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Full Length Research Paper

Isolation of two *Kocuria* species capable of growing on various polycyclic aromatic hydrocarbons

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Different samples collected from crude oil contaminated beach were enriched for isolation of bacterial strains capable of growing on naphthalene, phenanthrene and fluoranthene. Respiratory reduction of WST-1{4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} to a colored formazan showed that one isolated strain CMG2028, identified as *Kocuria flava* by 16s rRNA, grew on naphthalene and phenanthrene while CMG2042, identified as *Kocuria rosea* grew on all three polycyclic aromatic hydrocarbons (PAHs). In naphthalene degradation test, 64 and 47% residual naphthalene was extracted after ten days of incubation from culture medium of *K. rosea* CMG2042 and *K. flava* CMG2028, respectively, when provided with 0.5 mg ml^{-1} concentration as sole carbon source. Due to addition of 0.5 mg ml^{-1} yeast extract as additional carbon source, residual naphthalene extracted was 41 and 55% from *K. rosea* CMG2042 and *K. flava* CMG2028, respectively. Both strains exhibited growth on 0.01 mg ml-1 phenanthrene and fluoranthene in yeast extract added or omitted medium but only *K. rosea* CMG2042 degraded 9% phenanthrene as a sole carbon source. Both strains had growth on minimal agar plates coated with Iranian light crude oil as sole carbon source and on agar medium added with yeast extract.

Key words: Biodegradation, crude oil, *Kocuria*, polycyclic aromatic hydrocarbons, yeast extract.

INTRODUCTION

Anthropogenic inputs of hydrocarbons, especially aromatic compounds, are common stress to any site contaminated by crude oil or its refinery products. Due to the toxic, mutagenic and carcinogenic properties of hydrocarbons, their existence in any part of the ecosystem is of great environmental concern (Bastiaens et al., 2000; Boonchan et al., 2000; Zhou et al., 2006; Zhong et al., 2007). Research carried out over the last decades has shown that these hazardous compounds, especially polycyclic aromatic hydrocarbons (PAHs) are very often persistent. This persistence is very hazardous for the environment and can bring a drastic change in the biota where they spill (Pritchard, 1984; Cerniglia, 1992; Kanaly and Harayama, 2000; Andrea et al., 2001; Mikael et al., 2003).

By the consent of nature, there are microorganisms ubiquitously distributed in soil and aquatic environment which have hydrocarbons degrading capabilities and considered to be the major agents for remediation of contaminated sites (Leahy and Colwell, 1990; Boonchan et al., 2000; Widada et al., 2002; Zhong et al., 2007; Lin and Cai, 2008). Contamination of hydrocarbons, either terrestrial or aquatic, truly acts as selection pressure for these indigenous microorganisms. Microorganisms possess the greatest enzymatic diversity which they use to mineralize millions of organic compounds to capture the chemical energy for their growth (Dagley, 1987; Lawrence and Lynda, 1999). So far, major information of PAH degradation pathways is from studies on *Pseudomonas* and most of the reported PAH degrading bacteria are found to possess PAH degradation gene clusters highly homologous to the naphthalene gene (*nah* gene) cluster from NAH7 plasmid of *Pseudomonas putida* G7 (Cerniglia, 1993; Widada et al., 2002; Chadhain et al., 2006). Although recently, studies have been carried out on genetic and biochemical analysis of PAH degradation by gram positive bacteria such as *Rhodococcus, Myco- bacterium,*

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Abbreviations: PAHs, Polycyclic aromatic hydrocarbons; **YE**, yeast extract; **Nap**, naphthalene, **Phe**, phenanthrene; **Fla**, fluoranthene; **ASW**, artificial sea water medium; **MSM**, minimal salt medium; **PMM**, phosphate minimal medium; **NA**, nutrient agar; **LB**, Luria Bertani.

Terrabacter and *Nocardioides,* but still less information is available on PAH degradation by these organisms (Chadhain et al., 2006). Gram-positive bacteria can be divided into two major sub divisions, the phylum Actinobacteria (high G+C gram positives) and the phylum Firmicutes (low G+C gram positives) (Gontang et al., 2007). *Kocuria* is one of the genera of actinobacteria that was dissected from genus *Micrococcus* in 1995 (Stackebrandt et al., 1995). Harwati et al. (2007) first time reported degradation of components of Arabian light crude oil by *Kocuria rosea* and *Kocuria aegyptia*. Nazina et al. (2002) isolated a strain *Kocuria erythromyxa* (*K. rosea* according to reclassification) from an oil field. Rauch et al. (2006) isolated *Kocuria rhizophilia* from aviaton fuel tank. Tumaikina et al. (2008) isolated *K. rosea* E1 - 4.3 from the pondweed surface that grew on agar medium with crude oil as carbon source. Mariano et al. (2008) isolated *Kocuria palustris* from soil of a petrol pump and first time reported degradation of commercial diesel oil by *K. palustris*.

Addition of some covalent carbon sources in growth medium might aid in reducing the toxicity and growth inhibition of xenobiotics on cells, thereby increasing the transformation rates of xenobiotics. Several investigators showed the facilitation of chlorophenol degradation by using conventional carbon sources such as glucose, sodium glutamate and yeast extract (YE). YE may serve as a source of carbon, nitrogen and energy for the microbes. As YE contains complex sources of nutrients, it might enhance hydrocarbons degradation by promoting cell growth and survival of xenobiotic degrading organisms (Fakhruddin and Hussain, 2007; Mukred et al., 2008; Sabzali et al., 2009). In the present study, two strains of *Kocuria*, belonging to the species *rosea* and *flava*, were isolated and evaluated for growth and degradation of naphthalene (Nap), phenanthrene (Phe) and fluoranthene (Fla) as sole carbon source. YE was added in the medium as additional carbon source to enhance degradation of PAHs in both strains. Iranian light crude oil was used as mixture of hydrocarbons to grow both strains on agar medium coated with oil.

MATERIALS AND METHODS

Chemicals and reagents

All PAHs used were of gas chromatography (GC) or high performance liquid chromatography (HPLC) grade while purity of each of them was 98% or higher. Nap was purchased from Fluka. Phe and Fla were purchased from Wako, Japan. Cell proliferation reagent WST-1{4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} was obtained from Roche Molecular Biochemical. Sterile 96 well flat bottom microtiter plates were purchased from Nalge Nunc International Denmark (4-69078 polysorp F8).

Media and stock solutions

Artificial sea water medium (ASW) containing g l^{-1} of NaCl 23, $MgCl_2.6H_2O$ 5, $CaCl_2$ 1.1, KCl 0.66, H_3BO_3 0.026, $SrCl_2$ 0.024 in distilled water (pH 7.0-7.2) was used to enrich samples. Screening assay of 96 well microtiter plate was carried out in phosphate minimal medium (PMM) (Johnsen et al., 2002) while minimal salt medium (MSM) was used in PAHs degradation test (Wongsa et al., 2004). Stock solutions of Nap (5%), Phe (1%) and Fla (1%) were prepared by dissolving their crystals in hexane.

Enrichment and isolation

Hydrocarbons contaminated soil and water samples were collected from different sites at Clifton beach and adjoining areas of Karachi, Pakistan. Sampling was done right after the accidental oil spill of Iranian light crude oil from" Tasman spirit" at Sea view, Karachi. Samples collected were oil contaminated sea water, moist mud from mangrove area close by the spill site, water sample from Kemari Jetty, washed ashore plants, oil contaminated beach sand, oil sludge, sea weeds, crab, star fish and jelly fish. Samples collected were enriched in ASW at 37°C and 100 rpm. Nap (1 g l^{-1}) was added in ASW as sole carbon source as it has long been known for isolating PAHs catabolizing bacteria from soils and freshwater (Hedlund et al., 1999). After subsequent transfers of aliquots into same medium, minimal agar plates were spreaded with dilutions of enriched samples. Spreaded plates sprinkled with Nap crystals on lid were sealed with parafilm and incubated until growth appeared (Hedlund et al., 1999). Bacterial colonies picked from minimal agar plates were streaked and restreaked on nutrient agar (NA) plates until they became pure (Weissenfels et al., 1990). Isolated bacterial strains were characterized for colonial and cellular morphology.

Screening for growth on PAHs and respiration measurement with WST-1

Nap, Phe and Fla were selected as model PAHs containing 2, 3 and 4 rings, respectively. These compounds were dissolved in hexane to make solutions of 5 mg ml^{-1} concentration. Bacterial strains were screened for growth on PAHs by the method described by Johnsen et al. (2002) with a little amendment. Briefly, 20 µl of a PAH solution added in a well was evaporated in sterile flow cabinet to obtain coating of compound on walls. For each PAH solution, separate microtiter plate was used to avoid cross contamination. Control microtiter plate was treated in same way with hexane only. 200 µl of PMM, pH 6.8, was added in each well. Overnight grown cultures of bacterial strains in nutrient broth were washed twice with PMM and resuspended in 1 ml PMM to maintain optical density (OD) 0.4 at 600 nm. 10 µl of this suspension was used as inoculum per well. For a single PAH, isolated strains were inoculated in quadruplicate. The microtiter plates were wrapped in aluminum foil and incubated for 10 days at 30°C. PMM filled wells at the edges of plates were not used for calculation to avoid edge effect. An electron donor (e-donor) solution of pH 6.5 was prepared by dissolving glucose and pyruvate (16.6 mM each) in Tris buffer (40 mM). 50 µl of this solution was added in each well of 10 days incubated microtiter plate along with 10 µl of WST-1 reagent. Plates were incubated at room temperature on shaker table (gradually maintained till 300 rpm within 5 min). Absorbance was measured with a microplate autoreader (VERSAmax tunable microplate reader 9859) at 450 nm with reference wavelength 630 nm right after addition of WST-1 and after 90 min of incubation. The absorbance at time zero was subtracted from subsequent readings and the resultant change in absorbance was compared between control and PAHs treated wells.

16S rRNA identification of selected bacterial strains

Two bacterial strains, CMG2028 and CMG2042, were selected for

quantitative degradation analysis of PAHs. 16S rRNA sequencing of both selected strains was done commercially by Macrogen, USA. Sequences obtained were BLAST matched on NCBI web page.

PAHs degradation as sole carbon source

Stock solutions of Nap, Phe and Fla dispensed separately into 50ml capacity Teflon lined screw cap vials to give final concentration of Nap 0.5 mg ml^{-1} while Phe and Fla were both at concentration of 0.01 mg ml^{-1} as sole carbon source. 10 ml MSM was added in vials after hexane evaporation on clean bench and sonicated for 3 min to dissolve crystals of Nap, Phe and Fla. Two selected strains, CMG2028 and CMG2042, were grown overnight in Luria Bertani (LB) at 33°C and 160 rpm and inoculated in vials to give OD 0.1 at 600 nm. Cultures were shaken at 180 rpm and 33°C for four days, centrifuged at 2000 rpm for 10 min at room temperature, washed twice in MSM, resuspended in MSM and used to inoculate 10 ml MSM with the respective PAHs as described earlier. Control without inoculum was set as well to see abiotic loss of hydrocarbons. All treatments, in triplicate, were incubated at 180 rpm and 33°C. Residual Nap, after incubation of 10 days, was extracted with hexane: acetone (3:1) solvent in three steps (6, 2, 2 ml respectively). Fla (0.5 g l^{-1}) was employed as internal standard. 2 µl aliquots were analyzed by GC-FID. Residual Phe and Fla were extracted in 6ml chloroform/methanol (2:1) followed by 2 ml (2x) of chloroform after addition of n-dodecane (0.010 g l^{-1}) as internal standard. 10 ml extract was concentrated to 1 ml by purging with nitrogen and 2 µl aliquots were analyzed by GC-FID.

PAHs degradation with additional carbon source

Effect of additional carbon source on degradation of PAHs was carried out through addition of 0.5 g l^{-1} YE in MSM. Vials containing YE added medium with Nap, Phe and Fla were inoculated with cultures of CMG2028 and CMG2042 as described above. Incubation, extraction and analysis were done on same parameters.

Gas chromatography conditions

GL Sciences GC-353B equipped with HR-17 column (25 m long, 0.25 mm i.d.) was operated in split mode. Initial GC oven temperature was set at 150°C for Nap and 100°C for Phe and Fla that gradually increased to 300°C at the rate of 10°C/min. Injector and detector temperatures were set at 300°C. Carrier gas used was nitrogen.

Growth on Iranian light crude oil

Minimal agar plates with and without yeast extract were coated with 65 µl Iranian light crude oil. Overnight LB grown cultures of CMG2028 and CMG2042 were streaked on oil coated agar plates and incubated at 30°C for one week. Cultures picked from these plates were subculture on agar plates coated with 85 and 100 µl oil, respectively. Plates were incubated at 30°C for one week to observe growth of strains.

RESULTS AND DISCUSSION

From enriched samples, 41 bacterial strains were isolated and characterized for cellular and colonial morphology (data not shown). The cells of selected strain CMG2028 were cocci, gram positive, exhibited mucoid colonies with production of yellow pigment on LB agar plates and had 99% similarity with *K. flava* in BLAST (accession number GQ255646). Cells of CMG2042 were cocci, gram positive, exhibited mucoid colonies with orange pigment on LB agar plates and had 98 - 99% similarity with *K. rosea* in BLAST (accession number GQ255645). In screening assay, increase of 0.05 in absorbance was considered as a significant increase indicating true growth of strain on PAHs (Table 1). After 90 min of incubation, growth of five strains were detectable on Phe only whereas the four strains that grew on Phe could also grow on Fla while there was one strain that grew on Fla only. *K. flava* CMG2028 had growth on Nap and Phe. Although it also had growth on Fla but reduction of WST-1 was less than the control. *K. rosea* CMG2042 grew on all three PAHs (Table 1). In Nap degradation pathway, enzymes converting Nap to salicylate can convert Phe to salicylate and shuttle in the Nap degradation pathway; however isolates degrading Phe through phthalate pathway cannot grow on Nap as sole carbon source (Habe and Omori, 2003). This can be a possible reason why *K. flava* CMG2028 had growth on Nap and Phe. However, *K. rosea* CMG2042 that had growth on all three PAHs as well as those strains which grew on Phe and Fla might have enzymes involved in more than one pathway (Johnsen et al., 2007). In the screening assay, *K. flava* CMG2028 reduced more WST-1 in Nap coated wells than *K. rosea* CMG2042 which is in accordance with the results of Nap degradation as sole carbon source.

In minimal medium with Nap as sole carbon source, residual Nap extracted from *K. rosea* CMG2042 was 64% while from *K. flava* CMG2028, 47% residual Nap was extracted in ten days (Figure 1a and b). Enhanced utilization of PAH in the presence of a growth substrate has been frequently observed (Dean-Ross et al., 2002). Ambrosoli et al. (2005) observed better PAHs degradation when acetate or glucose was used as additional carbon source. Chang et al. (2008) observed enhancing effect of YE on degradation of Phe and Pyrene by microbial consortium. In this study, due to the addition of YE, more Nap was degraded by *K. rosea* CMG2042 and 41% residual Nap was extracted from medium after ten days of incubation; but in *K. flava* CMG2028 due to YE addition, degradation of Nap decreased and residual Nap extracted was 55% (Figure 1a and b). This result has shown that *K. flava* CMG2028 could better use Nap as sole carbon source. However failure of Nap degradation enhancement by YE in K. flava CMG2028 can be due to many reasons. YE might not be a suitable carbon source for K. flava CMG2028 or became competitive substrate being equal in concentration to Nap and decreased Nap metabolism in *K. flava* CMG2028. It is reported in some studies that tested substrates either did not enhance PAHs degradation or inhibited it completely while in some other cases, they supported high growth rate of bacterial community but caused loss of degradation or enhanced less efficiently or did not enhance below and higher to particular concentration (Juhasz et al., 2000; Wong et al.,

Table 1. Comparison of growth of bacterial strains on PAHs after 90 min of incubation determined as change in absorbance (A_{450}-A_{630}) indicating reduction of WST-1.

S/N	Strain	No PAH	Naphthalene	Phenanthrene	Fluoranthene
1	CMG2001	0±0	0±0	0.03±0.07	0.01±0.01
2	CMG2002	0±0	0.02±0.02	0.02±0.03	0±0
3	CMG2003	0±0	0.03±0.04	**0.77**±1.43	0.02±0.02
4	CMG2004	0.01±0.01	0.01±0	0.01±0	0±0
5	CMG2005	0±0	0±0	0.03±0.03	0.01±0.02
6	CMG2006	0±0	0.03±0.03	0.01±0.01	0.02±0.03
7	CMG2007	0±0.01	0±0	0.01±0.01	0±0
8	CMG2008	0±0	0±0	0.02±0.04	0.01±0.01
9	CMG2009	0±0	0±0	**0.61**±1.23	**0.07**±0.09
10	CMG2010	0.09±0.18	0.01±0.01	**0.82**±0.64	0±0
11	CMG2011	0±0	0±0	0±0	0.01±0.02
12	CMG2012	0±0	0±0	**0.79**±1.55	0±0
13	CMG2013	0±0.01	0±0	**0.27**±0.54	**0.12**±0.16
14	CMG2014	0.01±0.01	0±0.01	0±0	0±0
15	CMG2015	0±0	0±0	**0.06**±0.03	0±0
16	CMG2016	0.01±0.01	0.02±0.02	0.01±0.02	0±0
17	CMG2017	0±0	0±0.01	0±0	0±0
18	CMG2018	0±0	0±0	0±0	0±0
19	CMG2019	0±0	0.02±0.02	0.04±0.06	0.01±0.01
20	CMG2020	0±0	0±0	**0.28**±0.55	**0.28**±0.4
21	CMG2021	0±0	0±0	**0.36**±0.59	0±0
22	CMG2022	0±0	0.02±0.03	0.03±0.04	0±0
23	CMG2024	0±0	0±0	0±0	0.01±0.02
24	CMG2025	0±0.01	0±0	0.01±0.02	0.01±0.01
25	CMG2026	0.14±0.09	0.13±0.09	**0.34**±0.16	**0.45**±0.21
26	CMG2027	0±0	0±0.01	0±0	0±0
27	***K. flava*CMG2028**	0.52±0.1	**1.03**±0.51	**1.07**±0.65	0.49±0.5
28	CMG2029	0±0	0±0	0.01±0.01	0±0
29	CMG2030	0±0	0±0.01	0±0.01	0±0
30	CMG2031	0.07±0.15	0.09±0.05	0.04±0.07	0±0
31	CMG2032	0±0	0±0	0±0	0±0
32	CMG2033	0±0	0.01±0.02	0±0	0±0
33	CMG2035	0±0	0±0.01	0±0	0±0
34	CMG2036	0.06±0.04	0.01±0.02	0±0	0±0
35	CMG2037	0±0	0±0	0±0	0±0
36	CMG2038	0±0	0.01±0.02	0.01±0.02	0.03±0.04
37	CMG2039	0±0	0±0	0±0	0±0
38	CMG2040	0±0	0±0	0±0	0±0
39	***K. rosea*CMG2042**	0.38±0.17	**0.82**±0.28	**0.63**±0.39	**0.53**±0.74
40	CMG2043	0.03±0.03	0±0	0.05±0.03	**0.08**±0.01
41	CMG2044	0±0	0±0	0±0.01	0±0

Numbers in bold are significantly higher than those for control. Each number is average of 4 observations± standard deviation.

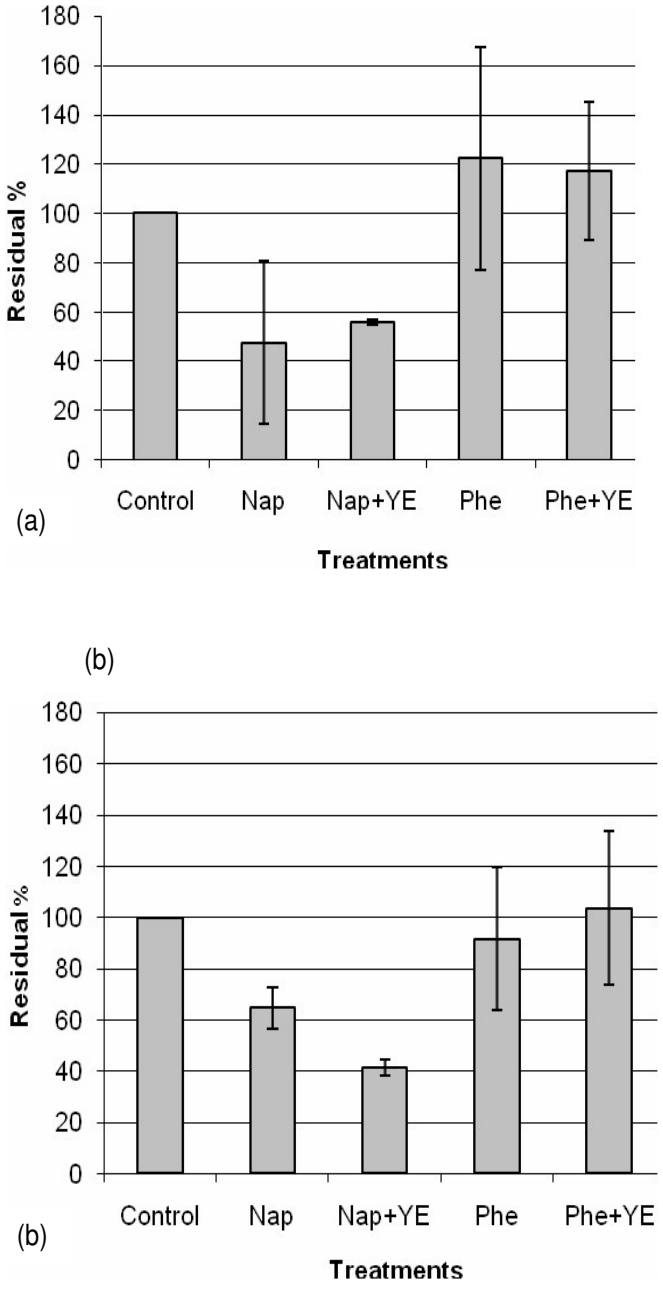

Figure 1. PAHs biodegradation by (a) *K. flava* CMG2028 and (b) *K. rosea* CMG2042.

2002; Marcoux et al., 2000; Chang et al., 2008). Although both strains grew on Phe to a different extent in YE added or omitted medium, small amount of it was degraded only by *K. rosea* CMG2042. 91% residual Phe was extracted from *K. rosea* CMG2042 when provided as sole carbon source. However, addition of YE inhibited the degradation of Phe completely which might be due to its availability as competitive substrate being higher in concentration than Phe in the medium. Fla was not degraded either provided with or without YE in the medium by both strains while residual Fla extracted was higher than the amount extracted from control. Muller et al. (1997) reported a phenomenon of less volatilization of semi volatile PAHs from culture grown medium than control due to their adsorption to cell biomass. Both strains did not degrade Fla and exhibited reduced growth on it. It has been reported that organic solvents and aromatic hydrocarbons cause cell growth inhibition and the destruction of microorganisms (Sikkema et al., 1994). Fujita et al. (2006) reported a bacterial strain *K. rhizophila* DC2201 which failed to cause cytolysis by various organic solvents and showed no significant changes in both cytomorphology and cytoplasmic membrane fluidity when exposed to alcohols even when lethal concentrations were used which proved that *K. rhizophila* had potentially robust cell structure against organic compounds. Ability of these two strains to grow in presence of Phe and Fla and not carry out degradation may be due to the possibility of having robust cell structure against these compounds.

Tumaikina et al. (2008) reported growth of *K. rosea* E1-4.3 on agar medium with crude oil as the sole carbon and energy source while there was no report of growth of *K. flava* on crude oil. In our study, both strains exhibited growth on MSM agar with crude oil either as sole carbon source or with additional carbon source till second subculturing (Figure 2). On LB agar plates both strains had mucoid pigmented colonies but on minimal plates with crude oil as the only carbon source, both strains had non-mucoid and non-pigmented colonies while on minimal agar with YE and same amount of oil, colonies of *K. flava* CMG2028 as well as *K. rosea* CMG2042 retrieved their mucoid growth pattern but not pigment. On plates of MSM either with or without YE, colonies of both the strains had accumulated oil around them. *K. rosea* CMG2042 had higher growth and oil accumulation in comparison to *K. flava* CMG2028. It is concluded that *K. flava* CMG2028 and *K. rosea* CMG2042 were able to utilize Nap as sole carbon and energy source. Additional carbon source YE, at tested concentration, can be used to enhance Nap degradation in *K. rosea* CMG2042 but not in *K. flava* CMG2028. Although both strains had growth on Phe but only *K. rosea* CMG2042 degraded less amount of it as sole carbon source. Fla containing medium had poor growth of both strains but Fla could not be degraded. Iranian light crude oil was consumed as sole growth substrate in minimal agar medium by both strains whereas addition of YE helped in retrieving mucoid colonial growth of both strains on crude oil.

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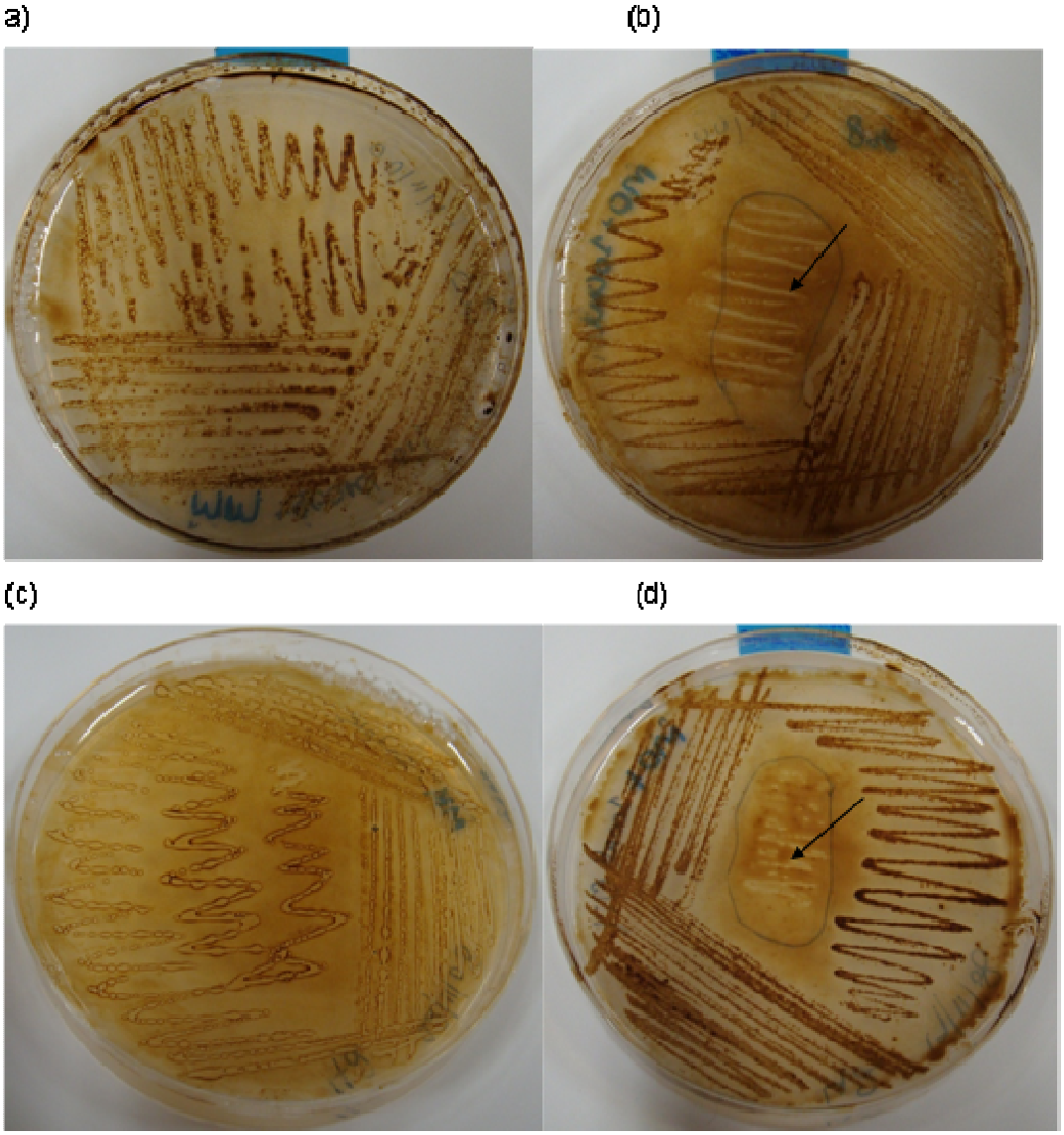

Figure 2. Growth of bacterial strains on MSM agar coated with 100 µl crude oil. (a) *K. flava* CMG2028 with yeast extract and (b) without yeast extract. (c) *K.rosea* CMG2042 with yeast extract and (d) without yeast extract. Arrows showing streak without culture.

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