COMPARATIVE SCREENING OF DIFFERENT MINERAL SALT MEDIA FOR BIOSURFACTANT PRODUCTION BY HALOPHILIC ORGANISMS

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Abstract: Biosurfactants produced by microorganisms, are amphipathic surface-active molecules containing hydrophilic and hydrophobic moieties that act by emulsifying hydrocarbons increasing their solubilisation and subsequently rendering them available for microbial degradation. A variety of microorganisms are capable of producing biosurfactants, but halophilic organisms deserve special attention as the biosurfactants obtained from halophilic organisms are capable of sustaining in saline, hypersaline and varied conditions of temperature and pH. In this research, an attempt was made to screen different Mineral Salt Media to produce biosurfactant by halophilic organisms. Halophiles are living organisms capable of growth in saline environment. Different versions of Mineral Salt Media such as Mukherjee media, Makkar and Cameotra media, modified indigenous media, inorganic salt media and modified M9 media were studied and modifications were made with respect to carbon and nitrogen sources along with different inducers such as kerosene and diesel. Following inoculation with different halophilic isolates and incubation in different media for one week, it was observed that inorganic salt media, modified indigenous and M9 media proved to give better results of emulsification test as compared to the other media studied. Out of different carbon and nitrogen sources tested, molasses proved to be a relatively better carbon source and ammonium chloride, ammonium nitrate proved to be relatively better nitrogen sources. Inducer such as kerosene enhanced production of biosurfactant.

Index Terms: Biosurfactant, halophiles, inducer, molasses.

I.INTRODUCTION:

Surfactants are the compounds that lower the surface tension between two liquids that differ in their chemical nature. There are many chemically synthesized surfactants available that are being used in removal of chemical pollutants and other industrial purposes such as microbial enhanced oil recovery (MEOR), but the chemically derived surfactants are accompanied by problems of toxicity and long persistence in nature. Problems associated with chemical derivatives of surfactant compounds leads to a need for an alternative, promising and eco-friendly agent such as biosurfactant.

Biosurfactants are secondary metabolites,surface active agentsproduced by a diverse group of living organisms. They have the ability to reduce surface tension in both aqueous and hydrocarbon mixtures (Banat,1995;Desai,1997;Mulligan,2005;Nitschke,2006;Yin and Qiang,2008 and Fang *et al*., 2007). Biosurfactants have the ability to aggregate at the interface between fluids which have different polarities such as water and oil, eventually leading to reduction in interfacial tension. Primarily they have been classified into different groups based on their chemical composition and microbial origins such as glycolipids, lipopeptides, phospholipids, polymeric compounds and neutral lipids(Ron and Rosenberg, 2001;Sen, 2010). Many microorganisms from diverse groups have been reported(Shoham*et al.*, 1983) to produce biosurfactants, but the biosurfactants derived from halophilic or halotolerant organisms deserve special attention, because they possess significant features of being able to work under saline, hypersaline,

higher temperatures and pH values. These conditions are characteristics of a general industrial process where conditions are predominantly on the higher side of the scale.
Organisms producing biosurfactants, can be isolated from different saline habitats and enriched in selective and controlled conditions. Different Mineral Salt Media have been reported in the scientific literature(Silva *et al*., 2010; Zajic, 1972) but the optimum media will be the one, which is suitable for the production and its choice will be based upon factors such as source of organisms, carbon and nitrogen sources, temperature and pH.

II.MATERIALS AND METHODS:

A) Collection of samples:

Saline water and soil samples were collected from saline lake and from the oil contaminated greasy soil near the wooden tracks used for transport of trollies carrying crude salt for processing, at Sambhar saline lake, Rajasthan, India. Samples were collected in sterile screw capped bottles, petri-plates and test tubes and bought to the Laboratory.

B) Enrichment and Isolation of Halophilic organisms:

Samples were subjected to enrichment in different media such assterile Sehgal and Gibbons broth, sterile Halophilic broth at 37^0C for one week and sterile Sabouraud's broth at room temperature for one week.

The enrichment of the samples for bacterial isolates was done in sterile Halophilic broth with NaCl (4.3M), sterile Sehgal and Gibbons broth with different NaCl concentrations:[0.2M, 0.5M, 1M, 1.5M, 2M, 2.5M,3M, 3.5M, 4Mand 4.3M] and for fungal isolates in sterile Sabouraud's broth incorporated with 100µg ml^{-1} ampicillin to avoid bacterial contamination and different NaCl concentrations:[0.2M, 0.5M, 1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M and 4.3M].

One gram of soil sample eachcollectedfrom different locations was added to 10ml of sterile saline,it was mixed properly and allowed to remain stable for 10 minutes. The initial inoculum density was adjusted to 0.1 (10^8 CFU/ml) according to McFarland standard and 1 ml of the saline suspension was aseptically inoculated into 100ml of following sterile broths. Similarly 1ml of saline water from different locations was taken and added to 100 ml of following sterile broths separately in other flasks.

Sterile Halophilic broth: [(g/L**)**10g of casein acid hydrolysate,10g of yeast extract,5g of proteose peptone,3g of trisodium citrate,2g of potassium chloride,25g of magnesiumsulphate and 250g of sodium chloride. The initial pH was adjusted to 7.2], incubated at 37^0C, for 1 week.(Quadri*et al*.,2016;Thombre and Oak, 2015)

Sterile modified Sehgal and Gibbons broth[(g/L):2 g of potassium chloride,20g of magnesium sulphate,0.023g of ferrous chloride,3g of sodium citrate, 7.5g of cas-amino acids,10g of yeast extract and with different NaCl concentrations:[0.2M, 0.5M,1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M and 4.3M] was incubated at 37^0C, for 1 week.(Quadri*et al*.,2016;Thombre and Oak, 2015)

Sterile Sabouraud's dextrose broth [(g/L):10g of peptone,40g of dextrose with different NaCl concentrations: [0.2M, 0.5M, 1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M and 4.3M] and incorporated with 100µg ml^{-1} ampicillin to avoid bacterial contamination and was incubated at room temperature, for 1week.(Quadri*et al*.,2016;Thombre and Oak, 2015)

The enriched samples were then subjected to serial 10fold dilutions and isolated on the respective solid media with same NaCl concentrations. Isolates obtained were maintained on sterile nutrient agar slants with appropriate NaCl concentration.

C) Screening of potential biosurfactant producers:

The isolates obtained were screened by enrichment in sterile Mineral salt medium (MSM).Activation of the isolate was done by growth in sterile nutrient broth at 37^0C for 18 hours with an inducer-1 % (v/v) kerosene in order to adapt the isolate for growth in the presence of inducer,followed by centrifugation at 2000 rpm for 20 minutes to obtain the pellet of the biomass. The inoculum density was adjusted to 0.1 (10^8 CFU/ml) according to McFarland standard and 1 ml was inoculated in 100ml sterile Mineral Salt medium (MSM) with an appropriate NaCl concentration. MSM was supplemented with a concentration of 1% (v/v) kerosene as an inducer in a 250 ml conical flask and was incubated at 37ºC for 1 week for enrichment. After that 1 ml was serially diluted till $10^{-5}$and then 0.1 ml was surface spread on the MSM plates. After incubation at 37ºC for 1week, plates were observed for colonies which were most probably biosurfactant producers

Isolates were screened by the use ofdifferentversions of MSMs. Most of the versions of MSM have this basal composition: Solution A and solution B as Trace mineral solution with minor differences.

[(solution A: 2.5g of NaNO3; 0.4 g of MgSO4.7H_2O; 1.0 g of NaCl; 1.0 g of KCl, 0.05g of $CaCl_2.2H_2O$; 10 ml of conc. Phosphoric acid (85%) pH of solution adjusted at 7.2 with KOH pellets. Solution B (per litre): 0.5 g of $FeSO_4.7H_2O$, 1.5 g of $ZnSO_4$. $7H_2O$, 1.5 g of $MnSO_4.2H_2O$, 0.3g of K_3BO_3, 0.15g of CuSO4.5H_2O, 0.1 g of $NaMnO_4.2H_2O$. One ml of Solution B to be added to 1000 ml of Solution A).1% crude oil to be used as carbon source.](Pandey *et al*., 2012).
MSMs were modified with respect to appropriate salt concentration, kerosene was used as an inducer and different carbon sources were studied.
Different types of MSMs which were studied were:

Mukherjee media(Mukherjee and Sen, 2009),Makkar and Cameotra media(Makkar and Cameotra, 1997),Modified Indigenous media (Mukherjee and Sen, 2009), Inorganic salt mediaType 1(Mukherjee and Sen, 2009),Inorganic salt media Type 2(Mukherjee and Sen, 2009), Modified M9 media(Xu *et al*., 2007).

D) Post-incubation processing of MSM:

After incubation for one week, MSM broths were harvested and the broth aliquots were dispensed aseptically in sterile tubes and subjected to centrifugation at 1000 rpm for 15 minutes. The cell-free supernatants obtained were individually evaluated for presence of biosurfactant byEmulsification index.

E) Emulsification index (E_{24}):

One ml of cell-free supernatant was added to one ml of kerosene oil and subjected to vortex for one minute followed by incubation at room temperature for 24 hours. SDS was used as a positive control, distilled water as a negative control along with sterile uninoculated MSM broth as medium control.(Cooper *et al.*, 1981)

III.RESULTS AND DISCUSSION:

A) Collection of samples:

Figure 3.1-After collecting the saline water samples from lake and soil from oil contaminated greasy soil near the tracks of trollies for crude salt, they were taken insterile screw capped bottles, petri-plates and test tubes.

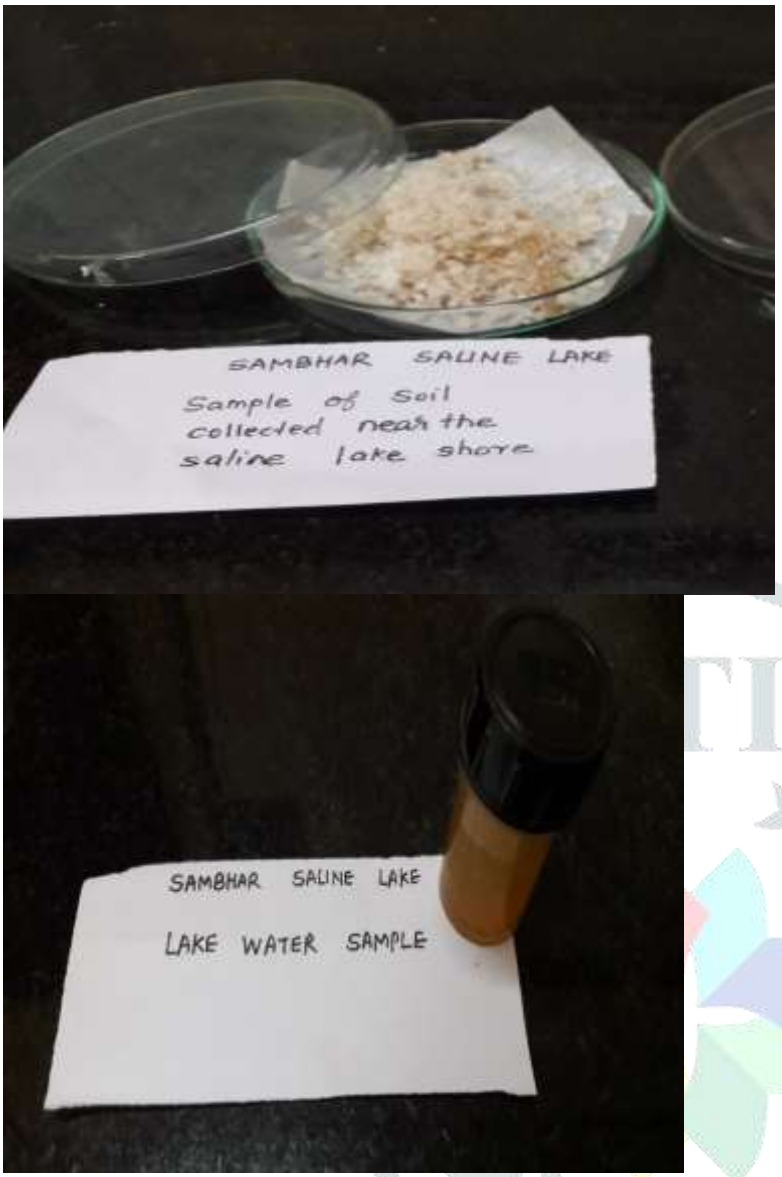

Fig:3.1-Soil and water samples collected from Sambhar Saline Lake.

B) ***Enrichment and Isolation of Halophilic organisms:***During incubation it was observed that there was visible growth in Sehgal and Gibbons broths having low concentration of NaCl, and relatively lesser growth at higher salt concentrations **(Fig:3.2)**This is relatively promising, and deserves attention, as most reported MSMs in scientific literature reports low concentration of NaCl in the broth. Also with respect to Halophilic broths some isolates were obtained, but did not give a visible positiveEmulsification test whereas Sehgal and Gibbons media gave better results.

Different isolates SH1, SH2, SH3, SH4, SH5, SH6, SH7, SH8, SH10, SH11, SH12 were screened in MSMs Some fungal isolates obtained from Sabouraud's broth with 0.2M, 0.5M and 1M NaCl **(Fig: 3.3)**were also screened in different MSMs but as compared to bacteria fungal response was weaker in terms of Emulsification test. Also fungus required longer time to show visible growth which is in accordance with the current literature.

Fig:3.2-Enrichment from Sambhar Saline Lake Sample in Sehgal Gibbons (SG) broth from left to right with differentNaCl concentrations and in Halophilic broth extreme right.

Fig:3.3-Enrichment from Sambhar Saline Lake Sample in Sabourauds-dextrose (Sab) broth from left to right with different NaCl concentrations.

C) Screening of potential biosurfactant producers:

SH1, SH2, SH3, SH4, SH5, SH6, SH7, SH8, SH10, SH11, SH12 isolates were screened in different MSMs such as Mukherjee media(Mukherjee and Sen, 2009), Makkar and Cameotra media(Makkar and Cameotra, 1997), modified indigenous media(Mukherjee and Sen, 2009),both types of inorganic salt media (Mukherjee and Sen, 2009)and modified M9 media(Xu *et al*., 2007). All MSMs gave positive results but both types of inorganic salt media, modified indigenous media and modified M9 media were relatively better for SH6, SH7, and SH8 isolates**(Table-1)** which were taken from 0.5M, 1M and 1.5M NaCl containing Sehgal and Gibbons media which is relatively promising from an industrial perspective as compared to other MSMs tested simultaneously.It was observed that in Inorganic salt media and modified

indigenous media there was relatively less precipitate as compared to Mukherjee media and MakkarCameotra media.This feature reduces additional steps and relatively makes it better to process faster.Also the biosurfactant production was observed in Makkar and Cameotra media but was relatively less. Many research papers report of glucose, sucrose as carbon sources(Makkar and Cameotra, 1997), but the use of molasses in this research showed a better alternative as modified indigenous media relatively shows better results as compared to other MSMs. Most probably halophilic organisms are better adapted to use molasses as a carbon source. Attempt was also made to test whether the presence of inducer such as kerosene has any impact on the production, it was observed that the presence of inducer kerosene enhances biosurfactant production, because if only kerosene is provided as a carbon source it would be initially a challenging state for a cell to utilize it and no other energy source would be initially present.Urea in inorganic salt media as well as in modified indigenous media highlights its significance.Ammonium chloride, ammonium nitrate provides the necessary nitrogen source. Presence of some other source of energy would initially serve to provide energy initially.So both carbon source and an inducer is better to have better biosurfactant production.

Table-1: Emulsification index (E_{24}) of isolates in different MSMs

MSM	Inorganic salt media Type-1	Inorganic salt media Type-2	Modified indigenous media	Modified M9 media
Isolate				
SH6	60%	65%	64%	63%
SH7	58%	61%	62%	59%
SH8	52%	53%	54%	55%

D) ***Post-incubation processing of MSM and Emulsification index (E_{24}):***Isolates were enriched in different respective MSMs and then processedfor Emulsification index determination. Methods differing in volumes are available in literature but in all the cases equal volume of cell-free supernatant and substrate oil (kerosene or some other oil) are being used.

IV.CONCLUSION: Halophiles are living organisms which have the ability to survive in saline conditions and in accordance with their ability to survive at higher salt concentration, the compounds derived from them have immense industrial and biotechnological potential. In this research, an attempt was made to screen different Mineral Salt Media (MSM) to produce biosurfactants using halophilic and halotolerant microorganisms.Out of many different MSMs it was found that both types of Inorganic salt media, modified indigenous media and modified M9 proved to be promising media. Shortlisted carbon and nitrogen sources open new horizons for further optimization of other parameters such as temperature, pH, duration etc.

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