

Production of L-asparaginase by the marine luminous bacteria

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Received 12 September 1991, revised 16 April 1992

Fortythree strains of luminous bacteria, belonging to 4 species, (*Vibrio harveyi*, *V. fischeri*, *Photobacterium leiognathi* and *P. phosphoreum*) isolated from different marine samples, were examined for the production of L-asparaginase. Presence or secretion of this enzyme was not a uniform trait among the strains of the former 3 species and none of *P. phosphoreum* strains possessed this enzyme. A wide difference was discernible in the production of L-asparaginase among the strains of *V. harveyi* and *P. leiognathi*. On a comparative basis, however, amounts of this enzyme secreted by the luminous prokaryotes is generally higher than those reported for other bacterial species.

Amidohydrolases, viz. asparaginase, glutaminase and urease play an important role in the biogeochemical cycling of carbon and nitrogen in natural waters¹ and sediments². Several marine microorganisms are potential sources of such important enzymes^{3,4}. Bioluminescent bacteria, which are unique and ubiquitous occupying a variety of ecological niches in the marine environment, are known to possess several enzymes⁵. Although Benny and Kurup⁶ recently reported varying amounts of L-asparaginase (L-asparagine amidohydrolase E.C. 3.5.1.1) in many genera of marine bacteria, there is no study available on the production of this enzyme by the luminous bacteria. This paper deals with the quantitative determination of L-asparaginase from the marine luminous bacteria.

A total of 43 strains of luminous bacteria belonging to 4 different species were examined for L-asparaginase production. These strains were isolated from various sources in the Arabian Sea such as water (estuarine, coastal and oceanic), sediment, algae, light organs of leiognathid fishes and gut contents of green mussel *Perna viridis*. Seawater complete (SWC) agar⁷ was used for isolation and maintenance of these bacterial strains and all these isolates were characterised by following an identification scheme⁸. Psychrotrophic strains of *Photobacterium phosphoreum* were grown at 20°C while all the other strains were grown at room temperature (28°C).

Presence of L-asparaginase in these strains was tested by growing them in a medium containing L-asparagine as the sole source of carbon. Strains

that grew in this medium were selected for the quantitative assay of L-asparaginase activity. For the assay of this enzyme, cells grown in complex liquid medium for 24 h were concentrated by centrifugation (at 10000g), washed twice with sterile seawater and the suspension was adjusted to a constant optical density. This cell suspension was used as the enzyme preparation, and protein content from such suspensions of all the strains was determined¹⁰. Viable counts of these preparations were also estimated following serial dilution and spread plate techniques. In principle, the assay for L-asparaginase activity from the bioluminescent bacterial strains was carried out by determining the amounts of ammonia liberated in the Conway microdiffusion chamber, when the assay mixture was treated with a saturated solution of potassium carbonate¹¹. The assay mixture consisted of 1 ml of 0.1 M tris-HCl buffer (pH 8.5), 0.5 ml of 0.04 M L-asparagine and 0.5 ml of enzyme preparation. This mixture was incubated at 37°C for 30 min and 0.5 ml of 1.5 M trichloroacetic acid was added to stop the enzyme reaction. The liberated ammonia was then estimated by Conway microdiffusion method¹¹. A standard curve was generated by using ammonium sulphate. Suitable blanks and controls were also maintained. Under these experimental conditions¹², one IU of the enzyme activity (IU mg^{-1} protein) is equivalent to $1 \mu\text{mole NH}_3$ liberated. min^{-1} .

Of the 43 strains examined for their ability to utilise L-asparagine as the sole source of carbon and energy, only 26 showed this property which was evident from the good growth in the complex medium containing

L-asparagine. This growth was further confirmed by streaking a loopful of inoculum on the SWC agar. Notably, presence of this activity was rather random among the strains of luminous bacteria isolated from various sources. Twelve of the 16 *Vibrio harveyi* isolates were found to possess L-asparaginase and the quantity of NH_3 liberated by different strains ranged from 4.71 to 39.22 $\mu\text{mole}\cdot\text{h}^{-1}\cdot\text{ml}^{-1}$ (Fig. 1), which in terms of L-asparaginase activity ranged from 0.33 to 2.62 $\text{IU}\cdot\text{mg}^{-1}$ protein (Table 1). Strains isolated from the coastal seawater samples were found to liberate more NH_3 than those isolated from algae or sediment samples. Generally, offshore strains showed moderate activity. Strains belonging to *Vibrio fischeri* positive (9 out of 14) for L-asparaginase were found to secrete moderate quantities with their activity varying from 0.439 to 1.332 $\text{IU}\cdot\text{mg}^{-1}$ protein. Five of the 8 strains of *Photobacterium leiognathi* were positive for L-asparaginase. However, the quantities of NH_3 liberated by these strains were generally higher ranging from 10.2 to 38.33 $\mu\text{mole}\cdot\text{ml}^{-1}\cdot\text{h}^{-1}$. This, in terms of activity ranged between 0.739 and 2.674 $\text{IU}\cdot\text{mg}^{-1}$ protein.

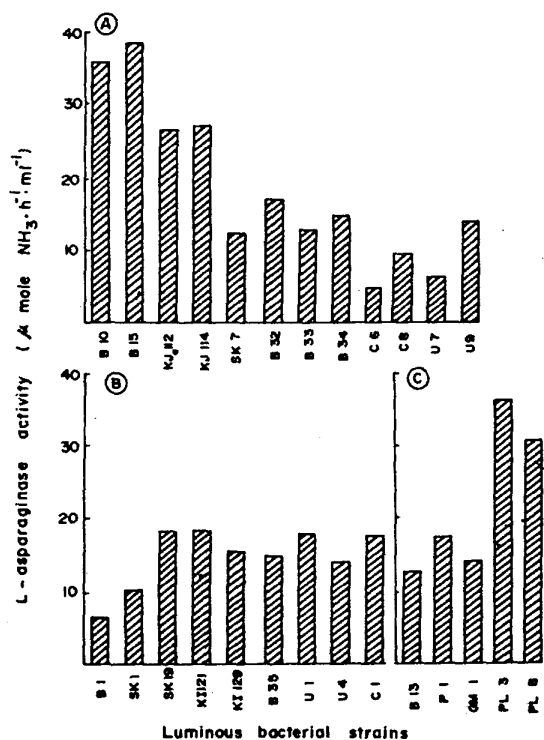


Fig. 1—L-asparaginase activity of luminous bacterial species: (A) *Vibrio harveyi*, (B) *V. fischeri* and (C) *Photobacterium leiognathi*

All the 5 tested strains of *Photobacterium phosphoreum* were found to be lacking this enzyme. This species, being a psychrotroph¹³, has a temperature optimum around 20°C. So all of them were confirmed for the absence of this enzyme by incubating their assay mixtures at 20°C and by determining NH_3 . This experiment was done for all strains which did not utilise L-asparagine as the sole source of carbon in order to rule out the possibility of missing this trait.

Table 1—L-asparaginase activity of different species of luminous bacteria

Strains	Source*	NH_3 released ($\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$)	Specific** activity ($\text{IU}\cdot\text{mg}^{-1}$)
<i>Vibrio harveyi</i>			
B10	1 (<30m)	0.607	2.468
B15	1 (<30m)	0.650	2.620
KJ112	2 (100m)	0.445	1.839
KJ114	2 (200m)	0.450	1.785
SK7	2 (150m)	0.209	0.864
C6	3	0.079	0.330
C8	3	0.155	0.698
U7	4	0.105	0.646
U9	4	0.230	0.432
B32	5 (30m)	0.280	1.167
B33	5 (30m)	0.248	1.038
B34	5 (30m)	0.220	0.921
<i>V. fischeri</i>			
B1	1 (<30m)	0.105	0.439
SK17	2 (200m)	0.175	0.729
SK19	2 (200m)	0.321	1.332
KI121	2 (300m)	0.321	1.088
KI129	2 (400m)	0.271	1.289
C1	3	0.307	1.253
U1	4	0.316	1.306
U4	4	0.247	1.017
B35	5	0.260	1.066
<i>Photobacterium leiognathi</i>			
B13	1 (<30m)	0.178	0.739
P1	2 (100m)	0.309	1.277
PL3	6	0.639	2.674
PL8	7	0.542	2.258
GM1	8	0.249	1.025

* Sources are: 1) coastal waters off Bombay, 2) oceanic waters, 3) marine alga *Chaetomorpha media*, 4) marine alga *Ulva fasciata*, 5) sediment sample off Bombay, 6) light organ of *Leiognathus bindus*, 7) light organ of *Secutor ruconius* and 8) gut contents of *Perna viridis*. Figures in the parentheses indicate water depth from where the strain was isolated.

** Product of the protein content of crude extract and the amount of NH_3 liberated by the strain.

Viable cell counts in the enzyme preparations examined in the present study ranged between 10^{11} and 10^{12} .ml⁻¹. Protein content of *Vibrio harveyi* was in the range of 0.484 to 0.504 mg.ml⁻¹. It ranged from 0.478 to 0.498 in *Vibrio fischeri* and from 0.478 to 0.486 mg.ml⁻¹ in the preparations of *Photobacterium leognathi* strains.

Besides being important in the biogeochemical cycling of organic material, bacterial asparaginases have shown appreciable activity in the clinical trials¹⁴ for antitumour/antileukaemic treatments. Therefore, several groups of microorganisms from various habitats have been screened to obtain potent strains^{6,11,12}. All the enzyme (crude) preparations of the luminous bacterial strains positive for L-asparaginase, showed higher activity than those reported for *Escherichia coli*¹⁵, *Citrobacter freundii*¹⁶ or for *Pseudomonas*, *Micrococcus*, *Bacillus*, *Flavobacterium* or for a marine *Vibrio*¹⁷. Hvozyak *et al.*¹⁸ and Makarova *et al.*¹⁹ after screening large number of bacterial strains from different sources concluded that selection of effective producers depended also on the habitat. Generally, possession or secretion of L-asparaginase by luminous bacteria examined in this study, did not seem to be influenced by the habitats. However, strains isolated from seawater, particularly from the offshore regions appear to be potential sources of asparaginases.

It appears that not all strains of a particular species possess asparaginases. Like in many other studies, it was seen in this study that differences do occur in the quantities of L-asparaginase produced by different strains of the same species. It may be mentioned that the strains of *P. leognathi* isolated from the light organs of different species of leognathids were identical in biochemical characteristics²⁰. But they appear to differ in the characteristic of asparaginase production. Selvakumar¹⁷ had shown that the presence of this enzyme in the natural isolates was random regardless of the source of their origin. Studies directed to understand ecophysiological

aspects as well as those involving strains improvements are needed to evaluate the potential of marine bacteria in the production of L-asparaginase and other useful bioactive enzymes/compounds.

Authors thank Dr. B N Desai and Dr. A H Parulekar for encouragement.

References

- 1 Frankberger WT (Jr) & Johanson J B, *Pol Arch Hydrobiol*, 30 (1973) 319.
- 2 Sallis P J & Burns R G, *Microb Ecol*, 17 (1989) 159.
- 3 Williams S T & Vickers J C, *Microb Ecol*, 12 (1986) 43.
- 4 Colwell R R, Praiser E R & Sinskey A J (eds), *Biotechnology of marine polysaccharides*. (Hemisphere Publishing Company, New York) 1985, pp.594.
- 5 Hastings J W, Potrikus C J, Gupta S C, Kurfust M & Makemson J C, *Adv Microb Physiol*, 26 (1985) 236.
- 6 Benny P J & Kurup G M, *Indian J Mar Sci*, 20 (1991) 36.
- 7 Rheinheimer G (ed), *Microbial ecology of brackishwater environment*, (Springer-Verlag, Berlin) 1977, pp.291.
- 8 Neelson K H, *Methods Enzymol*, 57 (1978) 153.
- 9 Baumann P & Baumann L, in *The prokaryotes*, edited by M P Starr, H Stolp, H G Truper, A Balows & H G Schelgel, (Springer-Verlag, Berlin) 1981, pp.1302.
- 10 Lowry O, Rosebrough N J, Farr A L & Randall R J, *J Biol Chem*, 193 (1951) 265.
- 11 Selvakumar N, Chandramohan D & Natarajan R, *Curr Sci*, 46 (1977) 287.
- 12 Nair G B, Selvakumar N, Chandramohan D & Natarajan R, *Indian J Mar Sci*, 6 (1977) 172.
- 13 Waters P & Llyod D, *J Gen Microbiol*, 131 (1985) 2865.
- 14 Salah F M, Ekram Z K & Samir M B, *Lanwirt Tech Microbiol*, 129 (1974) 525.
- 15 Zhen M G & Jun Q S, *Acta Microbiol Sin*, 15 (1975) 227.
- 16 Davidson L, Sam Ahn M B, Chang L & Kitto J B, *Biochem Biophys Acta*, 480 (1989) 282.
- 17 Selvakumar N, *Studies on L-asparaginase (anti-leukaemic agent) from coastal areas of Porto Novo, South India*, Ph D thesis, Annamalai University, 1979.
- 18 Hvozyak R I, Ozolin R K, Hryvin P P, Yakoviyeva L M, Volkva V P, Zuikova N I & Babych A K, *Mikrobiologiya*, 35 (1973) 683.
- 19 Makarova R A, Chaikovskaya S M, Tochenaya N P, Meshkov A N, Vishanskaya F L, Sterligova N D & Levitov M M, *Antibiot*, 19 (1974) 198.
- 20 Ramaiah N, *Studies on marine luminous bacteria*, Ph D thesis, Rani Durgavati University, 1989.