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Full Length Research Paper

Biodegradation of used lubricating and diesel oils by a new yeast strain *Candida viswanathii KA*-2011

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Disposal of the automotive lubricating oil after usage can cause environmental hazards. This study aimed to isolate a microbial isolate is that able to biodegrade lubricating oil effectively. A new yeast strain, identified by 18S-rRNA gene sequencing as *Candida viswanathii* KA-201l, was isolated from used lubricating oil, showed high biodegradation efficiency for different used lubricating oils. Capability of this isolate to degrade different high and low molecular weight hydrocarbons, castor oil, diesel oil and grease was tested. It showed high degradation efficiency for most of the tested compounds. The biodegradation products were analyzed by gas chromatography-mass spectroscopy (GC-MS) in most cases. The biodegradation efficiency under high osmotic pressure was studied. It effectively biodegrade lubricating and diesel oils (58.6 and 93.9%, respectively) at 6% salt concentration after four days only. *C. viswanathii* KA-2011 can be effectively used for removal of lubricants, diesel or vegetative oils pollution from soil, wastewater and sea water. Use of *C. viswanathii* KA-2011 in the bioremediation of lubricant or gasoline contaminated sea-water save the aquaculture from these pollutants, as well as it open new horizons in using of contaminated soil and wastewater in agriculture.

Key words: Lubricating oils, diesel oil, biodegradation, *Candida viswanathii.*

INTRODUCTION

A huge amount of used lubricating oils are produced worldwide. The different sources of used lubricating oils are railway workshops, industries, ship garages and automobiles workshops. All types of the used lubricants become contaminated and lose their performance due to changes in some of their properties. Therefore, such oils must be removed, as used oil, from the service as frequently as necessary (Shakirullah et al., 2006). How to handle and what to do with the used lubricating oils are serious concerns to environmentalists. Used lubricating oils disposal using traditional techniques such as land filling, road oiling, track side foliage etc., create serious environmental problems. The common disposal technique of used lubricating oil is burning for energy generation. In recent studies; the loss of lubricants to the environment was estimated to be 600,000 tons (Heitzman et al., 1985). This causes not only an un-quantified hazard to the environment, but also a potential hazard to the long–term health of the population (Wright et al., 1993).

The biodegradation of water–insoluble organic compounds of high molecular weight such as lubricating oils, industrial crude oil residues or petroleum compounds has been the subject of great interest for many years but has not been studied in detail. Michael et al. (1993) isolated an isolate of *Micrococcus roseus* able to biodegrade a synthetic lubricant. Jose et al. (2009) used an isolate of *Candida viswanathii* for biodegradation of biodiesel/diesel blends. This isolate was able to biodegrade approximately 50% of this blend in the contaminated soil. Adriano et al. (2010) reported that C. viswanathii was the best microorganism among the tested microbial isolates for biodegradation of biodiesel/ diesel blends.

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Abbreviation: GC-MS, Gas chromatography-mass spectroscopy.

Biodegradation using microorganisms is usually the preferred and major rout due to its cost effectiveness and comparatively higher cleanup. Therefore, several studies were performed for isolation of microorganisms able to biodegrade different types of lubricants, lubricant's constituents, petroleum products in addition to other hydrocarbon pollutants. The isolated microorganisms include bacteria and fungi (Michael et al., 1993; Taganas and Doyungan, 2002; Nwaogu et al., 2008; Park et al., 2008; Matthew et al., 2008; Hesham et al., 2009; Itah et al., 2009; Eze and Eze, 2010; Vieira et al., 2009; Sadouk et al., 2009; Xue et al., 2010).

In the present study, isolation and identification of a microbial isolate capable to utilize the used lubricating oils, gasoline as well as other hydrocarbon pollutants as sole sources of carbon has been carried out. Furthermore, identification of the biodegradation products was also studied in most cases.

MATERIALS AND METHODS

Sampling, chemicals and microbial media

Two lubricant oil samples were obtained from shops of lubricants exchange service, from two Governorates (Cairo and Alexandria), Egypt. Each of them was a mixture of different lubricating oils. Benzene, n-hexane, stearic acid and glycerol were obtained from El-Nasr Pharmaceutical Chemicals, Egypt. Cyclohexane was obtained from Koch-Light Laboratories Limited, Colnbrook, Bucks, Engalnd. Diesel oil was obtained from (Shell Co. petroleum products). Waste sludge was obtained from treatment of used lubricants with solvent mixture as described by Shakirullah et al. (2006). The soil sample was obtained from a lubricant contaminated soil of a car service workshop. The mineral salt medium (MSM) consists of (g l^{-1}): NH_4NO_3, 0.5; K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.2g; NaCl, 0.5; $FeSO_4$, 0.02g; $CaCl_2$, 0.05; pH 7 (Chao et al., 2006). This medium was used as liquid or solid by adding 2% agar. Potato dextrose agar (PDA) was obtained form International Diagnostics Group, United Kingdom.

Isolation of lubricant biodegrading microorganisms

The isolation was performed from both lubricating oils and the soil sample. Isolation from lubricant was performed by the enrichment technique, in which, the methylsulfonylmethane (MSM) was supplemented with 10% of the lubricant oil as a sole source of carbon. Conical flasks (250 ml) containing 50 ml of MSM and 5 ml of each lubricant were shaked in shaker incubator at 25°C for five days.

For isolation from soil, 1 ml from the 10^{-4} soil dilution was mixed with 10 ml of fused solid sterilized MSM supplemented with 1 ml of each lubricant. The dishes were incubated at 28°C for four days.

Purification of the microbial isolates

The obtained colonies were purified on solid MSM supplemented with lubricating oil (10%).

Biodegradation of lubricating oils

In 250-ml conical flasks, 50-ml of sterilized liquid MSM medium and 5 ml of lubricating oil were added. The flasks were inoculated by a loop from either one-day old bacterial or fungal slant. The flasks were incubated at 25°C in a rotary shaker at 150 rpm for seven days. At the end of the incubation period, the cells were separated by centrifugation. Volume of the remaining un-degraded lubricating oil (the upper layer) was measured.

Identification of the most active isolate

Isolate KA-2011 was identified by its morphological characteristics in addition to the molecular level by combination of polymerase chain reaction (PCR) amplification of its rDNA genes corresponding to unique sequences within the internally transcribed spacer (ITS) (Isogai et al., 2010). A single colony from the purified isolate was grown overnight in potato dextrose broth (PDB) liquid culture. Genomic DNA was isolated using Fermentas kit, Catalogue No. K0721. Primers (ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'), ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') for PCR were designed for two separate areas of the DNA encoding the rRNA. The pair of primers ITS1 and ITS4 targeted the conserved regions between 18S and 5.8S rDNAs (ITS1). The pair of primers ITS3 and ITS4 also targeted the conserved regions between 5.8S and 28S rDNAs of fungi (ITS2). PCR reaction conditions were optimized and mixed in 50-µl total volume. Amplification was carried out in a Hybaid PCR express, programmed for 40 cycles as follows: 94°C/4 min (1 cycle); 94°C/1 min, 55°C/1 min, 72°C/2 min (38 cycles); 72°C/8 min (1 cycle); 4°C (infinitive). Agarose (1.2%) was used for resolving the PCR products. A 1 Kb plus DNA Ladder was used as a standard DNA. The run was performed at 80 V in Bio-Rad submarine (8 X 12 cm). Bands were detected on ultra-violet (UV)-transilluminator and photographed by a Polaroid camera. The PCR products were purified using Gene Jet Gel Extraction Kit (Fermentas, k0691). The chain termination method was performed with the ABI PRISM Big Dye terminator kit (PE Applied Biosystems, USA) in conjunction with ABI PRISM (310 Genetic Analyzer). The nucleotide sequences were compared with sequence database using the BLASTN algorithms (Altschul et al., 1997).

Phylogenetic analysis

The genetic relationships among worldwide isolates were inferred by the neighbor-joining method. Homology analyses were performed with the BLASTN program (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/BLAST/]). These sequences were edited using Ugene software version 1.9.5 (http://ugene.unipro.ru/download.html). Comparisons of the new isolate sequences with those of homologs and orthologs sequences available in the GenBank database were performed using the online BLAST program. Sequence identities of nucleotides were analyzed using ClustalW2 Program (http://www.ebi.ac.uk/Tools/msa/clustalw2/), default parameters were accepted. Phylogenetic relationships were inferred from this alignment by using phylogeny program (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), default parameters were accepted. A distance matrix was generated using DNADIST under the assumptions of Jukes and Cantor (1969) and Kimura (1980). Phylogenetic trees were derived from these matrices using neighbor joining method.

Preparation of seed inoculum

A 250-ml conical flask containing sterilized MSM medium supplemented with 10% of lubricant oil was inoculated with a loop from one-day old slant of the yeast isolate KA-2011. The flask was

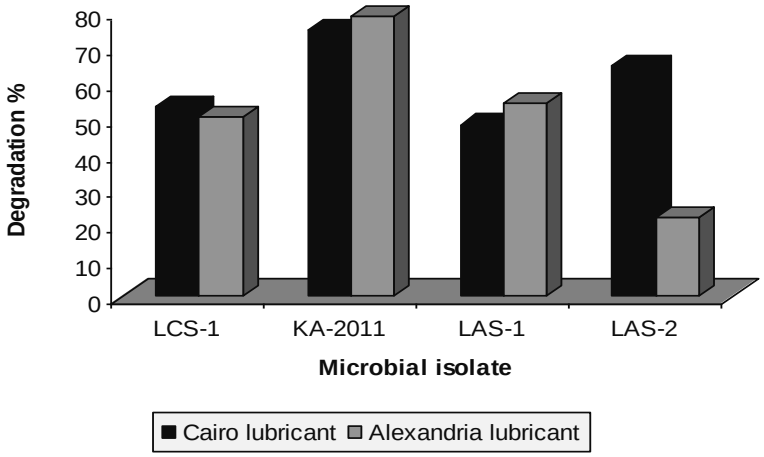

Figure 1. Efficiency of the isolated microbial isolates for biodegradation of two lubricant mixtures.

incubated on rotary shaker at 150 rpm, at 25°C for five days.

Biodegradation of various compounds

Ability of the yeast isolate KA-2011 to utilize different hydrocarbons and hydrocarbon derivatives as sole carbon sources was tested by using different compounds: n-hexane, cyclohexane, benzene, n-hexanol, glycerol, stearic acid, castor oil, diesel oil and grease waste sludge. In a Teflon screw caped (14 ml) culture tube containing 8 ml of MSM, 0.8 ml of n-hexane, cyclohexane, benzene, n-hexanol or glycerol was added. In conical flasks (100-ml) containing 25 ml of sterilized MSM, one of the following was added: 5 ml of diesel oil, 0.25 g of stearic acid; 2 ml of either castor oil or lubricant oil or 1 g of grease waste sludge. The media were then inoculated with 1 ml of the seed inoculum. A control was made from each experiment containing the same mixture of MSM and the tested carbon source without inoculation. Three replicates were made from each experiment. The cultures were incubated at 25°C for one week on a rotary shaker at 150 rpm. At end of the incubation period, the volume of the remaining un-degraded liquid compounds was measured.

Determination of microbial growth

The microbial growth was determined spectrophotometrically by measuring the optical density at 600 nm.

Extraction of the biodegradation products

The biodegradation products of n-hexane, cyclohexane, benzene, stearic acid and castor oil were extracted three times from the cell-free supernatant by diethyl ether. Both saponified and non-saponified compounds were determined in the ether extract of both stearic acid and castor oil by GC-MS as described by (Kinsella, 1966). The un-degraded amount of grease waste sludge was separated by centrifugation from both control and the inoculated flasks. The floated sludge was collected, air dried and weighted for calculation of the degradation percentage.

Gas chromatography - mass spectrometry (GC-MS) analysis

The biodegradation products were analyzed by HP-5890 GC equipped with HP-5972 mass detector. The analysis was performed by using HP-innowax column 30 m X 0.25 mm id X 0.25 µm film thickness. Mobile phase: helium, flow rate 1 ml/ min. Oven temperature was initiated at 50°C for 2 min up to 200°C at a rate of 10°C/min., 200°C for 15 min., 200 to 250°C at a rate of 15°C/ min. The mass detector temperature was 300°C. The fragmentation pattern of the obtained mass spectra was analyzed by Wiley7N mass library software.

Effect of salinity on the biodegradation efficiency of *C. viswanathii* KA-2011

Conical flasks (250 ml) containing 50-ml of MSM medium supplemented with 0, 3 and 6% NaCl were sterilized at 121°C for 15 min, and 5 ml of either lubricating or diesel oils was added. The flasks were inoculated with a loop of one day old slant of the isolate KA-2011 and then incubated at 25°C on a rotary shaker at 150 rpm for four days. The remaining amount of lubricant and diesel oils was extracted three times by petroleum ether.

RESULTS

Four microorganisms with different biodegradation efficiency were isolated in this study. One yeast isolate (named KA-2011) was isolated from the lubricant oil that has been obtained from Alexanderia, while no micro-organisms were isolated from that obtained from Cairo. On other hand, one fungus only was isolated from soil in the MSM medium supplemented with the lubricant obtained from Cairo (named LCS-1), while two bacteria were isolated from soil in the medium supplemented with the lubricant oil obtained from Alexandria (named LAS-1 and LAS-2). Each of these isolates was tested for its biodegradation efficiency of the two lubricating oils.

As shown in Figure 1, isolate KA-2011 showed the highest degradation percentages (75 and 79%) after one week only for both lubricant oils mixtures which were obtained from Cairo and Alexandria, respectively. This was followed by the fungal isolate LCS-1 that showed

Figure 2. Morphology of the KA-2011 isolate.

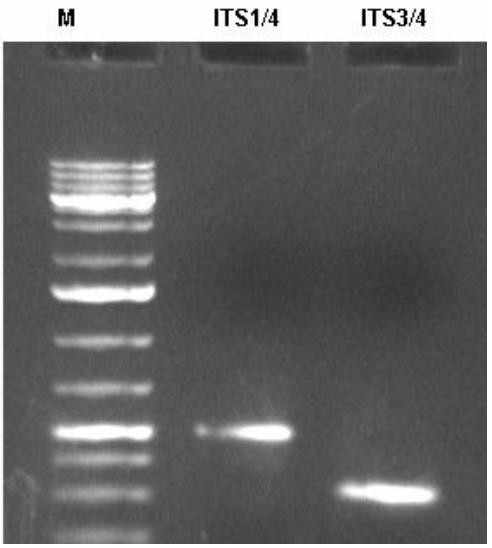

Figure 3. Molecular identification based on the amplification of the intergenic spacer region (ITS). M, 1 Kb plus ladder, Fermentas. ITS1/4, PCR products generated by primer combinations ITS1 and ITS4; ITS3/4, PCR products generated by primer combinations ITS3 and ITS4.

53.4 and 50.6% degradation percentages for the two lubricants, respectively. Bacterial isolate LAS-1 showed biodegradation percentages of 48.4 and 54.3% for the two lubricants, respectively. Bacterial isolate LAS-2 showed higher degradation percentage for the lubricant obtained from Cairo (65%), while it showed only 22.4% degradation percentage for that obtained from Alexandria.

Identification of the isolate KA-2011

Microscopic examination clearly showed pseudohyphae forming yeast cells, which are characteristic for *Candida* sp., (Figure 2). The differences of sequences in the intergenic spacer regions have been used to detect and identify fungi (Isogai et al., 2010). Two PCR products were obtained using two ITS specific primer combinations (Figure 3). The combination ITS1/4 generated a product length 532 bp, while the combination ITS3/4 generates a product length 322 bp. In a previous work, Isogai et al. (2010) found that the molecular weights of PCR products were near among the *Candida* species and there is a risk for miss identification when species were determined by only a PCR amplification method. Therefore, the obtained PCR products were purified and sequenced. According to the BLAST analysis, the resulting sequence had high similarity with the 18S ribosomal RNA gene sequence of *Candida viswanathii* strain SN40 (GenBank accession no. FJ542757) with sequence identity of 92%. A phylogenetic tree was constructed (Figure 4) based on the ITS rDNA gene sequence of KA-2011 and related strains. The sequences of both genes were submitted to the Genebank and we got the accession number JN714128 for the 28S *rRNA* gene (324 bp) and the accession number JN714127 for the 5.8S *rRNA* gene (532 bp).

Biodegradation of various compounds

Due to the higher biodegradation efficiency of the yeast isolate KA-2011 of both lubricant mixtures, it was selected for studying its ability to degrade different hydrocarbons, grease waste sludge, diesel oil, vegetative oil and other compounds. Among the tested compounds castor oil, diesel oil and stearic acid were highly utilized by the yeast isolate KA-2011 as a sole carbon source than the other tested compounds (hexane, cyclohexane and benzene). As shown in Figure 5, castor oil and diesel oil were 100% degraded by *C. viswanathii* KA-2011 after one week. This was followed by lubricating oil (75.9%), stearic acid (55%), n-hexane (55%), benzene (50%), sludge (46.2%) and cyclohexane (0.62%), which was the most resistant compound against degradation followed by n-Hexanol, which cannot be utilized. On other hand,

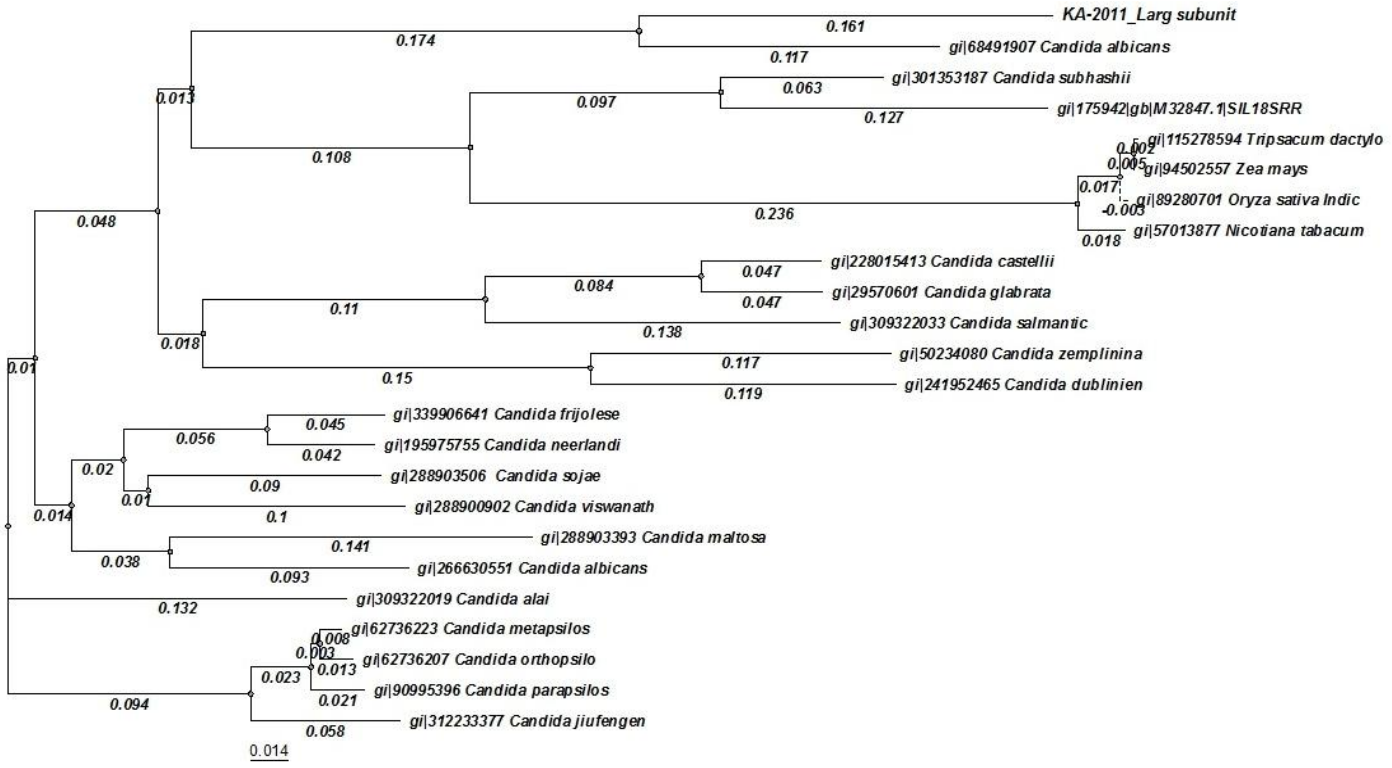

Figure 4. Phylogenetic tree showing the relationship of the isolate KA-2011 to the most closed related species.

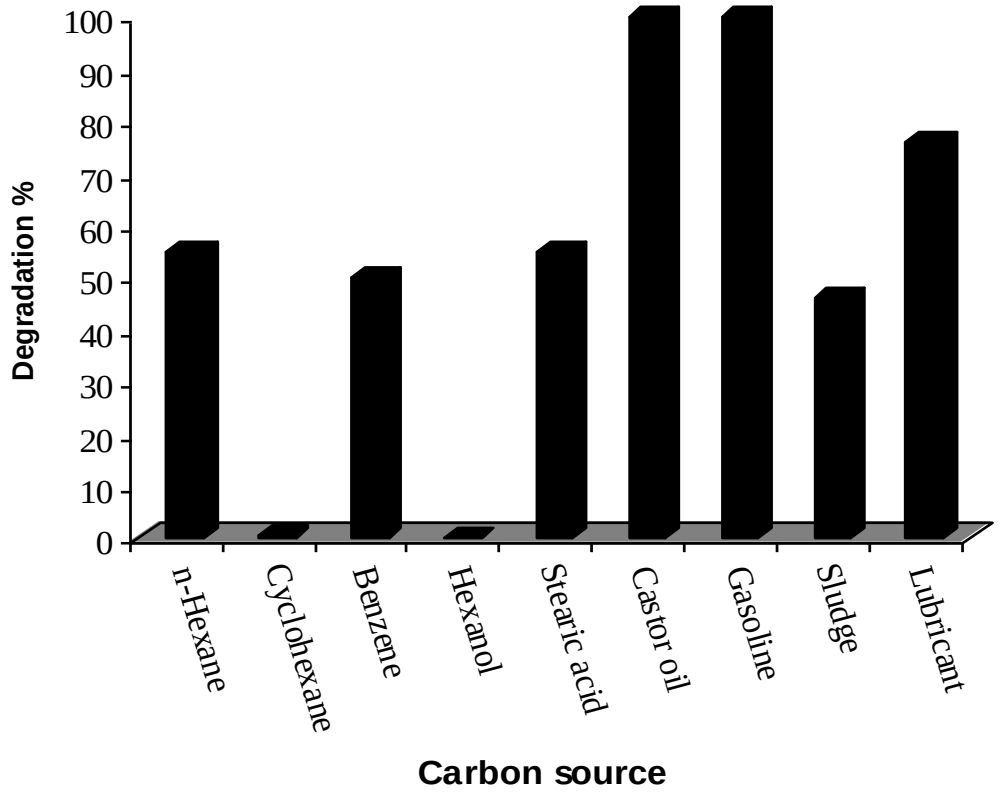

Figure 5. Biodegradation percentages of different compounds by *Candida viswanathii* KA-2011.

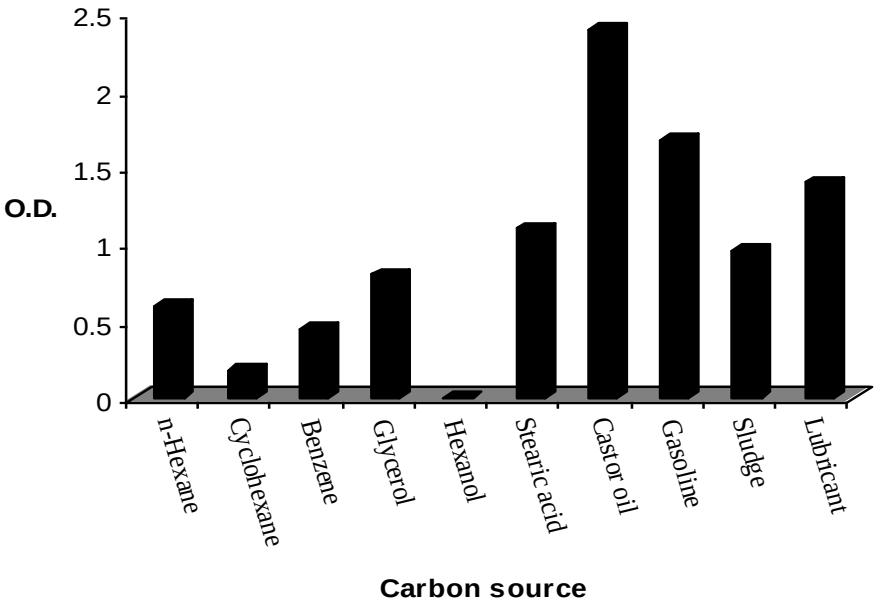

Figure 6. Effect of different carbon sources on growth of *Candida viswanathii* KA-2011.

the highest growth of this yeast isolate was obtained from castor oil, followed by diesel oil, lubricant oil, stearic acid, grease waste sludge, glycerol, n-hexane, benzene, cyclohexane and naphthalene, respectively (Figure 6).

Identification of the biodegradation products

GC-MS analysis showed no degradation products of hexane. This may be due to volatility of main products that were produced such as CO_2, ethane or propane. The biodegradation product of cyclohexane was 2-hydroxy hexanedial, while benzene showed two major compounds, 1,5-hexadiene-3-yne and 1,3-hexadiene-5-yne.

Biodegradation of diesel oil

GC-MS analysis of gasoline (control) and a remaining amount after biodegradation showed no difference in the components types. However, the percentages of the components were varied (Table 1). Among the gasoline components, octane and nonane compounds that are the lowest molecular weight components were the most degraded compounds.

Biodegradation products of stearic acid

The saponified fraction showed presence of a little amount of oleic and linoleic acids in addition to stearic acid. Analysis of the un-saponified fraction showed only two compounds: hexanol, 2-ethyl and un-identified compound.

Biodegradation products of castor oil

The saponified fraction showed presence of fatty acids hexadecanoic (C16), stearic (C18), oleic (C18: 1), linoleic (C18:2), linolenic (C18:3), ricinoleic (12-hydroxy-9-cis-octadecenoic acid) and ecosanoic acid (C20). The un-saponified compounds were dodecane, tetradecane and four un-identified compounds.

Effect of salinity on the biodegradation efficiency of *C. viswanathii* KA-2011

As shown in Figure 7, the biodegradation percentages of both lubricating oil and diesel oil increased as the salt concentration increases. The biodegradation percentages increased from 35 to 58.6% and 59.8% for lubricating oil and from 50.6 to 78.3 and 93.9% for diesel oil at salt concentrations 0, 3 and 6%, respectively, after only four-days. These results indicate that the efficiency of this yeast isolate for degradation of diesel oil is higher than that for lubricating oil. Furthermore, the high salt concentration enhances the biodegradation efficiency of this isolate. Therefore, it can be emphasized here that *C. viswanathii* KA-2011 can be effectively used for removing of diesel and lubricating oils pollution from sea water or salty soil.

DISCUSSION

Removal of the hydrocarbons pollution from water and soil has been a major target for the environmentalists worldwide. Therefore, several studies were performed to

Table 1. GC-MS analysis results of gasoline before and after biodegradation by *C. viswanathii* KA 2011.

Gasoline component	Component (%)	
	Control	Biodegraded
Octane	0.20	0.02
Octane 2,3-dimethyl	0.41	0.02
Nonane	0.91	0.35
Nonane, 2-methyl	0.98	0.97
Decane	2.41	1.93
Cyclohexane, 1-methy-2-propyl	0.77	0.97
Decane, 2-methyl	1.56	1.22
Undecane	3.51	3.55
Undecane 2-methyl	2.06	2.71
Dodecane	4.19	4.03
Naphthalene decahydro, 2-methyl	0.37	0.68
1-dodecene	1.58	2.12
Tridecane	5.08	4.77
Dodecene 2,7,10-trimethyl.	2.35	3.04
Tetradecane	5.40	5.57
Tridecane, 6-propyl	1.97	2.70
Dodecane, 5,8-diethyl	1.65	2.29
Pentadecane	6.70	6.43
Hexadecane	5.89	5.14
Pentadecane 2,6,10,14-tetramethyl.	3.07	3.90
Heptadecane	6.33	4.75
Benzene, octyl	1.01	1.74
Naphthalene	1.72	2.77
Hexadecane, 2,6,10,14-tetramethyl	1.65	1.98
Octadecane	5.13	4.28
Cyclohexane, butyl	0.33	0.68
Benzene, nonyl	0.49	0.82
Naphthalene 2-methyl	2.06	2.07
Nonadecane	5.22	4.45
Naphthalene 2, 6-dimethyl	1.26	1.29
Ecosane	4.28	3.44
Naphthalene 2, 3-dimethyl	2.17	2.24
Heni-ecosane	4.08	3.43
Naphthalene 2,3,6-trimethyl	1.22	1.27
Docosane	4.17	3.69
Naphthalene 1,6,7-trimethyl	0.92	1.41
Tricosane	2.75	2.38
tetracosane	2.70	2.45
Heptacosane	0.26	1.51
Octacosane	1.17	0.92

isolate microorganisms able to degrade the different types of hydrocarbons which include petroleum compounds, lubricating oils and other compounds with high efficiency in a short period of time. Ability of the yeast strain (*C. viswanathii* KA-2011) to degrade hydrocarbons with various complexity and high efficiency shows promise for using of this isolate in bioremediation of the contaminated soil, wastewater and sea water. Biodegradation of various organic compounds (aliphatic, cyclic and aromatic hydrocarbons, fatty acids, vegetative and mineral oils as well as grasses by one microbial isolate (*C. viswanathii* KA2011) reflects presence of various enzymatic systems in this yeast that requires further studies to be identified to enhance and control the

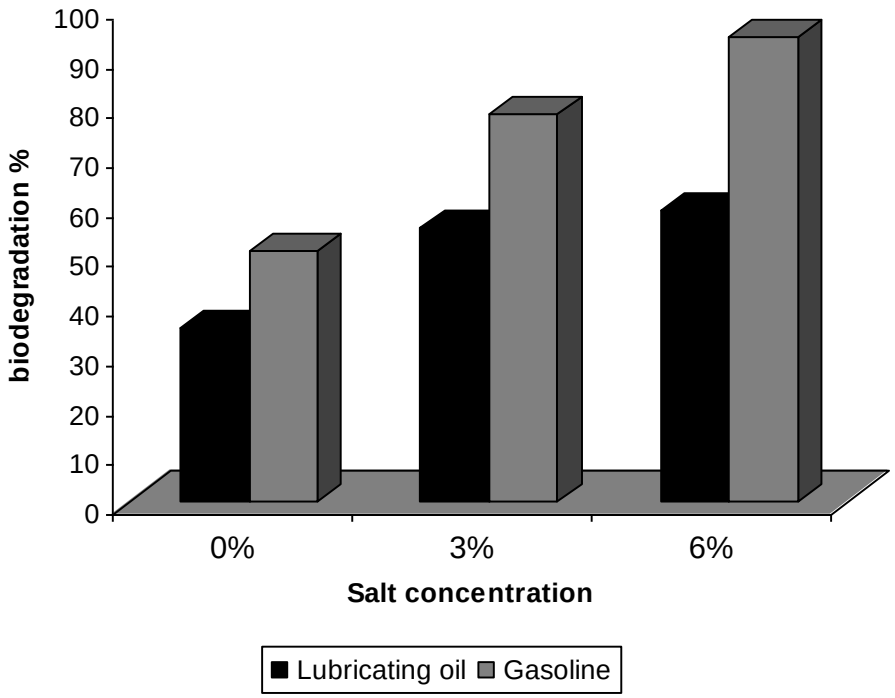

Figure 7. Effect of salinity on the biodegradation percentages of lubricating oil and diesel oil by *Candida viswanathii* KA-2011.

biodegradation efficiency of this isolate. Absence of any differences between the gasoline composition before and after biodegradation reflects the ability of *C. viswanathii* KA2011 to utilize all of the gasoline hydrocarbons with equal efficiencies. In addition, the high biodegradation percentages for both lubricating oil (75.9%) and diesel oil (100%) after a short period of time (one week) by one yeast isolate was firstly reported in this study. The biodegradation efficiency was increased in the high salt concentration medium (6%), where the diesel oil was approximately completely degraded (93.9%) after only four days. The reason for enhancement of biodegradation efficiency in the high salt concentration medium is not clear and requires further studies. It was noticed that diesel and lubricating oils highly induce pseudohyphae formation by this yeast isolate, as well as high biomass yield was obtained. It reached to 3.2 g dry weight/ L of the MSM supplemented with 10% of diesel oil. The pseudohyphae were floated on the surface of supernatant after centrifugation. This reflects the high lipid content of these hyphae.

Use of yeast isolates in biodegradation of lubricating and diesel oils was previously studied but with lower efficiency and longer time than *Candida viswanathii* KA-2011. The yeast *C. viswanthii* was also used by Hesham et al. (2009) for biodegradation of mixture of low and high molecular weight poly aromatic hydrocarbons (PAHs). José et al. (2009) also used an isolate of *C. viswanathii* to degrade biodiesel/diesel blends.

Candida viswanathii KA-2011 can be used effectively for biodegradation of gasoline, lubricating and vegetative oils from the contaminated soil, wastewater and sea water.

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