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RESEARCH ARTICLE

BIO DEGRADATION AND CYTOTOXIC POTENTIAL OF BIOSURFACTANT FROM MARINE BACTERIA ASSOCIATED WITH ALGAE *ULVA LACTUCA*.

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Abstract

Background: The use of biosurfactants to protect the marine environment seems possible since a number of marine bacterial strains can produce biosurfactants during growth on hydrocarbons (Bertrand *et al.*, 1993). For the sake of the environment, the use of biosurfactants is preferable to those of synthetic surfactants. However, little information on either biosurfactant produced by marine microorganisms or biosurfactants active in saline has been reported so far.

Objective: The isolated colonies were identified, potential biosurfactant producer was screened out. Biosurfactant extraction was done by lowering pH using 5M HCl followed by the detection and purification. The extracted biosurfactants were also used to study the effect on metal removal and anticancer activities.

Materials and method: The antibacterial activity was carried out by disc diffusion method, screening of potential biosurfactant producers by hemolytic assay, drop collapsing test, oil displacement test and emulsification activity, detected and purified by silica gel plate (TLC). Anticancer activity was done by *Dimethyl thiazolyl diphenyl tetrazolium bromide*, (MTT). Effects on metal removal were studies using the media with salts of CrSO₄ and ZnSO₄.

Result: Antimicrobial activity of SP2 showed highest activity against *Pseudomonas aeruginosa* (1cm) and *Bacillus megaterium* (2.2cm). Species 2 showed highest activity in the screening methods. TLC showed the presence of lipopeptide and rhamnolipid. The biodegradation using same concentration of bio surfactants produced by SP2, increased with increased concentration of the salts. SP2 microbial extracts showed cytotoxic activity in L929 cell line. These findings suggest that the identified sponges are source of pharmaceutical important compounds.

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Introduction:-

The number of natural products, discovered from several organisms that include plants, animals, and microorganisms, overcomes millions of compounds. Forty to sixty percent derives from terrestrial plants, from

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which twenty to twenty-five possess bioactive properties such as antibacterial, antiviral, anticancer and anti-inflammatory activity (Berdy, 2005). Bio surfactants are categorized mainly by their chemical composition and microbial origin. Major classification includes glycolipids, phospholipids, fatty acids, polymeric bio surfactants and particulate bio surfactants (Desai and Banat, 1997). They have many advantages, such as biodegradability, low toxicity (Poremba *et al.*, 1991) and environmental compatibility (Georgiou *et al.*, 1990). The use of biosurfactants to protect the marine environment seems possible since a number of marine bacterial strains can produce biosurfactants during growth on hydrocarbons (Bertrand *et al.*, 1993). They have the same physical and chemical properties as the synthetic surfactants. However, little information on either biosurfactant produced by marine microorganisms or biosurfactants active in saline has been reported so far. This article aims to provide an overview on the production of biosurfactants by marine microorganisms and to discuss their potential use in bioremediation.

Materials And Methods:-

Collection of algae:-

The algae were collected from Muttom Beach, Kanyakumari District, Tamilnadu. They were found attached to the rocks. Unwanted components were removed from the algae by washing with sea water and then preserved in refrigerator to carry out further studies.

Isolation and Enrichment of Bacterial Species from Marine Algae:-

Frozen algae were cut into small pieces. Mixed colonies of bacterial species were isolated from the algae by growing one of the small pieces of algae in Zobell Marine Broth for 24-48hrs at 37°C. The bacterial colonies were then isolated by spread plate technique (Shirling and Gottlieb, 1966). Characterization of bacterial strains was performed based on Bergey's manual of determinative bacteriology (Bergey *et al.*, 1994).

Screening Methods for Potential Biosurfactant Producers:-

The potential biosurfactant producer was screened by different method such as Hemolytic assay (Carillo *et al.*, 1996), Drop collapsing test by Bodour and Maier (1998) and Oil displacement test (Morikawa *et al.*, 1992). Maximum biosurfactant producing marine bacterial species was maintained on Zobell Marine Agar medium for further study. Those species with highest activity is selected for further studies.

Extraction of Biosurfactant:-

The extraction procedure was carried out based on the method described by (Kingsley *et al.*, 2004). After 96 hrs cultivation, each culture was centrifuged at 8000 rpm, 4°C for 10 min to harvest the cells. The culture supernatant was taken and pH of the culture supernatant was lowered to 2 with 5M HCl and incubating at 4°C for 24 hrs. The precipitate was separated by centrifugation at 8000 rpm for 20min. White precipitate formed culture was selected for further experimentation

Detection and Purification of Bioactive Bio surfactant:-

Thin layer chromatography was carried based on the procedure described by Rashedi (2005), using silica gel plate. A small quantity of crude extract was dissolved in chloroform: methanol (15:35 v/v). 10µl of the crude extract was added onto a TLC plate (silica gel 60) and apply at point of origin near the bottom of the plate. Once dried, develop plate in solvent system of chloroform: methanol: water (65:25:4). When developed remove plate and allow air drying in a fume cupboard. Spray the plate evenly with water or a solution of 5% sulphuric acid and place in an oven at 110°C for 20 min to visualize spots. At preparative scale, remove lipopeptide spots by scraping the silica from plate into a flask. The extract was dissolved in chloroform: methanol (2:1), incubated overnight.

Effect of Bio surfactant on Metal Removal:-

The extracted bio surfactants were used for the removal of metal such as chromium and zinc ions. The nutrient broth medium containing the salts of Chromium sulphate and Zinc sulphate was prepared and sterilized. The salts of Chromium and Zinc were added to the medium at a concentration of 60mg, 80mg, 100mg at 50mL of broth respectively. The pH of the medium was adjusted to 7 and sterilized in an autoclave at 15Lbs pressure for 15 minute. Then the extracted bio surfactants (about 50µL) was inoculated into the medium and incubated for 24hrs, 48hrs, 72hrs at 30°C to study the degradation effects. The tubes were analyzed for the concentration of metals present after treatment using a spectrophotometer. The absorbance can be read at 650nm (Perez *et al.*, 2007)

Antimicrobial Activity:-

The crude bio surfactant was tested for antimicrobial activity using Kirby Bauer disc diffusion method (Bauer *et al.*, 1996). The filter paper disc of uniform size 6mm were impregnated with the bioactive compound and then placed on the surface of the agar plate seeded with the microorganism to be tested. All the plate culture was incubated in an inverted position at 26-28°C for 24-48 hrs. The disc which showed maximum zone of inhibition was selected for further studies.

Invitro cytotoxic assay:-

MCF cells were purchased from NCCS Pune was maintained in Dulbecco's modified eagle's media (HIMEDIA) and grown to confluency at 37°C and 5% CO₂ in a humidified atmosphere in a CO₂ incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ EDTA solution) for 2 minutes and passaged to T flasks in complete aseptic condition. Extracts were added to grown cells at a concentration of 100µg, 500µg and 1000µg from a stock of 100mg/ml and incubated for 24 hours.

MTT assay:-

MTT assay was performed based on the method followed by (Arung *et al.*, 2000). MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilised formazan product was measured at 540nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

The cell culture suspension was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT - 5mg/ml dissolved in PBS). It was then incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 200µl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. OD was read at 540 nm using DMSO as blank.

Results:-**Isolation and Identification of Marine Bacteria associated with Ulva lactuca:-**

Four individual marine bacteria have been isolated by Zobell Marine Agar and were named as SP1, SP2, SP3 and SP4. The marine bacteria were identified as *Bacillus* species 1, *Bacillus* species 2, *Pseudomonas* species and *Staphylococcus* species based on Bergy's manual of determinative bacteriology (Bergey *et al.*, 2004).

Screening of biosurfactant:-

Hemolytic test was done for all the four biosurfactants produced from marine bacteria. The highest activity is shown by SP2 which is represented by a clear zone around the colony. In Drop collapsing test, the culture supernatant was added to the surface of oil droplet placed in a 96 well micro titer plate lid. The flat drops shown by the bio surfactant producing culture were scored as positive. More positive results were shown in vegetable oil and petrol by SP1 and SP2 (TABLE-1). Oil biodegradation activity was checked by oil displacement test. Of the four-culture species SP1, SP3, SP4 showed degradation against vegetable oil. SP2, SP3, SP4 showed degradation against kerosene. SP3 and SP4 showed degradation against petrol. SP1, SP3, SP4 shows degradation against diesel (TABLE-1).

Table 1:- Screening By Drop Collapsing And Oil Displacement Test.

Isolates	Drop collapsing test				Oil displacement test (cm)			
	Vegetable oil	Kerosene	Petrol	Diesel	Vegetable oil	Kerosene	Petrol	Diesel
SP1	+	+	+	-	1.3	-	-	1.2
SP2	+	-	+	+	-	1.5	1.9	-
SP3	+	-	-	-	1.5	2.1	1.3	1.6
SP4	+	+	-	-	1.4	1.6	1.5	1.8

Screening of antimicrobial activity:-

The antimicrobial activity was done against pathogenic bacteria *Salmonella typhi* (MTCC-733), *Klebsiella pneumoniae* (MTCC-4030), *Enterobacter aerogenes* (MTCC-111), *Mycobacterium tuberculosis* (MTCC-300), *Streptococcus pyogenes* (MTCC-928), *Pseudomonas aeruginosa* (MTCC-4676), *Bacillus megaterium* (MTCC-453). SP1 showed activity against *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, and *Bacillus megaterium*. SP2 showed high activity against *Pseudomonas aeruginosa* and *Bacillus megaterium*. SP3 showed high activity against *Mycobacterium tuberculosis* and also have activity against *Salmonella typhi*, *Streptococcus pyogenes* and *Bacillus megaterium*. SP4 showed activity against *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Bacillus megaterium* (GRAPH-1)

Graph 1:- Antimicrobial activity of biosurfactants isolated from the algae.**Separation of Biosurfactant:-****Thin Layer Chromatography:-**

The biosurfactant produced by SP2 were characterized by using TLC plates. The sediments obtained was placed in the TLC plate and the plates when sprayed with 5% sulphuric acid reagent it showed pinkish red spots and yellowish spots in the plates. This shows the lipopeptide and rhamnolipid biosurfactants production by the organism (TABLE-2)

RF Value =

Table 2:- rf value of the compounds separated by tlc.

Species	Rf value		Name of compound
SP1	Pink	0.93	Rhamnolipid
	yellow	0.73	Lipopeptide
SP2	Pink	0.86	Rhamnolipid
	Yellow	0.76	Lipopeptide
SP3	Pink	0.95	Rhamnolipid
	Yellow	0.75	Lipopeptide
SP4	Pink	0.91	Rhamnolipid
	Yellow	0.82	Rhamnolipid

Effect of Biosurfactant on Metal Removal:-

The biodegradation of heavy metal salts ZnSO_4 and CrSO_4 using same concentration of biosurfactants produced by SP2 and different concentration of salts at pH 7 revealed that there was significant degradation of the heavy metals by the surfactant. The degradation increased with the increased concentration of the salts which is illustrated in the following tables (TABLE-3)

Table 3:- Efficiency Of Biosurfactant On Reduction Of Zinc And Chromium.

Concentration of ZnSO_4 (mg)	OD at 650nm				Concentration of CrSO_4 (mg)	OD at 650nm			
	0 hrs	24 hrs	48 hrs	72 hrs		0 hrs	24 hrs	48 hrs	72 hrs
60	0.172	0.143	0.121	0.101	60	0.013	0.018	0.041	0.245
80	0.172	0.148	0.111	0.087	80	0.018	0.029	0.170	0.576
100	0.211	0.011	0.009	0.005	100	0.029	0.035	0.219	0.707

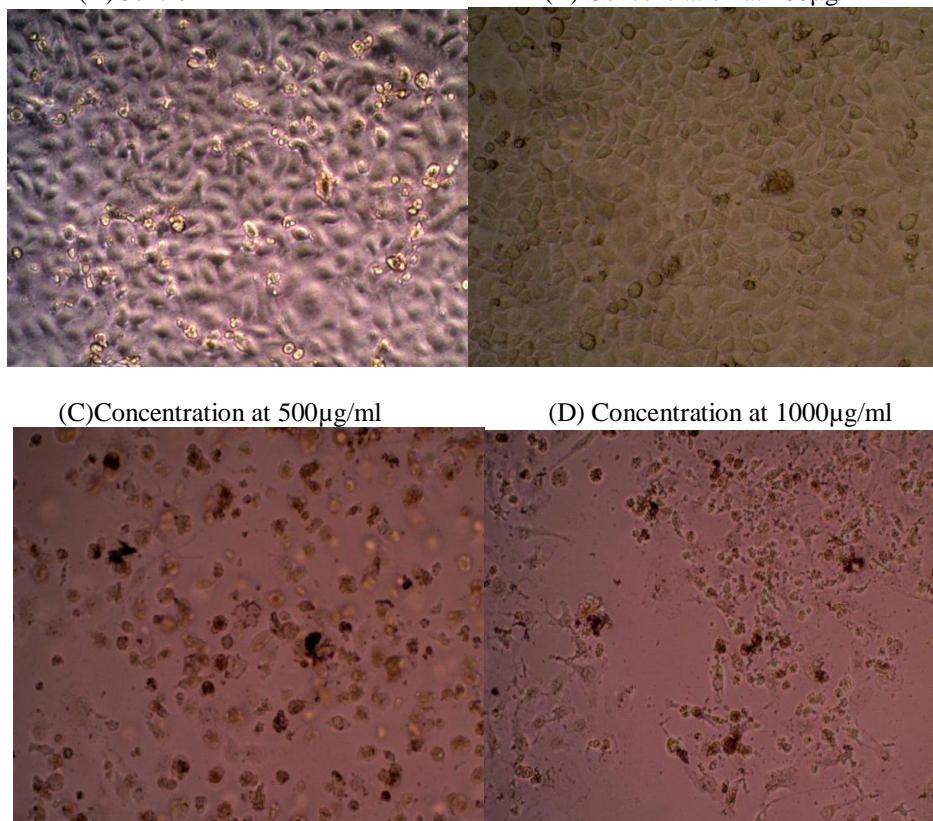
Anticancer activity of bioactive compound produced from algae on MCF cell line:-

The cytotoxic effect of SP2 microbial extract on MCF (Breast cancer) cell line was tested by MTT cell viability assay. The percentage of viability of MCF cell line by 1000 μg of microbial extract showed 54.9% viable cell. Results were showed in (TABLE-4, PLATE-1)

Table 4:- Anticancer Activity Against Cell Line MCF.

Sample concentration	OD at 540nm	% viability
Control	1.2	100
100 $\mu\text{g}/\text{ml}$	0.7844	65.36
500 $\mu\text{g}/\text{ml}$	0.6840	57.0
1000 $\mu\text{g}/\text{ml}$	0.6588	54.9

Plate 1:- Anticancer Activity Of Microbial Extract On Mcf Cell Line.
 (A)Control (B) Concentration at 100µg/ml



Discussion:-

During the last decade, they have been under investigation as potential replacements for synthetic surfactants and are expected to have many potential industrial and environmental applications (Banat *et al.*, 2000). Among them lipopeptides represent a class of microbial surfactants with increasing scientific therapeutic and biotechnological interests. Currently there is a limited offer of commercially available biosurfactants e.g., surfactin, sophorolipids and rhamnolipids. A variety of new biosurfactants respectively producing strains are the key issue in overcoming the economic obstacles of the production of biosurfactants. Therefore, increased efforts in the discovery of new biosurfactant producing microbes must be made by applying a broad range of different screening methods.

Biosurfactants were first discovered as extracellular amphiphilic compounds of fermentation bacteria (Kitamoto *et al.*, 2009). Initially they were seen interesting due to their ability to increase solubility of insoluble or poorly soluble hydrocarbons. However, the more and more popular trend of using renewable resources in industry (especially in food and pharmaceutical industries) have led to relentless interesting in gaining and application of natural surfactants, mainly biosurfactants. Biological degradation of light crude dispersed in sea water by a surfactant produced by a hydrocarbon degrading microorganism has been monitored in laboratory tests and it showed that oil dispersed by a biosurfactin was more easily degraded than chemically dispersed oil (Francesco Crescenzi *et al.*, 1971) Many bacterial strains have been isolated from coastal and oceanic environments; these bacteria, including the genera *Pseudomonas*, *Vibrio*, and *Flavobacterium*, has been considered to be representative of marine bacteria (Harayama *et al.*, 2004). In this work, four different species of microorganisms have been isolated namely *Bacillus sp. 1 and 2*, *Pseudomonas* and *Staphylococcus*.

In the past few decades, biosurfactants have gained attention because they exhibit some advantages over chemically synthesized surfactants, such advantages include bio degradability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates and effectiveness at extreme temperature and pH values (Cameotra and Makkar., 1998). The range of industrial applications of biosurfactants includes enhanced oil recovery, crude oil drilling, lubricants, bio remediation of pollutants, health care and food processing. The

antibacterial, antifungal and antiviral activities of biosurfactants make them relevant molecules for applications in combating many diseases and as therapeutic agents (Rodrigues *et al.*, 2006).

Biological methods for the removal of heavy metals from industrial waste may provide an attractive alternative to the physico-chemical process; bio surfactants are one of the compounds that aid in alleviating the heavy metals. Several microbes such as *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *arthrobacter* sp., are reported to carry out the process of bioremediation. In this work, it has been proved that the lipopeptide bio surfactants isolated from the fungal species are also capable of degrading heavy metals like chromium and zinc salts. Hemolysis may occur in vivo or in vitro (inside/outside the body). In vivo hemolysis can be caused by a large number of medical conditions including many gram-positive bacteria (e.g., *Streptococcus*, *Enterococcus* and *Staphylococcus*). There are at present several methods for the detection of hemolytic activity (Arimi *et al.*, 1990; Fricker *et al* 1985). It is the preliminary test for anticancer activity studies. Anticancer activity of the bioactive compound from algae had been performed which results in 54.9% viability in 1000g/ml concentration of SP2 microbial extract. This shows that the compound produced by SP2 cause destruction of half of the total viable cells. The results of the present study thus suggest that isolated biosurfactant may prove to be a promising agent and requires further investigation of its potential cytotoxic activity.

Conclusion:-

Biodiversity of marine algae and its associated microorganisms have been reported to produce several pharmaceutically and industrially important compounds. This work has been done concentrating mainly on the antimicrobial screening and heavy metal degradation of the lipopeptide bio surfactant. It was found that the bacterial species isolated from marine algae *Ulva lactuca* have antimicrobial activity. It is thus concluded that the algal associated bacterial strains are a good source for the treatment of infectious diseases and also it is a good candidate for the treatment of breast cancer (MCF cell line). It was also confirmed that the bio surfactant produced from the antagonistic bacterial strains are a good vector for bioremediation. In future, these properties can lead to the production of novel drugs for the treatment of various diseases including cancer treatment.

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