Studies on L-asparaginase Activity in *Bacillus* of Retting Ground

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Cultural conditions for the production of L-asparaginase enzyme by a *Bacillus* strain isolated from the sediment sample from the retting ground were studied. Two pH optima for enzyme synthesis were observed at 5.6 and 8. The growth was maximum at pH 8. Optimum temperature for this enzyme was at 37°C. The sodium chloride optima for L-asparaginase production was at 0.2% and for growth at 0.5% concentration. The incorporation of inorganic sulfate even at the minimum concentration inhibited the enzyme activity while inorganic phosphate showed inhibition only at high concentration. Methionine and cysteine enhanced the enzyme activity whereas threonine inhibited the enzyme activity. Sucrose was found to enhance both the activity and growth. Of the four heavy metals, the divalent cation copper at 2 ppm concentration induced the enzyme activity and mercury showed inhibition on growth of bacterium at the same concentration.

L-asparaginase (L-asparagine amido hydrolase 9.5.1.1) is an enzyme used in the treatment of tumour, and of acute lymphatic leukaemia of human beings. Broome (1961) established the link between the antilymphoma activity of guinea pig serum and the enzyme L-asparaginase present in it. Since then many reported the occurrence and activities of these enzymes in various bacteria, yeast, fungi and actinomycetes (Wade *et al.*, 1968; Imada *et al.*, 1973 Nakahama *et al.*, 1973, Dejong, 1972). However it was found that not all the L-asparaginase from different sources did exhibit both antitumour and antileukaemic properties. The marine environment is an untapped and potent source for this anticancer enzyme. Only limited literature is available from Indian coastal waters. Distribution and activity of L-asparaginase in fungi have been carried out in the marine environment at Porto Novo on the east coast (Balakrishnan Nair *et al.*, 1977). Selvakumar (1979) isolated the L- asparaginase producing bacteria from sediment and shellfish resources and characterised the antitumour activity in the mouse. Quite interestingly the extremely halophilic bacteria are also associated with the mangrove sediments as well as with the bivalve mollusc *Anadara rhombe* in the east coast exhibiting antileukaemic activity (Sudha, 1981). Information on the occurrence and activity of L-asparaginase producing microorganisms on the west coast of India is lacking. The present study aims at understanding the distribution of L-asparaginase producing microorganisms in the polluted retting ground and the cultural conditions to optimise the enzymatic activity in the selected *Bacillus* strain.

Materials and Methods

Water, sediment and coconut husks were collected from Anchuthengu coconut husk retting ground. Peterson grab and water samplers were used for collecting sediment and water respectively. The three month old retting husks could also be collected by hand picking. All the samples were brought to the laboratory in sterile containers making all necessary precautions to prevent any contamination. Approximately one gram of the sediment sample was asceptically removed from the central portions of sediment and was mixed

with 100 ml of sterile blank of appropriate salinity. Similarly 1 ml of water sample and 1 g of the coconut husk were mixed separately with 100 ml of sterile blank as mentioned earlier. The standard serial dilution technique was followed for sampling. For pour plating nutrient asparagine medium (peptone - 0.1 g; sodium chloride - 0.5 g; potassium dihydrogen phosphate - 0.2 g; L-asparagine - 0.2 g; phenol red - 0.2%; Agar - 2 g; 50% sea water - 100 ml; pH - 7) was used. The petridishes were incubated at room temperature for five days. The appearance of the pink colour around the colonies were noted as L-asparaginase positive culture. The randomly selected bacterial isolates were subcultured and stocked in the refrigerator for further characterisation. The bacterial isolates were identified according to the method adopted by Simidu & Aiso (1962) and the methods in the Bergey's manual of systematic bacteriology (Staley *et al., 1989).* All these cultures were screened by the conventional method to confirm the L-asparaginase activity (Wriston 1971).

The packed cells were suspended in distilled water to a population of 1×10^8 C.F.U./ ml (colony forming unit). From this stock culture 0.2 ml of the young cultures were independently inoculated into 5.5 ml of the nutrient broth. The medium was incorporated with 0.2 ml of 1% L-asparagine. They were incubated at room temperature (29 ± 2^{o}C) for 48 h. After the incubation period was over the bacterial biomass was estimated by measuring the turbidity in Spectronic-20 at a wave length of 600 nm. L-asparaginase activity was measured by adding 0.5 ml of the Nessler's reagent into 5.5 ml of culture broth after growing about 48 h. Within five minutes the colour was developed. The whole sample was centrifuged and supernatent was read in the Spectronic-20 at a wave length of 490 nm (Selvakumar *et al.*, 1977) and suitable control was maintained throughout the period of investigation. The standard was prepared with ammonium chloride. The activity was expressed as µg ammonia released per ml per h.

To study the cultural conditions on the enzymatic activity in the bacterial culture the medium was prepared in the buffered solution. To study the effect of temperature, the innoculated tubes were incubated at 4°C, room temperature (29 ± 2^{o}) and higher temperature.

To study the tolerance of bacterial strain towards the sodium chloride, the medium was incorporated with it at various concentrations raging from 0.1 to 5%. Simultaneously a control was maintained without sodium chloride to measure the growth as well as activity of bacteria.

The influence of pH on the enzymatic activity and growth of the bacterium was studied by growing the cultures in the buffered medium at various pH ranged from 4-11. After 48 h the activity was terminated by the addition of Nessler's reagent. The growth was also measured simultaneously.

In order to understand the influence of inorganic as well as organic compounds on the enzymatic activity and growth of bacterium selected inorganic salts such as magnesium sulfate and sodium dihydrogen phosphate at 0.05 to 2 molar were selected. For the influence of amino acids and carbon sources, 0.08 mg/ml of amino acids such as methionine, threonine, cysteine, L-glutamic acids and tryptophan were selected. The carbon sources such as glucose, lactose, sucrose and starch were taken at 1% concentration. The heavy metals such as mercury, copper, zinc and arsenic were incorporated into the growth medium at 2 ppm concentration to understand the inhibition on growth and activity of bacteria.

Results and Discussion

Asparaginases can be obtained from a variety of sources but the enzymes obtained from *E.coli, Erwenia caratovora, Mytobacterium tuberculosis, Acetobacter vinelandii,* guinea pig serum and the serum from other members of super family *Caviodia* have shown antitumour activity (Mashburn & Wriston 1964, Wade *et al.*, 1968, Subba Reddi *et al.*, 1969, Gaffer & Sethna 1975, Clementi, 1922, Yellis & Wriston, 1966, Old *et al.*, 1963). Except one report on the presence of this enzyme in three marine bacteria namely *Aeromonas hydrophylla, A. liquifacians* and *Vibrio* (Wade *et al.*, 1968, Selvakumar, 1979), nothing is known about other sources from

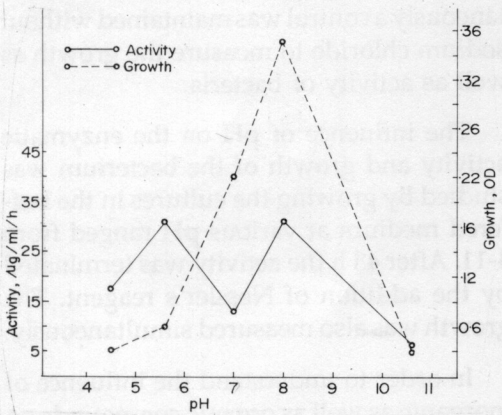

Fig. 1. Effect of pH on *L. asparaginase* activity

bacteria, fungi and actinomycetes from the marine environment. Asparaginase activity as a function of pH at 37°C is shown in Fig. 1. It is evident from the results that there are two pH optima one at 5.6 and the other at 8. However, the growth is maximum at pH 8. This clearly indicates that the growth of the bacterium and enzyme activity are independent. Selvakumar *et al.* (1977) and Selvakumar (1979) observed two pH optima in mangrove sediments and single optimum pH in bacterium *Vibrio* Possibly the extracellular secretion of microbial cell or the intracellular enzymes released after the death of the cell may be the reason for the peak at 5.6. The second optimum showed close similarities with pH optimum of *Streptomyces griseus* (Dejong, 1972).

The effect of temperature on enzyme activity and on growth of bacterium is shown in Fig. 2. Maximum activity and growth were recorded at 37°C. However, at 45°C more than 75% of maximum activity was reduced. The temperature of the environment was ranged between 28 and 33°C. In the aquatic environment, the stability of the enzyme at higher temperature was lost as in the case of urease activity (Rotini, 1935). However, Selvakumar *et al.* (1977) observed

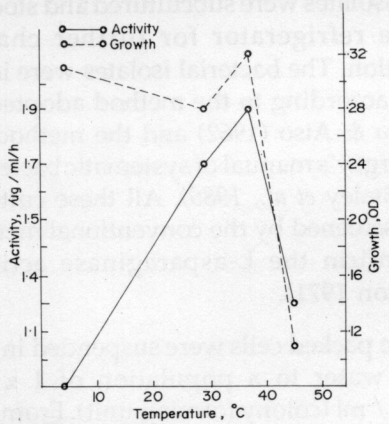

Fig. 2. Effect of temperature on *L.asparaginase* activity

optimum temperature at 40°C for L-asparaginase activity in the marine sediment and 68°C in marine *Vibrio.*

Fig. 3 shows the tolerance of enzyme activity and growth towards sodium chloride. The results indicated that the activity was noticed without sodium chloride and with two peaks of activity at 0.2 and 3% concentration. It could be presumed that the bacterium might synthesize more than one components of L-asparaginase enzymes and showed affinity towards sodium chloride at various concentrations as observed in the two pH optima of this inves-

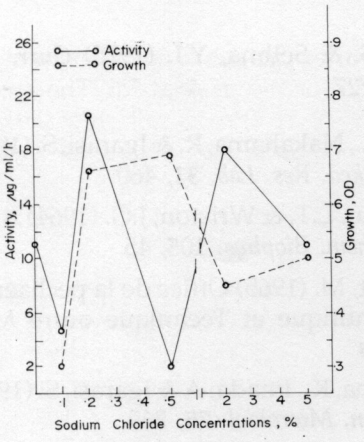

Fig. 3. Effect of sodium chloride on *L.* asparaginase activity

tigation. This suggests that the asperaginase activity is not affected by changes in salinity under natural conditions. The reduced activity at 0.5% sodium chloride may be due to the formation of certain inhibitors along with the sodium chloride during growth. It may be mentioned that the phosphate and nitrate at higher concentration are known to inhibit soil asparaginase (Mouraret, 1965). Similarly Selvakumar *et al.* (1977) noticed similar pattern of enzyme activity in marine sediments.

Tables 1 & 2 showed the influence of sulfate and phosphate on enzyme activity and growth. It is understood that the minimum concentration of sulfate (0.05 m) drastically inhibited the activity whereas the phosphate at 2 m concentration showed the total inhibition of activity. However, the growth was not much affected as in the case of enzymatic activity. Selvakumar (1979) screened the various inorganic salts on the synthesis of L- asparaginase in marine *Vibrio* and had similar observation.

Tables 3 & 4 showed the effect of amino acids and carbon sources on L-aspasraginase activity and growth of *Bacillus.* Among the amino acids cysteine enhanced the activity whereas threonine and tryptophan inhibited the enzymatic activity.

Table 1. *Effect of magnesium sulfate on L-asparaginase activity*

Concentration of mangesium sulfate, molar	Enzyme activity, µg/ml/h	Growth, OD at 600 nm
0.05	-	0.60
0.10	-	0.34
0.2	-	0.45

Table 2. *Effect of sodium dihydrogen phosphate on L-asparaginase activity*

Concentration of Phosphate molar	Enzyme activity, µg/ml/h	Growth, OD at 600 nm
0.05	9.5	0.42
0.10	3.5	0.41
0.2	-	0.25

Table 3. *Effect of Amino acids on L-asparaginase activity*

Amino acid (0.08 mg/ml)	Activity, µg/ml/h	Growth, OD
Methionine	2.0	0.26
Threonine	0	0.16
Cysteine	0.3	0.04
L-glutamic acid	0.6	0.13
Tryptophan	0.035	0.06

Table 4. *Effect of carbon sources on L-asparaginase activity*

Carbon sources (1%)	Activity µg/ml/h	Growth, OD
Glucose	2.10	0.95
Lactose	0.31	0.69
Sucrose	4.1	1.0
Starch	1.9	0.33

Among carbon sources the enzyme activity was more enhanced by sucrose than lactose. Selvakumar (1979) tested the L-asparaginase activity with 24 carbon sources and found that lactose had inhibiting effect in marine *Vibrio*.

The effect of various divalent metal ions on enzyme activity were also studied (Table 5). The metal ions were in contact with the

Table 5. *Effect on heavy metals on L-asparaginase activity*

Heavy metals,	Activity µg/ml/h	Growth, OD
Hg	0.5	0.31
Cu	9.8	0.34
Zn	5.8	0.59
As	4.1	0.35

enzyme during the incubation period of 48 h. The enzyme did not show any requirement for added metal ions. Arsenic, zinc, copper and mercury exhibited considerable inhibition. These divalent cations have strong inhibiting effect on L-asparaginase activity in the marine *Vibrio* associated with shellfish *Anadara rhombea* (Selvakumar, 1979).

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