



SCREENING AND ISOLATION OF L-GLUTAMINASE PRODUCING MARINE BACTERIAL CULTURES

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ABSTRACT:

Microbial glutaminases have found applications in several fields. L-Glutaminase has received significant attention recently owing to its potential applications in medicine as an anticancer agent and flavor enhancing agent in food industries. Screening of extra cellular L-glutaminase producing marine bacterial strains isolated from sediment and water samples collected at different places along the beach of Bay of Bengal in Visakhapatnam were done by dye based procedure. Among the fifteen isolates LG₂₄ gave highest activity of 18.82 U/ml with in 48 h where L-glutamine supplemented as sole carbon and nitrogen source in the media.

Key words: L-glutaminase, enzyme, extra cellular, marine, submerged fermentation, anticancer agent.

INTRODUCTION:

Microbial glutaminases have found applications in several fields. They had been tried as therapeutic agents in the treatment of cancer^[1,2] and HIV^[3], as an analytical agent in determination of glutamine and glutamate^[4-6], and in the production of specialty chemicals like threonine by gamma glutamyl transfer reactions^[7]. L-glutaminase is the enzyme deaminating L-glutamine. The action of glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes. In recent years glutaminase has attracted much attention with respect to proposed applications in pharmaceuticals as anti-leukemic agent^[8] and in food industry as flavor enhancing agent^[9]. L-glutaminase in combination with or as an alternative to asparaginase could be of significance in enzyme therapy for cancer especially acute lymphocytic leukemia^[8]. Its commercial

importance as anticancer and flavor enhancing agent demands not only the search for better yielding viable strains, but also economically viable bioprocesses for its large scale production^[10]. Another important application of L-glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid^[11]. Marine microorganisms hold significance in food industry by virtue of their ability to produce salt tolerant L-glutaminase, as salt and thermo tolerant glutaminases are needed in soy sauce fermentation^[12]. Renu and Chandrasekhar^[11] isolated extra cellular L-glutaminase-producing bacteria from seawater and marine sediments from environments at Cochin and observed that their populations were on the order of 10⁵ cells/per ml or gm. Species of *Pseudomonas*, *Vibrio*, *Bacillus*,

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Moraxella, *Aeromonas*, and *Acinetobacter* producing L-glutaminase were also recovered^[11, 13]. Different methods of fermentation technology can be applied for the production of L-Glutaminase. Commercial production of L- Glutaminase is carried out using SmF (Submerged fermentation) technique. Since the present source for this enzyme is limited to marine bacteria alone, importance is given to SmF (Submerged fermentation) technique only.

MATERIALS AND METHODS

Screening Procedure

Soil and water samples were collected from different places along the beach of Bay of Bengal in Visakhapatnam. All the samples were collected in sterile screw capped tubes and care was taken to see that the points of collection had as widely varying characteristics as possible as regard to the organic matter, moisture content, particle size, colour of soil and geographical distribution.

About 1 g of each of above samples were taken into 50 ml of sterile water. The suspension was kept on rotary shaker for 30 min and kept aside to settle the soil matter. One ml of the suspension was serially diluted five times with sterile water and 0.5 ml of each of these dilutions was added to 50 ml of sterile isolation medium (Yeast Extract 0.5 g, Peptone 0.5 g, Agar 20 g, and Aged Sea Water 1000 ml)

This media, after sufficient mixing were plated in 6-inch dia sterile petridishes and incubated at 37°C. Similarly, water samples were also diluted five times individually and plated as above. (Cyclohexamine 50 µg/ml is used as antifungal agent). Total thirty individual bacterial colonies have different macroscopic characters were selected from the plates after 40 h of incubation at 37°C. After preliminary screening, thirty five bacterial strains were selected to study enzymatic activity depending on the colony size, texture and macroscopic characteristics. After isolation the bacterial strains were maintained on the Nutrient agar slants.

Detection of L-glutaminase positive cultures

Minimal agar medium (KCl 0.5 g, MgSO₄.7H₂O 0.5 g, FeSO₄.7H₂O 0.1 g, ZnSO₄.7H₂O 1.0 g, KH₂PO₄ 1.0 g, L-Glutamine 0.5%, Phenol red 0.012 g, Aged Sea Water 1000 ml) contains 0.5% L-glutamine as the sole carbon and nitrogen source and phenol red as pH indicator. The colour change of the medium from yellow to pink is an indication of the extra cellular L- glutaminase production by the colony. This colour change is due to change in the pH of the medium, as L- glutaminase causes the breakdown of amide bond in L- glutamine and liberates ammonia. All the bacterial strains collected were streaked in small Petri plates with minimal agar medium individually. After two days of incubation at 37°C the plates turned pink indicating positive response. Out of 30 bacterial strains, 15 are positive out of which 5 from sediment, 5 from water collected in tenneti park area and 5 from water collected in sagar nagar area. These strains were used for further study.

Inoculum preparation

Sterile sea water 5 ml was aseptically added to 48 h old Nutrient agar culture slants and cell suspension was prepared. This suspension was added to 45 ml of inoculum medium (Peptone 5 g, Yeast extract 1 g, NaCl 2.45 g, Aged Sea water 1000 ml). The inoculated medium was kept on a rotary shaker at 200 rpm for 48 h. Then 10% inoculation medium is used as inoculum.

Production

From the prepared inoculum 5 ml was transferred aseptically to 45 ml of production medium (SWG) Sea water glutamine medium: L-Glutamine 20 g, D-Glucose 10 g, Aged Sea water 1000 ml, pH 8. The flasks were kept on an orbital shaking incubator at 35°C and at 120 rpm for 48 h. The samples withdrawn were centrifuged at 1500 rpm for 30 min and the clear supernatant was used for enzyme estimation.

Glutaminase activity

The activity of glutaminase is determined by estimating the amount of NH₃ liberated from glutaminase. The activity of an enzyme was

determined by following Imada et. al.^[17] method using ammonium sulfate as the standard. Enzyme preparation of about 0.5 ml was added to 0.5 ml of 0.04M L-glutamine and 0.5 ml of distilled water. To this 0.5 ml of 0.5M tris buffer (pH-8) was added and incubated at 37°C for 15 min. After incubation 0.5 ml of 1.5M trichloroacetic acid was added to stop the enzyme reaction. Blank tubes were run by adding the enzyme preparation after the addition of trichloroacetic acid. Then 0.1 ml of above mixture was taken and added to 3.7 ml of distilled water. Then 0.2 ml Nessler's reagent was added to it. Absorbance was measured at 450 nm using a visible spectrophotometer.

Standard graph was prepared by treating 1 ml of various concentrations (0.5mM, 1mM, 1.5mM, 2mM, 2.5mM etc.) of ammonium sulphate with 0.5 ml of 0.5M tris buffer, 0.5 ml of 1.5M TCA, and Nessler's reagent. One international unit of glutaminase was defined as amount of enzyme that liberates one micromole of ammonia under optimum conditions. The enzyme yield was expressed as units/ml (U/ml).

RESULTS AND DISCUSSION

The selected 15 isolates were used for glutaminase production in medium containing L- glutamine, D-glucose dissolved in sea water and pH adjusted to 8 at 35°C and at 120 rpm. The samples were withdrawn for every 24 h up to 120 h and assayed for glutaminase activity. The isolate LG₂₄ exhibited maximum glutaminase activity of 18.82 U/ml among all 15 isolates. The results showing the yields of all 15 isolated strains were given in table 1.

The present work was carried out to identify L-glutaminase producing cultures isolated from marine samples. A semi quantitative plate assay is used for screening of L-glutaminase producing microorganisms. The plate assay was devised using this principle by incorporating the pH indicator phenol red in the medium containing glutamine (sole carbon and nitrogen sources). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around the microbial colonies producing L-glutaminase.

Table 1: Glutaminase production by the selected isolates

Isolate number	Yield (U/ml)
LG ₁	11.76
LG ₃	9.41
LG ₄	3.53
LG ₁₂	4.71
LG ₁₃	7.53
LG ₁₄	10.59
LG ₁₇	7.29
LG ₁₈	5.88
LG ₂₃	7.29
LG₂₄	18.82
LG ₂₅	10.94
LG ₂₆	7.86
LG ₂₇	5.04
LG ₂₈	4.24
LG ₃₀	5.27

The plate assay used for screening the isolates to determine their glutaminase activity (equal to diameter of the pink zone) is also an indication of the amount of glutaminase produced by the colony or isolate (fig. 1). The different isolates exhibited pink zones around them. The size or diameter of these zones was proportional to the glutaminase produced by the colony. This revealed that isolated LG₂₄ had the maximum glutaminase activity. However all the 15 isolates were employed for glutaminase production by submerged fermentation and the results were reported in Table 1.



Detection of colonies

Fig. 1: Glutaminase positive colony on agar medium

As expected isolate LG₂₄ which exhibited a pink zone with maximum diameter also produced the maximum amount of glutaminase in submerged fermentation.

CONCLUSION

The results of the preliminary study indicates that L-glutaminase positive marine bacterial strain LG₂₄ could be a source for extracellular L-glutaminase production with possible utilization as potential source for anti-leukemic agent and flavor enhancing agent in the food industries after the extensive therapeutic activity determination. As it is of marine isolate it could tolerate the high salt concentrations in food industries which may affect the enzyme activity. Extensive bioprocess parameter studies should underway for microbial production of L-glutaminase for the isolated strain.

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