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Biosurfactant Production by a Moderate Halophilic *Aspergillus niger* HF-1 Isolated from a Saline Lake

Shabib Khan[1]* and Lolly Jain[1]

[1]*Department of Microbiology, K.J. Somaiya College of Science and Commerce, Vidyavihar Mumbai, (Autonomous- Affiliated to the University of Mumbai), Maharashtra, India*

**Corresponding Author Email*: *shabib@somaiya.edu*

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ABSTRACT

Biosurfactants are obtained from living organisms and reduce the surface tension observed in the case of liquids. They possess an advantage over synthetic counterparts as they are non-toxic and biodegradable. As compared to bacteria, there is relatively lesser scientific investigation on fungal biosurfactants; and that too from saline habitats. In this research, an attempt was made to isolate and identify a halophilic fungus capable of producing a biosurfactant. The research was based on hypothesizing the ability of halophilic fungus to utilize and emulsify Soybean and Olive oils. Using a combination of preliminary morphological studies along with 18S rRNA-based molecular methods, the fungus was identified to be *Aspergillus niger* HF-1. The data was uploaded on NCBI and the GenBank Accession number obtained was PP568077. The fungus could grow optimally at pH-7, at 37°C and 3% NaCl with 2.93cm±0.11cm oil displacement, 60.33%±0.57% emulsification index, 42%±1.73% reduction in surface tension, and an initial biosurfactant yield of 0.28±0.02 g% (2.8g/L).

Keywords: Biosurfactants, Halophilic fungus, NaCl, Emulsification, Surface tension

INTRODUCTION

Surface active chemical agents that reduce the tension-active force of liquids are surfactants (Asghari *et al*., 2017) . Majority of the surfactants in routine use are synthetic, and culminate in environmental hazards and toxic consequences. Biosurfactants are amphipathic in nature and secreted by living organisms with the potential to alter the tension-active forces in liquids. Biosurfactants appear to be a better alternative to the synthetic surfactants, as they are biodegradable, compatible with the environment, and capable of being synthesized using the approach of "Green technology" (Asghari *et al*., 2017). Biosurfactants have the capacity to partition at the interface zone observed in the case of oil/water or air/water, that too with a gradient of polarity and modifications in hydrogen bonding predominantly leading to foam formation, emulsification and detergency (Desai and Banat, 1997).

In nature, many xenobiotic compounds have permeated major domains of life. At present, a number of physical and chemical methods are used to treat contaminated habitats, but each of them has its merits and demerits. Predominantly the contaminated sites are found to be saline with challenging conditions and require a safer and eco-friendly method for treatment. Hence, there is a need to investigate saline contaminated habitats, in order to isolate and characterize halophilic organisms capable of synthesizing biosurfactants, and hypothesizing on their ability to emulsify different oils and hydrocarbons. Based on the level of optimal requirements of NaCl, the halophilic organisms are classified into different groups: slight (1-3%), moderate (3-15 %) and extreme (15-30 %) halophiles (Ventosa *et al*., 2015; Corral *et al*., 2019).

There is substantial research conducted on biosurfactants from various non-halophilic microbial sources (Uzoigwe *et al.*, 2015; Bouassida *et al.*, 2018; Shahwar *et al.*, 2019; Adu *et al.*, 2020). Biosurfactants contribute in the domains of therapeutics, detergents, textile, pesticides and herbicides, agriculture, paper and petrochemical industries (Banat *et al*., 2000).

In this research, an attempt was made to investigate the unexplored world of halophiles capable of producing biosurfactants. As compared to bacterial cells, there is relatively less data on halophilic fungal cells (Hasani Zadeh *et al*., 2018) and their potential to contribute in the mitigation of environmental issues. Halophilic fungal isolates from greasy oil studded soil and water samples from saline lake were obtained by enrichment in saline broth and isolation on respective solid

media. Isolates obtained were further enriched in mineral salt media followed by their isolation and screening for production of biosurfactant.

MATERIAL AND METHODS

Throughout the initial steps of enrichment, isolation and production of biosurfactant, 3% NaCl concentration, temperature 30^0C and initial pH in the acidic range was maintained.

Collection of samples

Greasy oil studded soil and water samples were collected from Sambhar saline lake near salt work (Rajasthan, India). Soil samples were collected up to a depth of approximately 10cm and water samples were collected from the shore of the saline lake habitats. Soil and water samples were taken from different locations at these places, and collected in sterile screw-capped bottles and plastic packets. Samples were brought to the laboratory within 10-15h and were subjected to enrichment for halophilic fungus.

Enrichment and isolation of halophilic fungus

A 1ml volume of saline lake water sample was aseptically inoculated in 250mL Erlenmeyer flask containing 100mL of sterile Sabouraud-dextrose broth with 3%NaCl. Similarly, 1g of soil was added to 10mL sterile saline and subjected to a vortex for a minute, and supernatant (inoculum density adjusted to 0.1 (10^8 CFU/ml) as per McFarland standard) was inoculated into 250mL Erlenmeyer flask containing 100mL of sterile Sabouraud-dextrose broth with 3% NaCl. Chloramphenicol, a broad-spectrum antibiotic (250 mg/L) was added into the sterile Sabouraud-dextrose broth and solid media to avoid bacterial contamination (Sena *et al*., 2018). Inoculated media were incubated on a temperature-controlled shaker (Classic Scientific) 30^0C (100rpm) for 7 days. Procedure was repeated with a new sterile Sabouraud-dextrose for second round of enrichment after which the enriched broth was subjected to serial 10-fold dilution and 100µL was used for surface-spreading on Sterile Sabouraud-dextrose agar media. Direct isolation on respective solid media was also adopted. A temperature of 30^0C and a duration of 4 days was adopted for incubation. The different fungal isolates obtained were subjected to enrichment and screening for biosurfactant production. Halophilic nature of the isolates was assessed by comparing their growth on media with different higher NaCl concentrations and better isolates with respect to growth at relatively higher salt concentration were selected (Ventosa *et al.,* 2015; Corral *et al.,* 2019).

Enrichment and isolation of prospective fungal producer of biosurfactant

Halophilic fungal isolates were subjected to enrichment. For seed inoculum, fungal isolates were initially cultured in sterile Sabouraud-dextrose agar with 3% NaCl, amended with 1% (v/v) Soybean oil and incubated at 30^0C for 4 days. Fungal mycelial disc (6 mm) in diameter was agitated in 10mL sterile distilled water and kept on shaker for 18h for dispersion of spores and fungal growth suspension was prepared by adjusting the spore density to $1x10^4$/ml using a Haemocytometer.

First round of enrichment

A modified Mineral Salt media (MSM) was amended with 1% (v/v) Soybean oil as an inducer and 3% NaCl and 0.1% Ammonium chloride (Sperb *et al.*, 2018). Soybean oil was initially sterilized by dry-heat method at 140^0C for 1h for three consecutive days. In order to supply minimum carbon in the initial stages for survival 0.1% glucose was added (Haque *et al*., 2020). To this MSM with pH 6.0, 1mL of the fungal growth suspension with a spore density $1x10^4$/ml was aseptically added. Inoculated media was subjected to agitation using shaker (100rpm) at 30^0C for 7 days.

Second round of enrichment

A 10 % inoculum was aseptically transferred from flask of first enrichment to the new sterile MSM for the next round of enrichment.

After the enrichments, the enriched broth of fungal isolates was used for surface spreading on sterile MSM agar containing 3% NaCl and 1% (v/v) Soybean oil. During the preparation of solid media for plates, the media were seeded with Chloramphenicol (250mg/L). Temperature of 30^0C and a duration of 5 days was adopted. Morphologically different fungal isolates obtained were purified by repeated streaking onto Sabouraud dextrose agar with 3% NaCl and 1% (v/v) Soybean oil. In further experiments, olive oil

and kerosene were also tested as alternative carbon sources. More growth was observed using olive oil.

Screening of biosurfactant producers

Positive isolates obtained were subjected to determination of emulsification index, followed by determination of surface tension of the media supernatant. Wherever applicable, as a positive control 0.1% SDS detergent was used and as a negative control distilled water was used.

Fungal isolates were activated in sterile Sabouraud-dextrose broth amended with NaCl, 1% (v/v) Olive oil for 24h, and biomass was subjected to filtration and the filtrate was used. Fungal growth suspension was also used for screening tests wherever applicable.

After 7 days-time period, the Erlenmeyer flask was observed for initial changes like turbidity (indicating growth) and foam formation (indicating emulsification of inducer) both changes could serve as primary indicators of biosurfactant formation. In this initial enrichment, the purpose was to enrich hydrocarbon degraders, with this intention no other carbon source was additionally added and hydrocarbon served as sole source of carbon as well as an inducer. Centrifugation of the enriched broth was carried out at 10,000g for 20min and further screening procedures were employed to investigate the biosurfactant producing ability using the supernatant.

Stage I screening

Along with the fungal isolates, two biosurfactant producing standard bacterial strains *Bacillus* sp. MTCC 2473 (SS1) and *Pseudomonas aeruginosa* MTCC 10636 (SS2) were procured from CSIR Institute of Microbial Technology, Chandigarh, India, and were tested in parallel. This served the purpose of keeping a biotic positive control. The purpose was to observe how a positive test appears from a living organism. Experiments were conducted in triplicate and the average value with standard deviation was noted.

Tilted glass slide (TGS) test

A small amount of fungal growth was brought in contact of a saline sample at one border of a sterile slide, and the saline drop was observed, if it begins dipping down it could be considered as a positive result (Satpute, 2008).

Haemolytic test

Filtrate was spot inoculated on sterile superimposed blood agar media (SIBA), incubated for 24h at 37^0C; and assessed for haemolysis of the red-blood cells. Zone of clearance served as an indicator of biosurfactant production (Youssef *et al*., 2004).

Parafilm M test

A drop of filtrate was bought in contact with a Parafilm M sheet and compared with a drop of distilled water placed on the same sheet, if its diameter is greater than water, it could be considered as a positive result. (El-Shahed *et al*., 2022). The diameter of the drop (cm) was measured.

Lipolytic test

Fungal isolate suspension was streaked on sterile Gorodkowa's Tributyrin media and assessed at 30^0C for a 48h duration for a zone of clearance surrounding the growth indicating lipase activity of the fungal isolates (Cha *et al*., 2008)

Stage II screening

CTAB agar method

A detergent Cetyl trimethyl ammonium bromide CTAB (0.2g/L) and Methylene blue stain (0.005g/L) were added to MSM media (Sperb *et al.,* 2018). Wells in the plate were filled with filtrate samples, and plates were incubated at 30^0C for 48-72h. After incubation, plates were observed for bluish halo concentric rings in the vicinity of the well, serving as an indicator of an anionic surfactant.

Bacterial adhesion to hydrocarbons (BATH) test

Fungal suspension from MSM with inducer was utilized for the BATH test. A 2mL of cell suspension was mixed with 100µL of n-hexane and the preparation was vortexed for a minute and allowed to settle under static conditions at 30^0C for 1h. Two layers were formed and the aqueous layer was pooled. Using a spectrophotometer in visible range, the factor evaluated was absorbance at a wavelength of 600nm. In order to estimate the percentage of the hydrophobicity, the ratio of the absorbance of suspension after and before incubation was subtracted from 1 and multiplied by 100 (Rosenberg *et al*., 1980).

Oil-displacement test

In a large petri dish, 50mL of distilled water was taken. To this 20 µL of Soybean oil was added, followed by adding 10µL filtrate on the oil layer. Oil displacement diameter was observed (Morikawa *et al*., 2000).

Oil drop collapse test

A 96 well microtiter plate was used. In the beginning 2µL of Soybean oil was loaded in the well and kept to stabilise for 1h at room temperature. After the stabilization, 5µL of the filtrate was added to the oil surface. It was observed for 1min. The change in the shape of the drop to a flat drop was considered as positive. (Bodour *et al*., 2003). For the sake of observation, + to +++ to denote partial to complete spreading; wherein if drop becomes flat after 1 minute it was denoted as '+++"; if drop becomes less flat after 1 minute it was denoted as '++'; and if drop becomes flat in a negligible manner it was denoted as '+'.

Emulsification index

A volume of 2mL of Kerosene and 2mL of filtrate was mixed and vortexed for a minute. It was observed for formation of an emulsion and its maintenance for 24h. Determination of emulsification index was based on a ratio of height of emulsion layer to the total height of the mixture and the value obtained was multiplied by 100 (Youssef *et al*., 2004). For this test 0.1% SDS was used as a positive control and distilled water as a negative control.

Determination of surface tension

Du-Nouy ring Tensiometer (Ganga Scientific, Kolkata), was duly calibrated using distilled water (72 mN/m). Initial uninoculated MSM with oil as abiotic control was maintained for initial surface tension of the broth and taken as control for comparison. Percentage reduction in surface tension was calculated (Qazi *et al*., 2014).

The above tests were conducted for all the isolates, and it was noted that HF-1,2 performed relatively better, out of these isolates HF-1 consistently maintained good results in terms of better emulsification index and a better percentage reduction in surface tension, so henceforth the promising isolate HF-1 was investigated further.

Fungal morphology of the isolate

Wet mount of the better prospective fungal producer of the biosurfactant was done using Lactophenol cotton blue stain and observed microscopically.

Identification of the fungal isolate

Along with the basic morphological studies using wet mount, the further steps of the identification of the fungal isolate HF-1 were done using partial sequencing of the 18S rRNA gene at HiMedia laboratories Pvt. Ltd., Thane, Maharashtra, India.

Initial medium for production of biosurfactant

The original composition of the MSM (g/L) as used for initial enrichment and then used for production was as follows: 2.0 KH_2PO_4, 1.0 $MgSO_4$, 0.01 $MnSO_4$, 0.63 mg $FeSO_4.7H_2O$, 0.62 mg $ZnSO_4$ (Sperb *et al*., 2018) . The media was amended with 1% (v/v) Olive oil, 3% NaCl and 0.1 % Ammonium chloride, the medium's initial pH was 6.0.

Production of biosurfactant using MSM

Fungus was activated by growing in sterile Sabouraud broth for 24h, and fungal growth suspension with a spore density 1 x 10^4 /ml was inoculated in initial MSM. It was incubated at 100rpm at 30^0C for 5 days.

Recovery of biosurfactant

In order to obtain biomass and cell-free supernatant with crude biosurfactant, the broth was centrifuged for 20min at 10,000rpm. Using 6N HCl, the supernatant pH was acidified to pH-2 and refrigerated at 4^0C over 24h to precipitate proteins and lipids. It was subjected to agitation using solvents chloroform and methanol (2:1) and vigorous extraction. Extracts were dried, rinsed with distilled water and again extracted with solvents. Organic layer with biosurfactant was taken and kept for evaporation followed by washings of the residue with n-hexane to remove any bound oil. Rotary evaporator was used to concentrate the biosurfactant and recover the solvents used. The viscous biosurfactant was thus obtained, dried and weighed to determine g% (Cha *et al*., 2008) .

Estimation of biomass

Cellular biomass obtained was agitated with 5ml of 3:1 acetone and n-hexane to remove bound hydrocarbons. It was centrifuged at 10,000rpm for 10min, supernatant was discarded and cellular biomass was dried in hot air Owen for 24h to know dry-weight as g% (Pruthi and Cameotra, 2000).

RESULTS AND DISCUSSION

Enrichment and isolation of halophilic fungus

Enrichment and isolation of halophilic organisms was initiated, taking cognizance of the fact that there is a requirement to explore saline habitats with better prospects for obtaining halophilic isolates. Chen *et al*. (2017) in their research, suggested screening the soil contaminated with organic content as they have higher probability of harbouring biosurfactant producing organisms. After the second round of enrichment fungal isolates were subjected to enrichment and screening. In this research, optimum fungal growth was observed on media containing 3% NaCl. It was observed that HF-1 could exhibit optimum growth at 3% NaCl amended media, but showed scanty growth on media containing lower NaCl concentrations, this implies that HF-1 was a moderate halophile in nature (Ventosa *et al*., 2015; Corral *et al*., 2019).

Enrichment and isolation of prospective fungal producer of biosurfactant

Enrichment and isolation of halophilic biosurfactant producing organisms was initiated hypothesizing that the isolates might emulsify oils and other hydrocarbons which are predominant at polluted habitats. Bodour *et al*. (2003) could isolate organisms capable of emulsifying hydrocarbons and using phylogenetic studies could reveal *Flavobacterium* species. In this research also, greasy oil studded soil and water samples near shore of saline lake were taken in initial enrichment for halophilic organisms. Researchers observed the biosurfactant producing organisms could be obtained relatively faster when enrichment conditions are equipped with inducers such as olive oil, mill wastewater (Meneses *et al*., 2017). In this research also, we investigated different vegetable-based oil sources which is in agreement with other researchers. From enrichment for halophilic fungi, each of the isolates were enriched in NaCl and Soybean oil or Olive oil amended MSM for production of biosurfactant respectively. After enrichment in liquid mineral salt media, they were assessed by growing on solid MSM media. Isolates could grow on MSM media containing 3% NaCl and 1% (v/v) Soybean oil along with an antibiotic Chloramphenicol. Plates supplemented with olive oil were also assessed in parallel. In liquid media, there was foam formation observed in the production flasks with Soybean and olive oil respectively, which served as a significant indicator of oil being used and production of biosurfactant **(Figure 1).**

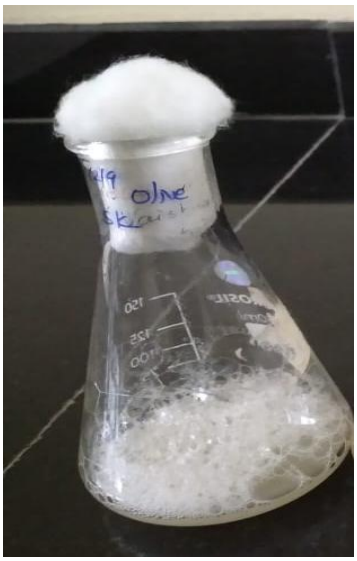

Figure 1: Foam production

Screening of biosurfactant producers

The filtrates obtained from fungal isolates were subjected to primary screening tests to obtain

a relatively better fungal isolate.

Tilted glass slide (TGS) test

Isolates were observed for dipping of the liquid. Results are shown in **(Table 1)**. As per scientific literature, tilted glass slide test in combination with other tests could serve in the screening of the biosurfactant producers (Satpute, 2008).

Table 1: Tilted glass slide test

Isolate	Observation
HF-1	+
HF-2	+
SS-1	+
SS-2	+

+, indicates a positive test; -, indicates a negative test.

Haemolytic test

Researchers in their review have mentioned haemolytic test as a significant preliminary test (Banat *et al*., 2000; Satpute *et al*., 2010). Similar to our research, the researchers could isolate a variety of haemolytic positive isolates such as *Serratia* sp., *Bacillus* sp. (Satpute, 2008; Nalini and Parthasarathi, 2013). However, we cannot rely totally on the haemolytic tests as the zone of clearance on SIBA-Super imposed blood agar could be due to other factors like virulence factor haemolysin or due to divalent ions. Hence, it is better to supplement the haemolytic test with other tests. Haemolytic activity results are mentioned in **(Table 2)**.

Table 2: Haemolytic test

Isolate	Observation
HF-1	++
HF-2	+
SS-1	+++
SS-2	++

++, clear zone of haemolysis would be less than 1cm; +++, clear zone would be greater than 1cm and less than 3cm; -, indicates a negative test indicating absence of haemolysis

Parafilm M test

After taking a drop of filtrate on the Parafilm-M paper strip the drop diameter was evaluated for isolates as shown in **(Table 3)**. Similar investigation was undertaken for the screening tests for the heavy metal tolerant biosurfactant producing isolates from Egyptian soil. In their research most of the isolates had a diameter in the range of 0.4-0.7cm. Our isolates also exhibited a similar response which in turn corroborates the data in the literature (El-Shahed *et al*., 2022).

Table 3: Parafilm-M test

Isolate	Diameter of the group (cm)
HF-1	0.55±0.04
HF-2	0.43±0.02
SS-1	0.57±0.01
SS-2	0.61±0.01

Lipolytic test

The isolates growing on Gorodkowa's Tributyrin agar were evaluated for a zone of clearance. The results are shown in **(Table 4).** Lipase test could be considered as a significant aspect. As per the literature, lipase production has been associated with the synthesis of compounds such as glycolipids, lipopeptides and neutral lipids (Desai and Banat, 1997).

Table 4: Lipolytic test

Isolate	Observation
HF-1	+
HF-2	+
SS-1	++
SS-2	++

++, indicates a prominent zone of clearance; + indicates a weaker zone of clearance

Fungal isolates (HF-1,2) could exhibit relatively better results for oil displacement and oil drop collapse test as shown in (**Table 5**) and were considered for secondary tests. Oil drop collapse tests basically work on the fact that surface as well as interfacial tension gets reduced due to biosurfactant and this test was widely employed (Bodour *et al*., 2003; Youssef *et al*., 2004). Oil displacement test serves as more sensitive approach as compared to drop collapse (Hamza Ainon, 2013) as an indirect approach to measure surface activity of the surfactant against oil. If the diameter of the zone of clearance is large, it indicates a higher activity of the biosurfactant (Rodrigues *et al*., 2006).

Table 5: Primary screening tests for producers of biosurfactants

Isolate number	Oil displacement test (Zone diameter in cm)	Oil drop collapse test
HF-1	2.93±0.11	+
HF-2	1.3±0.17	+
SS-1	2.9±0.17	++
SS-2	2.36±0.3	++

+ to +++, to denote partial to complete spreading; wherein if drop becomes flat after 1 minute it was denoted as '+++"; if drop becomes less flat after 1 minute it was denoted as '++'; and if drop becomes flat in a negligible manner it was denoted as '+'.

Isolates with better results were subjected to secondary tests for screening producers of biosurfactants as shown in (**Table 6**). Overall, the tests employed by us for the primary screening of the isolates are initially reliable, efficient and in alignment with other researchers (Amaral *et al*., 2006). Using the CTAB-based approach, isolates were assessed for the appearance of bluish halo surrounding the growth. Formation of blue colour indicates biosurfactant production (Sumathi, 2016).

The response to the BATH test indicates the hydrophobicity of biosurfactants. The parameter of E_{24} is dependent on biosurfactant and emulsification index above 30% is considered as starting base value for biosurfactant production (Satpute, 2008), and if the emulsification is equal to or more than 50% it could be considered as stable (Ahmad *et al*., 2016). Taking these aspects into consideration, it could be observed that HF-1 exhibited relatively better emulsification index (60.33%±0.57%) as compared to HF-2 (20.66%±1.15%). If surface tension is reduced to less than 40mN/m by a microbial cell (Ruggeri *et al*., 2009), it is significant and could be taken as a reliable aspect for further investigation. Data as mentioned in (**Table 6)**, reveals that HF-1 could exhibit 42%±1.73% reduction in the surface tension of the MSM, (initial surface tension of the uninoculated MSM was 65mN/m). HF-1 is capable of decreasing the surface tension to 37±1.15 mN/m whereas HF-2 could reduce to 40±0.58mN/m.

Table 6: Secondary screening tests for producers of biosurfactants

Isolate number	CTAB: Diameter of halo zone (mm)	BATH: %hydrophobicity	Emulsification index (E_{24})%	Percentage reduction in surface tension
HF-1	14±1.0	37.33±1.15	60.33±0.57	42±1.73
HF-2	11.33±1.15	44±1	20.66±1.15	38.97±0.88
SS-1	15.33±0.57	56.91±0.62	62±1	52.08±1.8
SS-2	17.33±1.15	60.33±0.57	64.33±0.57	48.95±0.9

Morphology of the fungal isolate

Microscopic observation of the Wet-mount of the fungus revealed the septate mycelial mass along with a conidiophore stalk, a tube-like structure. These features resemble the characteristics of filamentous fungi.

Identification of the fungal isolate

The pure culture was sent to Hi-Media Laboratory, Thane. When the isolate HF-1 was subject to 18S rRNA gene analysis, the amplicon was retrieved and sequenced using the Sanger sequencing machine. The phylogenetic analysis was undertaken. The isolate HF-1 was identified to be *Aspergillus niger*. On further analysis with BLASTN algorithm, the respective sequence files were uploaded on NCBI and the GenBank Accession number PP568077 was obtained.

Production of biosurfactant using initial MSM

Using initial MSM, 0.32 g%±0.02 g% biomass and 0.28 g% ±0.02 g% biosurfactant was obtained. The yield of biosurfactant is dependent on the microbial strain used, carbon and nitrogen sources used in media. Another research with a native strain of *Aspergillus niger* reported an initial yield of 2.3 g/L which increased to 3.3 g/L after sequential mutagenesis using ethidium bromide (Asgher *et al*., 2020). Our research with an initial biosurfactant yield of 2.8 g/L, is in alignment and corroborates these findings.

CONCLUSION

Research undertaken investigated the saline habitat so as to obtain a halophilic fungus capable of producing a biosurfactant. Saline conditions and inducers used during the enrichment step created selective conditions for the propagation of halophilic organisms producing biosurfactants. Screening methods adopted were sensitive enough and could substantiate the rationale behind the screening tests. Production was carried out using mineral salt media with an external carbon source, which substantiated the production of biosurfactant.

FUTURE PROSPECTS

There seems to be immense scope for optimization and characterization of biosurfactant. Further statistical studies could be undertaken using response-surface approach to evaluate the interactions between the components of the media. Studies with halophilic fungus for biosurfactant production might present a unique niche to be explored for broad applications in cosmetics, agriculture, medicine, food and dairy industry.

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