



Original article

Isolation and identification of crude oil-degrading yeast strains from Khafji oil field, Saudi Arabia

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ABSTRACT

Twenty five yeasts isolated were isolated from Khurais oil field in Saudi Arabia and assayed to evaluate their biodegradability. Only five isolates (namely, A1, A2, A3, A4 and A5) showed potential use of oil as sole carbon source. During incubation period, highest growth rate were recorded for A1, A2 and A3 isolates. Low growth distinguished A4 isolate; A5 isolate could not degrade oil.

Spectrophotometrical analysis for four yeast isolates biodegradation activities indicated that, A1 isolate was superior for oil degradation (61%) comparing with A4 isolate which reflected lowest degradation % (33%). A2 and A3 isolates showed moderate biodegradation activity (56 and 51% respectively).

D1/D2 domain of the 26S rRNA gene sequence was used as molecular marker to identify five yeast isolates. After comparing 26S rRNA gene sequences of five yeast isolates with highly similarity isolates, five yeast isolates (A1, A2, A3, A4 and A5) were submitted to database as *Candida tropicalis* (MW488263), *Candida tropicalis* (MW488264), *Rhodotorula mucilaginosa* (MW488265) and *Rhodospiridium toruloides* (MW488266) respectively. Using OXF1/ACR1 primer, specific lipase gene amplicon with 250 bp were detected with in all four yeast isolates.

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1. Introduction

Oil and oil-related compounds consider the main contaminant for Soil and water. Many strategies are developed to control environmental petroleum contamination.

Mechanical or physicochemical processes were applied firstly for oil spill disappearing. Then, varied physicochemical techniques are applied to clean-up water surface. (Cerniglia 1993). Hydrocarbons biodegradation by employing natural micro flora consider the main process for eliminating hydrocarbon-pollutions. Thus, biodegradation process. Microbial biodegradable activity consider a principle tool to eliminate petroleum contaminant has been studied and monitored extensively (Atlas, 1984; Leahy and Colwell, 1990; Vam Hamme et al., 2003; Pinzon-Martinez et al., 2010).

Recently, fungi have more concerns about its biodegradable capability through enzymes production which applied for degrada-

tion lignin and different environmental contaminants like hydrocarbons and pesticides (Bumpus et al., 1985). Fungi are superior bacteria for biodegradation process via hyphae production which penetrates soil and biodegrade hydrocarbon in deep contaminated soil surface (Novotny et al., 1999; April et al., 2000).

Many recent studies have been accomplished for isolation and identification fungi biodegradable hydrocarbons as a key role for biodegradation process (Clemente et al., 2001, Prenafeta-Boldu et al., 2001; Santos et al., 2008). D1/D2 domain of the 26S rRNA and 18S rRNA subunit sequences were studied extensively through polymerase chain reaction to compare with available nucleotide databases (Kurtzman and Robnett 1998, James et al. 1997). Furthermore, D1/D2 domain of the 26S rRNA has approved as a helpful tool for yeast identification and detecting biodegradation activity. D1/D2 domain includes different marker genes like, actin-1 (Daniel et al., 2001; Kurtzman and Robnett 2003), RNA polymerase II (Kurtzman and Robnett 2003) and mitochondrial encoded genes (Kurtzman and Robnett 2003, Belloch et al. 2000; Kurtzman and Robnett 2003).

Recently, GC-MS consider as effective tool for detecting degradation activity different microorganisms (Prachi and Mishra, 2015).

The objective of this investigation was to isolate and identify yeast isolates capable to utilize the oils as sole carbon sources.

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Additionally, biodegradation end products were recorded and detected. Furthermore, a lipases gene was detected for yeast isolates to clear correlation between gene presence and oil biodegradation activity.

2. Materials and methods

2.1. Sampling site and isolation of yeast

Oil polluted soil samples were collected from January – April 2019 from Khafji oil field (25.9333°N 49.6667°E) located in Eastern Province, Saudi Arabia (Fig. 1). Samples were collected from containers in sterile plastic and immediately transported to the laboratory and stored at 4 °C until further use.

2.2. Yeast isolation from oil contaminated soil

To isolate oil degrading yeast, enrichment method was applied by using mineral basal salts (MBS) medium supplemented with 1% (v/v) oil. After incubation culture for seven days at 30°C with agitation 200 rpm (Hesham et al., 2006). To purify yeast colonies, different dilutions (10^{-3} – 10^{-5}) of the growing cultures were streaked over oil-MBS media plates. Then, recitative streaking was performed for pure yeast isolates on coated agar plates with oil and refrigerated at 4°C on MBS agar plates.

2.3. Dcpi assay

DCPIP method was employed to select yeast strains according to its biodegradability. This method based on supplemented media with electron acceptor such as 2, 6-dichlorophenol-indophenol (DCPIP). Reducing DCPIP from blue (oxidized) to colorless (reduced) indicated yeast ability for utilizing hydrocarbon substrate. After added 250 µl Büshnell-Haas (BH) medium, 30 µl of each yeast suspension (108 CFU/mL) and 10 µl of diesel oil. After incubation for two weeks at 30 °C, changing color from dark blue to colorless indicates oil biodegradability for yeast isolate (Hanson et al., 1993).

2.4. Evaluation of degradation ability

Biodegradation test was carried out over 15 days in Erlenmeyer flasks of 500 mL for each flask, 60 mL of the Bushnell Haas medium containing KH_2PO_4 (1 g), K_2HPO_4 (1 g), NH_4NO_3 (1 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2g), FeCl_3 (0.05 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.02 g), H_2O distilled 1L (Atlas, 1995b) and 15 mL of oil which added as sole Carbon source and 25 mL of yeast inoculums. Samples without yeast were used as control. Also, three replicates were taken for each control and sam-

ples. Finally, Yeast growth was evaluated spectrophotometrically at 600 nm.

2.5. GC–Ms

Mass Spectra Gas chromatography (Shimadzu GCQ2010, Japan)-flame ionization detector (FID) was employed to detect the end metabolite products of oil degradation by four yeast isolates according to Hassanshahian et al., (2012). In the presence of dichloromethane, separating funnel was used to extract residual oil which remaining after yeast growth (in Bushnell haas media at 30 °C with 180 rpm for two weeks). To eliminate remaining moisture, Calcium sulfate was applied as drying agent. GC–MS program as follows 60 °C for 2 min and increase 6 °C until reaches 300 °C for 15 min. Operating temperature for the injector was 300 °C and detectors were 320 °C. The carrier gas for this run in Nitrogen.

2.6. Molecular identification of the yeast isolate

To identify yeast isolates, 26S rRNA gene sequence was employed as molecular marker. 1 g of fungal hyphae was incubated in 100 µl lyticase solution at 30 °C for 1 h. After that, 20 µl proteinase K were supplemented and incubated at 55 °C for 60 min to eliminate protein. Then, samples were incubated for two hours at 65 °C (El Hanafy et al., 2015). NL1 (5'-GCATATCAA TAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTCAAGACGG-3') primers were applied to amplify Specific D1/D2 domain of 26S rDNA region and PCR condition was optimized according to () with. Some modifications (Kurtzman et al., 1998). For detecting amplicons, 1.5% agarose gel electrophoresis (Ausubel et al., 1999) was used and documented with Digi-doc it gel documentation system (UVP, UK). Gene JET PCR Purification Kit (Thermo Scientific) was used for DNA purification. ABI PRISM® 3100 Genetic Analyzer was applied for PCR products and performed by Macrogen In. Seal, Korea. Aligned sequences were analyzed on NCBI website (<http://www.ncbi.nlm.nih.gov/webcite>) using BLAST to affiliate the yeast isolate. Multiple sequence alignment, and molecular phylogeny was performed using ClustalW software analysis (www.ClustalW.com). Nucleotide sequence accession numbers D1/D2 domain of the 26S rRNA gene sequences were submitted to NCBI database to get the accession numbers.

2.7. Amplification and detection of lipase genes

For detecting lipase genes, total genomic DNA for four yeast isolates were purified through Yeast DNA Extraction Kit (78870,



Fig. 1. Khafji oil field, Dhahran, Saudi Arabia.

Thermo Fisher Scientific) according to manufacturer protocol. As shown by table (), primers OXF1, ACR1 primers were selected to flank active-site and oxyanion hole regions of putative lipase genes from the related yeast *Candida albicans* (Rodríguez-Mateus et al., 2018). Green taq (DreamTaq) master mix (Thermo scientific) was used for lipase gene amplification according to manufacture protocol. Thermal cycler (Creacon, Holand), conditions were applied according to Rodríguez-Mateus et al., (2018) with some modifications. Lipase amplicon (approx, 869 bp) was loaded on 1.5% (w/v) Agarose gel and documented via gel documentation system (Dig-doc, UVP, INC, England). Gene JET PCR Purification Kit (Thermo Scientific) was used for DNA purification. ABI PRISM® 3100 Genetic Analyzer was applied for PCR products and performed by Macrogen In. Seal, Korea. Aligned sequences were analyzed on NCBI website (<http://www.ncbi.nlm.nih.gov/webcite>) using BLAST to confirm their identity by comparing with available lipase gene sequences in the GenBank.

3. Results

3.1. Dcpip

Depending on redox indicator reduction and changing redox indicator color, among twenty five yeast isolates which tested for utilizing crude oil as a sole carbon source only five yeast isolates were characterized with crude oil biodegradable activity.

3.2. Estimation of hydrocarbons degradation

By using mineral basal salts (MBS) medium supplemented with 1% (v/v) oil as the sole carbon source as enrichment method, twenty five yeasts isolated were tested to evaluate their biodegradability against oil. Only five isolates (namely, A1, A2, A3, A4 and A5) reflected distinguishable growth rate with oil after incubation for seven days. A1, A2 and A3 isolates were superior for oil degrading comparing with A4 isolate which showed low growth with oil degrade decreasing ability comparing with 0.4681 OD600 growth rate at zero time for all isolates. Interestingly, A5 isolate characterized with no ability for oil biodegrading (Table 1).

Regarding to Table 2, four yeast isolates showed varied crude oil degradation capabilities. A1 isolate was superior for oil degradation (61%) comparing with A4 isolate which reflected lowest degradation % (33%). Furthermore, all of isolates A2 and A3 showed moderate biodegradation activity (56 and 51% respectively).

3.3. GC-MS analysis

Depending on retention times for standard compounds (which present in crude oil), four major peaks were identified as Tetradecane, pentadecane, Heptadecane and Tetracosane. As shown by Table 3 and Fig. 3, four yeast isolates reflected varied biodegradation activities comparing with standard compounds. A1 isolate was superior for degrading major four oil compounds (59.5, 59.1, 45.6 and 51.4 of reduction area (%) comparing with A4 isolate which reflected lowest degradability for major four oil compounds (39, 23, 27.6 and 32.9 of reduction area (%) for Tetradecane, pen-

Table 1
growth rates (OD600) of five yeast isolates in the presence of 1% (v/v) oil.

Isolates	Code	Growth rate (OD 600)
1	A1	0.988
2	A2	0.873
3	A3	0.833
4	A4	0.688
5	A5	0.232

Table 2
Degradation % for five yeast isolates in the presence of 1% (v/v) oil.

Isolates	Identifying	Accession No.	% degradation
A1	<i>Candida tropicalis</i>	MW488263	61
A2	<i>Candida tropicalis</i>	MW488264	56
A3	<i>Rhodotorula mucilaginosa</i>	MW488265	51
A4	<i>Rhodospiridium toruloides</i>	MW488266	33

tadecane, Heptadecane and Tetracosane, respectively). A2 and A3 yeast isolate showed nearly the same patterns for degrading major four oil compounds.

3.4. D1/D2 domains of the 26S rRNA gene molecular identification marker

D1/D2 Domains of the 26S rRNA was applied as molecular marker. After purified total genomic DNA for all five yeast isolates, D1/D2 domain of the 26S rRNA gene was amplified. After that, eluted amplicons were sequenced. Based on D1/D2 domain of the 26S rRNA partial sequence, all five yeast isolates (A1, A2, A3, A4 and A5) has been deposited into GenBank under accession numbers MW488263, MW488264, MW488265, MW488266 and MW488267 respectively. According to alignments results in GenBank, five yeast isolates showed similarities ranged from 98 to 100% against different yeast species. A1 and A2 strains had identity to *Candida tropicalis* with 99% of genetic similarities. Moreover, strains A3, A4 and A5 showed 99% identity to *Rhodotorula mucilaginosa*, *Rhodospiridium toruloides* and *Sporobolomyces lactosus* respectively. Regarding to Fig. 2, MEGA version 4 software was used to construct Phylogenetic tree of five isolates against highest 10 similarity isolates from GenData Bank (<http://www.ncbi.nlm.nih.gov>).

3.5. Lipase gene detection

As shown in Fig. 4. A, B and C, by using OXF1/ACR1 primer, lipase gene amplicons were detected with 250 bp in all four yeast isolates. Based on semi quantitative expression data, lipase gene reflected different expression level for four yeast isolates. Yeast isolate A1 (*Candida tropicalis*) showed highest lipase gene (1.01) comparing with isolate A4 which expressed lowest expression level (0.55). Similar expression levels were detected for A3 (*Rhodotorula mucilaginosa*) and A4 (*Rhodospiridium toruloides*) yeast isolates (0.67 and 0.62 respectively). Our previous data for lipase gene semi quantitative expression levels for four yeast isolates added more light on oil biodegradation activities for four yeast isolates. Highest semi quantitative expression level for A1 (*Candida tropicalis*) was corresponded to increasing in oil degradation (61%). Furthermore, suppression of semi quantitative expression level for A4 (*Rhodospiridium toruloides*) could explain oil degradation decreasing activity (33%).

4. Discussion

Regarding to changing redox indicator color caused by reduction of redox indicator which referred to capability of crude oil biodegradable activity for five yeast isolates, more light was added to our findings by Hussein (2012). He cleared that, There are three main indicators could be use to identify capability of fungal isolates to degrade crude oil. Converting color of culture media from blue to colorless which corresponding to reduce crude oil quantity, indicate fungal biodegrading capability. In accordance with our results for using DCPIP reaction to screening fungal biodegradation activity, Hanson et al., (1993) explained relation between

Table 3
GC–MS analysis results of oil biodegradation compounds for four yeast isolates

Compound	Retention Time (min)	Peak Area (Control)	Peak Area For yeast isolates				Reduction Area (%) For yeast isolates			
			A1	A2	A3	A4	A1	A2	A3	A4
Tetradecane	6.250	356,377	211,988	187,992	162,278	138,832	59.5	52.8	45.5	39.0
pentadecane	6.945	986,523	582,771	488,277	387,716	226,672	59.1	49.5	39.3	23.0
Heptadecane	8.225	1,156,421	527,718	471,187	387,726	318,826	45.6	40.7	33.5	27.6
Tetracosane	9.380	970,288	498,818	418,562	389,928	3,185,821	51.4	43.1	40.2	32.9

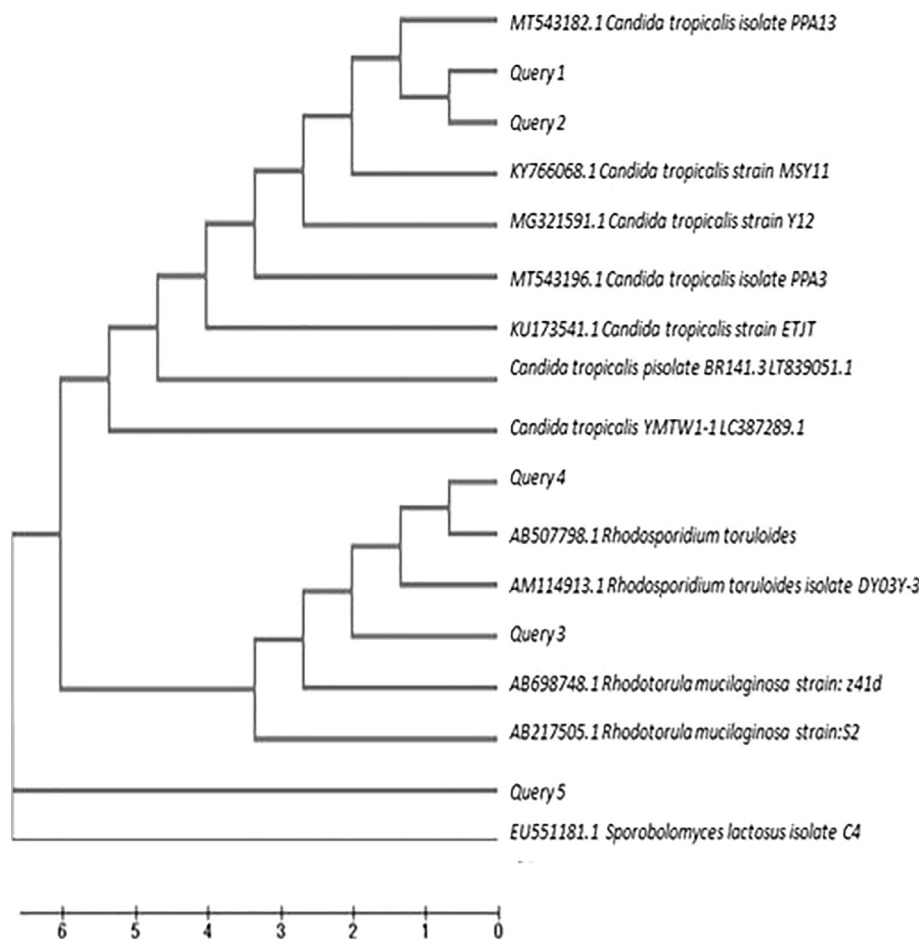


Fig. 2. Phylogenetic tree for five yeast isolates based on D1/D2 Domain sequences of the 26S rRNA.

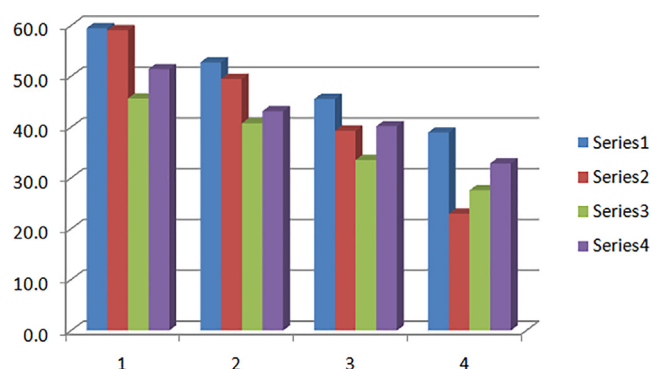


Fig. 3. GC–MS analysis of oil biodegradation compounds reduction area (%) for four yeast isolates.

degrading oil and changing DCPIP color. DCPIP reduction process which corresponded to changes color from blue (oxidized) to colorless (reduced) consider an electron lost mechanism. Fungi crude oil biodegrade processing was performed via incorporation of an electron acceptor.

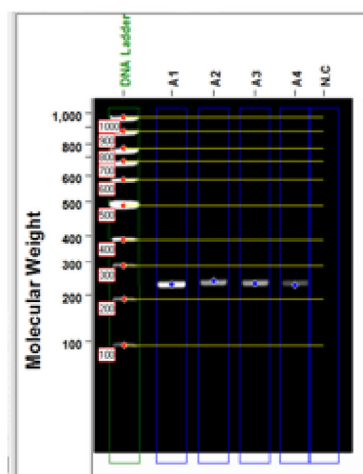
Four yeast isolates were showed varied growth rate on mineral basal salts (MBS) medium supplemented with 1% (v/v) oil. Furthermore, 61, 56, 51 and 33% of crude oil were degraded by A1, A2, A3 and A4 yeast isolates respectively. Recently, using natural microbial flora for biodegrading varied xenobiotic compounds, including oil consider the most powerful methodology (Cappello et al., 2007). With accordance of our results for yeast biodegradability, Shumin et al., (2012) indicated diesel oil biodegrading activity for yeast isolates which belong to Candida. Increasing oil biodegradation activity for yeast after incubation for seven days explained by bire et al., (2008). They explained that, it could be because increasing special receptor sites for binding hydrocarbons

1	<i>Candida tropicalis</i>
2	<i>Candida tropicalis</i>
3	<i>Rhodotorula mucilaginosa</i>
4	<i>Rhodosporidium toruloides</i>

	Tetradecane
	pentadecane
	Heptadecane
	Tetracosane



(A)



(B)

A1 (2)	A2 (3)	A3 (4)	A4 (5)
Lane %	Lane %	Lane %	Lane %
1.01	0.67	0.62	0.55

(C)

Fig. 4. Lipase gene specific amplicon with 250 bp (A), fragment length calculation (B) and semi quantitative lipase gene expression (C) for four yeast isolates.

which uptake hydrocarbon uptake and may be play a key role for transfer hydrocarbons into the cell. Furthermore, catabolic pathway was accelerated via energy which generate via enzymes that introduce oxygen into the carbon. Our findings for using yeast as biodegradation tool for crude oil was in accordance of Chandran et al., (2011). They indicated that, 97% of diesel oil was degraded through 10 days by applying formed yeast biofilm on gravels. Efficiency of use yeast for oil biodegradation could be explained in the light of fact that yeast was enriched with amino acids and vitamins which act as co-factors for important enzyme productions which consider a key role for oil biodegrading process (El-Helow et al., 2000). More support was added to our finding for evaluating oil biodegradation spectrophotometrically by Farag and Soliman (2012). Used turbidity measurement (OD600 nm) as indirect indicator for assaying yeast growth rate via visible spectrophotometer. More light was added by Leahy et al., (2003) for using spectrophotometrical method to detect hydrocarbon degrading capability

through estimate yeast growth at 600 nm. In accordance with our results, Leahy et al., (2003) indicated that bacterial hydrocarbon biodegrading was evaluated spectrophotometrically.

Depending on GC–MS analysis, four yeasts isolates reflected different biodegradation activities against four major derivatives were characterized as Tetradecane, Pentadecane, Heptadecane and Tetracosane. Our obtaining spectrophotometrical results for crude oil degrading by yeast were corresponded with GC–MS interrelated data. More support was added to our results for applying GC–MS to detect biodegradability of yeast for different hydrocarbons compounds by Norman et al., (2004). In accordance with our findings for using yeast for crude oil biodegradation, Abioye et al., (2013) indicated that, *Saccharomyces cerevisiae* showed around 49% of hydrocarbons biodegrading which present in crude oil. Our results for employing gas chromatography-mass spectroscopy (GC–MS) for detecting yeast biodegradability were supported by Ramadan, (2012). They the same technique to prove biodegrading efficiency

of *Candida viswanathii* KA-201 L against lubricating and diesel oils at salt concentration only after four days.

For identified four yeast isolates, 26S rRNA domains D1/D2 was employed as molecular identification method. Five yeast isolates were identified as *Candida tropicalis* (A1 and A2 strains), strains A3, A4 and A5 were identified as *Rhodotorula mucilaginosa*, *Rhodospiridium toruloides* and *Sporobolomyces lactosus* respectively. More support was added to our results for identifying yeast isolate via molecular sequencing marker by Shumin et al., (2012). They used 18S rDNA gene sequence to identify yeast hydrocarbon degrading as *Pichia ohmen*, which belong to *Candida*. With accordance with our findings for using D1/D2 domain of the large sub-unit 26S ribosomal DNA to identify oil biodegrading yeast isolate, Hashem et al., (2018) used the same technique to molecularly identified new six yeast isolates with degrade both aliphatic and aromatic hydrocarbons ability as *Yamadazyma mexicana* KKUY-0160, *Rhodotorula taiwanensis* KKUY-0162, *Pichia kluyveri* KKUY-0163, *Rhodotorula ingeniosa* KKUY-0170, *Candida pseudointermedia* KKUY-0192 and *Meyerozyma guilliermondii* KKUY-0214.

Four yeast isolates expressed varied levels of lipase gene depending on semi quantitative method. More light was added to our results for applied OXF1/ACR1 primer combination to detect lipase gene by (Rodríguez-Mateus et al., 2018). They amplified lipase gene through different primer combinations as a results to shortage of available sequences for lipase genes in yeast and indicated that, successful lipase gene amplicons of approximately 250 bp were obtained by using OXF1/ACR1 primer combination. Furthermore, the same primer combination was used successfully by Bell et al. (2002). They detect 250 bp lipase gene amplicons by sequencing and reflected highly similarity to lipases and phospholipids sequences. With accordance of our findings, Terahara et al. (2010) used OXF1-ACR1 primers to prove putative esterase genes from environmental DNA samples. Sequencing results showed that 32–80% similarity was detected for lipases gene.

5. Conclusion

Twenty five yeasts which isolated from contaminated soil of Khafji oil field (Eastern Province, Saudi Arabia) were evaluated for hydrocarbon biodegradative abilities. 61, 56, 51 and 33% of biodegradation % were characterized four yeast isolates A1, A2, A3 and A4 respectively. GC analysis for oil degradation Spectrophotometrical analysis for four yeast isolates biodegradation activities showed that, A1 isolate was superior for oil degradation (61%) comparing with A4 isolate which reflected lowest degradation % (33%). A2 and A3 isolates showed moderate biodegradation activity (56 and 51% respectively). Through D1/D2 domain of the 26S rRNA gene sequence which applied as molecular identification method, four yeast isolates identified and submitted as *Candida tropicalis* (MW488263), *Candida tropicalis* (MW488264), *Rhodotorula mucilaginosa* (MW488265) and *Rhodospiridium toruloides* (MW488266) respectively

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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