

PRODUCTION AND OPTIMIZATION OF L-GLUTAMINASE FROM *VIBRIO* SP. M9 ISOLATED FROM MAHABALIPURAM MARINE SEDIMENTS

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ABSTRACT

In the present study L-glutaminase producing bacteria were isolated from sediment samples collected from Mahabalipuram, Parangipettai and Sundarban mangrove ecosystems. L- glutaminase from selected 15 bacterial isolates was produced by submerged fermentation. Bacterial strain M9 isolated from Mahabalipuram marine sediments showed maximum L- glutaminase activity in semiquantitative assay. The crude enzyme from potential strain M9 was produced and partially purified by ammonium sulphate precipitation and dialysis. In quantitative assay, the partially purified preparation showed 28.7U/ml of enzyme activity. In SDS PAGE the enzyme showed two bands with 45 and 250 kDa respectively. Of the various Physic-chemical parameters

tested, pH 7, temperature 35°C and 3.5% NaCl concentration was found to influence L- glutaminase production by the strain M9. The potential marine bacterial isolate was identified as *Vibrio* sp., based on its morphological, cultural, biochemical and physiological characteristics.

Keywords: Marine bacteria, L-glutaminase, *Vibrio* sp.

INTRODUCTION

L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) catalyses the hydrolysis of L-glutamine into L-glutamic acid and ammonia. This is an essential enzyme for the synthesis of various nitrogenous metabolic intermediates [1]. In recent years, L-glutaminase has attracted much attention in both pharmaceutical and food industrial applications. In food industry, L-glutaminase is used as a flavor enhancer by increasing glutamic acid content in food through hydrolysis of L-glutamine to L-glutamic acid and ammonia. It also used in enzyme therapy for cancer especially for acute lymphocytic leukemia. Another important application of L-glutaminase is in biosensors for monitoring the glutamine levels in mammalian and hybridoma cells [2]. It also found to be active against human immunodeficiency virus (HIV) [3]. The tremendous 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 plant as well as animal sources, microbes are generally meeting the growing demands. Reports showed that the majority of microbes producing L-glutaminase have been isolated from soil and aquatic (marine) environment [4, 5]. The terrestrial bacterial source of L-glutaminase is currently used for the treatment of leukemia but this is known to cause a lot of side effects and hence there is a need for an alternative enzyme drug which is more compatible to human blood and induces less or no side effects in patients. The marine environment particularly sea water, which is saline in nature and chemically closer to human blood plasma, can provide microbial enzymes that are safe with no or less side effects when administered for human therapeutic application. Yet another important fact about marine microbial enzyme is that they show high level of salt tolerance [6]. Hence there is an increasing interest in the marine microorganism for therapeutic purposes. This paper describes the isolation and characterisation of L-glutaminase from selected marine bacteria.

MATERIALS AND METHODS

Collection of sample

Sediment samples were collected from three different marine environments in Tamilnadu such as Mahabalipuram (lat. 12.63° N, long. 80.17° E), Parangipettai mangrove vegetation (lat. 11.49° N, long. 79.7600° E), and Sundarban mangrove vegetation (lat. 21°31'-22°30' N and long. 88°10'-89°51' E). Samples were collected in sterile plastic bags and transported to the laboratory.

Enrichment and Screening for L-glutaminase producing bacteria

Each one gram of sediment sample was serially diluted using sterile sea water upto 10^6 dilutions. Hundred microliter of aliquot from 10^3 to 10^6 dilutions were transferred to minimal glutamine (MG) agar (grams/litre) include 0.5 KCl; 0.5 MgSO_4 ; 1.0 KH_2PO_4 ; 0.1 FeSO_4 ; 0.1 ZnSO_4 ; 10 glutamine and 0.12 Phenol red) plates and spreaded using sterile L-rod. About 50 μg /ml of nystatin was added into the medium, after sterilization, inorder to retard the growth of fungal colonies. Plating was done in triplicate and all the plates were incubated at 28°C for 48-72 hours. Only the bacteria which synthesise L-glutaminase can develop colonies on minimal glutamine agar plates and the extracellular production of L-glutaminase was confirmed by the formation of pink color zone around the colonies. After incubation, morphologically different bacterial colonies were selected, purified and subcultured on nutrient agar slants. All the isolates were preserved at refrigerated conditions as slant culture [2].

Production of L-glutaminase

L-glutaminase from all the bacterial cultures were produced by shake flask fermentation using seawater glutamine medium (SMG - g/l) L-Glutamine 20, D-Glucose 10, pH 8). Five ml of inoculum was transferred aseptically to 45ml of SMG medium [7]. All the flasks were incubated in rotary shaker at 28°C . After incubation, the fermentation medium was removed and centrifuged using cooling centrifuge at 10,000 rpm for 30 minutes at 4°C . The cell free supernatant was collected in screw cap tube and stored at 4°C to use as crude enzyme.

Semi quantitative assay of L- glutaminase

Minimal glutamine agar medium was prepared and a well with 5 mm diameter was made at the centre of the agar medium by using well puncher. About 50 μl of crude enzyme was loaded in to the well and incubated at 28°C for 24 hours [6]. Bacterial strain with its crude L-glutaminase enzyme showed maximum zone of color change (from yellow to pink) on minimal glutamine agar medium was selected as potential strain for further studies.

Production and partial purification of L-glutaminase from potential strain

L-glutaminase enzyme was produced in large quantities from potential bacterial strain by submerged fermentation method using SMG medium. Finely powdered ammonium sulphate (Himedia) was slowly added in to cell free supernatant (crude enzyme) so as to reach 40% saturation. The whole content was stirred at 4°C using magnetic stirrer. The precipitated crude enzyme was removed by centrifugation at 10,000 rpm at 4°C for 20 minutes. Fresh

ammonium sulphate was added to the supernatant to increase the saturation to 50%. The obtained precipitate was resuspended in a minimal volume of 0.01M phosphate buffer (pH 8). Precipitated protein was removed by centrifugation as described earlier. Once again the fresh ammonium sulphate was added to the cell free supernatant to increase the concentration to 80%. The obtained enzyme precipitate was resuspended in a minimal volume of 0.01M phosphate buffer (pH 8) and precipitated protein was recovered by centrifugation [2]. The enzyme precipitate obtained after ammonium sulphate precipitation was dialyzed against 0.01 M phosphate buffer (pH 8) for 24 hours at 4°C with stirring. The buffer was changed occasionally. The dialysate was collected and stored at 4°C.

Quantitative assay of L-glutaminase

L- glutaminase was assayed according to the procedure given by Imada *et al.*, [8]. The reaction mixture containing 0.5ml of L-glutamine (0.04M), 0.5ml of phosphate buffer 0.01M (pH 8.0), 0.5 ml of distilled water and 0.5 ml of enzyme solution was incubated at 37°C for 15 minutes. The reaction was stopped by addition of 0.5ml of 1.5M Trichloroacetic acid. Then to 3.7 ml of distilled water, 0.1ml of the reaction mixture and 0.2ml of Nessler's reagent were added. The OD value was recorded at 450 nm in a UV spectrophotometer. Enzyme and substrate blanks were used as controls. One unit of glutaminase was defined as the amount of enzyme that liberated 1 micromole of ammonia under optimal assay conditions [9]. Determination of the molecular weight of L-glutaminase, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 12% polyacrylamide gel and stained with silver staining [5].

Effect of pH, temperature and salinity on the production of L-glutaminase

Bacterial inoculum was prepared by inoculating the cells of bacterial strain M9 into nutrient broth and kept in shaker for 48 hours with 120 rpm. Each 5 ml of nutrient broth culture was used as inoculum. 5 ml of potential bacterial strain was inoculated in to series of flasks containing 100 ml of SWG medium with different pH (5,6,7,8 and 9). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours. To study the effect of temperature, 5 ml of inoculum was inoculated in to 100 ml of SWG medium and incubated at different temperature (25°C, 30°C, 35°C, 40°C and 45°C) for 120 hours in rotary shaker with 120rpm. To study the effect of salinity on glutaminase production, SWG medium was prepared with different concentration of sodium chloride (1, 2, 2.5, 3 and 3.5 %). Five ml of inoculum was added in to each flask and incubated at 28°C in rotary shaker with 120 rpm for

120 hours. Cell free supernatant was prepared from all the flasks and quantitatively assayed by adopting the procedure as described above.

Characterization of potential bacterial strain

The phenotypic characteristics such as microscopic appearance (gram's staining, motility), cultural and biochemical characters of the potential strain was studied by adopting standard procedures recommended by Bergey's Manual of systematic Bacteriology.

RESULT AND DISCUSSION

Marine environments are unique by virtue of their salinity and wide range of mineral contents. The marine bacteria have not been much experimentally tried for their potential in many of the human endeavours in which they would have a major role to play, for example in the field of health and medicine and in industry. Hence an attempt has been made in the present study to L-time glutaminase producing bacteria from the marine environments.

Isolation of L-glutaminase producing marine bacteria

Totally 15 morphologically different bacterial isolates were selected from the sediment samples collected from selected marine ecosystems. All the isolates produced pink color zone around the colony on minimal glutamine agar (MGA). Only the bacteria which produce L-glutaminase enzyme extracellularly, can grow in the MGA medium as L-glutamine is the only carbon and nitrogen source available in the media. Further the MGA media 0.012% Phenol red (pH indicator) supplemented in the medium gives a yellow colour to the media. The bacteria utilize the L-glutamine by hydrolyzing it with L-glutaminase and this leads to the production of glutamic acid and ammonia. The accumulation of ammonia in the media changes its pH to alkaline and due to the presence of phenol red in the media; the bacterial colonies are surrounded by pink zone. Balagurunathan *et al.*, [6] isolated the L-glutaminase producing actinomycetes by the same method described above. They also reported that this method is speedy, consistent and reproducible when compared to the earlier method that involves the isolation of microorganisms from certain environments by routine isolation procedures and then screened for enzymatic activity [10]. Thus the use of selective media and the presence of antibiotics, NaCl and pH indicator makes the media suitable for direct and selective isolation of L-glutaminase producing marine isolates.

Production of L-glutaminase

L-glutaminase from selected bacterial isolates was produced using seawater minimal

glutamine agar. Kiruthika and Saraswathy[1] reported that, sea water glutamine medium was found to be the best medium for L-glutaminase production. Hence, in our study SWG was used for L-glutaminase production and also for optimization studies.

Semi quantitative assay of L-glutaminase and selection of potential strain:

In semi quantitative assay, pink color zone with varying size was observed around the well on minimal agar medium. Two strains viz. M3 and M9 produced maximum of 18 mm and 29 mm diameter zone of color change, respectively. Based on the results of semi quantitative assay, bacterial strain M9 was selected as potential strain for further studies. In general enzymes are measured by estimation of enzyme activity by qualitative estimation [10] and also quantitatively by using UV spectrophotometer or colorimeter [11]. There is also one more semi quantitative method recommended by Gulati *et al.*, [10] for L-asparaginase activity in which the enzyme producing organism was spotted on medium supplemented with L-asparagine and phenol red as pH indicator. Based on the color change around the spot, the enzymatic activity was assayed semi quantitatively. But this is not accurate since the variations in the number of cells or the quantity of the crude enzyme when placed on the medium. So in the present study L-glutaminase activity was semi quantitatively assayed based on well diffusion method.

Partial purification and quantitative assay of L- glutaminase

L-glutaminase produced from M9 strain was purified by ammonium sulphate precipitation & dialysed and tested for L-glutaminase activity by quantitative assay. The dialysate produced 28.7 U/ml of L-glutaminase. Further chromatographic purification was needed to achieve more purity and also for its chemical characterization and structure elucidation. The partially purified L-glutaminase from the potent strain M9 was subjected to separation by SDS-PAGE which showed two bands of 45kDa and 250kDa. The result showed that the band represented the subunit of native enzyme from glutaminase class A. This result was similar to the result obtained by Yulianti *et al.*, [5].

Effect of pH, temperature and salinity on the production of L-glutaminase

The effect of physicochemical parameters such as pH, temperature and salinity was tested on the production of L-glutaminase from the strain M9. The maximum L-glutaminase was at pH 7, temperature 35°C and 3.5% NaCl concentration (Figure 1, 2 & 3). These results are same as glutaminase from *Streptomyces avermitilis* reported by Abdallah *et al.*, [9].

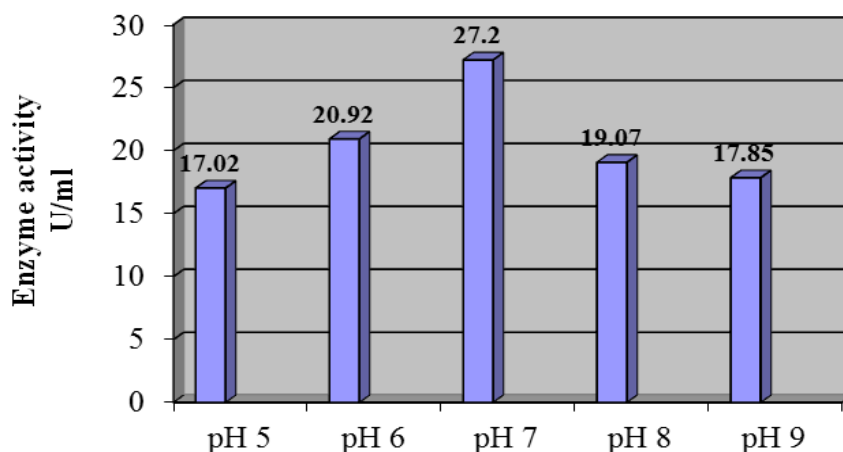


Figure I: Effect of pH on L-Glutaminase production

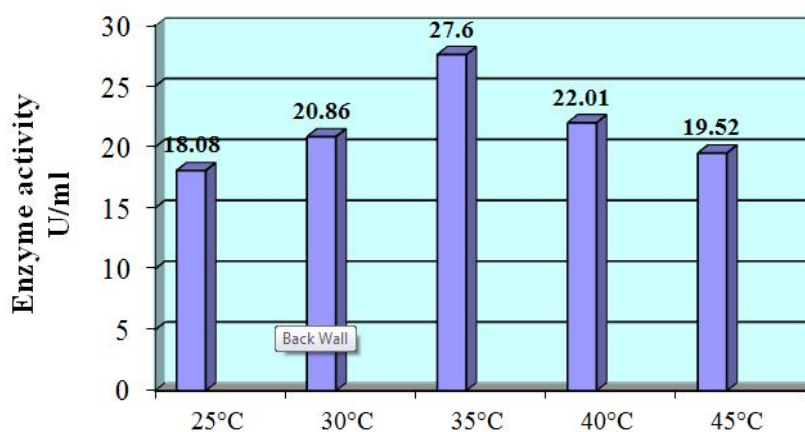


Figure II: Effect of temperature on L-Glutaminase production

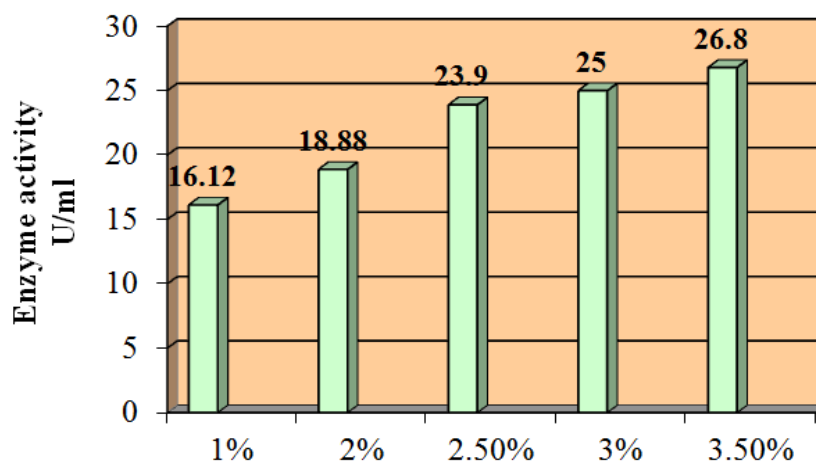


Figure III: Effect of Salinity on L-Glutaminase production

Identification of potential strain

The phenotypic characteristics such as microscopic appearance (gram's staining, motility), cultural and biochemical characters of the potential strain M9 were given in table 1. These results showed that the strain M9 was found to be the species of the genus *Vibrio*.

Table I: Characteristics of potential strain M-9

S.No	Test	Result
1	Microscopic appearance	
	Shape	Rods
	Gram's reaction	Gram negative
	Motility	Motile
2	Cultural Characteristics	
	Nutrient agar	Moist, translucent, round discs colonies.
	TCBS	Green color colonies
	Macconkeys agar	Colourless colonies
3	Biochemical Characteristics	
	Catalase	+
	Oxidase	+
	Indole	-
	Methyl red	-
	Vogesprousker	+
	Citrate	+
	Nitrate reduction	+
	Urease	-
4	Carbohydrate utilization	
	Glucose	+
	Cellobiose	+
	Xylose	-
	Rhamnose	-
	Lactose	-

CONCLUSION

The present study focuses on the selective isolation of the potent L-glutaminase producing marine bacteria. The results of the preliminary study indicates that L-glutaminase producing marine bacterial strain M9 *Vibrio sp.*, 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. Extensive bioprocess optimization studies is underway for microbial production of L-glutaminase from the *Vibrio sp.* M9.

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