Bull. zool. Surv. India, 8 (1-3) : 237-243, 1987

CULTURING GYMNAMOEBAE FROM SALINE ENVIRONMENTS OF HOOGHLY ESTUARY, INDIA.

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ABSTRACT

Small free-living gymnamoebae of the genus *Acanthamoeba*, isolated from typical intertidal benthic habitat of the coastal belts of Sundarbans, India, may be cultured on saline distilled water agar (SDWA) media with habitat associated food microbiota. Other media include sea-water agar (SWA), cerophyl sea-water agar (CSWA) and soil-extract distilled water agar (SEDWA). Highest high tide isolate from mangrove litter-soil exhibited optimal growth in media with high organic components e. g., CSWA. While isolates from mid-littoral and lowest low tide belts favoured SWA and SDWA media more.

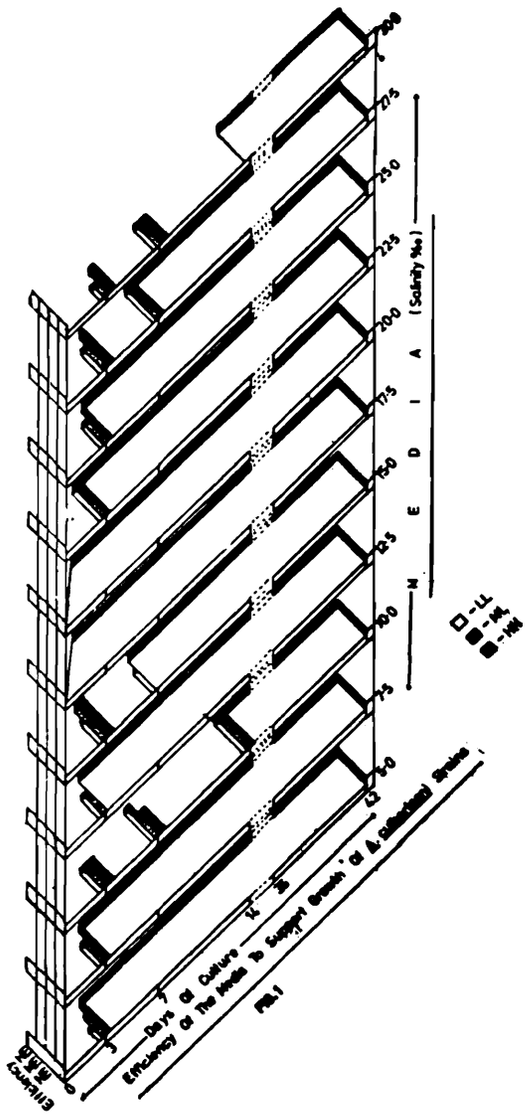
INTRODUCTION

Small naked amoebae are ubiquitous in their distribution and have been seen to be most well adapted to the interstitial milieu due to their plasticity. They are frequent in marine benthic situations (Sawyer, 1980) and make up most of the protozoa in terrestrial litters and soils (Bamforth, 1980 ; Coleman *et al.*, 1978). These amoebae form an essential component of the soil microbial ecosystem. It is worth mentioning that direct microscopic observations for quantitative as well as qualitative study from any definite amount of soil is not feasible with amoebe. Thus to initiate a comprehensive investigation, be it their abundance in a natural habitat, both in the active or trophic form, or inactive or cystic form ; tolerance to particular physico-chemical parameters including temperature, salinity, pH, etc. ; or food preference study, requires one suitable method of culturing them under standardized laboratory conditions. Most of the reported cultural methodologies are either the original propositions of Singh (1945, 55) or a simplified version thereof (cf : Page, 1976). These include their growth monoxenically on non-nutrient agar media with suitable bacterial food ; either *Escherichia coli* or *Aerobacter aerogenes*. Additionally, Page (cf : Page, 1976) has extensively

Key words : *Acanthamoeba*, Intertidal belts, Culture, Population growth, Comparison.

used a number of media requiring sea water for culturing isolates from marine or semi-marine environments.

In the present investigation, *Acanthamoeba culbertsoni* (Singh & Das, 1970) strains (designated as strains HH, ML and LL in sequence for highest high tide, mid-littoral & lowest low tide areas respectively) isolated



during monsoonal periods (July to October) from the south of Sagar Island (21°3.' to 21°50'N and 88°04' to 88°08'E) facing Bay have been cultured in a wide range of saline distilled water agar media. The physical texture as well as the chemical components of soils from the three different areas are different. The highest high tide belt area is a fair examle of typical mangrove litter-soil ecosystem. The mid-littoral belt is characterized by hard loamy-clay and roots of recently deforested 'Mangal'. While the lowest-low tide belt is a sand-silt subsystem. The overall growth on optimal artificial media have been compared with that on three other media involving either the sea water, the soil extracts or specific sea weed extract as essential components.

Materials and Methods

Sampling : Samples from 2-6 cm depth of different littoral belt areas were collected aseptically. All the samples were transported to the laboratory and processed within 24 hr.

Isolation and identification of the strain :

Samples of all the three regions were inoculated on central microorganismic patch in sea water agar petri-dishes. Establishment of *A. culbertsoni* clones corroborated the observations of Singh and Hanumaiah (1979).

Food source : Associated microorganisms from respective samples were grown on saline adjusted bacterial nutrient agar slopes. It was made as follows : to 1 lit of glass distilled water (pH 6.5) were added 20g NaCl, 20g Bacto agar (Difco Laboratories, U.S.A.), 2.60g Na_2HPO_4, 2g glucose, and 20g Bacto tryptone (Difco Laboratories, U.S.A.). It was then boiled in water bath, dispensed suitably and autoclaved. A very small quantity of the sample was inoculated aseptically on to the agar slopes and kept overnight at 37°C incubator. Repeated subcultures were made to avoid unwanted

particles etc. A mixed culture of food microbiota thus grown were used as food for the maintenance of respective clonal isolates.

The Supporting Media :

(a) Media of different salinity gradients : Saline Distilled Water Agar (SDWA) media :

Each of the 3 sets of experiments require 11 media of different salinity strengths ranging from 5-30‰ (W/V) at 2.5‰ intervals. To each of the 11 containers, 1 lit of glass distilled water was taken and pH was adjusted to 8.3 which was found to be the approximate mean value of monsoonal littoral water. NaCl of the quantities 5.0g, 7.5g, 10.0g, 12.5g, 15.0g, 17.5g, 20.0g, 22.5g, 25.0g, 27.5g, and 30.0g were then added separately to each container along with 15.0g Bacto agar. They were boiled, autoclaved and dispensed in sterile 7.5 cm dia petri dishes in 5 replicates for each medium. Media plates were dried for 7 days at room temperature before use.

(b) Sea Water Agar (SWA) medium :

To a lit of filtered monsoonal bay water 15g of agar was added, boiled and autoclaved. pH was adjusted as before.

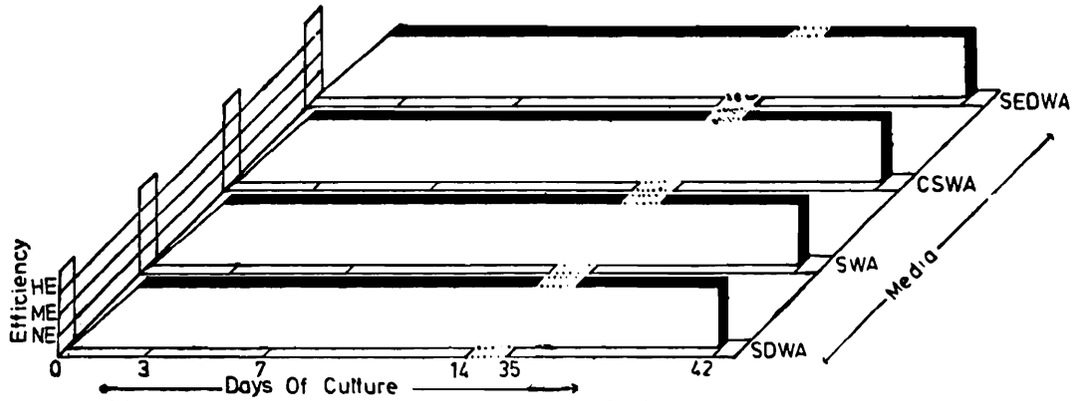
(c) Cerophyl Sea Water Agar (CSWA) medium :

Prepared after Page (1970) with minor modifications. 1g of cerophyl (Cerophyl Lab. Inc., U.S.A.) was boild in 1 lit of filtered water as SWA medium, filtered thrice through glass wool. 15g of agar was then added, boiled and autoclaved. pH was adjusted as before.

(d) Soil Extract Distilled Water Agar (SEDWA) medium :

Prepared after Singh (1975) with slight modification.

400g of seasonal sand/mud samples were boiled in 1 lit of glass distilled water for 30 min, extracts were decanted, filtered and finally made to 1 lit. 15g agar was added to each medium, boiled and autoclaved, pH was adjusted as before.



Efficiency Of The Media To Support Growth Of Strains FIG 2

Modified Page's Amoeba Saline (PAS) (Page, 1966) :

The chemical ingredients were same except NaCl which was adjusted as per the salinity of the respective fluid base of SDWA medium. To 1 lit of glass distilled water were added the following ingredients in g :

$MgSO_4 . 7H_2O$, 0.004 ; $CaCl_2 . 2H_2O$, 0.004 ; Na_2HPO_4, 0.142 ; KH_2PO_4, 0.136.

For other media all the chemicals other than NaCl were added to respective fluid bases. The pH value 8.3 was maintained throughout the experiment.

Experimental methodology :

Microorganisms from young cultures were suspended in as many tubes as sets of experiments containing respective modified sterile PAS. After centrifugation and triple wash the pellets were resuspended to make a final wet weight suspension of 0.05g/ml and stored at 4°C.

To 5 replicate plates for each experiment, suspensions were added so that each received about 0.01g wet weight of microorganisms. Excess fluid was allowed to absorb. Cysts form clonal culture, one in number, were inoculated on media plates. After absorption of the transporting modified PAS the plates were inverted and kept at 25 ± 1°C BOD incubator.

Efficiency of the media of use to support the growth of strains under study was assessed as follows : One medium was considered non-efficient if the mean number of growing organisms of 6 fields of 2 replicate plates lies less than 25 per microscopic field (100x) after desired periods of 3, 7, 14, 28, 35 and 42 days. One moderately efficient medium would show a range between 25 and 100 under such condition. While highly efficient medium exhibits more than 100 organisms per field. The overall growth was assessed on the basis of total population growth on 100 day old cultures and expressed in counts/petri-dish (mean of three replicate plates) using haemocytometer.

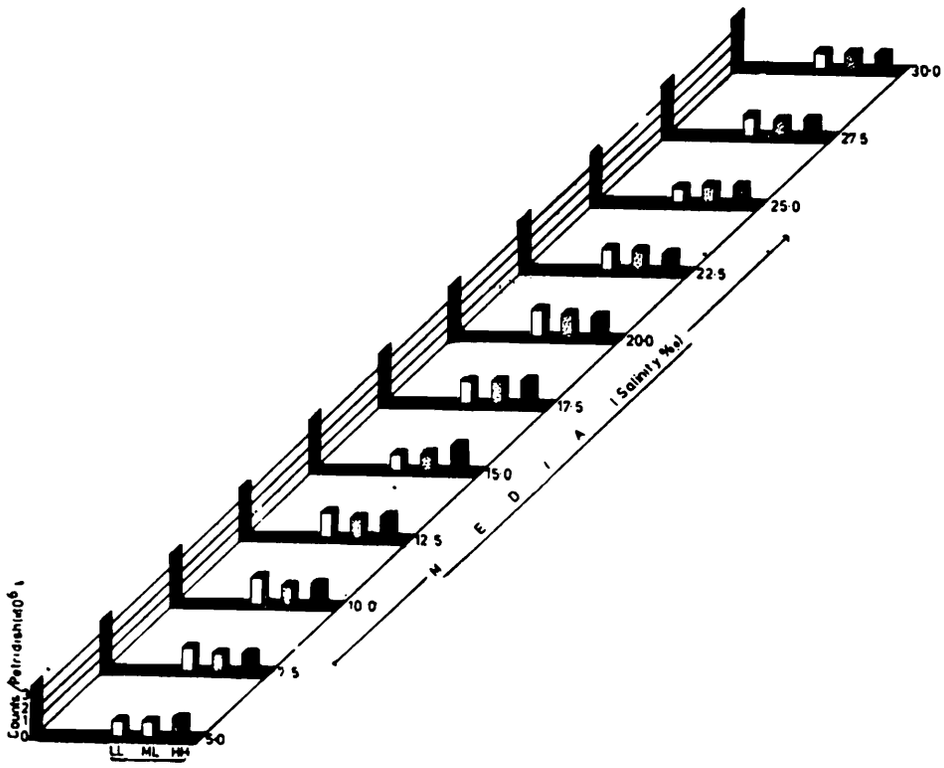
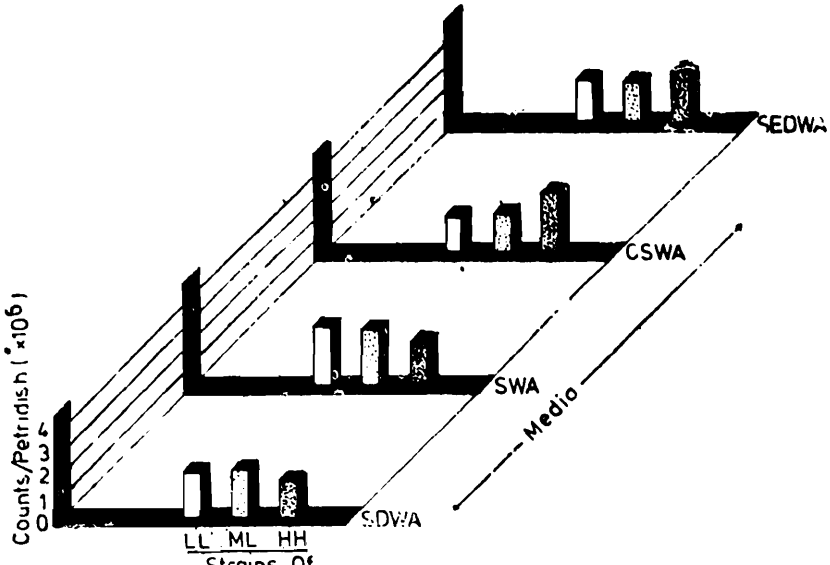


Fig. 3. Strains of *A. culbertsoni* showing overall growth.

Results

Growth curves of all the three strains in different media have been documented. Figure 1 shows the comparison of different

SDWA media to prove their efficiency to support growth. After 3 days media of 7.5, 10.0, 12.5, 15.0, 25.0 and 27.5‰, salinities show moderately efficient growth for LL strains while those of 17.5-22.5‰, exhibit behaved in the same fashion is perhaps unexplained on this ground but may be due to some other factors of the media petri dishes. On the 7th day most of the media exhibit highly efficient growth except 15.0‰



A culbertsoni showing overall growth

FIG 4

highly efficient growth. Media of 5.0 and 30.0‰ only could support growth in non-efficient way. ML strains on the other hand, grew in highly efficient manner on media ranging from 12.5-20.0‰, salinities. Other media ranging from 5.0-10.0‰ and 22.5-27.5‰ during the same period could support only moderately efficient growth. The only medium found non-efficient for this strain was 30.0‰ salinity one. Most of the media supported highly efficient growth of HH strains on 3rd day of culture. In only two, namely 10.0‰ and 30.0‰ media, they grew moderately. The reason for this in 30.0‰ medium may be explained on the ground of its high salinity for isolate from highest high tide area where the same in most of the time is considerably low.

But why then medium of 10.0‰ strength and 30.0‰ for LL isolate ; 10.0‰ and 27.5-30.0‰ media for ML one and 10.0‰ and 30.0‰ again for HH strains. Then onwards all the media were seen to support highly efficient growth for all the strains.

In figure 2 comparisons were made for four selected media. These were SDWA (20.0‰), SWA, CSWA and SEDWA. All the media were shown to be equally efficient to initiate highly efficient growth within the shortest periods of 3 days and maintain their efficiency throughout the periods of study.

Figure 3 represents a bar diagram to show the overall population growth of all the strains maintained for 100 days in each SDWA medium. This bar diagram depicts that the average mean values of the counts per petri-dish normally range between 1.2-2.0 x 10^6. The greater values have been

seen to concentrate around media of moderate salinities.

In figure 4 population comparisons were made for four selected media. The average mean values of the counts per petri-dish range between 1.7-2.8 x 10^6. One interesting feature is this, greater values for HH strains were obtained from CSWA and SEDWA media while the same for LL and ML strains were recorded from SDWA and SWA media. Probable explanations have been attempted later on. CSWA medium was found to support maxium growth, being exhibited by HH strains.

Discussion

The fact that encysted gymnamoebae can withstand the most adverse conditions for long periods combined with their microscopic size and the many avenues of transport which are open to them, ensure that there can be no topographic barrier against their distribution. Thus, the present study material, *A. culbertsoni* which is common inhabitant of most of the Indian soil types, is also a predominant member of saline water environment. Their way of life is ecologically very successful because crawling forms can move in-shallow water film with a large benthic surface in relation to volume of water and thus favours interface locomotion.

Thus to use this organism as a tool in planned works, establishment of optimal cultural conditions is a prerequisite. Media requiring distilled water as fluid base and sodium chloride in addition to Bacto-agar and habitat associated microbiota have been proposed as alternate to other more complicated media. Habitat associated microbiota, initially used as food organisms, may be replaced by *E. coli*. To start with, one can make a number of media of different salinities from as low as 5.0‰ to as high as 30.0‰. Within two weeks it is always possible to find one moderate tolerable zone for any isolate from specific environments. Thus, while screening for the suitable media for organisms from a specific habitat, it is possible to study their salinity tolerance limits. Optimal cultural conditions are also required to enumerate their numbers in soil. Of the four media used in comparative study, CSWA and SEDWA media are the two organically enriched ones. *A. culbertsoni* strains from highest-high tide belt have been observed to favour these two media, perhaps in consequence to their existence in habitat with high organic content, characteristics of litter-soil. Strains from lowest-low tide and mid-littoral environments normally prefer SWA and SDWA media more. The primarily sand-soil of the former and the paucity of leaf-litter in the later make the habitat less enriched with organic components as expected. Thus, it will be quite safe to use the saline-distilled-water-agar media for isolates from wide saline environments.

Acknowledgement

We are grateful to the Director, Zoological Survey of India, for his keen interest throughout the work. We further wish to record our appreciation to the Staff Members of Sushama Devi Choudhurani Marine Biological Research Institute, Sagar Island, West Bengal, for their assistance during field studies. Finally one of us (M.G.) is grateful to Department of Environment, New Delhi for the grant of Senior Research Fellowship.

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