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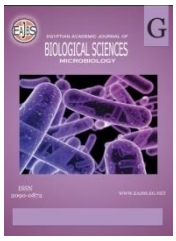

Screening of Actinomycetes for Asparaginase Production and it's Optimization Conditions

Dina M. Baraka [1, †], Mervat G. Hassan [1], Mohamed E. Elawady[2], Ahmed M. Abdelaziz[2] and Randa M. Hassen [1]

1- Department of Botany and Microbiology, Faculty of Science, Benha University, Benha 33516, Egypt.

2-Department of Microbial Biotechnology National Research Center.

*E.Mail:**dina.barakah@fsc.bu.edu.eg**

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ABSTRACT

The enzyme Asparaginase, known as L-asparagine amidohydrolase, has anti-tumor properties and is widely used as a chemotherapeutic treatment for acute lymphoblastic leukemia. Many different organisms, including plants and terrestrial and microbes such as bacteria, fungi, algae and actinomycetes, have produced this enzyme. In this study, 34 isolates of the actinomycetes were collected from soil and water from different governorates in Egypt. Only nine Streptomyces isolates were identified by the presence of a pink hue around their colonial growth as an indication and had the best ability for the synthesis of extracellular Asparaginase. Glucose and peptone were effective carbon and nitrogen sources, respectively. The ideal pH for Asparaginase is 7, and the ideal temperature is 30 ^{0}C. and an incubation time of 7 days with a 0.4 % asparagine concentration.

INTRODUCTION

All living things, including animals, plants, and microbes, contain Asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1), which catalyses the hydrolysis of the amide group of the sidechain of L-asparagine into aspartic acid and ammonia (Verma *et al*., 2007). ASNases have been a defining characteristic of multidrug chemotherapy treatments widely employed for the treatment of Cancers of the lymphoid system, including Hodgkin's lymphoma, lymphosarcoma, and melanosarcoma, can be treated (Stecher *et al*.,1999)

L-asparagine is a non-essential amino acid that can be produced by the human enzyme asparagine synthetase from intermediates in the central metabolic pathway without coming from outside the cell. The cells' primary need for L-asparagine in order to produce protein
To keep up with their rapid malignant growth, tumour cells, more specifically lymphatic tumour cells, require enormous amounts of asparagine. As a result, they use both the asparagine from their diet and what little asparagine they can produce on their own (which is minimal). L-asparagine is therefore an important amino acid for the development of tumour cells.

The idea overdue using asparaginase as an anti-tumor agent is that its incomes benefit from the fact that all leukemic cells lack the ability to synthesise asparagine, a non-vital amino acid that is important for the development of tumour cells, while normal cells are able to do so. As a result, leukemic cells need a lot of asparagine to survive. These leukemic cells get all of their food and nutrition from the bloodstream's asparagines. However, asparaginase facilitates the transformation of L-asparagine into aspartic acid and ammonia. This stops the leukemic cell from gaining access to circulating asparagine and slows its rapid malignant proliferation. (Verma *et al*., 2007)

ASNase is used in the food industry as well as Hydrolyzing L-asparagine, it aids in lowering the amount of acrylamide in baked food products. (Mario *etal.,2007)*. Microorganisms' Asparaginase has been successfully employed as a treatment for lymphosarcoma and acute lymphoblastic leukemia. The commercial synthesis of this enzyme has been carried out using *Escherichia coli, Erwinia carotovora*, *Bacillus sp., Enterobacte*r *aerogenes, Corynebacterium glutamicum, Pseudomonas stutzeri*, and *Candida utilis.*

Currently, Asparaginase from the bacterium *E. coli* and *E. carotovora* is used in clinical settings to treat acute lymphoblastic leukemia (Savitri and Azmi,2003). Actinomycetes are a reliable source of Asparaginase as well. Several Streptomyces species, including *S. venezuelae, S. longsporus flavus, S. karnatakensis*, and a marine Streptomyces are able to produce Asparaginase in measurable amounts (Dhevagi and Poorani,2006).

A protein must first be purified before its physical and biological characteristics may be determined. Furthermore, a protein is essential to be free of all toxins and filths in order to be used therapeutically. However, individuals who receive Asparaginase in group therapy often face serious allergic reactions (Duva*etal.,2002)*. These effects are mostly caused by bacterial endotoxins and Asparaginase related L-glutaminase action in enzyme provisions (Kotzia and Lbrou,2005).

The current study focuses on the isolating and characterisation of ASNase, a unique source of the enzyme that is efficient against leukemia, from isolates of actinomycetes found in soil and water samples. By analysing the sample's 16S rDNA, *Streptomyces albidoflavus* was determined to be the isolate. The optimization of various production limitations for L asparaginase is the subject of further investigation

MATERIALS AND METHODS

Isolation of Streptomycetes:

Streptomycetes isolates were isolated from marine sediment from soil and water from different governorates in Egypt. Water samples were taken from Alexandria and the South Sinai Sea, while soil samples were collected from Giza, Dakahlia, and Gharbia. Using serial dilution method on marine media containing: starch 10 g/L, K_2HPO_4 1.0 g/L, $MgSO_4.7H_2O$ 0.5 g/L, NaCl 0.5 g/L, KNO_3 2.0 g/L, $CaCO_3$ 2.0 g/L, $FeSO_4.7H_2O$ 0.01 g/L, agar 20.0 g/L, dissolved in 750 mL seawater completed to 1 L for distilled water and adjusted pH. Streptomycetes were isolated using agar slants that had the same constitution as the main plating medium and then purification to obtain single colonies

Screening of Bacterial Isolates for Asparaginase:

The rapid plate assay technique was used to test the bacterial isolates for their aptitude to yield ASNase (Li and Lu, 2011). Asparagine agar medium containing 0.3 ml of 0.07% phenol red, pH 7.0, was used as the testing media. The pinkish zone surrounding the gatherings was measured after two to three days of incubation at 37 °C, and an enzyme index was determined using the formula: Enzyme index = Pink zone (mm)/Colony diameter (mm).

Optimization of ASNase Production:

The broth media were optimised for the media elements needed for the *streptomyces* strain to produce the most asparaginase. The effects of different

asparaginase concentrations (0, 0.4, 0.8, 1.2 and 1.6%), different temperatures (20, 25, 30, 35 and 40 C), different incubation times (4, 5, 6, 7, 8 and 9 days), different pH levels (3, 4, 5, 6, 7, 8 and 9 adjusted with 1N HCl or 1N NaOH), and different additional carbon were used to optimise the enzyme.

RESULTS

Isolation and Screening of *Streptomycetes* Strains:

34 random isolates of streptomycetes were discovered in sediment, marine water, soil, and other environments. (Table 1). Streptomycetes isolates were most frequently discovered in Dakahlia, next in Gharbia and Giza, and least frequently in Alexandria and South Sinai (Sharm El-Sheikh). Only nine Streptomyces isolates were identified by the presence of a pink hue around their colonial growth as an indication and had the best ability for the synthesis of extracellular ASNase. when the release of ASNase by 34 Streptomyces isolates was evaluated using a quick plate assay test procedure Table (2).

Table 1: Total number of streptomycetes isolates.

Source	Locality	No. of isolates	Incidence percent (%)
Soil	Dakahlia	11	**32.35**
	Gharbia	8	**26.47**
	Giza	9	**23.52**
Marine	Alexandria	4	**11.76**
	South Sinai (Sharm El-Sheikh)	2	**5.88**
Total		**34**	**100**

Table 2. Screening for production of ASNase from streptomycetes isolated by qualitative rapid plate assay test.

Sample	Results	Sample	Results	Sample	Results
D 1	-	**G 2**	+	**GH 5**	+
D 2	-	**G 3**	-	**GH 6**	-
D 3	-	**G 4**	+	**GH 7**	+
D 4	-	**G 5**	-	**GH 8**	-
D 5	+	**G 6**	-	**Alex 1**	-
D 6	-	**G 7**	-	**Alex 2**	+
D 7	-	**G 8**	-	**Alex 3**	-
D 8	+	**G 9**	+	**Alex 4**	-
D 9	-	**GH 1**	-	**SH 1**	-
D 10	-	**GH 2**	-	**SH 2**	-
D 11	+	**GH 3**	-		
G 1	-	**GH 4**	-		

Screening for Asparaginase -Producing Marine *Streptomycetes*:

The release of extracellular ASNase was quantitatively screened for nine *Streptomyces* isolates (D5, D8, D11, G2, G4, G9, GH5, GH7, and Alex 2), which were identified by the presence of pink color and their colonial growth. The isolates that showed characteristic ASNase -producing ability were then subjecte8d to further identification, characterization, and optimization procedures.

The Outcome of Features Affecting ASNase:

The Outcome of Time: ASNase production by Streptomycete(G9) was affected by an incubation time test. Results in (Fig. 1) reveal that ASNase synthesis gradually increases until 7 days, at which point it reaches its maximum (5.84 U/ml), and subsequently enzyme activity declines.

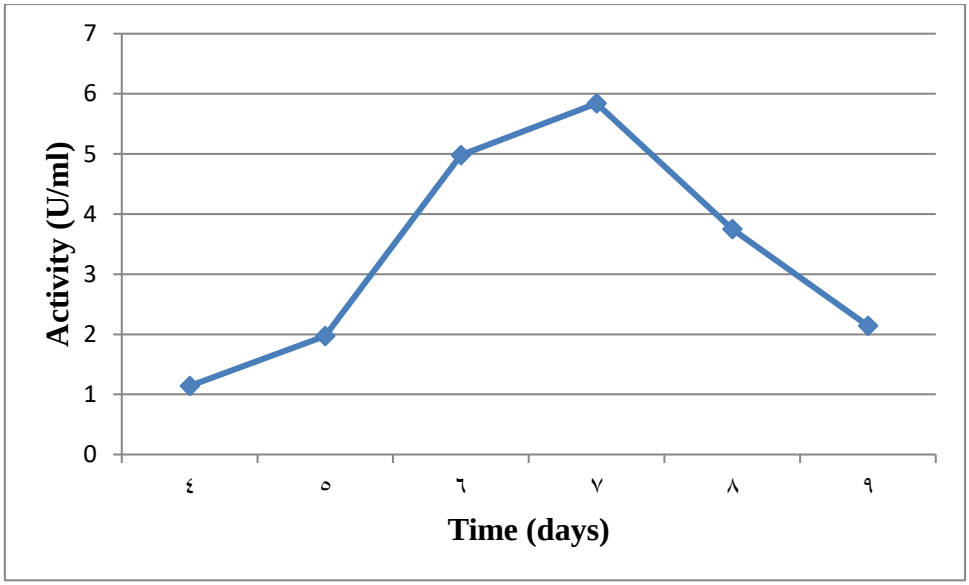

Fig.1. The outcome of incubation time on L- asparaginase production

The Outcome of pH: Streptomycete(G9) synthesis of ASNase is influenced by pH. The pH of the fermentation medium has an impact on the synthesis of enzymes, as shown in (Fig. 2). Thus, at pH 7.0, the maximum enzyme yield was observed to be (5.99 U/ml). Reduced enzyme synthesis was brought on by either an increase or a drop in the medium's pH.

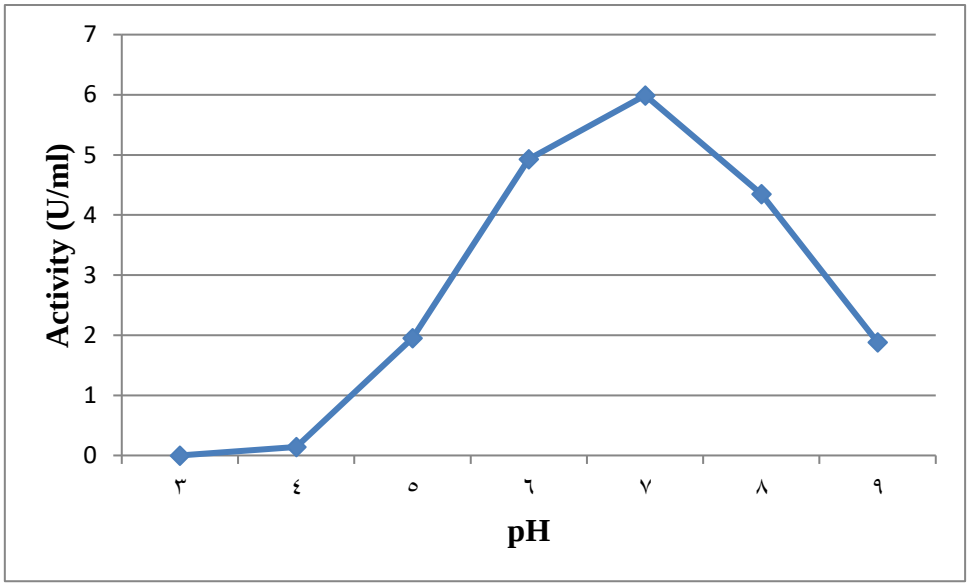

Fig.2. The outcome of pH on L- asparaginase production

The Outcome of Temperature: The results validated a substantial correlation between ASNase synthesis and incubation temperature up to 30 °C (Fig. 3), where an ASNase output of 6.01 U/ml was recorded. Compared to the optimal temperature value, there was a significant drop in enzyme synthesis at 35 and 40 °C, respectively.

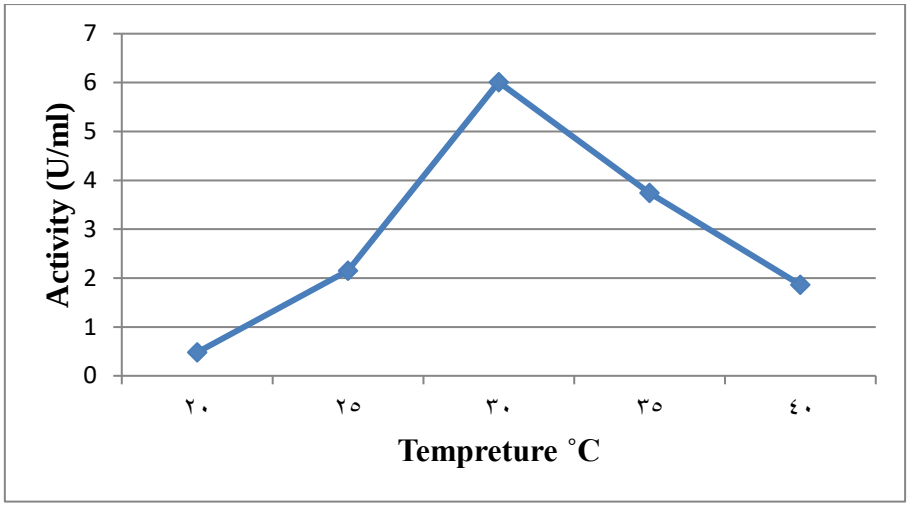

Fig.3. The outcome of Temperature on L- Asparaginase production.

The Outcome of Asparagine Concentration: Asparagine is a substance that stimulates the formation of ASNase. As a result, the medium for producing enzymes was mixed with asparagine in a variety of quantities. The results in (Fig. 4) demonstrated that a 0.4% concentration produced the most enzymes (6.1 U/ml). However, the production of enzymes was inhibited as a result of the rise in asparagine concentration.

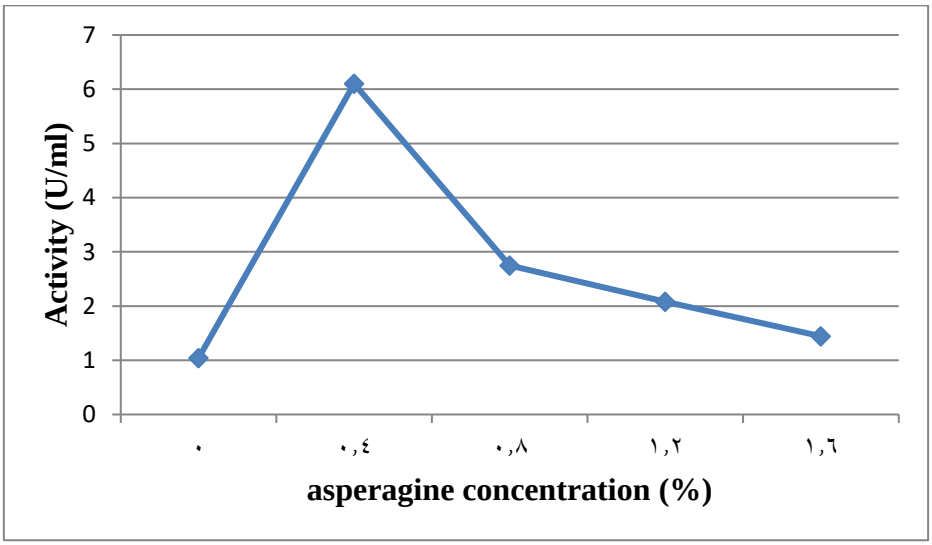

Fig.4. The outcome of asparagine concentration on L- asparaginase production.

The Outcome of Different Carbon Sources: In our data, the ability of the Streptomycete(G9) isolate to utilize several carbon sources to produce ASNase was examined. These sources included mannitol, glucose, fructose, maltose, galactose, starch, and arabinose. The carbon source employed significantly affected the synthesis of ASNase, with glucose being the favored carbon source and yielding enzymes with an activity of 6.14U/ml (Fig. 5). It is interesting to note that the other carbon sources examined couldn't sustain bacterial growth and enzyme synthesis, with the lowermost production of ASNase using maltose and mannitol as a source of carbon, reaching 0.5 and 0.13 U/ml, respectively—less than the highest recorded amount.

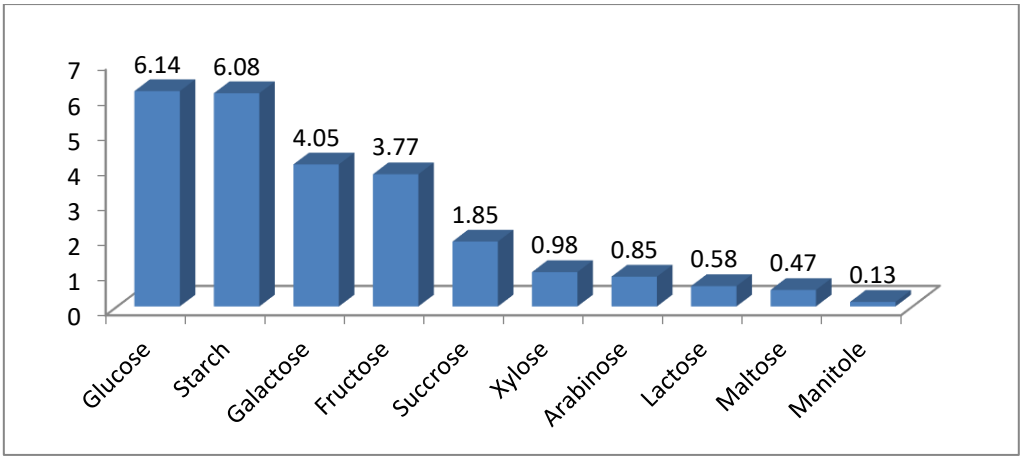

Fig.5. Effect of different carbon sources on L- asparaginase production

Effect of Different Nitrogen Sources: One of the nitrogen sources studied for its possible impact on ASNase synthesis was yeast extract, along with peptone, beef extract, ammonium sulfate, and ammonium chloride. The results displayed that peptone was the greatest source of nitrogen for ASNase production (Fig. 6). For Streptomycete(G9), peptone was found to be the optimum nitrogen source, increasing the maximum enzyme output (6.90 U/ml). Beef extract, ammonium chloride, or yeast extract had no discernible impact on the synthesis of enzymes, while ammonium sulfate drastically reduced it by 1.55 U/ml.

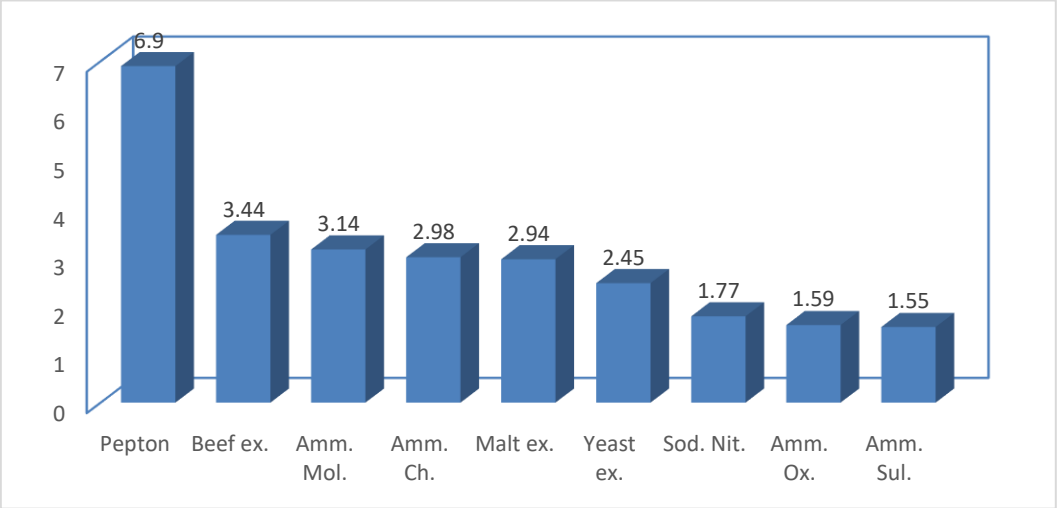

Fig.6. Effect of different nitrogen sources on L- asparaginase production

DISCUSSION

ASNase is an anticancer drug used for the treatment of acute lymphoblastic leukemia and acute myelocytic leukemia. The actinomycetes isolates that produce the anti-leukemic enzyme ASNase have been isolated from a variety of sources, including water, soil, and sediment. 34 isolates of actinomycetes from different Egyptian governorates could produce asparaginases by serial dilution method. Several studies have shown that Egypt's soil and marine waters are abundant in microbial species that may be useful in the search for new bioactive compounds. (Debbab*etal*.,2010),(El-Gendy*etal*.,2000)

The plate assay method was selected as a suitable method for semi-quantitative and rapid enzyme screening processes for actinomycetes that produce ASNase. Our analysis revealed that nine isolates from 34 actinomycetes isolates produce asparaginase

to varied degrees by producing pink zones. The ASNase-containing media is mixed with the pH indicator phenol red in the plate assay. ASNase hydrolyzes ASNase to produce L-aspartic acid and ammonia (Shi et *al.,2017)*.

At acidic pH, the phenol red is yellow, while at alkaline pH, it turns pink. The pH of the medium increases due to the production of ammonia, turning the dye pink and causing a colour shift around the enzyme-positive colony. Our findings are in line with research done by Desai *et al.* (Desai and Hungund, 2018), who established that Streptomyces is the organism that produces the most ASNase. However, our research with larger isolates from soil and marine materials, as well as the benefits of a fast test approach that allows for the immediate observation of ASNase synthesis on plates without the need for time-consuming assays, has revealed several advantages. The results of the current investigation, which concur with those of Abdelrazek *et al.* (Abdelrazek*etal*.,2019) found no relationship between the width of the pink zone and enzyme activity. This fact could be explained by the changes in enzyme levels might be caused by the isolate's characteristics or the fermentation environment.

The optimal conditions for Streptomycete(G9) ASNase production found in this study were consistent with those found in a study by El-Hadi et al. (El-Hadi *et al*.,2019), which found that ASNase production is significantly inclined by the conformation of fermentation media and the cultural circumstances, with time, temperature, pH, and various carbon and nitrogen sources. The maximum synthesis of the ASNase under investigation in this work was seen at 72 hours, during the stationary phase of bacterial development, demonstrating a relationship between enzyme production and bacterial growth [35]. The strain used for ASNase production will determine the best pH, with variations perhaps caused by the fermentation process or the unique genetics of the microbial species (Alrumman *et al*.,2019). This study's finding revealed that ASNase synthesis peaked at pH 8.5 is consistent with that reported by Jalan *et al*. (Jalan *et al*.,2022), who found that streptomyces produced ASNase most efficiently at this pH. Additionally, Streptomyces Pilatus's ASNase activity peaked at pH 7.0 according to Taube et al. (Taube *et al*.,2014), but Streptomycete(G9) ASNase production peaked at pH 7.5, according to Elshafei et al. (Elshafei and El-Ghonemy,2021) and Narayana et al. (Narayana *et al*.,2008). Streptomycete(G9) was found to be most active and flourish at a temperature of 30 C (Singh *et al*.,2019). Extreme temperatures had no favorable effects on this strain's capacity to produce ASNase or develop cells 12(El-Naggar,2015). When cultivated at 28 to 30 C, Streptomycete(G9) generated a significant quantity of ASNase. This result corroborated the claims made by Narayana et al. (2007) (Narayana *et al*.,2008), that Streptomycete(G9) generated the most enzyme when it was cultivated at 35 °C. Different bacteria have different nutritional needs for maximum ASNase synthesis, and even within the same organism, the rate of synthesis might vary depending on the culture and circumstances (Erenler and Geckil,2014). Different carbon sources were included in the medium formulations to promote growth and the production of primary metabolites. It was shown that several circumstances affect how much ASNase is produced in Streptomycete(G9) cells (Amena *et al.,2010)*. Similar to the findings for Streptomycete(G9), for bacterial growth and the production of ASNase, glucose was the best carbon source (El-Naggar,2015).

Conclusion

Streptomyces sp. was isolated from soil and marine environments obtained from different governorates in Egypt for maximum ASNase production with optimum pH 7, temperature 30, and an incubation time of 7 days with a 0.4 % asparagine concentration. Glucose was the best carbon source, and peptone was the best nitrogen source.

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