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Optimization studies of L-glutaminase production from *Halomonas hydrothermalis* B-15-9-2 isolated along Gujarat coasts

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L-glutaminase (EC 3.5.1.2) is an enzyme grouped under amidohydrolase and a member of serine-dependent β-lactamases & penicillin-binding proteins. The present study focuses on increasing the yield of L-glutaminase from marine bacteria isolated from coastal regions of Gujarat. Using enrichment culture technique marine samples were enriched and physicochemical analysis was done. A total of 12 bacteria isolated from Gujarat coasts were screened for L-glutaminase biosynthesis. Out of 12, most potent one isolate B-15-9-2 was selected for cultural, biochemical, and molecular identification by 16S rRNA analysis, which reveals the nearest homolog is *Halomonas hydrothermalis* with 99.87% similarity. To the best of our knowledge this is the first report of *Halomonas hydrothermalis* as L-glutaminase producer from Gujarat coasts. Further study deals with the optimization of medium components for higher yield of L-glutaminase by one variable at a time & response surface methodology. There was a significant increase in the L-glutaminase activity about 3.4-fold from 29 ± 0.8 U/mL to 98.95 ± 0.5 U/mL upon media optimization *via* statistical method.

Keywords: Halophiles, Marine enzymes, Molecular identification, Physicochemical analysis, Response surface methodology

Three-fourths of the Earth's glaze is covered by marine environment. It contains a high concentration of saline water with various salt and mineral combinations[1]. The sea environment is rich in varied living forms, from micro to macro. Marine microorganisms can be found anywhere in the marine environment, from sea ice in Polar Regions to temperatures as high as 100°C, in deep-sea hydrothermal vents, and from coastal to offshore regions[2,3]. Because of the dynamic system in the ocean, marine microorganisms have evolved unique physiological and metabolic capacities that allow them to survive in extreme environments such as pressure, salinity, and temperature[4]. As a result, in recent years, there has emerged an interest among academics in harnessing the potential of marine organisms for their novel primary and secondary metabolites. Terrestrial ecosystems have been filled with an abundance of bacteria that produce bioactive substances, but their potential for new metabolites is declining. Thus, there is an urgent need to search for the variety of microbial species that exist in marine habitats, opening the way for the finding of a novel molecule with unique properties[5].

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Marine habitat provides a suitable environment for extremophiles to possess unique biochemical pathways and characteristics[6]. Due to various adaptation strategies employed by extremophiles, it has become a focused area of research amongst the scientific community due to its vast applications. These demands enhance the requirement for biotechnology refinement and the discovery of new enzymes from unusual environments, such as marine ecosystems[7].

L-glutaminase, one of the numerous metabolites produced by marine microorganisms, has received a lot of attention for its potential applications in the food and pharmaceutical industries. The salt-tolerant ability has received the greatest attention due to its usefulness in the food industry for producing flavorful protein molecules at elevated salt concentrations. Marine microorganisms are a prospective source of novel thermal and salt-stable enzymes[8].

L-glutamine amidohydrolase (EC 3.5.1.2) belongs to the family of serine-dependent β-lactamases & penicillin-binding proteins. The enzyme is widely distributed amongst numerous life forms & it catalyzes the deamination process in which L-glutamine is transformed into L-glutamate and ammonia. L-glutaminase improves the distribution of

adequate nitrogen and carbon sources for the synthesis of different structural units of DNA and proteins. However, there is a significant increase in L-glutaminase enzymes with distinctive features that tolerate harsh industrial processes. The halophilic L-glutaminase retains more glutamic and aspartic acid residues than non-halophiles.

L-glutaminase has a wide range of applications in various industries. Commercially, most of the glutaminases in the market are produced for analytical purposes few are used in the food industry as a flavor enhancer. Use of this enzyme in cancer therapy depletes essential amino acids that retard the progression of cancerous cells, The *in vitro* anti-proliferative activity of L-glutaminases against two cancer cell lines having IC_{50} value less than 10 µg/mL & antioxidant properties as well (our unpublished work), indicates the potential use of unique L-glutaminases from Gujarat coasts in cancer therapy. The product of this enzyme, glutamic acid is a precursor for the synthesis of glutathione, an antioxidant[9]. The anti-retroviral activity of L-glutaminase has also been reported by Roberts & McGregor[10]. In biosensors development for examining the glutamine levels in hybridoma cell cultures, L-glutaminase is used[11]. Application of L-glutaminase in food for enhancing soy sauce quality with desirable seasoning increases glutamic acid content which subsequently results in enhancement of flavor due to umami content. The food production processes work at extreme temperatures and varied ranges of pH and salt concentration, so for the application of L-glutaminase in the food industry[12] thermos and salt-stable L-glutaminase enzymes are needed.

Materials and Methods

Materials

L-glutamine, Zobell marine broth, Trichloro acetic acid, Agar-Agar, Sodium Chloride, Nessler's Reagent, and Dialysis Membrane (Hi Media Pvt. Ltd., Mumbai, India); K_2HPO_4, KH_2PO_4, Na_2CO_3 (Sisco Research Laboratories Pvt. Ltd., Mumbai, India). All the other chemicals and reagents used in the present study were of analytical grade unless otherwise stated.

Collection of the samples

For isolation, the marine soil samples were collected from the Bhavnagar coastal region of Gujarat, India. The samples were collected in sterile bags & were refrigerated at 4°C till further processing. The Physicochemical parameters were studied as per the method BIS (Bureau of Indian Standards) (Table 1).

Isolation & enrichment

Enrichment culture technique is generally done to support and enrich the growth of marine bacteria. For the isolation of halophiles generally Zobell marine medium containing variety of salts in trace amount used, that favors the growth of halophiles. By employing the enrichment culture technique, the marine samples proceeded using Zobell marine broth (ZMB), Composition (g/L) Peptone 5.0, Sodium chloride 19.45, Sodium sulphate 3.24, Magnesium chloride 8.80, Calcium chloride anhydrous 1.80, Potassium chloride 0.55, Sodium bicarbonate 0.16, Potassium bromide 0.08, Strontium chloride 0.034, Boric acid 0.022, Sodium silicate 0.004, Yeast extract 1.0, Ferric citrate 0.10, Sodium fluorate 0.0024, Ammonium nitrate 0.0016, Disodium hydrogen phosphate 0.008, Final pH 7.6 ± 0.2. The 5% *w/v* soil samples were inoculated in ZMB with varying NaCl (5-25%); with pH 7-10, later on, all flasks were incubated on a rotary shaker at 150 rpm for 72 h at 37±2°C. Later on, enriched samples were serially diluted and isolated using the spread plate technique. Zobell marine agar plates were incubated at 37±2°C for 24-48 h & observed for a morphologically distinct colony.

Table 1 — Physicochemical analysis of marine soil samples

Physico-chemical parameters	Sample-1 Concentration	Sample-2 Concentration	Unit	Method
E.C. (Electrical Conductivity)	110.60	249.50	dS/m	EC Meter
pH	8.02	6.86	----	pH Meter
O.C	2.25	4.85	%	Walkley & Black
P (Phosphorus)	28.50	5.08	ppm	Olsen's Method
K (Potassium)	875	195	ppm	Flame Photometer
S (Sulfur)	859.3	532.2	ppm	Turbidity
N (Nitrogen)	6.93	23.68	ppm	Alkaline $KMnO_4$ Method
Na (Sodium)	1,25,500	8,23,750	ppm	Flame Photometer

Screening of L-glutaminase-producing marine bacteria

The primary screening was done using Minimal glutamine broth (MGB) Composition (g/L) L- glutamine 5, NaCl 50, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, $ZnSO_4.7H_2O$ 0.1, $FeSO_4.7H_2O$ 0.1, Phenol red 0.0012, pH 6.5 adjusted using 20% *w/v* Na_2CO_3, which contains L-glutamine as a sole carbon and nitrogen source. For the primary screening of 18-24 h, an active culture of isolates was inoculated into minimal glutamine broth and incubated at 37±2°C for 24-48 h. A marked color change of the medium from yellow to pink indicates a positive result for L-glutaminase secretion[13]. In the process, L-glutaminase acts on the substrate and releases L-glutamate along with ammonia into the reaction medium which results in a color change of the medium from yellow to pink due to alkaline pH.

Characterization & molecular identification

Morphological & biochemical characterization

The cultural and morphological characteristics were studied from Zobell marine agar medium & by Gram staining method as invented by Hans Christian Gram[14]and also by Potassium hydroxide test (KOH /String Test). The biochemical studies were performed as described by researchers Cappuccino & Sherman[15],which involves citrate utilization, triple sugar iron, methyl red, Voges-Proskauer, urea hydrolysis, deamination, indole production, hydrogen sulfide, tests catalase, oxidase, ammonia, nitrate reduction, Ox-Ferm (Oxidative Fermentative Test), Starch hydrolysis, Casein hydrolysis, Gelatin, and Lipid utilization as well as the fermentation of the various sugars such as Raffinose, Dulcitol, Salicin, Galactose, Fructose, Rhamnose, Lactose, Dextrose, Mannose, Mannitol, Trehalose, Melibiose, Inulin, Inositol, Xylose, Maltose, Adonitol, Cellobiose, Arabinose, Sucrose, Sorbitol. Because of the halophilic nature of the organism, all the biochemical media were supplemented with 5% (*w/v*) NaCl. The 100 µL active culture was inoculated to the respective biochemical medium and incubated at 37±2°C for 24-48 h and results were subsequently observed.

Molecular identification

The 16S rDNA sequence analysis of the B-15-9-2 isolate was done using a 27F & 1492R set of primers. Analysis of sequence is done in the National Center for Biotechnology Information (NCBI), Gene Bank Database using the Basic Local Alignment Search Tool for Nucleotide[16] BLASTn tool (http://www.ncbi.nlm.nih.gov/blast). B-15-9-2 Sequence is submitted in NCBI GenBank & Phylogenetic tree was constructed by Neighbor-Joining Method using MEGA 11 Software[17].

Inoculum preparation

A loop full of culture was inoculated into a 250 mL flask containing 50 mL modified Zobell marine broth containing 1% glutamine as an additional supplement & incubated at 37±2°C, 150 rpm for 18-20 h. Then inoculum is added into the production medium under aseptic conditions after measuring the optical density (OD) of 0.9 ± 0.1 at 600 nm.

L-glutaminase assay & protein estimation

L-glutaminase activity was measured by the Nesslerization assay method determined[18]with minor modifications using L-glutamine as a substrate. A 500 µL of the suitably diluted enzyme was incubated with 500 µL of 0.2 M L-glutamine & 1000 µL of phosphate buffer (0.01 M) with pH 7.0. Then it is incubated at 37±1°C for 30 min. Later on, the reaction was arrested with the addition of 500 µL, 1.5 M Tri-chloro-acetic acid (TCA). Now from the previous system take 100 µL reaction mixture, add 3700 µL of distilled water & 200 µL of Nessler's reagent. Finally, at 480 nm the absorbance was measured for ammonia produced. One unit was defined as the amount of L-glutaminase that produces one micromole of ammonia per minute under standard assay conditions. All the assays were performed in triplicates and enzyme activity was expressed as U/mL & measured using ammonium chloride (0-100 µg/mL) as a standard.

Total protein content was estimated using the method described by Lowry and co-workers[19]. The calibrated curve of protein was obtained using pure bovine serum albumin (0-100 µg/ mL) as a standard.

Optimization of L-glutaminase production

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

It is the method that optimizes the various parameters by keeping the previous optimum concentration constant. The optimization studies by one-variable-at-a-time isolates were done in a 250 mL flask containing 100 mL minimal glutamine broth. The flasks were inoculated and incubated at 37±2°C, 150 rpm & samples were withdrawn at a regular time interval of 24 h continuous monitoring of flask conditions for growth and enzyme production for 72-96 h was done. The various components of MGB were optimized. The parameters studied include the Inoculum size (1,2,3) % *v/v*; Organic nitrogen sources

such as beef extract, peptone & yeast extract at (1% *w/v*) concentration; L-glutamine (1 to 5 % *w/v*) concentration; Carbon sources such as glucose, sucrose, fructose (1% *w/v*) concentration; Inorganic nitrogen sources *i.e.*, ammonium sulfate, potassium nitrate, sodium nitrate (1% *w/v*) concentration; Sodium chloride concentration (1 to 5 % *w/v*).

Statistical optimization of media components

Based on the preliminary results, threesignificant variables viz. L-glutamine, Ammonium sulfate, and NaCl affecting L-glutaminase production were selected for optimization studies using CCD. The three independent variables were selected for CCD. The Central composite design (CCD) was generated using Design-Expert Version 13 (State Ease Inc. Minneapolis, MN, USA) to test the significance of variables on the production of L-glutaminase. Twenty experimental flasks containing sterile 100 mL production medium (pH 9 ± 0.2) with different nutrient compositions were prepared in 250 mL Erlenmeyer flask according to the design generated for CCD (Table 2). We inoculated all the flasks with 2% (*v/v*) active culture & incubated at 37±2°C, 150 rpm for 72 h. The ammonia produced (U/mL) in each flask was measured using the L-glutaminase assay method as described in the previous section. The response data obtained were analyzed using Design-Expert version 13 and analysis of variance (ANOVA) was calculated to check the linear as well as interaction effects of each medium component in the tested range. To determine the statistical significance of the model; the p-value and the coefficient of determination (R2) value were studied. To avoid systematic bias, the experiment was directed in a randomized order.

Validation of the experiment

For the validation of the study, an experimental run no. 2 suggesting response *i.e.*, 5.0 (g %) L-glutamine, 4.0 (g %) ammonium sulfate, 8.0 (g %) NaCl was kept for L-glutaminase production and incubated at 37 ± 2°C temperature, 150 rpm for 72 h by the numerical optimization process. Based on the optimized medium concentration partial purification of L-glutaminase was done.

Statistical analysis

All the experiments were performed in triplicates and data were represented as mean ± Standard deviation/error of the values.

Results

Isolation & enrichment

Two different marine soil samples were collected from the Bhavnagar region and designated as sample-1 (pH 8.02) (21.59 °N 72.29 °E) Near Nishkalank Mahadev temple & sample-2 (pH 6.86) (21.13°N, 71.97°E) Ucha Kotda Beach. The electricity conductivity, pH & salinity vary from 110.60-

Table 2 — L-glutaminase production by *Halomonas hydrothermalis* B-15-9-2 using Central composite design

Run No.	Components			L-glutaminase (U/mL)	
	A: L-glutamine (g%)	B: Ammonium sulfate (g%)	C: NaCl (g%)	Actual	Predicted
1	2.75	2.25	12	55	56.37
2	5	4	8	98.95	98.66
3	2.75	2.25	12	55	56.37
4	2.75	2.25	12	57	56.37
5	2.75	-0.693137	12	58.9	60.99
6	2.75	2.25	12	56	56.37
7	2.75	5.19314	12	76.94	77.94
8	0.5	0.5	16	73.1	71.15
9	0.5	0.5	8	58.65	57.32
10	6.53403	2.25	12	48.79	49.59
11	-1.03403	2.25	12	22.5	24.79
12	0.5	4	8	37	35.17
13	2.75	2.25	18.7272	68.9	70.03
14	2.75	2.25	12	57.13	56.37
15	5	0.5	8	55.3	53.64
16	5	4	16	80.3	79.44
17	5	0.5	16	37.5	37.15
18	2.75	2.25	5.27283	72.6	74.56
19	2.75	2.25	12	58.6	56.37
20	0.5	4	16	46.8	46.28

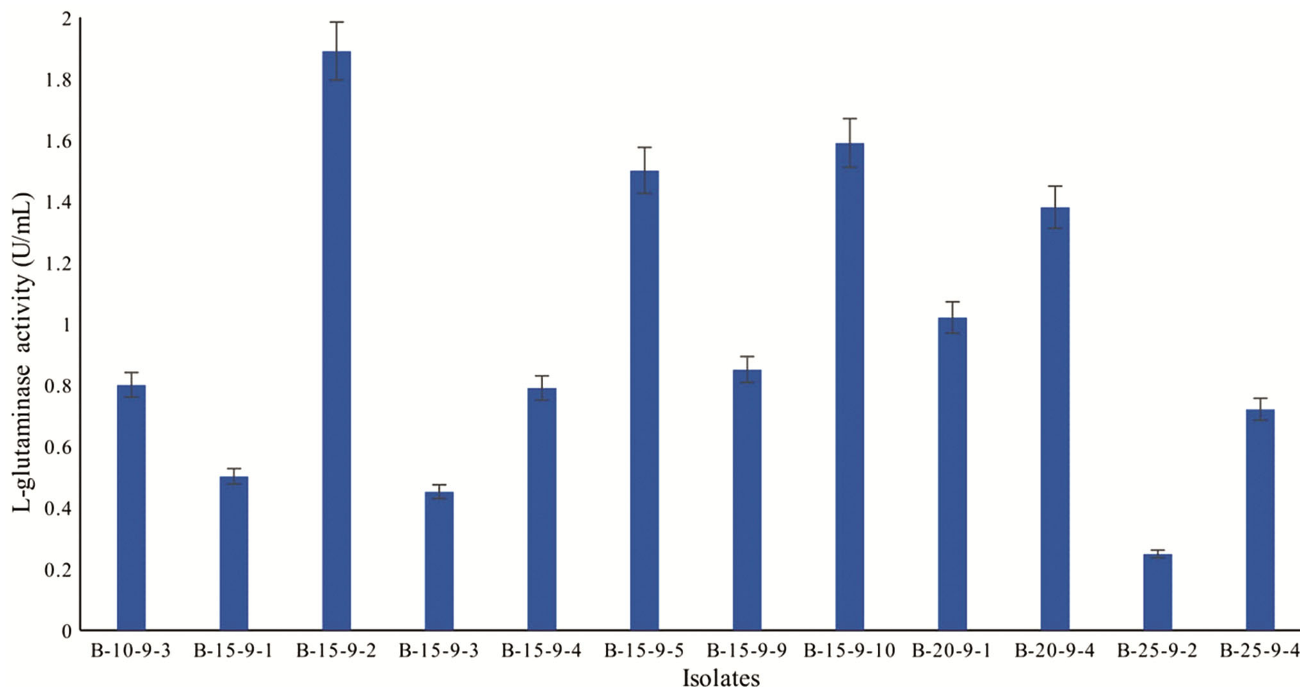


Fig. 1 — Secondary screening Values are mean ± standard deviation; n=3 samples

249.50 dS/m; 8.02-6.86; 1, 25,500 -8, 23,750 ppm, respectively. Both samples were dark brownish to moderate brownish in appearance. In both samples concentration of other parameters such as E.C. (Electrical Conductivity), Organic carbon content, P (Phosphorus), K (Potassium), S (Sulfur), and N (Nitrogen) also varies (Table 1). The higher pH and salinity indicate the alkaline nature of the samples. For enrichment Zobell marine broth with different pH (7-10) & NaCl ranges from (5-25% *w/v*) was done up to 72 h. Then the enriched samples were diluted serially and later spread onto Zobell marine agar of respective pH & NaCl concentration, and plates were incubated at 37±2°C for 24-48 h. Morphologically distinct 12 colonies were selected for further studies.

Screening of L-glutaminase-producing marine bacteria

Primary screening of marine bacteria secreting L-glutaminase was done using the broth assay method. A total of 12 distinct bacteria were screened out for their ability to utilize L-glutamine & produce ammonia along with glutamate. 100 µL of active cultures were inoculated into a tube containing 10 mL MGB and incubated at 37±2°C, 150 rpm on a rotary shaker for 48-72 h. After incubation, a visible color change from yellow (pH 6.5) to pink (pH up to 10) was observed, which indicates positive results for L-glutaminase secretion. Based on the growth rate, pH change, and color intensity 6 most potent isolates were selected for secondary screening by shake flask method using a minimal glutamine medium (Fig. 1). However, based on the results of secondary screening most potent isolate B-15-9-2 shows the maximum L-glutaminase production *i.e.*, 1.89 ± 0.5 U/mL is selected.

Table 3A — Cultural and morphological characteristics of B-15-9-2

Size	Small
Shape	Round
Margin	Entire
Elevation	Pulvinate
Consistency	Moist
Pigmentation	Creamish
Texture	Smooth
Opacity	Semi-Translucent
KOH Test	String formation
Gram's Reaction	Gram Negative; Thick Rods single & in chain

Characterization & molecular identification

The isolate B-15-9-2 cellular morphology *i.e.*, Gram-negative thick rods and colony and colony morphology is round with entire margin, moist consistency, smooth texture, semi-translucent, and creamy appearance with string formation in the KOH test (Table 3A). Further characterization is done by

Table 3B — Biochemical characterization of B-15-9-2

Biochemical Tests	Results
Citrate utilization test	+
Methyl red test	-
Voges-Proskauer test	+
Urea hydrolysis test	-
Deamination Test	-
Indole production	+
Hydrogen sulfide production	-
Triple sugar iron agar test	Butt: Alkaline Slant: Acidic Gas & H_2S: -
Nitrate production	+
Ammonia Production	+++
Starch Hydrolysis	-
Gelatin Hydrolysis	++
Casein Hydrolysis	-
Lipid Hydrolysis	-
Catalase	+
Oxidase	+++
Oxidative Fermentative Test	++
Sugar Fermentation Test	
Raffinose	-
Dulcitol	-
Salicin	-
Galactose	-
Fructose	-
Rhamnose	-
Lactose	-
Dextrose	-
Mannose	-
Mannitol	-
Trehalose	+++
Melibiose	-
Inulin	-
Inositol	-
Xylose	-
Maltose	-
Adonitol	-
Cellobiose	-
Arabinose	-
Sucrose	++
Sorbitol	-

Key: (+++) Highly Positive; (++) Moderate Positive; (+) Positive; (-) Negative

studying the biochemical and metabolic activities of the B-15-9-2 isolate (Table 3B). The isolate was able to utilize only two sugars *i.e.* trehalose & sucrose out of 21 sugars tested. The positive result was obtained for ammonia, citrate utilization, nitrate, oxidase, and catalase while all the other biochemical tests showed negative results.

The B-15-9-2 isolate from the Bhavnagar region was identified based on the 16S rRNA gene. The sequencing analysis report shows an alignment of the query sequence with that of the available database. The consensus sequence is further analyzed by NCBI BLAST for the nearest homologs by comparing the rRNA sequence with reference strains. The nearest homologs of isolate B-12-9-2 rRNA sequence is *Halomonas hydrothermalis,* to the best of our knowledge this is the first report of this genus as glutaminase producer from Gujarat coasts. The phylogenetic tree of the isolate was constructed using Neighbor-Joining Method by MEGA 11 software (Fig. 2). The 16S rDNA sequence of B-15-9-2 Sequence is submitted in NCBI GenBank with Accession number ON261395.

Optimization of L-glutaminase production by *Halomonas hydrothermalis* B-15-9-2

OVAT (one variable at a time)

The method involves the optimization of various parameters (Fig. 3A-E) by keeping previously optimized concentration constant. The inoculum size plays a pivotal role in enzyme production. Therefore, the effect of inoculum size (1% to 3%) on L-glutaminase production from *H. hydrothermalis* B-15-9-2 was studied & 1% inoculum was found to increase the enzyme activity from 1.89 ± 0.5 U/mL to 3.45 ± 0.2 U/mL (Fig. 3A), further enzyme activity decrease as the inoculum size increases. The L-glutaminase enzyme is involved in nitrogen metabolism so it is very important to study the effect of various nitrogen sources. In this context variations in the organic nitrogen sources beef extract, peptone, and yeast extract (1% *w/v*) were studied. As a result, in the present study, the beef extract increases the enzyme activity from 3.45 ± 0.2 U/mL to 4.55 ± 0.4 U/mL (Fig. 3B).

For L-glutaminase production, the most important component is L-glutamine which acts as a growth inducer and as a source of vital amino acids. The effect of L-glutamine on L-glutaminase production by *H. hydrothermalis* B-15-9-2 is studied from (1% to 5%), & 3% L-glutamine concentration is the optimum concentration with maximum production *i.e.*, 7.63 ± 0.3 U/mL (Fig. 3C). Further increase in concentration results into decreasing activity. Many carbon sources affect L-glutaminase production. In this context, various carbon sources such as glucose, sucrose, and fructose (1% *w/v*) were tested for L-glutaminase production, and fructose is the most preferable carbon source which increases the enzyme activity from 7.63± 0.3 U/mL to 10± 0.2 U/mL

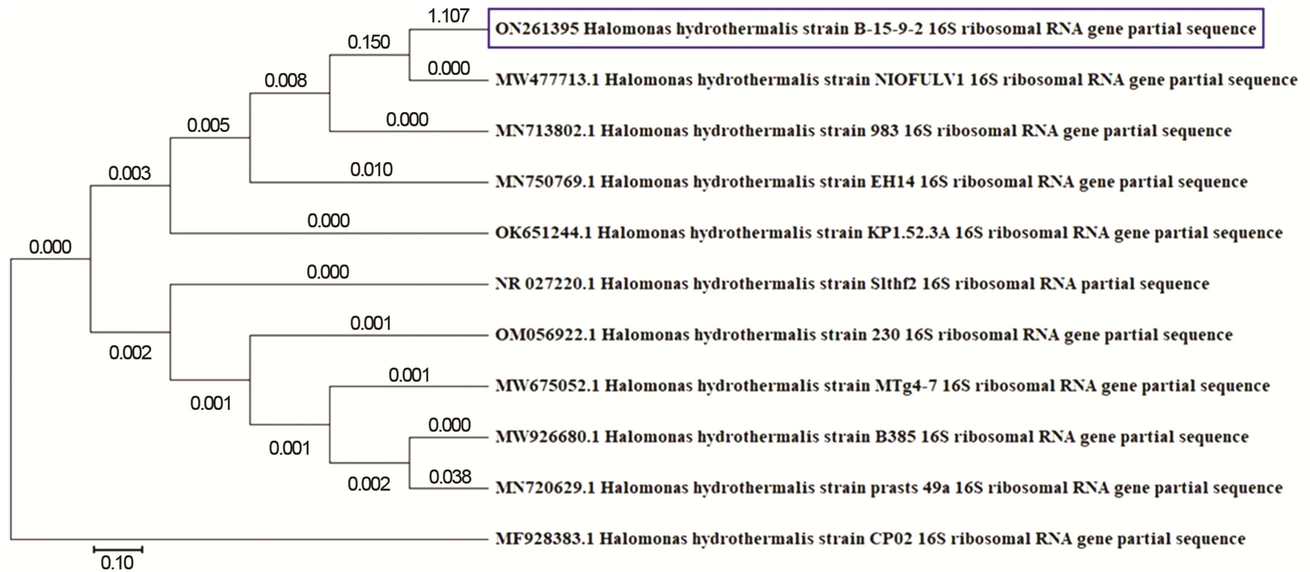


Fig. 2 — Phylogenetic tree of B-15-9-2 isolate constructed using Neighbor-joining method by MEGA 11 Software

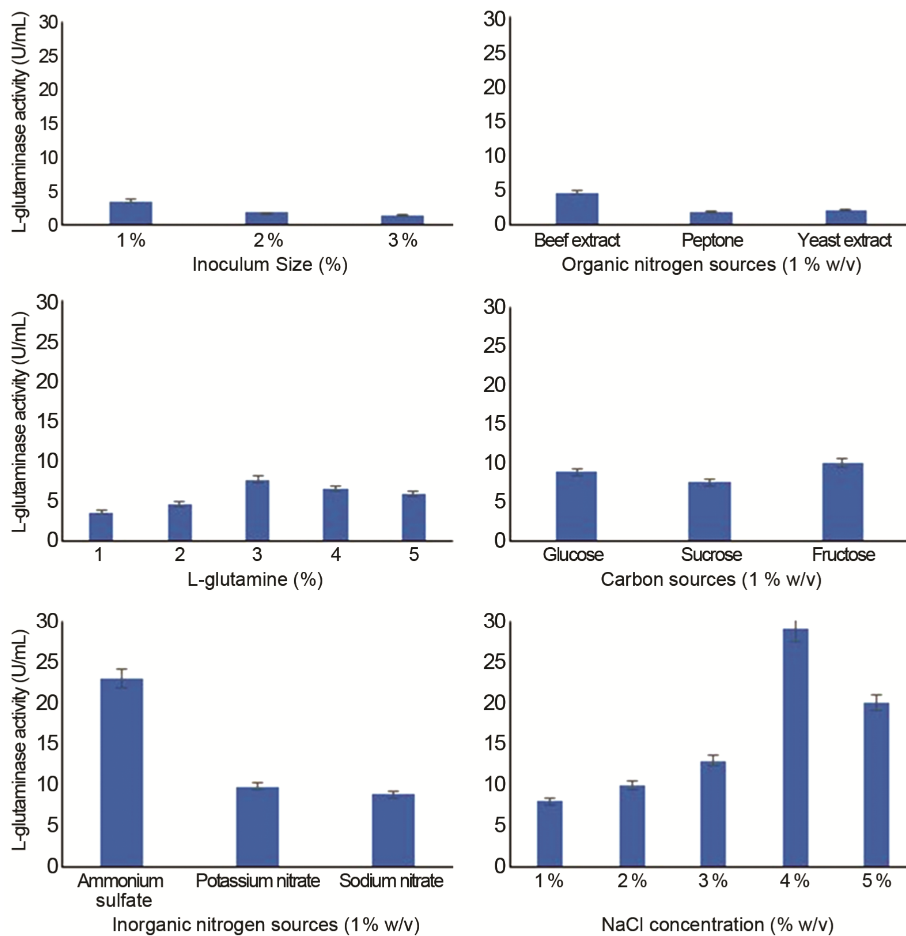


Fig. 3 — Effect of various parameters by one-variable-at-a-time approach on L-glutaminase production by *H. hydrothermalis* B-15-9-2. (a-Inoculum size; b-Organic nitrogen sources; c-L-glutamine concentration; d-Carbon sources; e-Inorganic nitrogen sources; f- Sodium chloride concentration). Values are mean ± standard deviation; n=3 samples

Table 4 — ANOVA for the experiments

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5402.97	9	600.33	153.44	< 0.0001	Significant
A-L-glutamine	741.99	1	741.99	189.65	< 0.0001	
B-Ammonium sulfate	346.49	1	346.49	88.56	< 0.0001	
C-NaCl	24.72	1	24.72	6.32	0.0307	
AB	2256.24	1	2256.24	576.69	< 0.0001	
AC	459.80	1	459.80	117.53	< 0.0001	
BC	3.71	1	3.71	0.9490	0.3530	
A^2	662.37	1	662.37	169.30	< 0.0001	
B^2	309.12	1	309.12	79.01	< 0.0001	
C^2	457.11	1	457.11	116.84	< 0.0001	
Residual	39.12	10	3.91			
Lack of Fit	29.33	5	5.87	2.99	0.1270	Non-significant
Pure Error	9.79	5	1.96			
Cor Total	5442.09	19				

(Fig. 3D) in the present study.Nitrogen source is considered to be the most important factor for L-glutaminase production. There for the effect of various inorganic nitrogen sources such as ammonium sulfate, potassium nitrate, and sodium nitrate (1% *w/v*) on L-glutaminase production was tested, and ammonium sulfate is the most significant for L-glutaminase production from *H. hydrothermalis* B-15-9-2. The enzyme activity increased and reached 29 ± 0.8 U/mL (Fig. 3E) which is higher as compared to a minimal glutamine medium. Due to the halophilic nature of *H. hydrothermalis* B-15-9-2, the effect of sodium chloride concentration on L-glutaminase production was checked & as a result, 4% *w/v* NaCl is the optimum concentration (Fig. 3F).

Statistical optimization of media components

The RSM was done using CCD design which shows the interactive effects of three independent variables (L-glutamine, Ammonium sulfate, and NaCl). The 20-run experiment was performed using the CCD model by Design-Expert Version 13. The results show as the L-glutamine concentration increases, the L-glutaminase production increases. The L-glutaminase production of 98.95 ± 0.5 U/mL was observed with a higher concentration of L-glutamine, and average concentration of ammonium sulfate and NaCl (run no.2); whereas 76.94 ± 0.6 U/mL was observed with the highest concentration of ammonium sulfate, NaCl and average concentration of L-glutamine (run no.7). The average L-glutaminase production of 80.3 ± 0.8 U/mL was obtained with a higher concentration of L-glutamine and NaCl *i.e.*, 5 & 16 g%, respectively, and average concentration of 4 g% in case of ammonium sulfate (run no.16). The final yield found in all the experiments was quite nearby to the predicted values; therefore, the media is optimized using the experimental results.

The ANOVA shows 153.44 Model F-value which implies the significance of the model and there is only a 0.01% chance for a larger F value to occur due to noise (Table 4). A p-value of less than 0.05 that indicates the model terms are significant. In the present study, A, B, C, AB, AC, A^2, B^2, and C^2 are the significant terms, whereas values greater than 0.100 indicate the non-significant effect of model terms. The Lack of Fit/ F-value is 2.99 which implies the non-significant Lack of Fit value relative to pure error, there is only a 12.70% chance that the large F-value could occur due to noise. The F-value of 2.99 is good as we want the model to fit. The model fitness can be expressed by the coefficient of determination R^2 value. The highest R^2 value is generally acceptable to correlate between independent variables used in the study, *viz*. Generally, an R^2 value ≥ 0.9 is considered to be significant for correlating experimental and predicted values, which reflects the accuracy and applicability of the model for optimization studies. In the present model, the R^2 value is 0.9928 which shows a reasonable agreement with that of Adjusted and Predicted R^2 values *i.e.*, 0.9863 & 0.9562, respectively. However, adequate precision measures the signal to noise ratio. The ratio greater than 4 is desirable, in the present model design ratio is 52.812 which indicates an adequate signal and further, this can be applied to navigate design space. The equation used for actual factors can be used to make predictions regarding the response for levels given for each factor. However, the equation for each specified level of the original units for factors is as follows:

X= +89.09826 + 11.15330 × A - 14.48817 × B - 6.24881 × C + 4.26508 × A × B - 0.842361 × A × C - 0.097321 × B × C -1.33916 × A^2 +1.51230 × B^2 +0.351998 × C^2

where X= L-glutaminase; A= L-glutamine; B= Ammonium sulfate; C= NaCl

The observation showed that among the various tested medium components, the cumulative effect of L-glutamine & ammonium sulfate is highly significant with p=value < 0.0001 & higher F-value 576.69; as well as the linear effect of L-glutamine is also significant with p=value < 0.0001 & F-value 189.65. This shows as we increase L-glutamine & ammonium sulfate concentrations L-glutaminase production would be increased in case of cumulative effect of components & in case of linear effect L-glutamine can increase the production level of L-glutaminase.

The Response surface plots and 2D contour plots were generated, which show the individual and cumulative effect of independent variables as well as probable correlations occurring between L-glutamine, ammonium sulfate, and NaCl on L-glutaminase production by keeping other at its central point (Fig. 4). The Interactive effect of two independent variables *i.e.*, L-glutamine & Ammonium sulfate shown in (Fig. 4A & B) indicates that by increasing L-glutamine and ammonium sulfate concentration has a favorable impact on L-glutaminase production, it gradually increases with concentration. However, the significant interactive effect of L-glutamine and NaCl on L-glutaminase production was found (Fig. 4C & D). No significant interaction between the two variables Ammonium sulfate & NaCl was found in the ANOVA (Fig. 4E & F). At the lower level of L-glutamine and ammonium sulfate, only 76.94 ±

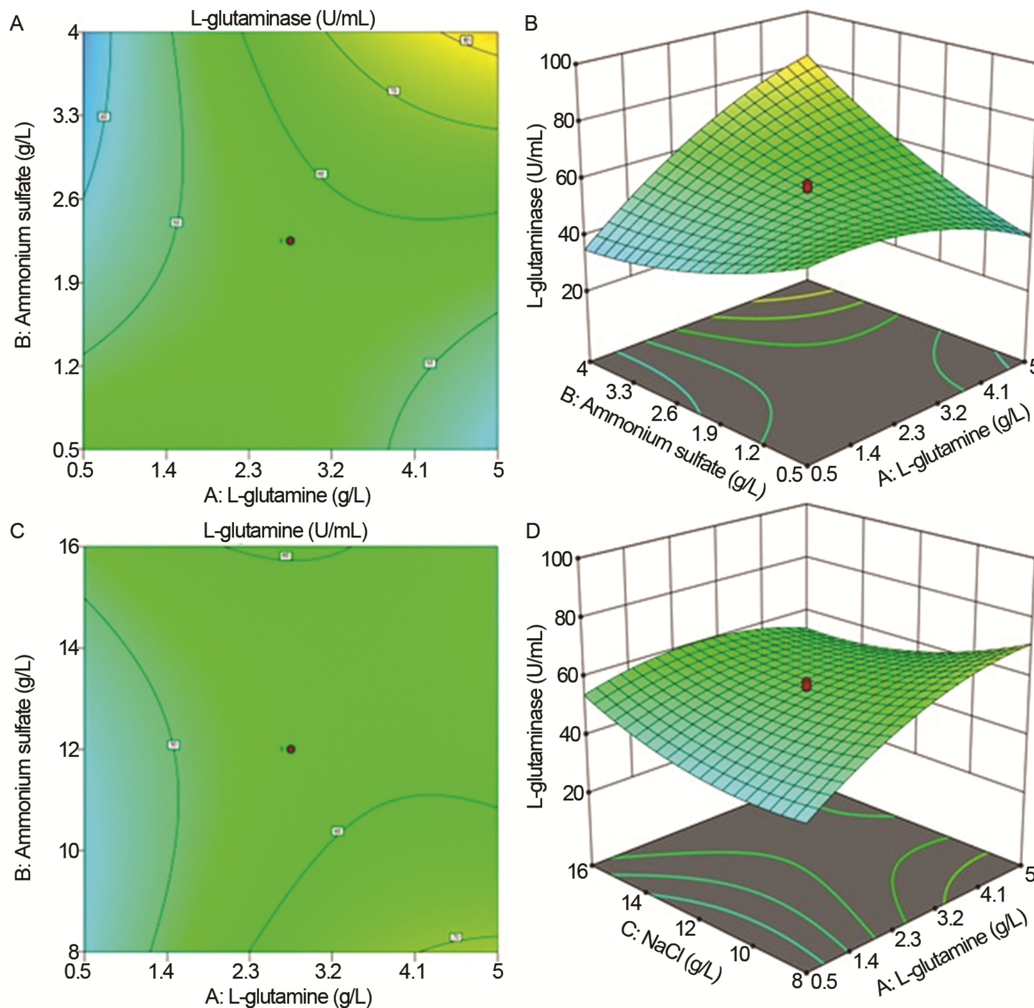
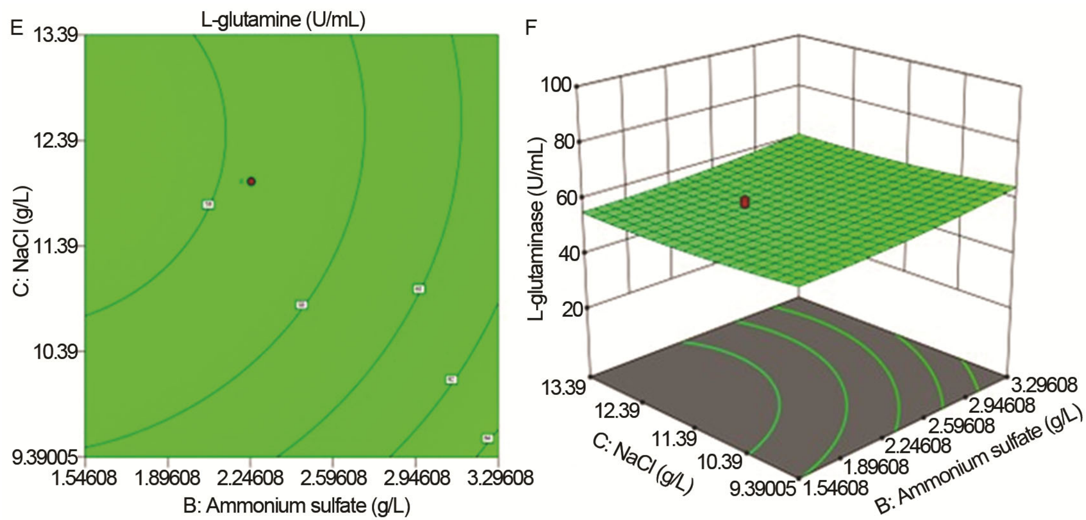


Fig. 4 — 3D Contour plots show the interaction between independent variables. Values are mean ± standard deviation; n=3 samples (*contd.*)



(*contd.*)

Fig. 4 — 3D Contour plots show the interaction between independent variables. Values are mean ± standard deviation; n=3 samples

0.6 U/mL was obtained, whereas 98.95 ± 0.5 U/mL of L-glutaminase production was found with higher concentration of above variables.

After CCD analysis, we kept an experiment run no.2 with L-glutamine, 5.0 g%; ammonium sulfate, 4.0 g% and NaCl, 8.0 g% for L-glutaminase production by *Halomonas hydrothermalis* B-15-9-2, which predicted 98.66 ± 3.0 U/mL L-glutaminase. Upon validation, *Halomonas hydrothermalis* B-15-9-2 produces 98.95 ± 0.5 U/mL L-glutaminase, which increases 3.4 times as compared to optimized media by the OVAT method. Therefore, the CCD design is successfully used for screening various medium components that enhance the L-glutaminase yield about 3.4 times.

Discussion

The present study deals with the isolation & screening of L-glutaminase-producing scarcely reported marine bacteria from Gujarat coasts[13]. The characterization and molecular identification studies reveal the nearest homolog of potent isolate B-15-9-2 is *Halomonas hydrothermalis*. Further various approaches for optimizing L-glutaminase production are done. By the OVAT method inoculum size is optimized and obtained results in the present work are contrary to marine *Halomonas meridiana* having 10% *v/v* inoculum size[20]. Whereas 4% *v/v* is the optimum inoculum size in the study[21]for marine *Bacillus subtilis* OHEM11 bacteria. The L-glutaminase production using other *Bacillus* sp. has optimal inoculum size (18% *v/v*)[22] & 20% *v/v*[23]. The organic nitrogen sources were tested in the present work and beef extract (1% *w/v*) is the optimum in the case of *Kosakonia radicincitans*[24] which can be comparable with the present study. Peptone is found to be optimum in the case of *Bacillus* sp.([24,25]). The yeast extract is found to be optimum in the case of *Bacillus subtilis* JK-79[26] which is contrary to the present study.

For L-glutamine concentration, 3% is optimum in the case of *Zygosaccharomyces rouxii*[27] which can be comparable to the present study. Whereas in the case of *Vibrio* sp.[28,29] 1% is optimum; 0.3% is the optimum in the case of *Bacillus* sp.[25,30] which is contrary to the present study. Further fructose (1% *w/v*) is the preferable carbon source in the case of marine *Bacillus subtilis* JK-79[26], which can be comparable to the present study. There have been no reports of L-glutaminase-producing bacteria for sucrose utilization till now. Glucose is the favorable carbon source in the case of marine *Providencia sp.*[31]; *Brevundimonas diminuta*[32]; *Kosakonia radicincitans*[24]. In the case of marine *Halomonas meridiana* there is a significant decrease in activity found[20] which shows the negative effect of inorganic nitrogen sources on L-glutaminase production this is contrary to the present study. Anotherstudy[24] shows the negative effect of ammonium ions on enzyme production by *Bacillus* sp. In the same study, sodium nitrate enhances the L-glutaminase yield at 0.01% concentration, which is contrary to results obtained in *H. hydrothermalis* B-15-9-2. Sodium chloride is known to play an important role in halophiles and the study reported[20] 3% *w/v* &[23] 5% *w/v* concentration as optimum for L-glutaminase production which is very similar & comparable with the present work.

Table 5 — L-glutaminase production comparison using statistical method

Sr No.	Microorganism	Significant variables	Fold enhancement	Reference
1	*Halomonas hydrothermalis B-15-9-2*	L-glutamine, ammonium sulfate and NaCl	3.4	Present study
2	*Serratia marcescens*	Galactose and L-glutamine	4	s
3	*Zygosaccharomyces rouxii*	Yeast extract	2.94	34
4	Providencia spp.	Urea	67 %	31
5	Bacillus subtilis RSP-GLU	Glutamine	1.5	35
6	*Alcaligenes faecalis* KLU102	Arabinose, Skim milk and sodium chloride	2	36
7	*Aeromonas veronii*	Peptone and L-glutamine	4	37
8	*Bacillus subtilis* JK-79	Sea water glutamine	3.48	38

For CCD the earlier report[33] shows the interactive effects of independent variables on L-glutaminase production by *Serratia marcescens*. Amongst the variables, Galactose and L-glutamine were the most significant factors that enhanced the L-glutaminase yield considerably, this can be compared with the present study. Many other authors also reported the application of RSM for enhancing L-glutaminase yield. The face-centered central composite design was employed in the case of *Z. rouxii*[34] to enhance L-glutaminase yield by 2.94 folds. The authors in the study of *Providencia* spp.[31] reported the optimization of the enzyme by OVAT and FCCCD. By using the hybrid methodology, a significant enhancement in L-glutaminase yield of about 47% is reported[35] in the case of *B. subtilis* RSP-GLU. The improvement in L-glutaminase production is obtained by the statistical method for optimization of medium composition in the case of marine *Alcaligenes faecalis* KLU102[36]. The statistical optimization of L-glutaminase by PBD and CCD was reported by mutant *Aeromonas veronii*, with about a 4-fold enzyme yield increase as compared to the native strain[37]. A recent study[38] reported the optimization of L-glutaminase production by PBD and CCD, with about 3.48-fold enzyme yield increases by *Bacillus subtilis* JK-79.The present study is very similar to the report[39] (Table 5).

Conclusion

From the present work, we can conclude that the marine coastal region particularly the Gujarat coasts is a unique habitat that harbors salt-tolerant microbes. To the best of our knowledge this is the first report of L-glutaminase producing marine bacteria from Gujarat coastal regions which is the unique counterpart of our work. The minimal glutamine medium used contains a very high salt concentration that supports the production of L-glutaminase from marine bacteria. A total of 12 marine bacteria were isolated from the Bhavnagar region using the enrichment culture technique & were screened using a minimal glutamine medium. Based on the growth rate, pH, and visible color change most potent isolate B-15-9-2 was selected for further studies. Morphological and Biochemical characterization reveals the nearest homolog ofB-15-9-2 rRNA sequence of the isolate is *Halomonas hydrothermalis* with 99.87% similarity. The optimization studies by One variable at a time and statistical approach optimized the medium components that enhance L-glutaminase yield up to 98.95 ± 0.5 U/mL. Further in our work, we found the *in vitro* anti-proliferative activity of L-glutaminases from Gujarat coasts having IC_{50} less than 10 µg/mL & antioxidizing properties as well (unpublished work), this would seem to be a promising candidate for deleterious cancer therapeutics.

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Conflict of interest

All authors declare no conflict of interest.

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