

Emulsifying Activity of Hydrocarbonoclastic Marine Yeasts

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Marine yeast growth on four petroleum hydrocarbons induced the production of extracellular emulsifying agents (biosurfactants). Out of the 17 marine yeast isolates tested, 7 isolates, i.e., *Candida parapsilosis*, *C. cantarelli*, *C. membranae-faciens*, *Candida* sp. *Debaryomyces hansenii*, *Rhodotorula glutinis* and *R. graminis* exhibited this activity on kerosene and 3 of them i.e., *Candida membranaefaciens*, *Candida albicans* and *Candida* sp. on diesel. The emulsification of the hydrocarbons provided larger surface area on the hydrocarbon for the yeast to attack, degrade and grow on them. The marine yeasts were isolated from Central Indian Ocean Basin and EEZ of India.

Alarming increase in hydrocarbon pollution in the marine environment due to accidental spills as well as human activities is affecting the marine living resources very badly. Methods to combat this pollution have been attracting the attention of scientists for quite some time. Microbiological methods for this have been tried by several workers. Atlas (1984) has compiled most of the work done on different aspects of petroleum microbiology. Detailed studies on microbiological degradation of hydrocarbons have been reported by Walker *et al.*, (1974), Crow *et al.* (1980), Rambeloarisoa *et al.* (1984), Goutx *et al.* (1987 & 1990), Sorokhoh *et al.* (1990), Schulz *et al.* (1991), Porembe *et al.* (1991) and Porembe (1993).

Apart from mechanical devices to combat hydrocarbon pollution, chemical dispersants, emulsifiers, surfactants etc. are also widely used. But the toxicity and poor degradability of these compounds have turned the attention of researchers to biosurfactants produced by microorganisms. It has been established that biosurfactants have stimulatory effect

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on oil degradation. Biogenic surfactants (emulsifiers) have proven environmental compatibility (Bernem, 1984; Lang *et al.*, 1986).

Considering all these, a study has been initiated on the production of emulsifying agents (EAs) by marine yeasts which are ideally suited for effective degradation of hydrocarbons spilled in marine environments. This will save the living marine resources from the toxic effects of hydrocarbon pollutants. In this study 17 marine yeast isolates obtained from the Central Indian Ocean Basin and EEZ of India were screened for their capacity to produce emulsifiers at different time intervals when grown in the presence of different hydrocarbons individually.

Materials and Methods

Yeast isolates were taken from the stock cultures maintained at the Regional Centre of National Institute of Oceanography, Cochin. They were identified according to Kreger van Rij (1984). Seventeen yeast isolates were screened for their production of EAs in fortified sea water medium (Sorokhoh *et al.*, 1990) with 1% diesel in one batch and 1% kerosene in the other batch and they were grown in culture of flasks on a rotary shaker at 95 r.p.m. at $20 \pm 1^\circ\text{C}$ for 4 days.

The cell free culture filtrates were tested for their opacity after 5 min mechanical shaking with 0.1% kerosene or 0.1% diesel for the corresponding batches. Depending on their opacity they were rated as ++++ to + (Shenoy & Karunasagar, 1990).

The promising cultures from the screening tests were selected. Inoculum was prepared as by Crow *et al.* (1980). The isolates were grown in Yeast Extract Nitrogen Base (YNB) with 1% glucose under rotary shaking at 110 r.p.m. and $26 \pm 1^\circ\text{C}$. After 48 h growth the cells were harvested by centrifugation, washed twice in sterile distilled water and resuspended in water to an optical density of 0.4 at 450 nm. This inoculum was used to inoculate media containing 1.0% (v/v) kerosene for kerosene batch and 1% diesel for diesel batch.

The optical density (O.D) was measured in Hitachi Model U-200-20 double beam spectrophotometer. For kerosene batch

NIOC-My* - 3, NIOC-My - 11, NIOC-My - 13, NIOC-My - 14, NIOC-My - 34 were tested. For diesel batch, NIOC-My - 6, NIOC-My - 11, NIOC-My - 13, NIOC-My - 34 were tested.

Emulsifying activity of the supernatant at 6000xg was measured according to the procedure followed by Goutx *et al.* (1987) modified as follows: 10 ml of the supernatant and 0.1 ml of kerosene or diesel, respectively were used. The stability of the emulsion for one hour was measured as O.D. at 610 nm.

The comparison of EAs production in the presence of three hydrocarbons were done with: (1) Kerosene (mixture); (2) Toluene (aromatic) and (3) Hexadecane (aliphatic). The potential cultures tested were NIOC-My - 3, My-11, My - 13, My - 14 and My - 34. Production of EAs was tested at 24 h, 48 h and 72 h of growth on rotary shaker. The procedure was the same as above except that three batches were run with 3 different hydrocarbons.

Results and Discussion

The details of production of EAs by the marine yeasts under study are shown in Table 1. As per Table 1, kerosene could induce production of EAs in 12 out of 17 isolates and diesel in 5. For kerosene, NIOC - My - 11 and My - 14 were excellent producers; My - 3, My - 13 and My - 34 were good; My - 1, My - 7 and My - 8 were moderate while My - 10 and My - 15 were poor. For diesel, My - 6, My - 11 and My - 14 were good producers of EAs while My - 13 and My - 34 were moderate.

Comparison of the ability of the yeast isolates to produce extracellular EAs in presence of kerosene and diesel at different phases of culture growth are given in Fig.1 and Fig.2 respectively. The graphs indicate that production of EAs in My - 3 and My - 14 for kerosene was highest at 9 h growth. O.D. was 1.34 and 0.94. At 12 h My - 13 showed its highest activity with 0.5 O.D. EAs for My - 11 and My - 34 were high from 48 h to 72 h. There was a rise in EAs production from 24 h to 48 h in most of the isolates.

* NIOC-My - National Institute of Oceanography, Cochin - Marine Yeast

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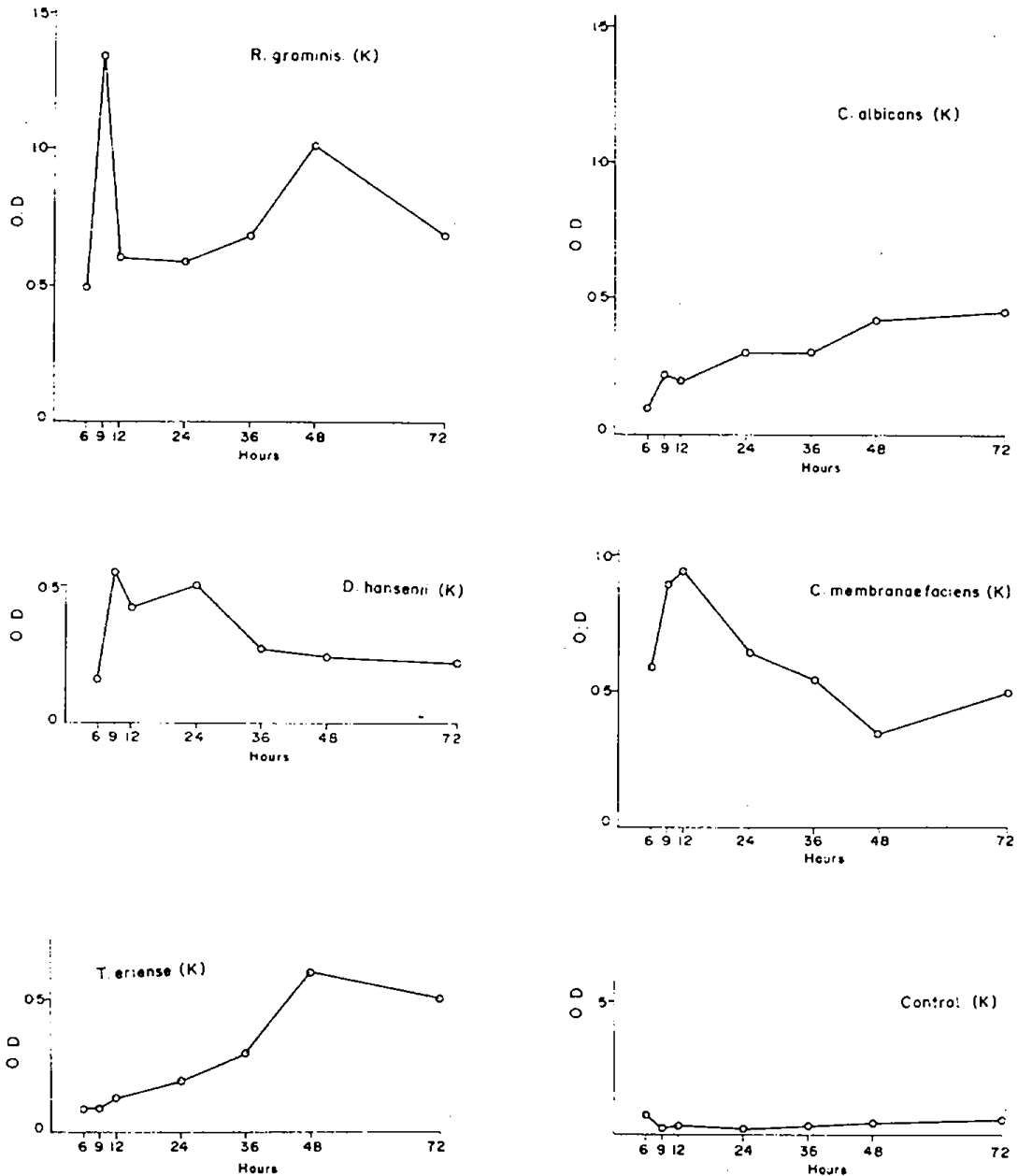


Fig. 1. Emulsifying activity (as O.D) of culture filtrates of 5 marine yeast isolates grown in medium containing kerosene. Samples were taken at different hourly intervals. Controls contained sterile medium with Kerosene. O.D - Optical Density, (K) Kerosene

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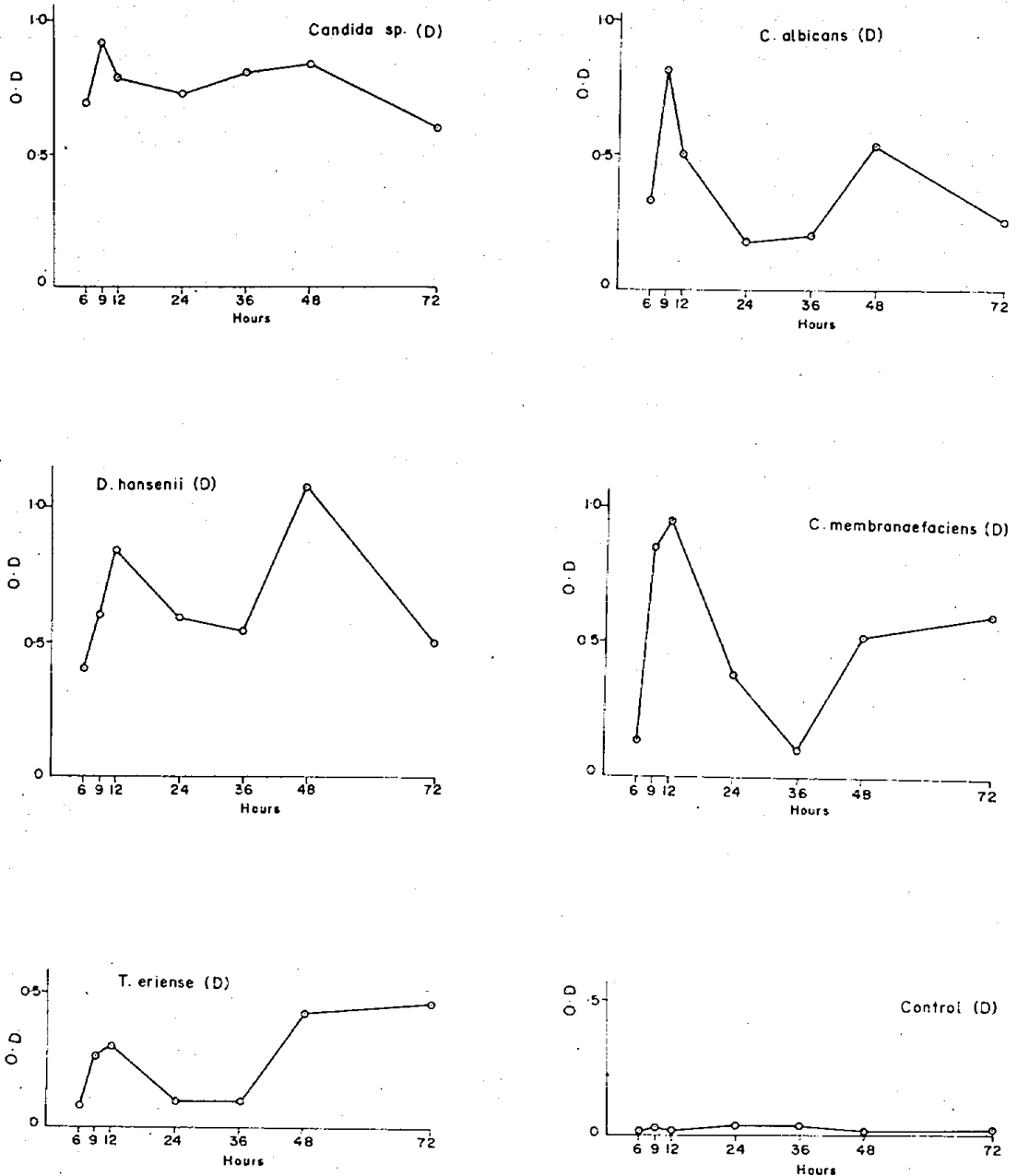


Fig. 2. Emulsifying activity (as O.D) of culture filtrates of 5 marine yeast isolates grown in medium containing diesel. Samples were taken at different hourly intervals. Controls contained sterile medium with diesel. O.D - Optical Density, (D) Diesel

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Table 1. Emulsifying activity of 17 marine yeast isolates after 4 days growth on shaker (95 rpm) at $20 \pm 1^\circ\text{C}$.

		Kerosene	Diesel	
1.	<i>Rhodotorula glutinis</i>	NIO.C-My-1	++	—
2.	<i>Rhodotorula minuta</i>	NIO.C-My-2	—	—
3.	<i>Rhodotorula graminis</i>	NIO.C-My-3	+++	—
4.	<i>Rhodotorula rubra</i>	NIO.C-My-4	—	—
5.	<i>Candida</i> sp.	NIO.C-My-6	+++	+++
6.	<i>Candida parapsilosis</i>	NIO.C-My-7	++	—
7.	<i>Candida parapsilosis</i>	NIO.C-My-8	++	—
8.	<i>Candida guilliermondii</i>	NIO.C-My-10	+	—
9.	<i>Candida albicans</i>	NIO.C-My-11	++++	+++
10.	<i>Rhodotorula</i> sp.	NIO.C-My-12	—	—
11.	<i>Debaryomyces hansenii</i>	NIO.C-My-13	+++	++
12.	<i>Candida membranaefaciens</i>	NIO.C-My-14	++++	+++
13.	<i>Rhodotorula</i> sp.	NIO.C-My-15	+	—
14.	<i>Rhodotorula rubra</i>	NIO.C-My-16	—	—
15.	<i>Trichosporon ericense</i>	NIO.C-My-34	+++	++
16.	<i>Ambrosiozyma monospora</i>	NIO.C-My-33	—	—
17.	<i>Cryptococcus</i> sp.	NIO.C-My-29	—	—

++++ Excellent growth, +++ Good growth, ++ Moderate growth, + Poor growth, — Nil growth. Nos. 13,14,15,16 and 17 were isolated from water samples from Central Indian Ocean Basin, the rest from EEZ of India.

In diesel the pattern was different: My - 6, My - 11, My - 13 and My - 14 exhibited equally high EAs production at 6 h, 9 h and 12 h, respectively, the O.D. being 0.92, 0.81, 0.84 and 0.95. My - 13 produced EAs more at 48 h, O.D. 1.08. After a lag period there was a rise in EAs at 48 h growth in the other isolates.

Controls of kerosene and diesel contained the uninoculated broth with 0.1% kerosene or 0.1% diesel. As in other cases, the O.D. was taken after 1 h stabilization of the emulsion.

Comparison of EAs production in the presence of 3 different hydrocarbons was carried out and the results are shown in Fig. 3.

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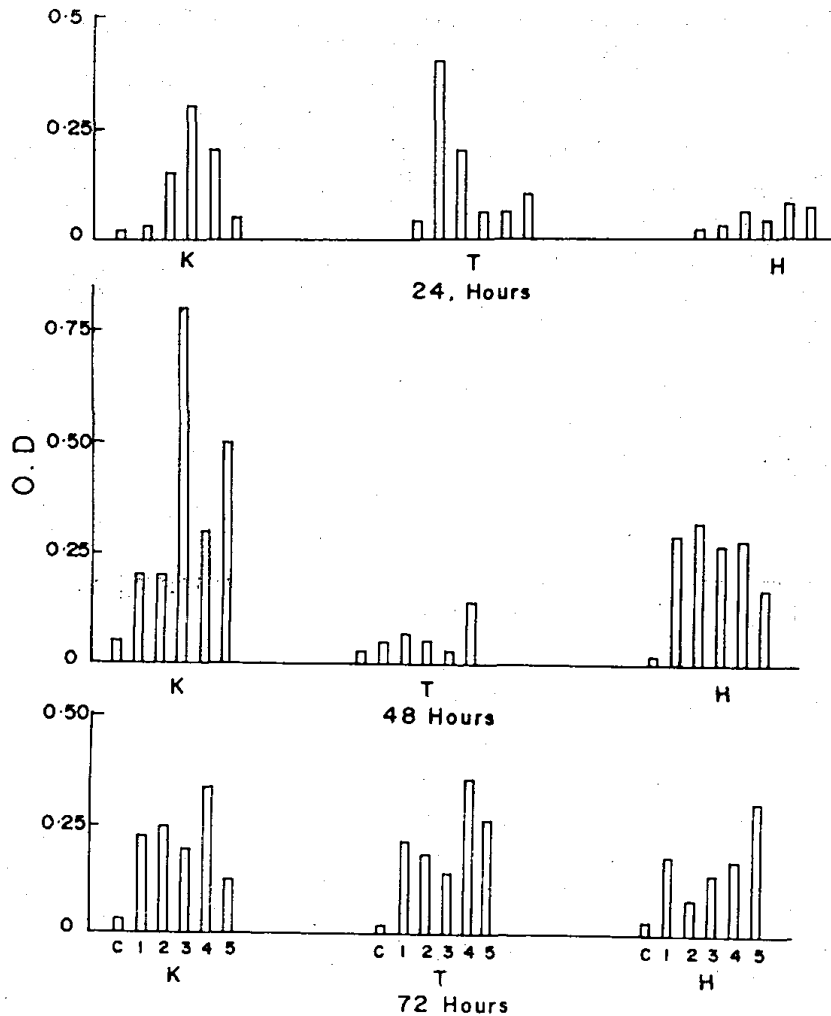
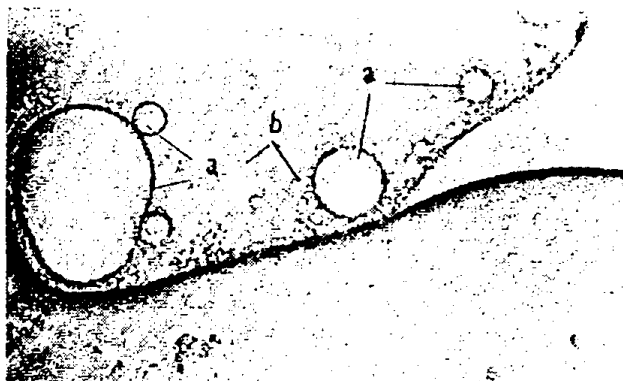


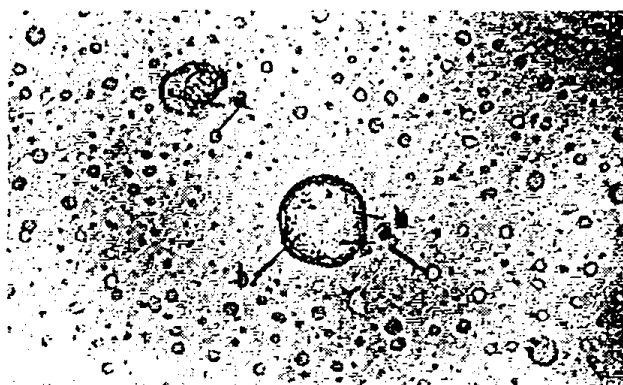
Fig. 3. Comparison of emulsifying activity (as O.D) of 5 marine yeasts on Kerosene, Toluene and hexadecane at 24 hours, 48 hours and 72 hours growth with the above hydrocarbons. C - Control, 1 - *Rhodotorula graminis*, My-3; 2 - *Candida albicans*, My-11; 3 - *Debaryomyces hansenii*, My 13; 4 - *Candida membranefaiens* My-14; 5 - *Trichosporon eriense*. My-34; K - Kerosene, T - Toluene, H - Hexadecane, C - Controls contained sterile medium with the corresponding hydrocarbon.

At 24 h growth the EAs production for kerosene was induced in My - 11, My - 13 and My - 14 to a reasonable extent. Toluene induced maximum production in My - 3, moderate in My - 11 and low in others. Hexadecane induced very low EAs production in all the yeast extracts.

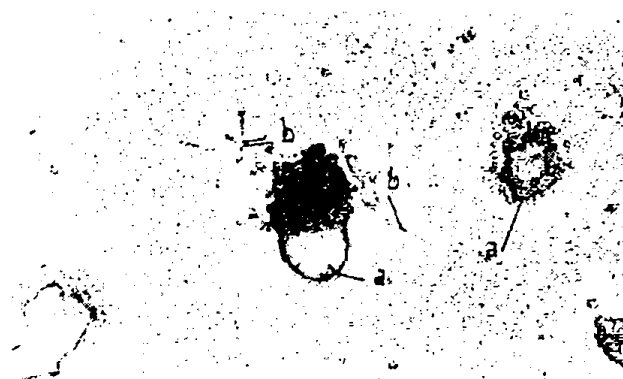
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A. Emulsified K in NIOC - My - 11 culture showing increased interfacial area on K (600 x magnification)



B Emulsion of K in NIOC - My - 3 culture



C Emulsion of D in NIO - My - 14 culture (400 x magnification)
a - oil droplets, b - yeast cells and pseudomycelia. Numerous emulsified droplets of K and D oils can be seen in B and C

Fig. 4. Photomicrograph of emulsions produced by marine yeasts in presence of kerosene (K) and diesel (D) in their culture broths.

At 48 h growth, EAs production in kerosene was highest in My - 13 followed by My 34. Others were also moderately active. Toluene induced only low EAs production in all except My - 34. Hexadecane could induce good EAs production in all.

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At 72 h growth, EAs production in kerosene was lower than that at 48 h. In toluene, all the isolates showed good EAs production. Hexadecane induced lower EAs production for all the isolates except My - 34 which was at its maximum production.

In this study, it is seen that a good number of marine yeasts are potent producers of emulsifying agents (surfactants) and the production was effective at different phases of growth for different hydrocarbons. *Trichosporon ericense* (NIOC - My - 34) was isolated from the water samples of the Central Indian Ocean Basin where the presence of hydrocarbon pollutants is extremely remote. Yet, strangely enough, it had the potential for good EAs production for all the 4 hydrocarbons tested.

The study indicates that some of the marine yeasts are potential producers of emulsifying agents (EAs) - the biosurfactants, effective on mixed (kerosene, diesel), aromatic (toluene) and aliphatic (hexadecane) hydrocarbons. The production of EAs was intermittent. Maximum production in most cases was at the early stages of exponential growth phase. A detailed study may be rewarding in view of the fact that EAs play an important role in stimulating biodegradation of hydrocarbon pollutants in aquatic environments - which is the ultimate aim of abatement strategies. These EAs produced by marine yeasts are important as it has been established that biogenic surfactants have much less toxicity and better degradability than the synthetic ones. As they are biogenic in origin, they have good environmental compatibility.

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