BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, October 2014. Vol. **11**(Spl. Edn. 1), p. 59-67

Production and Purification of Biosurfactant from Marine Yeast Isolated from Kelambakkam Salterns

R. Muthezhilan[1]*, R. Ragul[1], Angelin C Pushpam[1] and A. Jaffar Hussain

[1]Department of Marine Biotechnology, AMET University (U/S 3 of UGC Act 1956), Kanathur, Chennai 603112, India
[2]Centre for Marine Bioprospecting, AMET University (U/S 3 of UGC Act 1956), Kanathur, Chennai 603112, India.

doi: http://dx.doi.org/10.13005/bbra/1392

(Received: 15 August 2014; accepted: 10 October 2014)

The use of synthetic surfactants, derived from petroleum sources are usually toxic to the environment and soil living things. Nowadays, the research on biological surfactant production has grown significantly due to the advantages they present over synthetic compounds such as biodegradability, low toxicity and huge diversity. The biosurfactants are surface-active compounds from biological sources. In general, microbes especially yeast from extreme conditions like hypersaline environment are not fairly reported for biosurfactant production. Thus the present study was aimed to isolate the biosurfactant producing marine yeast from Kelambakkam Salterns, East Coast of Tamil Nadu, India. Morphologically 30 different Marine Yeast strains were isolated from saltern water and sediment samples using SGA, YMA, YPD and YM medium by spread plate and pour plate technique and they were named as AMBY101 to AMBY130. While screening all the marine yeast for biosurfactant production by Oil displacement Test using five different oils such as, Crude oil, Olive oil, Palm oil, Coconut oil and groundnut oil, the three strains namely AMBY109, AMBY117 and AMBY124 have showed a maximum activity and in all the tested oils. While checking the effect biosurfactant in crude oil degradation and emulsification assay using four different hydrocarbons (such as, waste motor lubricant oil, crude oil, diesel and kerosene), the strain AMBY109 have showed a maximum activity in all the experiments. The biosurfactants from the strain AMBY109 was extracted and purified. The purified biosurfactant was checked for antimicrobial activity and showed maximum zone. Further, based on the microscopic and morphological characteristics the marine yeast strain AMBY109 was identified

Key words: Marine Yeast, *Rhodotorula* sp, Biosurfactant, Crude oil degradation.

Surfactants are amphiphilic agents which, by accumulating at interface between immiscible phases, can reduce surface and interfacial tension. (Bodour *et al.*, 2002, Cameotra *et al*., 1998, Banat *et al*., 2000). Chemically-synthesized surfactants are not biodegradable and can be toxic to the environment (Desai *et al.*,1997). Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms. Due to their interesting properties such as lower toxicity, higher biodegradability, higher foaming capacity and higher activity at extreme temperatures, pH levels and salinity, biosurfactants have been increasingly attracting the attention of the scientific community as promising candidates for the replacement of a number of synthetic surfactants (Cirigliano *et al.*,1984). These compounds are biological molecules with noticeable surfactant properties similar to the well known synthetic surfactants and they also include microbial compounds with surfactant properties (Zinjarde

* To whom all correspondence should be addressed.
E-mail: mycomuthu@gmail.com

et al 2002.,Sarubbo *et al* 2007., Amaral *et al* 1894). Microbial surfactants are not yet competitive with those produced by the chemical industry, but efforts should be made on the different production aspects to find suitable and economic substrates and to develop new strategies to increase the volumetric productivity. The biosurfactant adhesive mechanism is based in the inhibition of microorganisms to different surfaces can interact with interfaces of the molecule. In this sense, they are an alternative to synthetic surface-active agents because of their low toxicity and biodegradability (Singh.P *et al* 2004, Das. P*et al* 2009) The biosurfactants have widely been applied in various fields like food and agriculture, detergents and cosmetics, environmental cleanup and oil recovery, biomedical and therapeutics during recent years (Khopade A *et al* 2012, Banat I M *et al* 2000, Lima TMS *et al* 2011)Various microorganisms such as bacteria, fungi and yeast are known to produce specific kind of biosurfactants (Kiran *et al*., 2009).The majority of microbial biosurfactants described in literature is of bacterial origin and the genders most reported as biosurfactant producers are *Pseudomonas sp.*, *Acinetobacter sp., Bacillus sp.* And *Arthrobacter sp*. However, due to the pathogenic nature of the producing organisms, the application of these compounds is restricted, not being suitable for use in food industry, among others (Shepherd *et al.,1995*). The great advantage of using yeasts in biosurfactant production is the GRAS (generally regarded as safe) status that most of these species present, for example *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Organisms with GRAS status are not toxic or pathogenic, allowing the application of their products in the food and pharmaceutical industries (Barth. G *et al*.,1997) micro organisms that exclusively produce biosurfactants during growth on water soluble compounds. Some species of yeast *Rhodotorula* produce a mixture of mannitol and pentitol esters of beta-D-hydroxypalmitic acid and beta-D-hydroxystearic acid during growth on a complex medium with glucose as carbon source. The glycolipid is partly acetylated. A promising method that can improve the effectiveness of bioremediation process is the use of biosurfactants, so the present work deals with the production of biosurfactants from marine yeast isolated from salterns and characterized by application in different oil degradation.

MATERIALS AND METHODS

Selection of Media for Marine Yeast isolation

Samples were collected from the Kelambakkam salterns, East coast of Tamil Nadu, India. Samples were serially diluted and plated on SGA, YMA, YPD and YM Medium using spread plate technique. Incubation was done for 7 days. Results were observed and the media was selected based on the growth of the Marine Yeast (Yi-Sheng Chen *et al.*, 2009). The SGA and YPD were prepared in different pH level and spread plate technique was done with the serially diluted sample and incubated for 7 days.

Biosurfactant assays

Oil Displacement Test

The culture in the MS medium was centrifuged at 6000 rpm for 20 minutes and supernatant was collected. 25 ml of distilled water and 50 µl of oil (crude oil, olive oil, palm oil, coconut oil and groundnut oil) were taken in as petri plate, oil was added drop by drop in the distilled water. 50 µl of supernatant was added to the center of oil, among the 30 strains three strains produce maximum zone against different oil these strains were taken for further characterization. (Nilanjana *et al.*, 2010)

Crude oil degradation

Shake flask biodegradation experiments were carried out in 500 mL Erlenmeyer flasks with 100 ml of mineral salt medium containing (g L_1) 1.0 K_2HPO_4, 0.2 $MgSO_4$ $7H_2O$, 0.05 $FeSO_4$ $7H_2O$, 0.1 $CaCl_{22}H_2O$, 0.001 Na2MoO_4 $2H_2O$, 30 NaCl and crude oil (2.0%, w/v). Sterilized culture medium was inoculated with 1% (v/v) inoculum containing yeast cells and the culture flasks were maintained in a shaker for 16-18 h. Biodegradation experiments were conducted in five different sets using above culture medium

Crude oil degradation

Set 1	yeast + medium + crude oil (normal)
Set 2	yeast + medium + fertilizer + crude oil
Set 3	yeast + medium+ biosurfactant + crude oil
Set 4	yeast + medium + fertilizer + biosurfactant+ crude oil
Set 5	Crude oil + medium (No yeast cells, control)

Emulsification assay

500 µl of cell free culture broth was added to 5 ml of Tris buffer (pH 8.0) in a 30 ml screw capped test tube. Hydrocarbons such as waste motor lubricant oil, crude oil, diesel and kerosene were tested for emulsification activity. 5 mg of hydrocarbon was added to the above solution and vortexed for 1 min and the mixture was allowed to stand for 20 min. The OD of the emulsified mixture was measured at 610 nm.

Purification of Biosurfactants

The culture broth was centrifuged at 10,000 rpm for 10 mins supernatant was collected in a beaker. 1M H_2SO_4 was prepared to adjust supernatant to pH 2. Chloroform: methanol (2:1) was added to the supernatant and kept in a beaker containing aluminium foil with holes and kept for overnight. White colored sediment was obtained as a result i.e. the biosurfactant. **(**Sekar *et al.*, 2010) Antimicrobial activity of biosurfactants

The crude biosurfactant was tested for antimicrobial activity using well diffusion method. The human pathogens were collected from the AMET Microbial culture collection center (*Escherichia coli*, *Enterococcus faecalis*, *Vibrio cholerae*, *V. parahaemolyticus*, *Salmonella* sp., and *Shigella* sp) were swabbed with sterile cotton swabs in petriplates. Then 30 µL of purified biosurfactants, synthesized from AMBY109 were dropped onto the wells. The plates were incubated at 37°C for 24 hours. After incubation the results were observed.

Identification of the potential pigment producing Marine Yeast

The pigment producing strains were suspended in sterile water and a loopful of the suspension streaked onto the YM agar. Incubate the plate at 30ºC for 4-6days and observed microscopically. The strains were identified by using standard manuals. The potential strains were characterized morphologically and biochemical characterization was done.

RESULT AND DISCUSSION

Normally, microbial produced compounds are easily degraded (Mohan *et al.*, 2006) and mostly suited for the environmental applications compared to the synthetic compounds (Mulligan, 2005). The microbial derived Biosurfactants have some of the potential applications of in pollution and environmental control, hydrocarbon degradation heavy metal removal, hexa-chloro cyclohexane degradation and antimicrobial activity (Singh, *et al.*, 2007). The marine yeast isolation was done in SGA, YMA, YPD and YM media, growth was observed in SGA and YPD medium and no colonies were found in YMA and YM media. A total of 30 yeast strains were isolated from the samples taken from Kelambakkam salterns, East Coast of Tamil Nadu, India and they were named as AMBY101 to AMBY130. Majority of the colonies were white, small and circular in shape. They are isolated in different pH concentration of SGA and YPD medium and found that the Marine Yeast grow abundantly in pH level 8.0 (Fig 1). Zinjarde and Pant studied the influence of initial pH in the production of a biosurfactant by *Y. lipolytica*. The authors observed that the best production occurred when the pH was 8.0, which is the natural pH of sea water.

The oil displacement test is an indirect measurement of surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution (Rodrigues *et al*., 2006).Displacement of oil was observed in the plate due to the Biosurfactants produced by the organism among the 30 Strains 3 strains produce maximum surfactant activity compared to other strains (Table 1& Fig 2). It was found that biosurfactants produced by yeast species on diesel were growth associated similar to that for some bacterial species like *Bacillusstearo thermophillus* VR-8 (Gurjar, 1995), *Pseudomonas aeruginosa* (Ilori and Amund, 2001) and *Aeromonas* sp.(Ilori *et al*., 2005). So, in this present study these three strains are used in crude oil degradation.

Among the 30 strains the strain number AMBY109, AMBY117 and AMBY124 showed maximum activity initially a zone was appeared and then it was degraded.

The crude oil degradation was done using crude oil and was kept in 5 different set up for observation (Fig 3). Each set up was taken OD value everyday to optimise the amount of crude oil degradation (Fig 4).

Each set up was taken reading and the degradation activity was observed in all the set up. The emulsification activity was done with waste

Table 1. Screening of Biosurfactant activity by oil displacement method using 5 different oils (olive oil, crude oil, palm oil, groundnut oil and coconut oil)

S. No	Strain No	Different oils				
		Olive oil	Crude oil	Palm oil	Groundnut oil	Coconut oil
1	AMBY101	-	+	+	-	-
2	AMBY102	-	-	+	-	+
3	AMBY103	+	-	-	-	+
4	AMBY104	-	+	+	+	-
5	AMBY105	-	+	-	+	-
6	AMBY106	+	-	-	-	+
7	AMBY107	-	+	+	-	-
8	AMBY108	+	-	-	-	+
9	AMBY109	+	+	+	+	+
10	AMBY110	-	-	-	-	+
11	AMBY111	-	+	-	-	+
12	AMBY112	-	+	-	-	+
13	AMBY113	+	-	-	+	-
14	AMBY114	+	-	-	+	-
15	AMBY115	+	+	+	-	-
16	AMBY116	-	+	+	-	-
17	AMBY117	+	+	+	+	+
18	AMBY118	+	-	-	-	+
19	AMBY119	-	+	-	-	-
20	AMBY120		+	-	-	-
21	AMBY121	+	+	-	+	-
22	AMBY122	+	-	+	+	+
23	AMBY123	-	-	+	+	-
24	AMBY124	+	+	+	+	+
25	AMBY125	-	+	+	-	+
26	AMBY126	+	-	-	-	+
27	AMBY127	-	-	-	-	-
28	AMBY128	-	+	-	-	-
29	AMBY129	-	+	-	-	-
30	AMBY130	+	-	+	-	-

“ = no inhibition zone, + = inhibition of zone

Table 2. Antimicrobial activity of biosurfactants

S. No	Organisms	Antimicrobial activity Biosurfactant synthezised by AMBY109
1.	*Escherichia coli*	++
2.	*Enterococcus faecalis*	++
3.	*Vibrio cholerae*	±
4.	*V. parahaemolyticus*	+
5.	*Salmonella* sp	±
6.	*Shigella*s	+

+ Positive
± Intermediate
- Negative

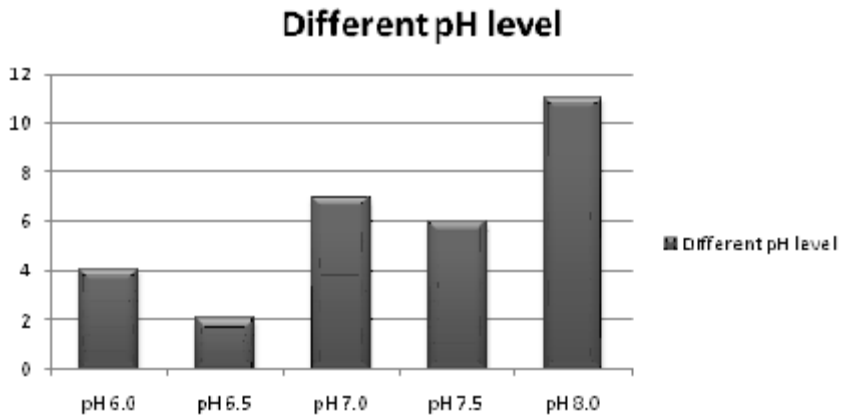
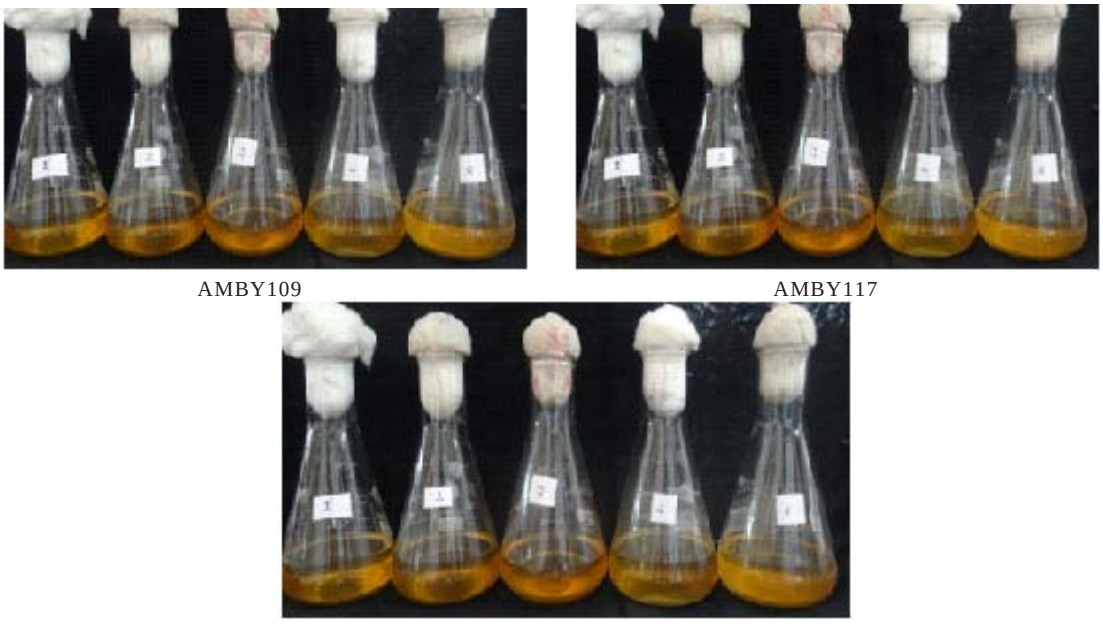


Fig. 1. Isolation of Marine Yeast in Different pH level

AMBY109 AMBY117 AMBY124

Fig. 2. Screening of oil displacement by oil displacement method
and AMBY 109, AMBY 117 and AMBY 124 showed maximum activity



AMBY109 AMBY117

AMBY124

Fig. 3. Crude oil degradation

motor lubricant oil, crude oil and diesel. These emulsification results showed that, biosurfactant produced from a substrate can emulsify different hydrocarbons to a greater extent which confirmed its applicability against different hydrocarbon pollution (Thavasi *et al.*, 2010) The strain AMBY109 is capable of degrading all the oil and showed maximum activity in all the assays.

From the point of view of microbial degradation, dissolution and emulsification of hydrocarbons appear to have positive effect on degradation rate (Amund and Adebiyi, 1991).

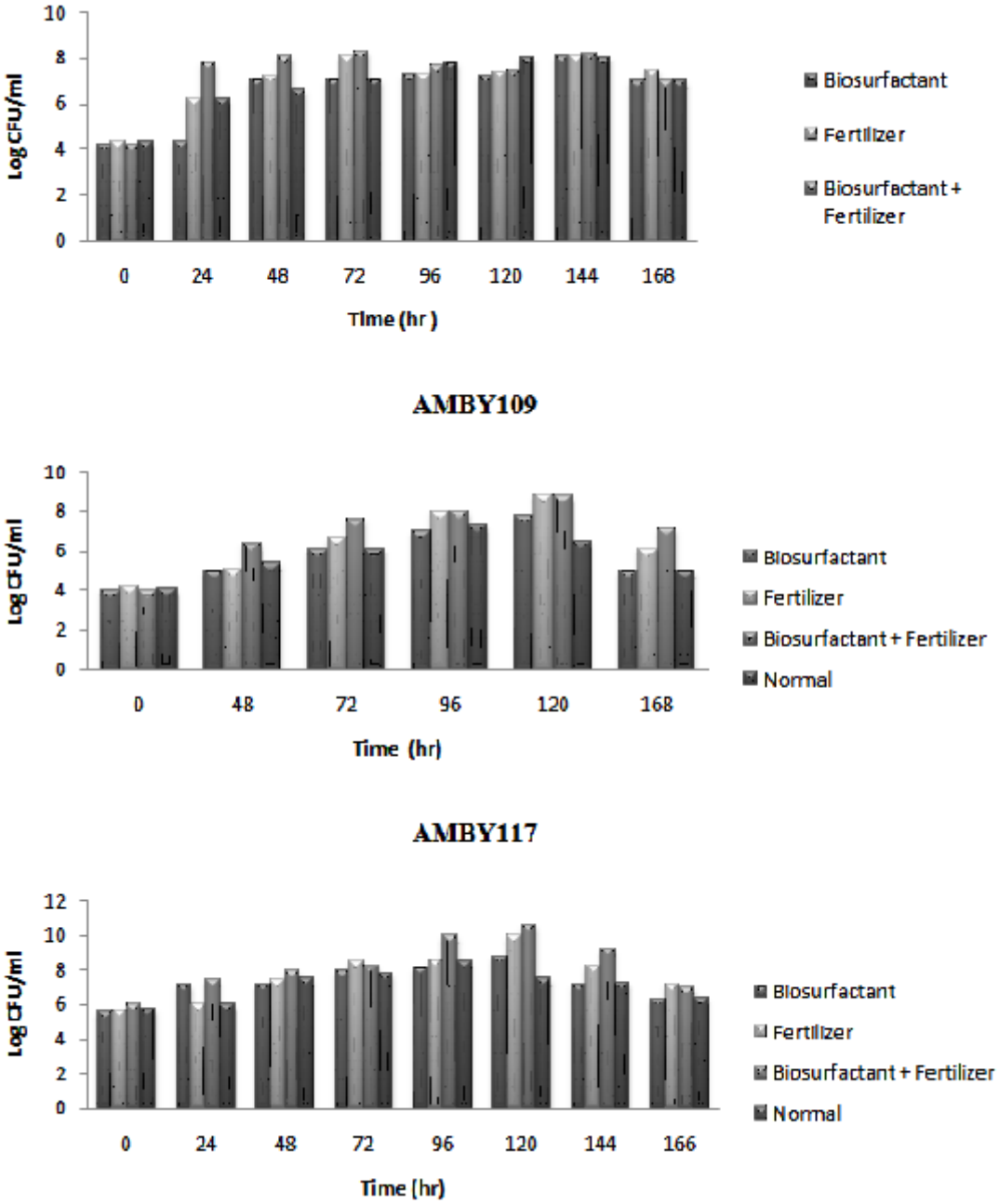


Fig. 4. Crude oil degradation

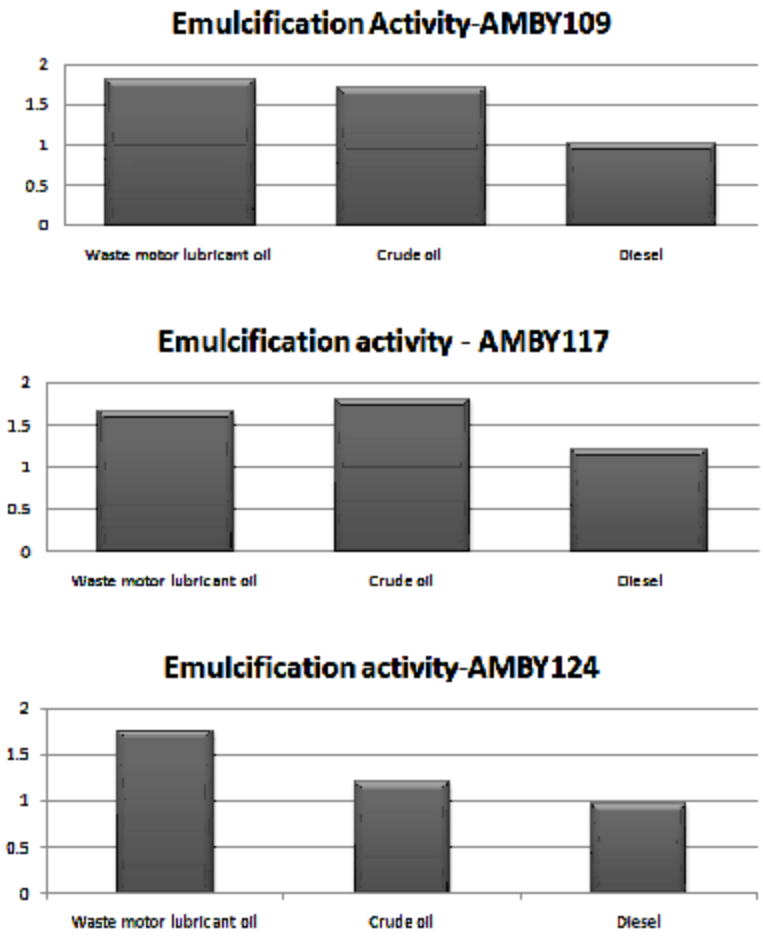


Fig. 5. Emulsification activity

The biosurfactants from the potential stain AMBY109 was purified and extracted a white sediment was obtained which is the pure form of biosurfactant (Fig 3).Higher concentration of biosurfactant at the early stationary phase may be due to the release of cell-bound biosurfactant in to the culture broth which led to the raise in extracellular biosurfactant concentration (Goldman *et al*., 1982).

The antimicrobial activity of biosurfactants was evaluated against a variety of bacterial pathogens. The biosurfactant synthesized by AMBY109have the ability of antimicrobial activity against all microbial cultures and showed maximum activity. The results were shown in (Table2)

The potential biosurfactant producing yeast AMBY109 was identified as *Rodotorula sp*

Fig. 6. The Microbial Biosurfactant extracted and purified from the Marine Yeast AMBY109

by using lab manual for yeast study (Sung-OuiSuh, Ning Zhang, Nhu Nguyen, Stephanie Gross &Meredith Blackwell 2008) and it was characterized morphologically and biochemical characterization was done for confirmation.

Morphology

Colour	Orange
Nature	Smooth
Features	Glistening
Surface	Ovoidal to globose
Shape	Convex
Reproduction	Multilateral budding
Pseudomycelium	absent

In conclusion, Biosurfactants find applications in a wide variety of commercial areas and industrial processes such as bio remediation of oil polluted soil and water (Volkering *et al*., 1997). The negative effects of the synthetic biosurfactant can be overcome by the microbial biosurfactants. Mostly all the microbes are capable of producing surfactants among which Yeast are readily grown and are easy to cultivate in large scale level. Thus the current study was focused on isolation of biosurfactant from marine yeast and found that 3 strains AMBY109, AMBY124 and AMBY130 are capable of producing biosurfactants. These biosurfactants were purified and extracted. Finally these strains were subjected to crude oil degradation and emulsification assay and found that the strain AMBY109 is more effective in the process of degradation. Morphological and biochemical analysis shows that the strain AMBY109 is *Rhodotorula sp.* Further study of this strain can lead to an effective microbial biosurfactant and can be applied in agro based industry.

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