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Degradation of diesel oil by immobilized *Candida tropicalis* and biofilm formed on gravels

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Abstract The performance of diesel oil degradation by *Candida tropicalis* immobilized on various conventional matrices (sodium alginate, carboxyl methyl cellulose, chitosan) and biowaste materials (wheat bran, sawdust, peanut hull powder) was investigated using the method of entrapment and physical adsorption. The yeast species immobilized in wheat bran showed enhanced efficiency in degrading diesel oil (98%) compared to free cells culture (80%) over a period of 7 days. Copious amount of exopolysaccharides were also produced in the presence of diesel oil. The biofilm forming ability of *C. tropicalis* on PVC strips was evaluated using XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) reduction assay and monitored by scanning electron microscopy and atomic force microscopy. Yeast biofilm formed on gravels showed 97% degradation of diesel oil over a period of 10 days. The potential use of the biofilms for preparing trickling filters (gravel particles), for attenuating hydrocarbons in oily liquid wastes before their disposal in the open environment is suggested and discussed. This is the first successful attempt for 'artificially' establishing hydrocarbon degrading yeast biofilm on solid substrates.

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Introduction

Diesel oil is a toxic compound found as a component of petroleum hydrocarbons. It is a complex mixture of paraffins, cyclic alkanes and aromatic compounds having low water solubility, high adsorption coefficient and high stable aromatic ring (Van Hamme et al. 2006). It can cause biohazardous effects on human beings as well as other living organisms in the environment (Refaat et al. 2008). Diesel oil can bind moderately to aquatic sediment and soils thereby leading to soil as well as ground water contamination if released to land. There are several methods for the remediation of diesel contaminated sites such as mechanical, chemical and biological. Mechanical (e.g. skimming) and chemical (e.g. chemical surfactants and dispersantants) methods have limited effectiveness and can be expensive (Vieira et al. 2007). Biological methods like bioremediation gaining importance due to their simplicity, higher efficiency and cost effectiveness when compared to other remediation methods (Ojo 2006). Bioremediation mainly rely upon biodegradation, the natural ability of microorganisms to carry out the mineralization of pollutants and ultimately leading to the formation of CO_2, H_2O and biomass.

The use of native biomass (such as bacteria, yeast, fungi and algae) for degradation of toxic compounds in freely suspended state is limited owing to their inherent disadvantages such as small particle size, possible clogging and low mechanical strength of the biomass (Godjevargova et al. 2004). When immobilized microbial cells are used, the efficacy of biodegradation is often improved. Under many conditions, immobilized microbial cells have the following advantages in comparison with the freely suspended cells: (1) it can increase the biodegradation rate through a higher cell loading; (2) the bioprocess can be controlled more easily; (3) the continuous process can take place at a high dilution rate without wash-out; and (4) the catalytic stability of biocatalysts as well as the tolerance against toxic compound can be improved (Wang et al. 1997; Baskaran and Nemati 2006). In addition, immobilized microbial cells offer several potential advantages over non-immobilized cells for the bioremediation of anthropogenic wastes, because the immobilized cells are less likely to be adversely affected by predators, toxins or parasites, in comparison with free cells (Sathesh Prabua and Thatheyus 2007). Biodegradation using immobilized cells has been widely investigated for numerous toxic compounds (Wang and Qian 1999; Wang et al. 2002).

Biofilm is a kind of immobilization of microorganisms in a solid matrix and can be applied for bioremediation of waste waters. During the last few decades, biofilm reactors have become a focus of interest for researchers in the field of bioremediation for xenobiotic compounds, including hydrocarbons. Hydrocarbon-degrading microbial consortia (bacteria and microalgae) in biofilms naturally coating gravel particles in the intertidal zone of the Arabian Gulf coast was reported (Radwan and Al-Hasan 2001). So far, there is no report regarding the application of yeast biofilm for degradation of hydrocarbons.

Diesel oil is an excellent model for studying hydrocarbon degradation since it contains variety of hydrocarbons. There are reports on degradation of diesel oil using free cells of yeast (Ilori et al. 2008; Miranda et al. 2007) while no report is available using immobilized yeast species as potent degrader of diesel oil. Therefore, in the present study, the yeast species, *Candida tropicalis* isolated from petroleum hydrocarbon contaminated soil was immobilized in different matrices and used for degradation of diesel oil. The application of yeast biofilm towards remediation of diesel oil has also been examined.

Materials and methods

Materials

Diesel oil used in this study was obtained from local petrol pump, Tamil Nadu, India was filter sterilized and used throughout the studies. Immobilization matrices viz. carboxymethyl cellulose, sodium alginate, chitosan were purchased from Sigma-Aldrich (USA) and biowaste material i.e. wheat bran was collected from local market Vellore, India. Peanut hull and sawdust were obtained from respective mills from Vellore.

Yeast and growth conditions

Candida tropicalis was isolated from petroleum hydrocarbon contaminated soil from Haldia Petrochemicals, Kolkata, India. The yeast was phenotypically characterized and identified to the species level by Vitek 2 Compact Yeast card reader with the software version V2C 03.01 from Council for Food Research and Development (CFRD), Kerala, India. The isolate was maintained in Bushnell Haas (KH_2PO_4, 1 g l^{-1}; K_2HPO_4, 1 g l^{-1}; NH_4NO_3, 1 g l^{-1}; $FeCl_3$, 0.05 g l^{-1}; $CaCl_2{\cdot}2H_2O$, 0.02 g l^{-1}; and $MgSO_4{\cdot}7H_2O$, 0.2 g l^{-1}; Miranda et al. 2007) agar slants with 2% diesel as sole source of carbon. The broth culture was prepared in Bushnell Haas medium containing 2% diesel, pH maintained at 7.5 and incubation temperature at 35°C.

Immobilization of *Candida tropicalis* in different matrices

Preliminary experiments were performed to screen the different immobilization matrices for efficient degradation of diesel oil. Preparation of beads using immobilization matrices like CMC and sodium alginate were done following the methodology reported by Gummadi et al. (2009) with minor modifications. Chitosan beads were prepared following the method of Barreto et al. (2010) with minor modifications. Viability of yeast cells immobilized in

beads was estimated intermittently for a period of 10 weeks by total plate count method.

Carboxyl methyl cellulose

Carboxyl methyl cellulose (CMC) solution of 100 ml (2%) was thoroughly mixed with yeast suspension at a concentration of about 1×10^8 cells ml^{-1}. The CMC-yeast mixture was injected drop-wise to $FeCl_3$ solution (0.05 M) using an injector to form beads. The yeast immobilized beads were cured in the $FeCl_3$ solution for 1 h to enhance the mechanical stabilities.

Sodium alginate

Sodium alginate solution of 100 ml (2%) was prepared and mixed with yeast suspension at a concentration of about 1×10^8 cells ml^{-1}. The sodium alginate–yeast mixture was gently dropped into 0.2 M $CaCl_2$ solution. The beads were cured in 0.2 M $CaCl_2$ solution for 2 h to enhance their mechanical stabilities.

Chitosan

Yeast cells (1×10^8 cells ml^{-1}) were transferred to 30 ml of sterilized 2% chitosan solution prepared in 1% acetic acid at pH 7.5. This mixture was added drop-wise through a 1.0 ml plastic tip into an 8% NaOH solution for coagulation and formation of the beads. To control the size of the beads, a constant height from the solution to the plastic tip was maintained. After 30 min, the beads were separated from the solution and washed twice with 200 ml of sterile water for 15 min three times under agitation for inoculation.

Sawdust, wheat bran and peanut hull powder

Sawdust, wheat bran and peanut hull carrier materials (0.3 g) were sterilized by autoclaving at 15 lbs for 15 min. Each vial containing the sterile carrier material was inoculated with 1 ml cell suspension (16 mg dry wt ml^{-1}) in 10 mM phosphate buffer prepared from 24 h yeast extract peptone dextrose (YEPD) broth culture. Vials were incubated for 4 h and its contents washed by resuspending the carrier cultures in 2 ml of Bushnell Haas medium and the supernatants were drained. This was to remove the cells which were not sufficiently adhered to the carrier surfaces. Subsequently, the vials containing the carrier cultures were incubated at 35°C for various periods (4, 24 and 96 h) to determine the optimum incubation period necessary for the development of a well-established (immobilized) culture on the carriers. After each incubation period, replicate cultures were withdrawn and dried in a sterile hood in a stream of dry air at room temperature for 4 days and viability of dried carrier cultures in room temperature were tested during different time intervals over a period of 10 weeks by total plate count technique.

Screening of different matrices for diesel degradation compared to free cells

To screen the best matrix for immobilization, *Candida tropicalis* immobilized on various matrices were transferred into 50 ml of Bushnell Haas medium containing 2% diesel oil and incubated at 35°C on a rotary shaker at 120 rpm. At regular time intervals, samples were collected and degradation was monitored by gas chromatographic (GC) analysis. The above procedure was also followed for studying the biodegradation of diesel oil by free cells by inoculating free *Candida tropicalis* (1×10^8 cells ml^{-1}) to the Bushnell Haas medium containing 2% diesel oil and incubated under same conditions stated above.

Analytical methods for diesel oil analysis

Diesel oil from culture broth was extracted using hexane as the solvent. A control system was incubated with substrates without immobilization of yeast to monitor abiotic losses of the diesel substrate. Hexane extracts (1.0 µl) were analyzed with Hewlett Packard 5890 Series II Gas Chromatograph equipped with flame ionization detector (FID) and 30 m long HP-5 column (internal diameter, 0.25 mm, film thickness, 0.25 mm). The carrier gas was nitrogen. The injector and detector temperatures were maintained at 250 and 350°C, respectively. The column was programmed at an initial temperature of 70°C which was held for 2 min, then ramped at 10°C min^{-1} to 320°C and again held for 10 min. The degradation of diesel was expressed as the percentage of diesel degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples. The biodegradation efficiency (BE)

was calculated based on the decrease in the total concentration of hydrocarbons using the expression described by Michaud et al. (2004):

$$\text{BE}\ (\%) = 100 - \left(\frac{\text{As} \times 100}{\text{Aac}}\right)$$

where As is the total area of peaks in each sample; Aac the total area of peaks in the appropriate abiotic control; and BE is the biodegradation efficiency.

Growth study of free and immobilized yeast cells in diesel oil

The beads were separated from the medium at regular time intervals and washed twice with 200 ml of sterile water for 15 min three times under agitation. The collected washing solutions were analyzed by viable cell count. The carrier cultures in biowaste materials like sawdust, wheat bran and peanut hull powder were suspended in 10 ml sterile normal saline water and left to rehydrate for 2 min. Subsequently, each suspension was agitated vigorously on a vortex mixer for 10 min to suspend the adhered cells. Aliquots containing 0.1 ml were plated on YEPD agar plates and incubated at 30°C for 72 h before final counts were taken. The viability count of free cells were estimated by directly plating aliquots from media onto YEPD agar plates and incubated at 30°C for 72 h.

Biosurfactant production

The surfactant production was monitored by the emulsification assay proposed by Cooper and Goldenberg (1987). Briefly, 2 ml of cell-free supernatant, and equal amount of diesel oil were vortexed for 2 min. After 24 h, the height of emulsion layer was measured. The emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100. All tests were run in triplicate and repeated twice.

Biofilm formation

Biofilm was formed on PVC strips (1 cm^2), coated with albumen and diesel oil as well as on non coated strips. All the strips were dipped in 1 ml of culture with 5×10^8 CFU ml^{-1} of 72 h grown *Candida tropicalis* and placed for 90 min of adhesion phase at 37°C. The PVC strips were then washed with sterilized phosphate buffer saline (PBS) to remove loosely adherent cells. One millilitre of Bushnell Haas medium was added to the washed pieces and incubated at 37°C for 72 h. The biofilm thus formed was then quantified using XTT reduction assay (Lal et al. 2010) from standard graph with known viable cells versus OD_{492} nm. Biofilm formation by yeast species was monitored by scanning electron microscopy (FEI Sirion, Eindhoven, Netherlands) and atomic force microscopy (NTEGRA; NT-MDT, Moscow, Russia).

Scanning electron microscopy

Scanning electron microscopy (SEM) was prepared in accordance with the procedure given by Hawser and Douglas (1994). Briefly, yeast biofilms formed on PVC pieces (1 cm^2) were fixed with 2.5% glutaraldehyde in phosphate buffer for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h and washed with distilled water. The samples were dehydrated in ethanol series (50, 80, 90 and 100%). All samples were dried to critical point by polaron critical point drier, gold coated and viewed under SEM.

Atomic force microscopy

Images of the biofilms formed by yeast species were obtained using commercial atomic force microscopy (AFM). All images were collected in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 Nm^{-1}. The cantilevers had an amplitude range of 5–15 nm, tip radium of 10 nm and cone angle of 22°. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz (Lal et al. 2010).

Formation of biofilm on gravels

Biofilm was also formed artificially on gravels by immersing the gravels in YEPD medium for a period of 30 days. After the 30 days of incubation, the biofilm formed on gravels were washed with phosphate buffer to remove the loosely attached cells. The washed gravels were then immersed in 50 ml

sterilized Bushnell Haas medium containing 2% diesel for a period of 10 days and diesel oil was extracted for GC analysis as described before.

Results and discussion

Development of immobilized cells and viability

Candida tropicalis was immobilized in various substrates. The viability of dried carrier cultures in biowaste materials like wheat bran, sawdust and peanut hull powder was checked at room temperature. The cells immobilized in wheat bran were found to retain the maximum viability over a period of 10 weeks (Fig. 1a). Viability of yeast cells immobilized in sodium alginate, CMC and chitosan was also checked over a period of 10 weeks. The yeast cells immobilized in chitosan was found to be more stable and retained maximum viability compared to sodium alginate and CMC (Fig. 1b).

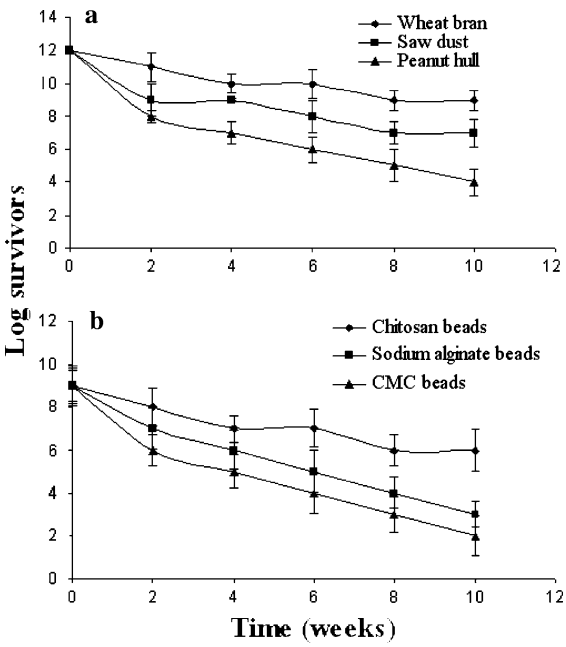


Fig. 1 Time-course of the changes in the viable number of *Candida tropicalis* immobilized in **a** biowaste materials and **b** beads during storage at room temperature for a period of 10 weeks

Screening of different matrices for diesel degradation

Diesel oil degradation by immobilized *Candida tropicalis* in different matrices were performed in batch cultures. Table 1 showed that yeast species immobilized in wheat bran showed maximum diesel oil degradation (98%) over a period of 7 days compared to free cells. The data was supported by the reduction in the GC peaks of diesel oil (data not shown). In GC analysis of diesel oil (control), 26 peaks of *n*-alkanes (C9–C26) and branched alkanes such as 2,6,10,14-tetramethyl pentadecane (pristine) and 2,6,4,10-tetramethyl hexadecane (phytane) were detected. In test system, the yeast species degraded all the hydrocarbons extracted with hexane from diesel oil after the incubation period of 5th and 7th day. Rapid reduction in peaks and disappearance of some peaks could be observed after 7th day of degradation of diesel oil by *Candida tropicalis*. Abiotic loss of diesel oil components was measured at the time of last extraction from control flasks, which was 2–3% within 7 days. In our previous study using free cells of yeast *Trichosporon asahii*, diesel oil degradation was found to be 95% over a period of 10 days (Chandran and Das 2010). Whereas the present work using immobilized yeast, *Candida tropicalis* on wheat bran, degraded diesel oil 98% within a period of 7 days. Therefore, in the present study we report for the first time that it has been possible to develop self immobilized yeast cultures of *Candida tropicalis* in wheat bran having the potential for application as ready-to-use seeds towards diesel oil bioremediation.

Growth study of free and immobilized yeast cells in diesel oil

Growth of immobilized yeast species in different matrices were monitored by total plate count method. Growth of yeast species immobilized in different matrices was found to be increased over a period of 7 days (Table 1). *Candida tropicalis* immobilized in wheat bran grew more rapidly than immobilized in other substrates. This also proved that wheat bran provided suitable support for the growth of yeast species on diesel containing media.

Table 1 Biosurfactant production, growth and diesel oil degradation potential of free cells and immobilized *Candida tropicalis* in different substrates

	E_{24} index (%)		TPC (CFU/ml)		Diesel degradation (%)	
	5th day	7th day	5th day	7th day	5th day	7th day
Free cells	65 ± 0.8	72 ± 0.9	2.4×10^8	5.5×10^8	73	80
Immobilized cells						
Immobilization matrices						
Sodium alginate	70 ± 0.9	79 ± 0.6	4.6×10^8	8.5×10^8	67	72
CMC	69 ± 0.9	72 ± 0.8	3.5×10^8	6.0×10^8	58	67
Chitosan	75 ± 0.8	80 ± 0.7	4.4×10^7	8.8×10^7	72	78
Sawdust	85 ± 0.7	92 ± 0.6	5.9×10^8	9.8×10^8	88	90
Wheat bran	89 ± 0.8	98 ± 1.2	6.7×10^8	7.2×10^9	94	98
Peanut hull	80 ± 0.9	82 ± 1.5	5.5×10^7	6.9×10^8	85	88

Biosurfactant emulsification activity

Biosurfactant produced by *Candida tropicalis* could effectively emulsify and stabilize emulsions with diesel oil (Table 1). The formed emulsion was found to be stable at room temperature for a period of one month without changing emulsifying activity. Emulsification of hydrocarbons appeared to have positive effect on degradation rate (Amund and Adebiyi 1991).

Biofilm formation

The adherence and subsequent biofilm formation by *Candida tropicalis* on PVC strips, coated with diesel and albumin as well as non coated were studied using XTT reduction assay. The biofilm formation was found to be more without any adhesive or pre coatings in PVC strips (Table 2). The production of the soluble colored formazan salt from biofilm forming yeast cell on non coated PVC strips was a direct reflection of cellular metabolic activity which increased with time (Fig. 2). The sequence of biofilm formation in different time intervals (24, 48 and 72 h) and exopolymer production (biosurfactant) could be clearly seen in SEM images (Fig. 3a–c) which might be the important mechanism of adhesion of yeast cells to an inert material to form biofilm. Increase in biofilm formation was also observed in AFM after 24 and 72 h showing thick biofilm formation (Fig. 4a, b).

It was possible to establish thick and mucilaginous yeast biofilm on gravels after 30 days of incubation. The degradation of diesel oil (97%) in Bushnell Haas medium was noted by biofilm formed on gravels on 10th day. GC analysis showed significant peak

Table 2 Estimation of biofilm on PVC strips non coated and coated with albumen and diesel oil by XTT reduction assay

PVC strip	Biofilm estimation	
	Initial (0 h)	Final (72 h)
Non coated	4.45×10^4	1.8×10^8
Coated with albumen	2.5×10^4	2.3×10^6
Coated with diesel	4.8×10^4	1.76×10^7

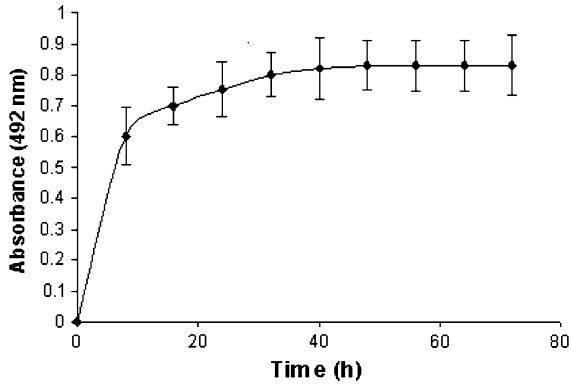
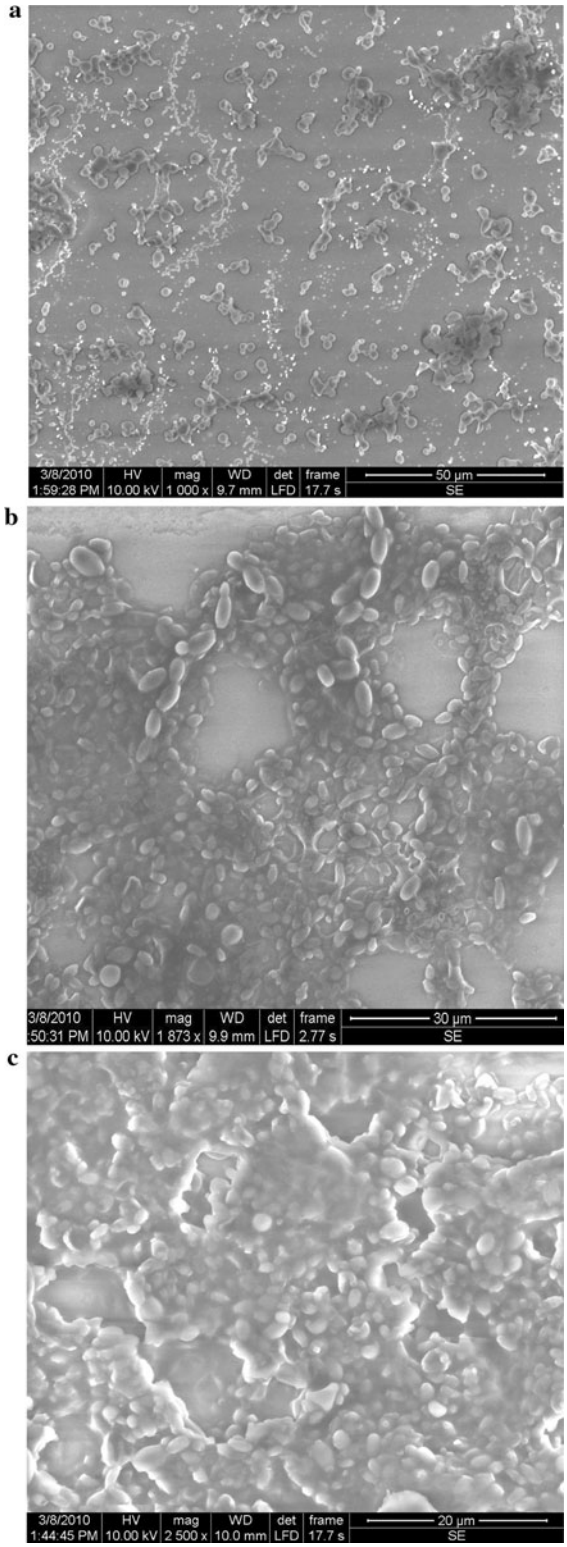


Fig. 2 *Candida tropicalis* biofilm formation on PVC strips (non coated) as determined by XTT reduction assay



◀ **Fig. 3** SEM images showing sequential biofilm formation by *Candida tropicalis* in PVC strips during different time intervals **a** 24 h, **b** 48 h and **c** 72 h. Production of exopolymers can be clearly visible on 72 h as sticky colorless matrix adhering all yeast cells

reduction compared to control suggesting the degradation of all hydrocarbons extracted with hexane from diesel oil by the biofilm (data not shown). Thus the present study showed that the yeast biofilm systems could efficiently degrade diesel oil in liquid wastes within short period of time. Biofilm systems appeared ideal for the degradation of xenobiotics because of their reported advantages over planktonic cultures. Most microorganisms that have the ability to degrade xenobiotic compounds have comparatively slow growth rates, and biofilm reactors allow the enrichment of these microorganisms independent of hydraulic retention times (Wobus et al. 1995). It has been shown in numerous studies that biofilms are less susceptible than suspended bacteria to changes in environmental conditions such as temperature and pH and the presence of metabolic products and toxic substances (Wobus et al. 1995; Ohandja and Stuckey 2006). The high cell concentrations that could be achieved in biofilm systems in combination with high volumetric flow rates could potentially result in high biodegradation rate without the risk of cell washout.

Conclusions

By using the natural ability of the yeast species *Candida tropicalis* to attach to surfaces, the use of extraneous chemical substances in immobilization has been avoided and, therefore, presents a potentially low cost-technology, cheaper option to chemical immobilization. The extensive exopolysaccharide formation (biosurfactant) would protect the cells from environmental chemical toxicity. Since the 'artificially' formed yeast biofilms could efficiently degrade the diesel oil under laboratory condition, they can also play an important role in the bioremediation of liquid wastes containing hydrocarbons.

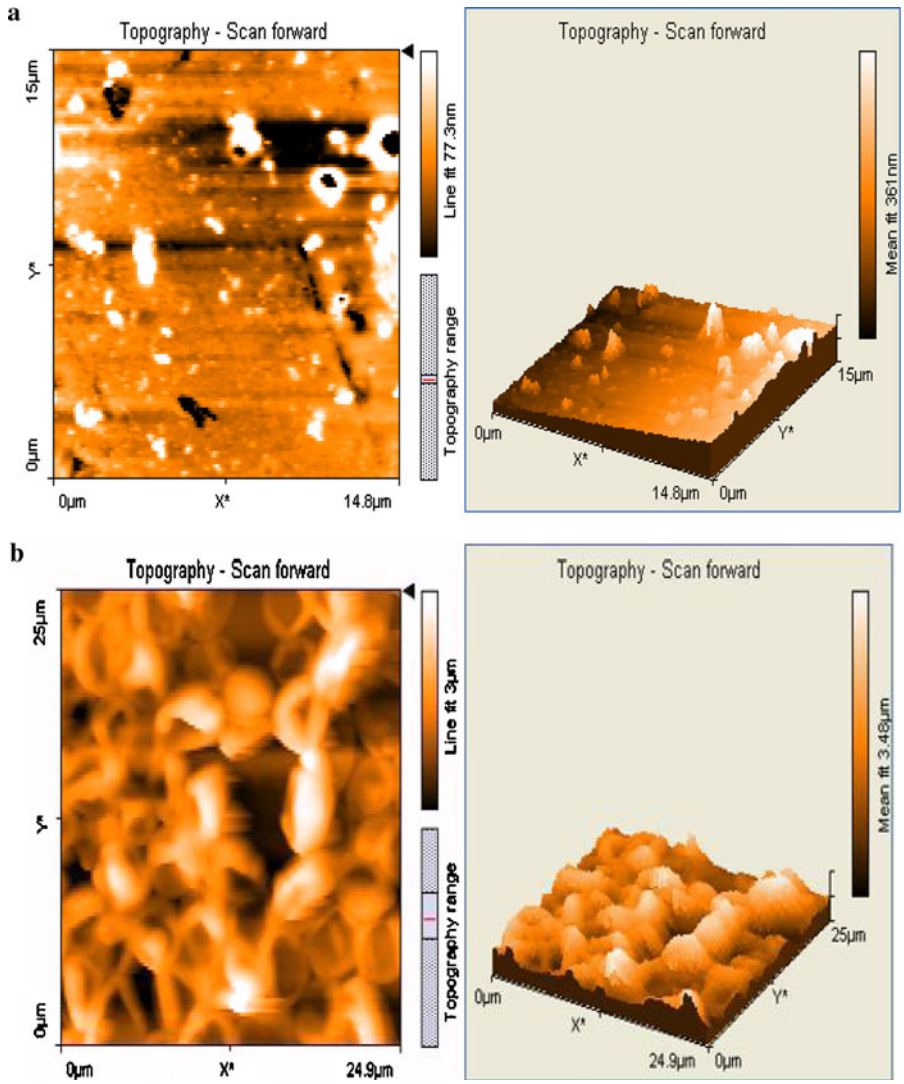


Fig. 4 AFM images of *Candida tropicalis* biofilm formation in PVC strips during 24 h (**a**) and 72 h (**b**) of growth

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