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Article

Optimization Studies on L-glutaminase Production from the New Marine *Pseudomonas* sp. RAS123 Isolated from Burullus Lake, Kafr El-Sheikh, Egypt

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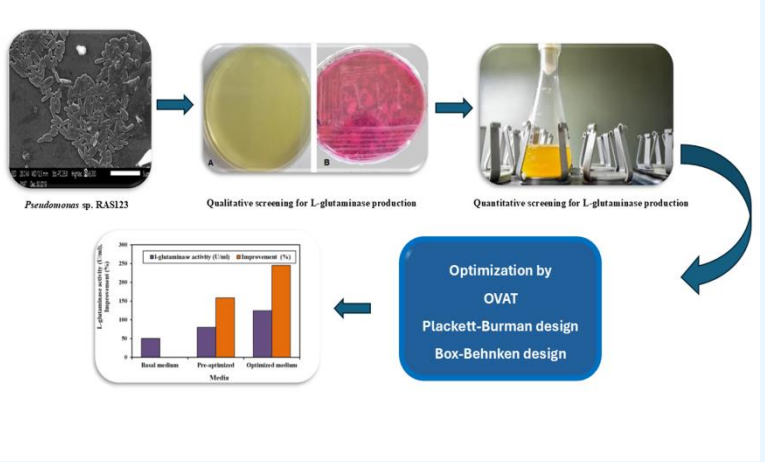
ABSTRACT: It has been demonstrated that microbial anti-cancer enzymes are efficient and cost-effective cancer therapeutic agents. The current study used statistical methods and one-variable-at-a-time (OVAT) methodology to optimize the production medium for maximum L-glutaminase synthesis. *Pseudomonas* sp. RAS123, which was isolated from Brullus Lake, Kafr El-Sheikh, Egypt, and produced the highest L-glutaminase activity (40.826 U/mL) among the tested isolates, was selectively chosen for more studies. OVAT studies showed that after two days of incubation, the maximum enzyme synthesis was achieved. Moreover, the findings showed that the best pH and inoculum size were 7 and 0.5 mL/50 mL medium, respectively. To produce the maximum L-glutaminase activity, glucose was the most effective carbon source, and L-glutamine was the preferred nitrogen source. Plackett-Burman design (PBD) was utilized to further optimize the fermentation conditions to achieve the maximum production. The findings showed that $MgSO_4.7H_2O$, NaCl, and $CaCl_2$ had a detrimental impact on the enzyme, while L-glutamine had the opposite effect. The activity reached 80.364 U/mL, which indicated a 1.59-fold increase in enzyme synthesis. Interactions between factors such as L-glutamine, $MgSO_4.7H_2O$, and $CaCl_2.2H_2O$ were also investigated by Box-Behnken designs (BBD) factorial experimental designs. Compared to the basal condition, the synthesis of L-glutaminase increased by approximately 2.45 times. The model's accuracy and validity were also confirmed by matching the measured L-glutaminase activity (124.25 U/mL) under ideal conditions with the anticipated one (126.19 U/mL).



1. INTRODCTION

Many researchers have recently become interested in L-glutaminase due to its diverse applications, particularly in medicine and food technology. In the medical field, this enzyme shows promise as an anticancer agent, while in the food industry, it serves as a flavor-enhancing additive in fermented products such as sufu, miso, and soy sauce. Additionally, it can be utilized as a biosensor to monitor glutamine levels in mammalian and hybridoma cell cultures. Moreover, theanine and other fine compounds are produced by it [1, 2]. This enzyme, classified as an amidohydrolase (EC 3.5.1.2),

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facilitates the hydrolysis of L-glutamine into L-glutamate and ammonia. It works well as a treatment for acute lymphocytic leukemia and has anticancer effects by removing L-glutamine from malignant cells, which use it more often than healthy cells for energy needs and proliferation. Additionally, a key vulnerability of cancer cells is their limited capacity to produce L-glutamine endogenously, which many anticancer drugs that deplete amino acids take advantage of, as mentioned by Jesuraj et al. [3] and Cruzat et al. [4].

The most promising cancer therapies are marine-derived biomolecules because of their special characteristics [5].Compared to equivalent terrestrial bacteria, the enzymes of these microorganisms tend to remain more stable and resistant to variations in temperature, pressure, pH, and salinity [6].Since human blood plasma and seawater have a closer chemical relationship, marine microbe-derived enzymes are considered promising candidates for medical use, potentially offering therapeutic benefits with fewer side effects. These enzymes are also more compatible with human physiology because they have evolved to work in these environments [7, 8]. Despite these advantages, research on marine-derived L-glutaminase is still limited compared to studies on terrestrial microorganisms, particularly regarding production optimization strategies for new marine strains.

Optimizing the physical characteristics and components of the culture medium is essential for increasing the output yield. Statistical or conventional methods are used for optimization. The statistical method is used to screen variables and choose the important elements for additional optimization procedures, while the traditional method only modifies one element at a time while maintaining the other elements fixed and constant [9]. For medium optimization, several statistical techniques are available. Fractional factorial designs, like the Plackett-Burman design (PBD), are among them. While the screening is performed using a linear technique, this design fails to show the interaction between factors [10]. Numerous benefits of statistical designs were mentioned by many researches, which include their speed, ability to prevent results from being misinterpreted, and ability to save time and chemicals. According to Carvalho et al. [11], these techniques also reduce the overall number of tests and long-listing of nutrients [11,12]. Additionally, a statistical and mathematical optimization technique called response surface methodology (RSM) is used with 3D images on the x, y, and z axes. The three key components selected by PBD can be optimized using it [12]. Although these statistical methods are more efficient to minimize experimental trials and define major factors, the best culture conditions for this specific strain for enhancing enzyme yield have not been extensively investigated in any previous research. By methodically adjusting the physical and chemical characteristics of the culture medium, this study seeks to close this gap and increase the production of L-glutaminase from this promising new source.

The current study aims to optimize the L-glutaminase production by the newly identified marine strain *Pseudomonas* sp. RAS123 by employing statistical techniques like the Plackett-Burman and Box-Behnken designs, as well as one variable at a time.

2. Materials and Methods

2.1. Bacterial strains isolation

Marine water samples were collected from various sites in Brullus Lake, Kafr El-Sheikh, Egypt, as listed in Table 1. 15 samples were taken throughout the summer season. At each site, surface water samples were collected using sterile 500-mL bottles made of polyethylene at a depth of 20–30 cm below the surface of the water. To reduce contamination, the bottles were rinsed with lake water prior to sampling. Samples were received in the laboratory in an icebox and handled within 24 h of their collection.

One mL of each water sample was diluted in a 2% (w/v) sterile sodium chloride (NaCl) saline solution before being plated on top of a ZoBell agar medium supplemented with L-glutamine [13, 14]. ZoBell agar medium was prepared according to ZoBell [13] and contained the following components (g/L): yeast extract, 1.0; peptone, 5.0; agar-agar, 15.0; $FeSO_4 \cdot 7H_2O$, traces; filtered seawater, 800 mL; distilled water, 200 mL, with the final pH adjusted to 7.5. L-glutaminase production was screened for using the rapid plate test. The following components (g/l) were added to modified M-9 medium, which was used to cultivate the colonies that were obtained in the previous step: In 1000 mL of distilled water, $Na_2HPO_4.2H_2O$, 6.0; KH_2PO_4, 3.0; NaCl, 20.5; L-glutamine, 5.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.15; glucose, 2.0; and bacteriological agar, 18.0. To serve as a pH indicator, 2.5 mL of 3% (w/v) phenol red solution prepared in ethanol and buffered to pH 7 was incorporated. The plates were kept at 35°C for 72 hours. L-glutaminase synthesis was shown by the formation of pink zones surrounding colonies during incubation [15]. Following purification, those colonies were then selected for additional screening in a liquid media. One mL of a culture that had been grown overnight in ZoBell medium was added to aliquots of 50 mL of mineral medium to produce L-glutaminase. Then, the mixture underwent shaking at 35°C for 72 h.

Eight distinct bacterial isolates that were found to produce L-glutaminase were obtained through preliminary screening. One isolate was chosen for more research for L-glutaminase production, though. The GeneJET Genomic DNA Purification Kit (Thermo Scientific, K0721) was used to extract the genomic DNA of the chosen bacterial isolate according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the 27f (5′-AGA GTT TGG ATC MTG GCT CAG-3′) and 1492r (5′-CGG TTA CCT TGT TAC GAC TT-3′) (20 pmol/µL each). A 1 µL mixture containing primers, 5 µL template DNA, nuclease-free water, and Taq DNA polymerase (1 U) was used for PCR reactions. The thermal cycling parameters consisted of 95°C for 5 minutes, 20 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 90 seconds, and a final extension at 72°C for 7 minutes. The GeneJET PCR Purification Kit (Thermo Scientific, K0701) was used to purify the PCR products, and GATC Biotech (Germany) used an ABI 3730xl DNA sequencer to sequence them. 454 pyrosequencing was used combined with Sanger sequencing to assure high accuracy and coverage. BLAST (NCBI) was utilized to analyze the sequences, and BioEdit was used to align them. Our prior publication [14] provides full sequencing details and methodology. It was determined that the isolate was *Pseudomonas* sp. RAS123 (GenBank accession number

MN900616).

For optimum L-glutaminase production, one factor at a time and statistical methods were applied for maximum enzyme production.

Table 1. Collection Sites of Bacterial Isolates

Site Name	Isolate symbols
Drain 7	Brs2, Brs3, Brs5
Drain 8	Brs1
Khasha drain	Kh3a, Kh2a
West Brullus drain	Me1a, Me2a

2.2. Measurement of Bacterial Growth

Bacterial growth was assessed spectrophotometrically by measuring the optical density (OD) of cultures at 600 nm using a Pharmacia Biotech Novaspec II spectrophotometer.

2.3. Crude enzyme preparation and assays

2.3.1. Crude enzyme extraction

Following incubation period, the culture underwent centrifugation for 15 min at 5000 rpm using a cooling centrifuge set at 4°C. The clear supernatant was thought to have created the crude enzyme.

2.3.2. Protein estimation

The protein concentration in the enzyme extract was assessed using the approach described by Lowry et al. [16]. In this procedure, 1 mL of the enzyme sample was combined with 5 mL of Lowry reagent C and left undisturbed at ambient temperature for 10 min. Subsequently, 0.5 mL of the diluted Folin-Ciocalteu phenol reagent was promptly added, followed by immediate mixing. The blue color that emerged at 750 nm after 20 min was measured using a spectrophotometer. a protein standard curve where the standard is crystalline bovine serum albumin.

2.3.3. Enzyme activity assay

Nesslerization was used to determine enzyme activity [15]. After adding 0.5 mL of distilled water, 0.5 mL of 0.1 M phosphate buffer (pH 8), 0.5 mL of supernatant, and 0.5 mL of 0.04 M L-glutamine solutions, the test was mixed and incubated for 30 minutes at 37°C. 1.5 M trichloroacetic acid (0.5 mL) was added to halt the reaction. To extract the precipitated proteins, centrifugation was employed (10000×g for 20 min). Following the addition of trichloroacetic acid, enzyme preparation was also added to prepare control tubes. The Nesslerization procedure was conducted using the supernatant. To 3.7 mL of distilled water, 0.1 mL of the abovementioned mixtures and 0.2 mL of Nessler's reagent are then introduced. After the color developed for 20 minutes at room temperature, the solution's optical density was measured at 450 nm. The ammonia amount released per milliliter of enzyme preparation per minute was defined as the amount of L-glutaminase (U/mL), which was determined using the standard plot of ammonium chloride.

2.4. Optimization of L-glutaminase production

2.4.1. OVAT (one-variable-at-a-time)

To determine optimal production conditions, one parameter was varied at a time while keeping all other variables constant:

Incubation time: Cultures grew at 35°C under shaking conditions (120 rpm) for a range of times up to three days to investigate the impact of the incubation period. Optical density, L-glutaminase activity, and extracellular proteins were all measured [17].

pH: Also, the medium was adjusted at different pH levels between 5.0 and 9.0 and incubated for 48 h at 35°C under shaking conditions to assess how the pH affects L-glutaminase production [17].

Inoculum size: Different volumes of inoculum, from 0.2 to 3.0 mL of bacterial suspension, were introduced to 50 mL of modified M-9 medium adjusted at pH 7 and incubated for 48 h at 35°C under shaking conditions to determine the impact of the size of inoculum [18].

Carbon sources: Sucrose, dextrose, starch, fructose, and mannitol were introduced to the modified M-9 medium one at a time. The media were adjusted at pH 7, inoculated with 0.5 bacterial suspension, and incubated for 48 h at 35°C under shaking conditions to find out the effects of various carbon sources [18].

Nitrogen sources: A source of nitrogen at equivalent weight as sodium nitrate, potassium nitrate, ammonium nitrite, ammonium chloride, ammonium sulfate, casein, peptone, yeast extract, and beef extract was also introduced one at a time to examine the effects of different nitrogen sources [18]. The media was adjusted at pH 7 and incubated for 48 h at 35°C under shaking conditions.

2.4.2. Plackett-Burman design

The relative importance of various factors that impact the activity of the L-glutaminase enzyme to break down L-glutamine was demonstrated using PBD [10]. Limiting the number of experiments while retaining insights into the main effect of tested variables is possible by applying a fractional factorial design. Using a full factorial design may necessitate 2n experiments if n components need to be examined. The design matrix is used to arrange the nine combinations of seven independent variables that are assessed in this experiment. A low (-) and high (+) level was examined for every variable (Table 2). The averages of the degradation data from observations were utilized as the response, and each trial was carried out in triplicate. The given-below equation was utilized to evaluate each variable's main effect:

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

Exi denotes the variable main effect, N is the number of trials divided by 2, and Σpi+ and Σpi– are the responses of L-glutaminase synthesis in trials when the independent variable (Xi) is present in high and low levels, respectively. The ideal medium's validity is additionally verified using the basal medium and the Plackett-Burman reverse medium.

2.4.3. Box-Behnken design

Depending on the PBD findings, the BBD was utilized in Response Surface Methodology to assess the impact of the significant independent variables, L-glutamine (X1), $MgSO_4.7H_2O$ (X2), and $CaCl_2$ (X3), on the L-glutaminase production by cultures of *Pseudomonas* sp. RAS123. The results and the two levels used by PBD led them to further investigate at three levels (Table 3). The three most significant

variables were selected after the relative significance of the independent variables was estimated in order to ascertain their ideal level in response to enzyme activity (U/mL) [19]. The three main steps in this optimization method are carrying out statistically designed trials, calculating the structured mathematical model's coefficients, forecasting the outcome, and determining the suitability of the model.

Table 2. Experimental variables at upper and lower levels used in PBD

Variables (g/l)	Symbol code	Experimental values		
		Lower (-1)	Basal (0)	Higher (+1)
Na_2HPO_4	Na_2	3	6	9
KH_2PO_4	K	1.5	3.0	4.5
NaCl	Na	10.25	20.5	30.75
L-glutamine	Glut	2.5	5.0	7.5
$MgSO_4.7H_2O$	Mg	0.25	0.5	0.75
$CaCl_2$	Ca	0.07	0.15	0.225
Glucose	Glu	1	2	3

Table 3. The three levels of significant independent variables used in BBD

Symbol code	Factors	Actual levels of coded factors		
		Higher (+1)	Basal (0)	Lower (-1)
X1 (g/l)	L-glutamine	11.25	7.50	3.75
X2 (g/l)	$MgSO_4.7H_2O$	0.375	0.250	0.125
X3 (g/l)	$CaCl_2$	0.1125	0.075	0.0375

2.5. Statistical analysis of data

Microsoft Excel 97 was used to carry out multiple linear regressions on the enzyme activity data in an attempt to estimate the t-value, P-value, and confidence levels. The students' t-test was employed to calculate the significance level (P-value). The P-value was expressed as a percentage for the confidence level. The Microsoft Excel tool's solver function was applied to assess the optimum value of L-glutaminase activity. The simultaneous effects of the three most important independent factors on each response are displayed in three-dimensional graphs produced using STATISTICA 5.0. Each experiment was carried out in triplicate, and the average findings are displayed.

3. Results

3.1. Screening for L-glutaminase enzyme production

The eight selected isolates were quantitatively tested for L-glutaminase production using glutamine as the nitrogen source. The results represented in **Figure 1** show that all isolates exhibited the capability to synthesize the L-glutaminase enzyme in varying amounts. The most potent glutamine degrader was the Brs5 isolate, producing the maximum yield of L-glutaminase enzyme (40.826 U/mL), followed by the Brs1 isolate (32.514 U/mL).

The extracellular protein content of the tested isolates ranged from 0.1 to 0.8 mg/mL. According to the previous results, Brs5 isolate obtained from Brullus Lake, Kafr El-Sheikh, Egypt, was then identified as *Pseudomonas* sp. RAS123 and chosen for additional L-glutaminase production optimization research in the current research.

3.2. OVAT (one-variable-at-a-time)

3.2.1. Production of L-glutaminase at different incubation periods

The length of incubation is a key element in maximizing L-glutaminase synthesis and cell growth. Following 48 hours of incubation, *Pseudomonas* sp. RAS123 produced the highest amount of L-glutaminase (49.811 U/mL), as shown by the results in **Figure 2**. The simultaneous observation of growth and an increase in extracellular protein may suggest the involvement of growth-associated enzyme synthesis. Based on the present results, the 48-hour incubation period was selected for the next experiment.

3.2.2. Effect of some physio-chemical conditions

The results recorded in **Figure 3a** indicated that an initial pH of 7.0 was the optimum pH, yielding maximum L-glutaminase production (49.617 U/mL), extracellular protein (0.288 mg/mL), and growth (1.201). The initial pH value level below or above 7.0 had an adverse effect on L-glutaminase production as well as growth. At acidic pH (4.0), the production of L-glutaminase declined to around 47.7% of that acquired at the initial pH of 7.0. The results recorded in **Figure 3b** revealed that the growth and L-glutaminase activity, together with extracellular protein, were affected by the inoculum volume of bacterial suspension. The maximal L-glutaminase activity (49.845 U/mL) was obtained in cultures inoculated with 0.5 mL bacterial suspension. However, the lowest L-glutaminase activity (28.248 U/mL) was obtained in cultures inoculated with 3.0 mL bacterial suspension. The data presented in **Figure 3c** showed that *Pseudomonas* sp. RAS123 cultures can grow on different sugars as a sole carbon source and produce L-glutaminase enzyme with different levels. Glucose was the optimal carbon source, yielding a maximal L-glutaminase activity (49.678 U/mL). However, the minimal L-glutaminase activity (24.543 U/mL) was in cultures growing on mannitol.
The results recorded in **Figure 3d** showed that all the investigated nitrogen sources exhibited L-glutaminase activity, lower than that acquired when using L-glutamine (50.179 U/mL) in the modified M-9 medium. Sodium nitrate was the most preferable nitrogen source after L-glutamine, giving approximately high L-glutaminase activity (42.922 U/mL). Meanwhile, the minimal L-glutaminase activity (4.561 U/mL) was found in cultures using yeast extract as a nitrogen source.

3.3. Plackett-Burman experimental design

The design-derived data are listed in Table 4, where the enzyme activity is in the range from 34.545 to 70.56 U/mL. To highlight the most significant, the t-test for two unequal variances was computed. The difference between the mean values obtained at the high (+1) and low (−1) settings for each element was referred to as the main effect. The main effect of the independent variables was computed using these data, and the findings are shown graphically in **Figure 4**.

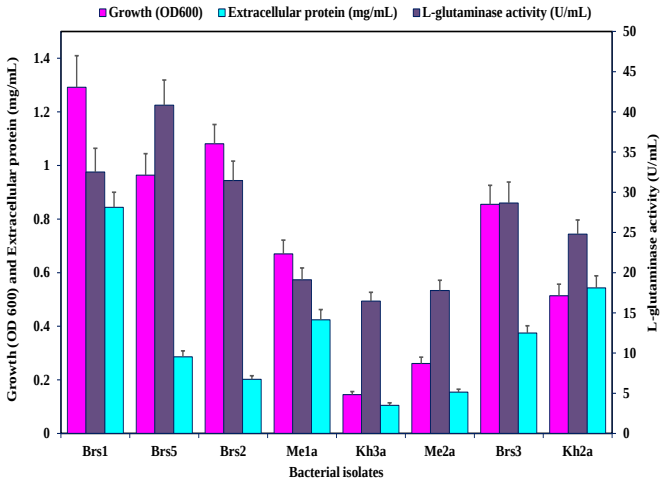

Figure 1. L-glutaminase production by bacterial isolates. Cultures were incubated at 35°C under shaking conditions for 72 h of incubation. Growth was measured spectrophotometrically at 600 nm, extracellular protein was quantified (mg/mL), and L-glutaminase activity was determined (U/mL). Error bars indicate standard deviations (n = 3).

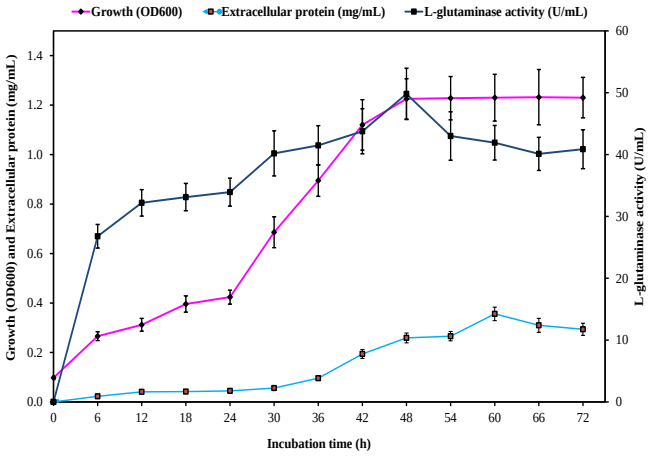

Figure 2. Effect of different incubation times on bacterial growth, extracellular protein production, and L-glutaminase activity by *Pseudomonas* sp. RAS123 cultures. Cultures were incubated at 35°C under shaking conditions over 72 h of incubation. Growth was measured spectrophotometrically at 600 nm, extracellular protein was quantified (mg/mL), and L-glutaminase activity was determined (U/mL). Error bars indicate standard deviations (n = 3).

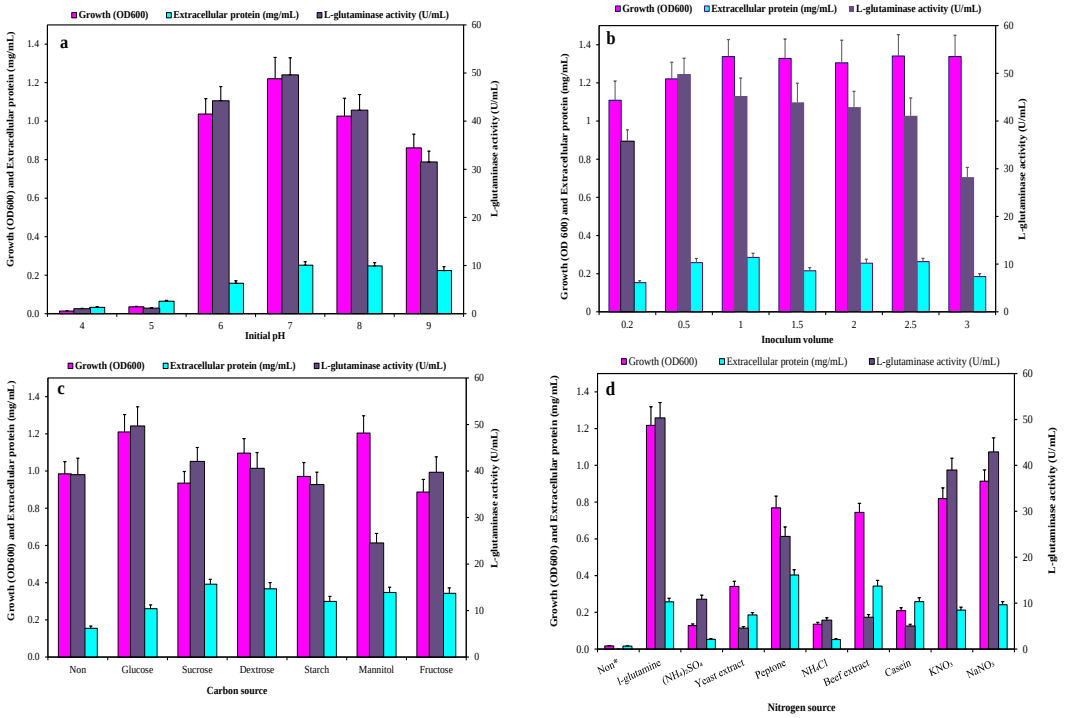

Figure 3. Effect of initial pH value (a), inoculum volume (b), different carbon sources (c), and different nitrogen sources (d) on bacterial growth, extracellular protein production, and L-glutaminase activity by *Pseudomonas* sp. RAS123 cultures. Cultures were incubated at 35°C under shaking conditions for 48 h of incubation. Growth was measured spectrophotometrically at 600 nm, extracellular protein was quantified (mg/mL), and L-glutaminase activity was determined (U/mL). Error bars indicate standard deviations (n = 3).

Table 4. Results of PBD experiment for *Pseudomonas* sp. RAS123's synthesis of L-glutaminase

Trial	Na_2	K	Na	Glut	Mg	Ca	Glu	Extracellular protein (mg/mL)	L-glutaminase activity (U/mL)
1	-1	-1	-1	1	1	1	-1	0.414	56.154
2	1	-1	-1	-1	-1	1	1	0.179	43.071
3	-1	1	-1	-1	1	-1	1	0.155	46.305
4	1	1	-1	1	-1	-1	-1	0.410	65.562
5	-1	-1	1	1	-1	-1	1	0.419	70.560
6	1	-1	1	-1	1	-1	-1	0.172	34.545
7	-1	1	1	-1	-1	1	-1	0.248	41.307
8	1	1	1	1	1	1	1	0.229	46.893
9	0	0	0	0	0	0	0	0.254	50.450

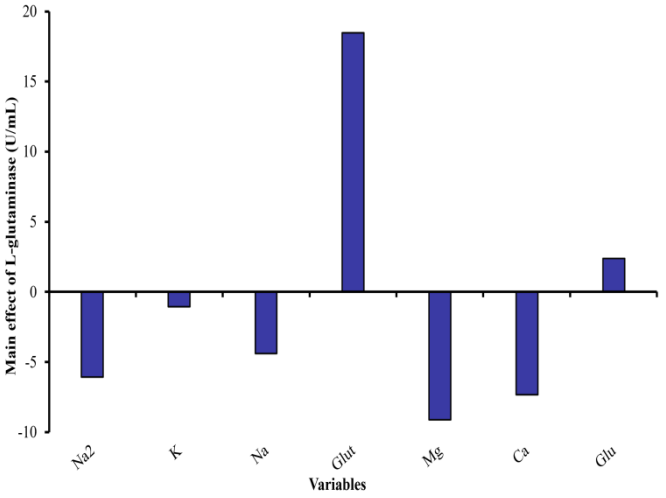

Figure 4. Identification of the fermentation parameters that influence the synthesis of L-glutaminase. The y-axis is the main effect of variable on L-glutaminase activity (U/mL). A positive bar means that the variable has a positive influence on the enzyme activity and a negative bar means that the variable has a negative influence on the enzyme activity. As the effect is of a greater size, the height of the bar increases accordingly.

A positive main effect value implies that a higher concentration of the variable is near optimum, while a negative primary effect value suggests that a lower concentration yields better results. If the mean effect is near zero, it signifies minimal or no impact from the factor; these findings are confirmed by **Figure 5**. The most significant factor that favorably impacted L-glutaminase activity within the test ranges, according to the computed t-test Table 5, was L-glutamine, whereas $MgSO_4.7H_2O$ and $CaCl_2$ had a negative impact on L-glutaminase production, while glucose, Na_2HPO_4, NaCl, and KH_2PO_4 have little influence **Figure 4**. Coefficient (R2) determination was employed in assessing the model's goodness of fit. The R2 value in such a case was determined to be 0.99997, meaning that this model may explain 99.997% of the response's overall variability and that only 0.003% of it could not be explained. A regression model is said to have a very strong correlation if its R2 value is near 1.0. As a result, the current R2 value indicated that the model is dependable for predicting L-glutaminase synthesis and showed a very excellent fit between the observed and anticipated responses.

The following formulation (g/l) is considered near-optimal according to the information collected from PBD experimental findings: Na_2HPO_4 , 6.0; KH_2PO_4, 3.0; NaCl, 20.5; L-glutamine, 7.5; $MgSO_47H_2O$, 0.25; $CaCl_2$, 0.075, glucose, 2.0, After adjusting the medium to pH 7, the flask was incubated at 35°C for two days in a rotary shaker operating at 120 rpm.

A confirmation experiment was conducted to ascertain the correctness of the PBD screening approach that was used. Following the analysis and comparison of the expected near-optimal levels of independent variables with the baseline settings, Table 6 shows the L-glutaminase production. Approximately 80.364 U/mL of L-glutaminase was produced at the near-optimal conditions that were employed.

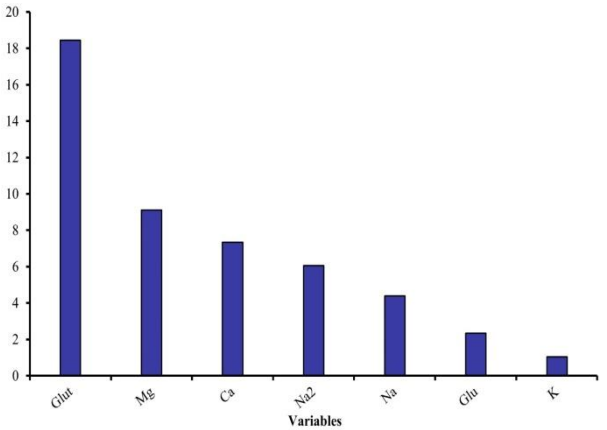

Figure 5. Packett-Burman Pareto chart illustrating how media elements affect L-glutaminase activity produced by *Pseudomonas* sp. RAS123. The x-axis displays the different variables that influence L-glutaminase activity. They are ranked in descending order of their effect.

3.4. Box–Behnken design

The effects of L-glutamine, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ concentration on L-glutaminase activity were evaluated. These variables' levels were adjusted to maximize L-glutaminase. Every variable was investigated at three distinct levels through a 15-trial experimental design: low (-1), middle (0), and high (+1). The response data obtained from the design following the measurement of the L-glutaminase activity was fitted to a second-order polynomial model. The following is the form of the polynomial equation:

$$Y = \beta0 + \beta1X_1 + \beta2X_2 + \beta3X_3 + \beta12X_1X_2 + \beta13X_1X_3 + \beta23X_2X_3 + \beta11X_1^2 + \beta22X_2^2 + \beta33X_3^2,$$

Where Y was the predicted response, β0 was the model constant; X1, X_2 and X_3 were independent variables; β1, β2 and β3 are linear coefficients; β12, β 13 and β 23 were cross product coefficients and β 11, β 22 and β 33 were the quadratic coefficients. The variables' design matrix and each trial's response, which is the activity of the L-glutaminase enzyme, are shown in Table 7.

The highest L-glutaminase production was found in trial number 1 (123.403 U/mL), then 7 and 11 with an improvement percentage of 153.56%, 122.52% and 123.41%, respectively, as shown by Table 7 as opposed to the control, which was derived from the PBD that had been previously optimized.

The analysis of variance (ANOVA) for the fitted quadratic polynomial model is also presented in Table 8, while the analysis of variance (ANOVA) for the response quadratic model is shown in Table 9.

Table 5. Statistical analysis of PBD experiment

Intercept	Coefficients	Standard Error	t- Stat	Main effect	P-value
	50.539	0.03130	1614.18		0.00039
Na_2	-3.0320	0.03320	-91.2986	-6.064	0.00697
K	-0.5330	0.03320	-16.0464	-1.066	0.03962
Na	-2.2230	0.03323	-66.9523	-4.402	0.00951
Glut	3.000	0.03320	278.3225	18.44	0.00229
Mg	-4.5750	0.0332	-137.778	-9.111	0.00469
Ca	-3.6930	0.03320	-111.218	-7.342	0.00579
Glu	1.1576	0.03320	34.85947	2.36	0.01826

Table 6. Power generation verification experiment comparing pre-optimized and basal media

Media applied in verification experiment	L-glutaminase activity (U/mL)	Improvement (%)
Basal medium	50.450	0
Pre-optimized medium	80.364	159.29

Table 7. Experimental results of the BBD for producing L-glutaminase by *Pseudomonas* sp. RAS123

Trial	Glut	Mg	Na	X1X2	X1X3	X2X3	$X1^2$	$X2^2$	$X3^2$	L-glutaminase activity (U/mL)
	X1	X2	X3							
1	0	1	-1	0	0	-1	0	1	1	123.403
2	1	1	0	1	0	0	1	1	0	45.990
3	1	-1	0	-1	0	0	1	1	0	54.948
4	-1	-1	0	1	0	0	1	1	0	26.098
5	1	0	1	0	1	0	1	0	1	58.667
6	1	-1	1	-1	1	-1	1	1	1	39.736
7	0	0	-1	0	0	0	0	0	1	98.465
8	1	0	1	0	1	0	1	0	1	60.904
9	-1	0	-1	0	1	0	1	0	1	50.945
10	-1	1	0	-1	0	0	1	1	0	38.253
11	-1	-1	-1	1	1	1	1	1	1	99.178
12	0	1	1	0	0	1	0	1	1	76.133
13	0	0	0	0	0	0	0	0	0	80.139
14	0	0	0	0	0	0	0	0	0	81.729
15	0	0	0	0	0	0	0	0	0	81.503

Table 8. Analysis of variance for the fitted quadratic polynomial model

	Coefficients	Standard Error	t- Stat	P-value
Intercept	81.1235	7.0053	11.5802	0.0001
X1(L-glutamine)	-20.3446	4.2899	-4.7425	0.0051
X2($MgSO_4.7H_2O$)	-15.5105	4.2899	-3.6156	0.0153
X3($CaCl_2$)	18.3848	4.2899	4.2856	0.0078
X1X2	12.1411	6.0668	2.0012	0.1018
X1X3	-4.6574	6.0668	-0.7677	0.4773
X2X3	-2.5879	6.0668	-0.4266	0.6874
X1X1	-10.0990	6.3145	-1.5993	0.1706
X2X2	-8.4147	6.3145	-1.3326	0.2402
X3X3	-6.5815	6.3145	-1.0423	0.3450

Table 9. Analysis of variance (ANOVA) for the response quadratic model

	Df	*SS*	*MS*	*F*	*Significance F*
Regression	9	9339.537	1037.726	7.048602	0.022302307
Residual	5	736.1221	147.2244		
Total	15	10075.66			

*R2= 92.69 %, R2 (pred) = 99.93 %, R2 (adj) = 99.62 %

Df degrees of freedom, SS sum of squares, MS: mean square.

The R2 value (0.9269), a reliable indicator of being near unity, describes the behavior of all experimental data. The high degree of accuracy of the polynomial model, which indicates a high degree of fitting between anticipated and experimental data, was shown by the statistical assessment of the design.

By applying quadratic regression analysis to the experimental data, the synthesis of L-glutaminase was shown to be explained by the following equation:

$$\mathbf{Y}=81.1235-20.3446*X_1-15.5105*X_2+18.3848*X_3+12.1411*\mathbf{X_1X_2}-4.6574*\ \mathbf{X_1X_3}-2.5879*\mathbf{X_2X_3}-10.0990\ \mathbf{X_1^2}-8.4147*\ \mathbf{X_2^2}-6.5815*\mathbf{X_3^2}$$

where X1, X2, and X3 are the concentrations of the independent variables as indicated in Table 7, and Y is the dependent variable (L-glutaminase synthesis) in U/mL.

An optimal response was found at the concentrations of L-glutamine 0.375%, $MgSO_4.7H_2O$ 0.0125%, and $CaCl_2$ 0.01125%, with a predicted activity of 126.19 U/mL, when the model was solved using the values from Table 8.

Another approach to solving the problem was the graphical method, which is shown in **Figure 6 (a, b, & c)**. This method was used to create a three-dimensional drawing of the polynomial equations using the Statistical 10 software.

Figure 6a showed the impacts of $MgSO_4.7H_2O$ and L-glutamine levels on the production of L-glutaminase (U/mL). The highest activity was clearly observed at 2.5 and 6.75%, respectively. **Figure 6 (b & c)** revealed the response surface plot for the activity of L-glutaminase (U/mL) as a function of $CaCl_2$ with L-glutamine and $MgSO_4.7H_2O$ concentration, respectively. A positive effect of $CaCl_2$ level can be seen. Maximum activity of L-glutaminase (U/mL) was obtained at 2.5%.

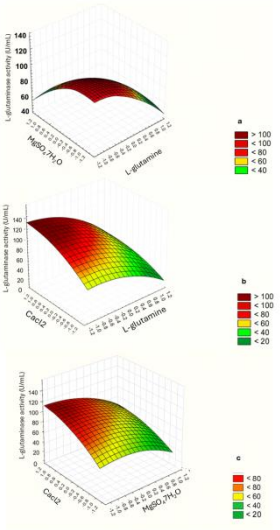

Figure 6. 3D response surface plots showing the combined effects of medium components on L-glutaminase production by *Pseudomonas* sp. RAS123. (a) Increasing $MgSO_4{\cdot}7H_2O$ up to ~2.5 g/L with L-glutamine (~6.5–7.0 g/L) resulted in maximum enzyme activity (>100 U/mL). **(b)** A similar positive interaction was observed between $CaCl_2$ and L-glutamine, though higher $CaCl_2$ concentrations led to inhibition. **(c)** The interaction between $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ showed an optimum at moderate levels of both salts, beyond which enzyme activity declined. The legend next to each plot shows the activity values corresponding to different colors. Red and orange indicate the highest L-glutaminase activity, green and blue indicate lower activity.

According to PBD and BBD results, the following medium composition is anticipated to produce an optimal response for L-glutaminase activity (g/L): L-glutamine, 3.75; Na_2HPO_4, 6.0; NaCl, 20.5; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.125; $CaCl_2$, 0.1125; and glucose, 2. After adjusting the pH to 7 and inoculating the medium with 0.5 mL of bacterial suspension, the flasks were incubated at 35°C at 120 rpm on a rotary shaker. Following two days of incubation, L-glutaminase output was calculated using the basal culture media as a control in a confirmatory experiment to determine the maximum L-glutaminase synthesis under the anticipated ideal conditions. Under the optimum conditions, enzyme activity was 124.25 U/mL. The model's accuracy and validity were further confirmed by matching the measured L-glutaminase activity (124.25 U/mL) under optimal conditions with the anticipated one (126.19 U/mL) **(**Table 10**).**

Table 10. Verification experiment for L-glutaminase production on basal versus optimized medium

Media	L-glutaminase activity (U/mL)	Improvement (%)
Basal medium	50.612	0
Pre-optimized	80.364	159.41
Optimized medium	124.25	245.495

4. Discussion

One of the most common microbial enzymes in anticancer treatment is L-glutaminase, which has a growing market share of 16.5%. The need for microbial enzymes in the pharmaceutical and medical industries has grown because of the new options they have created for dealing with many cancers, such as breast cancer, lymphosarcoma, and leukemia [20]. The use of microbiological sources (bacteria and fungi) to produce L-glutaminase has expanded in recent years because of its value as a cost-effective agent in cancer treatment, the food industry, and biosensors for monitoring L-glutamine levels. To identify the best enzyme producers that provide a greater yield with special qualities for usage in industrial and medicinal applications, L-glutaminase production from microorganisms is screened [21, 22].

Marine bacterial isolates from Brullus Lake, Kafr El-Sheik, were selected in this study. They were tested for synthesis of L-glutaminase using the M-9 medium, a selective medium that contains L-glutamine and phenol red as an indicator. The pink area, which is developed around the colony in the eight isolates, indicated positive findings [23]. After secondary screening, the isolate that gave the best L-glutaminase activity was chosen in this study and identified as *Pseudomonas* sp. RAS123 [14]. It then underwent additional testing. The strain also showed several other benefits compared to other marine isolates screened in this work. It grew and secreted enzymes successfully over a broad range of pH conditions and has a relatively short incubation time to achieve optimal enzyme production, minimizing the time and cost of production. In addition, the genomic identification and deposition in GenBank can serve as a reference in future optimization and recombinant expression attempts. Yet, a drawback is that species of *Pseudomonas* can be a biosafety concern in large-scale applications, as they are opportunistic pathogens, requiring a high level of strain safety assessment prior to pharmaceutical application.

It is thought that *Pseudomonas* spp. are a good L-glutaminase source. Divatar et al. [24] isolated L-glutaminase from *Pseudomonas* sp. KLM9, Kumar and Chandrashekaran reported the isolation of *Pseudomonas* BTMS-51 [25], and Jyothi et al. described the isolation of *Pseudomonas* VJ6 [26].

The nature of marine microorganisms is unique to that of their terrestrial counterparts.Many investigators reported L-glutaminase production from marine bacteria [25, 27–30]. The marine microorganisms have been adapted to tolerate harsh environmental factors such as salinity, changing pH, low nutrient levels, and changing temperatures. Such selective pressures can commonly lead to remarkably halotolerant, thermostable, and catalytically efficient enzymes useful in industrial as well as therapeutic uses [31]. It is also noted that marine-derived enzymes are becoming widely appreciated due to their biocompatibility, environmentally friendly generation, and use in pharmaceutical activities, especially cancer therapy and biotransformations [32].

Pseudomonas sp. RAS123 was tested for L-glutaminase enzyme production at different incubation times. The late exponential phase (48 h) exhibited the maximum enzyme activity; after that, the activity declined. The decreased activity might be owed to either the buildup of autotoxic metabolites of the bacteria in the media or the depletion of nutrients in the medium. Another possible explanation is the release of proteolytic proteins, which break down peptide links between amino acids and produce protein denaturation [33]. In a partial concurrence with our findings, *Bacillus cereus* MTCC 1305 cultures produced the maximum L-glutaminase activity after 40 h of incubation, according to Singh et al. [34]. However, *Bacillus* sp. required a fermentation time of 24 h to achieve the best L-glutaminase activity as investigated by Sinha and Nigam [35], while *Pseudomonas* VJ6 required 72 h [26]. L-glutaminase production by *Pseudomonas* sp. RAS123 is highly affected by the fermentation medium's pH, which has a crucial role for the passage of different elements throughout the cell membrane and for regulating the cell's metabolic activities, or due to their role in medium substrate solubility, ionization, and availability for microbial development and, consequently, product production [36]. Our findings agreed with those of earlier studies that revealed that the synthesis of L-glutaminase is favorable at a pH between 6.5 and 7.5 [23, 37, 38]. On the contrary, Bülbül and Karakuş [39] found that the optimal pH for maximum activity of L-glutaminase from *Hypocrea jecorina* was pH 8. The size of the initial inoculum can also influence growth and a variety of metabolic processes that result in the production of extracellular products and total biomass [40]. High inoculum densities can either accumulate specific non-volatile self-inhibiting chemicals that prevent the synthesis of products or produce excessive biomass that exhausts the substrate of nutrients, while a low inoculum density might yield insufficient biomass, resulting in decreased product formation [41]. Similarly to our finding, for the highest L-glutaminase production, Patel et al. [30] utilized 1% of the inoculum size of *Halomonas hydrothermalis* B-15-9-2 cells. This result is different than that reported by Sathish and Prakasham [42] for *Bacillus subtilis* RSPGLU (2%), Jambulingam *et al*. [43] for *B. subtilis* JK-79 (3%), and Orabi

et al. [44] for *B. subtilis* OHEM11 (4%). Sources of carbon and nitrogen provide the building blocks required for the synthesis of nucleic acids, proteins, and components of cell walls [45]. Our outcomes align with Jyothi et al. [26] and Desai et al. [37], who utilized glucose as a highly efficient carbon source to increase the L-glutaminase production. On the other hand, other sugars such as maltose were used by other investigators [46]. Also, the data showed that extracellular protein and enzyme activity were affected differently by the nitrogen source used. The results obtained aligned with several findings that reported the stimulatory effect of L-glutamine as a nitrogen source for *Pseudomonas aeruginosa* BGNAS-5 L-glutaminase synthesis [46] and *Streptomyces enissocaesilis* DMQ-2 [47].

Various optimization strategy phases, such as determining the best conditions for screening tests and targeted response(s), involve the use of statistical designs. They have been in use for many decades [12, 48- 49]. The results of statistically designed experiments are more widely convincing than those of the traditional one-variable-at-a-time method. PBD and response surface approaches with different designs are two examples of statistical designs that are applied. Li et al. [50] demonstrated that the surface restricted in the contour diagram's smallest ellipse represented the highest expected value.

In a small number of studies, the PBD was utilized to screen the most important variables for maximum L-glutaminase synthesis and determine their likely best levels [51]. Of the seven factors, L-glutamine had a significant favorable impact on the production of L-glutaminase. Alternatively, L-glutaminase synthesis by *Pseudomonas* sp. RAS123 cells had a significantly negative effect from the addition of mineral salts ($CaCl_2$ and $MgSO_4.7H_2O$) to the substrate. A previous study showed that Ca^{2+} and Mg^{2+} stimulated L-glutaminase activity to a small degree (5 and 12 mM, respectively), suggesting that the stimulation may reach a limit or even decrease with increasing ion concentration [52]. Several other reports indicated that low millimolar concentrations of divalent cations (Mg^{2+} and Ca^{2+}) can activate L-glutaminase; however, higher concentrations can be inhibitory for a variety of enzymes, and this perturbation can be rationalized by changes in binding, assembly, or ionic imbalance [53, 54]. This result showed a 1.59-fold increase in enzyme synthesis relative to the average of the values reported under the ambient condition. In accordance with our results, Jesuraj *et al*. [3] used PBD for optimizing fermentation medium and reported that L-glutamine and glucose positively influenced L-glutaminase production by *Aeromonas veronii*.

The concentrations of the three independent variables were further examined using BBD in the second optimization step. Many investigators used BBD for optimization of microbial enzymes by various microorganisms [12, 55–58]. Our findings unequivocally show that the BBD's fitted model offered a sufficient approximation of the actual system. Parity plots, a validation experiment, and the regression coefficient's value of 0.9269 were used to determine this. When compared to the pre-optimization settings, the average output of L-glutaminase by *Pseudomonas* sp. RAS123 culture has increased by 2.45 times under these optimal conditions, reaching about 124.25 U/mL, which is thus competitive or even superior to several bacterial and fungal producing strains, showing that it can be a good, efficient source of biomedical enzyme product. As an example, *S. griseorubens* [51] created an extracellular output of L-glutaminase in the range of about 35.96 U/mL, whereas *Halomonas hydrothermalis* B-15-9-2 [30] reached an extracellular L-glutaminase output of about 98.95 U/mL under optimal conditions. While *Streptomyces sp.* strain 5M tends to yield less (approximately 6.33 U/mL) [59].

5. Conclusion

The present study precedes our previous work on the purification and application of L-glutaminase from *Pseudomonas* sp. RAS123 by introducing systematic optimization of culture conditions for the first time to the investigated strain to improve enzyme production, thus closing the gap between characterization in the laboratory and scalable bioprocess development. OVAT and statistical techniques were used to enhance the L-glutaminase production; these techniques assisted in identifying the significant production media variables and their optimal levels for the greatest yield of extracellular L-glutaminase. Allowing the bacterial strain to grow with 1% inoculum on an optimized medium containing L-glutamine, glucose, $MgSO_4$, and $CaCl_2$, at the following concentrations, respectively 3.75, 2, 0.125, and 0.1125 gm, while other nutrients were at basal conditions, at pH 7.0, and incubating for two days at 35°C on a rotary shaker (120 rpm) produced the highest L-glutaminase yield. The enzyme activity increased to 124.25 U/mL following optimization. These findings showed that compared to the basal condition, the optimized conditions enhanced the L-glutaminase production by almost 2.45 times. The findings showed that the potent strain's extracellular L-glutaminase production may be raised by using the statistically optimized fermentation technique.

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