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Biodegradation of Crude Petroleum Oil and Environmental Pollutants by *Candida tropicalis* Strain

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ABSTRACT

A local yeast isolate named A was isolated from polluted area of Abou-Qir gulf (Alexandria, Egypt), identified according to a partial sequence of 18sRNA as Candida tropicalis*. The isolate showed a high potency in petroleum oil biodegradation as well some hydrocarbons. Morphological changes in cell diameter of this yeast were recognized upon growing the target cell in sea water medium supplemented with petroleum oil as sole carbon source in comparison to the growth in enriched medium. Statistically-based experimental design was applied to evaluate the significance of factors on petroleum oil biodegradation by this yeast isolate. Eleven culture conditions were examined by implementing Plackett-Burman factorial design where aeration, NH_4Cl and K_2HPO_4 had the most positive significance on oil degradation.*

Key words: **Key words**: petroleum oil, *Candida tropicalis*, 18Sr RNA, biodegradation, Plackett-Burman

INTRODUCTION

In recent years, various technologies have emerged in order to manage oil residues and effluents contaminated with hydrocarbons. Bioremediation is one of the most extensively used because of its low cost and high efficiency (Alxander, 1999; Van Hamme et al., 2003). Biodegradation of hydrocarbons by natural populations of microorganisms is the main process acting in the depuration of hydrocarbon-polluted environments. The mechanism has been extensively studied and reviewed (Atlas, 1984; Leahy and Colwell, 1990; Korda et al., 1997; Kanaly and Harayama, 2000; Vam Hamme et al., 2003; Pinzon-Martinez et al., 2010).

The utilization of n-alkanes by yeast as a sole carbon and energy source has been reviewed by Obuekwe et al. (2005) and Ashraf and Ali, (2006). In many reports, bacteria have been identified as more efficient crude oil degraders than yeast.

On the contrary, there are scanty information that yeasts is better crude oil degraders than bacteria (Walker et al., 1978). Additionally, a consortium of symbiotic bacteria or supporting materials can be used to enhance the biodegradation process as described by Tejo-Hernandez et al. (2007); Nievas et al. (2008); Hii et al. (2009); Wang et al. (2010) and Zhang et al. (2010). Some reviews focused on the examination of factors, are including nutrients, physical state of the oil, oxygen, temperature,

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salinity and pressure influencing petroleum biodegradation rates, with a view to developing environmental applications (Atlas, 1981; Jonathan et al., 2003).

Factorial design techniques present a more balanced alternative to the one-factor-at-a-time approach to fermentation improvement (Haaland, 1989; Myers and Montgomery, 1995). Plackett-Burman design comprises one type of two level screening designs and can be constructed on the bases of factorial replication of a full factorial design (Plackett and Burman, 1946). This design allows reliable short listing of small number of ingredients for further optimization and allows to obtain unbiased estimates of linear effects of all the factors with maximum accuracy being the same for all the effects (Akhnazarova and Kafarov, 1982).

Recently, Renato et al. (2009) used respirometry technique to measure a biodegradation process, then applied a mathematical models for studying the bioegradation kinetics of different oils.

The purposes of this work were to (i) collect a number of aerobic culturable HC (Hydrocarbons)-degrading microorganisms from petroleum polluted sites in Abou-Qir gulf of Egypt, (ii) identify the isolates to species level using molecular techniques, then examine under SEM (Scanning Electron Microscope), (iii) investigate the biodegradation activity of selected yeast strain and explore the variables affecting the crude oil biodegradation using statistical design.

MATERIALS AND METHODS

Sampling site and isolation of yeast

The yeast isolate used in this study was isolated from a motor oil polluted area of Abou-Qir gulf, Alexandria, Egypt. Samples were collected from sea water and slurry sediments. Nutrient Broth (NB) medium supplemented with 3.4% (w/v) NaCl was used for enrichment purposes. Cultures were incubated at 30˚C with agitation 200 rpm for three days. Different dilutions (10^{-3}-10^{-5}) of the growing cultures were taken and streaked out over two different media: Nutrient Agar (NA) supplemented with 3.4% (w/v) NaCl, and Natural Sea Water agar (NSWA) supplemented with 1% (w/v) glucose after that, the plates were incubated at 30◦C for overnight (O.N). Further purification was carried out for the yeasts colonies showing distinctive morphological features.

Preliminary test for degradation ability

The degradation capabilities of the purified yeast isolates was carried out by growing them on NSWA plates containing petroleum oil 0.5% (v/v). The plates were incubated at 30◦C for one week. The yeast strain showing the highest oil degradation capability was selected as a good candidate for extensive study.

DNA (Deoxyribonucleic acid) isolation from the yeast strains

Yeast strain was grown in GPY (Glucose Peptone Yeast Extract) medium containing (g/l) glucose 20, peptone 10 and yeast extract 5. Yeast culture was incubated at 30◦C for ON with agitation at 200 rpm. After that, the cells were harvested by centrifugation at 7,000 rpm for 3 min. in a microcentrifuge. The pellets containing the cells were washed with SET buffer and were used for isolation of genomic DNA according to manufactuer's instruction of EZ-10 Spin Column Kit (Bio Basic Inc. Canada).

Identification of the yeast isolate

18SrRNA (Ribonucleic acid) sequencing technique was employed to identify the yeast isolate on a molecular level. The universal primers 18SF149:5'-GGAAGGG(G/A)TGTAT TATTAG -3'and 18SR 701: 5'-GTAAAAG TCCTGGTTCCC-3', were used to amplify a partial fragment (522bp) of the 18SrRNA from yeast isolate PCR.

The PCR mixture contained 25 pmol of each primer, 10 ng of chromosomal DNA, 200 mM dNTPs and 2.5 U of Taq polymerase in 50 µl of polymerase buffer. The PCR thermocycler (Eppendorf) was programmed as follow: 95°C for 5 min for initial denaturation, 30 cycles 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. and a final extension of at 72◦C for 10min. Five microliters of the obtained PCR product were analyzed on 1% agarose gel electrophoresis (Ausubel et al., 1999) and were visualized on UV-transilluminator. The PCR product was purified using QIAquick PCR purification reagent (Qiagen). The amplified 18SrRNA fragment (552 bp) was sequenced in Both direction. BLAST (www.ncbi.nlm.nih.gov/blast) sequence analysis was carried out to affiliate the yeast isolate. Multiple sequence alignment and molecular phylogeny was performed using BioEdit (Hall, 1999).

Monitoring yeast growth and degradation ability

Microbial growth was measured spectrophotometrically as optical density (OD) at $\lambda_{600\ nm}$ wavelength. The percent of oil consumption was determined gravimetrically. A preliminary test for oil degradation by the yeast strain A was carried out in triplicates by cultivating it under shaking condition (200rpm) at 30˚C for 72h on NSW amended with 0.5% crude petroleum oil (M1); NSW amended with 1% crude petroleum oil (M2); NSW amended with 0.5% glucose and 0.5% crude petroleum oil (M3); NSW amended with 0.5% yeast extract and 0.5% crude petroleum oil (M4); NSW amended with 0.5% glucose; 0.5% yeast extract and 0.5% crude petroleum oil (M5); and Malt extract 2% and peptone 0.5% (M6). Media pH was adjusted to 7.0 before autoclaving. Microbial growth (OD_{600}) was measured spectrophotometrically, and the percent of oil consumption was measured gravimetrically using chloroform extraction method in acidified medium (APHA, 1998).

Physiology and phenotypic characterization

Some physiological tests were carried out on the isolate such as ability to grow at different pH of the medium (4-11). The ability of yeast to grow at medium containing different salt concentrations (1-12%) was tested as well. Cell morphological features were examined under SEM (Jeol analytical Scanning Electron Microscope/ 6360 LA), upon growing the yeast candidates on three media namely, M2, M5 and M6.

Hydrocarbons and some derivatives biodegradation potentials

The ability of the yeast isolate to biodegrade some aromatic derivatives hydrocarbons (naphthylamine, phenol, naphthalene ethyldiamine, phenanthrene, naphthalene 2-sulfonate and naphthalene) and aliphatic (n-hexane, n-heptane and n-pentadecane) was taken as an indication for the biodegradation potential of this isolate. Each hydrocarbon or derivative was tested at a concentration of 500 mg/l in NSW medium. Two percents of mother culture of the yeast isolate pre-grown in GPY medium for O.N was used to inoculate different sets of NSW supplemented with different aromatic hydrocarbons separately. All yeast cultures were grown at 30^{o}C for three days with agitation at 200 rpm. Yeast growth was monitored spectrophotometrically as mentioned above. However, the residual non-degraded hydrocarbons were estimated by GC and HPLC. The degradation efficiency of the isolate was studied using different concentrations (500 to 3000 mg/l) for naphthalene and phenol under the same cultural conditions.

Analytical methods

Extraction of residual crude oil

Cell– free supernatants of yeast cultures containing petroleum oil was extracted three times and the hydrophobic phase (organic phase) was separated by separating funnel. The chloroform extracts were evaporated and were weighed. Negative controls of the non-inoculated medium was extracted using the same extraction procedure. The residual oil was quantified gravimetrically after subtracting the reading from the negative control (APHA, 1998).

Determination of residual hydrocarbon and some derivatives

The treatments containing naphthalene, phenanthrene and phenol as carbon sources were extracted by *n*-hexane, other aliphatic hydrocarbons were extracted by dichloromethane. The extracts were analyzed by Gas Chromatography (GC), under the following conditions: GC Shimadzu GC-17A equipped with Shimadzu Aoc-zoi autosampler, column parameter 15 m, 0.25 mm ID–Rtx-5, 1.0 µL split injection with split ratio of 50:1. The injector and Detector temperatures were 250ºC. The oven temperature for naphthalene, naphthylamine, n-hexane, n-heptane and n-pentadecane detection was 150ºC, whereas for phenanthrene, it was 200ºC.

The residual phenol, naphthalene ethyldiamine, and naphthalene 2- sulfonate were analyzed using High Pressure Liquid Chromatography (HPLC) on Beckman system Gold 126 Solvent Module, 168 Detector (Dioale array) and outosampler (507e), under the following conditions: column 250 X 4.6mm, hyper clone 5µL ODSC18, automatic injection 20 µL/ wave length 254nm. (75% acetonitryl, 25% water) mobile phase.

A sterile negative control was being included in each experiment to calculate the percentage removal of tested compounds.

Experimental design

Fractional factorial deisgn

A Plackett-Burman design was employed to screen, evaluate and determine the key determinates affecting the oil biodegradation by the yeast isolate. Based on this design, each factor was studied at two levels: -1 for a low level and 1 for a high level (Plackett and Burman, 1946). This design is practical specially when the investigator is faced with a large number of factors and is unsure which settings are likely to be nearer to optimum responses (Strobel and Sullivan, 1999).

Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta i x_i$$

where Y is the response (% oil consumption), β_0 is the model intercept and β_i is the linear coefficient and x_i is the level of the independent variable. This model does not describe interaction among the variables and it is used to screen and evaluate the important variables that influence the response. Eleven independent variables were tested in 12 combinations. Trials were performed in triplicate and the average of the observations was taken as the response (Y). The main effect of each variable was calculated simply as the difference between the average of measurements made at the high setting (+) and the average of measurements observed the low setting (-) of the factor.

Statistical analysis of the data

Multiple regression analysis was carried out using Microsoft Excel 97 to determine the *p*-value, *t*-value and the confidence level. Factors (independent variables) showing the highest *t* value were chosen as the key determinants in the oil biodegradation process.

RESULTS

Isolation, identification and phylogenetic analysis

Only one yeast isolate, namely A, was selected as a good candidate oil degrader in this study. Molecular identification of this isolate based on 18srRNA sequencing was carried out. According to sequencing similarities and multiple alignment the isolate A was found to be in a close relation to *Candida tropicalis* NRRL Y-12968 (ac: EU348785) with a 99% identity. The partial sequence of 18Sr RNA of this isolate (A) has been deposited into GeneBank under accession numbers EU921327.

Morphological and oil biodegradation behavior of *C. tropicalis* A in different media

The effect of different media (M1-M6) on % of oil consumption and growth of *C. tropicalis* A was tested (Table 1). In this experiment, six media (mostly, FNSW: Filtered Natural Sea Water) based were used to explore the petroleum oil degradation potency in relation to growth morphology by the tested strain after 72h incubation time. The results revealed that M5 was the best medium among the tested media in respect to growth and oil consumption, while M2 was not favored either for growth or oil consumption.

Also, cellular dimensions were significantly altered as affected by medium composition as shown in elecrtron micrographs, where, a maximal cell diameter was attained in M6 and decreased in M5 and M2 (data not shown).

Table 1- Utilization of crude oil by *Candida tropicalis* strain A.

Tested medium	OD_{600}	Oil consumption %
M1	0.266	5.3
M2	0.213	2.1
M3	0.353	6.0
M4	2.580	6.1
M5	5.170	7.7
M6 (without oil)	7.990	0.0

Hydrocarbons and some derivatives degradation potentials of *C.tropicalis* strain A

The ability of *C. tropicalis* strain A to utilize different hydrocarbons and some derivatives at concentration of 500 mg/l was measured in submerged culture after 72h incubation time in NSW containing the tested compounds as a sole carbon source. Significant differences in degradation potentials were noticed as could be seen in Table 2. The tested strain showed a high

utilization tendency towards naphthalene and phenol. It showed a preference to degrade the short aliphatic chain while the degradation pattern as well as cellular growth decreased with increasing the chain length from n-hexan to n-pentadecane. A weak degradation potential was measured for naphthylamine. In addition, strain A showed a relatively high potency in degradation of naphthalene 2-sulphonate in comparison to phenantherene and naphthyl ethyl diamine. To evaluate the degradation potency of the strain for naphthalene and phenol, the NSW medium was amended with different concentrations ranging from 500 to 3000 mg/l and the results were illustrated in Fig. 1 and 2, respectively.

Table 2 - Utilization of different hydrocarbons and some derivatives by *Candida tropicalis* strain A.

Tested hydrocarbons	OD_{600}	Hydrocarbons/ derivatives consumption %
Phenol	0.491	53.60
Naphthalene	0.541	97.85
n-Hexane	0.234	23.40
n-Heptane	0.220	20.20
n-Pentadecane	0.151	15.80
Naphthalene 2-sulphonate	0.171	19.00
Naphthyl ethyl diamine	0.207	20.00
Naphthylamine	0.039	0.000
Phenantherene	0.279	22.00

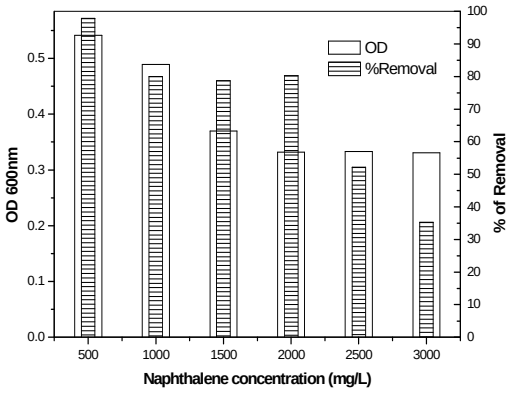

Figure 1 - Growth and utilization of naphthalene by *Candida tropicalis* strain A.

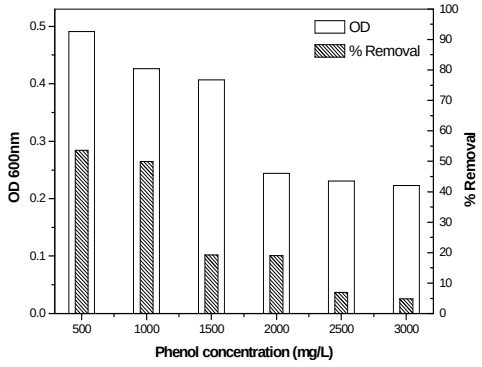

Figure 2 - Growth and utilization of phenol by *Candida tropicalis* A.

Results showed a progressive decrease in growth and percentage of naphthalene degradation with increasing naphthalene concentrations. Almost complete removal of naphthalene at concentration

500 mg/l was obtained and the removal efficiency decreased by increasing the naphthalene concentration and reached to 35% at 3000 mg/l naphthalene (Fig. 1). Similarly, a progressive decrease in growth and percentage of phenol degradation has observed with increasing phenol concentrations, while at moderate concentration of phenol (1000 mg/l), the strain utilized 49.8% (Fig. 2).

Factors affecting petroleum oil degradation by yeast strain A

In order to find out the most significant factors affecting petroleum oil degradation by the *C. tropicalis* strain A, Plackett-Burman factorial design was applied. The independent variables examined in the Plackett-Burman experiment and their settings along with the measured percentage of petroleum oil consumption are shown in Table 3; the degradation percentage ranged from 0.85 to 13.8 %. The main effect of the examined variables on petroleum oil consumption was calculated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor, and presented graphically in Fig. 3.

Table 3- Randomized Plackett–Burman experimental design for evaluating factors influencing oil degradation by *Candida tropicalis* strain A.

Trial	Petrolum crude Oil	Glucose	$(NH4)_2SO4$	NH4-Cl	NH4HPO4	Yeast-Extract	KH2PO4	$K2HPO_4$	Inoclum size	pH	Medium volume	Percentage of consumed crude petroleum oil mg%
1	1(1.0)	-1(0.5)	-1(0.5)	1(1.0)	-1(0.5)	1(0.5)	1(0.5)	1(0.5)	-1(1)	-1(5)	-1(50)	6.39
2	1(1.0)	-1(0.5)	1(1.0)	1(1.0)	1(1.0)	-1(0.1)	-1(0.1)	-1(0.1)	1(3)	-1(5)	-1(50)	0.85
3	1(1.0)	-1(0.5)	-1(0.5)	-1(0.5)	1(1.0)	-1(0.1)	-1(0.1)	1(0.5)	-1(1)	1(7)	1(30)	5.37
4	1(1.0)	1(1.0)	1(1.0)	-1(0.5)	-1(0.5)	-1(0.1)	1(0.5)	-1(0.1)	-1(1)	1(7)	-1(50)	5.11
5	-1(0.5)	-1(0.5)	1(1.0)	-1(0.5)	-1(0.5)	1(0.5)	-1(0.1)	1(0.5)	1(3)	1(7)	-1(50)	5.97
6	-1(0.5)	1(1.0)	-1(0.5)	1(1.0)	1(1.0)	1(0.5)	-1(0.1)	-1(0.1)	-1(1)	1(7)	-1(50)	2.56
7	1(1.0)	1(1.0)	-1(0.5)	-1(0.5)	-1(0.5)	1(0.5)	-1(0.1)	-1(0.1)	1(3)	-1(5)	1(30)	9.72
8	-1(0.5)	-1(0.5)	1(1.0)	-1(0.5)	1(1.0)	1(0.5)	1(0.5)	-1(0.1)	-1(1)	-1(5)	1(30)	5.63
9	1(1.0)	1(1.0)	1(1.0)	1(1.0)	1(1.0)	1(0.5)	1(0.5)	1(0.5)	1(3)	1(7)	1(30)	3,56
10	-1(0.5)	1(1.0)	-1(0.5)	-1(0.5)	1(1.0)	-1(0.1)	1(0.5)	1(0.5)	1(3)	-1(5)	-1(50)	4.26
11	-1(0.5)	-1(0.5)	-1(0.5)	1(1.0)	-1(0.5)	-1(0.1)	1(0.5)	-1(0.1)	1(3)	1(7)	1(30)	13.80
12	-1(0.5)	1(1.0)	1(1.0)	1(1.0)	-1(0.5)	-1(0.1)	-1(0.1)	1(0.5)	-1(1)	-1(5)	1(30)	13.30

Variable levels are presented between brackets expressed as g%.

Based on these results, the low level of NH_4HPO_4, petroleum oil, NH_4SO_4 and yeast extract enhanced the oil consumption. On the other hand, the high level of aeration, NH_4Cl and K_2HPO_4 improved the oil consumption by the yeast strain and recorded to have the highest positive significant effect. The analysis of the regression coefficient and *t*-values for the eleven variables are presented in Table 4. The *t*-test for an individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Therefore, when neglecting the insignificant terms, the equation for oil consumption % could be reduced as follows:

$$Y_{\%\ \text{oil consumption}} = 6.376667 + 0.36667\ X_4 + 0.098333\ X_8 + 2.18666\ X_{11}$$

Where, X_4, X_8 and X_{11} are NH_4Cl, K_2HPO_4 and aeration as medium volume, respectively.

Application of the suggested near–optimum culturing condition in NSW based medium resulted in about 2 folds increase in oil consumption after 72h incubation under shaking at 30°C.

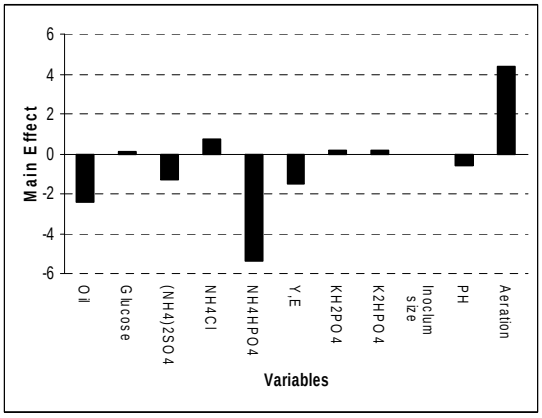

Figure 3 - Effect of environmental and nutritional factors on crude petroleum oil degradation by *Candida tropicalis* strain A.

Table 4- Statistical analysis of Plackett-Burman experiment for crude petroleum oil degradation by *Candida tropicalis* strain A.

Variable	β	Main effect	*t*-stat	*P*-value	Confidence level %
Intercept	6.376670	-	-	-	-
Petroleum crude oil	-1.210000	-2.420000	-1.270460	0.250958	74.9042
D-Glucose	0.041667	0.083340	0.043749	0.966524	3.34760
$(NH4)_2SO4$	-0.640000	-1.280000	-0.671980	0.526610	47.3390
NH4Cl	0.366667	0.733400	0.384989	0.713533	28.6460
NH4HPO4	-2.671670	-5.343340	-2.805170	0.030952	96.9048
Yeast-Extract	-0.738330	-1.476670	-0.545860	0.608623	39.1377
KH2PO4	0.081667	0.166334	0.060337	0.954194	4.58060
K2HPO4	0.098333	0.196666	0.072699	0.944864	5.51360
Inoclum size	-0.016670	-0.033340	-0.012320	0.990645	0.39550
pH	-0.315000	-0.630000	-0.232880	0.825084	17.4900
Medium volume	2.186667	4.373340	1.616635	0.166880	83.3120

DISCUSSION

One major obstacle that has slowed the implementation of microbial enhanced oil recovery has been the difficulty in isolating and/or engineering microorganisms that can survive the harsh environment of the oil reservoir. Therefore, in this study, a marine yeast strain A isolated from Abou-Qir gulf of Alexandria, Egypt was selected due to its pronounced crude petroleum oil biodegradation ability. The selected strain was identified as *Candida tropicalis* with a 99% of similarity. Morphological examination under SEM and simple characterization of the tested strain was performed and proved its ability to grow over a wide range of pH (4-11) with optimal growth at pH 8. In addition, the isolate tolerated a wide range of salt concentrations (1-12%). These properties, in addition of being a natural isolate of petroleum oil contaminated area, motivated to investigate the potentiality of this isolate to be used for petroleum biodegradation in sea water.

The ability of crude oil degradation by the investigated strain was monitored in different media with respect to cellular growth, where M5 containing glucose and yeast extract supported the growth, and consequently maximum oil consumption (7.7%). On the other hand, M3 which contained glucose and petroleum oil showed lower levels of oil consumption (6 %). However, the addition of yeast extract to the medium enhanced the biodegradation capacity. This could be due to the fact that yeast extract was enriched with amino acids and vitamins which acted as co-factors for enzyme productions (El-Helow et al., 2000; Pedersen and Nielsen, 2000; Konsoula et al., 2004).

The cellular morphology of the yeast strain A was examined under SEM, when grown in three different media: Malt-peptone medium as a

complex medium without adding petroleum oil (M6), NSW+ 1% petroleum oil (M2) and NSW supplemented with glucose and yeast extract amended with 0.5% crude petroleum oil (M5). The morphological shape (cellular diameter) was changed in relation to medium composition, where a progressive decrease in the cell diameter from complex medium (M6) to medium with additives (M5) to the poorest medium (M2) was observed. This showed the importance of organic nitrogen source for enhancing initially the growth as described previously (Konsoula et al., 2004).

Crude petroleum oil and hydrocarbon degradation has been analyzed for several fungal and bacterial species, showing variable potentialities (Ijah, 1998; Chaillan et al., 2004; Elshafie et al., 2007; Nievas et al., 2007). However, only few works refered to the isolation and characterization of yeast strains able to utilize hydrocarbons (Ijah, 1998). Therefore, this study focused initially on measuring the ability of the yeast strain A to degrade different aliphatic and aromatic hydrocarbons and some derivatives as sole carbon source in natural sea water medium.

Among tested compounds the investigated yeast showed a great ability to degrade most of them but, with distinctive preference for naphthalene and phenol.

Regarding the aliphatic hydrocarbons, the strain showed adequate potential of degradation which decreased with increasing the chain length (C6, C7 and C15).

In contrast, *Serratia marcescens* OCS-21 and *Acinetobacter calcoaceticus* COU-27 attacked long chains n-alkanes, probably because of the less toxic nature of the long chain n-alkanes rather than short chain as reported by Atlas and Bartha (1981) and Ijah (1998). On the other hand, the tested yeast could not metabolize or degrade naphthylamine and naphthalene 2- sulphonate, due to their lethal effect as reported by Nynes et al. (1968). The ability of *C. tropicalis* A, to degrade long and short chain hydrocarbons could be attributed to having a very efficient degradative enzyme system. This was in agreement with the data reported by Ijah (1998). The utilization abilities of fungi to different hydrocarbons showed significant differences (Elshafie et al., 2007). Prince (1993) reported that microorganisms show a distinct preference for some hydrocarbons over other.

To evaluate the potency and tolerance of the selected strain to degrade different naphthalene and phenol concentrations, the natural sea water was supplemented with naphthalene and phenol at different concentrations (500-3000 mg/l). Strain A showed good potency in degradation and tolerance of naphthalene over phenol.

At a concentration of 1500 and 1000 mg/ml of naphthalene and phenol the utilization reached 78 and 49.9%, respectively.

Medium optimization is generally a time consuming and labour-intensive process. The Plackett-Burman experimental design proved to be a valuable tool for the rapid evaluation of the effects of the various medium components. Since this design is a preliminary optimization technique, which tests only two level of each variable, it can not provide the optimal level of each component required in the medium. This technique, however, provides indications of how each component tends to affect petroleum oil degradation by the tested yeast. The variables tested in the design were contributed differently on petroleum bioremediation by *C.tropicals* strain A. Aeration, NH_4Cl and K_2HPO_4 contributed positively. However, NH_4HPO_4, oil concentration, $(NH_4)_2SO_4$ and yeast extract contributed negatively. This showed NH_4Cl was preferred and the other inorganic and/or organic nitrogen sources repressed the biodegradation activity. Glucose showed a positive effect. The limited glucose concentration is essential to avoid catabolite repression. Aeration plays an important role in oil bioremediation and is considered a rate limiting factor in the biodegradation of marine oil spills where, oxygen is necessary for initial breakdown of hydrocarbon and subsequent reactions (Haines et al., 2003). The presence of phosphate plays a critical role and its inadequate supply may result in slowing the rate of biodegradation (Haines et al., 2003). Also, phosphorus can be used as a source of storage energy in the form of ATP. Lacke of nitrogen and phosphorus is most likely to limit biodegradation as reported by Prince et al. (1994).

This work might eventually help in bioremediation studies to clean up marine oil spills in an affected environment by the use of marine yeast strains with desirable degradative activities. Bioremediation in expected to play an important role as environmentally safe and cost-effective response to marine oil spills.

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