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# Screening and Selection of Marine Isolate for L-Glutaminase Production and Media Optimization Using Response Surface Methodology

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**Abstract** The current work details the screening of about 400 marine isolates from various marine niches, from which one isolate was finally selected based on the productivity of glutaminase (71.23 U/l). Further, biochemical identification tests and 16S rRNA sequencing identified this isolate to be *Providencia* sp. This isolate was taken up for further media optimization studies by using one-factor-at-a-time approach and subsequently by response surface methodology. A face centered central composite design was employed to investigate the interactive effects of four variables, viz., concentrations of glucose, methionine, urea, and succinic acid on glutaminase production. A significant influence of urea on glutaminase production was noted. Response surface methodology showed that a medium containing (g/l) glucose 10.0, urea 5.15, methionine 3.5, succinic acid 6.0, ammonium sulfate 2.5, and yeast extract 6.0 to be optimum for the production of glutaminase. The applied methodology was validated using this optimized media and enzyme activity 119±0.12 U/l and specific activity of 0.63 U/mg protein after 28 h of incubation at 25 °C was obtained.

**Keywords** Glutaminase · Response surface methodology · Marine isolates · Screening

## Introduction

Marine microorganisms known to produce a diverse spectrum of novel metabolites are a hereto-untapped source for discovery of new bioactive compounds. Marine microorganisms are unique in nature and differ in many aspects from their terrestrial counterparts. They are not fully understood with respect to their biology, which perhaps is the major reason for the lack of adequate recognition to their potential [1]. Due to a recent deceleration in natural product research in terrestrial habitats, there is an increasing interest in the exploration of

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marine microorganisms for novel metabolites using biological and chemical methods developed for such studies in terrestrial environments. It is estimated that marine environments including the subsurface harbors about $3.67\times10^{30}$ microorganisms that represent an extraordinary and dynamic gene pool of biodiversity. The majority of these microbes have never been cultured, identified, or classified, and their enormous chemical richness has remained untapped. However, in the past two decades, this situation has changed as a result of the rapid progress in phylogenetic studies based on small rRNA and genome-related technology independent of standard microbial cultivation [2].

The marine environment is characterized by the hostile parameters such as high pressure, salinity, low temperature, and absence of light. Marine heterotrophic bacteria have adapted themselves to survive in this environment—they require sodium ions for their growth so as to maintain the osmotic environment for protection of cellular integrity. Oligotrophy is also one more adaptation because of the small amount of available nutrient. However, heterotrophic bacterial action promotes organic degradation, decomposition, and mineralization in sediments and in the overlying water and releases dissolved organic and inorganic substances [3].

Halotolerant marine organisms provide an interesting alternative for therapeutic purposes. They have a diverse range of enzymatic activity catalyzing various biochemical reactions with novel enzymes. Hydrolytic enzymes from these microbes are capable of functioning under conditions that lead to precipitation or denaturation of most proteins from mesophilic (terrestrial) microorganisms. Further, it is believed that sea water, which is saline in nature and chemically closer to the human blood plasma, could provide biomolecules, in particular enzymes that could have lower or no toxicity or side effects when used for therapeutic applications [4]. It was against this background that we undertook screening of marine isolates showing higher expression of glutaminase.

Glutaminase or glutamine aminohydrolase (EC 3.5.1.2) is an enzyme catalyzing the hydrolysis of glutamine to stoichiometric amounts of glutamate and ammonia. In most mammalian tissues where the enzyme is expressed, it is involved in the generation of energy using glutamine as a major respiratory fuel. Thus, many types of tumor cells as well as actively dividing normal cells exhibit high rates of glutamine utilization through this pathway. In the brain, glutaminase also plays a key role in the synthesis of the excitatory neurotransmitter glutamate, which is a precursor of the inhibitory neurotransmitter, $\gamma$-aminobutyric acid. Furthermore, the enzyme regulates the cerebral concentrations of glutamine and glutamate, which are very important in processes such as ammonia detoxification [5]. Besides its use in therapeutics, glutaminase is a key flavor-enhancing additive in the production of fermented foods. Its commercial importance demands the search for new and better yielding microbial strains and economically viable bioprocesses for its large-scale production [6].

Microbial screening techniques generally involve the screening of microbes from specific ecological sites (soil and/ or water) or screening already identified microorganisms [7]. One of the most efficient and successful means of finding new enzymes is to screen a large number of microorganisms due to their characteristic diversity and versatility [8].

16S rRNA sequencing in combination with the biochemical characterization of organisms based on taxonomic scheme as described in Bergey's Manual of Systemic Bacteriology is being increasingly used for bacterial identification. Identification of bacteria based on its morphological and biochemical characterization is a difficult task since new species are frequently added with consequent phylogenetic realignment. 16S rRNA gene sequence analysis can confirm the genera and species of the isolate.

Improvement in microbial metabolite production is generally attempted by manipulating the nutritional and incubational parameters of the organism. Further, a manipulation in its metabolic pathway and/or mutation selection can also be done as these can significantly

alter the product yield. The statistical methodologies are preferred for media optimization because of obvious advantages in their use. Response surface methodology (RSM) is an efficient experimental strategic tool used for industrial purposes to obtain optimum conditions for a multivariable system and is suitable for describing a near optimum region for investigating process parameters and conditions for a multifactorial system. RSM allows for the reduction of the number of experiments without neglecting the interaction among the parameters. This methodology involves the design of experiments to characterize the response as a function of several factors, the fitting of empirical or theoretical models to the experimental data, the interpretation of the nature of the fitted response, and the judgment of the adequacy of the model [9].

In the present study, we detail the isolation, screening, and biochemical identification of a new marine isolate toward glutaminase production. Further, 16S rRNA sequence analysis of the same is described. Identification of incubation parameters and media components for glutaminase production was done by the one-factor-at-a-time approach. Subsequently, RSM was employed to obtain a media for increased production of glutaminase.

## Materials and Methods

### Medium Components

Sucrose, yeast extract, glutamine, glutamic acid, and other media components were procured from Hi-Media Limited, Mumbai, India. Buffer salts like Tris, dipotassium hydrogen phosphate, sodium chloride, HCl, etc. were purchased from Merck India Limited, Mumbai, India. L-Glutamate dehydrogenase, nicotinamide adenine dinucleotide (NAD), and adenosine diphosphate (ADP) were purchased from Sigma Aldrich Pvt. Ltd., Steinheim, Germany. UV transparent 96-well microplates were a gift sample from Corning, NY, USA.

### Isolation and Primary Screening of Glutaminase Producers

Over 400 different bacterial strains were isolated from marine niches covering 300 km of the western seacoast of Maharashtra (India). A variety of samples were collected, viz., sand from clear beaches, mud from creeks and sea floors exposed by low tide, algal scrapings and barnacles from rocky surfaces, muddy clay from mangroves, seaweeds, fish and crab scales, and seawater. The collection of samples was done about 50 ft away from the shore and to a depth of about 25 ft. At some places, water samples were collected by boat up to 5 km from the shore. The samples were collected in presterilized bottles and refrigerated (4 °C) till processing. All the samples were collected from sites relatively away from habitation. Approximately 2 g of each sample was collected. Seawater was collected from each site for chemical analysis and also for preparation of media.

In the laboratory, the samples were homogenized in seawater and microbes were isolated using spread plate and streaking methods on various media, which essentially contained seawater and diluted nutrients. These isolates were initially grown in an oligotrophic environment to simulate conditions in seawater and then used in the screening medium. Initially, all isolates to be screened were incubated in media containing (g/l) meat extract 0.33, yeast extract 0.66, and peptone 1.67 dissolved in artificial seawater (ASW). ASW used throughout the study contained (g/l) NaCl 24.7, KCl 0.66, $MgCl_2.6H_2O$ 4.7, $CaCl_2.2H_2O$ 1.9, $MgSO_4.7H_2O$ 6.3, and $NaHCO_3$ 0.18 with pH adjusted to 8.1 using 0.1 N NaOH filtered through #3Whatman before use.

For primary screening, a media containing (g/l) glucose 5.0, glutamine 5.0, $Na_2HPO_4.2H_2O$ 6.0, $KH_2PO_4$ 3.0, $MgSO_4$ 0.49, $CaCl_2$ 0.002, and a 2.5% *w*/*v* ethanolic phenol red solution 0.06 ml with pH adjusted to 7.6 was used. Appropriate controls were taken wherein the same cultures were grown in medium containing (a) $NaNO_3$ at 5.0 g/l instead of glutamine, (b) media devoid of any carbon source and contained only glutamine 10.0 g/l as both carbon and nitrogen source dissolved in ASW, and (c) same aforementioned media without the dye. The isolates were grown in 100 ml flasks containing 20 ml media on orbital shaker at 180 rpm maintained at 27±3 °C for 48 h. The cultures that showed positive utilization of glutamine and production of glutamic acid (with or without concomitant production of other organic acids) indicated by a decrease in pH were taken up for secondary screening of glutaminase. Some cultures further increased the pH indicating the production of a basic compound or breakdown of media to release basic ingredients such as, for example, ammonia. These were also included for secondary screening. The entire screening was also done simultaneously on solid agar medium using procedure of Gulati et al. [10].

## Secondary Screening for Glutaminase

The 75 selected isolates from primary screening were grown in media containing (g/l) glucose 5.0, $Na_2HPO_4.2H_2O$ 6.0, $KH_2PO_4$ 3.0, $MgSO_4$ 0.49, and $CaCl_2$ 0.002 for 48 h at 180 rpm at 27±3 °C. The intracellular and extracellular glutaminase contents were estimated as described under "Analytical Determinations" section of this paper. The cultures showing higher glutaminase production were taken up for identification and further media optimization was done for the isolate giving maximum production.

## Biochemical Identification and 16S rRNA Sequencing and Analysis of Selected Isolate

Biochemical tests were done as given in Bergey's Manual of Systemic Bacteriology. 16S rRNA sequencing was done at National Centre for Cell Science, Pune, India. The sequence obtained was searched against the GenBank database and homology studies undertaken to identify the isolate. Sequence was initially analyzed at National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (blastn) tool and corresponding sequences were downloaded and the sequence homology analysis done. Phylogenetic tree was constructed by the neighbor-joining method using the Ribosomal Database Project-II website (http://rdp.cme.msu.edu). The sequence was deposited at GenBank with accession no. EU596455

## Microorganism and Culture Maintenance Conditions

The isolate giving maximum glutaminase yield was maintained on agar medium slants containing glucose 10 g/l dissolved in ASW. Inoculated slants were grown in an incubator at 30 °C for a day. The slants were kept at 4 °C and subcultured every 20 days in the aforementioned media.

## Inoculum Preparation

Inoculum was prepared in 250 ml Erlenmeyer flasks containing 50 ml of liquid media containing glucose 5.0, yeast extract 1.0, $Na_2HPO_4.2H_2O$ 6.0, $KH_2PO_4$ 3.0, $MgSO_4$ 0.49, and $CaCl_2$ 0.002 with pH maintained at 8.0. The media so prepared was autoclaved at 121 °C (15 lb) for 20 min and then inoculated with selected isolate designated UICT/T5. The inoculated flasks

were kept on a shaker at 180 rpm for 24 h and used as the inoculum. This inoculum ($2.34\times 10^6$ cells/ml) was used for all subsequent inoculations unless otherwise mentioned.

## Optimization of Fermentation Parameters

Initially, optimization of various physicochemical parameters required for maximum glutaminase production by the isolate was evaluated on shake flask level in 250 ml Erlenmeyer flasks. The parameters studied were incubation temperature (25–55 °C), pH (1–11) and incubation time (0–60 h), and inoculum size (0–10% *v*/*v* of a 24-h culture having $2.34\times 10^6$ cells/ml).

## Media Component Selection by a One Factor at a Time

Initially, media components were screened by one-factor-at-time approach. These were the effect of different carbon sources (glucose, galactose, sucrose, lactose, mannitol, citric acid, rhamnose, fructose, glycerol, soluble starch, and sorbitol at 5 g/l), effect of complex nitrogen sources (urea, peptone, soybean meal, biopeptone, malt extract, beef extract, yeast extract, and corn steep liquor 5 g/l), inorganic nitrogen sources (ammonium dihydrogen phosphate, ammonium bicarbonate, triammonium citrate, ammonium phosphate, ammonium nitrate, ammonium acetate, ammonium molybdate, ammonium bicarbonate, ammonium sulfate, ammonium chloride, ammonium oxalate, and diammonium hydrogen phosphate at 0.05 M), amino acids (phenylalanine, proline, glutamic acid, glutamine, asparagine, arginine, and methionine 0.5 g/l), trace elements (potassium chloride, calcium chloride, magnesium chloride, manganese chloride, cobaltous chloride, cupric chloride, nickel nitrate, mercuric chloride, ferric chloride, sodium sulfate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium dihydrogen phosphate, barium chloride, zinc chloride, ethylenediaminetetraacetic acid (EDTA), succinic acid, and boric acid at 1 mM), and finally the effect of NaCl concentration replacing the NaCl in the ASW (0–240 g/l). ASW was used as the basal solution to dissolve the media components. The procedure adopted was to evaluate the effect of independent parameters keeping others constant and to incorporate it at the optimized level in the next experiment while optimizing the next parameter. All the experiments were carried out in at least triplicate and the mean values taken. All the assays were carried out after 24 h of incubation. Further screening for concentrations of glucose (5–30 g/l), urea (2.5–10 g/l), ammonium sulfate (0.5–3 g/l), succinic acid (10–50 g/l), and methionine (0.5–6 g/l) was done.

## Statistical Experiment Design

Following identification of the variables affecting glutaminase production by one factor at a time, the four most important variables, glucose, urea, succinic acid, and methionine, were selected for optimization using RSM. The medium contained 2.5 g/l ammonium sulfate and 6 g/l yeast extract in ASW as basal medium. RSM using face centered central composite design (FCCCD) was adopted for improving glutaminase production using the software Design-Expert Version 6.0.10, Stat-Ease Inc. Minneapolis, MN, USA to find the interactive effects of four variables. Central composite design at the given range of the above parameters in terms of coded and actual values is presented in Table 1. The average maximum glutaminase activity (U/l) and specific activity (U/mg protein) were taken as dependent variables or responses $Y_1$ and $Y_2$. Regression analysis was performed on the data obtained. A second-order polynomial equation was then fitted to the data by multiple

**Table 1** Experimental range and levels of the independent variables used in RSM in terms of actual and coded factors employed in the FCCCD.

| Media component | Range of levels | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Actual (g/l) | Coded | Actual (g/l) | Coded | Actual (g/l) | Coded | Actual (g/l) | Coded | Actual (g/l) | Coded |
| Glucose | 0 | −2 | 10 | −1 | 20 | 0 | 30 | 1 | 40 | 2 |
| Urea | 0 | −2 | 2.5 | −1 | 5 | 0 | 7.5 | 1 | 10 | 2 |
| Methionine | 0.5 | −2 | 1.5 | −1 | 2.5 | 0 | 3.5 | 1 | 4.5 | 2 |
| Succinic acid | 0 | −2 | 2 | −1 | 4 | 0 | 6 | 1 | 8 | 2 |

regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a four-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

where $Y$ is the predicted response; $\beta_0$ is the intercept; $\beta_1$, $\beta_2$, $\beta_3$, and $\beta_4$ are the linear coefficients; $\beta_{11}$, $\beta_{22}$, $\beta_{33}$, and $\beta_{44}$ are the squared coefficients; $\beta_{12}$, $\beta_{13}$, $\beta_{14}$, $\beta_{23}$, $\beta_{24}$, and $\beta_{34}$ are the interaction coefficients; and A, B, C, and D are independent variables while $A^2$, $B^2$, $C^2$, $D^2$, AB, AC, AD, BC, BD, and CD are the corresponding functions of the independent variables. Analysis of variance (ANOVA) was performed. The proportion of variance explained by the polynomial models obtained was given by the multiple coefficient of determination, $R^2$. The fitted polynomial equation was expressed as three-dimensional response surface and contour presentations to find the concentration of each factor for maximum glutaminase production and visualize the relationship between the responses and the experimental levels of each factor used in the design. To optimize the level of each factor for maximum response, "numerical optimization" process was employed. The combination of different optimized parameters, which gave maximum glutaminase yield, was tested experimentally to validate the model.

## Growth and Production Profile Studies of Glutaminase

The RSM optimized media was used to study the growth profile in 250 ml Erlenmeyer flasks. About 50 ml of media containing (g/l) glucose 10.0, urea 7.5, methionine 1.84, succinic acid 5.0, ammonium sulfate 2.5, and yeast extract 6.0 was adjusted to pH 8.1±0.1 and inoculated with 5% *v*/*v* inoculum (containing $2.34 \times 10^6$ cells/ml) prepared as described earlier. The inoculated flasks were kept on a shaker at 180 rpm for 48 h, and samples were taken every 4 h to determine biomass, glutaminase activity, and protein content.

## Effect of NaCl on Crude and Purified Glutaminase

The enzyme obtained was purified to homogeneity using affinity chromatography (methodology and data not reported). The crude as well as the purified enzyme was studied for their ability to tolerate NaCl. The salt dependence of glutaminase was determined by adding 0–3 M NaCl to the reaction medium. Relative activities of the enzyme were calculated and expressed as percentage of the activity without salt (0% *w*/*v* NaCl) considered as 100%

activity. Further, the crude and purified glutaminase was also studied for long-term stability in 2 M NaCl at 25 °C and reported as % residual activity of the original activity.

### Analytical Determinations

#### *Enzyme Extraction*

At appropriate time intervals, the fermentation flasks were harvested for the enzyme. The broth was centrifuged at 8,000 rpm, 4 °C, and the supernatant obtained was used for the determination of extracellular glutaminase. The biomass pellet was washed twice with 0.9% saline and then suspended in 10 ml saline. This suspension was sonicated using a probe sonicator (Branson Sonifier 450) for 3 min at 4±2 °C. The suspension was then centrifuged at 8,000 rpm, 4 °C, and the supernatant obtained was used for intracellular enzyme determination

#### *Glutaminase Activity and Specific Activity*

For estimation of glutaminase activity, a modification of the method of Moriguchi et al. [11] was used. In the first step of the assay, the glutaminase produced in the fermentation broth converts glutamine to glutamate. This glutamate formed is converted in the second step of the assay to α-ketoglutarate by L-glutamate dehydrogenase and in the process, NAD is reduced to NADH. This is measured as a change in the absorbance at 340 nm. The final reaction mixture contained 184 mM potassium phosphate, 44 mM Tris-HCl, 0.18 mM EDTA buffer pH 8.6, 20 mM L-glutamine, and crude enzyme extract (0.1 ml) in a final volume of 1.0 ml. After reacting for 35 min at 37 °C, the reaction was terminated by adding 0.1 ml 3M HCl and kept at 4 °C for 5 min. Later, 130 mM Tris-HCl buffer (pH 9.4), 20 mM β-NAD, 0.5 mM ADP, and 25 U/ml of L-glutamate dehydrogenase were added in a total volume of 0.295 ml to 10 μl of reaction mixture from the earlier step. The reaction was conducted in UV transparent 96-well plates, which was read on a μQuant Elisa plate reader (Bio-tek Instruments Inc., USA). The absorbance at 340 nm was measured before and after incubating the mixture for 1 h at 37 °C. One unit of glutaminase activity was defined as enzyme required for deamination of 1.0 μmol of glutamine per min at pH 8.6, 37 °C [12]. The protein concentration was determined by the Folin Lowry method using bovine serum albumin as the standard [13].

#### *Biomass Estimation*

The bacterial biomass in the fermentation broth was quantified by dry-cell weight analysis and by measurement of optical density of the broth [14]. For dry weight determinations, the cells were recovered by centrifugation at 8,000 rpm, 4 °C, and washed twice with 0.9% saline and once with distilled water. The recovered biomass was dried to constant weight in an oven (80 °C) for 24 h. For optical density measurements, the absorbance was read at 600 nm using UV/Vis Hitachi Spectrophotometer.

## Results and Discussion

### Isolation and Screening of Glutaminase Producers

The diversity of marine environment in terms of temperature, pressure, and nutrient concentrations make marine microorganisms encompass a complex and diverse assemblage

of microscopic life forms. The unique habitats in marine environment provide microbes with novel physiological and metabolic capabilities for survival and a great potential for the production of metabolites not found in terrestrial environments [15], validating the pharmacological value of biodiversity studies in this area. With this perspective, marine niche was selected for isolation of possible glutaminase producers.

In the current study, the organisms were isolated from crab shells, fish scales, brine, sand, breathing roots of weeds, clay, mud, etc. These isolated organisms were maintained as glycerol stocks and revived in appropriate media. Primary screening showed that approximately 75 of the 400 cultures screened produced glutaminase. These cultures were taken up for secondary screening wherein the intracellular and extracellular enzyme activity was determined. It was found that the culture designated UICT/T5 gave extracellular glutaminase production of about 71.23 U/l. Thus, UICT/T5, an isolate from the breathing roots of weeds growing in the Ghodbunder creek area, Thane, India, was selected for further studies.

## Morphological, Physiological, and Biochemical Characteristics and 16S rRNA Based Identification of Isolate

The culture gave cream colored flat colonies with smooth margin on nutrient agar. Microscopically, they were seen as Gram-negative nonmotile coccobacilli. The culture showed swarming growth after 18 h on plates with seawater containing nutrient agar. Growth period on solid agar is approximately 18–20 h, while that in liquid broth was approximately 24–28 h. Initial biochemical identification tests showed that the culture could use glucose, galactose, sucrose, adonitol, inositol, rhamnose, mannitol, sorbitol, but not maltose, trehalose, raffinose, xylose, fructose, and lactose. It showed growth on cetrimide agar, reduced nitrate to nitrite, was urease positive, indole positive, methyl red positive, Voges–Proskauer negative, oxidase negative, citrate negative, and lysine decarboxylase negative. It showed growth in aerobic conditions with negligible growth under anaerobic conditions. The 16S rRNA sequence analysis gave high homology with *Providencia* sp. The sequence homology studies showed 99.0% similarity with *Providencia stuartii* (AY803746).

By comparing differences in the nucleic acid sequences of homologous genes from different organisms, a molecular genealogy (phylogeny) can be constructed [16]. The distance between the two sequences is indicative of their relatedness to each other. Figure 1 shows the phylogenetic relationship between different members of genus *Providencia* and our bacterial isolate UICT/T5. On the basis of 16S rRNA gene sequence analysis, the isolate (UICT/T5) was identified as *Providencia* sp. In fact, a recent publication has identified the gene responsible for glutaminase production in *Providencia* [18].

## Optimization of Fermentation Parameters

The optimum incubation temperature was found to be approximately 25 °C (79.23 U/l), while optimum pH adjusted before autoclaving was found to be pH 8.0 (79.54 U/l). The pH increased to 9.3–9.5 with the progress of fermentation, which was maintained throughout the stationary phase. Temperatures above 45 °C decreased the biomass as well as the enzyme activity. The incubation period was optimized to 24 h, beyond which the enzymatic activity and biomass were found to be constant. Inoculum size was optimized to 5% *v*/*v* ($2.34\times10^6$ cells/ml) of the fermentation broth. An inoculum size above 5% *v*/*v* decreased both the biomass and the enzyme activity, probably due to nutrient limitation.

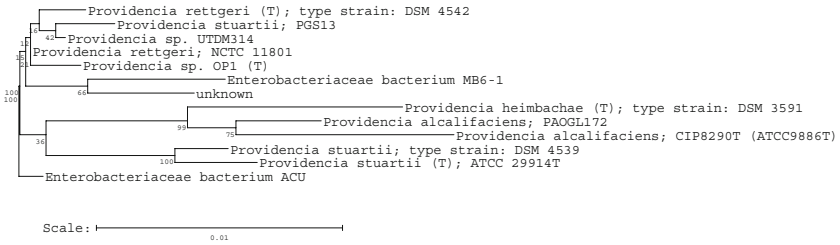



**Fig. 1** Phylogenetic tree based on 16S rRNA gene sequence comparisons showing the relationship between members of genus *Providencia* and isolate UICT/T5. *Enterobacteriaceae* bacterium ACU has been taken as outgroup. The sequences have been retrieved from NCBI database and the tree has drawn using neighbor-joining method using the RDP-II [17]. *-unknown indicates the marine *Providencia* sp. (EU596455)

## Media Component Selection by a One Factor at a Time

Among the various carbon sources, glucose and galactose promoted both biomass and glutaminase production (78.32 U/l). This is in accordance to earlier reports on the preference of marine organisms for slowly utilizable sugars like galactose [19]. Lactose, citric acid, mannitol, and maltose decreased both the glutaminase activity as well as the biomass. When free sugars were eliminated from the medium, there was production of both biomass and activity (65.74 U/l). This indicated the ability of the organism to utilize other media components for energy.

The effect of various nitrogen sources was evaluated using 0.5% glucose as the carbon source. Ammonium sulfate gave an activity of 80.95 U/l and hence was selected among the inorganic nitrogen sources studied. Urea and corn steep liquor both increased the activity (83.23 U/l) when included with ammonium sulfate as inorganic nitrogen source. A further evaluation with respect to concentration showed urea to give a better response at lower

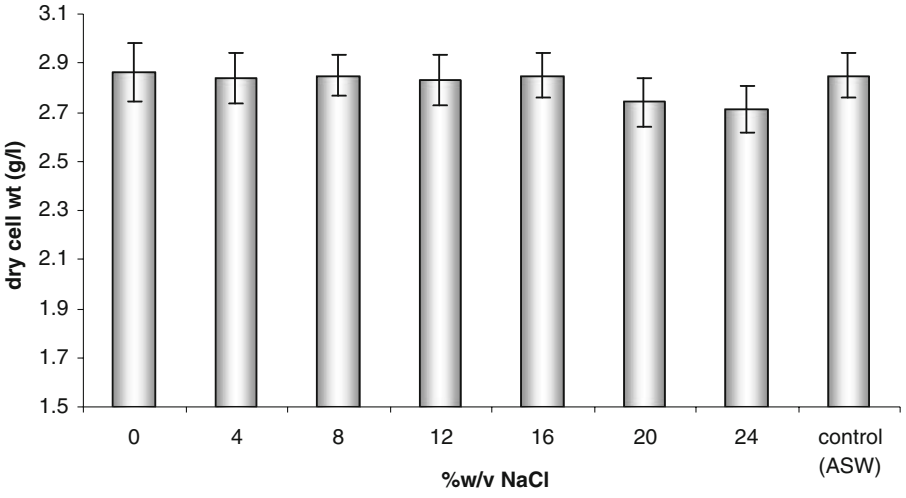



**Fig. 2** Effect of increasing NaCl concentration in the growth medium on growth of *Providencia* isolate (UICT/T5)

concentration of 0.25% *w/v*. Hence, it was selected to be used in combination with ammonium sulfate. Among the amino acids evaluated, methionine substantially increased the glutaminase production. Succinic acid also increased the biomass and, consequently, the production of glutaminase. There have been earlier reports of succinic acid and boric acid having a favorable effect on glutaminase production by *Pseudomonas* sp [20].

As seen in Figure 2, the organism showed similar growth from 0% to 16% *w/v* NaCl. Beyond 16% *w/v* NaCl, there was a slight decrease in growth (as evidenced from viability count, optical density, and dry cell weight) even in 24% *w/v* NaCl (though perceptibly less compared to growth in RSM optimized medium) indicating that the organism may be halotolerant rather than halophilic. Thus, from the "one-factor-at-a-time" approach, 5% *v/v* inoculum ($2.34\times10^6$ cells/ml) in a media containing (g/l) glucose 10.0, urea 2.5, ammonium sulfate 2.5, succinic acid 2.0, and methionine 0.25 showed good glutaminase production after 48 h.

## Statistical Experiment Design

Optimum levels of the aforementioned significant factors and the effect of their interactions on glutaminase production were determined by the FCCCD of RSM. Table 1 gives the details of the actual and coded values employed in the FCCCD. The results obtained by FCCCD were analyzed by standard ANOVA, and the mean predicted and observed responses are presented in Table 2. The second-order regression equation provided the levels of glutaminase production as a function of initial values of glucose, urea, succinic acid, and methionine, which can be predicted by the following equation:

$$\begin{aligned}\text{Enzyme activity } (Y_1) = {} & 100.43 + 0.62 \times \mathrm{A} - 4.76 \times \mathrm{B} + 1.68 \times \mathrm{C} + 4.43 \\ & \times \mathrm{D} - 1.84 \times \mathrm{A}^2 - 1.93 \times \mathrm{B}^2 - 0.16 \times \mathrm{C}^2 - 0.63 \\ & \times \mathrm{D}^2 - 0.87 \times \mathrm{AB} + 0.43 \times \mathrm{AC} - 0.56 \times \mathrm{AD} - 1.89 \\ & \times \mathrm{BC} + 5.37 \times \mathrm{BD} + 0.47 \times \mathrm{CD}\end{aligned}$$

$$\begin{aligned}\text{Specific activity } (Y_2) = {} & 0.51 - 0.064 \times \mathrm{A} + 0.016 \times \mathrm{B} + 7.511\mathrm{E}{-}003 \times \mathrm{C} \\ & + 0.027 \times \mathrm{D} + 0.019 \times \mathrm{A}^2 + 0.025 \times \mathrm{B}^2 + 0.013 \times \mathrm{C}^2 \\ & + 0.030 \times \mathrm{D}^2 + 2.242\mathrm{E}{-}003 \times \mathrm{AB} + 4.062\mathrm{E}-003 \\ & \times \mathrm{AC} - 0.021 \times \mathrm{AD} - 0.015 \times \mathrm{BC} - 5.584\mathrm{E}{-}003 \\ & \times \mathrm{BD} + 8.818\mathrm{E}{-}003 \times \mathrm{CD}\end{aligned}$$

where A—glucose, B—urea, C—methionine, and D—succinic acid.

Table 3 gives the model coefficients and regressional analysis of the FCCCD and Table 4 gives the ANOVA values for the two responses, viz., enzyme activity and specific activity from the RSM experiments. The *P* values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The *F* value and the corresponding *P* values, along with the coefficient estimate, are given in Table 3. The smaller the magnitude of *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.050 indicate the model terms to be significant. In case of enzyme activity, A, B, C, D, $A^2$, $B^2$, $D^2$, AB, BC, and BD were significant model terms. As seen in Table 4, the model *F* value of 75.65 implied the model to be significant. Values of "Prob > *F*" less than 0.0500 indicate model terms to be significant, while those greater than 0.1000 indicate the model terms to be insignificant.

**Table 2** Results of FCCCD using four independent variables and six centre points showing observed and predicted response.

| Standard order | Glucose | Urea | Methionine | Succinic acid | Enzyme activity (U/l) | | Specific activity (U/mg) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Predicted | Observed[a] | Predicted | Observed[a] |
| 1 | −1 | −1 | −1 | −1 | 96.86 | 97.08±1.61 | 0.59 | 0.59±0.011 |
| 2 | 1 | −1 | −1 | −1 | 100.09 | 98.57±1.52 | 0.49 | 0.48±0.012 |
| 3 | −1 | 1 | −1 | −1 | 82.12 | 80.43±2.14 | 0.66 | 0.65±0.015 |
| 4 | 1 | 1 | −1 | −1 | 81.87 | 80.68±2.41 | 0.57 | 0.57±0.011 |
| 5 | −1 | −1 | 1 | −1 | 102.20 | 101.55±1.66 | 0.61 | 0.62±0.012 |
| 6 | 1 | −1 | 1 | −1 | 107.17 | 107.01±2.56 | 0.52 | 0.52±0.014 |
| 7 | −1 | 1 | 1 | −1 | 79.88 | 79.19±2.11 | 0.61 | 0.59±0.011 |
| 8 | 1 | 1 | 1 | −1 | 81.37 | 80.93±1.56 | 0.54 | 0.54±0.016 |
| 9 | −1 | −1 | −1 | 1 | 95.16 | 94.10±1.44 | 0.67 | 0.67±0.016 |
| 10 | 1 | −1 | −1 | 1 | 96.15 | 96.33±2.09 | 0.49 | 0.50±0.015 |
| 11 | −1 | 1 | −1 | 1 | 101.91 | 101.55±1.89 | 0.72 | 0.72±0.012 |
| 12 | 1 | 1 | −1 | 1 | 99.43 | 98.57±1.56 | 0.55 | 0.53±0.013 |
| 13 | −1 | −1 | 1 | 1 | 102.37 | 103.04±2.66 | 0.73 | 0.71±0.011 |
| 14 | 1 | −1 | 1 | 1 | 105.10 | 105.27±2.14 | 0.56 | 0.56±0.014 |
| 15 | −1 | 1 | 1 | 1 | 101.54 | 101.55±2.88 | 0.72 | 0.72±0.019 |
| 16 | 1 | 1 | 1 | 1 | 100.79 | 100.06±2.63 | 0.56 | 0.55±0.015 |
| 17 | −2 | 0 | 0 | 0 | 91.84 | 92.60±2.12 | 0.72 | 0.72±0.018 |
| 18 | 2 | 0 | 0 | 0 | 94.32 | 95.59±2.45 | 0.46 | 0.47±0.017 |
| 19 | 0 | −2 | 0 | 0 | 102.23 | 102.29±2.22 | 0.58 | 0.57±0.017 |
| 20 | 0 | 2 | 0 | 0 | 83.19 | 85.15±2.4 | 0.64 | 0.66±0.02 |
| 21 | 0 | 0 | −2 | 0 | 96.43 | 98.57±2.66 | 0.55 | 0.55±0.016 |
| 22 | 0 | 0 | 2 | 0 | 103.14 | 103.04±2.41 | 0.58 | 0.59±0.005 |
| 23 | 0 | 0 | 0 | −2 | 89.06 | 91.11±2.78 | 0.58 | 0.58±0.011 |
| 24 | 0 | 0 | 0 | 2 | 106.79 | 106.77±2.55 | 0.68 | 0.69±0.009 |
| 25 | 0 | 0 | 0 | 0 | 100.43 | 100.42±1.69 | 0.51 | 0.51±0.011 |
| 26 | 0 | 0 | 0 | 0 | 100.43 | 100.43±1.56 | 0.51 | 0.51±0.018 |
| 27 | 0 | 0 | 0 | 0 | 100.43 | 100.43±1.55 | 0.51 | 0.52±0.014 |
| 28 | 0 | 0 | 0 | 0 | 100.43 | 100.43±1.52 | 0.51 | 0.51±0.015 |
| 29 | 0 | 0 | 0 | 0 | 100.43 | 100.44±1.49 | 0.51 | 0.50±0.015 |
| 30 | 0 | 0 | 0 | 0 | 100.43 | 100.42±1.88 | 0.51 | 0.51±0.014 |

[a] Results are mean ± standard deviation of three determinations

Similarly for specific activity, the model $F$ value of 93.36 implies the model is significant. In this case, A, B, C, D, $A^2$, $B^2$, $C^2$, $D^2$, AD, BC, and CD are significant model terms.

The $R^2$ value indicated by ANOVA is 0.9860 for enzyme activity and 0.9887 for specific activity. $R^2$ value being a measure of fit of the model indicates that the model could explain 95–98% of the variability in response. The adequate precision which measures the signal-to-noise ratio was 29.52 and 32.50 (Table 4) for responses $Y_1$ and $Y_2$, respectively, which indicates an adequate signal. A ratio of >4 is desirable. This model can be used to navigate the design space for both the responses $Y_1$ and $Y_2$. The closer the value of $R$ to 1, the better is the correlation between the observed and the predicted values reflecting the accuracy and applicability of the model to optimization. The "Pred $R$-squared" of 0.9196 and 0.9346 is in reasonable agreement with the "Adj $R$-squared" of 0.9730 and 0.9781 for $Y_1$ and $Y_2$, respectively.

**Table 3** Model coefficients estimated by multiple linear regression and significance of regression coefficient for glutaminase yield and specific activity.

| | Enzyme activity (U/l) | | | Specific activity (U/mg) | | |
|---|---|---|---|---|---|---|
| Factor | Coefficient estimate | Standard error | Prob > $F$ | Coefficient estimate | Standard error | Prob > $F$ |
| Intercept | 100.43 | 0.53 | | 0.5112 | 0.00474 | |
| A | 0.62 | 0.27 | 0.0343 | −0.0638 | 0.00237 | <0.0001 |
| B | −4.76 | 0.27 | <0.0001 | 0.0164 | 0.00237 | <0.0001 |
| C | 1.68 | 0.27 | <0.0001 | 0.0075 | 0.00237 | 0.0064 |
| D | 4.43 | 0.27 | <0.0001 | 0.0266 | 0.00237 | <0.0001 |
| $A^2$ | −1.84 | 0.25 | <0.0001 | 0.0195 | 0.00222 | <0.0001 |
| $B^2$ | −1.93 | 0.25 | <0.0001 | 0.0247 | 0.00222 | <0.0001 |
| $C^2$ | −0.16 | 0.25 | 0.5298 | 0.0134 | 0.00222 | <0.0001 |
| $D^2$ | −0.63 | 0.25 | 0.0240 | 0.0297 | 0.00222 | <0.0001 |
| AB | −0.87 | 0.33 | 0.0178 | 0.0022 | 0.0029 | 0.4520 |
| AC | 0.43 | 0.33 | 0.2032 | 0.0041 | 0.0029 | 0.1822 |
| AD | −0.56 | 0.33 | 0.1077 | −0.0207 | 0.0029 | <0.0001 |
| BC | −1.89 | 0.33 | <0.0001 | −0.0151 | 0.0029 | 0.0001 |
| BD | 5.37 | 0.33 | <0.0001 | −0.0056 | 0.0029 | 0.0737 |
| CD | 0.47 | 0.33 | 0.1745 | 0.0088 | 0.0029 | 0.0083 |

Figure 3 i–vi shows the response surface contours generated for the variation in the yields of glutaminase as a function of concentrations of two variables with the other two variables at their central value. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components. Evaluation of response surface curves and contour plots indicate the range of optimum conditions within the experimental area covered or show the way to conduct further experiments to achieve better results.

Urea at low concentrations was found to be the most important factor showing interaction with all the other factors as seen from Fig. 3. Urea is known to be a major nitrogen resource in the sea, but little molecular information exists about its utilization by marine organisms [21]. As seen from Fig. 3 i, the optimum activity is obtained at higher concentration of carbon source and lower concentration of nitrogen source. Similarly, interaction between methionine and urea was found to have a favorable effect on production (Fig. 3 ii). Figure 3 iii indicates succinic acid to exert a minor effect on

**Table 4** ANOVA for the experiments.

| Term | Response $Y_1$ | Response $Y_2$ |
|---|---|---|
| | Glutaminase activity (U/l) | Specific activity (U/mg protein) |
| $F$ value | 75.65 | 93.36 |
| $P > F$ | <0.05 | <0.05 |
| Mean | 96.79 | 0.58 |
| $R^2$ | 0.9860 | 0.9887 |
| Adjusted $R^2$ | 0.9730 | 0.9781 |
| Coefficient of variance | 1.35 | 2.0 |
| Adequate precision | 29.526 | 32.507 |

increasing the glutaminase. However, as seen from Fig. 3 iv–vi, glucose, methionine, and succinic acid decreased the specific activity, while urea had a favorable effect on specific activity. All the components studied were required for the normal growth and metabolism of the organism under study. Addition of urea gave better specific activity, probably due to

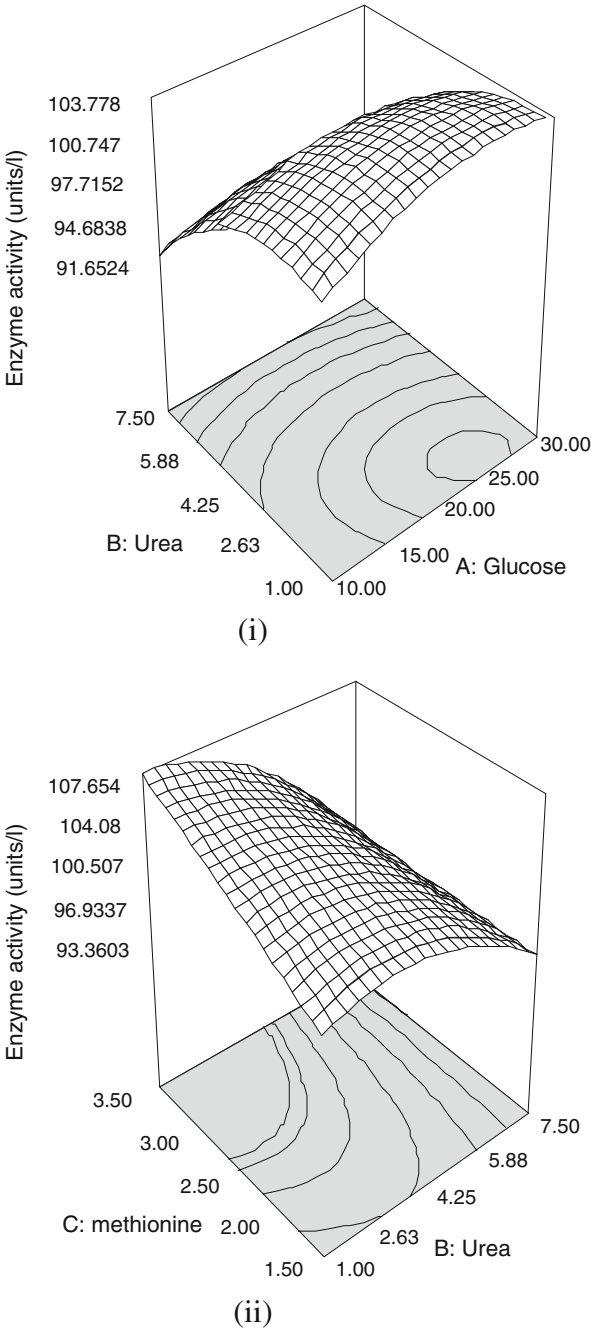
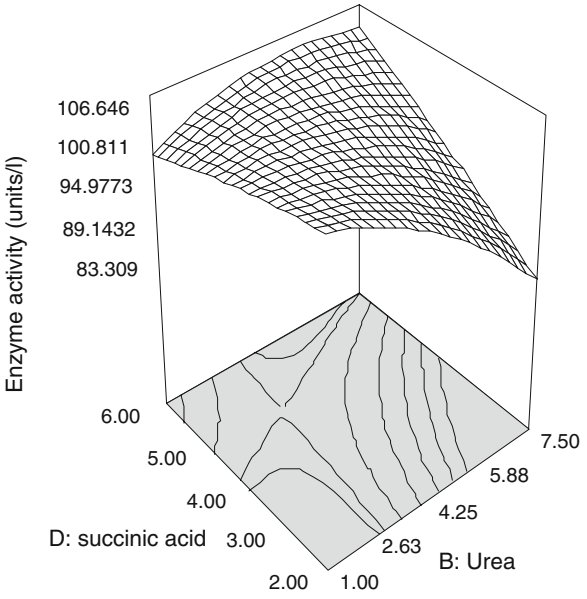
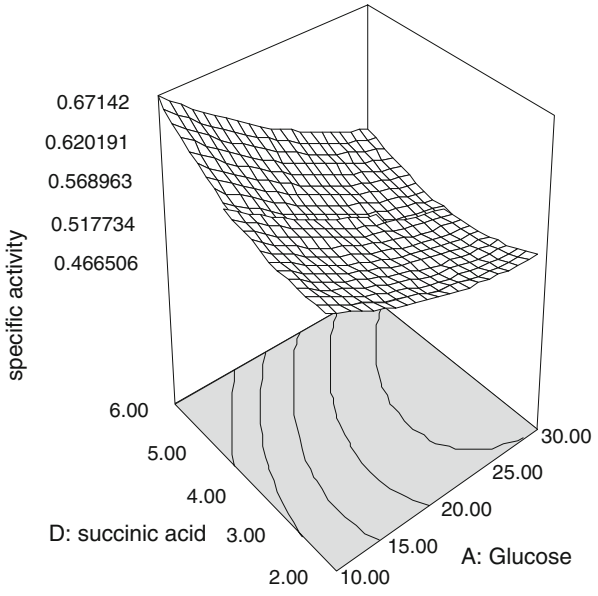



**Fig. 3** *i–vi* 3D response surface: interactive effects of (*i*) varied urea and glucose at 2.5 g/l methionine and 4 g/l succinic acid, (*ii*) varied urea and methionine at 20 g/l glucose and 4 g/l succinic acid, (*iii*) varied urea and succinic acid at 20 g/l glucose and methionine 2.5 g/l, (*iv*) specific activity interactive plot for glucose and succinic acid at 5 g/l urea and methionine 2.5 g/l, (*v*) specific activity interactive plot for urea and methionine at glucose 20 g/l and succinic acid 4 g/l, and (*vi*) specific activity interactive plot for methionine and succinic acid at glucose 20 g/l and urea 5 g/l, keeping ammonium sulfate (2.5 g/l) and yeast extract (6 g/l) constant throughout the RSM

**Fig. 3** (continued)




(iii)

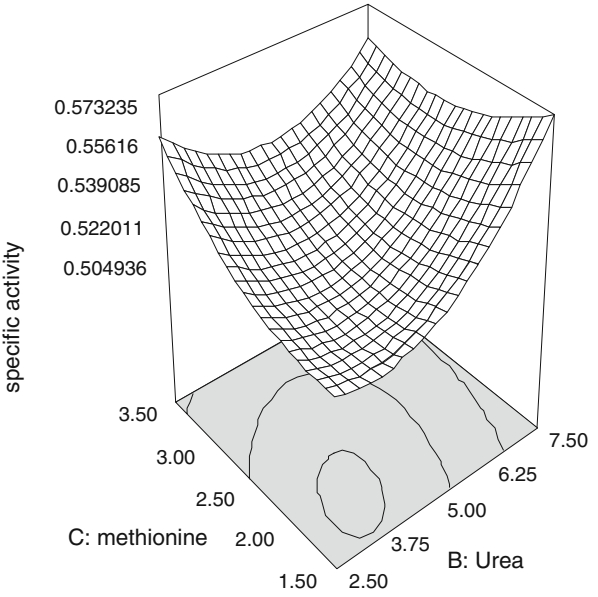



(iv)

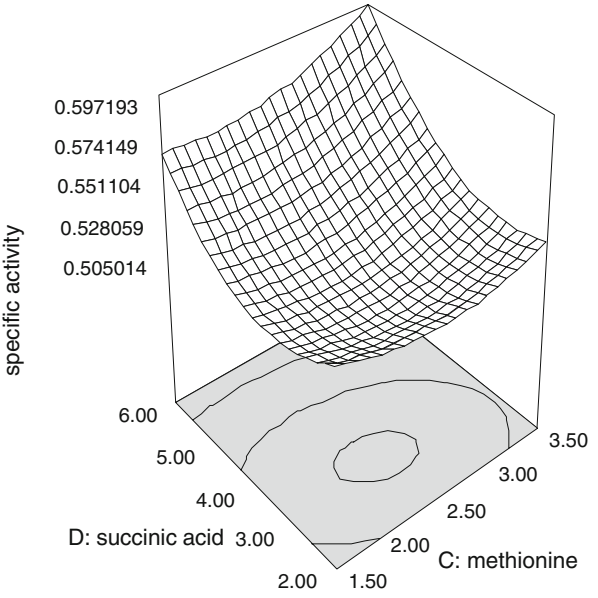
the regulation of glutaminase to some extent by the urea pathway. There have been earlier reports on the use of urea as a nitrogen source for glutaminase production. *Providencia* sp. in general shows an ability to utilize urea as nitrogen source.

Further, the predicted values by "contour plot generation" and "numerical optimization" were experimentally verified. Thus, a combination of media components containing (g/l)

**Fig. 3** Continued.




(v)




(vi)

glucose 10.0, urea 5.15, methionine 3.5, succinic acid 6.0, ammonium sulfate 2.5, and yeast extract 6.0 gave an enzyme activity of 109.23±1.34 U/l, which was almost equal to the actual predicted value of 103.85 U/l. This corresponded to a specific activity of 0.66 U/mg protein compared to the predicted value of 0.69 U/mg protein. Thus, the model was successfully validated.

## Growth and Production Profile Studies of Glutaminase

In order to confirm the optimization results, production profiles of the culture were studied using RSM predicted media composition and culture conditions. About 50 ml of media containing (g/l) glucose 10.0, urea 5.15, methionine 3.5, succinic acid 6.0, ammonium sulfate 2.5, and yeast extract 6.0 was adjusted to pH 8.1±0.1, inoculated with 5% *v/v* inoculum containing $2.34\times10^6$ cells/ml, and incubated on a shaker at 180 rpm for 48 h. Samples were withdrawn every 4 h to determine biomass, glutaminase activity, and specific activity. The cells grew rapidly and maximum cell growth was observed between 18 and 24 h. As can be seen from Fig. 4, a maximum rate of glutaminase production was observed during the late exponential and stationary phase, where it attained a peak of 119.23±0.12 U/l (0.63 U/mg protein) at 28 h. Glutaminase production remained unchanged between 28 and 32 h, beyond which the enzyme activity and specific activity both decreased.

## Effect of NaCl

The effect of NaCl concentration on the activity of the purified glutaminase was similar to the crude glutaminase. The crude glutaminase could tolerate higher concentrations up to 2.5 M (12% loss in activity), while the purified enzyme could tolerate 2 M NaCl (19% loss in activity) beyond which there was drastic loss in activity as seen in Fig. 5 i. Similarly, as seen from Fig. 5 ii, both crude and purified enzyme could tolerate up to 2 M NaCl for 20 days with negligible loss in activity at 25 °C. Thus, it can be inferred that *Providencia* glutaminase is halotolerant in nature. Glutaminases from various species of organisms behave differently in the presence of NaCl. Glutaminase I of *Micrococcus luteus* K-3 was activated by about 30% in the presence of 16% *w/v* NaCl [22], whereas glutaminase II was not [23]. Glutaminase of *Actinomucor taiwanensis* exhibited inactivation to some extent in the presence of NaCl [22]. Glutaminases from *Escherichia coli*, *Pseudomonas fluorescens*, *Cryptococcus albidus*, *Aspergillus oryzae*, and *Aspergillus sojae* showed 65%, 75%, 65%, 20%, and 6% residual activity, respectively, in 18% *w/v* NaCl [24].

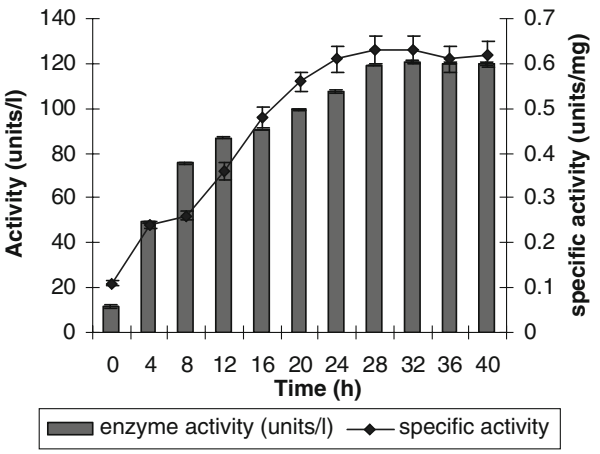
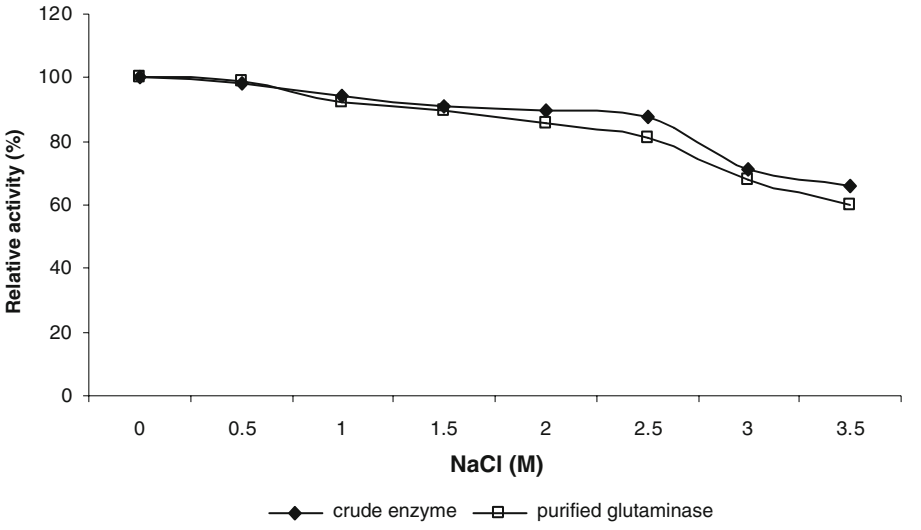
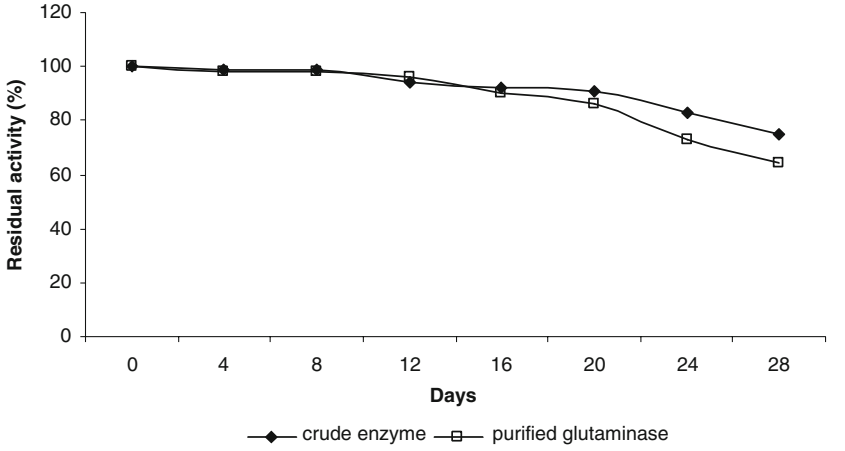



**Fig. 4** Production and growth profile of *Providencia* sp. in optimized medium




(i)




(ii)

**Fig. 5** *i* Effect of increasing NaCl concentration on glutaminase activity, *ii* residual activity of *Providencia* glutaminase in the presence of 2 M NaCl over 28 days at 25 °C

## Conclusions

In the present study, a Gram-negative coccobacilli of marine origin was isolated from breathing roots of weeds. This isolate, identified as *Providencia* sp by 16S rRNA sequencing, produced about 71.23 U/l of glutaminase extracellularly. Statistical optimization of media composition and culture conditions could increase the glutaminase yield by 67%. Urea had a substantial effect on glutaminase production showing interactions with all the other media components studied. This report is the first of its kind identifying *Providencia* sp. as a glutaminase producer. Further studies on purification and characterization of the enzyme from this strain are in progress.

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