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Crude oil biodegradation aided by biosurfactants from *Pseudozyma* NII 08165 or its culture broth

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HIGHLIGHTS

- Biosurfactants assisted crude oil biodegradation using *Pseudozyma* biosurfactants.
- Hydrocarbon degradation by *Pseudozyma* sp. NII 08165.
- Improved crude oil degradation with addition of *Pseudozyma* culture broth.

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ABSTRACT

The aim of this work was to evaluate the biosurfactants produced by the yeast *Pseudozyma* sp. NII 08165 for enhancing the degradation of crude oil by a model hydrocarbon degrading strain, *Pseudomonas putida* MTCC 1194. *Pseudozyma* biosurfactants were supplemented at various concentrations to the *P. putida* culture medium containing crude oil as sole carbon source. Supplementation of the biosurfactants enhanced the degradation of crude oil by *P. putida*; the maximum degradation of hydrocarbons was observed with a 2.5 mg L^{-1} supplement of biosurfactants. Growth inhibition constant of the *Pseudozyma* biosurfactants was 15 mg L^{-1}. It was interesting to note that *Pseudozyma* sp. NII 08165 alone could also degrade diesel and kerosene. Culture broth of *Pseudozyma* containing biosurfactants resulted up to ~46% improvement in degradation of C10–C24 alkanes by *P. putida*. The enhancement in degradation efficiency of the bacterium with culture broth supplementation was even more pronounced than that with relatively purer biosurfactants.

1. Introduction

Oil spillage is a major environmental problem with a serious health and environmental consequences and bioremediation is one of the effective clean-up strategies implemented for the restoration of oil polluted environments. Bioremediation makes use of the enzymatic capabilities of hydrocarbon degrading microbes, where the hydrocarbons is degraded to water and CO_2 by mineralization (Atlas, 1991). The bioavailability of hydrocarbon is the major challenge in bioremediation of recalcitrant hydrocarbons. Surfactants are the powerful tool for overcoming the low bioavailability of the pollutants. It has been found that the addition of surfactants reduces the interfacial tension, increases the emulsification of hydrophobic pollutants, improves the solubility of hydrocarbon, and thus, enhances the bioavailability of hydrophobic pollutants (Collina et al., 2007). Kaczorek and Olszanowski (2011) found that natural surfactants such as rhamnolipids and sophorolipids were more effective in promoting hydrocarbon degradation, when compared to chemical surfactants such as Triton X-100.

Several studies have demonstrated the efficacy and performance of biosurfactant assisted bioremediation (Banat, 1995). Rhamnolipids supplementation improved the bioremediation of crude oil contaminated soil with reduction of total petroleum hydrocarbon up to 86.97% (Zhang et al., 2011). Apart from the solubilization of hydrocarbons, biosurfactants can also change the surface properties of microbial cells, which leads to the attachment of hydrocarbon to bacteria and a consequent increase in hydrocarbon utilization. The *in-situ* bioremediation of hexadecane in a saturated sand column was promoted by the addition of low level of rhamnolipids (Herman et al., 1997). Supplementation of rhamnolipids at 15 mg L^{-1} concentration improved the efficiency of polyaromatic hydrocarbons (PAH) removal and soluble COD reduction to 90% and 99%, respectively indicating its potential to treat the waste water abundant in polyaromatic hydrocarbons (Sponza and Gok, 2010). Rhamnolipids affect the energy-dependent transport of hydrophobic compounds by *Pseudomonas aeruginosa* (Noordman and Janssen, 2002).

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Sophorolipids, the glycolipid biosurfactants produced by *Candida bombicola*, have been also reported for enhancing the biodegradation of crude oil where it resulted 80% biodegradation of saturates and 72% of aromatics (Kang et al., 2009). Challenges in soil bioremediation of phenanthrene such as longer bioprocess and residual pollutants can be avoided by the addition of surfactants such as sophorolipids (Schippers et al., 2000). Moran et al. (2000) reported that surfactin, produced by *Bacillus subtilis* could be used to promote the biodegradation of hydrocarbon waste by the indigenous microbial consortium. Whang et al. (2008) studied the effectiveness of rhamnolipids and surfactin to improve the diesel solubility, biomass growth and biodegradation efficiency. Similarly, trehalose lipids were proved to enhance the solubility and biodegradation of phenanthrene, which made them useful in the bioremediation of sites contaminated with polyaromatic hydrocarbons (Chang et al., 2004). Biosurfactants usually have an adverse effect on bioremediation when used at high concentration due to the antimicrobial activity. Hence, for enhancing hydrocarbon degradation, the optimal concentration of biosurfactant needs to be determined.*Pseudozyma* sp. NII 08165, a novel yeast isolate was capable of producing biosurfactants, which included mannosylerythritol lipids (MEL) A, B and C (Sajna et al., 2013a). This study was aimed at determining the effect of biosurfactants from this yeast on hydrocarbon degradation by a model microbial culture, *Pseudomonas putida* MTCC 1194. *Pseudozyma* biosurfactants were purified; their surface activity was evaluated and the potential to aid the biodegradation of crude oil was studied. The optimal concentration that supported the maximum hydrocarbon degradation was determined.

2. Methods

2.1. Culture medium and crude oil

Bushnell Hass (BH) medium was used for al e hyd arbon degradation studies. It contained (g L^{-1} H_2PO K_2HPO_4 – 1.0, NH_4NO_3 – 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, 0.05 a $CaCl_2$ – 0.02 and an initial pH of 7.0. Y extract (g L^{-1}) was supplemented to the medium as t ganic nitro source. Crude oil (Persian Gulf grade was procured fro Kochi Refineries Ltd., Kochi, India.

2.2. Screening of *P. putida* ains

Five *P. putida* strains p from t Microbial Type Culture Collection (MTC ndiga ndia e screened to use as the model strain hyd arbon tion studies. These were *P. putida* MTC 190, *P. tida* MTC 1192, *P. putida* MTCC 1194, *P. putida* MT 273 *a* MTCC 102. Two more strains, *viz. P. putida* 0822, *P. putida* NBTC 0823 were also used. Screening was ased on microtiter plate based INT (p-iodonitrotetrazo violet) indicator assay (Haines et al., 1996). Seed cultures of the *P. putida* strains (grown on nutrient broth at 30 °C, 200 rpm for 12 h) were inoculated into BH medium and incubated at 30 °C for seven days. To a microtitre plate, 200 µl of the culture broth from each strain was added along with 100 µl INT indicator (HiMedia, India, 7.5 g L^{-1} prepared in distilled water) and was incubated for 3 h. Scoring was performed based on the intensity of the red precipitate formed.

2.3. Preparation of Biosurfactants

Pseudozyma sp. NII 08165 was inoculated into production medium containing (g L^{-1}) soybean oil – 40, $NaNO_3$ – 3.0, $MgSO_4{\cdot}7H_2O$ – 0.3, KH_2PO_4 – 0.3, yeast extract – 1, and was incubated at 30 °C for nine days (Morita et al., 2008). Biosurfactants were extracted from the culture broth by adding two volumes ethyl acetate. The ethyl acetate extract was subjected to silica gel column chromatography for purification of glycolipid biosurfactants. During the chromatography, lipids were removed by 100% chloroform elution, followed by 4:1 (v/v) ratio chloroform: ethyl acetate. Finally, the glycolipids were eluted with 100% acetone.

2.4. Measurement of surface activity of *Pseudozyma* biosurfactants

Surface tension of an aqueous solution of the biosurfactants preparation was determined at diff ntrations by Wilhelmy plate method using Wilhelm ype auto c tensiometer (Dataphysics DCAT 21) at 25 °C. al micelle centration (CMC) was then determined fro the k point o he surface tension *versus* the log of bulk centratio rve.

2.5. Effect of *Pseudozyma* surfact on crude oil degradation by *P. putida*

Seed culture *putida* (5%, inoculated into BH medium contain 2% oil. Purifie biosurfactants was added to the culture medium a concentrations 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 8 d 100 mg L H medium with the crude oil inoculate *P. putida* as abo but without biosurfactants supplem tion was taken as control. All the flasks were incubated at 30 for 15 days d the cell growth and utilization of the hydrocarb were ana ed. Cell growth was measured by determining the co form g unit (CFU) mL^{-1}. After completing the incubation, 100 µl of a 10^{-5} dilution was spread on nutrient agar plates olonies formed were counted after 12 h of incubation at de oil biodegradation was studied by measuring the peak ea of alkane components of crude oil, using GC–MS. and comparing with the control. The alkane fraction in the medium was extracted with the equal amount of n-hexane. BH medium containing 2% crude oil was used as the control. Percent degradation was calculated by following the equation.

$$\text{Percent degradation} = \left(1 - \frac{\text{Peak area of the hydrocarbon in the sample}}{\text{Peak area of the hydrocarbon in the control}}\right) \times 100$$

GC–MS analysis was done with a Shimadzu QP 2010 Gas Chromatograph Mass Spectrometer, fitted with Rxi®-5 ms column (Restek) with 30 m × 0.25 mm ID. The instrument conditions were the following: helium column flow 1 ml/min, pressure 18.89 psi and split ratio 25:0. Injection volume was 1.0 µl. Initial temperature was kept at 70 °C for 5 min with a temperature ramp of 14 °C min^{-1} and the final temperature of 280 °C was kept for 10 min with a total run time 30 min (Malatova, 2005).

2.6. Growth inhibition of *P. putida* by the *Pseudozyma* biosurfactants

The growth inhibition kinetics of *P. putida* was studied in glucose medium containing varying concentrations of biosurfactants. The specific growth rate was calculated by sampling the cells at regular time intervals. Linearized Haldane growth inhibitory equation was used for representing growth inhibition kinetics and determination of growth inhibition constant (Gottumukkala et al., 2014).

Specific growth rate, $\mu = ((\log_{10}Z - \log_{10}Z_0)\ 2.303)/(t - t_0)$

Z and Z_0 corresponded to the cell growth at time t and t_0, respectively.

Linearized Haldane equation, $1/\mu = 1/\mu_{max} + (I/K_i\mu_{max})$

Linear graph was plotted with $1/\mu$ *versus* initial inhibitor concentrations (S). Maximum specific growth rate (μ_{max}) was obtained

from the slope of the curve, and inhibition constant (K_i) was derived from intercept on *X* axis.

2.7. Hydrocarbon degradation by Pseudozyma

Hydrocarbon degradation by *Pseudozyma* sp. NII 8165 was studied by growing the yeast in BH medium containing diesel, kerosene and petrol as the carbon source at 30 °C, 200 rpm for 35 days. BH medium containing no carbon source, inoculated with the *Pseudozyma* sp. was taken as negative control. Sampling was performed at seven days interval to measure the cell dry weight. At the end of incubation, INT assay was performed as indicated above. Hydrocarbon utilization was studied by analysing the hydrocarbon using GC–MS.

2.8. Effect of Pseudozyma culture broth on hydrocarbon degradation by P. putida

Seed culture of *P. putida* (5%) was inoculated into BH medium containing 2% crude oil. Culture broth from a nine day old *Pseudozyma* fermentation was supplemented at 2% (v/v) to the above culture medium. Improvement in the degradation was studied by measuring the cell growth and hydrocarbon analysis.

2.9. Statistical analysis

All the values were presented as the mean ± standard deviation from at least two experiments. Student's *t*-test was performed to analyze the statistical significance. A probability level of $P < 0.05$ was considered statistically significant (Daniel, 2000).

3. Results and discussion

3.1. Screening of P. putida strains

For selecting a model hydrocarbon degr[illegible] strains were screened based on the IN[illegible]ssay. [illegible] the cultures screened gave a red precipitate in [illegible] INT assay. *[illegible]utida* MTCC 1194 was selected as the most p[illegible] hydrocarbo[illegible]grader as it gave an intense red precipita[illegible] in th[illegible] assay. *P. putida* NBTC 0522 and *P. putida* NBTC 0[illegible] gave mode[illegible] red precipitate and all other strains gave a s[illegible]t red precipitate[illegible]ults not shown). INT assay is a routine[illegible]ased s[illegible]ning method to determine the potential hydrocarbo[illegible]gra[illegible]g microbes. Malatova (2005) had employed microtitre ba[illegible]T assay[illegible] investigate the hydrocarbon degradati[illegible]ntial [illegible]icrob[illegible]isolates. In the field studies, INT can be[illegible]d as [illegible]liable a[illegible]cient method to measure overall mic[illegible]al activ[illegible] associate[illegible] with hydrocarbon degradation (Mathew[illegible] Q[illegible]

P. putida [illegible]idely used microbe for bioremediation due to its hydrocarbon [illegible]adation potential and bio-safety. Successful clean-ups of oil c[illegible]minated sites employing *P. putida* along with fertilizers have been reported (Raghavan and Vivekanandan, 1999). The bacterium possesses both alkane and polyaromatic hydrocarbon degradation pathways, and hence can utilize complex substrates such as crude oil.

3.2. Pseudozyma biosurfactants

A number of glycolipids are produced by *Pseudozyma*, which have isomeric structure, and hence require multi-step purification. Since bioremediation is a cost-sensitive method, bio-augmentation with pure metabolites does not seem economically feasible. Hence, total biosurfactants produced by *Pseudozyma* sp. NII 08165 were extracted and used to study the improvement in biodegradation.

Pseudozyma sp. NII 08165 was reported to be a good producer of glycolipid biosurfactants, including all the three isomers of mannosylerythritol lipids (MEL) along with unknown glycolipids. MEL-C, the major glycolipid produced by *Pseudozyma* sp. NII 08165 contained a non-conventional hydrophobic structure, with shorter chain fatty acids like C2 or C4 at the C-2′ position and long chain fatty acids such as C14, C16 and C18 at C-3′ position of the mannose moiety (Sajna et al., 2013a). MEL-C having this particular structure has been proposed to be highly water soluble, and hence of application in the formulation of water- in-oil type emulsifiers and detergent formulations (Morita et al., 2008). The potential of these glycolipid biosurfactants as la[illegible]itives have been reported by fabric wash analysis. [illegible]addition[illegible] that, they are highly active at high temperatu[illegible]d alkaline [illegible] (Sajna et al., 2013a).

3.3. Measurement of surf[illegible]activity [illegible]f Pseu[illegible] biosurfactants

The critical mic[illegible]le c[illegible]tion (CM[illegible]) of *Pseudozyma* biosurfactants was [illegible]g L^{-1} [illegible] surf[illegible] tension at CMC was 33.67 mN M^{-1}[illegible] 1]. CMC is [illegible]ificant parameter to be considered fo[illegible]e [illegible]ication of s[illegible]ctants in bioremediation as it indicates the rela[illegible] concentration of surfactant at which impro[illegible]ent in biod[illegible]dation can be observed. Hence, the conce[illegible]tion of biosurfacta[illegible]s to be added for the improving the b[illegible]emediation should be studied above and below CMC, which l[illegible]es in the [illegible]ge of 1–100 mg L^{-1}. *Pseudozyma* biosurfactants e[illegible]ited high[illegible]rface activity when compared to the reported CM[illegible]lues [illegible]hamnolipids and surfactin, which were 50 and 45 mg [illegible]espectively. The application of surfactant above the [illegible]ncentration range of its CMC could lead to improvement in the [illegible]diation by increasing the bioavailability of hydrocarbons. Solubility of petroleum hydrocarbons was improved remarkably by the addition of biosurfactants such as rhamnolipids and surfactin above their CMC. However, at higher concentration, biosurfactants may have an inhibitory effect on the bioremediation as they exhibit antimicrobial activity (Whang et al., 2008). Therefore, it is necessary to study the effect of biosurfactants on the bioremediation at different concentration of biosurfactants.

Zeng et al. (2011) studied the effect of monorhamnolipids on the degradation of hexadecane by *Candida tropicalis*, where the CMC of rhamnolipids was determined to be 38.0 mg L^{-1} and enhanced biodegradation was observed with the supplementation of 19.0 mg L^{-1} of rhamnolipids. Adsorption of rhamnolipids to the cell surface of polyaromatic hydrocarbon (PAH) degrading bacteria depend on the CMC of rhamnolipids. An increase in the

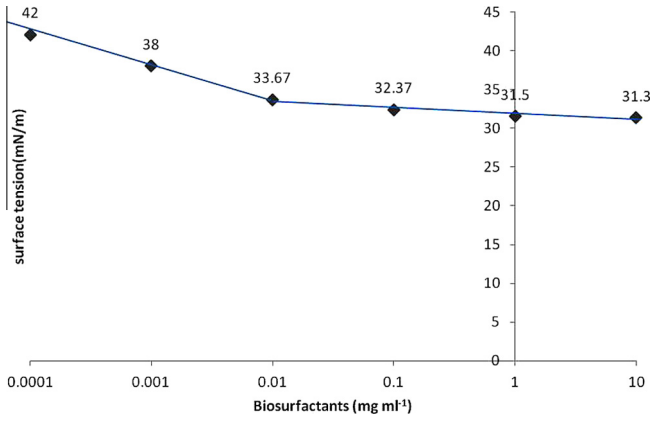

Fig. 1. Surface activity of *Pseudozyma* biosurfactants. CMC of *Pseudozyma* biosurfactants was 0.01 mg mL^{-1} (10 mg L^{-1}) and surface tension at CMC was 33.67 mN/m.

concentration of rhamnolipids resulted in increased adsorption until the concentration of rhamnolipids reached CMC, where the adsorption reached a plateau (Zhao et al., 2011). The addition of biosurfactants from *B. subtilis* below its CMC did not result in any increase in the bioremediation while bioremediation was stimulated by the addition of biosurfactants above CMC (Moran et al., 2000). Ron and Rosenberg (2002) reported that the addition of low molecular weight biosurfactants with low CMC value resulted in significant improvements in hydrocarbon solubility by incorporating them to the hydrophobic cavities of micelles.

3.4. Effect of *Pseudozyma* biosurfactants on crude oil degradation by *P. putida*

Efficiency in the degradation of crude oil was determined by measuring the cell growth and hydrocarbon utilization. Cell growth is indicative of hydrocarbon degradation as complete mineralization results in the formation of biomass, CO_2 and H_2O. The cell count of *P. putida* in control BH medium (without the addition of biosurfactants) was 2.6×10^6 CFU mL^{-1}. Highest cell count (10×10^6 CFU mL^{-1}), which is approximately 4× higher than the control was observed at a biosurfactants concentration of 2.5 mg L^{-1} and there was slight decrease in cell count with the increase in biosurfactant supplementation up to 10 mg L^{-1}. Cell count at 1, 2.5, 5 and 10 mg L^{-1} were 4.1×10^6, 10×10^6, 8×10^6 and 6.5×10^6 CFU mL^{-1} respectively. Thereafter, the cell count decreased rapidly with increase in biosurfactant concentration and no colonies were observed at the concentration of 100 mg L^{-1}. Cell count observed at the concentrations of 20, 40, 80 and 100 mg L^{-1} were 2.1×10^6, 9×10^5, 4×10^5 0 CFU mL^{-1} respectively, indicating the toxicity of biosurfactan higher concentrations.

Using GC–MS, crude oil utilization by *P. putida* in the presenc of *Pseudozyma* biosurfactants was studied. Fig. 2 e percentage degradation of alkanes present in the cr oil by *putida* with and without 2.5 mg L^{-1} biosurfactants pleme tion. A significant level of biodegradation of alkan obs on the addition of 2.5 mg L^{-1} urfacta ($P < 0.05$). Supplementation of the biosurfactan proved the adation of decane (26.7%), undecane (22.5 do ne (52.8%), decane (23.6%), tetradecane (20.2%), pentadecan 1.4%), hexadecane (10.9%), heptadecane (27.9 octadecane %), nonadecane (24%), icosane (19.7%), he osane (28.8%), doc ne (31.9%), tricosane (13.4%) and t cosan 3.2%). An average of 23.5% improvement in the on of C10–C24 alkanes was observed. Among the alkanes, dodecane was degraded most while tetracosane, followed by hexadecane was least degraded. The rate of degradation decreased with increase in the chain length of hydrocarbon. The preferential pattern of hydrocarbon utilization by the microbe when growing in a mixture of complex hydrocarbons suggested that lower chain molecules were utilized in the initial stage and higher chain alkanes towards the later stage of growth. Nevertheless, the rate of hexadecane utilization was low even though it was not a higher chain length alkane. The cleavage of carbon atoms during the degradation of higher chain alkanes might result in the accumulation of medium chain alkanes such as hexadecane, which could possibly e is observation. Both CFU determination and hydroca analy evealed that the addition of *Pseudozyma* biosurf ts at a co ntration of 2.5 mg L^{-1} improved the biodegradatio crude oil *P. putida.*

Most of the biosurfactants exhibit toxi t hig concentration, which affects the cell face properti etabolic pathways of microbes, leadi to the e ne in bi ass required for degrading the hydroc bo s hamp s the bioremediation. Since the CMC of *Ps ozyma* urfacta was 10 mg L^{-1} and the surfactant toxi was exh d concentrations above 10 mg L^{-1}, it s sible that formation of hydrocarbon micelles by *Pseudozy* biosurfactants were the reason for the decline in hydrocarbo gradation at higher concentrations. Micel ation may red the efficacy of bioremediation, since the queous phase concentration of the pollutants is reduced (Na nal Resear Council, 1993).

3.5. Growth inhibition kinetics of biosurfactants

kinetics of *P. putida* was studied by growing the organism in glucose containing medium at different concentrations of *Pseudozyma* biosurfactants (Fig. 3). Specific growth rate of *P. putida* t 1.0 or 2.5 mg L^{-1} supplementation of biosurfactants was almost similar to that of the bacterium grown without biosurfactants. However, specific growth rate decreased significantly at and above the concentration of 20 mg L^{-1} when it became negative. Plotting the linearized Haldane equation gave a line with the regression coefficient of 0.923. Growth inhibition constant (K_i) and maximum specific growth rate (μ_{max}) were 11.07 mg L^{-1} and 0.434, respectively. This indicated that the concentration of biosurfactants to be added should be less than 11.07 mg L^{-1}, and higher concentrations might negatively affect the growth of the organism and its hydrocarbon biodegradation efficiency.

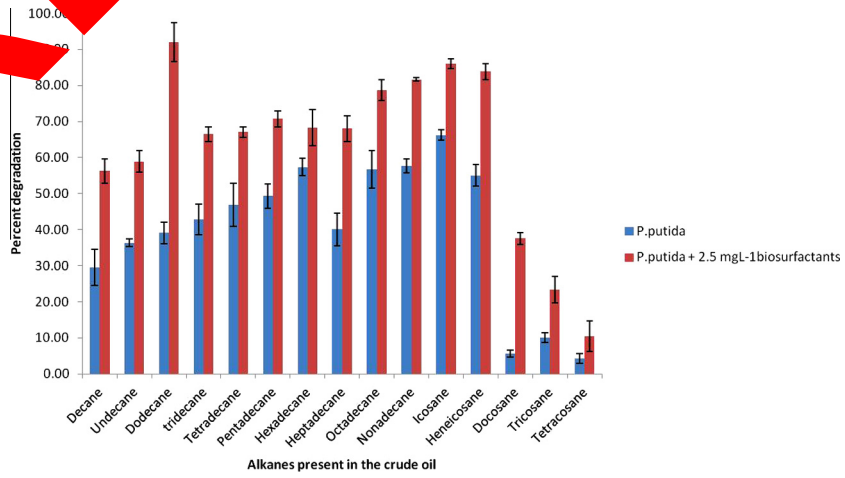

Fig. 2. Percent degradation of alkanes present in the crude oil by *P. putida* MTCC 1194 on supplementation of 2.5 mg L^{-1}biosurfactants.

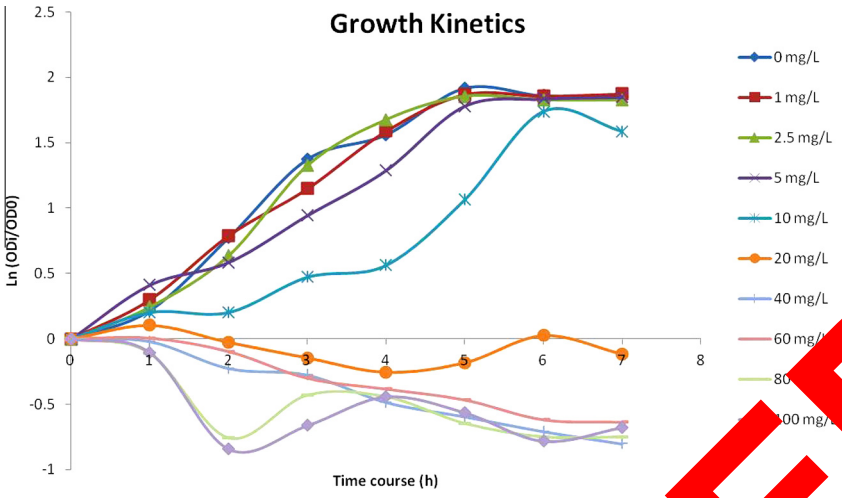

Fig. 3. Growth kinetics of *P. putida* MTCC 1194 at various concentrations of *P. ...dozy...* ...factants.

Many biosurfactants exhibit antimicrobial activity and the antimicrobial activity depend on the concentration of biosurfactant and type of strains used for bioremediation. Mannosylerythritol lipids A and B have antimicrobial activity against Gram-positive bacteria and *Pseudomonas* strains are relatively more sensitive to MEL than other Gram-negative bacteria (Kitamoto et al., 1993). Whang et al. (2008) reported that the addition of surfactin above 80 mg L^{-1} had an inhibitory effect on diesel biodegradation by a microbial consortium, which was attributed to cell disp... property of surfactin at higher concentrations. Similarly, the ... tion of rhamnolipids at a concentration of 240 mg L^{-1} had an ...i-bitory effect on the growth of two hydrocarbon degrading stra... thus preventing phenanthrene degradation by ...rganis... (Shin et al., 2005). Nevertheless, it is also kn... tha...osurfac... tants have significantly lower toxicity tha...emical ...rfactants (Lim et al., 2011).

3.6. Hydrocarbon degradation by Pse...yma sp. NI...65

Pseudozyma sp. NII 08165 gre... by fo... smaller emulsions of hydrocarbon in the mediu... containing d... and kerosene. Cell growth measurement o... *eudozyma* growi... different hydrocarbons is shown in ... 4. Hi... biomass yield was observed in the medium contai... di... followed by kerosene, and the growth was comparati... w in pe... Since *Pseudozyma* grew in diesel, wh... tained ... her... ain alkanes than petrol, the reduced g... h in ... mediu... taining petrol could be attributed to t... apid va...rization of petrol at 30 °C. INT assay was positive for ... the medium containing diesel and kerosene. G... analyzes revealed that in the medium with diesel, there was ... mplete utilization of lower chain alkanes (<C9) and a substantia... line in the medium chain alkanes with C9–C31, compared to the control (un-inoculated BH medium containing diesel). From these results, it was concluded that *Pseudozyma* sp. NII 0815 was capable of degrading the hydrocarbons.

Many yeast species have been reported to degrade hydrocarbon and some by producing biosurfactants. *C. antarctica*, during its growth on hydrocarbon produces biosurfactants, which alter the surface properties of microorganism and promotes the biodegradation of n-alkanes (Hua et al., 2003). However, *Pseudozyma* sp. NII 08165 did not secrete any biosurfactants during its growth on hydrocarbon, as no glycolipids spots were detected by the TLC. Kitamoto et al. (2011) had demonstrated the plastic degrading potential of *Pseudozyma antarctica* that could degrade plastic films made from *polybutylene succinate.*

3.7. Effect of ...yma culture ...n on hydrocarbon degradation by *P. putida*

...il was efficie... degraded by *P. putida* culture supplemented with the *Pseudozyma* culture broth. The cell count in *P. ...ida* culture ...plemented with the *Pseudozyma* culture broth ... 7.80×10... U mL^{-1}, whereas it was only 3×10^6 CFU mL^{-1} in ...n-sup...mented control. Microscopic observation revealed that t...oped colonies were *P. putida* only and there were no ...isible yeast cells. On plating also, the colonies developed were ...f *P. putida* and yeast colonies were not detected (results ...not shown). Percentage degradation of the alkanes in the crude oil by the *P. putida* cultures supplemented with or without *Pseudozyma* culture broth is shown in Fig. 5. GC–MS analyzes revealed that the supplementation of *Pseudozyma* culture broth improved the degradation of decane (48%), undecane (53.9%), dodecane (22.8%), tridecane (37.2%), tetradecane (32.9%), pentadecane (34.7%), hexadecane (27.4%), heptadecane (45%), octadecane (35.4%), nonadecane (34%), icosane (27.4%), heneicosane (34.8%), docosane (91.4%), tricosane (87.5%) and tetracosane (76.6%). This indicated that the addition of *Pseudozyma* culture broth resulted in improved degradation, even better than that observed for pure biosurfactants supplementation [$P < 0.05$]. On an average, 45.9% improvement in the degradation of C10–C24 was observed. Enhancement in the degradation could be attributed to an enhanced growth of *P putida* itself and the presence of biosurfactants in the *Pseudozyma* culture broth. The neutral lipids and fatty acids present in the culture broth could also have supported a higher biomass of *P. putida*. *Pseudozyma* culture broth might contain other metabolites that could aid the bioremediation; the yeast has been previously demonstrated to produce exopolysaccharides (EPS) (Sajna et al., 2013b). The EPS in the culture broth could also stabilize the hydrocarbon emulsion and improve the bioavailability. The addition of the culture broth could also surpass the inhibitory effect of biosurfactants, which was more prominent when biosurfactants were added in pure form. Biosurfactants assisted hydrocarbon degradation by the supplementation of culture broth containing the biosurfactant(s) could be a cost effective strategy, and thus ideal for large-scale bioremediation as it would avoid the cell removal and multi-step purifications for biosurfactants. The strategy would become more effective when the culture producing the biosurfactant itself is capable of the targeted bioremediation as was the case in this study. This could ensure that even if the cells of the biosurfactant producing microbe were present in the culture broth, this would only aid further the

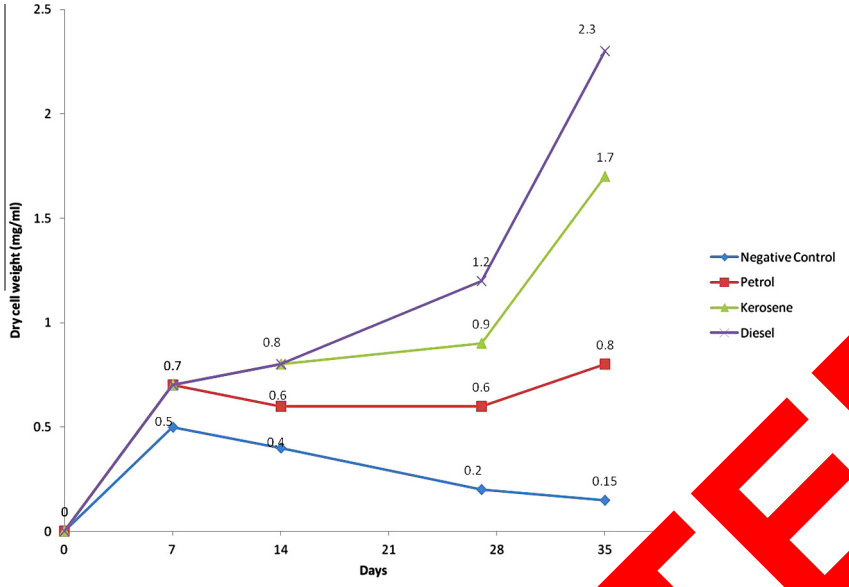

Fig. 4. Measurement of cell growth of *Pseudozyma* sp. NII 08165, in a medium containing petrol, diesel, kerosene at various time interval. Here negative control is the organism growing in B.H. medium without any carbon source.

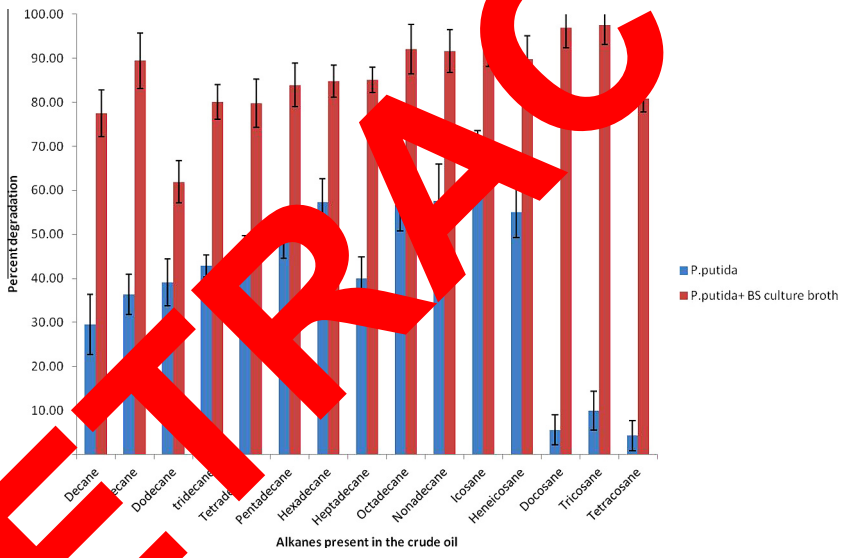

Fig. ent degradation of nes present in the crude oil by *P. putida* MTCC 1194 on supplementation of *Pseudozyma* culture broth.

bioremedia . Bana t al. (199 employed biosurfactant containing cu broth st-effective cleanup of the oil tank and obtained covery of rude oil from the sludge. The addition of biofertili could be avoided to a larger extent as biosurfactant containing h itself would improve the bioremediation, thus avoiding the environmental impact associated with the addition of fertilizer (Ron and Rosenberg, 2002).

4. Conclusions

Biosurfactant assisted bioremediation using biosurfactants from a *Pseudozyma* isolate was attempted for the first time. *Pseudozyma* sp. NII 08165 produced glycolipid biosurfactants, mannosylerythritol lipids. Crude oil degradation by *P. putida* was improved by the supplementation of biosurfactants, but at higher concentrations of biosurfactants, there was an inhibitory effect on the cell growth. Growth inhibitory kinetics revealed that K_i of *Pseudozyma* biosurfactants on the growth of *P. putida* was 11.07 mg L^{-1}. From these results, it was concluded that the supplementation of *P. putida* culture with *Pseudozyma* culture broth could be used as an effective strategy for the improved and cost-effective biodegradation of crude oil.

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