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Isolation, Conventional Identification and Pre-screening of the Yeasts Degrading Crude Oil and Its Aromatic Hydrocarbons from Turkey

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Forty-four yeast isolates were obtained by using various isolation methods from different environmental samples including soil and sea water either contaminated or uncontaminated with crude oil, activated sludge and waste water samples. The isolates were identified based on their microscopic, physiological and biochemical properties. As a consequence, eleven different species belonging to genus *Rhodosporidium* (18 strains), *Candida* (15 strains), *Pichia* (5 strains), *Rhodotorula* (5 strains), and *Cryptococcus* (1 strain) were classified. These isolates were then pre-screened for their degradation ability using aromatic hydrocarbons with different ring numbers such as benzene, naphthalene, phenanthrene, pyrene and flourene as the sole carbon and energy source. While benzene and fluorene were commonly used by the isolates, *Candida shehatae* was found to be the best growing yeast on each aromatic tested indicating that it has the potential for biological application to treat aromatic contaminants.

Key words: Yeasts, identification, crude oil, polycyclic aromatic hydrocarbons (PAHs), biodegradation.

Increasing demand for petroleum not only as an energy supply but also as raw materials in various industries such as petrochemical and polymer industries has also increased serious issues. According to the records, the total world oil production in 2008 was around 85 million barrels per day and approximately half of it was transferred via the international shipping lanes of the world's seas[1]. Offshore oil production, catastrophic tanker casualties, and accidental oil spills during transportation are of great importance since excessive discharge of crude oil into the aquatic and terrestrial ecosystems has resulted in ecological, commercial, aesthetic, and recreational problems for many years[2-5].

Petroleum or crude oil is composed of various organic and inorganic constituents such as aliphatic and aromatic hydrocarbons, asphaltenes, and resins. Among all, aromatic hydrocarbons have prolonged persistence in the environment because of their low solubility and bioavailability[6]. Polycyclic aromatic hydrocarbons (PAHs), that possess genotoxicity and carcinogenicity, consist of two or more aromatic rings in different structural configurations[7-9]. Their solubility decreases with an increase in their molecular size and results in bioaccumulation of those compounds in food chains[10,11]. In order to restore the original environmental conditions after an oil spill, bioremediation technologies are

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preferred most of the times for their efficiency and cost-effectiveness[12-16].

Microorganisms play a very crucial role in removal of environmental contaminants. To date, extensive researches have been carried out to reveal bacterial contribution to the biodegradation of aromatic hydrocarbons. On the other hand, although the yeasts are one of the common inhabitants of the polluted environments,[17-19] very few studies focused on the isolation of the yeasts with a potential biodegradation ability of PAHs. Thus, the purpose of this study was to isolate, identify, and pre-screen the indigenous yeasts with an innate capacity for PAHs utilization.

MATERIALS AND METHODS

Sample collection and isolation of yeasts

In order to isolate the yeast capable of degrading crude oil and its aromatic components, the samples (soils from Tupras and Atas Refinery, activated sludge from Tupras Refinery, sea water from Mersin harbour) contaminated with either crude oil or its derivatives were collected in screw capped sterilized bottles and subjected to the isolation procedures. Crude oil was kindly provided by Atas petroleum refinery.

Three different methods were applied for the isolation of the yeasts capable of degrading the crude oil and its aromatic hydrocarbons. In the first method, pre-enrichment technique was performed by adding 1% crude oil into Mineral Salt Medium (MSM) and pre-incubated for 1 week at 30 °C using rotary incubator at 200 rpm to increase the number of yeast isolates. Then, serial dilutions were prepared and spreaded onto Oil agar including 1% crude oil[20]. For the second method, the samples were suspended in Artificial Sea Water (ASW) supplemented with 1% crude oil and incubated for 5 days at 30 °C. Serial dilutions were made and transferred onto Malt Yeast Glucose Peptone (MYGP) agar prepared in ASW[21]. In the third method, samples were diluted in sterile distilled water and spreaded over modified Czapek agar plates[22]. The isolates were maintained on MYGP agar for further steps.

Identification of the yeast isolates

The yeasts were identified based on their physiological and morphological properties according to Barnett *et al.* [23] The tests included fermentation of sugars, assimilation of carbon and nitrogen compounds, growth at 25, 30, 35, 37 and 42 °C, growth on yeast extract agar supplemented with either %50 or %60 (w/v) glucose, growth in vitamin-free medium, growth in media containing %10 and %16 NaCl, resistance to 0,01% and 0,1% cycloheximide, growth on media with 1% acetic acid and urease activity.

Fermentation of sugars

Fermentation of D-glucose, sucrose, D-galactose, lactose, maltose, D-xylose, α,α-trehalose, Me-α-D-glucoside, melibiose, cellobiose, melezitose, starch, inulin, and raffinose were tested in accordance with the description of Barnett *et al*,[23]. A positive result was indicated by accumulation of gas in Durham tubes.

Assimilation of carbon and nitrogen compounds

Commercially produced Yeast Nitrogen Base (YNB, Difco) was used for the assimilation of various carbon compounds. Final concentrations of the carbon sources was 0.5%. The carbon compounds tested were galactose, glucose, sucrose, lactose, L-sorbose, D-glucosamine, D-ribose, L-arabinose, D-arabinose, maltose, D-mannitol, melibiose, raffinose, soluble starch, xylose, α-methyl-D-glucoside, cellobiose, erythritol, xylitol, citrate, DL-lactate, α,α- trehalose, salicin, arbutin, melezitose, inulin, glycerol, dulcitol, myo-inositol, D-glucono 1,5 lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-glucuronate, D-galacturonic acid, succinate, methanol, ethanol, propan 1,2-diol, butan-2,3-diol, quinic acid, D-glucorate, and D-gluconate.

For the assimilation of nitrogen compounds, Yeast Carbon Base (YCB, Difco) was used. The nitrogen compounds tested were included nitrate, nitrite, ethylamine, L-lysine, cadaverine, creatinine and tryptophan. The plates were incubated at 27 ^{0}C for 3 days in both groups of assimilation tests.

Hydrolysis of urea

Urea hydrolysis was tested using method of Van der Walt and Yarrow[24]. Suspension of actively growing yeast cultures were spreaded over urea agar plates with a sterile wire loop and incubated at 27 °C for 24 hours. A positive reaction was the development of a deep pink colour in the agar.

Diazonium Blue B (DBB) test

The test described by Van der Walt and Yarrow[24] was performed with some modifications to distinguish between the ascomycetous and basidiomycetous yeasts. The strains were initially grown at least for 21 days at 25 ^{0}C on GYP agar (2%, w/v glucose, 0.5%, w/v yeast extract, 1%, w/v peptone, 2%, w/v agar). A few crystals of Fast Blue B-Salt (Serva 21270) were placed onto the yeast cultures, and ice-cold Tris-buffer (12.1 g Tris in 1 L H_2O bidest. pH 7.0) was added. The appearance of a red colour within two minutes was recorded as a positive test reaction (basidiomycetous yeast).

Other tests

Colony morphologies of the isolates were determined on Malt Extract Agar (MEA) after 4 days of incubation at 30 °C[25] Colony colours, size and the shape of well-separated colonies were recorded. Cell morphology, type of vegetative reproduction, and growth characteristics of the isolates in broth medium were determined using Yeast Malt (YM) broth. Ascospore formation of the cultures was examined on McClary Acetate agar media. The inoculated media were incubated at 30°C for 3 weeks and examined 7-day intervals. Formation of pseudohyphae was examined on Potato Dextrose Agar (PDA).

Pre-screening tests for utilization of aromatic hydrocarbons

Aromatic hydrocarbon degradation ability of the yeast isolates were tested against different aromatic hydrocarbons with various ring numbers which are given in Table 1. Aromatic substrates (purchased from Fluka) were dissolved in methanol and filter sterilized using a 0.20 µm syringe filter (Sartorius AG, Goettingen, Germany). The substrates were then added to autoclaved Nitrogen base medium individually as sole carbon source. The plates were inoculated using modified replica plate technique and incubated at 27°C for 10 days. Colony formation was recorded as positive result for the utilization of relevant PAHs.

RESULTS AND DISCUSSION

A total of fourty four petroleum utilising yeast strains were obtained from environmental samples. To increase the number of the yeast isolates, three different isolation methods were followed. Among them, the methods described by Ijah[20] and Raymond *et al*.[22] gave a higher number of yeast isolates (Table 2).

On the bases of morphological and physiological features, the strains were conventionally identified according to the identification key by Barnett *et al*.[23] as shown in Table 3. DBB, urea hydrolysis, nitrate assimilation, and glucose fermentation tests were primarily performed since their results are used for genus-level identification. Twenty four yeast isolates were produce red colour with DBB indicating that these isolates belong to basidiomycetous yeasts. Yeasts vary in their ability to ferment sugar. Barnett and colleagues, therefore, classified the yeasts into two main groups in terms of glucose fermentation. Eighteen of the yeast isolates were defined as positive for glucose fermentation. On acetate agar, only one isolate produced ascospores.

Conventional identification showed that the strains belong to five genera, namely, *Candida*, *Rhodosporidium*, *Cryptococcus*, *Pichia*, and *Rhodotorula*. The genus *Candida* was represented by *C. shehatae* (6 strains), *C. pseudolambicola* (2 strains) ,*C. tropicalis* (2 strains), *C. zeylanoides* (2 strains), *C. glabrata* (1 strain), *C. ernobii* (1 strain), and *C. albicans* (1 strain) while the genus *Rhodosporidium* was represented by only one species, *R. toruloides* (18 strains). The isolation of *Rhodosporidium* and *Candida* species as the most predominant hydrocarbon degraders (40,9 % and 33,7 % isolation frequency, respectively) are well corroborated with a series of previous studies[21, 28-34].

Microbial growth on PAH substrates as a sole carbon and energy source indicates that the relative microbiota has capacity to assimilate and mineralize the parent molecules, at least partially. Pre-screening tests on agar media demonstrated that the strains identified as *Candida shehatae* were able to grow faster on each aromatic substrates tested when compared with the rest of the strains. Therefore, *C. shehatae* was selected as the best PAH degrader. Hesham and colleagues[35] reported that the use of phenanthrene as the sole carbon source is the best way for the selection of high molecular weight polycyclic aromatic hydrocarbon degrading yeasts. Consistent with this observation, phenanthrene utilizing *C. shehatae* could also use other low molecular weight aromatics. In addition, strains of *Candida*

tropicalis exhibited different growth patterns depend on the carbon substrate. One of the isolates from this genus could use all of the test aromatics. However, its growth was slower in comparison to that of *C. shehatae*. Similar pattern was also observed for *Rhodosporidium* species. One of the members from this genus could grow faster on media containing pyrene and phenantrene individually. The variation in growth pattern could be attributed to the strain differentiation even though the strains were assigned as the same species[29]. Furthermore, although *Pichia caribaea* and *Candida zeylanoides* were isolated as petroleum utilizer, they were not able to grow on any of the reference aromatics except a slow growth on monoaromatic substrate benzene. This probably

Table 1. Physical properties of aromatic hydrocarbons used in this study[26, 27]

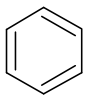
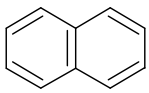
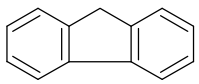
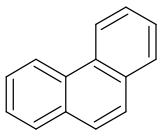
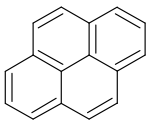
Aromatic hydrocarbon	Formula / Mw	Structure	Aqueous solubility at 25°C (mg/L)
Benzene	C_6H_6 / 78		1760
Naphthalene	$C_{10}H_8$ / 128		31
Fluorene	$C_{13}H_{10}$ / 166		1.9
Phenanthrene	$C_{14}H_{10}$ / 178		1.1
Pyrene	$C_{16}H_{10}$ / 202		0.132

Table 2. Yeast species identified by using the conventional methodology

Species	Identification number of isolates	Frequency (%)
Rhodotorula pilatii	1, 11, 21, 23, 24	11.3
Candida tropicalis	19, 33	4.5
Rhodosporidium toruloides	2, 5, 6, 7, 9, 10, 12, 14, 16, 17, 20, 25, 26, 28, 29, 30, 34, 43	40.9
Candida shehatae	32, 35, 36, 39, 42, 44	13.6
Candida glabrata	4	2.2
Candida albicans	3	2.2
Candida ernobii	22	2.2
Candida pseudolambica	15, 31	4.5
Candida zeylanoides	37, 38	4.5
Cryptococcus albidus	13	2.2
Pichia caribaea	8, 18, 27, 40, 41	11.3

Table 3. Compilation of the physiological data of all strains which were identified phenotypically

Yeast species	*Rp*	*Ct*	*Rt*	*Cs*	*Cg*	*Ca*	*Ce*	*Cp*	*Cz*	*Cra*	*Pc*
Fermentation											
D-Glucose	-	+	-	+	+	+	+	+	-	-	+
D-Galactose	-	+	-	+	-	+	-	-	-	-	-
Maltose	-	+	-	+	-	+	-	-	-	-	-
Sucrose	-	+	-	-	-	+	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	+	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	+	-	-	-	-
Raffinose	-	-	-	-	-	-	+	-	-	-	-
Xylose	-	-	-	+	-	-	+	-	-	-	-
Assimilation											
D-Glucose	+	+	+	+	+	+	+	+	+	+	-
D-Galactose	40	+	+	+	-	+	-	-	-	+	-
L-Sorbose	80	+	+	+	-	+	-	-	-	-	-
D-Glucosamine	-	-	-	+	-	+	-	+	-	+	-
D-Ribose	-	50	+	+	-	-	-	-	-	+	-
D-Xylose	+	+	+	+	-	+	-	-	-	+	20
L-Arabinose	-	-	+	+	-	-	-	-	-	+	-
D-Arabinose	-	-	+	+	-	-	-	-	-	+	-
L-Rhamnose	40	-	-	+	-	-	+	-	-	+	-
Sucrose	+	+	+	+	-	+	+	+	-	+	-
Maltose	+	+	+	+	-	+	-	-	-	+	-
Trehalose	60	50	+	+	-	+	-	-	-	+	-
Methyl α-D-glucoside	80	+	-	+	-	+	-	-	-	+	-
Cellobiose	-	+	+	+	-	+	-	-	-	+	-
Salicin	-	+	+	+	-	+	-	-	-	+	-
Arbutin	+	+	+	+	-	+	-	-	-	+	-
Melibiose	-	-	-	-	+	-	-	-	-	+	-
Lactose	-	-	-	-	-	-	-	-	+	+	80
Raffinose	60	-	+	+	-	-	-	-	-	+	-
Melezitose	+	50	+	+	-	+	+	-	-	+	-
Starch	-	+	-	-	-	+	-	+	-	+	+
Glycerol	+	-	+	+	-	-	+	+	-	-	-
Erythritol	-	-	-	+	-	-	-	-	-	+	-
Ribitol	-	-	-	-	-	+	-	-	-	+	-
Xylitol	-	+	+	+	-	-	-	-	-	+	-
L-Arabinitol	-	-	-	+	-	-	-	-	-	+	-
D-Glucitol	+	+	+	+	-	+	-	-	-	+	-
D-Mannitol	+	+	+	+	-	+	-	-	-	+	20
D-Galactitol	-	-	-	-	-	-	-	-	-	+	-
Myo-Inositol	-	-	-	-	-	-	-	-	-	+	20
D-Glucono 1,5 lactone	+	+	+	+	-	+	-	-	-	+	20
2-Keto-D-gluconate	-	+	-	+	+	+	-	-	+	+	-
5-Keto-D-gluconate	-	+	-	-	-	+	-	-	-	+	20
D-Galacturonic acid	40	50	83	-	-	-	-	-	-	+	20
DL-Lactate	-	-	-	+	-	-	+	+	+	+	+
Succinate	+	+	+	+	-	+	+	+	-	+	20
Citrate	-	+	16	+	+	+	-	-	+	-	80
Methanol	-	-	-	-	-	-	-	-	-	-	-
Ethanol	+	+	+	+	-	+	+	+	-	+	20

Propan 1,2-diol	+	-	11	-	-	+	+	-	-	-	+
Butan 2,3-diol	-	+	-	-	-	+	+	-	-	-	+
Quinic acid	+	-	-	-	-	+	-	-	-	-	-
D-Galactonate	-	-	-	-	-	-	-	-	-	+	-
Nitrate	+	-	+	-	-	-	+	-	-	+	-
Nitrite	60	-	-	-	-	-	-	-	-	-	-
Ethylamine	40	+	+	+	-	+	+	+	-	+	-
L-Lysine	80	+	50	+	-	+	+	+	-	+	-
Cadaverine	-	+	+	+	-	+	+	+	-	+	-
Creatine	80	+	-	+	-	+	+	-	-	+	-
Creatinine	-	+	_	+	-	+	+	-	-	+	-
D-Triptophan	+	+	+	+	-	+	+	+	-	+	-
Additional tests											
w/o Vitamins	+	+	+	+	-	+	+	+	-	+	20
w/o myo-Inositol	+	+	+	+	-	+	+	+	-	+	60
w/o Panthotenate	+	+	+	+	-	+	+	+	-	+	20
w/o Biotin	+	+	+	+	-	+	+	+	+	+	80
w/o Thiamin	+	+	+	+	-	+	+	+	-	+	20
w/o Biotin & Thiamin	+	+	+	+	-	+	+	+	-	+	20
w/o Pyridoxine	+	+	+	+	-	+	+	+	-	+	40
w/o Niacin	+	+	+	+	-	+	+	+	-	+	20
w/o PABA	+	+	+	+	-	+	+	+	-	+	60
T 25 °C	+	+	+	+	+	+	+	+	+	+	+
T 30 °C	+	+	+	+	+	+	+	+	+	+	+
T 35 °C	+	+	+	+	+	+	+	+	+	+	+
T 37 °C	+	+	+	+	+	+	-	-	+	-	+
T 40 °C	-	+	-	+	+	+	-	-	-	-	+
T 42 °C	-	+	-	+	+	+	-	-	-	-	-
T 45 °C	-	-	-	-	-	-	-	-	-	-	—
0.01% Cycloheximide	+	+	+	+	+	+	+	-	+	-	+
0.1% Cycloheximide	-	+	-	+	+	+	-	-	+	-	-
50% D-Glucose	-	+	+	+	-	-	-	-	-	+	-
60% D-Glucose	-	+	-	+	-	-	-	-	-	-	-
10% NaCl	-	50	+	+	-	-	-	-	+	-	-
16% NaCl	-	-	-	-	-	-	-	-	-	-	-
Urea hydrolysis	+	-	+	-	-	-	-	-	-	+	-
DBB reaction	+	-	+	-	-	-	-	-	-	+	-
Pink colonies	-	-	+	-	-	-	-	-	-	-	-
Budding cells	+	+	+	+	+	+	+	+	+	-	+
Filaments	-	-	+	-	-	+	+	-	+	-	+
Pseudohyphae	-	+	+	+	-	+	-	-	+	-	-
Septate hyphae	-	+	+	-	-	-	+	-	-	-	-

Rp: *Rhodotorula pilatii*; *Ct*: *Candida tropicalis*; *Rt*: *Rhodosporidium toruloides*; *Cs*: *Candida shehatae*; *Cg*: *Candida glabrata*; *Ca*: *Candida albicans*; *Ce*: *Candida ernobii; Cp*: *Candida pseudolambica*; *Cz*: *Candida zeylanoides*; *Cra*; *Cryptococcus albidus*; *Pc*: *Pichia caribaea*. The numbers show the percentage of the positive test results. +: 100%; -; 0%; nd: not determined.

indicates the consumption of aliphatic hydrocarbons present in crude oil.

CONCLUSION

Hydrocarbon contamination is one of the major problem around the world. Due to the hazardous effects on a wide range of living organisms, PAHs are of special interest. Degradation of PAHs is mainly achieved by microbial populations in nature. However, environmentally friendly technologies such as bioremediation is highly applied when the excess amount of hydrocarbon contaminants release into

the ecosystem. Unlike physical and chemical clean-up processes, bioremediation techniques use the microorganisms and can be implemented efficiently 'on site' where the contaminants present. Therefore, isolation of the microorganisms which have a versatile metabolic pathways related with degradation of recalcitrant environmental pollutants like PAHs is of paramount importance for the development of *in situ* bioremediation strategies. In this study, *Candida shehatae* was selected as the best degrader of aromatic hydrocarbons among a number of petroleum degrading yeast isolates from different contaminated sites. Thus, we proposed that *Candida shehatae* may have the potential for bioremedial applications.

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