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Determining the Optimum Conditions for L-Asparaginase Produced from a Local Isolate of *Pseudomonas Aeruginosa*

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ABSTRACT: L-asparaginase is an enzyme of significant medical importance that is extensively applied in the management of cancers. The enzyme catalyzes the degradation of the amino acid L-asparagine, thereby preventing cancer cells from utilizing it for proliferation. Organisms such as bacteria are crucial sources of the enzyme. Bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) are exceptionally adaptable across diverse habitats and can synthesize many biologically essential enzymes. The study aims to isolate and characterize *P. aeruginosa* from various sources in Baghdad, determine its capacity to synthesize L-asparaginase, and establish favorable conditions for enzyme synthesis. The total number of samples taken was seventy-two (sixty-nine soil samples and three water samples). The samples were cultured on appropriate nutrient medium after dilution. The isolates were characterized morphologically and biochemically, with further confirmation carried out using the VITEK 2 method. Enzyme production by the isolates was initially screened by culturing them on a nutrient medium supplemented with L-asparagine and phenol red indicator. Enzyme activity was estimated using the Nessler test, whereas protein was estimated using the Bradford method. Isolates that exhibited high efficiency were chosen for optimization studies. The results showed great ability in the biosynthesis of L-asparaginase with statistically significant differences. The number of isolates obtained was 72, where 30 showed a positive result for biosynthesis of the enzyme, accounting for 41.7%. According to the first screening test results, 78.1% of the isolates chosen produced the enzyme with different levels according to their ability and the amount of enzyme production index (EPI). The isolates (P6, P25, PW30) proved to be the most efficient ones regarding quality. The enzyme activity varied quantitatively from 4.76 to 76.90 U/mL. The isolate P1 proved to have high activity (76.90 U/mL) and high specific activity (49.30 U/mg), which means that its enzyme production rate is greater than that of other isolates. The best production medium was C1 (lacking asparagine) with the highest enzyme activity and specific activity of 20.47 U/mL and 23.27 U/mg respectively, implying that the deficiency of substrate induces the production of enzyme. In terms of carbon sources, sucrose was the most efficient carbon source with the highest specific activity of 84.32 U/mg. Yeast extract exhibited high efficiency with enzyme activity and specific activity of 75.39 U/mL and 110.87 U/mg respectively, followed by ammonium sulfate, while peptone had low efficiency. The optimum pH was eight with the highest enzyme production of 74.60 U/mL, while the highest specific activity was at 30°C. The highest enzyme activity and specific activity were 74.60 U/mL and 124.33 U/mg respectively.

INTRODUCTION

P. aeruginosa is a Gram-negative, rod-like, facultative anaerobic bacterium regarded as an opportunistic bacterium, characterized by its ability to adapt easily to a wide range of environmental conditions. This organism is widespread in nature; it is commonly found in soil, freshwater, saltwater, and even humid places like sewage and aquatic environments, as well as hospitals and medical institutions (Gellatly & Hancock, 2013; Mohammed et al., 2018; Mohammed et al., 2020; Jabaar et al., 2025; Hussein et al., 2026). Moreover, it should be noted that this bacterium produces numerous enzymes (protease, lipase, and amylase) (Costerton et al., 1999), indicating its high metabolism. The aforementioned attributes, when considered together, help to provide a rationale for the considerable research interest generated by this bacterium for use in the environmental and practical fields because it can be used as a valuable enzyme producer. Moreover, since the bacterium is found in soils and water samples, it can be used as a potential subject for studies involving the isolation of enzymes of therapeutic importance, like L-asparaginase. The microbial enzymes are some of the most significant biomolecules that have brought about changes in the field of medicine, industries, and the environment, owing to their great efficacy and specificity in biological processes (Verma et al., 2007). *P. aeruginosa* is one of the most crucial groups that can be considered a producer of L-asparaginase because of its fast growth rate, environmental control ability, and the possibility of increasing production capacity via gene manipulation or better culturing methods (El-Bessoumy et al., 2004). The processes of screening and isolation are basic procedures in the study of microorganisms, focusing on the selection of microorganisms that have the ability to produce the required enzyme from a wide array of microorganisms found in nature. It is common practice to obtain samples from different sources such as soil and water, mainly because of their diverse microbial composition that may serve as a rich source of enzyme-producing microorganisms (Cappuccino & Sherman, 2014). The initial screening process involves the use of specific media containing L-asparagine as a source of nitrogen. After the screening step, the isolation and purification steps come (Janda & Abbott, 2007). Enhancing the culture conditions is a very important stage in enhancing enzyme production since the quantity of enzymes produced depends on a number of factors such as temperature, pH level, substrate concentration, carbon, nitrogen sources, and the period of incubation (Pandey et al., 2000). In the case of Iraq, some studies showed the diversity of microorganisms in local habitats like rivers and agricultural soil, which is an excellent chance for isolating enzyme-producing bacterial strains with great significance. According to Al-Janabi (2015), there was a possible isolation of bacteria that could produce enzymes in the Tigris River, while Al-Khafaji et al. (2019) suggested the isolation of bacteria that produce different enzymes from Iraqi agricultural soil. L-asparaginase is still being explored, especially from those natural sources that have not been investigated much, with the intention of acquiring L-asparaginase that can be more effective and less toxic for clinical purposes. The other difficult task is optimizing the production process to attain maximum output at minimum cost. From the information presented above, this research paper will narrow down its scope to analyzing the isolation and optimization of the conditions required for the production of the L-asparaginase enzyme from the *Pseudomonas aeruginosa* bacteria found in environmental sources like soil and water in order to explore the biological capacity of the organism in producing the enzyme.

METHODS:

Isolation of *P. aeruginosa* from Soil Samples: The soil samples (69 samples) were taken from different regions surrounding electric generators within Baghdad as a result of the high amounts of lipids and oils found there. The samples were gathered at a depth of 3 cm beneath the surface layer. Following sampling, the soil samples were promptly placed into sterilized and tightly capped plastic tubes for safe transportation. These tubes were refrigerated with ice packs in case of any delays during transportation (Gaete et al., 2020). Serial dilution of soil samples was done. Serial dilution is a common method used in microbiology to prepare a bacterial culture from a sample that contains a high amount of microbes. The method entails the gradual dilution of the sample until the bacterial concentration in the sample becomes low enough to be measured (Kumar et al., 2016).

Isolation and diagnosis of *P. aeruginosa*: The water samples (3 samples) were obtained from a small river located within the University of Baghdad/Al-Jadriya using sterile glass bottles under aseptic conditions. The water samples were collected from a depth of 10-20 cm below the water level to eliminate contaminants found on the water surface. The samples were sealed tightly and kept in a refrigerated box (4°C). They were analyzed in the lab within 24 hours to preserve microbial life. Serial dilution of the samples was used to isolate microorganisms [APHA, 2017]. The bacteria were identified through morphological, biochemical, and cultural tests that are usually carried out in microbiology laboratories. This process entailed staining the organisms after fixation on the slides. This was followed by culturing of the isolated bacteria on different media like nutrient

agar, MacConkey agar, and Cetrimide agar. This was done to examine the growth of the organisms on the media. Growth of the organisms was achieved after incubation for 24 to 48 hours at 35-37 degrees Celsius. [2] Another special type of medium was King's B medium, which was employed to cultivate *Pseudomonas aeruginosa* in order to stimulate the growth of fluorescent dyes, for example, pyoverdine [3]. Moreover, biochemical tests, including the catalase test, were conducted. The lactose fermentation ability test was also done using MacConkey agar media for analysis of the metabolic growth pattern [4]. This is a combination of processes that have been accepted by relevant bodies for use in the diagnosis of *Pseudomonas aeruginosa*. It was confirmed by means of the VITEK 2 instrument (BioMérieux), which refers to the automated diagnostic systems that are integrated based on fast biochemical identification with high accuracy [Pincus, 2006].

SCREENING OF *P. AERUGINOSA* FOR L-ASPARAGINASE PRODUCTION

A. Asparagine medium preparation for preliminary (primary) screening (Sharma & Husain, 2015): The modified asparaginase medium plate (g/250 mL) comprised dissolving 10 g of asparagine, 2 g of glucose, 1.52 g KH_2PO_4 , 0.52 g KCl, 0.52 g MgSO_4 , 0.03 g FeSO_4 , 0.03 g ZnSO_4 , 0.05 g CuSO_4 , and 20 g agar-agar in 950 mL of distilled water. The solution was made up to 1 liter, mixed properly and adjusted to a pH level of 7.0. This was sterilized through an autoclave method at 121 degrees Celsius for 15 min at 15 psi with cooling to 45-50 degrees Celsius. Then, 0.09 g of phenol red was added to the filtered solution in sterilized medium and dispensed to sterile petri dishes.

B. Qualitative screening (primary screening): The qualitative primary screening for L-asparaginase production in *Pseudomonas aeruginosa* isolates was done according to the protocols of Gulati et al. (1997) and Sharma (2015), but modified slightly. For the primary screening, asparaginase medium agar was used. To perform inoculation, bacterial isolates were spread across the medium surface with spot inoculation by means of a sterile inoculation loop, or a small volume of bacterial suspension (10–20 μL) with a concentration of ≈ 0.5 McFarland was added to the well which was done by the sterile cork borer on the agar surface. After inoculation, plates were incubated in the incubator at 37°C for 24–48 h. The presence of the enzyme was determined by observing the colour changes in the medium around the colony from yellow to pink or red because of increasing pH and the release of ammonia.

Secondary Investigations (Quantitative Investigations): To prepare crude enzyme, thirty (30) bacterial isolates that produced high efficiency in producing L-asparaginase enzymes were chosen according to the results of the primary investigation of L-asparaginase enzymes in a solid medium consisting of L-asparagine. The isolated bacteria were inoculated in a pre-prepared submerged medium. Fifty mL of this medium was inoculated in 250-mL conical flasks and each of these flasks was inoculated with 2% (3×10^8 cells/mL) fresh bacterial culture (grown overnight), which had a 0.5 McFarland density (approximately 108 cells/mL). These conical flasks were kept in a shaker at 37°C and 150 rpm for 24 hours. After the incubation time, the cultures were centrifuged at 10,000 rpm for 10 minutes and the supernatant obtained was the source of crude enzyme. All experiments were performed in duplicates to ensure accurate readings (Al-Dulimi et al., 2020). The activity of L-asparaginase was estimated via Nessler's method (Good and Izawa, 1972; Mokrane, 2003; Khalaf, 2012; Dulimi et al., 2020; Park et al., 2020; Han et al., 2021). Asparaginase activity measurement was evaluated according to Park et al. (2020) and Han et al. (2021). The concentration of protein was calculated with the help of the Bradford assay (Bradford, 1976). The activity of the L-asparaginase enzyme was specifically determined by using the procedure recommended by Daboor et al. (2012), which is the ratio between enzyme activity and protein content as indicated below:

Optimization of Conditions for L-asparaginase Production: The conditions for the production of L-asparaginase from native strains of *P. aeruginosa* were optimized by submerged fermentation. This study evaluated the impact of factors such as medium composition, carbon source, nitrogen source, pH, temperature, and fermentation period. In all the optimization procedures, the enzyme was synthesized and isolated, and its activities were analyzed. Different culture media were studied in order to find out the most suitable media for enzyme production. 50 mL of different media was introduced to 250 mL of conical flasks that were sterilized and subsequently inoculated with 1% of freshly grown bacteria culture (approximately 0.5 McFarland or 10^8 cells/mL). Then, the mixture was incubated at 37°C in a shaker incubator with a speed of 150 rpm for 24 hours. Subsequently, the cells were isolated by centrifugation at 10,000 rpm for 10 minutes. Different types of media (pH 7-7.5) were utilized to study secondary screening of optimal media for asparagine production. Media A containing 1 g asparagine, 0.2 g glucose, 0.152 g KH_2PO_4 , 0.052 g KCl, 0.052 g MgSO_4 , 0.003 g FeSO_4 , 0.003 g ZnSO_4 , and 0.005 g CuSO_4 (Sharma and Husain, 2015). Media B is composed of 1 g asparagine, 0.2g glucose, 0.5 g peptone, 0.5 g NaCl and 0.01 g MgSO_4 . Media C is composed of

1g Asparagine, 0.2g glucose, 0.045 g KH_2PO_4 , 0.045 g NaCl, and 0.01g MgSO_4 (Dejong, 1972). A1 medium (without asparagine) containing 0.2g glucose, 0.152g KH_2PO_4 , 0.052 g KCl, 0.052g MgSO_4 , 0.003 g FeSO_4 , 0.003 g ZnSO_4 , and 0.005g CuSO_4 . B1(without asparagine) is composed of 0.2g glucose, 0.5g peptone, 0.5g NaCl, and 0.01 g MgSO_4 . C1(without asparagine) contains 0.2 g glucose, 0.045g KH_2PO_4 , and 0.045g NaCl, 0.01g MgSO_4 (Basha et al., 2009). To study the effect of different carbon sources (glucose, sucrose, maltose, fructose, and starch), they were separately introduced into the optimum medium at a concentration of 0.1 g/L after sterilizing the medium. Then, the solution was filtered through a 0.22 μm membrane filter, followed by addition to the media. The media were then inoculated with 1% fresh bacterial cultures and incubated at 37°C, 150 rpm for 24 hours. Following that, centrifugation was performed, and filtrates were used to measure enzyme activity, protein content, and specific efficacy. To determine the optimum nitrogen source (yeast extract, peptone, and $(\text{NH}_4)_2\text{SO}_4$) at a concentration of 0.05 g/L, experiments were conducted following the determination of the optimum carbon source. The media were prepared, and the pH was adjusted to 7.0, and sterilization was performed. After inoculating the medium with 1% bacterial isolate, it was incubated for 24 hours at 37°C using a shaker incubator set to 150 rpm.

The pH of the optimum growth medium was varied (6, 7, 8, and 9). The media were sterilized and inoculated with a 1% bacterial culture and grown at 37°C and 150 rpm for 24 hours. After the growth, the cultures were centrifuged, and enzyme activity, protein content and specific enzyme activity were measured. To study the effect of different temperatures (30, 37, and 40 °C) on the enzyme production, the medium with optimal pH was used. Then, 1% bacterial culture was added to the medium, and it was incubated for 24 hours at 150 rpm. After incubation, enzyme activity, protein content and specific activity were measured. The effect of different incubation periods (24, 48, and 72 hours) on the enzyme production was studied. The media were inoculated with 1% bacterial culture and were grown in 37°C conditions for each period. After each incubation period, the samples were centrifuged, and enzyme activity, protein content, and specific enzyme activity were measured.

RESULTS AND DISCUSSION

The findings from bacterial isolation from soil and water samples confirmed the effectiveness of serial dilution and plate culturing methods to isolate bacteria with different morphological features. In total, sixty-nine soil samples were gathered from areas near power generators in Baghdad, while three water samples were collected from a stream found in the University of Baghdad/Al-Jadriya. The isolated bacterial colonies with unique morphological features were chosen and cultured again to derive pure bacterial isolates. Initially, a considerable number of bacterial isolates presumed to be *P. aeruginosa* were obtained, considering their morphological traits like smoothness, circular, and greenish/bluish colors. In comparison with soil samples, water samples indicated a lower density of bacteria but this was expected because of their comparatively smaller amount of organic matter. Nevertheless, a few distinct bacterial colonies were produced and morphologically different colonies were picked up and purified, resulting in the isolation of three bacterial isolates from water samples. In general, it should be stated that a total of 72 bacterial isolates were isolated from both soil and water samples, including 69 from soil samples and three from water samples. Most of these bacteria revealed the primary characteristics similar to those of *P. aeruginosa* which proves the prevalence of these bacteria in organic environments such as oil-contaminated soil and, to some degree, in surface waters. In any case, these findings indicate that soil samples are better and richer sources for isolating *Pseudomonas aeruginosa* in comparison with water samples. It is known that much research confirms the adaptation and survival abilities of these bacteria in harsh environments containing organic pollutants. The multifaceted analysis provides a representation of the distribution and frequency of *P. aeruginosa* isolates from both soil and water samples. According to the bar graph (A), the proportion of positive isolates for soil was 40.6% (28/69), while for water, it was 66.7% (2/3). Nevertheless, the difference was not statistically significant ($P = 0.56$) because of the relatively low sample size. Based on the pie chart (B), it can be observed that the overall frequency of bacteria was 41.7%, while the prevalence rate of negative bacteria was 58.3%, signifying an average presence of bacterial populations in the investigated area.

Table 1: Isolation of *P. aeruginosa* from soil and water samples

Sample Type	Total Number of Samples	Positive Isolates (<i>P. aeruginosa</i>)	Negative Isolates	Remarks
Soil	69	28	41	Higher prevalence in oil-contaminated soil
Water	3	2	1	Lower detection rate compared to soil
Total	72	30	42	—

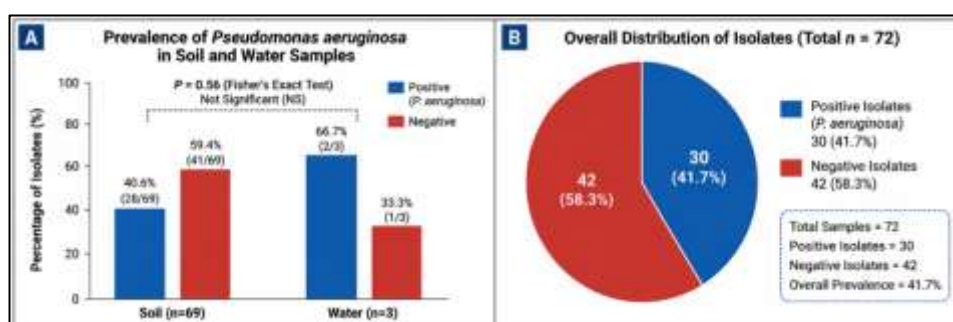


Figure 1: Isolation and prevalence of *P. aeruginosa* in samples

Results of this experiment, where 30 positive isolates of *P. aeruginosa* were isolated from 72 samples (41.6%), agree with many scientific works available in the literature, with small variations due to sample type and environmental factors. In the study conducted by Mena and Gerba (2009), it was established that *P. aeruginosa* is abundant in aquatic environments as well as in the soil, but the bacterial population is higher in rich soils. This result agrees with the findings in this experiment where a higher number of positive isolates was found in soil samples than in water. According to the findings of Kumar et al. (2016), the frequency of isolation of pseudomonads from soils lies between 30-50%, depending on the degree of soil contamination, and this percentage was not far from the percentage found in this study. In turn, the study conducted by Stelma et al. (2004) shows that the occurrence of *P. aeruginosa* in surface water is relatively rare and is dependent on the level of contamination. Its concentrations are lower when compared to soil; a similar trend was noted in this study, with only two out of three samples being positive. Therefore, this analysis demonstrates that the results of the present study fall within the natural range observed globally and further supports the assumption that soil, particularly organic soil, is a more favourable environment for the proliferation of *P. aeruginosa* compared to water. Soares et al. (2023) conducted a study that successfully and effectively isolated *P. aeruginosa* from the soil found in the Cerrado region of Brazil. They were able to obtain a significant number of bacteria capable of synthesizing phosphate, as evidenced by their solubility, indicating that the soil is an appropriate site for the growth and reproduction of these bacteria. Indeed, this is consistent with your research findings that there was a higher rate of isolation from soil than from water. As expected, this confirms that the soil (especially nutrient-rich or contaminated soil) is the primary source of this bacterium. Additionally, according to Jindal & Aggarwal (2024), the *P. aeruginosa* isolated from oil-contaminated soil had the highest tolerance of hydrocarbons, accounting for the high isolation rate in the soil samples taken from the vicinity of oil-rich power plants.

IDENTIFICATION OF *P. AERUGINOSA*:

Cultural characteristics: Regarding the colonies of *P. aeruginosa* on nutrient agar, the presence of pigments and the odour produced (grape-like odour) were used to characterize its growth. The colonies had a greenish colour, which is consistent with existing studies (DeBritto et al., 2020). The identification of *P. aeruginosa* was made by the morphological characteristics of

the bacteria on both selective and enriched culture media. The colonies of bacteria of *P. aeruginosa* on MacConkey agar were pale yellow because of the absence of lactose fermentation. This finding is consistent with the findings reported in earlier studies (Forbes et al., 2002). Regarding the culture on the Cetrimide agar plate, the colonies for the *P. aeruginosa* were observed to be greenish yellow due to their capacity to produce pigments such as pyocyanin which is a blue-green dye, and pyoverdine that gives off light after exposure to UV light; both pigments are water soluble (Sudhakar et al., 2015). Cetrimide agar is both a selective and differential medium that is used to evaluate whether the bacteria, *P. aeruginosa* have grown in the presence of 0.03% cetrimide, a detergent that is capable of inhibiting the growth of other microbes (Laine et al., 2009; Dianel et al., 2013). *Pseudomonas aeruginosa* colonies were seen in King A medium as greenish-blue. There was blue and green pigmentation (pyocyanin) observed, but none of the isolates grown on King B agar formed any pyocyanin. This is attributed to the adequate presence of salts of potassium and magnesium in the King A medium, thereby suppressing fluorescein (pyoverdine) formation observed on the King B medium; thus, King B is a unique characteristic of *P. aeruginosa* as compared to *Pseudomonas fluorescens* (Blondel-Hill et al., 2007; Vindeirinho et al., 2020).

Microscopic examination and biochemical characteristics of *P. aeruginosa* isolates: in the light microscope (100X) observation, Gram-negative bacilli were identified. In the case of biochemical tests, the results for all isolates were positive for the catalase test, which proved the ability of the bacteria to split hydrogen peroxide into water and oxygen gas (Orlandi et al., 2018); the oxidase test was positive, as the result revealed the presence of oxidase cytochrome in microorganisms (Yan et al., 2019); IMViC tests, including indole, the methyl red test, and the VP (Voges Proskauer test) were negative (-), citrate utilization was positive (+), resulting in an alkaline reaction (could not ferment glucose and lactose) and the formation of H_2S production (-). The identification of *P. aeruginosa* isolates was confirmed with the VITEK 2 compact system.

Table 2: Isolation and Identification of *P. aeruginosa* from Soil and Water Samples

Parameter	Soil (n=69)	Water (n=3)	Total (n=72)
Positive isolates (<i>P. aeruginosa</i>)	28 (40.6%)	2 (66.7%)	30 (41.7%)
Negative isolates	41 (59.4%)	1 (33.3%)	42 (58.3%)
Gram stain	Gram-negative rods	Gram-negative rods	—
MacConkey agar	Non-lactose fermenter	Non-lactose fermenter	—
Cetrimide agar	Green-blue colonies (pyocyanin)	Same	—
King's B medium	Fluorescent (pyoverdine)	Fluorescent	—
Catalase test	Positive	Positive	—
VITEK 2	Confirmed	Confirmed	Confirmed
Remarks	Higher prevalence in contaminated soil	Small sample size	—

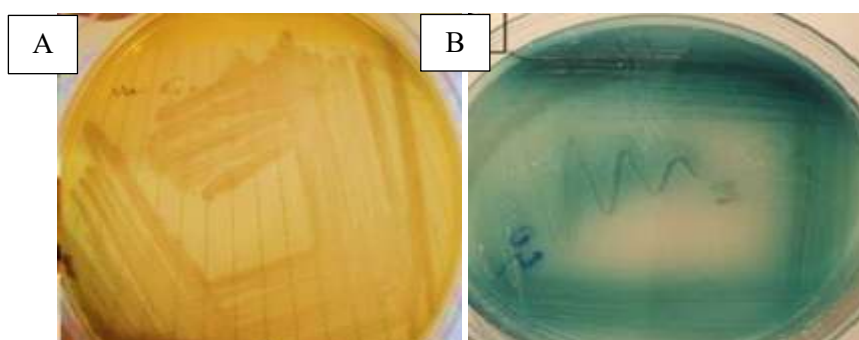


Figure 2: A- On MacConkey agar (distinguishing lactose fermenters), there are colorless colonies that are non-lactose fermenters, and this is a characteristic feature of *Pseudomonas aeruginosa*; B- Cetrimide agar is a selective medium for *Pseudomonas*. It produces blue-green colonies due to the production of pyocyanin, which is a strong diagnostic feature.

Primary Screening of *P. aeruginosa* for L-asparaginase Production: A total of 30 isolates of *P. aeruginosa* were tested for their ability to produce L-asparaginase under modified asparagine medium conditions. It was found that 25 isolates (78.1%)

produced enzymes very efficiently based on the intensity of color change, whereas the other isolates did not show any enzyme production. The preliminary screening through the use of Asparagine Medium with L-asparagine (substrate) and phenol red demonstrated the production of L-asparaginase by some isolates of *Pseudomonas aeruginosa*, which was evident from the change in color from yellow to pink/reddish around the colonies because of the liberation of ammonia, resulting in increased pH. According to the obtained results, the isolates were categorized as enzyme producers and non-producers based on the degree of colour change and halo formation. These findings suggest that there are different levels of enzyme activity produced by different isolates of *P. aeruginosa*. Also, the application of phenol red asparagine medium was found to be an effective method of detecting isolates with relatively high enzymatic activity, considering the time required to do so. The difference in the enzyme activity level could also be attributed to the different genetic makeup of the isolates. This finding could open doors to isolating strains with higher efficiency for therapeutic use against cancer.

Results obtained in this study agree with numerous studies done internationally that revealed the prevalence of *P. aeruginosa* as a major source of L-asparaginase. Most of the isolates were able to exhibit the enzyme activity in asparaginase medium added phenol red where color changes depend on ammonia release and pH increase as noted by Gulati et al. (1997). In addition, differences in color change and halo formation by the isolates showed variations in enzyme activity, which was reported before in many other studies where differences in enzyme production among bacterial isolates were attributed to genetic and environmental factors (El-Bessoumy et al., 2004; Sharma & Husain, 2015). In addition, modern scientific findings confirm those results by revealing the availability of properties of L-asparaginase in *Pseudomonas* sp. that allow it to be used in biomedical applications with increasing interest in improving the process of enzyme production from microbial sources (Naseem et al., 2023; Verma et al., 2025). Thus, besides validating the effectiveness of asparaginase as a first screening medium, the results obtained in this study revealed high potential in environmental isolates of *Pseudomonas aeruginosa* to be developed as a source of the enzyme.



Figure 2: The figure above represents an agar plate that has been used in the preliminary testing for the production of L-asparaginase. This process utilizes the degradation of L-asparagine to produce aspartic acid and ammonia, leading to an increase in pH level and a shift in color from red to pink/purple. In the upper part of the plate, a well has been prepared, and there is a formation of a ring of color from the diffusion of ammonia in the medium. In the lower portion of the plate, spot inoculation is observed, and there are clear rings of color and borders around it, showing that the production of enzymes occurs within the bacteria. This demonstrates the activity of L-asparaginase and its ability to diffuse in the medium, meaning that the enzyme is extracellular.

Enzyme Production Index (Qualitative Results): The calculation of the enzyme index is based on the measurement of the diameter (P) and the diameter of the colony (W) [Table 5]. The results clearly demonstrate that there is a distinction between the isolates. The EPI value presented in the table shows how there is a quantitative difference in the values of the P/W ratio among the various isolates of *P. aeruginosa*, while the graph provides a quantitative visualization of these values, making interpretation much easier. The isolates are ordered according to the decreasing order of the EPI values obtained from the table, and the isolates with the highest values are listed at the top of the list. Isolates P6, P25, and P30 have the highest values obtained (1.40, 1.32, and 1.28, respectively), as seen from the table too. The classification given in the table (High, Moderate, and Low) has a corresponding colour code as used in the graph, where those that cross the line drawn in the middle of the graph are those with the highest efficiency of enzyme secretion. For example, isolates P12, P17, P13, and P4 all have values greater than 1 as shown in the table, which is represented in the graph by the columns crossing the horizontal line. The isolates categorized as intermediate in the table (P3, P5, P10, P16) are depicted in the graph through bars that fall just under the line showing EPI = 1, implying moderate levels of enzyme production. Low producers (P9, P26, P11) can easily be identified in

both illustrations from their low numerical figures with bars not reaching above the dotted line. This comparison between the graph and table makes the findings more believable. While the former offers exact data, the latter enables one to visualize the results, especially when looking for the best isolates. Additionally, it is worth noting that the application of the reference line (EPI = 1) on the graph introduces another useful aspect of analysis through which the efficiency of the isolate can be readily distinguished. Thus, it becomes possible to conclude that the table reflects a quantitative analysis technique, while the graph is used for the interpretation of data visually. The combination of the two techniques makes it possible to present the results comprehensively, thereby strengthening the scientific research carried out. Moreover, the combination of the two techniques makes it easy to choose isolates to test, as the most efficient ones are selected in both graphs (e.g., P6, P25, and P30). However, the enzyme production index analysis (EPI) revealed notable variation in the capacity of *P. aeruginosa* strains for L-asparaginase production. Such variations can be directly linked to the origin of the isolates (soil vs. water) and the biological characteristics of the isolates. According to the obtained results, high producers of L-asparaginase were mainly soil strains, as described by Abdelrazek et al. (2020), whereby enzyme-producing strains isolated from soil were found to be highly productive as a result of nutrient availability and a diverse microbial population. Thus, stimulation of extracellular enzyme production becomes a competitive means. Also, soil samples are rich in organic matter, nitrogen-containing substances that stimulate the expression of genes coding enzymes like L-asparaginase. The aquatic P30 isolate showed high EPI values, which are similar to the findings reported by Badoe-Dalfard (2016) who studied the isolation of *Pseudomonas* strains from aquatic environments such as hot springs and found that they were able to produce L-asparaginase in large amounts. Therefore, aquatic environments might be hosts of highly specialized and efficient isolates. This is due to the efficient enzyme secretion processes in aquatic strains since nutrient content in aquatic environments is less than that of soil. This could be attributed to the differences in gene regulation in enzyme production among different strains, resulting in high variations in their EPI values. Alshamy et al. (2025) stated that enzyme production in *P. aeruginosa* relies on regulatory and molecular properties affecting stability and catalytic efficiency of enzymes. In addition, protein secretion system differences in strains affect the amounts secreted extracellularly. Additionally, the production of enzymes depends largely on environmental factors like temperature, pH, and substrate concentration. According to Ebrahiminezhad et al. (2011), isolates of bacteria from particular environments like saline or aqueous environments have the ability to produce enzymes with unique properties that include their adaptability to tough environmental conditions and efficient production of extracellular enzymes. Within the local context, a research study conducted by Sahar et al. (2023) involved the isolation of L-asparaginase producing bacteria from varied environments in Baghdad; the isolates produced diverse enzyme production levels. This is evidence enough that the origin of the environmental samples plays a vital role in influencing the efficiency of enzyme production. The variations in enzyme production levels can therefore be attributed to the fact that soils produce higher quantities due to their richness in nutrients and competitiveness. Water produces lower enzyme production levels with exceptions like P30 due to low nutrients. Intra-environment variation occurs because of variations in gene expression, secretion systems, and metabolic abilities. The data suggest that terrestrial environments are the most constant environments for obtaining L-asparaginase-producing isolates, whereas aquatic environments can be hosts for very efficient specialists. Additionally, it must be noted that intra-environment variation can occur not just because of the environment, but also depending on the genetic and physiological traits of the particular isolate; hence, EPI proves to be a useful technique for selecting more advanced organisms.

Secondary investigation (Qualitative) production of L-asparaginase: Quantitative analysis of L-asparaginase production showed considerable variations in enzyme activity, protein concentration, and specific potency in different bacteria isolated. The enzyme activity levels varied from 4.76 to 76.90 U/mL, where isolate P1 showed the highest enzyme activity level of 76.90 U/mL, followed by isolates P18 with 69.05 U/mL, isolate P20 with 63.81 U/mL, isolate P3 with 56.83 U/mL, and isolate P22 with 55.24 U/mL. The lowest values were shown in isolates P13 with 4.76 U/mL, isolate PW29 with 6.75 U/mL, and isolate P4 with 6.19 U/mL. On the other hand, the protein concentration level varied from 0.92 to 39.04 mg/mL, with isolate P9 having the highest protein concentration at 39.04 mg/mL, followed by isolate P8 with 35.36 mg/mL, isolate P6 with 34.80 mg/mL, and isolate P5 with 28.2 mg/mL. On the matter of specific activity, the range of values was between 0.53 and 49.30 U/mg, with the highest value recorded in isolate P1 (49.30 U/mg), followed by isolates P3 (35.52 U/mg), P18 (28.30 U/mg), and P16 (23.98 U/mg). On the other hand, isolates P6 (0.53 U/mg), P8 (0.56 U/mg), and P7 (0.57 U/mg) recorded the lowest values. In addition, there was a significant disparity noted in terms of enzyme activity and the amount of protein present. While some isolates showed a considerable amount of enzyme activity (P7 with 43.41 U/mL) and protein concentration (23.12 mg/mL), the isolates had a low specific activity rate (1.88 U/mg). Isolates such as P1 and P3 demonstrated a good correlation between enzyme activity and protein concentration, leading to high specific efficacy rates. It can be noted that isolates PW29

and PW30, which were aqueous in nature, had lower amounts of enzyme activity (6.75 and 13.25 U/mL) and thus had poor specific efficacy in comparison to the majority of other isolates. The overall findings from the experiment indicate that there are significant differences in the isolates' ability to synthesize the L-asparaginase enzyme, with efficiency-specific being the best criterion for determining enzyme efficiency, and isolates P1, P3, P18 and P16 are considered the best isolates. The quantitative assessment of L-asparaginase production showed that there were considerable variations between isolates in terms of enzymatic activity, protein concentration, and specificity efficacy. This trend is observed often in studies involving microbes, as there are notable dissimilarities in the regulatory processes and expression of genes in different strains. The isolates P1, P3, and P18 had the highest values for specificity efficacy, showing that their enzyme production was highly efficient compared to other proteins present in the cell. This is the most reliable measure of enzyme production quality, not quantity (Verma et al., 2014). However, it has been noted that while some of the isolates like P20 and P7 had considerable levels of enzyme activity, their specific activity was low because of the high amount of total protein concentration, suggesting the synthesis of non-specific proteins. This is consistent with the study by Izadpanah Qeshmi et al. (2022), which states that an increase in the nitrogen source would promote the growth of cells and synthesis of proteins, but not necessarily the production of the enzyme. In contrast, the considerable differences in specific activity despite having almost the same enzyme activity depend on the different efficiencies of their protein secretion mechanisms and the capability of the cells to secrete the enzyme outside the cell. Alshamy et al. (2025) have proven that there are numerous secretion systems found in *P. aeruginosa*, and they play a major role in determining how much enzyme can be secreted outside the cell. This is the reason why some isolates are more effective than others. The study also showed that the isolates that have the highest concentration of proteins (e.g., P9, P8, and P6) failed to attain high specific activity, meaning that their production efficiency does not correlate with their enzymatic efficiency. It is consistent with what Thakur et al. (2025) claim about the importance of using specific activity as the most reliable factor for assessing enzymes used in industries and medicine. In addition, it was noted that the isolates from the aqueous environment (PW29 and PW30) had lower efficiency and activity of the enzyme compared to those obtained from soil (soil isolates). This may be explained by the difference in environmental conditions because studies show that soil isolates usually have a high ability to produce extracellular enzymes due to severe competition in soils for nutrients, whereas aquatic isolates use other methods of surviving (Izadpanah Qeshmi et al., 2022). The variability in productivity of the studied isolates may be attributed to genetic differences. According to Ebrahiminezhad et al. (2011), L-asparaginase production is regulated genetically, depending on the growth condition and origin of an isolate, resulting in a variety of efficiencies in producing L-asparaginase between isolates of the same bacterial species. Based on the study results, it may be concluded that for selecting highly effective isolates, their effectiveness should be prioritized, and not their enzymatic activity only. The most productive isolates identified are P1, P3, and P18. From the research findings, it is clear that enhancing L-asparaginase production will involve many aspects. On the contrary, the range in the specific activity (0.53-49.30 U/mg) signifies a notable variation in efficiency in producing L-asparaginase among isolates, which was also observed in earlier studies from Iraq. The specific activity of isolates from the genus *P. aeruginosa* varied significantly since different isolates produced enzymes at varying efficiencies depending on enzyme secretion capacity and regulation of gene expression, according to Shehata and Ali (2022). Some isolates had high activity even if they had low protein levels, while others had high protein concentration but were inefficient in producing it. Khalaf NA (2012) supported this fact in his observation that a high protein level did not necessarily indicate high specific activity because non-specific proteins were produced by certain isolates, including P6 and P8 in this study. Overall, the similarity between this study and other studies from Iraq shows the validity of the data obtained from this experiment.

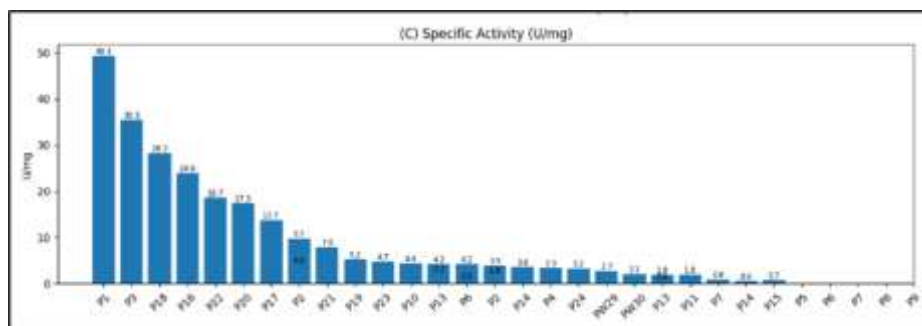


Figure 3: L-asparaginase enzyme production quantification in *P. aeruginosa* isolates. specific activity (U/mg). The values are represented above each column

OPTIMIZATION OF L-ASPARAGINASE PRODUCTION

Type of Production Medium: The impact of various culture media on the production of L-asparaginase by *Pseudomonas aeruginosa* strains was studied using six culture media (A, B, C, A1, B1, and C1), each subjected to constant incubation parameters (temperature 37°C, 150 rpm for 24 hours). The findings indicated that there were variations in enzyme activity, protein content, and specific efficacy, according to the medium used. The highest enzyme activity of 20.47 U/mL was observed in medium C1 (free from L-asparaginase), and it had a relatively low protein content of 0.88 mg/mL, producing the highest specific efficacy of 23.27 U/mg. Following medium C1, the enzyme activity in medium A was moderately high (14.52 U/mL), protein content (0.8 mg/mL), and specific efficacy (18.15 U/mg). Although the enzyme activity in medium B was high (15.63 U/mL), it also had high protein content (4.36 mg/mL), indicating a sharp decline in specific efficacy (3.58 U/mg). On the other hand, media without L-asparagine were not uniformly successful. Medium B1 recorded average enzyme activity (8.88 U/mL) and specificity efficacy (5.55 U/mg), whereas medium A1 was less productive in enzyme activity (1.74 U/mL) although the specificity efficacy was average (4.36 U/mg). In conclusion, it can be noted that culture media play an important role in enzyme production. The maximum efficiency in enzyme activity and specificity efficacy was shown by medium C1, followed by medium A, and the other media were less efficient. From the findings on the improvement in L-asparaginase production depending on the culture medium used, there was a notable difference in the enzymatic activity and specific potency in units per milliliter and milligram, respectively. The medium with C1 recorded high productivity compared to others with varying efficiencies. It implies that the composition of the culture medium significantly influences the gene expression and metabolic regulation of enzyme production. Based on the findings, the L-asparaginase-free medium (C1) recorded high enzyme productivity, indicating that the mechanism involved in enzyme production is an induction/repression regulation. Certain bacteria depend on the stimulation of enzyme production when substrate availability is low in the medium. According to Thakur et al. (2025), the production of L-asparaginase in microorganisms is regulated by complex mechanisms depending on the availability of carbon and nitrogen sources, whereby low substrate availability stimulates enzyme production as compensation. On the contrary, media with L-asparaginase (media B) recorded a higher concentration of total protein, with lower specificity, suggesting non-specific production of proteins. This finding is consistent with that of Izadpanah Qeshmi et al. (2022), who observed that L-asparaginase production by *P. aeruginosa* may depend on the production of other proteins due to the availability of nitrogenous substances within the medium, lowering the enzyme specificity. Additionally, the variation in concentrations of salts and trace elements in the media was found to influence production, because enzymes are activated or inhibited depending on the composition of these elements in the media. According to Alshamy et al. (2025), the activity of L-asparaginase depends on the presence of metal ions like potassium and calcium ions, which help activate and stabilize enzyme activity. Thus, the findings from this experiment could be seen as the uncontested superiority of medium C1 in terms of its performance in the production of L-asparaginase owing to the induction process under the substrate limitation regime. The non-existence of L-asparaginase induces the metabolic processes that promote the biosynthesis of the enzyme as compensation for the supply of nitrogen. The optimization of L-asparaginase synthesis is not only determined by the presence of substrates but also by the establishment of an equilibrium between the nutrient medium's components and the properties of the strain used. The optimal culture medium should not be rich but effective in stimulating enzyme expression (Castro et al., 2021; Abhini et al., 2022; El-Naggar et al., 2024; Saleh et al., 2025; Thakur et al., 2025).

Table 3: Impact of various media on the activity of L-asparaginase enzyme, protein concentration, and specific activity in *P. aeruginosa* isolates

Type of Production Medium	abs.625	Enzyme Activity (U/mL)	abs 595	Protein conc. (mg/mL)	Specific Activity (U/mg)
A	0.183	14.52380952	0.02	0.8	18.1547619
B	0.197	15.63492063	0.109	4.36	3.585990971
C	0.11	8.73015873	0.071	2.84	3.073999553
A1	0.022	1.746031746	0.01	0.4	4.365079365
B1	0.112	8.888888889	0.04	1.6	5.555555556
C1	0.258	20.47619048	0.022	0.88	52.26839827

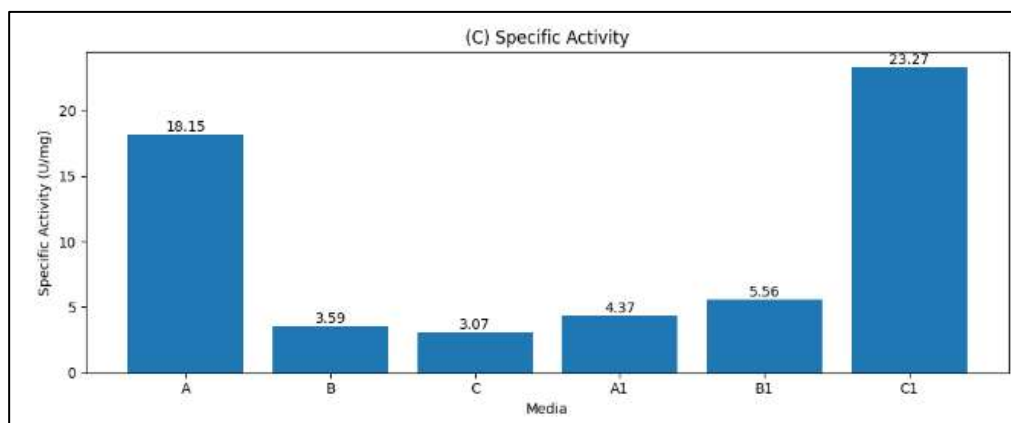


Figure 4: Effect of different culture media on the activity of the L-asparaginase enzyme, specific activity in *P. aeruginosa* isolates

Influence of Different Carbon Sources on L-asparaginase Synthesis: The influence of different carbon sources (glucose, fructose, maltose, starch, and sucrose) on the synthesis of L-asparaginase enzyme from *P. aeruginosa* bacteria cultures was analyzed under the optimum conditions of their growth. It was established that the activity of enzyme, protein content, and specific activity varied considerably depending on the kind of carbon source used. It was found that sucrose served as the best carbon source, showing high enzyme activity (13.49 U/mL) together with low protein content (0.16 mg/mL) and, consequently, the highest specific activity (84.32 U/mg), i.e., enzyme was synthesized in great amounts with little amounts of non-enzymatic proteins produced simultaneously. The use of fructose also gave good results with enzyme activity of 12.14 U/mL and low protein content (0.48 mg/mL) leading to high specific activity (25.29 U/mg). On the other hand, glucose displayed a relatively high enzymatic activity (10.31 U/mL), however, it showed the highest amount of proteins (1.4 mg/mL) thus giving low specific activity (7.36 U/mg). This phenomenon can be explained by the fact that the presence of readily available sugars causes a process known as catabolite repression, thereby reducing the enzyme production. Maltose and starch showed relatively low activity, although specific activity was moderate for both. Reduced efficiency for starch can be explained by the fact that it needs to undergo pre-hydrolysis prior to being used. The conclusions reached by this current study are similar to those from previous investigations concerning *Pseudomonas aeruginosa* (Sauvage et al., 2022), since it is evident that not only does the carbon source influence bacterial growth but it also leads to major changes in the expression of various proteins and enzyme production processes. For example, according to one of the recent proteomics experiments, the change in the carbon source used (glucose, citrate, succinate, glutamate), results in changes in the expression of up to 581 intracellular proteins. Some of the changes include the presence of an increased number of proteins in relation to metabolism, pathogenicity, and biofilm formation; therefore, it is evident that the nutrient medium plays a pivotal role in bacterial regulation. The experiment shows that bacteria are able to change their metabolic pathways in accordance with the availability of the carbon source. Concerning the results obtained in this study, the predominance of sucrose and fructose with respect to the efficiency of production of the L-asparaginase enzyme could also be justified based on this theory, since the former might favour more productive metabolic pathways in terms of enzymes than glucose, the latter being a possible cause of metabolic inhibition. Given that the current study proved that *P. aeruginosa* reacts positively to changes in the carbon source in terms of protein synthesis, this is further proof of how the difference in enzyme activity observed might depend upon changes in gene/protein expression (El-Bessoumy et al., 2004; Kumar et al., 2024; Thakur et al., 2025).

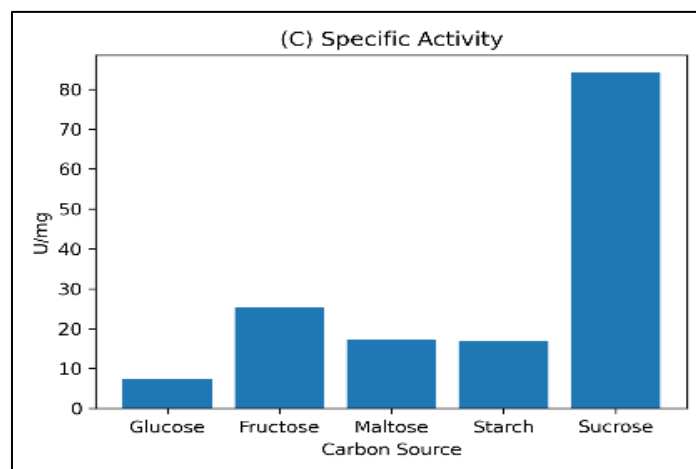


Figure 5: Influence of various carbon sources on specific activity (U/mg) in *P. aeruginosa*

Effect of Nitrogen Source on L-asparaginase Synthesis: The influence of three nitrogen sources (yeast extract, peptone, and $(\text{NH}_4)_2\text{SO}_4$) on the biosynthesis of L-asparaginase by *P. aeruginosa* was determined under optimum culture conditions. The experimental data revealed considerable variations in enzyme activity, protein content, and specific activity, which depended on the type of nitrogen source. The yeast extract appeared to be the best nitrogen source since it possessed the highest activity (75.39 U/mL) along with a relatively low amount of proteins (0.68 mg/mL), providing the maximum specific activity (110.87 U/mg). This is indicative of enzyme synthesis efficiency in connection with the reduced biosynthesis of unwanted proteins. The $(\text{NH}_4)_2\text{SO}_4$ was also found to have a good efficiency, where it produced 69.84 U/mL of enzymatic activity, with 1.04 mg/mL of protein concentration to give a specific activity of 67.15 U/mg. This proves the capability of inorganic nitrogen sources for enzyme production but with less efficiency compared to nutrient sources, which are organic. Peptone has been identified as the nitrogen source with the least enzymatic activity (3.65 U/mL), which occurred at a high protein concentration of 1.32 mg/mL and thus gave the lowest specific activity of 2.76 U/mg. The reason for this low activity is the induction of non-specific protein formation due to the rich nitrogen compounds in this medium, which hampers the formation of the L-asparaginase enzyme. From the above discussion, it is evident that the nitrogen source is very important for enzyme production, with yeast extract having the highest, $(\text{NH}_4)_2\text{SO}_4$ the second best, and peptone the least efficient. Based on this experiment, it can be concluded that organic nitrogen sources with growth factors facilitate enzyme formation more than any other form. According to the findings of this research, it is clear that the nitrogen source significantly influences L-asparaginase production in *P. aeruginosa*. The enzyme activity and specific potency were greatest for yeast extract (75.39 U/mL and 110.87 U/mg, respectively) and then $(\text{NH}_4)_2\text{SO}_4$, whereas peptone had the smallest value. Therefore, it can be said that the enzyme yield would be greater in the case of complex organic nitrogen sources, which include vitamins and amino acids. Similar results have been reported in many studies, where yeast extract was found to be the most effective nitrogen source for L-asparaginase production. For instance, in a study conducted on *Stenotrophomonas maltophilia*, the maximum enzyme production was recorded in the case of yeast extract, due to its growth factors that facilitate metabolism and protein synthesis (Abdelrazek et al., 2020). The findings of the present study have also been found to correlate with previous studies where the role of ammonium salts like ammonium sulfate in the promotion of enzyme production is evident but less pronounced when compared to organic sources. In a research work by Alrumman et al. (2019), it has been observed that *Bacillus licheniformis* is effectively stimulated with ammonium sulfate as a nitrogen source for enzyme synthesis. However, the substantial drop in the level of enzymes generated using peptone in this particular experiment partly correlates with other findings. According to research on fungi, peptone does not always provide the best medium for the synthesis of L-asparaginase; sometimes, the enzymes generated through the use of peptone are less active than enzymes produced using other materials like asparagine and yeast extract (Khalifa et al., 2026). This study also proves that there are differences in nitrogen sources depending on the type of organisms used. Asparagine proved to be more effective when applied to fungi, whereas other types of organic nitrogen sources were found to be less effective at times (Khalifa et al., 2026). Normally, the advantage of using yeast extract is due to the presence of organic nitrogen and growth substances that facilitate protein and enzyme production; however, the use of ammonia is based on simple metabolic reactions that might not contribute to protein production as much as organic nitrogen

does. Peptone is an organic source but is not likely suited to enhance enzyme production in *P. aeruginosa*.

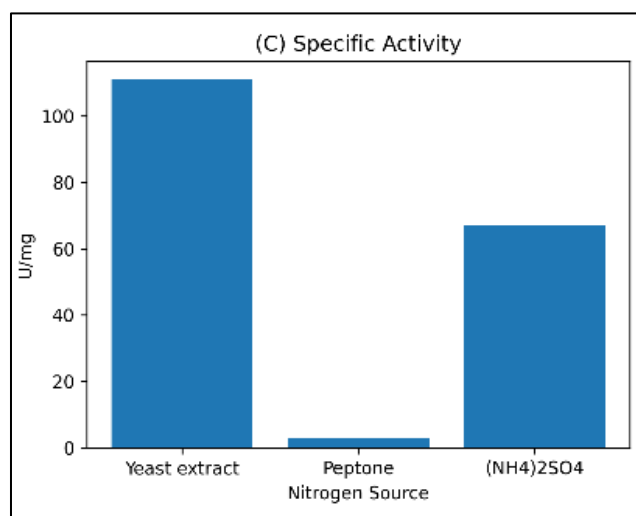


Figure 6: Influence of various nitrogen sources on specific activity (U/mg) in *P. aeruginosa*

Effect of pH on the production of L-asparaginase: The influence of various pH levels (6, 7, 8, and 9) on the production of L-asparaginase by *Pseudomonas aeruginosa* was studied. It was observed that pH had significant effects on enzyme activity, concentration of protein, and specific enzyme activity. Enzyme production had an optimum pH value of 8; at this value, enzyme activity was recorded to be 74.60 U/mL, having a protein concentration of 0.96 mg/mL and hence giving a high specific activity of 77.71 U/mg. A pH level of 7 was also efficient, as enzyme activity of 53.96 U/mL and low protein concentration (0.72 mg/mL) gave high specific activity (74.95 U/mg). At pH value 6, there was a drop in the enzyme activity to 31.74 U/mL with a specific activity of 41.77 U/mg, implying that an acidic pH inhibits the process of enzyme production. On the other hand, pH value 9 had the lowest enzyme activity of 5.71 U/mL but a very high protein content of 1.6 mg/mL, implying a marked reduction in specific activity of 3.57 U/mg. This is an indicator that enzyme production becomes inefficient under a highly alkaline environment due to its inhibiting effects on enzyme stability or gene expression. In general, the findings show that maximum production of L-asparaginase occurs at a pH that ranges from slightly alkaline to neutral (7-8) but not at highly acidic or alkaline pHs.

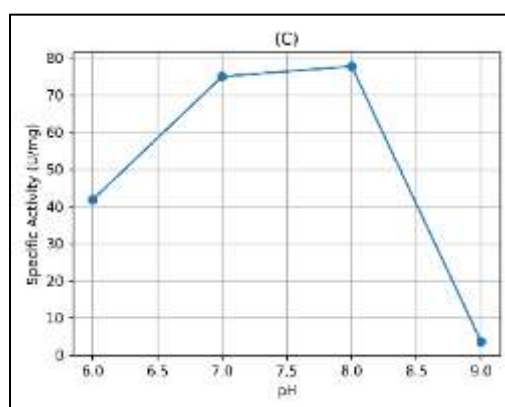


Figure 7: Effect of pH on specific activity (units/mg) in *P. aeruginosa* bacteria

Effect of temperature on the production of L-asparaginase: An experiment was carried out to find out how varying temperatures (30, 37, and 40 °C) affected the production of the L-asparaginase enzyme by bacteria known as *P. aeruginosa*. From the data, a significant effect was seen in the rate of enzyme activity, protein concentration, and specific activity as affected by temperature changes. The best temperature for enzyme production was seen at 37 °C, which had the highest enzyme activity (61.11 U/mL), protein concentration (0.8 mg/mL), and hence a very high specific activity of 76.38 U/mg. On the other hand,

enzyme activity at 30°C decreased to 14.68 U/mL, where protein concentration was low (0.12 mg/mL), although specific activity surprisingly increased to 122.35 U/mg, which shows efficient enzyme production compared to protein production. This could be due to reduced growth and stimulated enzyme production. Enzyme production was significantly reduced at 40°C with high protein concentration (1 mg/mL) with specific activity (11.74 U/mg). This could be due to enzyme instability caused by high temperature or inhibitory effects of temperature on bacterial growth. From the results, it can be seen that enzyme production is optimum at 37°C, while at lower or higher temperatures there is low enzyme production efficiency because of the effects of temperature on metabolic activities and stability of intracellular proteins.

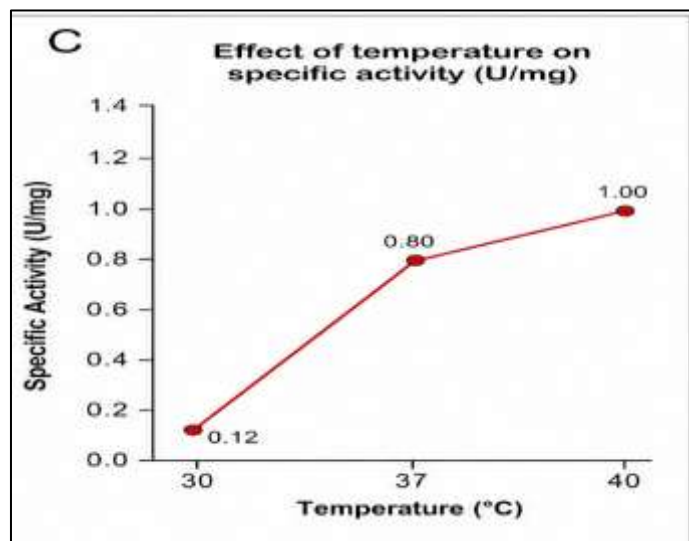


Figure 8: Effect of temperature on specific activity (units/mg) in *Pseudomonas aeruginosa*

Effect of Incubation Time on the production of L-asparaginase: The findings of the experiment indicate that the incubation time plays a definite role in determining the L-asparaginase level in *Pseudomonas aeruginosa* strains regarding enzyme activity, protein concentration, and specific activity. Differences occurred among the three periods (24 hours, 48 hours, and 72 hours). Maximum enzyme activity occurred after 48 hours (74.60 U/mL) rather than 24 hours (69.84 U/mL) followed by a dramatic reduction in enzyme activity after 72 hours (10.79 U/mL). This implies that the optimum time for enzyme synthesis is 48 hours since the bacteria have reached the log stage. This stage is associated with maximum enzyme activity due to maximum metabolism and enzyme synthesis. The reduction in enzyme levels after 72 hours can be attributed to exhaustion of nutrients and buildup of wastes, which causes a cell death. The protein concentration showed a distinct trend where it was low at 24 hours (0.017 mg/mL) and 48 hours (0.015 mg/mL), before drastically increasing at 72 hours (0.055 mg/mL). This is due to bacterial cell lysis resulting in the release of non-specific proteins into the medium, showing that protein concentration increases do not mean enzyme productivity increases. Specific activity peaked at 48 hours (124.33 U/mg) and 24 hours (102.70 U/mg), but significantly reduced to 4.90 U/mg at 72 hours. This means that the enzyme activity is at its peak in the middle stages of the bacteria's growth, where most of the protein produced is active enzymes, while prolonging the incubation time causes more non-active protein to be produced, reducing enzyme productivity. In summary, the data collected proves that the optimum incubation period for producing L-asparaginase in *P. aeruginosa* is 48 hours, because it is the point where both enzyme activity and enzyme efficiency are at their highest.

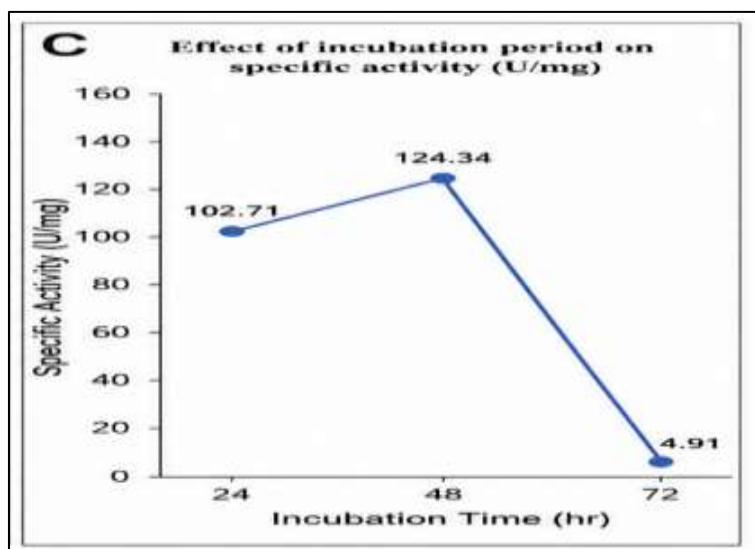


Figure 9: Effect of incubation period on specific activity (units/mg) in *P. aeruginosa*

According to the findings of this experiment, the best pH for producing L-asparaginase by *P. aeruginosa* is pH 8 due to the maximum enzyme activity observed, followed by pH 7, and a sharp decline in activity at pH 9. It is clear that the findings of this experiment agree with the work done by Abdel-Fattah & Olama (2002), who stated that the optimal pH for producing enzymes by *P. aeruginosa* was pH ≈ 7.9 . The findings are also in agreement with those reported by Pradhan et al. (2013) for *P. aeruginosa* F1, where the highest production was noted at pH ranging between neutral and alkaline (6.5 - 7), indicating that neutral to slightly alkaline conditions favor bacteria growth and enzyme production. It is worth mentioning that the findings obtained in the study conducted by Wakil & Adelegan (2015) revealed that many bacteria that produce enzymes, especially those related to the genus *Pseudomonas*, produce enzymes at pH 8. Biochemically, the highest activity and stability of L-asparaginase produced by *P. aeruginosa* were found to exist within the pH range of 7 to 8, thereby providing an explanation for the observed reduction in activity under high alkalinity. This is because the enzyme's structural conformation is altered when the solution becomes more alkaline. Also, Nikolaev et al. (1975) proved that the L-asparaginase enzyme in the *Pseudomonas* genus had the best pH of 8 to 9, which was slightly alkaline. In terms of temperature, the results indicate that maximum production is achieved at 30 °C, a temperature that aligns with the optimum growth temperature of the bacteria. According to a study conducted by Sheltagh & Ali (2022), the optimum activity of L-asparaginase enzyme obtained from *P. aeruginosa* is realized at 37 °C, while the enzyme remains thermally stable between 30–40 °C. This result is supported by the finding of El-Naggar et al. (2018), who found that the optimum temperature for the majority of bacterial L-asparaginase enzymes is 37 °C, where higher temperatures will lead to protein denaturation. Nevertheless, as indicated by Pradhan et al. (2013), some bacterial strains have the capacity to produce L-asparaginase enzyme optimally at 40 °C. Similarly, Sanghvi et al. (2016) established that increased temperature causes loss of structural stability in enzymes and consequently reduced enzymatic activities. As for the incubation period, the results have indicated the maximum production rate in 48 hours, followed by 24 hours, while there was a marked decrease at 72 hours. This finding is congruent with the study conducted by Abdel-Fattah & Olama (2002) in which it was found out that the optimum enzyme production is reached in the late logarithmic growth phase (24-48 hours). Another study by Sheltagh & Ali (2022) has shown that the maximal production of L-asparaginase from *P. aeruginosa* is achieved after two days of incubation (approximately 48 hours). Moreover, your findings are in line with the study by Sanghvi et al. (2016), according to which the activity of enzymes increases until reaching its peak at 48 hours and after that it declines due to enzyme degradation or depletion of nutrients. The same is confirmed by the study by El-Naggar et al. (2019) where it was found out that long incubation periods lead to a decline in activity due to the secretion of proteolytic enzymes causing the degradation of L-asparaginase.

CONCLUSION

The environmental isolates of *P. aeruginosa* bacteria are a good prospect for the enzyme L-asparaginase production. Improvement of culture conditions greatly helps to boost the productivity of such isolates, making them a viable choice for

potential future biological/medical applications.

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