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SCREENING OF L-GLUTAMINASE PRODUCING MARINE BACTERIAL CULTURES FOR EXTRACELLULAR PRODUCTION OF L-GLUTAMINASE

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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 extracellular 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 thirty isolates LG24 gave highest activity of 22.68U/ml with in 120h where L-glutamine supplemented as sole carbon and nitrogen source in the media.
KEY WORDS: L-glutaminase, enzyme, extracellular, marine, submerged.

INTRODUCTION

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 [1] and in food industry as flavor enhancing agent [2]. L-glutaminase in combination with or as an alternative to asparaginase could be of significance in enzyme therapy for cancer especially acute lymphocytic leukemia [1]. 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 [3].

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 [4]. Marine microorganisms hold significance in food industry by virtue of their ability to produce salt tolerant L-glutaminase, as salt and thermotolerant glutaminases are needed in soy sauce fermentation [5]. The production of extracellular L-glutaminase by marine *Pseudomonas* sp., *Vibrio costicola* [6-8] and *Micrococcus* sp., [9] was reported.

Dharmaraj *et al*., [10] reported that mangrove sediments on the east coast of India exhibited higher L-glutaminase activity than sediments of other biotypes. Renu and Chandrasekaran [5]isolated extracellular 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, Moraxella, Aeromonas,* and *Acinetobacter* producing L-glutaminase were also recovered [5,7]. As far as the occurrence of glutaminase producers with respect to the environment is concerned, majority of the glutaminase producing microorganisms were isolated from soil except few on aquatic marine environment [10].

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.

EXPERIMENTAL

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 1g of each of above samples was taken into 50 ml of sterile water. The Suspension was kept on rotary shaker for 30min 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 20.0g, and Aged Sea Water 1000 ml)

This media, after sufficient mixing were plated in 6-inch dia sterile petridishes and incubated at 37^0C. Similarly, water samples are also diluted five times individually and plated as above. (Cyclohexamine 50µg/ml is used as antifungal agent).

Total 35 individual bacterial colonies have different macroscopic characters were selected from the plates after 40 hrs of incubation at 37^0 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 0.5g, $MgSo_4.7H_2O$ 0.5g, $FeSo_4.7H_2O$ 0.1g, $ZnSo_4.7H_2O$ 1.0g, KH_2PO_4 1.0g, L-Glutamine 0.5%, Phenol red 0.012g , 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 librates ammonia.

All the bacterial stains collected were streaked in small petri plates with minimal agar medium individually. After two days of incubation at 37^0C the plates turned pink indicating positive response. Out of 35 bacterial strains, 30 are positive out of which 18 are from sediment, 9 are from water and 3 are from flora. These strains were used for further study.

Inoculum preparation:

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

Production:

From the inoculum prepared 5ml was transferred aseptically to 45ml of production medium (SWG) Sea water glutamine medium: L-Glutamine 20g, D-Glucose 10g, Aged Sea water 1000mL, pH 8. The flasks were kept on a orbital shaking incubator at 35^oC and at 120 rpm for120h. The samples withdrawn were centrifuged at 1500rpm for 30min and the clear supernatant was used for enzyme estimation.

Glutaminase activity:

The activity of glutaminase is determined by estimating the amount of NH_3 liberated from glutaminase. The method of Imada *et al* [11] followed which is as follows. Enzyme preparation of about 0.5ml was added to 0.5ml of 0.04M L-glutamine and 0.5ml of distilled water. To this 0.5ml of 0.1M phosphate buffer (pH-8) was added and incubated at 37°c for 30min. After incubation 0.5ml 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.

Then 0.1ml of above mixture was taken and added to 3.7ml of distilled water. Then 0.2ml Nessler's reagent was added to it. Absorbance was measured at 450nm using a visible spectrophotometer. Standard graph was prepared by treating 1ml of various concentrations (0.5mM, 1mM, 1.5mM, 2mM, 2.5mM etc.) of ammonium sulphate with TCA, NaoH 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 30 isolates were used for glutaminase production in medium containing L-glutamine, D-glucose dissolved in sea water and p^H adjusted to 8 at 35^oC and at 120 rpm. The samples are withdrawn for every 24h up to 120h and assayed for glutaminase activity. The isolate LG24 exhibited maximum glutaminase activity of 22.68 U/ml among all 30 isolates. The results showing the yields of all 30 isolated strains were given in table 1.

Table.1: Glutaminase Production by the selected isolates and pH of the cultures after production.

Isolate number	Yield (U/ml)	pH (Cultures filtrates)
LG1	22.4	8.00
LG2	18.68	7.89
LG3	19.7	7.67
LG4	16.64	7.94
LG5	16.12	7.88
LG6	19.2	8.56
LG7	18.88	8.20
LG8	17.53	8.15
LG9	17.02	8.82
LG10	20.92	8.13
LG11	17.28	8.62
LG12	19.07	8.09
LG13	22.20	8.74
LG14	16.00	8.61
LG15	17.85	8.90
LG16	19.71	8.46
LG17	21.18	8.08
LG18	20.67	8.10
LG19	20.28	8.18
LG20	15.61	8.69
LG21	16.06	8.06
LG22	20.86	8.21
LG23	16.76	8.80
LG24	22.68	8.53
LG25	19.07	8.66
LG26	18.49	8.93
LG27	22.01	8.06
LG28	18.81	8.54
LG29	19.52	8.03
LG30	19.00	8.07

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. L-glutaminase production is accompanied by an increase in the pH of the culture filtrates. 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. 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. 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 30 isolates were employed for glutaminase production by submerged fermentation and the results were reported in Table 1. 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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