Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology, 4 (4): 168-172, 2009
ISSN 1816-4935

L-Glutaminase Production and the Growth of Marine Bacteria

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Abstract: The search of salt-tolerant and thermo-stable bacterial L-glutaminase in the marine environment was done from Coleroon estuary, Muthupet mangrove and Mullipallam lagoon which possess different marine biotopes. The isolated and identified high potent strains were subjected in to comparative study between their growth and production to select the industrially potent strains. Within that the Mullipallam lagoon strain *Vibrio* sp. SFL-2 (Sethusamudharam Field Laboratory) had produced 352.4±0.23 IU (International Unit) of L-glutaminase in the 96 hrs of culture but their growth rate was more or less same as other strains.

Key words: Estuary, Mangrove, Lagoon, bacteria, growth, L-glutaminase production

INTRODUCTION

The L-glutaminase is the cellular enzyme deaminating L-glutamine and acts as proteolytic endopeptidase, which hydrolyses the peptide bonds present in the interior of the protein molecules. The enzyme L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia. Although L-glutaminase can be derived from plant as well as animal sources, microbial enzymes are generally meeting the industrial demands. They were potentially useful in the pharmaceutical and fine food industry also. But there must be need to produce L-glutaminase which have salt-tolerant (Wakayama *et al*., 2005; Yoshimune *et al*., 2006), thermo-stable (Kazuzki *et al*., 2003; Masayuki *et al*., 2007) and anti-leukemic (Robert *et al*., 2001) activities in both the food (Kyoko *et al*., 2004) and pharmaceutical industries. Thus the marine environment had the vast bacterial diversity and high fluctuations in their salinities and temperatures become the source for the salt-tolerant; thermo-stable L-glutaminase (Renu and Chandrasekaran, 1992). Even though many bacteria had the ability to produce the L-glutaminase enzyme, only few bacteria produce more L-glutaminase in their minimnm growth was selected for large-scale industries as high potent strains.

In bacteriology, the term growth refers to increasing changes in the mass population and mode of cell division in the usnal growth cycle of bacterial populations is transverse binary fission (asexnal reproductive process), which was calculated on the basis of time. The time taken for doubling of bacterial cells was called as generation time. On the basis of generation time, the growth curve was plotted with four phases *viz*., lag, log, stationary and decline (Tom and James, 2005). The growth of the bacteria was mainly dependent on the physical parameters and availability of nutrients, which was available in the specific environment. Each and every genus of bacteria had their own generation time, optimnm temperature, pH, sodinm chloride concentration and specific nutrients for their growth. In natural environment, the proficiencies of those enhance the growth were abundant for bacterial population but *in vitro* conditions, it was not easy to provide all these to several genera of bacteria.

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The production of L-glutaminase by the bacterial cells may be endo- or exo-enzymes, which was based on the metabolic activity of bacterial cells. Indirectly the substrate concentration, temperature and pH were also effects the production of L-glutaminase production, which can overcome in laboratory conditions. But, in large scale, this was a knotty process. Industrialists want ouly the enzyme products and they won't interest to invest their cost on the bacterial growth, this was prevented by using the lag phase culture as inoculants. So, economically the bacteria, which grow less and produce more enzymes, will be select as industrially potent through optimization of media. Thus the objective of the study was to select the industrially potent L-glutaminase bacteria by comparing the bacterial growth with their L-glutaminase production was proceeded as economically in the laboratory itself.

MATERIALS AND METHODS

Screening for L-Glutaminase

The sediment samples were collected from Coleroon Estuary (Lat. 11°20′58 N and Long. 79°48′40. 7′′ E), Muthupet Mangrove (Lat. 10°20′17.5′′ N and Long. 79°32′31. 1′′ E) and Mullipallam Lagoon (Lat. 10°20′19.71′′ N and Long. 79°32′53.65′′ E) intertidal region with the help of Peterson grab (0.0256 m^2) on the summer season of year 2007. In laboratory, one gram of wet sediment samples was transferred to a conical flask containing 99 mL of 50% sterile sea water as diluents. The dilutent sediment sample was once again tenfold diluted using sterile water blanks. The blanks were prepared with aged seawater (50%). The tenfold diluted sediment samples were enriched by transferred 1 mL of dilution in to conical flask containing 99 mL of sterile seawater complex broth (Parsons *et al*., 1984) and incubated at 30°C for a day in an incubator cum shaker. After incubation, 1 mL of enriched culture was streaked in nutrient agar plate incorporated with L-glutamine and once again it was incubated at 30°C for 48 hrs (Gulati *et al*., 1997; Rajeswari and Shome, 2001). The colonies which change the color of media around it were subjected to several biochemical tests for identification of bacteria (Shewan *et al*., 1960; Simidu and Aiso, 1962; Gibbs and Skinner, 1966; Buchanan *et al*., 1974; Oliver, 1982; James Cappuccion and Natalie, 2001).

Inoculum Preparation

The inoculum was prepared by inoculating the 2.5 mL of 8 hrs old specific selected pure culture (Nutrient broth) was aseptically transferred to separate 25 mL of nutrient broth in boilers tube and incubate it for day at 35°C. After incubation, a loop full of inoculum was inoculated in 25 mL of nutrient broth contained in 100 mL conical flasks and once again incubated in incubator-cum-shaker at 35°C. Each and every strain had five sets of L-glutamine incorporated nutrient broth flask. At the end of every 24 hrs of incubation period, every set of flasks were harvested to determine the L-glutaminase assay with the growth of selected bacteria.

Determination of Growth

The Optical Density (O.D) values were observed using UV- spectrophotometer (Systronics-Visiscan-167, India) at 650 um for the incubated bacterial culture in L-glutamine incorporated nutrient broth. The O.D. values were taken at every 24 hrs intervals until reaches 120 hrs of incubation time (Krishna and Reddy, 1987).

Extraction and Determination of L-Glutaminase

The bacterial cultures were lysed by sonicator (Systronics, India) and supernatant was centrifuged at 10,000 rpm at 4°C for 20 min in a cooling centrifuge (REMI). After the centrifugation, the pellets were incubated with 5 mL of modified potassium phosphate buffer at 30°C for 4 hrs and again

centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant (crude extracts) was collected and assayed for L-glutaminase. The L-glutaminase concentration was determined by assay method proposed by Imada *et al*. (1973) using UV- spectrophotometer (Systronics-Visiscan-167, India). L-glutaminase activity is expressed in International Units (IU). One IU is the amount of enzyme required to liberate 1 µM of ammonia in one minute under experimental conditions.

RESULTS AND DISCUSSION

The selection of L-glutaminase potent bacteria was steadily based on the total number of colonies, like *Bacillus* sp. SFL-4 as 11.63±0.89×10^5 CFU g^{-1} from Coleroon estuary, *E. coli* SFL-1 (21.74±2.25×10^5 CFU g^{-1}); *Micrococcus* sp. SFL-15 (10.14±1.03×10^5 CFU g^{-1}) from Muthupet mangrove and *Pseudomonas* sp. SFL-3 (12.5±1.86×10^5 CFU g^{-1}); *Vibrio* sp. SFL-2 as 31.25±2.25×10^{-5} CFU g^{-1} from Mullipallam lagoon. Overall the *Vibrio* sp. SFL-2 was recorded as maximum numbers within the selected strains. All these L-glutaminase potent bacteria were isolated in summer season itself.

After selection, the bacterial culture were processed for growth curve as the optical density values for growth ranged from 0.02±0.01 to 0.24±0.03, 0.09±0.01 to 0.34±0.01, 0.11±0.01 to 0.36±0.01, 0.07±0.02 to 0.56±0.02 and 0.18±0.02 to 0.61±0.02 for *Bacillus* sp. SFL-4, *E. coli* SFL-1, *Pseudomonas* sp. SFL-3, *Micrococcus* sp. SFL-15 and *Vibrio* sp. SFL-2 respectively. All the low OD values were recorded in the initial stage of culture. The peak values of OD were recorded in 72 hrs of *E. coli* SFL-1 and *Pseudomonas* sp. SFL-3 and in 96 hrs of *Bacillus* sp. SFL-4, *Micrococcus* sp. SFL-15 and *Vibrio* sp. SFL-2, respectively. The growth curve shows that the selected bacteria were gradually increase from 24 hrs to 96 hrs except *E. coli* SFL-1 and *Pseudomonas* sp. SFL-3, after that their growth was declined. Generally, the low growth OD value was recorded in *Bacillus* sp. SFL-4 and high growth OD value was observed in *Vibrio* sp. SFL-2.

The measurement value for the L-glutaminase arrayed from nil to 17.2±0.23 IU, 15.2±0.14 IU to 52.7±0.18 IU, 11.4±0.18 IU to 32.7±0.23 IU, 5.7±0.18 IU to 20.3±0.31 IU and 22.4±0.09 IU to 352.4±0.23 IU for *Bacillus* sp. SFL-4, *E. coli* SFL-1, *Pseudomonas* sp. SFL-3, *Micrococcus* sp. SFL-15 and *Vibrio* sp. SFL-2, respectively. The maximum L-glutaminase measurement reading was observed in *Vibrio* sp. SFL-2 of 96 hrs culture and there was no production was recorded in *Bacillus* sp. SFL-4 of 24 hrs culture. The high productions of L-glutaminase were recorded in 72 h for *Bacillus* sp. SFL-4, *E. coli* SFL-1, *Pseudomonas* sp. SFL-3 and *Micrococcus* sp. SFL-15 and in 96 hrs culture of *Vibrio* sp. SFL-2 but in *Pseudomonas* sp. SFL-3 only, the L-glutaminase production values was remain as stable in both 48 and 72 h. As usually, all the low production values were recorded in initial stage of culture as 24 h.

The comparative OD value for the growth and L-glutaminase concentration was shown in Table 1. On comparative of both the growth OD value and L-glutaminase activity showed their low

Table 1: Comparative value between the production of bacterial L-glutaminase and their growth

Name of the strain	Source	OD value	Time (h) 24	48	72	96	120
Bacillus sp.	Coleroon	Growth	0.02±0.01	0.07±0.02	0.15±0.03	0.24±0.03	0.18±0.02
SFL-4	Estuary	L-glutaminase	0.00	13.40±0.23	17.20±0.23	12.90±0.23	9.40±0.23
E. coli	Muthupet	Growth	0.09±0.01	0.24±0.01	0.34±0.01	0.32±0.02	0.30±0.01
SFL-1	Mangrove	L-glutaminase	15.20±0.14	36.40±0.18	52.70±0.18	46.50±0.27	40.20±0.27
Micrococcus sp.		Growth	0.07±0.02	0.15±0.03	0.24±0.02	0.56±0.02	0.34±0.03
SFL-15		L-glutaminase	5.70±0.18	16.40±0.18	20.30±0.31	18.40±0.10	12.70±0.23
Pseudomonas sp.	Mullipallam	Growth	0.11±0.01	0.28±0.02	0.36±0.01	0.30±0.01	0.26±0.02
SFL-3	Lagoon	L-glutaminase	11.40±0.18	32.70±0.23	32.70±0.23	26.90±0.18	22.70±0.14
Vibrio sp.		Growth	0.18±0.02	0.32±0.02	0.52±0.02	0.61±0.02	0.56±0.04
SFL-2		L-glutaminase	22.40±0.09	59.40±0.23	272.90±0.14	352.40±0.23	324.70±0.14

values in their initial stages. *Bacillus* sp. SFL-4, *Pseudomonas* sp. SFL-3 and *Micrococcus* sp. SFL-15 were the bacterial strains, which showed the low growth OD values when compared with their L-glutaminase concentration.

The selections of industrially potent L-glutaminase strains were done on the basis of their number of colonies and strong color change in the L-glutamine incorporated nutrient media. On the basis of these selections, the five bacterial strains were selected *viz*., *Bacillus* sp. SFL-4, *E. coli* SFL-1, *Pseudomonas* sp. SFL-3, *Micrococcus* sp. SFL-15 and *Vibrio* sp. SFL- 2 from Point Calimere coast, Muthupet mangrove and Mullipallam lagoon respectively. *Bacillus*, *Escherichia*, *Pseudomonas*, *Micrococcus* and *Vibrio* genera were native flora of marine, which were tolerate and grown even in the 45°C temperature, 3% of NaCl concentration and ferment the glucose as their source. Within the isolates, Gram negative rod shape bacteria were dominant in the production of L-glutaminase.

The five isolated had the different growth rate particularly the generation time. As *Vibrio* sp. SFL-2 was naturally occur in marine envirounent were highly halophilic and the soil texture in Mullipallam lagoon with high nutrients and minerals were enhance the population of *Vibrio* sp. SFL-2 when compared with any other isolates. The comparative study showed that the *Vibrio* sp. SFL-2 have the high ability to produce the L-glutaminase, when compared to other isolates. It produced about 352.4±0.23 IU in 96 hrs incubated culture can be used for producing the L-glutaminase in industries, which was supported by Sabu *et al*. (2000) found that the L-glutaminase production increased along with an increase in total cell protein, during the logarithmic phase up to 96 hrs followed by decline on extended incubation. But Marcus *et al*. (2002) and Alejandro *et al*. (2004) had different controversy as the highest specific activity of glutaminase in *B. pasteurii* during late exponential; stationary growth and that the exponential phase of *Rhizobium etli* growth produce more glutaminase-A, when compared to stationary phase, respectively.

CONCLUSION

The important of L-glutaminase obligate the biotechnological industries to produce in the large scale were crafted to search the high potent bacteria. Even though several bacteria have potent, they were growth related. But ouly the marine source L-glutaminase potent bacteria have the ability to produce more L-glutaminase when compared to the terrestrial L-glutaminase potent bacteria. Thus to construct the growth independent L-glutaminase potent bacteria, *Vibrio* sp. SFL-2 (which was non-pathogen to human beings) become the best source for the L-glutaminase production. This study had concluded that the L-glutaminase production by marine bacterial populations was growth independent.

ACKNOWLEDGMENTS

We thank to the authority of Annamalai University and Anna University for providing the facilities and financial supports. We also thank to the Dredging Corporation of India (DCI) for granting the project.

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