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PRODUCTION OF ANTI-TUMOR L- ASPARAGINASE BY FREE AND IMMOBILIZED MARINE CYANOBACTERIUM PHORMIDIUM FORMOSUM AS A NOVEL SOURCE

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ABSTRACT

L-asparaginase (L-AsnA) is cosmopolitan among microorganisms and has vital applications in medical and in food technology sectors. Therefore, the power of the assembly, of L-AsnA from *Phormidium formosum* was tested. Cyanobacterium *Phormidium formosum* were isolated from Eastern harbor of Mediterranean Sea coast of Alexandria, Egypt. Different incubation temperatures ranged from 20°C to 45°C were applied to get the optimum incubation temperature for the enzyme production using modified F/2 medium. The pH range between pH 6.0 to 10.0 was applied on the modified F/2 production medium to demonstrate the optimum pH for L-asparaginase production. Adsorption of *Phormidium formosum* on different solid porous supports such as pumice, coal, ceramic, luffa pulp and polyurethane foam for l-asparaginase production was investigated. *Phormidium formosum* cultures were grown in F/2 medium for 14th days. The cultures of *Phormidium formosum* showed a high L-AsnA enzyme activity (49.120 U/ml). the optimized pH and temperature were 8 and 30 ^{o}C, respectively. Immobilization using adsorbed Luffa pulp enhanced the enzyme production compared to the conventional free- cells repeated reuse of the adsorbed cells and achieved the maximum enzyme specific activity (52.562U/ml). Scans using electron microscopic showed the adsorption of *P. formosum* on the pores of luffa pulp. The study findings revealed that *Phormidium formosum* can provide a new and rich source of L-asparaginase enzyme and this can be the primary report on L-asparaginase production *P. formosum*.

KEYWORDS: ***Marine microalgae, Asparaginase, Optimization, Immobilization***

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INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase EU, 3.5.1.1) accelerator is used in the treatment of acute body fluid malignant neoplastic disease (ALL) in children's.[1] The accelerator deprives (L-asparagine amidohydrolase EU, 3.5.1.1) kinds of cancer of L-asparagine and as a result of it catalyzes the L-asparagine chemical action, a necessary organic compound for lymphocyte growth, to L-aspartic acid and ammonia and so shrinks these tumors.[2] L-asparagine is employed by each traditional and cancer cells for his or her survival. It is synthesized by traditional cells for metabolic activities; therefore, they will survive L-asparagine starvation. However, tumour cells were depended in the main on an exogenous offer of L-asparagine.[3] Man varieties of tumour cells needed this organic compound for macromolecule synthesis. They're bereft of a necessary protein within the presence of L-asparaginase accelerator leading to antineoplastic activity. The administration of this enzyme can digest its substrate resulting in starving and killing certain cancer cells. Due to this fact, this enzyme was considered a potent antitumor agent. All the forms of the enzyme have similar functionality and receive importance.[4-5] Recently, L-asparaginase is used in food technology as a potent mitigating agent for reducing the acrylamide (AA, CH2=CH-CO-NH2), a potential carcinogen, which is formed in the reaction of L-asparagine (L-AsnA) and reducing sugars contained in foods during heating processes. [6] L-asparaginase extraction from mammalian cells was found to be very difficult. So microorganisms are the best sources for enzyme production. The enzyme has been produced and characterized from several bacterial genera, as *Escherichia coli , Erwinia carotovora , Streptomyces albidoflavus*, *Helicobacter pylori* and *Pseudomonas aeruginosa*.[7-8] It was also produced by microalgae, filamentous fungi, and higher plants.[9-10] Recently, L-asparaginase production from blue green microalgae are receiving more attention because they have many advantages such as high nutrient contents, low cost of production, cost-effectiveness, no seasonal variation, high efficient producers, being easily cultured and harvested at large scales, and cheaper and easier extraction, and higher yields and purification of protein and enzymes by simple methods are available. However, few reports regarding production of enzyme by blue-green algae are recorded.[11] Optimization of enzyme production is very important before industrial-scale production can be considered. The medium and environmental conditions of the culture lead to modifications in cellular metabolism.[12-13] Cell immobilization was considered to be a promising approach for enhancing the enzymes production, fermentation processes, and bioremediation of toxic substances.[14-15] Immobilized cells have many advantages such as facilitating continuous operation over a prolonged period, offer possible recycling of immobilized cells and simple way for harvesting the products, reactor productivity, ensures higher efficiency of catalysis and development of economical methods focusing on lowering the cost of industrial process. Present investigation is aimed to produce l-asparaginase enzyme from a marine cyanobacterium *phormidium formosum* as a novel source. In addition to optimization of different factors as incubation time, pH and temperature for l-asparaginase production, immobilization of cells were also used for enhancement enzyme production.

MATERIALS AND METHODS

Reagents and chemicals

All reagents and other chemicals were chemically pure grades.

Algal isolation

Different Microalgae were isolated from three different locations, namely, El-Agamy (west of Alexandria), Eastern harbor (Alexandria), and Baltim (East of Alexandria) in the Mediterranean coast of Egypt (Figure 1).[16] The isolated strains were identified according to.[17-19] Samples were grown in F/2 medium.[20-21] Cells were cultured in a 250 ml Erlenmeyer flask containing 100 ml of sterilized medium and incubated at 30±2°C for 20 days. The flask was illuminated by fluorescent white lamps at an intensity 100 µmol photons m^{-2} s^{-1}. The algal growth determined by chlorophyll (a). Chlorophyll content was determined according to.[22] Harvesting took place by centrifugation at 5000 rpm for 15 min.

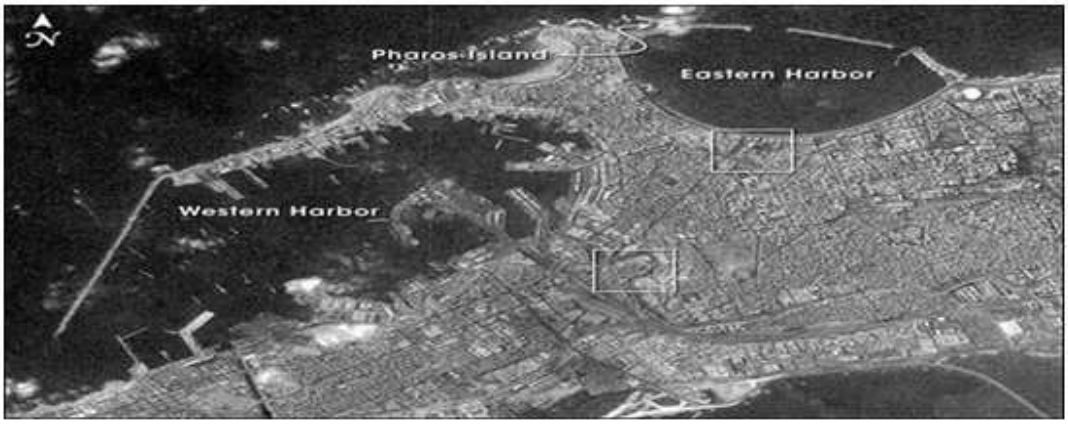


Figure 1

Locations for isolated microalgae I- Phormidum formosum and Oscillatory Spp. (Eastern Harbor) II- Chlorella marina (Baltim) and III- Navicula f. delicatula (El Agamy).

Screening for Microalgae L-asparaginase production

L-asparaginase production can be determined by two techniques namely Qualitative methods and Quantitative methods.

Qualitative method (Fast screening of L-asparaginase production)

Fast screening of L-asparaginase made up of algal cells was assessed by phenol red as a pH indicator among the medium.[23] Phenol red is yellow at acidic pH and turns to pink at base-forming pH; so a pink color is made by Algal cultures manufacturing L-asparaginase. Screening of potential L-asparaginase manufacturing algal was dispensed with the employment of asparagine pH adjusted to 6.8 and supplemented with phenol red as a pH indicator (0.009% final concentration). Tubes were examined for amendment in color of cultures from yellow to pink because of amendment in pH indicating the positive antineoplastic drug activity and are used for any study.

Quantitative method

The modified F/2 broth medium (containing phenol red as a pH indicator) served as a production medium (was adjusted to pH 7.0 before autoclaving). After sterilization, the medium (50 ml placed in 250 ml Erlenmeyer flasks) was inoculated by 5ml old seeded cultures of microalgae and incubated at 160rpm and 30°C for 20 days.[24] The L- asparaginase activity was determined by nesslerization method.

Preparation of cell-free extract

It was done by adding 10ml of sodium phosphate buffer (pH 7) to the algal cultures, incubated in rotator shaker for 45 min. 1ml of the extract was centrifuged at 6,000 rpm for 10 min. The obtained supernatant was used as crude enzyme extract for L- asparaginase assay.[25]

Estimation of L-asparaginase enzyme activity

L-asparaginase activity was carried out by measuring the amount of ammonia released by nesslerization according to emada et al**.** [26] The reaction mixture containing 0.5ml of 0.04M L-asparagine, 0.5 ml of 50 mM Tris-HCl (pH 7.8), 0.5 ml of crude enzyme and 0.5 ml deionized-water was incubated at 30°C for 30 min. The reaction was stopped by adding 50 µl of 1.5 M Trichloroacetic acid (TCA). The mixture was then centrifuged at 5000 rpm for 15 min. To 450 µl of pipetted supernatant, 125 µl of the Nessler solution was added. The solution was maintained for 15 min at room temperature to allow the appropriate reaction between the Nessler solution and ammonia. The results were obtained at OD 480 nm. A standard curve was prepared with dilution series of ammonium chloride, and the enzyme activity was expressed as units (U).[27] One international unit of asparaginase is defined as the amount of protein required to release one micromole of ammonia per minute.[28]

Optimization of Parameter for Higher Production of L- Asparaginase

The enzyme production by the most potent isolate was optimized under shacked condition. The effects of different parameters including pH and temperature on enzyme production were studied and L-asparaginase activity analyzed by standard assay as mentioned before. Different incubation temperatures ranged from 20°C to 45°C were applied to get the optimum incubation temperature for the enzyme production using modified F/2 medium. The pH range between pH 6.0 to 10.0 was applied on the modified F/2 production medium to demonstrate the optimum pH for L-asparaginase production.

Immobilization of cells by adsorption

Two ml of algal growth was added to 50 ml of sterilized modified F/2 medium dispensed in Erlenmeyer flasks (250 ml capacity) and support materials such as pumice, coal, ceramic, luffa pulp and sponge cubes. The flasks were incubated at 160 rpm at 30 °C for 20th days of incubation.[29]

Scanning Microscope analysis

(JEOL JSM – 5300)

. Magnification range between x15 and x200,000

. Fitted with an Orion digital image capture system

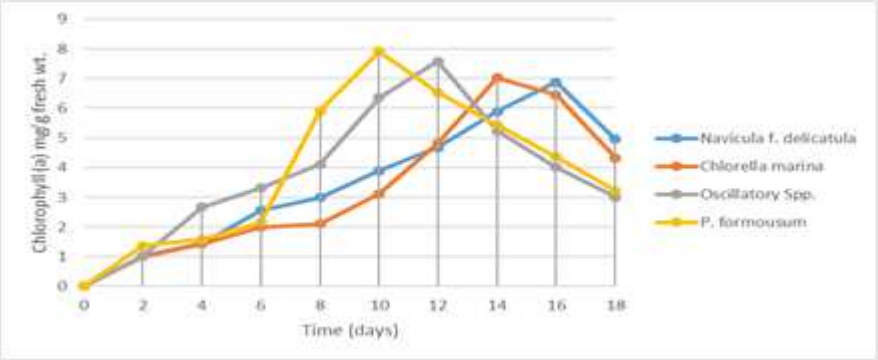
STATISTICAL ANALYSIS

All measurements were carried out in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range tests. Differences at $p < 0.05$ were considered statistically significant. The results were presented as mean values (±SD, standard deviations).

RESULTS AND DISCUSSIONS

The marine environment is a rich source of chemical and biological diversity. It consists of more than 70% of the Earth's surface and has features of wide diversity of habitats, displaying extreme conditions, where marine organisms thrive, offering a vast pool for different kinds of microorganisms and enzymes. Given the dissimilarity between marine and terrestrial habitats, enzymes and microorganisms, either novel or with different and appealing features as compared to terrestrial counterparts, may be identified and isolated. L-asparaginase (E.C. 3.5.1.1), is among the relevant enzymes that can be obtained from marine sources.[30] Cyanobacteria and eukaryotic microalgae have great economic and ecological importance. Cyanobacteria, formerly named blue-green algae are the only known prokaryotes capable of oxygenic photosynthesis.[31] They are considered to be among the oldest life forms on Earth and are the original producers of the Earth's oxygenic atmosphere.[32] The algal strains were identified as *Phormidium formosum, Oscillatory spp. Chlorella marina and Navicula f. delicatula*. It is evident from (Graph 1) that the growth of *Phormidium formosum and Oscillatory spp.* increased and reached their maximum value at stationary phase after 10th days and 12th days respectively , then, phase of stationary phase started to decrease but in *Chlorella marina and Navicula f. delicatula* the growth reached its maximum value after 14th days and 16th days respectively . In this respect**,** Becker[33] concluded that, the most studies on the biochemical production of algal and their analysis were carried out in stationary phase of growth period.

Graph 1
The growth of four microalgae measured as chlorophyll (a) mg/g fresh wt.



Data are expressed as mean ± SE (n=3).

Influence of incubation time

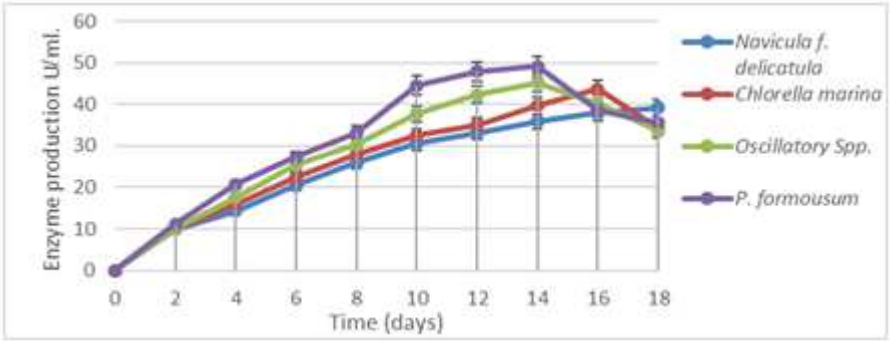
Four microalgae were tested for their ability to produce L-asparaginase enzyme. They grew on modified F/2 medium (containing phenol red as an indicator) at a temperature 30°C and pH 8 (Figure 2). The isolate *Phormidium formousum* was the most potent isolate for the L-asparaginase production by giving the dark pink colour. Also, nesslerization method confirmed the results.

(A) P. formosum, (B) Oscillatory Spp., (C) Chlorella marina, (D) Navicula f. delicatula and (E) control without L-asparaginase extract.

Figure 2
L-asparaginase production from microalgae

The isolate *P.formosum* had the highest activity of L-asparaginase 49.120 U/ ml, L-asparaginase activity increased gradually to the maximum peak after 14th days of incubation. On the other hand, the isolate *Navicula f. delicatula* produced the lowest enzyme activity (39.231U/ml) at 18th days of incubation period, above this period a gradual decrease was reported in the enzyme activity. So the algal isolate *P. formosum* was used for further study and identified (Graph 2 & Table 1). Ellaiah *et al*.[34] have reported that growth rate and enzyme synthesis of the culture are the two main characteristics which are mainly influenced by incubation time. Abd El Baky and El Baroty [35] found that 18th days incubation of cyanobacterium *Spirulina maxima* contained a significant various quantity of dry biomass yields and highest enzyme levels.

Graph 2
Effect of incubation time on production of asparginase.



Data are expressed as mean ± SE (n=3).

Table 1
Screening for algal L-asparaginase production

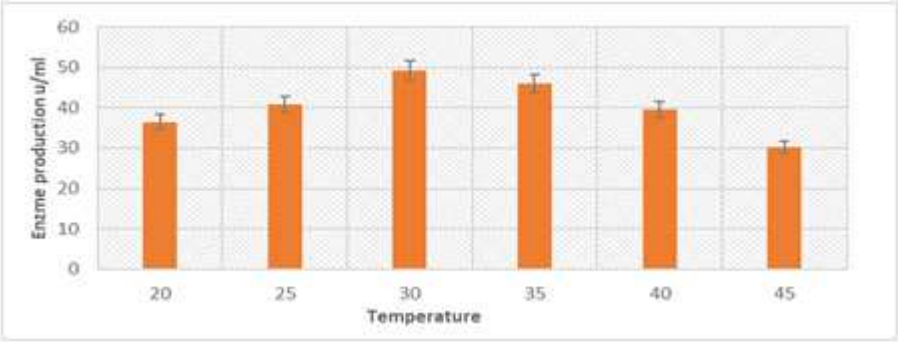
Algal isolate	L-asparaginase activity (U/ml)
Phormidium formosum	49.120±0.05
Oscillatory spp	45.220±0.09
Chlorella marina	43.682±0.08
Navicula f. delicatula	39.231±0.1

Values are mean ±SD; (n=3), P < 0.05 when compared with control

Effect of Different temperature

The results illustrated in (Graph 3) indicated that the highest L- asparaginase production was observed at incubation temperature of 30°C (49.120 U/ml) and lowest production was detected at 45°C (30.25 U/ml). Further increase in temperatures adversely affected the enzyme production. Our results in good line with Rani *et* al.[36] who reported that the optimum temperature for the highest production of L-asparaginase by microalgae *Arthrospira platensis* was 30°C.

Graph 3
Effect of different temperature on production of asparginase by marine cyanobacterium P. formosum.

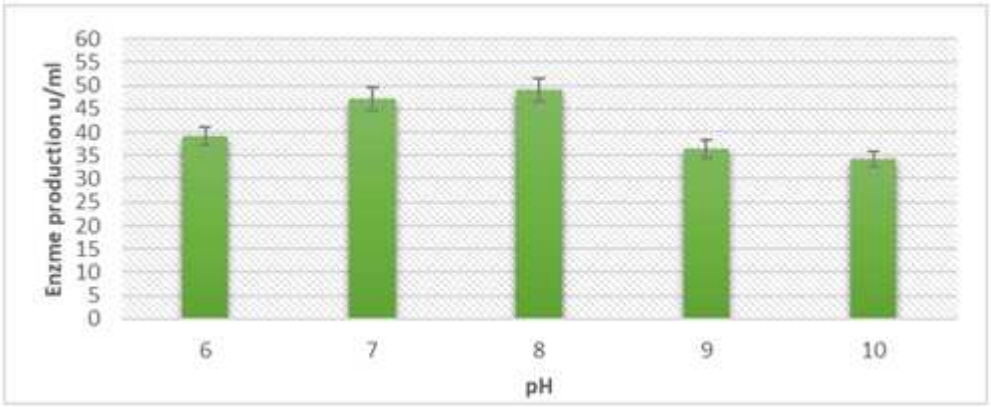


Data are expressed as mean ± SE (n=3).

Effect of different pH

The cyanobacterium *phormidium formosum* recorded the highest L-asparaginase activity (49.120 U/mL) at slightly alkaline culture medium with optimal pH value at pH 8.0. The enzymatic activity decreased to 34.221U/mL by increasing the medium pH value, the L-asparaginase activity continued gradually as the pH value increased. Also, the enzyme activity also decreased to 39.105U/ml by decreasing the medium pH to 6.0 (Graph 4). Similar results have been reported by De-angeli et al.[37] that pH 7.0 is the optimum pH for L-asparaginase production under submerged fermentation process.

Graph 4
Effect different pH on production of asparginase by marine cyanobacterium P. formosum.



Data are expressed as mean ± SE (n=3).

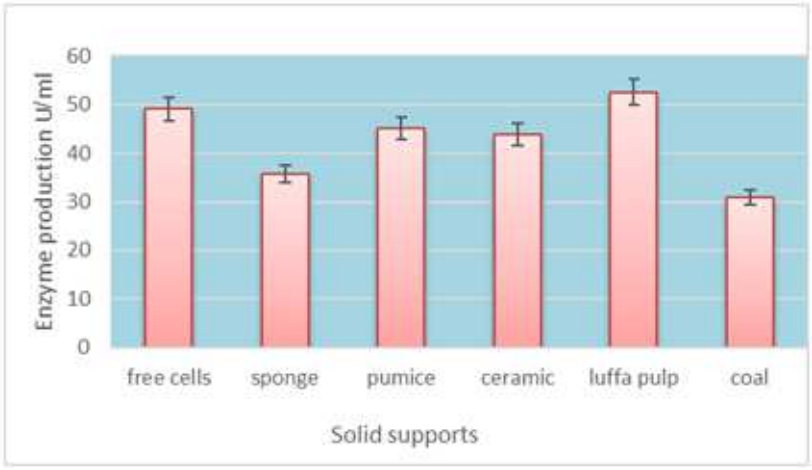
Production of L-asparaginase by immobilized Phormidium formosum

Adsorption of *Phormidium formosum* on different solid porous supports such as pumice, coal, ceramic, luffa pulp and polyurethane foam for l-asparaginase production was investigated. The results (Graph 5) showed a significant adsorption of algal cells on luffa pulp, pumice and ceramic supports. Particularly, luffa pulp showed a slightly higher algal adsorption when compared to the other supports. Cultures containing adsorbed algal cells on luffa pulp, pumice and ceramic showed a relatively high l-asparaginase activity (52.562,

45.094 and 43.875 U/ml, respectively), while polyurethane foam showed the lowest enzyme activity (30.911 U/ml). Thus, cultures adsorbed on luffa pulp had higher l-asparaginase specific activity (52.562 U/ml) than that of free cultures. Therefore, luffa pulp was selected for production of l-asparaginase.

Graph 5

Production of asparaginase on different solid support by marine cyanobacterium P. formosum.



Data are expressed as mean ± SE (n=3)

Electron microscopic photographs (Figure 3) showed the adsorbed *P. formosum* on the luffa pulp. The polyurethane foam was used as a best support material for immobilization of *Streptomyces gulbargensis* mu24 and l-asparaginase yield was increased by 30.2% as compared to free cells.[38] Many reports used various cell immobilization techniques for the biotechnological processes optimization, such as gel entrapment, adsorption and covalent attachment.[39-40] An ideal matrix for immobilization should be strong, resistant to operating conditions and preferably have an open structure.[41] Our results revealed that use of polyurethane foam as a support for immobilization of *Phormidium formosum* enhanced enzyme activity, and as a low cost, porous, non-toxic and biodegradable matrix, presenting many benefits.[42]

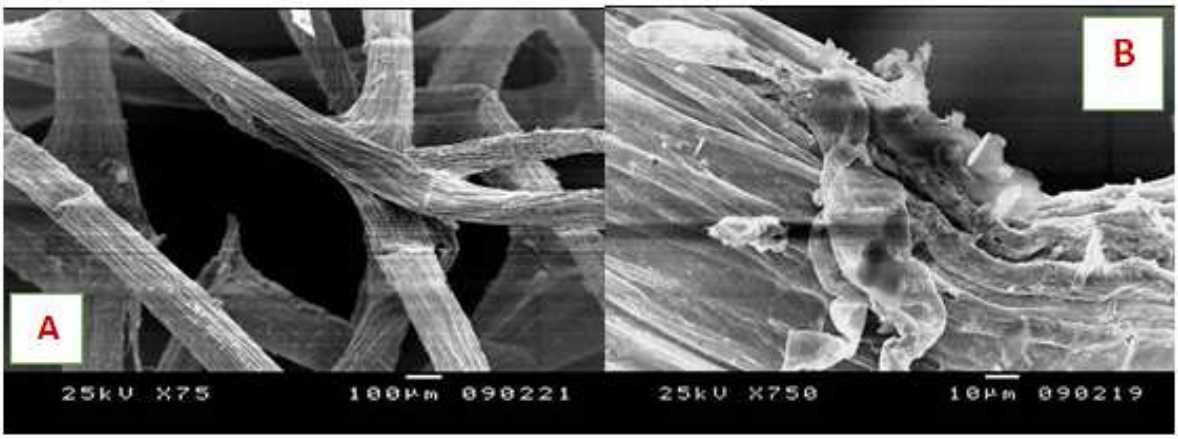


Figure 3

Electron microscope micrographs illustrate (A) Luffa pulp and (B) the adsorbed cyanobacterium P. formosum on Luffa pulp

CONCLUSION

From this present research work, it was found that marine cyanobacterium *Phormidium formosum* can provide a new and rich source of L-asparaginase enzyme. Also, this study reveals that maximum production of L-asparaginase enzyme after optimization parameters such as temperature and pH. Hence, *Phormidium formosum* appears to produce high amount of l-asparaginase enzyme which may be useful in many applications. The study may confirm the advantage of immobilization of the whole cells and reuse of them to increase the production of the enzyme.

AUTHORS CONTRIBUTION STATEMENT

Reham G. Elkomy and Aida M. Farag contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

CONFLICT OF INTEREST

Conflict of interest is declared none.

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