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Culturing of Marine Sponges and Extraction of Antibiotics from Sponges Collected from Bay of Bengal

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Abstract: Marine sponges are a rich source of bioactive compounds with pharmaceutical Potential. Since biological production is one option to supply materials for early rug development, the main challenge is to establish generic techniques for Small-scale production of marine organisms. We analyzed the state of the art for cultivation of whole sponges, sponge cells and sponge symbionts. To date, Cultivation of whole sponges has been most successful in situ; however, optimal Conditions are species specific. The establishment of sponge cell lines has Been limited by the inability to obtain an anemic inoculums as well as the lack of Knowledge on nutritional requirements in vitro. Approaches to overcome these bottlenecks, including transformation of sponge cells and using media base on yolk, are elaborated. Although a number of bioactive metabolite-producing Microorganisms have been isolated from sponges, and it has been suggested that the source of most sponge-derived bioactive compounds is microbial Symbionts, cultivation of sponge-specific microorganisms has had limited success.

The current genomics revolution provides novel approaches to cultiva these microorganisms.

Key words: sponge; cultivation; cell culture; symbiosis; natural product; Microorganisms; secondary metabolite; genomics

I. ACKNOWLEDGEMENT

I thank the Lord Almighty for being gracious and merciful unto me .He has been mindful of me and blessed me with abundance .I stand to testify His unchanging love and compassion towards, which has filled me with strength to endure. Glory to God, He Lifted me up, I thank "THE LORD ALMIGHTY" who is the owner of strength and confidence in my life.

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Last but not the least; I would like to thank my Parents for their continuous support and love.

II. INTRODUCTION

Among all metazoan phyla, sponges are known to produce the largest number of bioactive compounds, some of them are rich in human therapeutic value. Therefore, an increasing interest in basic cell biology research up to biochemical engineering can be observed, aiming at the production of bioactive compounds from sponges, under completely controlled conditions. Sponges are

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Sessile, soft-bodied marine invertebrates which lack obvious physical defenses and biosynthesize bioactive, non-primaryorsecondary metabolites to protect themselves and maintain homeostasis (Atta way and Zaborsky, 1993). Sponges have antibacterial,antifungal, antiviral, anti-helminthic, anticoagulant, antitumor, cytotoxic, antidiabetic, anti inflammatory, antimalarial, and antiplatelet, antiprotozoal, antileukemic, anti tuberculosis, and immunomodulatory activities (Butler, 2004).Sponges are an important source of secondary metabolites with pharmaceutical interest. This is the main reason for the increasing interest of sponge culture in recent years. The use of sponges dates back many centuries. As early as 700 BC, Homer and Aristotle described the use of sponges in medicine (Voultsiadou, 2007). Until the end of the nineteenth century, sponges had been widely used in surgery (Müller et al., 2004b). For example, sponges soaked with extracts of opium were used to anaesthetize patients prior to surgery, and extracts of Spongia tosta were found to be effective in the treatment of scrofula (Müller et al., 2004b). Besides their biomedical application, sponges also had commercial value as bath sponges, because of their absorptive properties. Until 1960, commercial bath sponge farming was a lucrative business spreading from the Mediterranean to the Atlantic and Pacific Ocean (Bernard, 1968; Duckworth, 2009). From 1960, due to the invention of the less expensive synthetic bath sponge, farming of the natural bath sponge decreased and now only occupies a small niche in the market for high-quality applications (Hogg et al., 2010). Current biotechnological applications of sponges are more directed towards the production of marine natural products and biomaterials. Besides pharmaceutical products many other applications of sponge derived biomaterials have been identified. For example, the silicon skeleton of glass sponges may serve as a blueprint for the production of very efficient fibre optics (Sundar et al., 2003) and biosilica-producing enzymes from sponges have been applied in nanotechnology (Schröder et.al.,2010).

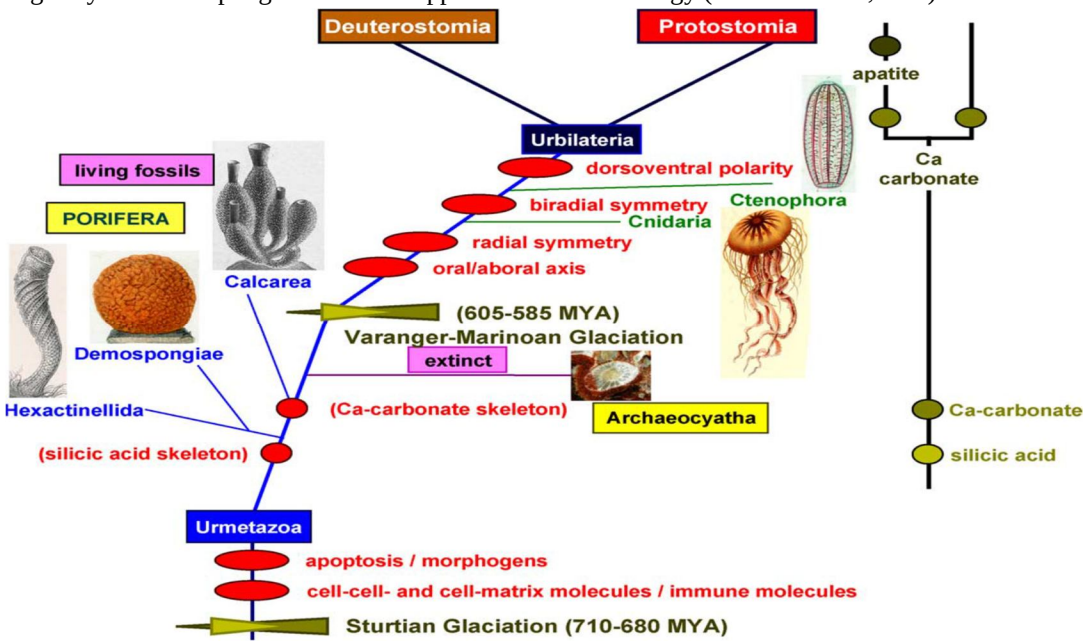

Fig: 1 Phylogenetic position of the Porifera between the Urmetazoa and the Urbilateria. The major evolutionary novelties which have to be attributed to the Urmetazoa are those molecules which mediate apoptosis and control morphogenesis, the immune molecules and primarily the cell adhesion molecules. The siliceous sponges with the two classes Hexactinellida and Demospongiae emerged first and finally the Calcarea, which possess a calcareous skeleton, appeared. These three classes of Porifera are living fossils that provide a reservoir for molecular biological studies. The Archaeocyatha, sponge related animals with a calcareous skeleton, became extinct. The Calcarea are very likely a sister group of the Cnidaria. From the latter phylum the Ctenophora evolved which comprise not only an oral/aboral polarity but also a biradial symmetry. Finally the Urbilateria emerged from which the Protostomia and the Deuterostomia originated. Very likely the Urmetazoa emerged between the two major ‘snowball earth events’, the Sturtian glaciation (710–680 MYA) and the Varanger-Marinoan ice ages (605–585 MYA). In the two poriferan classes Hexactinellida and Demospongiae the skeleton is composed of amorphous and hydrated silica, while the spicules of Calcarea are composed of Cacarbonate.The latter biomineral is also prevalent in Protostomia and also in Deuterostomia. In vertebrates the bones are composed of Ca-phosphate [apatite].

In fact, sponges are the only animal able to polymerize silica to generate massive skeletal elements in a single reaction at ambient temperature and pressure (biosintering) (Müller et al., 2009). According to a survey, sources have stated that there has been no

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recent research on sponge culture techniques in India. The optimal culture system depends on the species to be cultured, while some species easily produce sponge aggregates after dissociation (primmorphs), others show a great capacity to regenerate after fragmentation (explants).One major obstacle is the limited availability of larger quantities of defined sponge material, “the so-called supply problem” .In this Research Project, different approaches used so far for producing sponge biomass by culturing methods as well as some significant modifications are introduced for the maintenance of sponges are studied .In recent years, great efforts have been made to set up in vitro culture systems for the cultivation of sponge cells. One of the major advantages of cell cultures is the possibility to control and manipulate the cultivation conditions depending on the sponge species and the target metabolite. Successful attempts to produce sponge metabolites using culturing of epidermal cells of sponges by manipulating the mechanism of regeneration in sponges are discussed.

III. LITERATURE REVIEW

Shear effects on suspended marine sponge cells was done by F. Garc´ıa Camachoa, E.H. Belarbi a, M.C. Cer´on Garc´ıa a, A. S´anchez Mir´on a, T. Chile b, Y. Chisti b, E. Molina Grima states that, a four-step mechanistic model was shown to describe the kinetics of cell death and fragmentation. The damage to cells was not depending on cell–cell interactions. The forces in the agitated fluid killed the viable cells by impact, which was not accompanied by cell rupture (i.e. the cell was left dead, but intact). Biochemistry and Cell Biology of Silica Formation in Sponges done by: werner e.g. mu¨ ller, anatoli krasko, gae¨ l le pennec, and heinz c. schro¨ der states that The spicules occur in the cytoplasm and the extracellular space and also in the nucleus (as silicate crystals) of some sponge cells; the spicules are formed by the enzyme called Silicatein. The enzyme is dependent on ferric ion. Silicatein also has proteolytic (cathepsin-like) activity. The morphogenetic activity of silicate is underscored by the finding that this ferric ion increases gene expression of silicatein and collagen. Based on these findings, it is concluded that both ferric ion and silicate stimulate the activity of silicatein. Furthermore, it is proposed that the growing spicules are surrounded by the scavenger receptor which might be considered as a docking molecule for the collagen matrix into which the spicules are embedded. Cell culture from sponges: pluripotency and immortality so` nia de Caralt1, Marı´a J. Uriz and Rene´ H. Wijffels states that Sponges have an enormous potential for the Development of new medical drugs. Thus, although several Bottlenecks remain, efforts to develop a technology for continuous cell cultures, such as the use of embryonic stem

Cells to form a cell line and research into the control of apoptosis are worthwhile and might become successful in the interim. Establishment of a primary cell culture from a sponge: primmorphs from Suberites domuncula Werner e. g. miller,

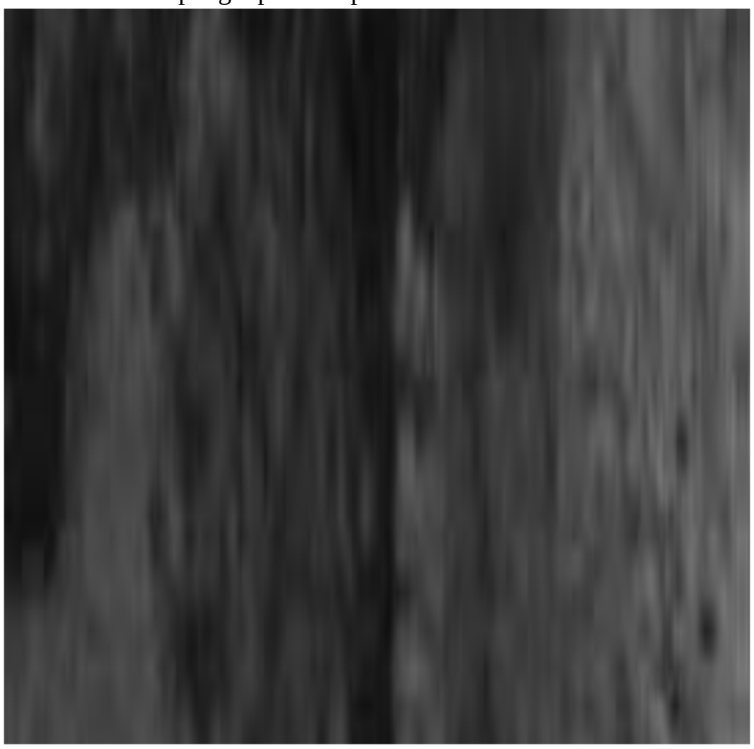

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Fig: 2.1 A large barrel sponge. Courtesy of Jonathan Bird, Oceanic Research Group

matthias wiensl, renato et.al steffen, heinz. schroder, adovan to conclude, the primmorph system described by Custodio et al. (1998) can be used in the future for a variety of applications in the following main directions:(1) As a bioreactor to produce bioactive compounds from sponges and (2) For the detection of potential cytostatic compounds causing a transition from telomerase-positive to telomerase-negative cells. Advances in the production of sponge biomass *Aplysina aerophoba* A model sponge for ex situ sponge biomass production Rudolf Haussmann, Marco P. Vitello b ,Frank Leitermann a, Christoph Syldatk a Obtaining that is at present extremely difficult because most sponges are relatively rare in nature and their mass cultivation in the laboratory has not yet been accomplished. In this study the possibility of culturing *Aplysina aerophoba* fragments in laboratory was examined. While a substantial biomass increase was not yet observed, we achieved fragmented sponge tissue to develop into a functional sponge as a first success. Antibacterial Activity of Marine Sponge Extracts against Fish Pathogenic Bacteria *G.* Annie Selva Sonlal, A.P. Lipton and R. Paul Raj. Suggest that fractionation and purification of the crude methanol extract of A. elongata has potential in the development of novel Antibiotic substances for managing common bacterial diseases in aquaculture. The most active species was A. elongata which inhibited 100% and 87.5% of the tested bacterial isolates at 20°C and 30°C respectively. For most sponge-derived bioactive compounds, it is not clear whether they are produced by the sponge or the symbiont (which can contribute up to 40% of the sponge volume). Metabolites known to be produced by the sponge are, for example, avarol, found in Dysidea avara (Uriz et al., 1996) and stevensine, found in Axinella corrugata (Andrade et al., 1999). On the other hand, it has been demonstrated that Oscillatoria spongelia, a cyanobacterial symbiont, produces antimicrobial polybrominated biphenyl ethers and might keep the sponge free of other bacteria (Unson et al., 1994).

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Fig: 2.2 A dye squirted around the base of a purple tube sponge colors the jet emerging from the osculum at the top of the sponge. Courtesy of Jonathan Bird, Oceanic Research Group.

IV. MATERIALS AND METHODOLOGY

A. Collection of sponges

Sponged were collected by, hand-picking at the coastal regions of Pamban Bridge (9°19' N latitude and 79°23' E longitude). Sponges were obtained as entangled specimens from fishing nets set at depths of 10-15 m off Pamban coast of Rameswaram. The churning seawater in this area during January-February and May-August lead to dislodging and entanglement of marine organisms in nets. The collected specimens were washed in filtered seawater. Eight species of sponges were collected, and, were identified by Dr.G.Sivaleela of Zoological Survey of India, Chennai, as Clathria Gargonoides, Haliclona fibulata, Sigmadocia petrosionides, Dysidea herbacea, Hyattella intestinalis ,Gelliodes pumila, Stellitethya repens and Suberitus carnosus all of which belong to the class Demospongiae Sollas.

B. Formation of sponge cell lines

In general, animal cell cultures are obtained by isolating cells from an axenic piece of tissue. These cells are cultured in medium that will support proliferation. This stage is called primary cell culture and most cell types will undergo a limited number of divisions and then enter senescence. Continuous cell lines are generally obtained from spontaneously immortalized primary cell cultures (e.g.,

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random mutagenesis) or are derived from cancerous tissue (Freshney, 2005). Another approach towards developing a continuous cell line is the insertion and expression of immortalizing genes (e.g., SV40LT, hTERT) in primary cells. Immortalizing genes interfere with the regulatory pathways for cell division, and in this way cause unlimited cell division, resulting in a continuous cell line (Freshney, 2005).

In principle, sponges have great potential for cell culture because of the presence of totipotent stem cells (i.e. archaeocytes) and because cells can be easily dissociated from its tissue, due to their very loosely packed cellular organization. However, despite efforts by several research groups, a continuous sponge cell line has not yet been developed, and the number of primary sponge cell cultures developed is very limited (Rinkevich, 2005; Pomponi, 2006; Schippers et al., 2012). This is related to the following aspects:

1) Due to the presence of symbionts inside the sponge, it is practically impossible to obtain an axenic piece of sponge tissue. Researchers have used antibiotics and antimycotics to reduce growth of contaminants (Pomponi and Willoughby, 1994), but still contamination occurs (Ilan et al., 1996).

2) Also obtaining proliferating starting material remains problematic, since most dissociated sponge cells go into apoptosis and die (Schippers et al., 2011 However, Pomponi and Willoughby (2000) were able to show sponge cell division when exposed to the mitogen, phytohaemagglutinin. Other options for obtaining proliferating starting material are the use of primmorphs, gemmules or larvae.

3) Primmorphs are reaggregated sponge cells which have the ability to proliferate *in vitro*, although no net growth has been observed (Sipkema et al., 2003). Gemmules and larvae both contain a package of stem cells, and can develop into a juvenile sponge *in vitro*.

4) Nutritional requirements of sponge cells are still poorly understood. Most of the sponge cell culture media are based on mammalian cell culture media, but certain components such as fatty acids or growth factors, are expected to be different for sponge cells.

The establishment of sponge cell lines is still hampered by three issues: the presence of contaminants in the starting material, the lack of knowledge on nutritional requirements and the lack of proliferating starting material. This thesis focusses on the last item; sponge cell proliferation).

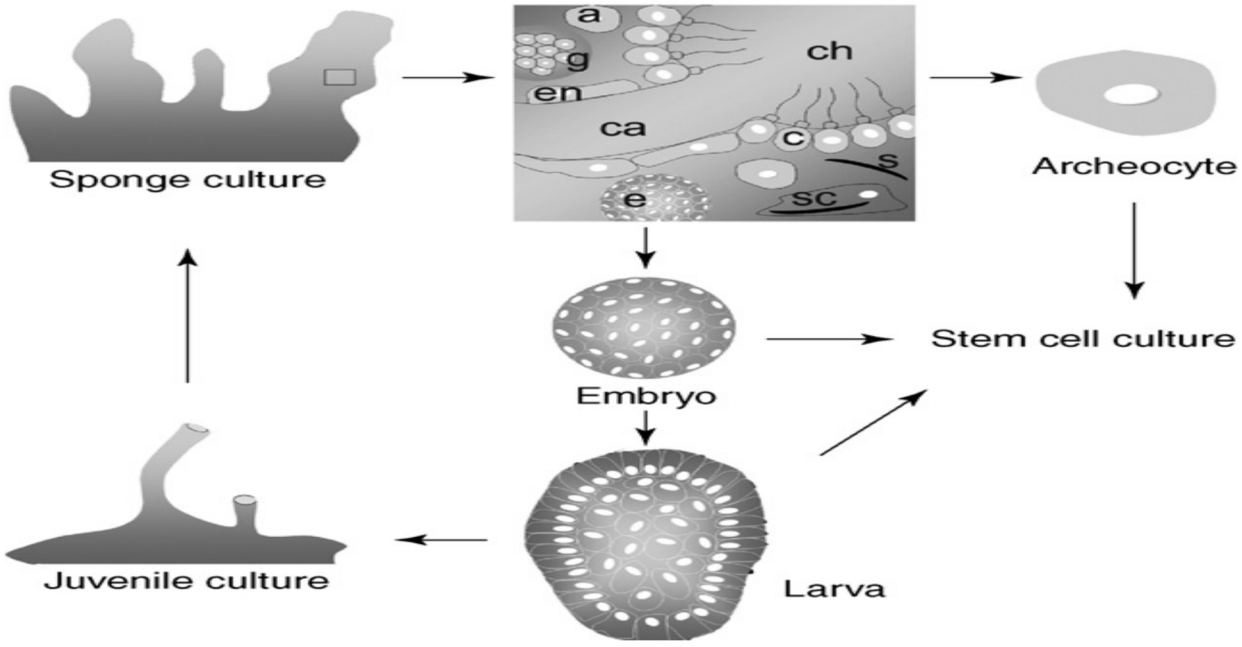

Fig: 3.1 Schematic representation of different sponge material for stem cell cultures. Adult sponges have differentiated cells (e.g. choanocytes, pinacocytes and sclerocyetes) and pluripotent cells (archeocytes). Ripe sponges harbour high numbers of embryos, which are composed of blastomeres (stem cells) that increase in number per sponge unit during the later stages of the reproduction cycle. The embryos are released as larvae, which can be cultured as a source of
(i)stem cells and (ii) sponge juveniles for obtaining sponge biomass.
Abbreviations – archeocytes; c – choanocytes; ca – canal; ch – choanocyte chamber; e – embryo;en – endopinacocyte; g – gemmule; s – spicules; sc – sclerocyte

5) Explants were cultured under two Methods as follows:

a) In vitro Culturing Technique.

b) Closed aquarium system.

In this study only the invitro culturing of marine sponges *Clathiria gorgonoides and Dysidea herbaceae* is performed

C. Invitro cell culture technique

Cell or tissue culture (both subsequently referred to as in vitro culture) is a preferred method because the system can be defined and controlled. Sponges have great potential for cell culture because of the presence of totipotent stem cells and because cells can be easily dissociated from its tissue, due to their loosely cellular organization. Despite efforts by several research groups, a continuous sponge cell line has not yet been developed, and the number of primary sponge cell cultures developed is very limited (Rinkevich, 2005). The only report of a continuous sponge cell culture was Klautau et al. (1993, 1994); however, this cell line was subsequently identified as a protozoan (Custodio et al., 1995).

V. DISSOCIATION OF SPONGE CELLS

The first step in obtaining a primary sponge cell culture involves dissociation of
the cells into a monodisperse suspension. Several dissociation techniques, Such as
Mechanical, chemical, enzymatic and spontaneous, have been studied

The most successful dissociation method is a combination of mechanical and chemical dissociation (Pomponi and Willoughby, 1994; Rinkevich et al., 1998). If a monodisperse cell suspension is needed, the method described by Pomponi and Willoughby (1994) is generally used , whereas the method described by Custodioet al. (1998) is applied when re-aggregation of sponge cells (i.e. to formprimmorphs) is needed .The dissociation method, will result in a mixture of cell types, for example, archaeocytes, choanocytes, pinacocytes, collencytes. Archaeocytes and choanocytes are considered to be the pluripotent stem cells in sponges. These stem cell-like cells have the capacity to proliferate and to differentiate into other cell types. Most cell types in a sponge are expected to be terminally differentiated and not able to divide. Consequently, selecting and enriching for archaeocytes and choanocytes may result in a proliferating sponge cell culture. By using a process which involving differential centrifugation, selective aggregation in low Ca2/Mg2 seawater, differential adherence in artificial seawater and by centrifugation, eventually obtained an archaeocyte-enriched fraction greater than 80%, and proliferation was demonstrated by BrdU incorporation

A. Steps which are involved in cell dissociation of marine sponges

1) Cut sponge in very small parts of 2–3 cm from the mother sponge in water with the help of sterile razor sharp scalpel.

2) Wash in fresh artificial sea water to Remove debris and mince into small pieces of 2-5mm in length.

3) Cut parts of sponge where transferred to Petri dishes containing CMFSW-EDTA in the ratio of 10:1.Soak for 20 minutes in sterile CMFSW-EDTA.

4) Filter through 70 mm nylon mesh /sterile gauze now the cells are easily released to remove spicules.

5) To form crude cell suspension centrifuge at 300rpm for 5minutes to enrich the sponge cell content which where in pellet and remove most of the bacteria in supernatant.

6) The supernatant was discarded and cells in the pellet were resuspended in CMFSW-EDTA.

Table1: Publications about Primary sponge cell culture

Sponge species	Reference	Proliferation	Comments
Ircinia Muscarum & Sarcotragus foetidus	De Rosa et al. (2001, 2003)	Yes, but. . .	No data of sponge cell growth over time are shown
Suberites domuncula	De Rosa et al. (2003) and Zhang et al. (2004)	Yes	Sterilized sponge cell proliferates approximately after 3 days

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Hymeniacidon heliophila	Pomponi and Willoughby (1994)	Yes	PHA-stimulated cells doubled after 72 h
Ephydatia muelleri	Imsiecke et al. (1995)	Yes	Cells were taken from hatched gemmules Eventually, cultures were dominated by bacteria
Acanthella acuta	Nickel et al. (2000)	No	-
Dysidea avara	Nickel et al. (2000) and De Rosa et al. (2003)	No	-
Hymeniacidon heliophila	Pomponi and Willoughby (1994)	Yes	PHA-stimulated cells doubled after 72 h

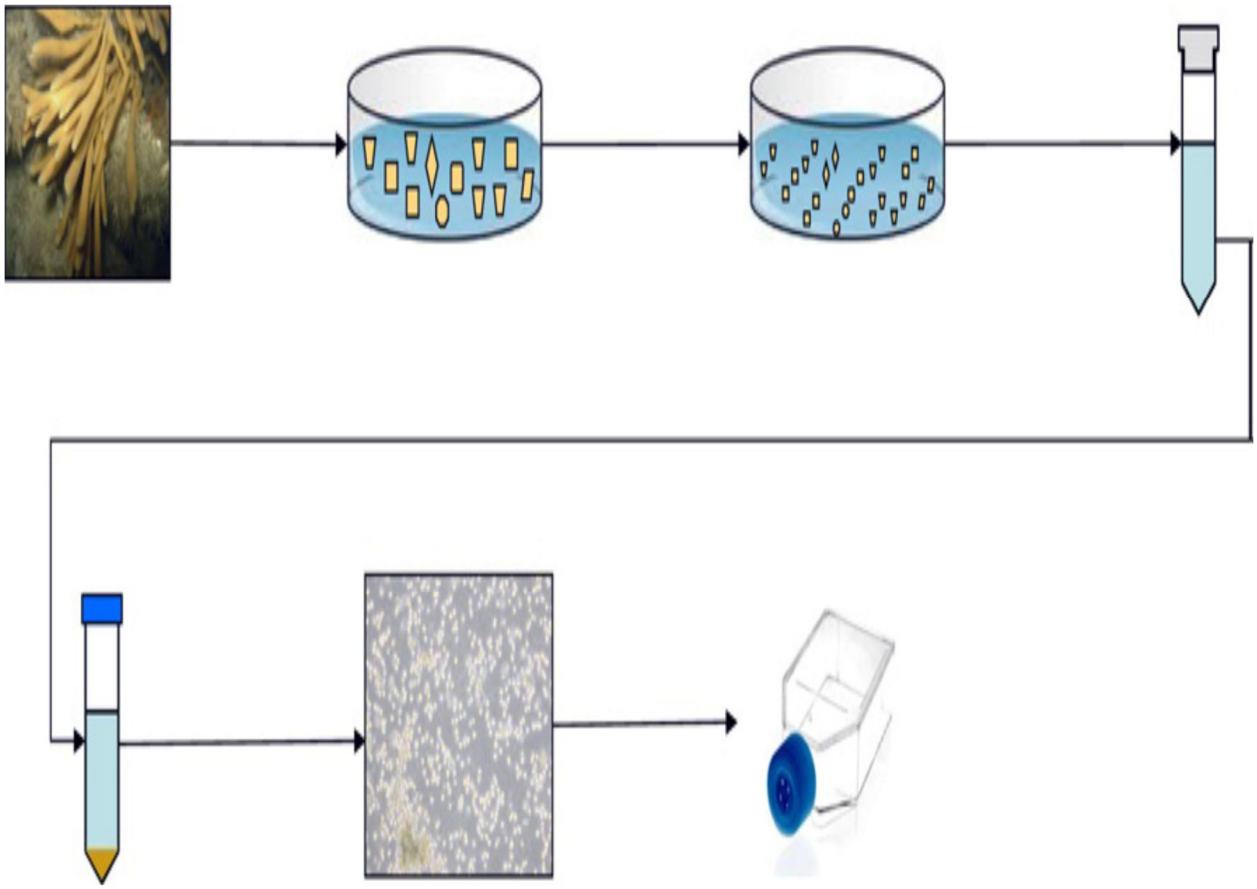

Fig: 3.2 Dissociation method described by Pomponi and Willoughby (1994).

7) Finally resuspend pellet into culture medium for further regeneration of cells. Phyto-haemogluttinin a mitosis inducing lectin was also added.

8) EDTA10mM was added which helps in dissociating cells by chelating any multivalent metal ions in the sponge surface.

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B. Medium for sponge cell culture:

Preparation of a culture medium is very essential to support the growth of Sponge cells. Generally, an axenic inoculum is used to initiate a primary cell Culture from animal tissue (Freshney, 2005). It is not very easy to isolate such

An axenic part from sponges, since both eukaryotic and prokaryotic symbionts are intermingled with the sponge in the tissue. This made it very difficult to develop a specific nutrient media for the growth of sponge cells, since adding nutrients for sponge cell growth will also stimulate growth of other contaminants present in the sponges. Therefore, measures have been taken to limit the growth of contaminants, such as bacteria, fungi, protozoa etc... To prevent growth of these contaminants, antibiotics and antimycotics can be added to the medium, but most of these only temporarily inhibit growth of bacteria and fungi. This may be due to the fact that some bacteria in sponges may have antibiotic resistance. In addition, high degree of antibiotics and antimycotics can also have a negative effect on the viability of the sponge cells. Despite these difficulties, researchers have tried to develop culture media for sponge cells. The most basic culture medium consists of filtered seawater which provides the appropriate salinity and pH, and contains some dissolved nutrients and trace elements which are generally present in the sea. Sponge cells could be maintained in filtered seawater for several weeks without contaminants overgrowing the culture. However, since no growth of the sponges a

Table 2: Publications about sponge cell culture media:

Media	Antibiotics	Supplements	Contaminations	Sponge species
SW	Penicillin, streptomycin	Glutamine, pyruvate, iron citrate,silicon, RPMI 1640, Marine Broth 2216	No	Suberites domuncula
SW	-	-	Yes	Xestospongia muta
M199 in CMF	Rifampicin	5% FBS, 1.5% PHA	No	Hymeniacidon heliophila
Modified ASW		Inorganic salts (e.g. ferric iron NaCl,) amino acids, sugars, vitamin C, DMEM, RPMI 1640, 1.5% PHA	No	Hymeniacidon perlevis
DMEM in ASW	Nystatin, rifampicin, gentamycin, Penicillin, Ciprofloxacin	-	Yes	Acanthella acuta Dysidea avara

Cell was observed (Schippers et al., 2011), it is concluded that the dissolved nutrients in seawater are not sufficient to support long-term growth of sponge cells.

Other sponge cell culture media (reviewed by Pomponi, 2006) have mostly been derived from existing animal cell culture media, which contain amino acids, vitamins and glucose. Since these media are rich in nutrients, the use of antibiotics and antimycotics is also essential. Despite the use of antibiotics and antimycotics, contamination can still occur (Ilan et al., 1996), and the addition of cell culture media alone does not support sponge growth.Growth factors is essential. For example, the addition of PHA (phytohemagglutinin), a mitogenic lectin, stimulated cell division (Pomponi et al.,1997; Sun et al. 2007) and the addition of cholesterol resulted in increased cell density compared to cell cultures without cholesterol (De Rosa et al., 2001,2003). Evidently, cells in vitro are unable to biosynthesize sterols, and the addition of sterols is needed to satisfy membrane requirements. Ferric iron was also found to significantly improve cell viability. So to prepare a new culture medium to culture sponge cells the methods are as follows, the medium consists of the parent sea water from where the sponges subjected for culture process is taken and it is sterilized by filter sterilization technique. The antibiotics like penicillin and streptomycin was also sterilized and added to the filtered fresh parent sea water. Now the substratum like mussels, rocks, and other calcareous fossil remains which found in-depth of the sea to which the sponge holds was placed inside the medium which is also sterilized by acid wash and finally with double

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distilled water to remove the acidified nature. Next is to provide the exact temperature, light intensity, Pressure and pH and make the water in the medium not to stagnant, provide a little motion to the water by the blower. By enabling this suitable environment for sponge culture process we can expect the proliferation of sponges will takes place.

C. Primmorph

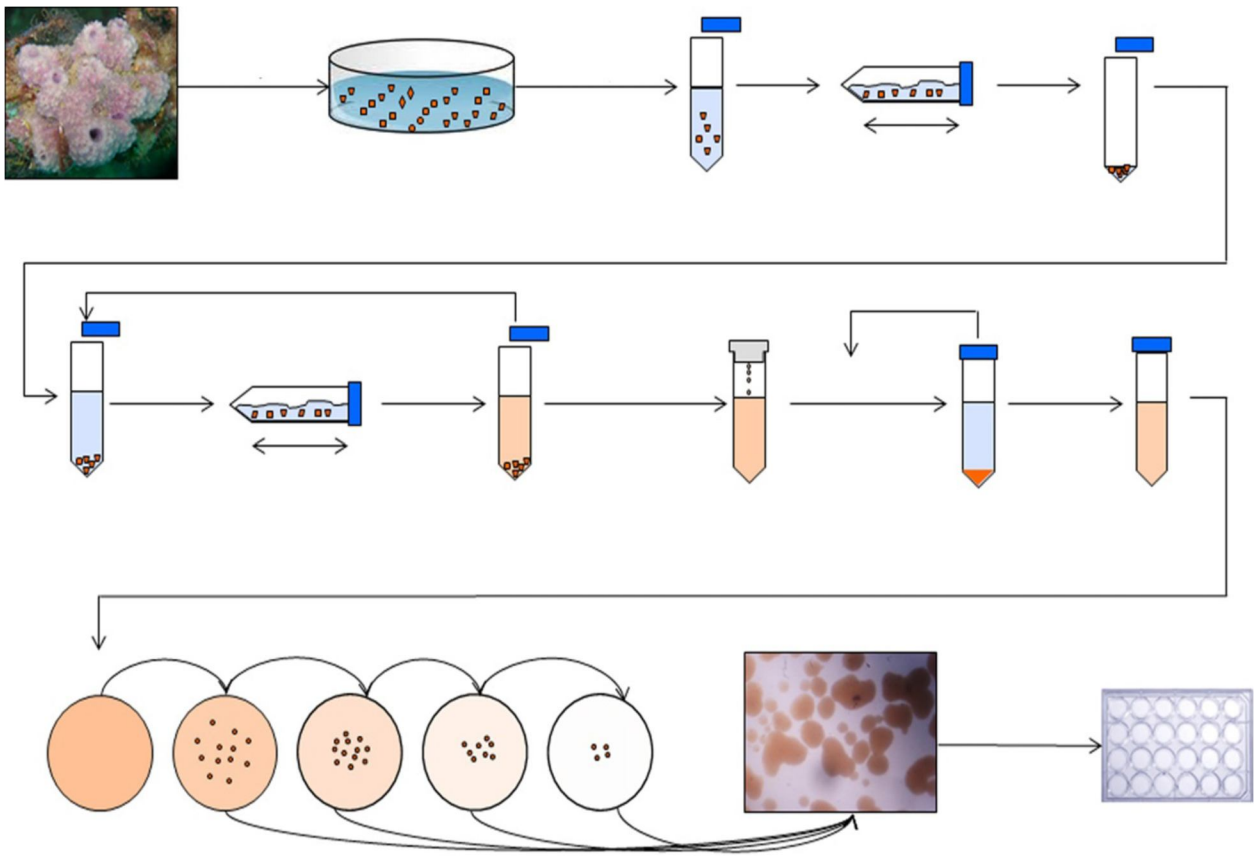

Fig: 3.3 Method to generate primmorphs, described by Custodio et al. (1998).

Abbreviations:
FSW- filtered seawater
CMFSW calcium- and magnesium-free seawater.
EDTA-Ethylene Diamine Tetra Acetic Acid
SW- seawater
AB- antibiotics

1) The sponge taken from the sea is cut into pieces of 1 mm.
2) Wash with sterile Calcium Magnesium free fresh sea water
3) Transfer the sponges into 50ml test tube containing 40ml CMF-EDTA solution.
4) Gentle shaking of the test tube for 30minutes.
5) Discard the solution and fill the test tube with new CMF-EDTA solution.
6) Gentle shaking for 30 minutes and the solution is collected.
7) Filter the collected water through 40micrometer nylon mesh and isolate the sponge cells.
8) Isolated sponge cells are subjected to centrifugation at 500rpm for 5minutes.
9) Repeat the centrifugation process for two times and resuspend the solution to sea water and antibiotic solution

Collect the primmorphs and wash with sea water and place them in well plates for further experiments.

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Fig: 3.4

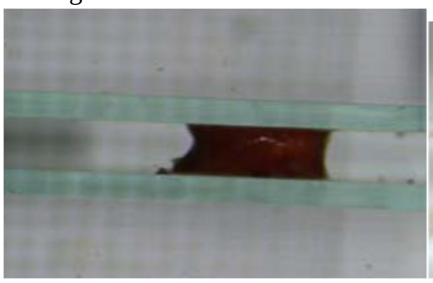
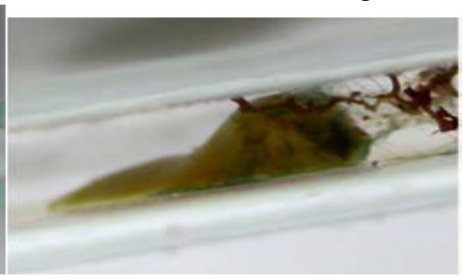


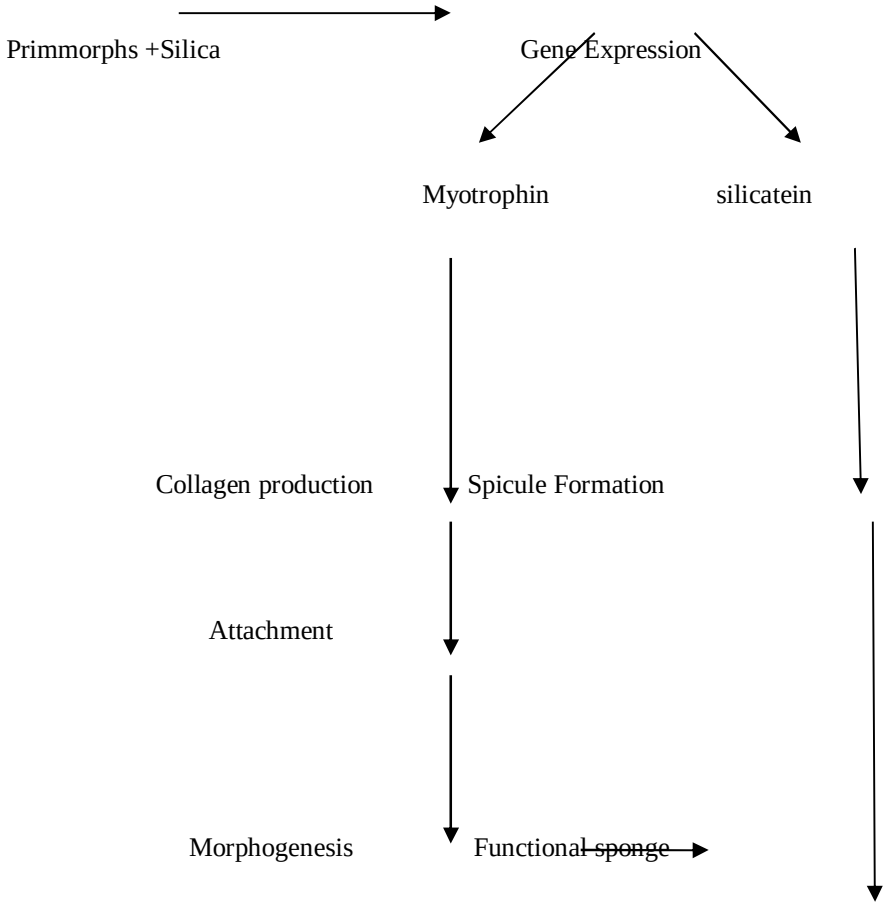
Fig: 3.5



D. Primmorphs

The monodispersed sponge cells lack the capacity to proliferate (Koziol et al. 1998). A possible explanation could be the loss of cell adhesion factors, such as cell–cell and cell–matrix contact, when cells are dissociated using calcium- and magnesium-free seawater (Koziol et al., 1998).They found that the activity of telomerase, a bio-marker for cell proliferation, in tissue of S. domuncula and Geodia cydonium was very high, while the telomerase activity of dissociated sponge cells was almost zero. When the dissociated sponge cells re-aggregated, the telomerase activity was recovered, confirming the hypothesis that cell adhesion factors are important for cell proliferation (Custodio et al., 1998). These multicellular aggregates from dissociated sponge cells are termed primmorphs. Primmorphs are dense sphere-shaped aggregates, 1–2 mm in diameter. They differ from aggregates that occur after dissociation of sponge tissue in that they have an external layer of pinacocytes and a central zone with primarily spherulous cells. The formation of primmorphs is based on the discovery of Wilson (1907) that sponge cells have re-aggregative properties and can form a newly functional sponge.

Fig: 3.6



Marine sponges produce secondary metabolites that can be used as a natural source for the design of new drugs and cosmetics. There is, however, a supply problem with these natural substances for research and eventual commercialization of the products. *In Vitro* sponge culture is nowadays one of the most reliable methods to supply pharmaceutical companies with sufficient quantities of the target compound.

E. Cell Proliferation of Sponges

Cell proliferation has been observed in primmorphs by means of BrdU incorporation (Custodio et al., 1998; Zhang et al., 2003b); however, an increase in total biomass over time has not been observed. Possibly there is equilibrium in the primmorphs between proliferating and apoptotic cells (Koziol et al., 1998). A possible explanation for the lack of increase in biomass is the deficiency of nutrients in the media. Primmorphs were generally cultivated in nutrient-poor medium, such as natural seawater or artificial seawater without the addition of any nutrients (Custodio et al., 1998; Nickel et al., 2001; Sipkema et al., 2003c; Zhang et al., 2003a, Valisano et al., 2006b). Only Muller et al. (2000) and Krasko et al. (2002) added 0.1% (v/v) marine broth 2216 or 0.2% RPMI 1640, respectively, to their media, but they do not show data for nutrient uptake by the primmorphs. To be able to obtain continuous biomass growth, it is important to supply appropriate nutrients and growth factors.The cell culture of sponge *Clathria gorgonoides* was not successful because from which the cells are dissociated, during dissociation it was damaged and lost its proliferating nature eventhough some callus like structure was arised but they are found to be the culture of symbiotic micro-organisms associated with sponges, but the culture of *Dysidea herbacea is* successful because the cell are not dissociated but as whole mass of 1-2mm of length as a tissue fragment or otherwise called as primmorph is cultured in this process the mass of sponge ex-plant placed in the medium was doubled in four days and further growth is not observed due to addition of antibiotics such as penicillin and streptomycin to control the contamination which eventually affects on the primmorphs of sponges which subjected to cultivation. So only the primary cell culture of sponges was obtained. For future investigation of sponge culture, extra care must be given to the methods of cultivation and addition of antibiotics to develop Immortal sponge cell lines.

F. Discussion on Genomic Based Approach for sponge cell culture

Sponges are a rich source of bioactive compounds which have the potential to provide future medicines, such as new antibiotics and anticancer drugs (Blunt et al., 2009; Sipkema et al., 2005a). However, the limited availability of sponge biomass hampers the development of these potential drugs into commercial products. The use of *in vitro* sponge cell cultures is a potential alternative for biological supply of sponge-derived products, but until now a continuous sponge cell line has not been developed (Pomponi, 2006; De Caralt et al., 2007b; Schippers et al., 2012).This is primarily due to significant gaps in our understanding of sponge cell proliferation and death, nutritional requirements and cultivation conditions. Recent developments in the field of genomics and transcriptomics can help us to get more insight in sponge growth and death as well as nutritional requirements. Recently, the first genome of a sponge has been sequenced, and information about genes specific for multicellularity, such as control of cell proliferation and death, were revealed (Srivastava et al., 2010). These genes play a significant role in regulating cell growth, which is crucial for multicellular animals, since unrestricted growth of cells (= cancer) is detrimental for the other cells and the organism as a whole. For the development of a continuous cell line, the possibility to attain unrestricted growth is needed, per definition. Accordingly, most mammalian cell lines are derived from cancerous tissue or are actively immortalized, e.g., by inserting viral genes. The occurrence of cancer in an organism shows that cells of that organism can, in principle, be immortalized and the tumors themselves can serve as a source of immortal cells. Although many genes involved in the development of cancer are also present in sponges, cancerous growth in sponges has not been observed yet, neither *in situ* nor *in vitro.* The genes of interest discussed in this study focus on genes involved in cell proliferation, cell death and cell adhesion, since these genes play a significant role in maintaining homeostasis in multicellular animals. Deregulation of these genes can cause unlimited growth of cells (i.e. stimulation of cell proliferation in combination with prevention of apoptosis), which is essential for the development of a continuous cell line. Most sponge cultivation studies observe the change of biomass (e.g., volume, weight, cell numbers, protein content) over time and do not measure expression of certain genes and their related products. We suggest to study gene expression (tools to study gene expression are mentioned in box 1) in proliferating and non-proliferating conditions to obtain insight in the role of known genes involved in cell proliferation and cell death and find other genes of interest. Following conditions could be compared:

G. Larvae and gemmules vs. adult sponge.

Juveniles of larvae and gemmules have the potential to proliferate *in vitro,* whereas an adult sponge in general lacks this capacity. A

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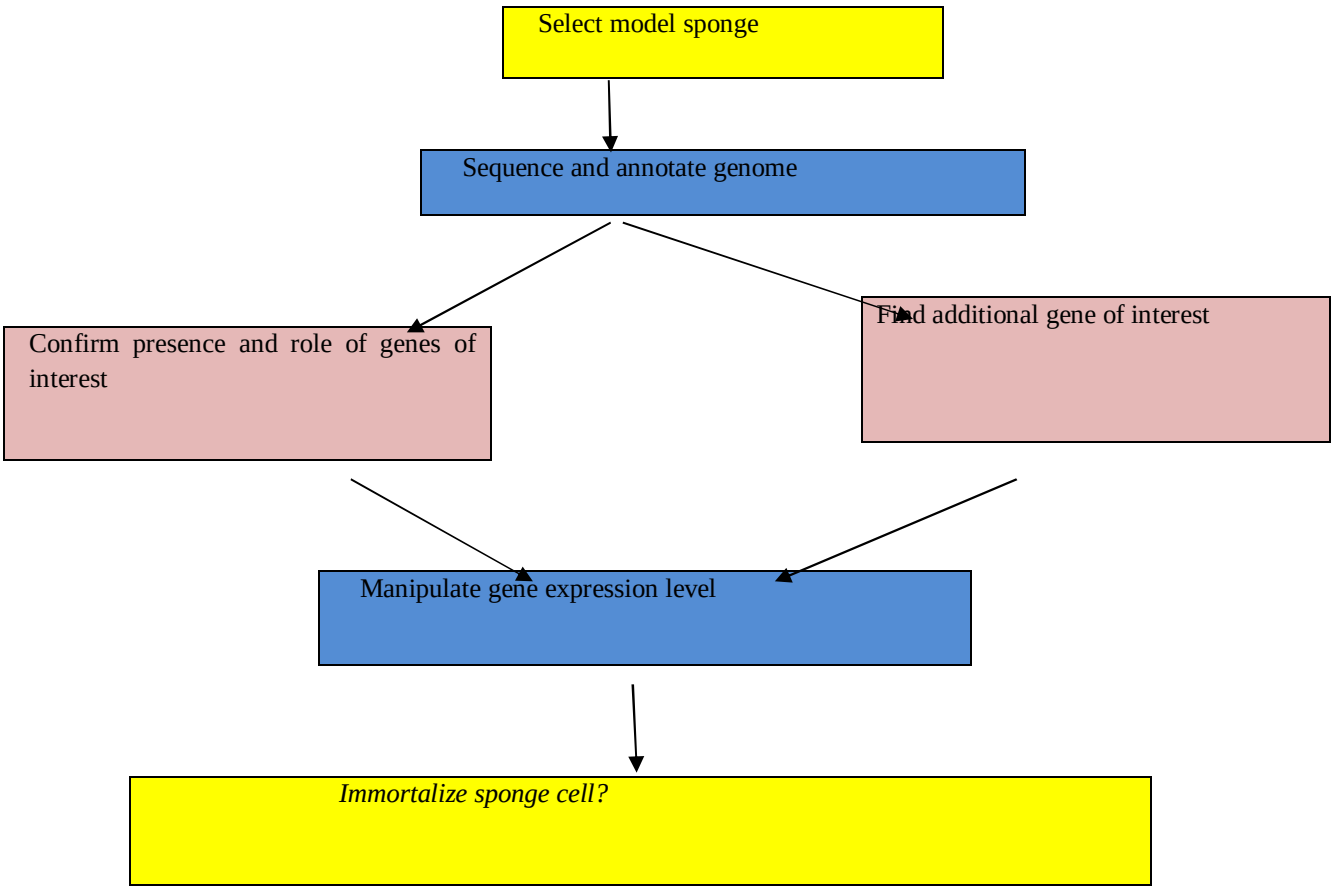
plausible explanation for this could be the large number of stem cells present in larvae and gemmules. Using sponge stem cells for cell culture has already been proposed by De Caralt et al. (2007b) and Rinkevich (2011). Since juveniles of larvae and gemmules are growing and developing into a sponge, genes related to cell proliferation are expected to be more actively expressed than in an adult sponge which is in homeostasis. By comparing gene expression profiles of juveniles with adult sponges, we can confirm if specific genes involved in cell proliferation are more actively expressed.

H. Primary sponge cell cultures vs. adult sponge.

In general, primary sponge cell cultures lack proliferative capacity. Previous studies already showed that dissociation of sponge cells resulted in increased caspase activity (Schippers et al., 2011) which is an indicator for apoptosis, and decreased telomerase activity (Koziol et al., 1998) which is a bio-marker for cell proliferation. Nevertheless, Pomponi et al. (1997) were able to demonstrate sponge cell growth when PHA was added to the growth medium. Subsequently Willoughby (2002) compared gene expression levels of sponge cells cultivated with and without PHA. She found that PHA effects expression levels of proliferative and anti-apoptotic genes in marine sponge cells. This research has not been followed up, but it would be of value to study the expression of the genes mentioned in primary sponge cell cultures stimulated with mitogens like PHA and to compare with gene expression in the adult sponge.

Another alternative is exposure of sponge cells to carcinogens or UV-radiation. Carcinogens are substances which can induce cancer, which is in most cases related to mutagenesis. Exposure to carcinogens can for example lead to increased oncogene expression and can therefore also be applied to transform cells. Examples of carcinogens which lead to immortal transformation in mammalian cells are UV-radiation (Wazer et al., 1994), heavy metals (Hamilton et al., 1998) and (Stampfer and Bartley, 1985). By exposing sponge cells to these substances, we can alter the activity of e.g. tumor suppressor genes and proto-oncogenes and possibly immortalize sponge cells.

Fig: 3.7 Proposed strategy of a genomic-based approach towards a sponge cell line.



In addition, for future study researchers can search for additional genes in sponges important for regulating cell proliferation and death by studying the transcriptome of proliferating sponge cells (e.g. larvae, gemmules, PHA stimulated sponge cells) in comparison with non-proliferating sponge cells (e.g. adult sponge, non-PHA stimulated sponge cells).

Finally, a promising method to develop a continuous sponge cell line is to insert immortalizing genes, such as SV40 LT together with TERT. To develop a continuous sponge cell line using immortalizing genes, the following prerequisites need to be met: metabolically active primary cells, a promoter that is recognized by the RNA polymerase of the sponge, effective DNA delivery method, functional immortalizing genes, and a method to obtain stable insertion of the genes (e.g. appropriate selectable marker for sponges.

I. Preparation of Methanol Extract of Sponge

The sponges collected are washed in deionised water and they are subjected to shade dry till they reach crispy condition then they are powdered in the blender. Now fifty (50 gm) gram of shade dried sponge powder is mixed with Methanol and homogenized. Keep the mixture at shaker at 100 rpm for 24 hours and then they are centrifuged at 12000 rpm for 10 minutes. Then the supernatant was filtered thorough Whatman No: 1 filter paper or syringe filter. The filtrate was dried to evaporate the solvents at room temperature. Te sediment extract i.e. pellet was weighed and dissolved in five percentage (5% DMSO), and refrigerated for future study

J. Qualitative Analysis of Active Metabolites from Sponge Extract

Terpenoids, steroids, Alkaloids, Saponins, Glycosides were screened from marine sponges by adopting following protocol

1) Terpenoid and steroid

a) Four milligram of the extract was treated with the0.5 ml of Acetic Anhydride and 0.5 ml of Chloroform.

b) Then concentrated solution was added slowly and red violet color was observed for terpenoid and green bluish color for steroids.

2) Alkaloid

a) The extract as evaporated to dryness and residue was heated with the boiling water bath with 2% hydrochloric acid.

b) After cooling the mixture was cooled and treated with few drops of Mayer's reagent.

c) The samples were then observed for the presence of turbidity or Yellow Precipitation.

3) Saponins

a) Frothing test was identified the presence of saponins.

b) 100ml of extract was added 5 ml distilled water.

c) Frothing Persistence indicated the presence of positive result ie.the presence of Saponin.

4) Glycoside

a) To the solution of the extract glacial acetic acid is added few drops of ferric chloride and concentrated sulphuric acid.

b) Observed for red brown colorations at the junctions of the tube and bluish green color in the upper layer.

K. Preparation for antibacterial sensitivity test

A cross- streak method was used for antimicrobial activity .Single streak of was made on surface of the modified Muller- Hinton Agar Plates Supplemented with 2% of NaCl and incubated at 28 °C. The pathogenic bacterial strains, such as E. coli, H. influenzae, Klebsiella pneumoniae, Enterococcus faecalis which were streaked and incubated at 28 °C and the incubation distance was measured after 24-48 hrs. A control plate was also maintained without inoculating the microbes to assess the normal growth of bacteria.

L. Antibacterial Assay

Antibacterial activity was determined against E. coli, K. pneumoniae, H. influenzaes , E. faecalis, using the paper disk assay method (12).Whatman No. 1 filter paper disk of 6-mm diameter was sterilized by autoclaving for 15 min at 120 0C. The sterile disks were impregnated with different extracts (500 Ag/ ml).Agar plates were surface inoculated uniformly from the broth culture of the tested

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microorganisms. In all cases, the concentration was approximately 1.2X108 CFU/ml. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37 0C for 24 h. Disk of Streptomycin (400 Ag/ml) was used as a posiive control. The diameter (mm) of the Zone of inhibition (halos) caused by the methanolic extracts of sponges was examined.

Table 3: shows the measurement of zone of inhibition i.e., Antibacterial activity of methanolic extract of different marine sponges against the different disease causing pathogenic bacteria

NAME OF THE SPONGE	Name of the tested micro-organisms (Zone of Inhibition in Diameters mm)			
	E.Coli	H.influenzae	K.Pneumoniae	E.faecalis
Clathria gorgonoides	5±2.58	_	10±1.91*	9±4.11
Sigmodocia petrosioides	9±0.36	8±1.49	12±0.09*	6.3±2.6
Haliclona fibulata	_	11±1.49*	3.41±0.21	13±3.68*
Suberitus carnosus	3.65±0.6	5±0.02	11±0.50*	_

(-) no inhibition of growth during testing Asterisks (*) indicates the values which are significantly different

Fig: 4.1

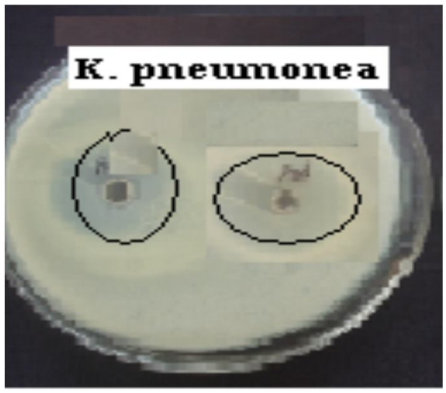

Fig: 4.2

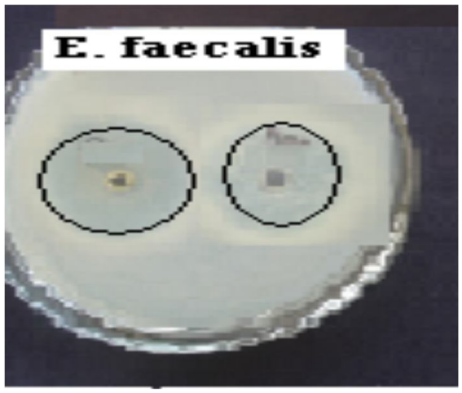

Fig 4.1 and 4.2 shows the Secondary screening of antibacterial activity of the Marine sponges against the various Bacterial Pathogens

The Present study clearly shows that the maximum peak of activity was noted on two sponge species such as Sigmodocia petrosioides and Haliclona fibulata crude extract showed the peak activity 12±0.09 and 13±3.68 bioactivity against the pathogen of K.Pneumoniae and E.faecalis respectively.Subsequently another maximum zone of inhibition (11±0.50) formed with the sponge Suberitus carnosus extract on K. pneumoniae. The present result denoted on both sponge extract antimicrobial activity at 5% level of statistically significant.

Research has indicated that the secondary metabolites of sponges play an important role in their defense against infectious pathogens. This systematic investigation of marine environments is reflected in the large number of novel compounds especially reported in the literature over the past decade. Marine natural products especially sponges could yield new drugs to cure the severe diseases. The quest for drugs from the sea has yielded an impressive list of natural products mostly from invertebrates such as sponges that are either in the late stages of clinical trials, or have already entered the market. Some of the Sponge derived bioactive compounds presently available in the market are Ara-A (antiviral), Ara-C (anticancer) and Manoalide (phospholipase A2 inhibitor), while IPL512602 (anti-inflammatory), KRN 7000 (anticancer), LAF389 (anticancer), Discodermolide (anticancer) and HTI286 (anticancer) are under clinical trial. Besides their pharmaceutical potential, sponges are an important to explain classification patterns and phylogenetic relationships. During the year 1998 Hattori demonstrated new ceramide compound from marine sponge. Possible Biogenetic Relevance with Manzamine portrayed novel Lipid contents of the sponge *Haliclona* sp. Sponges produce a wide array of secondary metabolites ranging from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides and sterols.

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M. Antibacterial Analysis

Marine sponges are potential sources of unique bioactive metabolites and many of these compounds are valuable for medicinal uses. The present study was also the part of the project, undertaken to assess the potential antibacterial spectrum of various marine sponges against pathogenic organisms. The evaluation of wide array of marine sponges for potential use in chemotherapy poses problems of procurement of materials and of techniques of screening for significant drug activity. From the present result clearly expressed the maximum peak antibacterial

Activity was noted on two sponges such as *Sigmodocia petrosioides* and *Haliclona fibulata* crude extract showed that the peak activity bioactivity against12±0.09 and 13±3.68 the pathogens of *K.Pneumoniae* and *E.faecalis* respectively. Subsequently another maximum zone of inhibition (11±0.50) formed with the sponge *Suberitus carnosus* extract on *K. pneumoniae*. In this study also concluded the dominant bioactivity denotes on the marine sponge *Haliclona fibulata* possessed an important Antimicrobial compound such as 2-Methoxy-1, 4-Benzenediol identified through a GC-MS analysis. Hence, the present results profounded the promising antibacterial effect on Four active marine sponges against pathogenic strains so this study concluded all the four marine sponges possessed excellent source of antibacterial bioactive compound thus they act as a excellent peculiar antibacterial

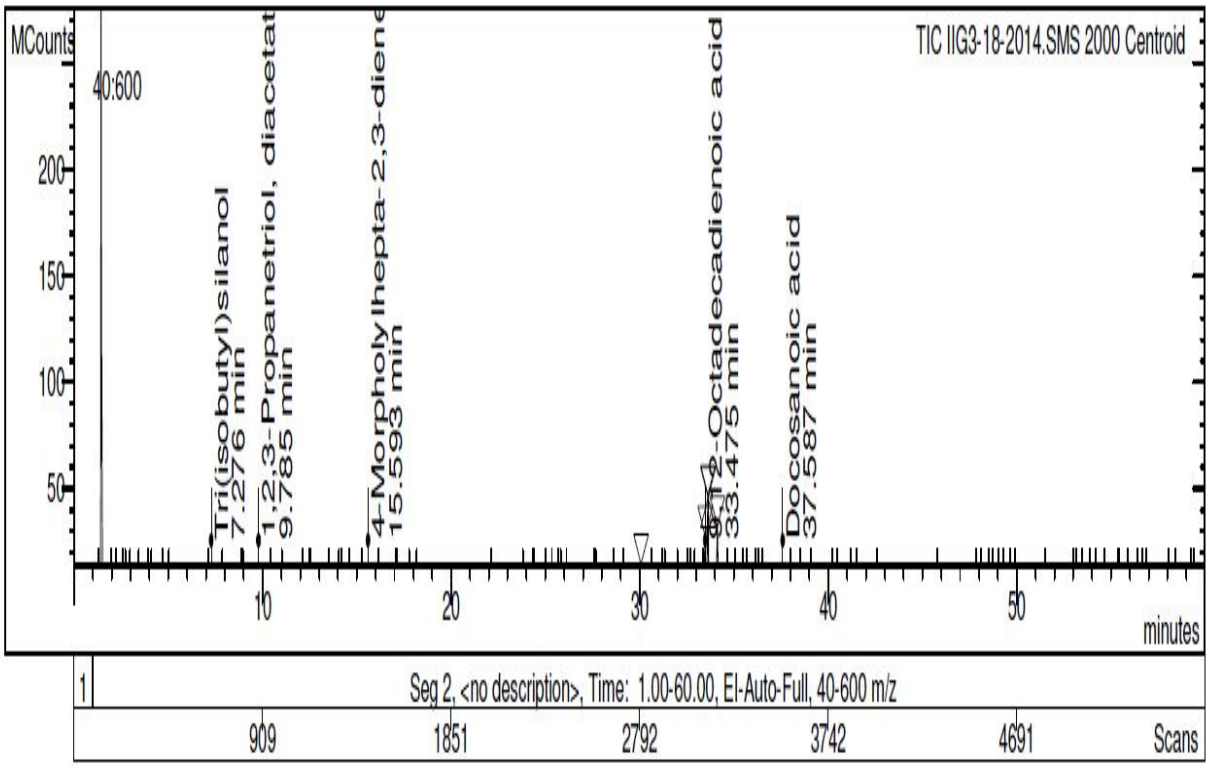

Fig: 4.3 GCMS spectrum of Methanol extract of Haliclona fibulata

VI. SUMMARY

In summary, although continuously proliferating cultures from immortalized sponge stem cells have not yet been developed, the information accumulated from sponge biology, cell requirements and genetic techniques offer good prospects for future developments in this field. However, once a continuous sponge cell culture is obtained, the question remains as to whether the cultured cells will continue to produce the bioactive molecules necessary for new medicines. The success of cell culture or tissue culture is species-dependent. Cell cultures and tissue cultures of Clathria gorgonoides were all unsuccessful, probably caused by the release of toxins, while tissue culture of Dysidea herbacea was successful. For the studied sponges in vitro tissue culture did not result in better cell proliferation and viability than cells in primary suspension culture, as was hypothesized. For Dysidea herbacea in vitro tissue culture was more favorable, which is probably caused by the constant supply of nutrients, dissolved oxygen and flushing away of waste products. This holds potential for development of primary cell cultures from these sponges. The cultured explants are

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subjected under several experimental conditions are studied to find the best conditions for obtaining functional explants, I assayed a range of conditions, including seasons of collection, culture temperature, filtered versus filtered-sterile seawater, addition of antibiotics. I monitored the changes in shape and ultra structure during the formation of explants. From 2 to 4 weeks later, the aquriferous system regenerated, and fragments became functional sponges (explants). Antibiotics were only added to the in vitro culture to assess their effect on the symbiotic bacteria, which remained healthy despite the presence of antibiotics. Two food requirements of sponges (marine bacteria and green algae) were assayed for their ability to satisfy the metabolic requirements of explants. I monitored explants survival and growth. Explants showed a high long-term survival rate (close to 100%). Growth rates were higher in the closed aquarium system, without antibiotic addition, and fed with algae. Explants cultures were hardly contaminated because manipulation was reduced to a minimum and by using sterilized seawater.

VII. CONCLUSION

The cultured sponge biomass produces bioactive molecules, which may play a defensive role in the sponge and may have pharmaceutical interest. The bioactivity of the explants was not similar to that of wild sponges i.e., same like that in the marine environment from where they are taken, because sponges are not encountered with any predators during laboratory culture processes. But in the natural marine environment due to attack of various external agents it produces bioactive compounds of higher degree it cannot be replicated in the in vitro culture process. Sponges and sponge symbionts produce numerous unique metabolites of potential commercial value. Producing many of these metabolites would require large quantities of sponge biomass that cannot be sustainably harvested from natural populations. Production of cultivated sponge biomass from sea-based farms is feasible, but productivity is variable. Biomass production in controlled environments of aquariums has the potential to provide consistent yields, but many aspects of aquarium cultivation remain unknown for most sponges. Culture of sponge cells and, more likely, primmorphs can become a future source of metabolites; however, cell and primmorph cultures are not feasible at present for producing large amounts of biomass. Major questions remain concerning the production of sponge-sourced bioactives, so, can methods be developed for culturing healthy sponge without its endosymbionts? Can endosymbiotic bacteria be cultured in the absence of live sponge tissue and cells, to produce metabolites of interest? Studies are needed of sponge nutrition and how nutrition can influence growth and metabolite production. What might be the influence of precursor feeding? All these and many other questions remain to be answered.

ANNEXURE 1

Fig:5.1 Dysidea herbacea:

Fig:5.2 Haliclona fibulata

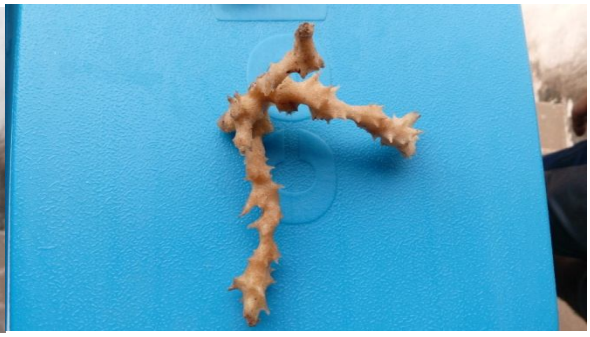

Fig:5.3 Sigmodocia petrosioides

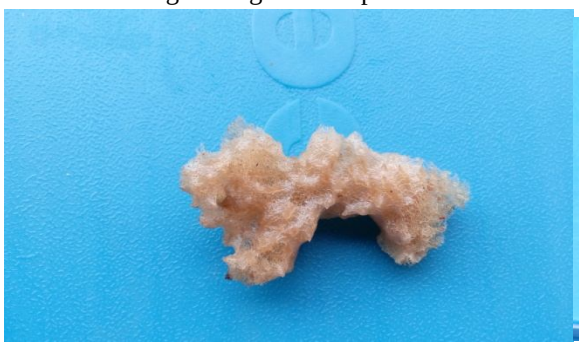

Fig:5.4 Stellitetheya repens

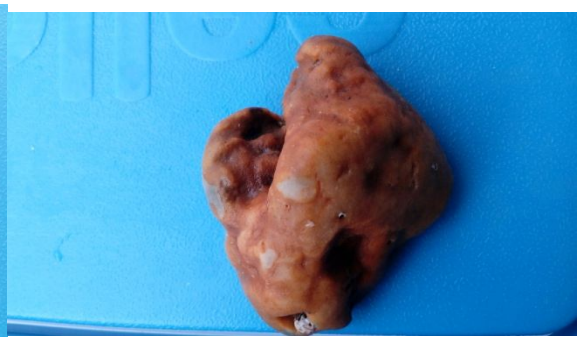

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Fig: 5.5 Hyattella intestinalis

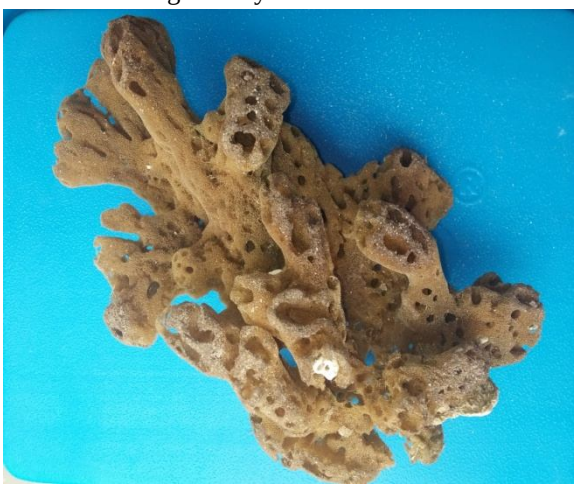

Fig: 5.6 Gellioides pumila

Fig: 5.7 *Suberitus carnosus*

Fig: 5.8 *Clathria gorgonoides*

ANNEXURE 2

Fig: 6.1

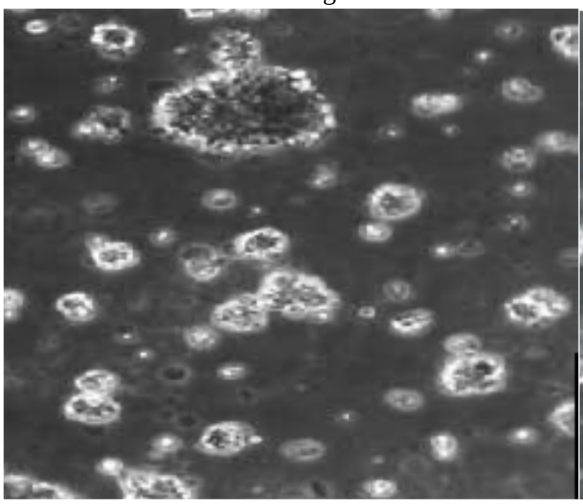

Fig:6.2

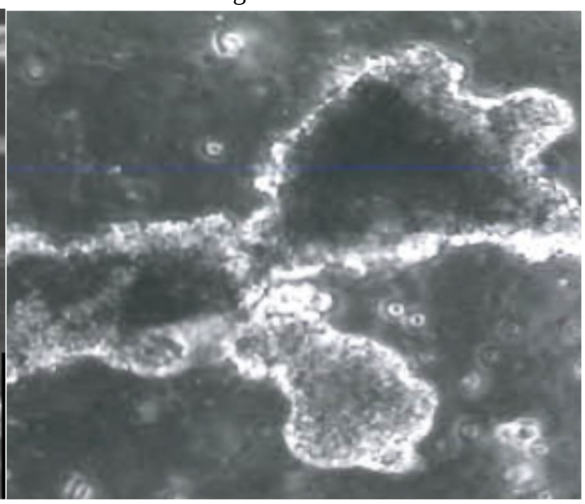

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Fig:6.3

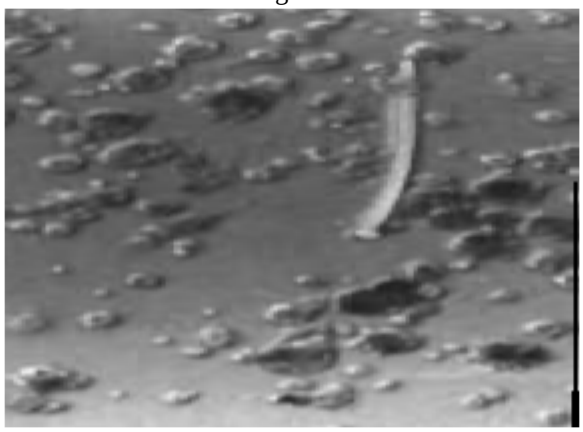
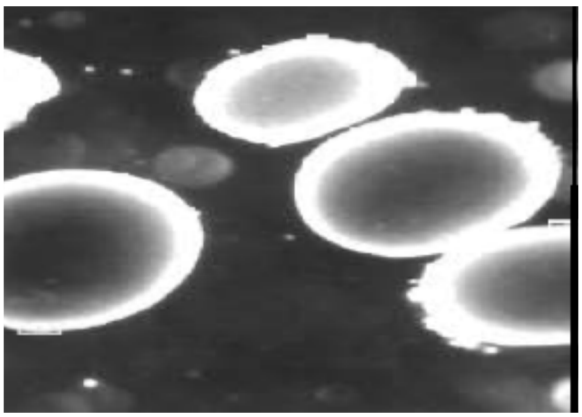


Fig:6.4



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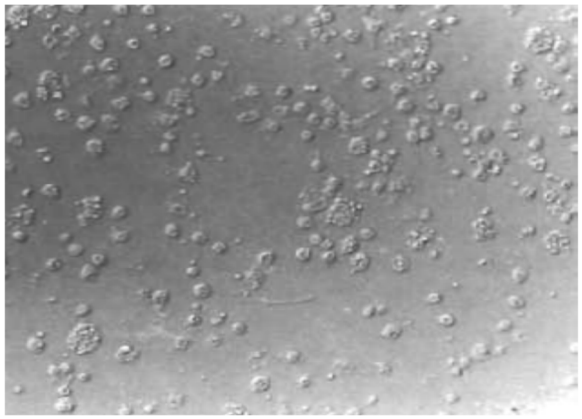
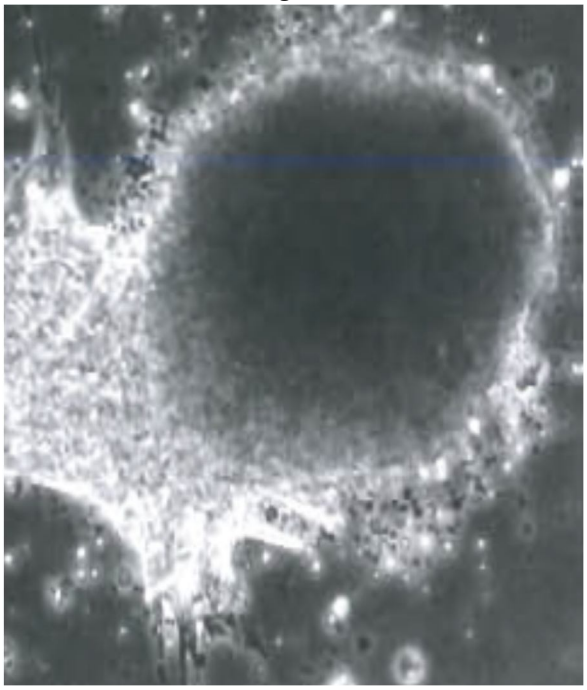


Fig:6.6



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ANNEXURE 3

Fig:7.1

Fig:7.2

ANNEXURE 4

Fig: 8.1

Figs: 8.2

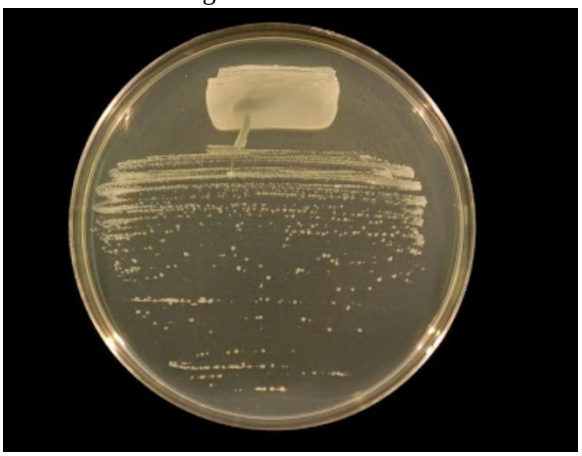

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Fig: 8.3

Fig: 8.4

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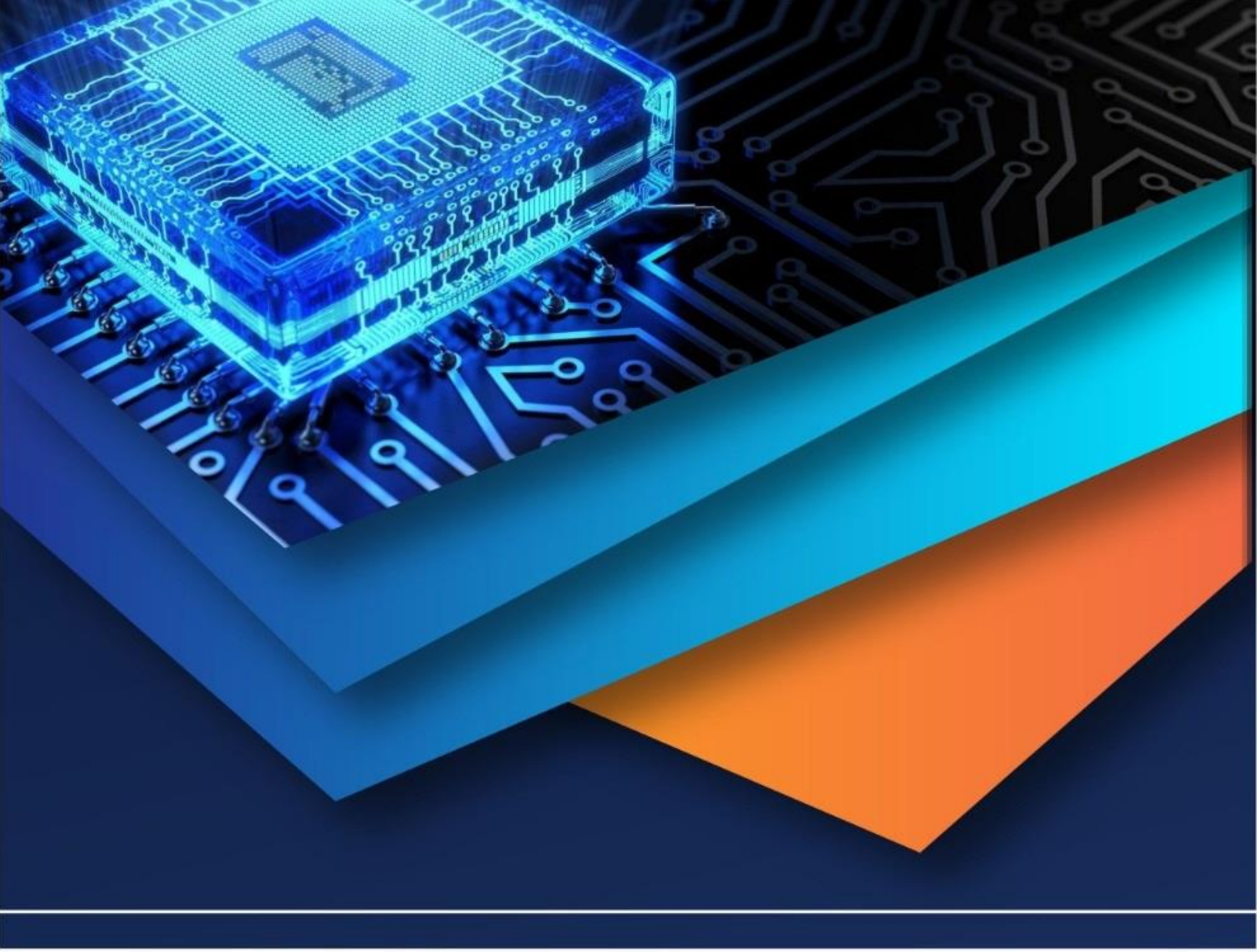

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