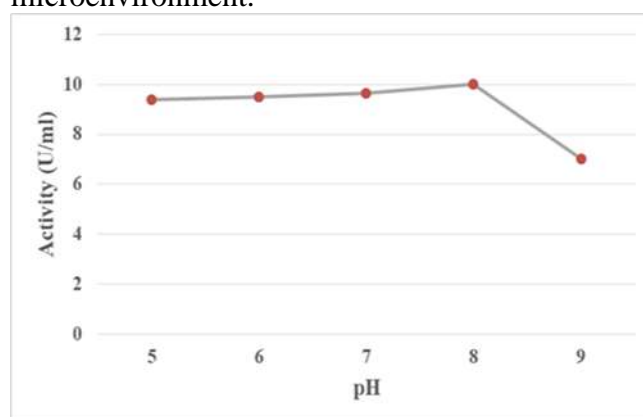


Figure 9. Influence of various values of pH (5.0-9.0) on partial purified L-asparaginase activity from *S. aureus* TG98

Several researches pointed that the enzyme reach its maximal activity at pH 7.0 and 8.0 (7).

Effect of pH values on stability of L-asparaginase: The results in (Figure.10) shows that 8.0 were the optimal pH for L-asparaginase stability, L-ASNase kept about 100% of its activity in pH 8.0, but kept about 93.3% in pH 7 .0. The enzyme activity was declined out of the optimal values of pH. The residual activities were 92% at pH 5.0, the activity of enzyme was reduced at nearly acidic pH and it was reached to 93% at pH 6.0. Also enzyme activity decreased toward alkaline pH like 93.2% for pH 9.0. The

asparaginases from *Aspergillus niger* LBA 02 and *A. oryzae* LBA 01 were stable at pH (7.0-9.0). Generally, the optimum ranges of pH for asparaginase were at pH 6.5–9.0 (25).

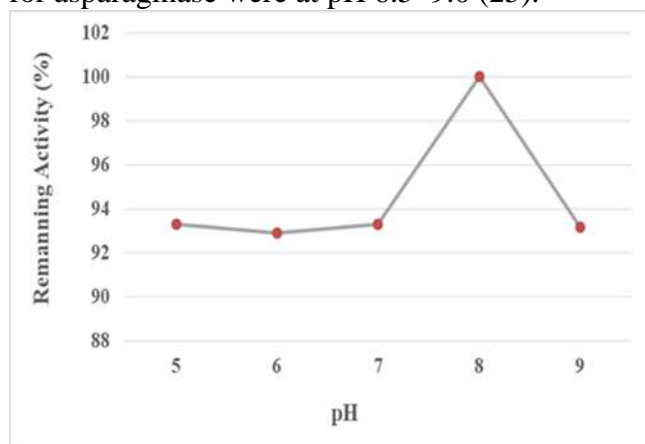


Figure 10. Influence of various pH values (5.0-9.0) on partial purified L-asparaginase stability from *S. aureus* TG98

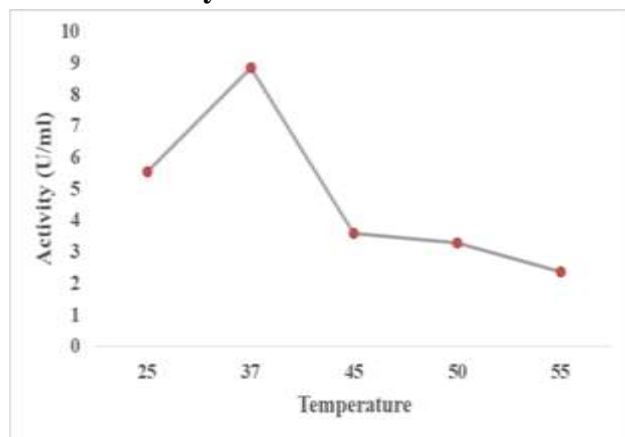


Figure 11. Effect of different temperatures on partial purified L-asparaginase activity from *S. aureus* TG98

Impact of temperature on activity of L-ASNase: The activity of partial purified L-ASNase was measured at ranges of temperatures (25°C-55°C). (Figure 11), shows an increase in activity to 8.86 U/mL at 37°C, then a drop in activity with rising temperature reached to 50°C, with a minimum activity of 2.37 U/mL at 55°C. At 25°C, the minimum activity of 5.56 U/mL was also recorded. The optimal temperature for activity of purified L-ASNase from *Aspergillus terreus* KLS2 was found to be 37°C (35).

Effect of temperature on stability of L-asparaginase: The L-ASNase stability at varied temperatures was determined via incubation of L-ASNase at temperatures ranging between 25°C to 55°C for 15min. Then determining the remained activity

percent. Activity of L-ASNase was maintained in the current investigation at ranges of temperature from 25 to 37°C. (Figure 12), but the activity initiates to decline with elevating at ranges between (45°C-55°C). The decreased activity of L-asparaginase at temperatures above 37°C is due to its susceptibility to high temperatures; however, the increased thermal stability of L-ASNase resulting from immobilization should be beneficial for treating effluents at high temperatures. The thermal stability studies of purified L-ASNase from *Fusarium Culmorum* ASP-87 detected that this enzyme have high stability for 120 min at (30°C-40°C), but it deactivated when the temperature become above of 40°C while retaining the 50% of activity at 60°C for 1 hr (29).

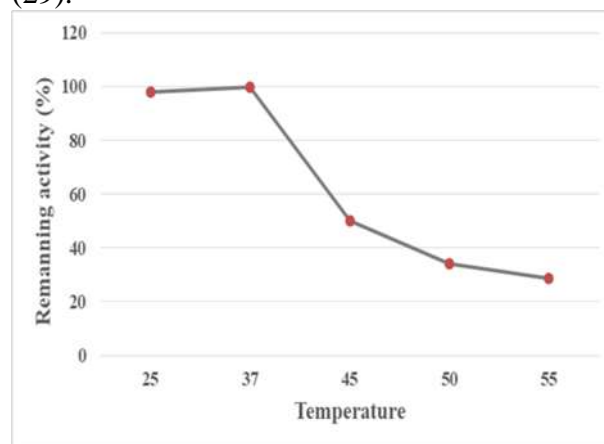


Figure 12. Effect of different ranges of temperature on stability of partial purified L-ASNase from *S. aureus* TG98

Substrates specificity of L-asparaginase

The best substrate needed for support the activity of L-ASNase was studied by incubation of the purified L-ASNase with variable substrates such as asparagine, collagen, gelatin and casein in pH 8.0 at 37°C. The findings in (Figure 13) shows an elevate in percentage of enzyme activity utilizing asparagine as a substrate, it was arrived to 100%, followed by using collagen 89.2% whereas the percentage of activity by using gelatin reached to 78.4% and casein (57.8%). Meghavarnam, and Janakiraman (29) reported that the high specificity of enzyme observed for its natural substrate (asparagine), but the very low specificity of this enzyme observed towards L-aspartic acid. However, there is no activity observed towards both of

L-glutamic acid and L-glutamine when utilized these materials as a substrate.

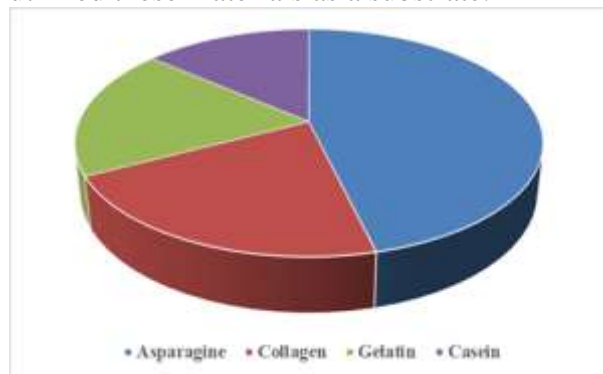


Figure 13. Influence of various substrates on purified L-asparaginase activity released from *S. aureus* TG98 at pH 8

Effect some chemical compounds on L-asparaginase activity: Chemical materials were used to treat purified L-asparaginase from *S. aureus* TG98. The results in Figure (14) reveal that all of these materials inhibited the L-asparaginase enzyme, while there were some variances. Metal ions' influence on activity of L-ASNase varies depending on the enzyme's origin, but at 1 and 5 mM, ZnSO₄, CuSO₄, CaCl₂, HgCl₂, KCl, EDTA, and cysteine were shown to suppress activity of enzyme to levels less than their original activity (value of control). At 1 mM and 5 mM, HgCl₂ suppressed L-ASNase, with residual activity of 52% and 60%, respectively. EDTA inhibits enzyme activity, with remaining activity equal to 62% and 61% at concentrations of 1 and 5 mM, respectively, Fig. (14). Also, the results show that enzymatic activity was decreased by using 1 and 5 Mm of ZnSO₄, CuSO₄, CaCl₂, KCl and cysteine, with (46, 46, 45, 49 and 55) % at 1mM and (45, 55, 49, 54 and 56) % at 5mM respectively. The results of a recent research showed that different types and concentrations of ions have different effects on enzyme function, and that these effects frequently increase with increasing concentration. The complex formation with the enzyme stops it from attaching to the substrate in order to generating the product, leading to a decline in activity (32). At 1mM and 5mM, HgCl₂ was able to inhibit the enzyme, demonstrating the existence of SH groups in the enzyme active region that were oxidized by the HgCl₂ existence. Furthermore, HgCl₂ in the substrate processing solution may impede substrate

binding and product formation by creating a complex with the enzyme (27). EDTA was utilized to test the inhibitors effect on the activity of L-ASNase. This enzyme was shown to have decreased enzyme activity and increased inhibitor concentration, as measured by the remaining enzyme activities. Also when incubation with EDTA, the findings revealed that the enzyme was a metallo-enzyme and that divalent ions played a key role in the generated activity, therefore EDTA was utilized to test the inhibitors effect on the activity of L-ASNase. When the enzyme was treated with cysteine, disulfide bonds in the structure of protein and conformation are decreased leading to the protein fragmentation into its component units and a reduction in its activity. These findings suggested that the enzyme under investigation included disulfide bonds (8).

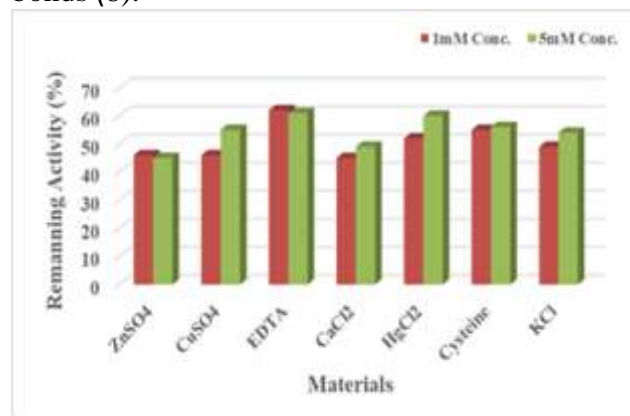


Figure 14. Influence of some chemical compounds and ions on purified L-asparaginase activity of *S. aureus* TG98 at 37 °C.

In vitro cytotoxicity assay of L-asparaginase: For testing the cytotoxicity index of the purified L-ASNase, varied doses of L-ASNase were utilized for treating the MCF7 cell lines for 24 hr. The findings demonstrated that the vitality of MCF7 cells viability was influenced by L-ASNase in a dose-dependent manner (Figure 15). MCF7 cells that treated with 1.35, 0.88, 6.59, 6.10, 3.31 U/mL of the purified L-ASNase was 97.15, 95.45, 79.50, 67.62 and 53.40, in order. This variation was significantly observed ($p < 0.0001$), which showed that the asparaginase effect varies between types of cell, and the purified ASNase can suppress the cancer cells reproduction in selective pattern. In another hand, non- carcinogenic cell line of human (WRL68 cells) was unaffected at the utilized

concentrations. The inverted microscope was utilized for assay the morphological modification in tumor cell line after treatment using L-ASNase. The cells separation from the wells surface indicated the death of cells. In addition, the apoptotic cells showed specific alternations, such as losing the adhesion of cell, rounding cell, blabbing the membrane and shrinking. The cytotoxicity of the L-ASNase suggested a noticeable toxicity (IC_{50} values of 152.5 U/mL) against MCF7 cells.

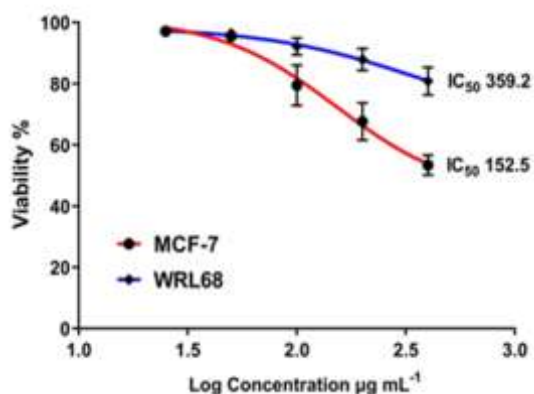


Figure 15. The influence of various concentrations of L- asparaginase on the viability of MCF-7 and WRL68 cell line by assay of MMT. The untreated group (control) is equal to 100%. Data are represented as the mean \pm SD of five repeat.

Cytotoxic effect on tumor cell lines: The L-ASNase cytotoxicity, combination on the tumor (MCF-7) and normal cell line (WRL68) was studied utilizing the MTT assay. Various concentrations of L - ASNase ranging from 25 to 400 $\mu\text{g mL}^{-1}$ for 24 hours were used to measure cell viability in MCF -7 cells and WRL68 cell lines (Table.2). Furthermore, the L-ASNase also has a dose dependent effect, the cell viability was 53.4% and 80.89% for MCF -7 cells and WRL68, respectively. IC_{50} values of L-ASNase 152.5%. On the other hand, all compounds revealed low and moderate cytotoxic effect against normal cell lines (WRL-68). Viability of cell (%) at a concentration 400 $\mu\text{g mL}^{-1}$ remained at 53.40% after treatment with L-ASNase. WRL-68 does not show any significant differences in the viability pattern of cells at lower concentrations 100 $\mu\text{g mL}^{-1}$ and for all treatments. For more than 40 years, the L-ASNase has been utilized for all treatment and inhibition of cell growth in solid tumors (10). This study reported that the impacts of *S.*

aureus purified L-ASNase was observed on breast cancer cell lines. The findings observed that therapy of tumor cells with *S. aureus* L-ASNase could considerably raise the rates of apoptosis to 68.6% and 76.6% in MCF7 cells, respectively. Indicating that *S. aureus* L-ASNase, could significantly suppress cancer cells proliferation via induction of apoptosis. Furthermore, these findings observed that nucleus fragmentation and losing the normal nuclear construction were stimulated after cells treatment by L-ASNase, but the intact nuclei existence in control (untreated) cells suggested no alteration of nuclear morphology. These findings suggested that L-ASNase stimulate damage of DNA, which could resulting in cell apoptosis (28).

Table 2. The conjugated concentrations of L-ASNase with their specific activity

MCF-7		WRL68	
Mean	SD	Mean	SD
53.40	3.31	80.89	4.54
67.62	6.10	87.92	3.58
79.50	6.59	92.21	2.78
95.45	0.88	96.49	1.29
97.15	1.35	96.95	1.14

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