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Isolation, Screening & Identification of L-Glutaminase Producer from the River Bank Soils of Andhra Pradesh

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Abstract : With an aim to isolate a potential enzyme producer from the marine sources, around fourteen marine colonies were isolated and were evaluated for the l-glutaminase production. The screening of l- glutaminase producing isolates were carried out using rapid agar plate technique by dye based method. Out of fourteen isolates the strain GA-07 showed highest potential for the l-glutaminase production. The enzyme activity was further confirmed by colorimetric method. The strain GA-07 was identified as Idiomarina sp. based on morphological and chemical characteristics and finally confirmed as *Idiomarina sediminum; H1695;* (Gene Bank Accession Number JF346667) by sequencing method.
Keywords : *Idiomarina sediminum; H1695*;l-glutaminase,mineral salt agar medium, DNA sequencing technique.

Introduction

L-Glutaminase also known as l-glutamine amidohydrolase(E.C 3.5.1.2) is an enzyme that catalyzes the deamidation of l-glutamine to l-glutamic acid and ammonia [1]. L-Glutamine plays an important role in nitrogen metabolism. L-Glutamine and l-glutamate are one of the important amino acids used for the production of proteins in cellular metabolism. It is either synthesized with in the cell by the action of the enzyme glutamine synthetase(GS) or obtained through diet.

Some of the tumor cells are auxotrophic(GS^-) in nature and they depend upon external supply of the amino acids (from diet) for their growth and development. Depletion of which results into the death of the cancer cells. L-Glutaminase depletes the levels of the amino acids available converting them into glutamic acid and ammonia leading to the death of GS^- cancer cells. In recent years l-glutaminase has received significant attention owing to its activity against wide range of cancer cells.

Another most promising application of l-glutaminase is its usage in the treatment of human immunodeficiency virus (HIV) [2].L-Glutaminase can also be used in biosensors to monitor the levels of glutamine in the cell culture broths [3,4] .It is also useful in the production of high marketed value speciality chemicals like threonine. The vast application of l-glutaminase in various fields always prompted a search for a better source of the enzyme. Although l-glutaminase can be derived from both plant and animal sources, microbial source is generally preferred for industrial production due to their economic production, consistency, ease of process modification and optimization [5].

L-Glutaminase activity was reported in various terrestrial micro-organisms such as *Escherichia coli, Pseudomonas sp, Acinetobactersp, Bacillus sp, Proteus morganni, Cryptococcus, Candida and Aspergillus*

oryzea[5].Apart from terrestrial sources, few marine micro-organisms were also known to synthesize l-glutaminase and include *Pseudomonas flurosence, Micrococcus luteus, Vibrio costicola and Beuveriabassiana*[6].

In spite of its demonstrated potential as anticancer agent, l-glutaminase is generally regarded as a key enzyme that controls the taste of fermented food such as soya sauce by increasing the glutamic acid content, there by imparting a unique flavor to the food [7]. Thus, salt tolerant and heat stable l-glutaminase demands not only search for potential strain, but also economically viable bioprocessfor its large scale production[8].From the literature, it is evident that only few reports are available on the extracellular production of l-glutaminase from marine bacteria and since there is an excessive requirement for salt and thermo tolerant l-glutaminases, a search for a potential marine strain that hyper produce this enzyme with novel properties and an economically viable bioprocess is pursued.

The present work deals with the isolation, screening and identification of potential l-glutaminase producers from marine sources.

Materials and Methods

Chemicals and reagents

The reagents employed in the current investigation were of high grade and quality. Media components used for the preparation of bacteriological medium were of Hi- Media Labs. Remaining chemicals belongs to Sigma Aldrich.

Sample Collection

Test microorganisms used in the current work were isolated from the marine samples collected from river banks A.P, India. Over all around 20 tests samples were collected from different places. Samples thus obtained were air dried, sieved to eliminate foreign particles, packed in a sterile containers and preserved under refrigeration for further use.

Enrichment and isolation of microorganisms

1g of the sieved air dried marine soil was suspended into a 50ml of sterile water and mixed well for 30 minutes .1ml of the superficial liquid was collected and diluted up to five times. 0.5ml of the sample was then inoculated into a 50ml of sterile enriched media and incubated at 37^0C for 24 -48 hours. Fluconazole was incorporated into the media to prevent the growth of the fungal colonies. Similarly marine water dilutions were prepared and added into the medium for the separation of the colonies.

Table 1: Composition of Enriched agar medium

Ingredients	Quantities
Yeast extract	0.5g
Peptone	0.5g
Agar	20g
Fluconazole	50µg/ml
Agar	20g
Aged sea water	1000ml
p H	7.4

Colonies thus separated from the marine samples were collected, sub-cultured on sterile NA slants and maintained in the laboratory at 4^0 C. Colonies differing in macro morphological characters were preferred for the screening process.

Screening of the marine bacterial isolates for l-glutaminase production

Primary screening

Bacteria thus isolated were tested for the enzyme production by dye based method on minimal mineral glutamine media. Each colony thus isolated were picked up (with the help of sterilized transferring needle) and inoculated onto slants containing sterilized minimal mineral l-glutamine medium. And labelled accordingly and incubated at 37^0 C for 48hrs. Since the medium composed of l-glutamine as the sole energy source, the microorganisms having capability to metabolize l-glutamine can only grow. L-Glutaminase producing microorganisms convert the amino acid present in the media to l-glutamine and ammonia. Generation of ammonia changes the p H of the medium which gives pink colour due to the presence of chloro phenol red. L-Glutaminase positive colonies are detected by pink colouration.

Table 2. Composition minimal mineral glutamine medium

Ingredients	Quantities g/ml
L-Glutamine	0.5g
KCl	0.5g
K_2HPO_4	1g
KH_2PO_4	0.1g
$MgSO_4.7H_2O$	1.0 g
$FeSO_4.7H_2O$	0.1g
Chloro-phenol red	0.012g
Agar	20g
Aged sea water	1000ml ; p H 7.4

The l-glutaminase production was in addition proved by estimating the levels of ammonia formed by method described by Imada*et al*. (1973)[9].

Inoculum preparation

Adequate amount of sterile water was poured into the NA slants containing glutaminase positive cultures and mixed well. 5ml of the resultant bacterial suspension was inoculated into the medium , (peptone - 5g; yeast extract 1g; NaCl -2.45g; aged sea water 495ml) and incubated for 24 hrs. 1% of the medium was used as an inoculum.

Production of enzyme l-gutaminase

5ml of the Inoculum was added into 100ml of erlenmeyer flasks containing 45ml of sterile production medium (d-glucose -10g, l-arginine -20g, aged sea water 1000ml, p H8). The flasks were incubated at 35^0C for 120hrs at 120rpm on an orbital shaker incubator. The samples were centrifuged for 30 minutes at 1500rpm and clear superficial liquid was used for the enzyme estimation.

Estimation of ammonia concentrations

Glutaminase was assayed according to the method described in the study of Imada *et al.* (1973). An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M l-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1 M, pH 8). The mixture was incubated at 37°C for 30 min and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloro acetic acid.

To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 450 nm using a UV-visible spectrophotometer. The liberated ammonia content was measured and one international unit of l-glutaminase was defined as the amount of enzyme that liberates one µmole of ammonia under optimal conditions. Assays were done in triplicates and the mean enzyme activity was expressed as units/ml of culture supernatant (U/ml).

Characterization of the isolate

Out of all the positive cultures, the one showing the highest enzyme yield was selected and identified by basic morphological screening and biochemical tests and finally confirmed by sequencing method.

Morphological screening

Macro-morphology was done by observing the characteristics of colonies such as size, shape, colour, texture etc. Colonies of the strain were cultivated on a fresh NA plates and incubated for 24hrs.Colonies thus generated upon incubation were examined. Micro morphological screening was done by observing the structure of strain under microscope by staining techniques.

Molecular characterization of GA-07

It was carried out by the following technique:

Preparation of template DNA:

Pure fresh cultures were used for the identification. Colonies were picked up with the help of sterile transferring needle and suspended in 0.5ml of sterilized saline solution and centrifuged. Supernatant was used for PCR. Insta Gene Matrix consists of a specially formulated 6% w/v chelex resin, which helps in easy quick preparation of good quality DNA that can be used for PCR.

PCR:

To the 20µl of PCR reaction solution about 1µl of template DNA was added. Amplification was done using 785F- GGATTAGATACCCTGGTA /907R CCGTCAATTCMTTTRAGTTT primers. Around 35 amplification cycles were performed at 94^0C for 45 sec,55^0C for 60 sec and 72^0 C for 60 sec. DNA fragments were amplified to about 1,400bp.Positive (E. Coli genomic DNA) and negative control were employed in the PCR.

Purification of PCR products:

Millipore PCR filter plates offers fast automatic, high degree purification process involving less number of steps.

1. PCR reactants were added.
2. Filtered with Millipore vacuum manifold for 5-10 minutes or until wells are dry.
3. Buffer or water was added to each well .Mixed and the sample was retrieved by aspiration.

Sequencing.

The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers. Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied Bio Systems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Bio Systems, USA).

Results & Discussion

Isolation of microorganisms

Around 20 samples containing both the water as well as soil samples were collected approximately from different locations, diluted and labelled accordingly. Several colonies with different macroscopic characters emerged on the enriched media within 24-48 hrs of incubation, out of which 35 colonies were selected for the testing (figure 1 a & b).

Figure 1 a. &1 b. Colonies on enriched media c. Positive culture showing pink color around the colonies.

1^0 screening: Dye based method

Screening is first step in the fermentation process. Thus the procedure adopted for the screening process should be perfect accurate, simple, easy and inexpensive. Testing for l-glutaminase production was done initially by dye based method which was later confirmed by colorimetry.

Semi-quantitative testing for l-glutaminase presented that during 48 hrs of incubation, marine isolates differed in growth on mineral salt agar medium, 9 isolates were seen during 24hr and 5 isolates arosed during 48hr, while 21 isolates couldns't grow. The glutamine degrading 14 isolates differed in color intensity originated from reaction of the dye. 14 isolates produced pink color around their colonies depicting the extracellular production of the enzyme. L-Glutamine negative cultures failed to raise on minimal arginine medium. These results are presented in fig 1.c.

Fourteen isolates showing pink coloration were selected, sub cultured on fresh NA slants and numbered as GA 1 to GA 14.

Semi-quantitative screening conferred that, mineral salt agar medium was very suitable medium for testing of glutaminase production because it had l-glutamine as a sole energy source. And the organisms having ability to digest l-glutamine will only grow. The enzyme converts (metabolism) l-glutamine present in the media to glutamic acid and ammonia. If the enzyme is produced by the test microorganism, it is seen in the form of pink colouration (fig 1.c). Thus the budding isolates proved that they produced this enzyme.

2^0screening:Colorimetric method

The selected 14isolates were employed for l-glutamine production in medium having l- glutamine, D-glucose solubilized in sea water and pH adjusted to 8 at 35^{o}C and at 120 rpm. The samples were withdrawn for every 24h up to 120h and tested for enzyme activity. The isolate GA-07 demonstrated maximum glutaminase activity among all 14 isolates. The results showing the yields of all 14 isolated strains were given in the figure 2.

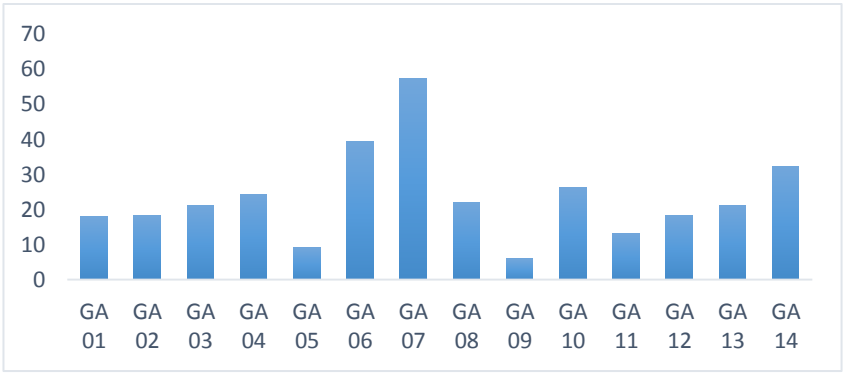

Figure 2: Enzyme activity of the fourteen bacterial isolates

Marine bacterial strain was preliminarily recognized by observing the colony characteristics. Morphological, biochemical and cultural characters revealed that the marine isolate GA-07 belongs to the *Idiomarina* genus. Gram staining and hanging drop technique revealed the strain GA-07 is a gram negative rod shaped non-motile bacteria. The colonies appear white, round shaped with entire edge on NA media .Endospores were absent.

It showed a positive reaction to indole production, methyl red, glucose and lactose fermentation, gelatin, casein and starch hydrolysis test, reduction from nitrate to nitrite, catalase test etc and growth in Nutrient broth with 6-10% NaCl. Negative findings included: Vogesproskauer test, growth on Simmons' citrate, growth in Nutrient broth with 0% NaCl, H_2S on tri-sugar iron agar (TSI), gas production, urea hydrolysis, sucrose, xylose, arabinose fermentation etc.

Morphological and biochemical characteristics of the strain reveals that the unknown GA-07 bacterial strain belongs to genus *Idiomarina*. Further the strain was confirmed as *Idiomarina sediminum; H1695* by sequencing method.

Conclusion

The present study focuses on the selective isolation of the potent l-glutaminase producing marine bacteria. The isolates potential in l-glutaminase production were evaluated colorimetrically. Based on the enzyme activity, the potent isolate was selected and further biochemical and molecular identification was carried out to identify the micro-organism. The isolate was found to be *Idiomarina sediminum; H1695*; (Gen Bank Accession Number GU726873). This strain isolated from the marine sediments has the potential for l-glutaminase synthesis and suggests that this can be used as a potent source of the enzyme. Further studies on the media optimization for the large scale production of the enzyme from this strain are in progress.

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Conflict of Interest: We have no conflicts of interest.

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