L-glutaminase Production from New Halophilic Marine *Streptomyces griseorubens* NAHE Isolated from Mangrove Sediment, Red Sea, Egypt

Aida M. Farag[1*] Sobhy E. Elsilk[2], Hasnaa E-B. Ghonam[1], Neveen M. El-Shafaey[2], Eman H. Zaghloul[1], Nanees G. Allam[2]

[1]National Institute of Oceanography and Fisheries (NIOF), Egypt
[2]Department of Botany and Microbiology, Faculty of Science, Tanta University, Egypt

[*]**Corresponding Author:** aidafarag92@yahoo.com

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ABSTRACT

L-glutaminase is an enzymatic catalyst that hydrolyzes glutamine, converting it into L-glutamic acid and ammonia. It has several biotechnological uses in the medicinal and food sectors. Moreover, its role in enzyme therapy for cancer treatment, particularly in acute lymphocytic leukemia, has been widely acknowledged. This study screened marine sediment-derived actinobacterial isolates for L-glutaminase. The most promising L-glutaminase producing strain was selected and identified through 16S rRNA gene sequence analysis as *Streptomyces griseorubens* NAHE (OR462786). The maximum L-glutaminase activity (20.777U/ mL) was detected in the growth medium containing sucrose and glutamine as the carbon and nitrogen sources, respectively. The Plackett-Burman experimental design was used to optimize and identify the factors that have the greatest impact on L-glutaminase production. This design revealed that the optimized medium increased L-glutaminase activity by 1.47-fold, higher than that recorded in the case of the basal cultural conditions. The current study also showed that L-glutaminase produced by adsorbed marine *S. griseorubens* NAHE enhanced enzyme activity by 3.31-fold compared to conventional free cells. Furthermore, the produced enzyme exhibited a promising antimicrobial activity against *Staphylococcus aureus*, *Fusarium oxysporum*, *Candida albicans*, and *Aspergillus flavus,* which indicates its suitability for numerous therapeutic applications.

INTRODUCTION

The L-glutaminase enzyme (E.C.3.5.2.1) is classified as an amidohydrolase enzyme and a member of the hydrolase class. The primary role of this enzyme is to facilitate the deamidation process of L-glutamine, producing ammonia and L-glutamic acid (**Orabi *et al.*, 2019**). In addition, L-glutaminase plays a crucial role in the cellular metabolism of both eukaryotes and prokaryotes (**Elshafei *et al*., 2014**). It is one of the most important therapeutic enzymes. Its prospective applications in pharmaceuticals as an anti-leukemic medication have attracted significant attention (**Orabi *et al*., 2019**). L-glutamine is the essential energy source for growing tumor cells (**Wise & Thompson,**

2010). L-glutaminase enzymatically converts L-glutamine into glutamate, impeding the growth of cancer cells by depriving them of their essential nutrient (glutamine). Hence, L-glutaminase is a promising cancer treatment agent **(Choi & Park, 2018)**. Moreover, it has potential use in biosensors for accurately measuring glutamine concentrations in mammalian and hybridoma cell cultures **(Ahmed *et al.*, 2016)**.

L-glutaminase is found everywhere in nature and has been detected in animals, plants, and many microbes **(Ito *et al.*, 2013; Hamed & Al-wasify, 2016; Lavanya Kothapalli, 2023)**. Microbial sources are highly favored for industrial applications of L-glutaminase production due to their cost-effectiveness, consistent output, ease of control, and optimization methods **(Aishwariyaa *et al.*, 2020)**.

The importance of marine environments has been determined as a promising source for producing numerous enzymes with outstanding properties, including stability under harsh environmental conditions and salt tolerance **(Pandian *et al.*, 2014)**. Various maritime environments have been investigated to isolate marine actinomycetes that produce L-glutaminase, such as *Streptomyces olivochromogenes, Streptomyces* sp. SBU1 **(Balagurunathan *et al.*, 2010)**, as well as *Streptomyces* sp. D214 **(Aly *et al.*, 2017)**. The mangrove ecosystem is recognized as a highly distinctive marine niche, characterized by the presence of promising strains that have evolved to thrive in this environment possessing the ability to produce enzymes and bioactive compounds with unique properties.

Statistical methods such as Plackett-Burman Designs **(Plackett & Burman, 1946)** have proven effective in conducting multivariable optimization analyses across various chemical, environmental, and biotechnological processes **(Farag *et al.*, 2015; Durthi *et al.*, 2019)**. Moreover, they are regarded as a potent and efficient instrument for evaluating crucial variables from a complex system with few experiments **(Majumder & Goyal, 2008)**.

Furthermore, immobilization technology provides numerous benefits in comparison to free cells. These advantages include increased cell density, continuous utilization, improved cell activity, retention of plasmid-bearing cells, prevention of interfacial inactivation, enhanced productivity, safeguarding against shear forces and acidification in the environment, and resistance to many environmental conditions **(Bayat *et al.*, 2015; Mitra & Mukhopadhyay, 2016)**. Various immobilization techniques have been employed to produce organic acids, enzymes, and amino acids and to carry out the bioremediation of hazardous pollutants **(Farag *et al.*, 2022)**.

Hence, the current research's objective was to isolate a novel marine actinomycetes strain exhibiting a high L-glutaminase production capacity. Additionally, this study aimed at optimizing the production yield and exploring bacterial cell immobilization. To our knowledge, this study represented the initial documentation of L-

glutaminase synthesis by free and immobilized marine *Streptomyces griseorubens* strains obtained from the distinctive mangrove habitats in Egypt.

MATERIALS AND METHODS

1. Isolation of L-glutaminase producing actinomycetes

Collected sediment samples were obtained from several areas within the mangrove ecosystems throughout the Red Sea coasts of Egypt. The specimens were obtained under aseptic containers, transferred to the laboratory in ice boxes, and stored at 4^oC until investigated. Various dilutions of each sample were prepared, and 100µL of each dilution was spread over a sterile plate with a modified minimum glutamine medium (MGM). The medium composed of (g/ L): glutamine, 10.0; KH_2PO_4 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.1; $ZnSO_4$, 1.0; agar, 20.0. The components were dissolved in 1000mL of aged, filtered seawater; the medium pH was adjusted to 7.0± 0.2 (using 1N HCl or NaOH) and supplemented with 0.009% (v/v) phenol red dye (as pH indicator) and antifungal cycloheximide (20µg/ mL). L-glutamine serves as the sole source of nitrogen and carbon **(Hymavathi *et al.*, 2009)**. Individual colonies were picked and streaked on fresh media until pure colonies were obtained.

2. Detection of L-glutaminase producing actinomycetes

2.1. Qualitative assay of L-glutaminase

Each of the obtained isolates was then streaked on the surface of solidified MGM medium and incubated at 30.0± 2.0ºC for 2– 7 days. A non-inoculated petri dish was incubated as a negative control. The transition of the medium hue from yellow to pink signified L-glutaminase synthesis. The alteration in hue results from variations in the medium's pH since L-glutaminase catalyzes the hydrolysis of amide bonds in L-glutamine, releasing ammonia. The trials were conducted in triplicates.

2.2. Quantitative assay of L-glutaminase

Seed cultures of different isolates were prepared by inoculating one mL of 7 days culture of each isolate into 20mL of sterilized broth MGM and incubated for 7 days at 30.0± 2.0ºC under shaking condition (JSSI-100T, JSR, Korea) at 160rpm. After cultivation, the conidia of isolates were collected by scraping them using autoclaved seawater, and the number of spores was then recovered and quantified using a hemocytometer. Subsequently, one milliliter aliquots (with a concentration of 1×10^6 spores/ mL) was cultured in 50mL of sterilized MGM. After incubation, the culture was collected by centrifugation (for 30min at 6,000rpm) to remove cells, and the clear cell-free filtrate was used as a crude L-glutaminase enzyme **(Dura *et al*., 2002)**.

3. L-glutaminase and protein content assay

The quantitative estimation of L-glutaminase activity was conducted using Nessler's reagent, following the method described by **Borek *et al*. (2004)**. The experimental procedure combined 0.5mL phosphate buffer (5mM, pH 7.0), 0.5mL of the crude enzyme, 0.5mL of a 0.04M glutamine solution, and 0.5mL of distilled water. The resultant mixture, with a total volume of 2.0mL, was incubated for 30min. Subsequently, the reaction was halted by adding trichloroacetic acid (TCA), and the resulting mixture was centrifuged. Afterward, a volume of 0.1mL from the obtained clear supernatant was mixed with 3.7mL of distilled water, and the reaction was initiated by adding 0.2mL of Nessler's reagent (Himedia, India). The developed color reaction was permitted to progress, and successively the absorbance was measured using a visible-light spectrophotometer (Alpha 1102, Laxco, USA). The quantification of ammonia released was determined by constructing a curve using ammonium chloride as the reference standard. An IU of L-glutaminase refers to the quantity of the enzyme that releases one μM of ammonia under ideal conditions, as stated by **Sabu *et al*. (2000)**. The protein content was assessed using the methodology outlined by **Lowery *et al*. (1951)**, with BSA (bovine serum albumin) serving as the reference protein standard.

4. Characterization of the most potent strain

The most potent L-glutaminase producing isolate was characterized using morphological, physiological, biochemical, and molecular approaches. Spore shape and morphology were determined using light and scanning electron microscope (SEM) after growing on a medium of starch nitrate agar. Classical classification methods described in the identification key by Bergey's Manual of Determinative Bacteriology were employed **(Cross, 1989)**.

4.1. Molecular identification of the most potent strain

The DNA extraction of the promising strain was performed in SolGent (Solution for Genetic Technologies), following the manufacturer's instructions. The polymerase chain reaction (PCR) was conducted utilizing the primer pairs 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') to amplify the 16S rRNA gene. The reaction mixture (50μL) used in the experiment is as follows: 5μL of Taq Polymerase (5 units/ μL), 5μL of $MgCl_2$ (50mM), 5μL of Taq Buffer, 5μL of dNTPs (10mM), 3μL of 27F primer (10mM), 3μL of 1492R primer (of 10mM), 19μL of nuclease-free H2O (ultrapure water), and 100ng/ μL of DNA template. The PCR was conducted with the following parameters: an initial denaturation step at 98°C for 3min, followed by 35 cycles of denaturation at 98°C for 10s, annealing at 60°C for 10s, and extension at 72°C for 60s. A final extension step at 72°C for 10min was conducted using the T100TM BioRad Thermal Cycler. The amplified products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. The species

identification was done by analyzing the 16S rRNA gene sequence using the BLASTn tool provided by NCBI. The research indicated a 99.8% similarity with *Streptomyces griseorubens*. Subsequently, the sequence was submitted to the GenBank and given the accession number OR462786 **(White *et al.*, 1990)**.

5. Optimization of fermentation conditions for L-glutaminase production

The effect of various parameters on L-glutaminase activity was explored through one factor at the time of studies.

5.1. Effect of incubation time

The marine isolate was cultivated in the MGM (pH of 7.0) at 30°C in a shaking incubator at 160rpm to promote a consistent growth. L-glutaminase activity, extracellular protein concentration, and biomass were regularly measured for 1- 12 days.

5.1.1. Influence of different carbon and nitrogen sources

Different sugars (glucose, fructose, lactose, maltose, mannose, sucrose, and starch) at 0.1% concentration were used to detect the best carbon source for growth and enzyme activity. Furthermore, seven nitrogen sources were examined, including organic (yeast extract, peptone, tryptone, casein) and inorganic (urea, ammonium chloride, and potassium nitrate) at an equivalent weight of nitrogen contained in L-glutamine used in MGM. One mL of culture aliquot from the 7-day culture was inoculated in 50mL of MGM broth supplemented with the different carbon or nitrogen sources mentioned above. Then, they were cultivated for five days at 30± 2ºC in a rotary shaker at 140rpm. At the end of the incubation period, L-glutaminase activity and extracellular protein values were estimated. All tests were conducted in triplicate, and the mean values and standard deviation (SD) were determined **(Desai *et al.*, 2016)**.

5.2. Optimization of nutritional factors using Plackett-Burman experimental design

The Plackett-Burman experimental design was employed to optimize the growth medium and incubation conditions **(Plackett & Burman, 1946)**. An experimental design incorporating eleven independent factors was established. The low (-) and high (+) levels of each independent factor were analyzed, as shown in Table (1). The trials were carried out in triplicates, and the mean of the obtained results was processed as the response of L-glutaminase activity. The primary impact of each factor was computed using the subsequent equation:

$$Exi= (\Sigma pi+ - \Sigma pi-)/N$$

The main effect, *Exi*, represents the impact of the independent factor (xi) on the L-glutaminase activity (responses), $\Sigma pi+$ and $\Sigma pi-$. $\Sigma pi+$ corresponds to the reaction when the independent variable is in high concentration, while $\Sigma pi-$ corresponds to the response when the independent variable is in low concentration. N represents the number of trials divided by 2.

Table 1. Independent factors and their levels in Placket Burman design

Factor	Symbol	Experimental values		
		Low (-1)	Basal (0)	High (+1)
Sucrose (g/L)	X1	1.0	2.0	3.0
Glutamine (g/L)	X2	7.5	10.0	12.5
KH_2PO_4 (g/L)	X3	0.50	1.0	1.50
KCl (g/L)	X4	0.25	0.5	0.75
MgSO4.7H_2O (g/L)	X5	0.25	0.5	0.75
$FeSO_4$ (g/L)	X6	0.05	0.10	0.15
Incubation temperature (^{o}C)	X7	25	30	35
pH	X8	6	7	8
Incubation time (day)	X9	4	5	6
Inoculum size (%)	X10	1	2	3
Volume of medium (ml)	X11	25	50	75

6. Production of L-glutaminase enzyme by immobilized *Streptomyces griseorubens* NAHE cells

6.1. Adsorption on different solid porous materials

A defined volume of pre-activated *S. griseorubens* NAHE cells was introduced into 50mL of autoclaved optimized MGM with different solid supports (luffa pulp, polyurethane foam (PF), pumice, and charcoal cubes) and incubated under shaken condition (160rpm) at 35°C for 6-days.

6.2. Entrapment in alginate and k-carrageenan

The cells were entangled in sterilized 3% calcium alginate or potassium k-carrageenan gel beads, as described by **Farag *et al*. (2022)**. Approximately, 3mL of cells was added to the sterile alginate or k-carrageenan solution. A sterile syringe was used to draw the gel-mixing cells, dropped into a cross-linking solution (sterilized 2% $CaCl_2$ and 2% KCl solution for alginate and k-carrageenan, respectively) to obtain spherical beads (3- 4mm diameter). The resulting beads were left for 2h to harden and then washed. Finally, the formed beads were added to sterilize MGM and incubated under shaken condition (160rpm) at 35°C for 6- days.

7. Antimicrobial assay for L-glutaminase

The antimicrobial activity of the produced enzyme was assessed through the agar well diffusion test **(Balouiri *et al*., 2016)**. The indicator bacterial pathogens Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC® 25923 and *Escherichia coli* ATCC® 8739, as well as the fungal pathogens *Candida albicans* ATCC® 10231, *Fusarium oxysporium*, and *Aspergillus flavus* **(Awad *et al.*, 2019)** were used. In this experiment, a

volume of Muller Hinton agar medium was inoculated with 50μL of an indicator pathogen after standardization using 0.5 McFarland standard solution (~1.5× 10^8CFU/ mL). The resulting mixture was then poured into sterilized plates. After solidification, agar wells were cut out utilizing a sterile cork borer with a diameter of 6mm. Then, 100μL of the sterilized crude L-glutaminase (30U/ mL) was distributed into each well. The plates were refrigerated for 2h to allow enzyme diffusion. Subsequently, the plates were incubated for 24h at 37.0± 2.0ºC for bacteria and 5 days at 30.0± 2.0ºC for fungi. After incubation, the inhibition zone diameters surrounding each well were expressed in mm. The previous procedures were repeated three times, and the mean and SD were calculated. Penicillin G, an antibacterial agent at a concentration of 200μg/ mL, and Tinidazole, an antifungal agent at the same concentration, were employed as positive controls.

RESULTS

1.   Isolation of L-glutaminase producing actinomycetes

In the current study, different actinomycetes isolates were recovered from various collected sediment samples from the Red Sea coasts of Egypt. Based on the usual culture traits of Streptomyces, a total of twenty possible Streptomyces strains were successfully isolated on MGM. These strains exhibit filamentous colonies with the production of dry, powdery-cottony aerial mycelium and non-fragmenting substrate mycelium. The isolates were assessed for their capacity to synthesize L-glutaminase using MGM as a screening method. Among all isolates, 12 strains exhibited visible pink zones (ranging from 11 to 32mm) surrounding the colonies on the MGM supplemented with phenol red (pH indicator). This change in color from yellow to pink (in the acidic and alkaline conditions, respectively) (Fig. 1) is caused by the breakdown of L-glutamine into ammonia and L-glutamic acid by the L-glutaminase. The isolates were purified by undergoing multiple rounds of subculturing and streaking on MGM plates.
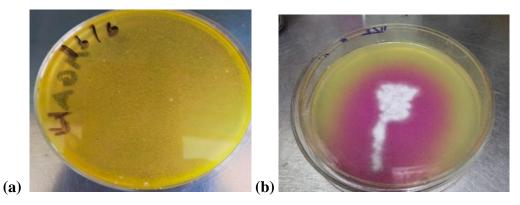

(a)   **(b)**

Fig. 1. Qualitative screening for L-glutaminase activity. The plates show that the color of MGM changed from **(a)** yellow to **(b)** pink due to L-glutaminase production

The quantitative enzyme activity screening by selected strains was conducted using submerged fermentation. The findings revealed that all 12 isolates exhibited varying levels of L-glutaminase activity, ranging from 0.556± 0.69 to 12.220± 0.95U/ mL (Table 2). The isolate NAHE (isolated from mangrove sediment, Safaga, Egypt) showed the highest L-glutaminase activity (12.220± 0.95U/ mL). Therefore, it was selected as the most potent isolate and used for the subsequent experiments.

Table 2. Screening of the most promising L-glutaminase producing marine isolates

Isolate	Extracellular protein (mg/mL)	L-glutaminase activity (U/mL)	Dry weight
NAHE	0.503±0.01	12.220±0.95	2.67
S2	0.474±0.02	11.968±0.68	1.22
S3	0.441±0.03	10.496±0.10	2.65
S4	0.361±0.01	9.496±0.09	1.976
S9	0.479±0.03	9.496±0.14	1.621
S10	0.674±0.02	9.086±0.28	1.543
S11	0.622±0.04	1.713±0.12	1.22
S12	0.325±0.04	8.114±0.15	1.72
S14	0.416±0.03	7.575±0.06	1.22
S16	0.216±0.04	5.604±0.40	1.87
S17	0.199±0.01	3.869±0.10	1.2
S19	0.132±0.01	0.556±0.69	1.11

2. Characterization of the most potent L-glutaminase producing strain

The morphological and biochemical characteristics of the most potent strain (NAHE) were conducted. The target strain (Fig. 2) exhibited well aerobic growth on ISP2 medium at 5% NaCl, pH 8.0, and temperature of 35°C. Additionally, it was able to utilize different carbon and nitrogen sources in a characteristic pattern. The selected isolate identification was further conducted through 16S rRNA gene sequence analysis performed through Real Bio Gene Company, Egypt. The obtained sequence showed similarity to the *Streptomyces* sp. by 99.8%. Consequently, the nucleotide sequence has been submitted to the GenBank database and given the accession number OR462786. The phylogenetic relation of the obtained isolate and the closely related relatives was analyzed (Fig. 3). Based on these results, the experimental marine *Streptomyces* isolate has been identified as *Streptomyces griseorubens* NAHE.
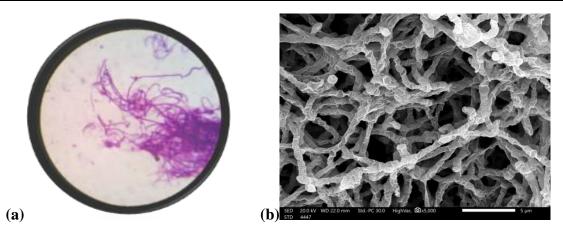
Fig. 2. (a) Gram staining of the marine isolate NAHE showing its cell shape and Gram-positive reaction, and **(b)** Scanning electron microscope image of NAHE at 5000X
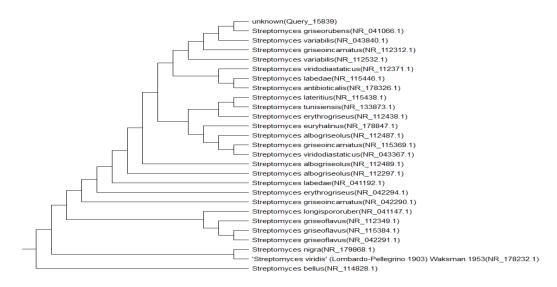

Fig. 3. Phylogenetic relationships among the representative experimental strain (NAHE) and the most closely related *Streptomyces* species

3. Optimization using sequential one factor at a time design (OFAT)

3.1. Effect of the incubation period

At several time points ranging from one to twelve days, the time course of L-glutaminase synthesis in conjunction with the growth of *S. griseorubens* NAHE was investigated. After 7 days of incubation, the findings suggested that the highest activity level was observed during the late exponential growth phase. In 5-days of cultivation, the highest levels of enzyme activity, extracellular protein, and dry weight were obtained, with values of approximately 15.141± 1.04U/ mL, 0.571mg/ mL, and 1.623mg, respectively. After reaching their maximum levels, all these variables gradually decreased, as shown in Fig. (4).
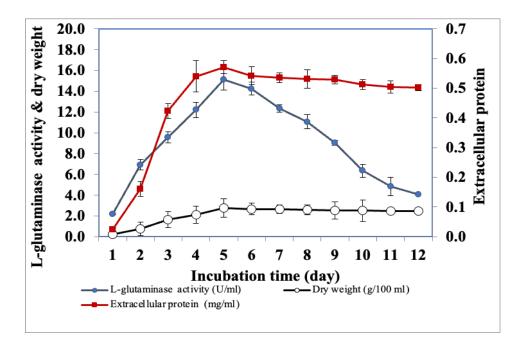
Fig. 4. Kinetics of cell growth, extracellular protein, and L-glutaminase activity by *S. griseorubens* NAHE cells. Data are the mean of triplicates ± standard deviation

3.2. Influence of carbon and nitrogen sources

As shown in Fig. (5a), the results revealed that incorporating all used carbon sources decreased the enzyme's activity except for sucrose, which enhanced L-glutaminase production (20.592U/ mL). In addition, the tested strain was able to utilize all the examined nitrogen sources for L-glutaminase production (Fig. 5b). The maximal L-glutaminase activity of *S. griseorubens* NAHE was detected when glutamine was used as a sole nitrogen source, showing 20.777U/ mL. Conversely, the minimal activity (3.867U/ mL) was recorded in the case of potassium nitrate.
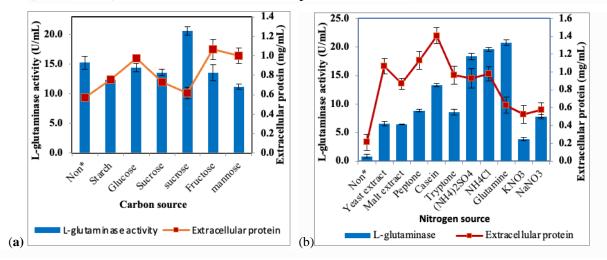

Fig. 5. The impact of **(a)** various carbon and **(b)** nitrogen sources on the activity of L-glutaminase and extracellular protein production by *S. griseorubens* NAHE cells

3.2. Optimization by Placket Burman design (PBD)

Eleven nutritional and physical independent variables were examined at the high (+) and low (-) levels for L-glutaminase production as a response, considering each variable's main effect and t-values. The data (Table 3) indicate that the highest enzyme activity (31.748U/ mL) was achieved in trial number 1, whereas the lowest enzyme activity (9.162U/ mL) was detected in trial number 8. It was found that glutamine, KH_2PO_4, $FeSO_4$, and medium volume have a negative effect on the production of L-glutaminase enzyme within the test ranges. In contrast, all other variables affected the activity of L-glutaminase positively. The suitability of the model and the significance of the medium components and cultural conditions on the production of L-glutaminase by *S. griseorubens* NAHE were evaluated by ANOVA, as listed in Table (4). Fisher's statistical test (F-test) evaluated the model's statistical significance. The statistical confidence was calculated as: Statistical confidence = $(1-p) *100$. Hence, any variable showing a confidence higher than 95% was considered significant.

Table 3. Matrix for eleven variables with coded levels using PBD

Trial No.	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	L-glutaminase activity(U/mL)	
												Actual	**Predicted**
1	-1	-1	1	1	1	-1	1	1	-1	1	-1	31.748	31.703
2	1	1	1	-1	1	1	-1	1	-1	-1	-1	16.748	16.703
3	-1	1	-1	-1	-1	1	1	1	-1	1	1	29.619	29.574
4	1	1	-1	1	-1	-1	-1	1	1	1	-1	31.021	30.976
5	-1	1	1	-1	1	-1	-1	-1	1	1	1	28.891	28.846
6	1	1	-1	1	1	-1	1	-1	-1	-1	1	15.346	15.301
7	-1	-1	-1	1	1	1	-1	1	1	-1	1	31.457	31.412
8	1	-1	1	1	-1	1	-1	-1	-1	1	1	9.162	9.117
9	1	-1	1	-1	-1	-1	1	1	1	-1	1	16.523	16.479
10	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	17.966	17.921
11	1	-1	-1	-1	1	1	1	-1	1	1	-1	12.357	12.312
12	-1	1	1	1	-1	1	1	-1	1	-1	-1	15.611	15.566
13	0	0	0	0	0	0	0	0	0	0	0	20.788	21.326

Table 4. Statistical analysis of PBD

Factor	Coefficient	Main effect	t-value	*P*-value	Confidence %
	21.326	42.652	137.398	0.005	99.5
Glutamine	-4.511	-9.022	-27.925	0.023	97.7
Sucrose	1.502	3.004	9.297	0.068	93.2
KH$_2$PO$_4$	-1.590	-3.180	-9.843	0.064	93.6
KCl	1.020	2.040	6.314	0.100	90.0
MgSO$_4$	1.387	2.774	8.585	0.074	92.6
FeSO$_4$	-2.212	-4.424	-13.691	0.046	95.4
Volume of medium	-1.170	-2.340	-7.243	0.087	91.3
pH	4.815	9.631	29.807	0.021	97.9
Incubation time	1.273	2.545	7.878	0.080	92.0
Inoculum size	2.429	4.858	15.035	0.042	95.8
Temperature	0.462	0.925	2.862	0.214	78.6

The main effects of the examined factors on L-glutaminase production were determined and are graphically illustrated in Fig. (6). Based on the statistical analysis of confidence level and *P*-value of the eleven variables, only 5 variables (sucrose, MgSO$_4$, FeSO$_4$, pH, and inoculum size) had confidence levels greater than 95% and a *P*-value ranging from 0.023- 0.024. These variables were considered the most significant parameters that influence L-glutaminase production. In addition, the model's goodness of fit was assessed using the coefficient of determination (R2). The R2 value in this instance was determined to be 0.99997, indicating that 99.997% of the overall variability in the response could be accounted for by this model. In comparison, just 0.003% of the entire variance remained unexplained. Pareto chart (Fig. 7) analyzed the impact of L-glutaminase production at a 95% confidence level.
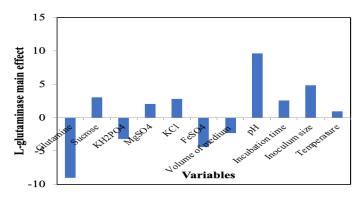

Fig. 6. The main effect of independent variables on L-glutaminase activity by *S. griseorubens* NAHE as a result of PBD
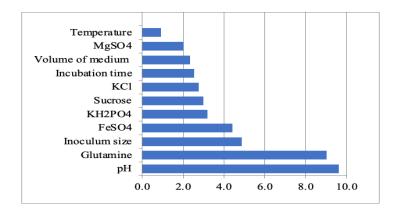
Fig. 7. Pareto chart of the medium components' effect on L-glutaminase yield

This optimization technique aims to minimize the expenses associated with specific processes by optimizing the parameters in a way that allows for the removal of insignificant parameters from the design. Glutamine is a primary nitrogen supply in this investigation, whereas MgSO4 and KCl, acting as metal ions, provide the most efficacy.

$$Y=21.326- -4.511*X_1 + 1.502*X_2 -1.590*X_3 + 1.020*X_4+1.387*X_5 -2.212*X_6-1.170*X7 + 4.815*X_8 + 1.273*X_9+2.429*X10+0.462X11$$

Where, Y represents L-glutaminase activity (U/ mL), and X_1: X_{11} are the independent variables concentrations, as listed in Table (4) under materials and methods.

According to the obtained results, the predicted optimized medium composition for the cultivation of *S. griseorubens* NAHE cells was as follows: (g/L): sucrose, 3.0; glutamine, 7.5; K_2HPO_4, 0.5; $MgSO_4.7H.O$, 0.75; KCl, 0.75; $FeSO_4.7H_2O$, 0.05; inoculum size, 3.0 %; adjusted to pH 8.0 and incubation period 6 days at 35^oC.

A verification experiment was conducted with three separate replicates to validate the precision of the implemented PBD screening test. The dependent variables predicted near-optimum levels were calculated and compared to the basal settings, and the L-glutaminase activity is demonstrated in Table (5).

Table 5. L-glutaminase activity on basal versus optimized medium

Medium	L-glutaminase activity (U/mL)	L-glutaminase specific activity (U/mg protein)	Improvement (%)
Basal medium	20.788	33.15	0
Optimized medium	35.96	48.59	46.58

Applying near optimum conditions resulted in L-glutaminase activity of approximately 35.58U/ mL (predicated) and an actual 35.96U/ mL. The correlation between the actual and predicted L-glutaminase activity is very close, revealing the

model's appropriateness. These results represented a 46.58% improvement in L-glutaminase activity compared to the activity in the case of the basal conditions.

3. Production of L-glutaminase by cells immobilization

Immobilization of *S. griseorubens* NAHE cells was performed by two techniques: entrapment by using various gel materials and adsorption of living cells and byusing different solid porous supports. As shown in Fig. (8), the formed biocatalyst by entrapment in alginate beads produced the highest L-glutaminase activity (87.660± 2.36U/ mL) among all gel materials and recorded 2.45-fold increases compared to free culture. Additionally, the maximum L-glutaminase activity was obtained from cells adsorbed on polyurethane foams (PF) (118.455± 3.32U/ mL), followed by luffa pulp (LP) (107.08± 2.01U/ mL), which exceeded the yield achieved with free culture (35.745± 0.37U/ mL) by 3.3 and 2.9 folds, respectively. On the other hand, the pumice cubes recorded a minimal L-glutaminase activity (22.84± 1.36U/ mL).
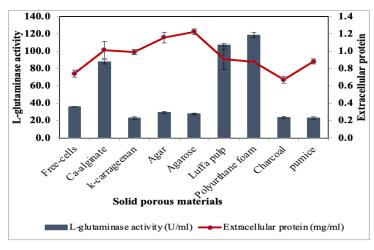

Fig. 8. L-glutaminase activity produced by immobilized *S. griseorubens* NAHE cells within different solid and porous materials

Based on the various micrographs acquired using SEM of cells adsorbed on PF, it is evident that there is a compact cluster of cells on the PF (Fig. 9), indicating a simple and efficient adsorption on this cost-effective solid support material
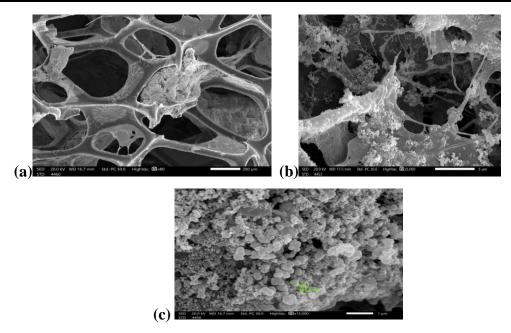
Fig. 9. Scanning electron micrographs showing **(a)** Polyurethane foam (PF), **(b)** *S. griseorubens* NAHE adsorbed on PF, and **(c)** Spores of *S. griseorubens* NAHE

4.   Determination of L-glutaminase antimicrobial activity

The potential of *S. griseorubens* NAHE L-glutaminase as an antimicrobial agent was studied (Table 6). The antimicrobial activity was evaluated against different bacterial and fungal pathogens. Data showed that the enzyme has promising antimicrobial activity against *F. oxysporium*, *A. flavus*, *C. albicans,* and *S. aureus*. On the contrary, the enzyme showed no antimicrobial activity against *E. coli*.

Table 6. Antimicrobial activity of produced L-glutaminase against pathogenic microorganisms

Agent	S. aureus (MRSA)	E. coli	C. albicans	F. oxysporium	A. flavus
	Diameter of inhibition zone (mm)[*]				
L-glutaminase	18±0.4	0	25±0.7	35±1.1	28±0.9
Penicillin G	35.0±1.4	11±0.5	0.0	0.0	0.0
Tinidazole	0.0	0.0	27.0±1.4	21.0±1.0	20±0.6

DISCUSSION

Streptomyces, a genus belonging to the Actinobacteria phylum, are microorganisms that live in soil and derive nutrients from decaying organic matter. They can synthesize several bioactive compounds **(Yang *et al.*, 2021)**. Nevertheless, the declining enthusiasm for soil-dwelling bacteria as a reservoir of new bioactive

compounds has occurred due to the rediscovery of previously identified bioactive chemicals **(Almeida *et al*., 2019)**. Hence, Streptomyces residing in alternative settings, such as marine ecosystems, has become highly esteemed due to the extensive range of variations and ability to adapt to extreme conditions, such as limited nutrients, elevated pressure, and high salinity. These conditions may alter the organisms' physiological state, promoting the synthesis of unique bioactive compounds **(Almeida *et al*., 2019)**. In addition, marine sediments are often considered dominant in origin concerning actinobacteria **(Girão *et al*., 2019)**. L-glutaminase is fateful in medical applications due to its potent anti-tumor properties, especially in treating acute lymphoblastic leukemia. Therefore, searching for novel L-glutaminase producing actinomycetes and selecting high-producer isolates for commercial and industrial applications is particularly interesting.

In the current investigation, different strains were isolated from the Red Sea coasts of Egypt. Preliminary screening of the L-glutaminase yield of the 20 potential isolates was conducted using MGM supplemented with phenol red as a pH indicator **(Raj *et al*., 2016)**. The production of a pinkish color around the colonies indicates L-glutaminase production **(Balagurunathan *et al*., 2010)**, as this color change is related to a change in the pH of the media resulting from the hydrolysis of L-glutamine and the release of ammonia **(El-Naggar *et al.*, 2015; Patel *et al*., 2020)**. Out of the obtained isolates, 12 strains gave pink zones, therefore they were considered as L-glutaminase procedures. The 12 isolates were purified and identified morphologically and microscopic as actinomycetes. The plate screening approach is only qualitative, without quantitative measurements, and the correlation between activity and the ratio of colonies to clean zones may be weak **(Maki *et al.*, 2009)**. Therefore, submerged fermentation was applied for the L-glutaminase production by the examined 12 isolates, and isolate NAHE (isolated from mangrove sediment, Safaga, Red Sea, Egypt) produced the maximum L-glutaminase activity (12.22± 0.95U/ mL) among all isolates. The marine actinobacterial isolate NAHE was characterized morphologically (mycelium production, pigmentation, and spores) using SEM. When cultivated in marine ISP4 medium, the isolate produced grey spores without any diffusible pigment. Analyzed using SEM, the spores were found to have a spherical morphology, with dimensions ranging from 0.5 to 1.0µm.

The current result revealed that the most potent isolate is marine-derived and identified as *Streptomyces griseorubens*. The obtained isolate's 16S rRNA gene sequence was deposited in GenBank under the accession number (OR462786). *S. griseorubens* is an actinobacterium derived from soil **(Pridham *et a*l., 1958)**. It plays a significant role in the recycling of carbon and nitrogen **(Feng *et al.*, 2014)**. Although *S. griseorubens* is terrestrial, it was also isolated from marine sediments **(Paderog *et al*., 2020)**. For example, *S. griseorubens* strain MMH 9 was isolated from marine sediment collected from the Suez Gulf, Egypt **(Al-Agamy *et al*., 2021)**. Similarly, **Lavanya Kothapalli**

(2023) isolated *S. luteogriseus* from marine sediment and assigned it as an L-glutaminase potential producer.

The production of L-glutaminase is affected by several variables, including the duration of incubation and the sources of carbon and nitrogen. The results revealed that *S. griseorubens* NAHE showed the highest L-glutaminase activity (15.141 ± 1.04U/ mL) after 5 days of incubation. The short incubation time for enzyme production may offer the opportunity for cost-efficient production. Furthermore, a more extended incubation period might reduce enzyme activity due to the exhaustion of nutrients in the fermentation medium, which stresses microbial physiology and ultimately leads to enzyme inactivation **(Gautam *et al*., 2011)**. Similar to our finding, **Desai *et al*. (2016)** elucidated that the maximum L-glutaminase activity produced from *Streptomyces* sp. had been detected after 5 days of incubation, and then no further increase was detected. The carbon source reflects the energy supply necessary for microbial growth and enzyme manufacturing **(Chitanand & Shete, 2012)**.

In this investigation, sucrose was utilized by *S. griseorubens* NAHE culture as an additional carbon source for an optimum L-glutaminase production. In contrast, several bacteria utilize glucose as the optimal carbon source for L-glutaminase synthesis **(Desai *et al*., 2016; Al-Zahrani *et al*., 2020)**. The choice of nitrogen supply impacts enzyme production significantly since it serves as the primary building block for protein synthesis. Moreover, the nitrogen type can alter the medium's pH, potentially impacting the enzyme's activity and stability. The present investigation shows that L-glutamine is the most effective stimulus to produce extracellular L-glutaminase. Reseach studies have utilized glutamine as a nitrogen source to increase L-glutaminase production as well **(Desai *et al*., 2016; Al-Zahrani *et al*., 2020)**.

For the commercial production requirements of L-glutaminase enzyme, marine S. *griseorubens* NAHE has been further studied to optimize the growth medium components and physicochemical parameters through PBD methodology for large-scale enzyme yield. The design was employed to specify the significant variables that have the greatest impact on achieving maximal L-glutaminase activity and to determine their most likely optimal levels via a limited number of experiments. Out of the examined 11 variables, glutamine, KH_2PO_4, $FeSO_4$, and medium volume only negatively influenced L-glutaminase production significantly.

Fisher's statistical test (F-test) evaluated the model's statistical significance. According to the statistical analysis of the confidence level (%) and *P*-value, only four variables (glutamine, $FeSO_4$, pH, and inoculum size) were considered the most significant parameters influencing the enzyme yield. Some researchers noticed that confidence levels beyond 90 or 95% affect the response significantly **(Abdel-Fattah & Olama, 2002; Niladevi *et al*., 2009)**. Furthermore, the Pareto chart serves as a useful tool for visualizing the outcomes of a PBD **(Hassan *et al*., 2018)**. It indicates the magnitude

of each variable estimated (irrespective of its impact, be it negative or positive). In addition, the R^2 = 0.9997, which is a considerable coefficient of determination that explains the variability of the data. These optimum conditions improved the average yield of L-glutaminase from *S. griseorubens* NAHE culture by 1.47-fold in comparison with the conditions implemented prior to optimization. The use of PBD in optimization studies has shown its efficacy in improving the yield of L-glutaminase **(Iyer & Singhal, 2010; Pandian *et al*., 2014; Jesuraj *et al*., 2017; Elborai *et al*., 2023)**. The synthesis of L-glutaminase by B. subtilis JK-79 was greatly improved with the use of PBD, resulting in much higher enzyme titers. The enzyme activity reached an impressive level of 691.27U/mL **(Jambulingam & Saranya, 2020)**.

Immobilized cells are often shielded from pressures that induce shearing and exhibit a specific resilience to different environmental stresses, such as changes in pH and temperature. Furthermore, they have the potential to remain alive and active for an extended period, hence facilitating continuous cultivation operations and enhancing operational stability **(Zhu, 2007)**. Hence, this research aimed to investigate the impact of immobilization by entrapment and adsorption on L-glutaminase production by *S. griseorubens* NAHE using the optimized medium derived from PBD. The obtained data showed that the optimum L-glutaminase activity was detected in case of adsorption of cells on PF and LP compared to free cells by 3.31 and 3.00-fold, respectively, followed by entrapping cells in alginate beads (2.45-fold). This can be due to the better availability of L-glutamine (substrate) to the microbial cells in case of adsorption. In addition, LP showed countless advantages, such as an inexpensive support, excellent adsorption surface, large porosity, strong mechanical qualities, and simplicity of handling **(Patil *et al*., 2006)**. In contrast, the cells that were trapped inside the Ca-alginate matrix resulted in slow diffusion of glutamine and air into the alginate beads, reducing the production of the L-glutaminase enzyme. It was suggested that sodium alginate is the most suitable matrix for immobilizing microorganisms to produce different enzymes **(Divatar *et al*., 2015; Shen *et al*., 2021; Elborai *et al*., 2023)**.

The antimicrobial efficacy of *S. griseorubens* NAHE L-glutaminase was assessed based on the inhibitory zone diameter, which varied between 18 and 35mm. The enzyme proved to have promising activity against Gram-positive and fungal strains. **Raj *et al*. (2016)** found that both L- glutaminase and L-asparaginase producing isolates have only antibacterial activity. In addition, **El-Borai *et al*. (2023)** examined the antimicrobial effect of *Pseudomonas* sp. RAS123, which showed an activity against only bacterial strains (*S. aureus*, *B. subtilis*, *Streptococcus mutants*, *Enterobacter cloacae,* and *E. coli*) and no activity against fungal strains.

CONCLUSION

The present data support the isolation of new promising L-glutaminase producing strain molecularly identified as *S. griseorubens* NAHE from the mangrove ecosystem in

the Red Sea, Egypt. NAHE free and immobilized celles were used for L-glutaminase production. The Plackett-Burman experimental design was used to obtimize L-glutaminase production medium and growth conditions and successfully increased it by 1.47-fold, higher than that recorded in the case of the basal cultural conditions. In addition, the adsorbed marine *S. griseorubens* NAHE enhanced the enzyme activity by 3.31-fold compared to the conventional free cells. Moreover, the produced L-glutaminase showed a promising antimicrobial activity against indicator pathogens. Future research would investigate the purification process and implement the immobilized L-glutaminase enzyme for use in the medicinal and food sectors.

Ethical Approval: Not applicable.

Authors Contributions

Aida M. Farag, Sobhy S. Mohamed, Hasnaa E. Ghonem, and Nanees G. Allam participated in the conceptualization, supervision, methodology, data curation, and writing the manuscript draft. **Neveen M. El-Shafaey** participated in methodology, data curation, and writing draft. **Eman H. Zaghloul** contributed to performing the methodology, data analysis, visualization, and manuscript writing. All authors read and approved the final manuscript.

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