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Isolation and Identification of Microorganisms from Crude oil Contaminated Soils of Dar es Salaam, Tanzania

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Authors' contributions

This work was carried out in collaboration between all authors. Author PEK did the laboratory work. Author EMS performed the statistical analysis and wrote the first draft of the manuscript. Author AMM managed the literature searches and all analyses of the study. All authors planned the study, wrote the protocol, read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to isolate and identify microorganisms in crude oil polluted soils in Eastern Tanzania.
Study Design: Isolation and identification of microorganisms using conventional and molecular techniques.
Place and Duration of Study: Department of Molecular Biology & Biotechnology, University of Dar es Salaam; October 2012 to June 2013.
Methodology: Soils were collected from Mlalakua (ML), University of Dar es Salaam (UDSM) and Ubungo (Ub) local garages in Dar es Salaam, Tanzania. The selective isolation of microorganisms was done based on colony morphological appearance on agar plates and Gram reaction. Three isolates (one from each site) were subjected to molecular methods for the identification purposes. Phylogenetic analyses of bacterial 16S rRNA gene and fungal ITS region sequences were employed in this study to identify and determine the evolutionary relationships of the ML, Ub and UDSM isolates.
Results: The 16S rRNA analysis revealed that the ML isolate belonged to the genus *Klebsiella*, while the ITS analysis on Ub and UDSM isolates assigned them to the genus

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Trichosporon and *Candida*, respectively.
Conclusions: From these findings, further studies are recommended to assay for the biodegradative potentiality of the identified isolates for bioremediation of crude oil contaminated soils.

Keywords: Isolation; identification; microorganisms; crude oil; soils.

ABBREVIATIONS

ATCC - American Type Culture Collection; BLAST - Basic Local Alignment Search Tool; Bp – Base pairs; CIB 1 –World Bank Sub-project (Strengthening of Teacher Training, Research and Public Services Capabilities) of the College of Natural and Applied Sciences (Formerly Faculty of Science); DNA - Deoxyribonucleic acid; EDTA –Ethylene Diamine Tetra Acetic acidg DNA - genomic DNA; GmbH – German for "company with limited; liability"/ "Gesellschaft mit beschränkter Haftung"; ITS – Internal Transcribed Spacer; ML – Mlalakua; NCBI - National Centre for Biotechnology Information; NJ – Neighbour - joining; rRNA – Ribosomal Ribonucleic Acid;SDS - Sodium Dodecyl Sulfate; STHEP - Science Technology and Higher Education Program; Ub – Ubungo; UDSM – University of Dar es Salaam; UV – Ultra –Violet.

1. INTRODUCTION

Soil contaminated by used lubricating oil is a common occurrence in most of developing countries. It is rapidly increasing due to global increase in the usage of petroleum products. Furthermore, presence of different types of automobiles and machinery results in an increase in the usages of lubricating oil [1].

Hydrocarbon contamination of the soil especially by Polyclic Aromatic Hydrocarbons (PAHs) attracts public attention because many PAHsare toxic, mutagenic and carcinogenic. Prolonged exposure to high oil concentration may cause the development of liver or kidney diseases, possible damage to the bone marrow and an increased risk of cancer [2,3].

Biodegradation is a foremost method that eliminates pollutants from the environment. Many strains of microorganisms, competent in degrading petroleum hydrocarbons have been isolated [4]. In this regard, microorganisms capable of degrading hydrophobic contaminants may have a distinct advantage over competitors in contaminated areas. Therefore isolates and samples from such sites are often rich in microorganisms with the desired characteristics for both in situ and ex situ bioremediation processes [2,5].

On the other hand, the major sources of land or soil pollution by oil include: Accidental breakage of pipelines and discharge of oil from industries. As far as Tanzania is concerned, means of bioremediation must be taken into account. Since reports on microorganisms with the ability to degrade the petroleum hydrocarbons in Tanzania are lacking, this study, aimed at isolation and identification of microbial strains from soils contaminated with crude oils, which could be employed for bioremediation of oil contaminated soils.

A number of studies from other parts of the world have been reported on isolation and identification of microbes from oil-contaminated soils. Some researchers have examined a better understanding of how groups of hydrocarbon degraders contribute to the catabolism of petroleum in soils [4]. In addition, Ameen et al. [1] reported the isolation and identification

of hydrocarbon degrading microorganisms from crude oil contaminated soils. Furthermore, Samaei et al. [4] reported isolation and characterization of bacteria degrading n-Hexadecane from soil. However, reports on the similar studies in Tanzania on indigenous microorganisms adapted to biodegrade crude oil contaminated soils are lacking.

We report for the first time the isolation and identification of microorganisms from crude oil contaminated soils of some garages in Kinondoni Municipality, Dar es Salaam, Tanzania, which in future could be used in bioremediation of crude oil contaminated soils.

2. MATERIALS AND METHODS

2.1 Sampling site

The samples were taken from 3 different garages (Mlalakua (ML), Ubungo (Ub) and University of Dar es Salaam (UDSM) in Kinondoni Municipality, Dar es Salaam, Tanzania.

2.2 Place and Duration of Study

This study was conducted at the Department of Molecular Biology & Biotechnology, College of Natural and Applied Sciences, University of Dar es Salaam, Tanzania from October 2012 to June 2013.

2.3 Sediment Collection

At each location (Ubungo, University of Dar es Salaam and Mlalakua garages), 2 soil samples were collected (approximately 100g) using a small hand-held stainless steel shovel at a depth of 0-10 cm within100m^2 area. The soil samples from each location were bulked and homogenized to prepare composite samples. Later on, all samples were kept in polyethylene bags on ice cool box during transportation from the field. The pH of the each soil sample was measured upon arrival in the laboratory at the Department of Molecular Biology and Biotechnology, UDSM and stored at 4ºC.

2.4 Enrichment and Isolation of Microbes from oil Contaminated soil and Gram Staining

2.4.1 Isolation media

Isolation media: Malt Extract broth and Agar (MEB/MEA) and Nutrient broth/agar (NB/NA) were prepared according to Guiraud et al. [6] and with the following compositions; Malt extract 5%, Saccharose 3% and NaCl 8%. The composition of a nutrient broth/agar was as follows: Peptone - 5g/L, Meat extract - 1g/L, Yeast extract - 2g/L, Sodium chloride - 5g/L, Agar - 15g/L and pH - 7.0±0.2. The recommended media storage temperature adopted was - 2 to 8ºC.

2.4.2 Fungal and bacterial isolation

1 g (weight) of soil sample (Fig. 1) was suspended in 9ml sterile saline solution (0.85% NaCl).Aliqouts of 200µl of soil sample were directly inoculated into 100ml Malt Extract (MEB) and Nutrient broth (NB). The Erlenmeyer flasks containing the cultures were incubated at 30ºCfor 12 hours in a laboratory orbital shaker at 200rpm (Edmund, Bühler, 7400 Tübingen,

West Germany). The broth was then aseptically streaked on the Malt Extract Agar (MEA) and nutrient agar (NA) plates and incubated for two days. In case there was no growth observed after this incubation period, plates were incubated for a further two to three days. Emergent colonies were purified by the serial transfer technique of Van der Walt and Yarrow [7]. The NA, NB, MEA and MEB were made selective after sterilization by supplementing with 50mg/L cycloheximide to inhibit fungi and with the antibiotic Ampicillin (0.02%) to suppress bacterial growth, respectively as successfully done in previous studies [8,9].



Fig. 1. A soil sample contaminated by oil

2.5 Isolates Maintenance

The pure cultures were maintained on MEA and NA slants. The isolates were stored at 4ºC after sub culturing after every fortnight as described by Sosovele [9].

2.6 Microscopic Observation

Microscopy (wet preparation and Gram staining) of the isolates was routinely carried out to determine the morphology of the fungal and bacterial isolates as well as ensuring purity of the cultures.

2.7 Molecular Techniques

2.7.1 Genomic DNA (gDNA) isolation from pure isolates

DNA was extracted using a protocol followed by [9]. Prior to DNA isolation, the pure culture isolates were cultivated in liquid medium (25ml) and incubated overnight at 30ºC in a shaking incubator set at 150rpm (Edmund, Bühler, 7400 Tübingen, West Germany). Suspension from overnight culture was dispensed in vials (1ml each) and biomass pelleted by centrifugation at 14,000rpm (Mikro 220 - Hettich machine: CH-8806 Bäch, Germany) for 5 minutes at 4ºC. The pellet was resuspended in a total of 50–75µl extraction buffer (2% Triton

X100, 1% SDS, 100mMNaCl, and 10mMTris-pH 8, 1mM EDTA-pH 8). The suspension was ground with a mini pestle in vials until homogenous, and then incubated at 60ºC for 20 minutes. 40 µl of potassium acetate was added and the whole mixture incubated on ice for 20 minutes, then centrifuged at 1,400rpm (Mikro 220 - Hettich machine: CH-8806 Bäch, Germany) for 4 minutes at 4ºC. The supernatant was then decanted and the pellet discarded. Equal volume of chloroform was added to the supernatant, and then vortexed followed for 10 seconds, then centrifugation done again for 4 minutes at 14,000 rpm at 4ºC. Carefully the upper phase was collected and put into fresh tubes. Two volumes of ice-cold Isopropanol were added to the supernatant, mixed thoroughly by repeated inversion (not vortexed) and then the mixture incubated for 15 minutes at –20ºC. The mixture was then centrifuged at 16,400 rpm for 10minutes and a DNA pellet obtained. The centrifugation was done at 16,400rpm for 10 minutes and the DNA was pelleted, then 100µl of 75% ethanol was added to the pellet to wash and centrifuged at 14,000rpm for 5 minutes (Mikro 220 - Hettich machine: CH-8806 Bäch, Germany).The DNA pellet was dried by incubating the tube inclined face down at room temperature. The DNA pellet was finally resuspended in 30µl of TE buffer and was stored at –20ºC until used. 5µl of the DNA sample was loaded on 0.9% agarose gel prepared in TBE buffer -Tris Borate EDTA (108g Tris base, 55 g Boric acid, 20 ml 0.5M EDTA pH 8 in 1000 ml sterile distilled water), stained with ethidium bromide (0.5 µg/ml), electrophoresed (Mini-Sub Cell, GT electrophoresis systems: USA 800 4BIORAD) and visualized on UV transilluminator (UV/White Light Transilluminator: Biometra GmbH Rudolf-Wissell-Str. 30D-37079 Goettingen, Germany), to check whether DNA was isolated successful then sent for PCR and sequencing to a commercial facility at Inqaba Biotech; South Africa.

2.7.2 Polymerase chain reaction (PCR) and sequencing

All obtained gDNAs were sent to a commercial facility at Inqaba Biotech Pretoria, South Africa for sequencing. Genetic identification of isolated bacterium was performed according to nucleotide sequence of 16S ribosomal DNA (rDNA). Bacterial 16S rDNA was amplified with universal primers previously described by Molina et al. [10] and Moreno et al. [11]: 16F275′-CCAGAGTTTGATCMTGGCTCAG-3′ and 16R14885'-AGAGTTTGATCMTGGCTCAG-3' respectively. Furthermore, ITS primers described by White et al. [12], which amplify a fragment of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions was used for the purpose. The primer sequences were ITS 1-5'-TCC GTA GGT GAA CCT GCG G -3' and ITS 4-5' -TCC TCC GCT TAT TGA TAT GC -3'.16S rRNA gene and ITS PCR products of the isolates were sequenced with an ABI PRISM 3100 genetic analyzer.

2.7.3 Basic Local Alignment Search Tool (BLAST)

The sequences obtained were initially monitored for vector areas using 'Bioedit' method available from the 'National Centre for Biotechnology Information' (NCBI). After eliminating all the dirty vector areas, the sequences were harmonized with the Gen Bank database using the Basic Local Alignment Search Tool (BLAST) algorithm [13] at NCBI. The ITS region sequence similarity and strain identification of all Sequenced PCR products were assembled and analyzed. The three isolates (Ub, UDSM & ML) were then ascribed to the con specific species showing the highest matched sequence identity (more or equal to 98-99%) and with the lowest expect value (e-value = 0.0).

2.7.4 Phylogenetic analysis

The bacterial 16S rRNA gene and fungal ITS regions sequences were used to generate phylogenetic trees. The phylogenetic analysis was conducted in MEGA5 as by Felsenstein [14], Saitou and Nei [15] and Tamura et al. [16].

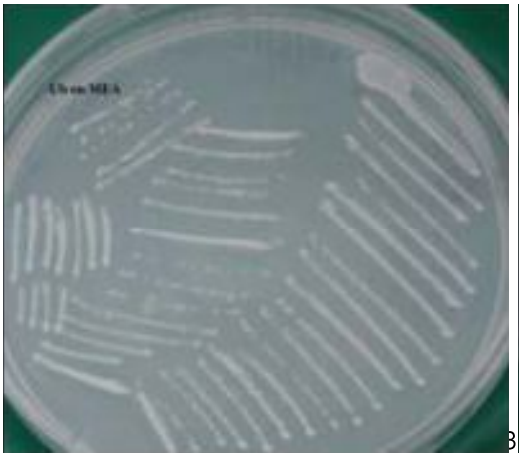
3. RESULTS

3.1 Enrichment and Isolation of Microbes

The two media (MEA + NA) employed for the isolation of soil microorganisms in this study were effective as intended. MEA produced the fungal colonies and the NA yielded a bacterium colonies. As it is shown in (Table 1), isolates of this study formed white and cream colonies (Figs. 2-4). Gram stain reactions through microscopic observations, revealed ovoid (Ub), round (UDSM) and rod (ML) shapes of the isolates. But also, the same reactions confirmed two strains (Ub and UDMS) as fungi and one (ML) as a bacterium (Figs. 5-7).

Table 1. Isolates colony features and their gram staining results

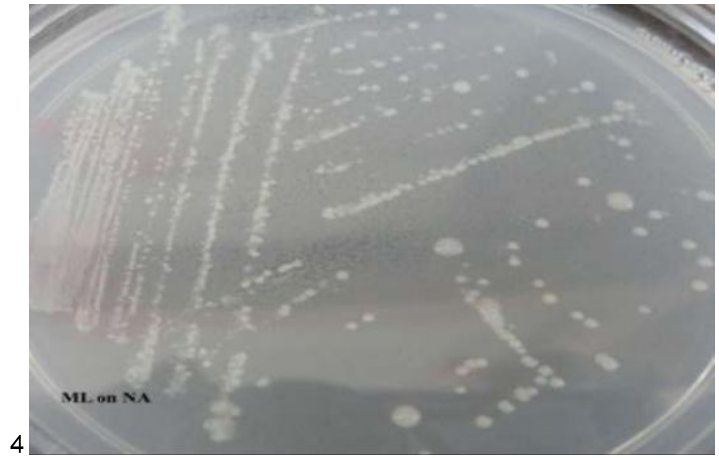
	Colony color + texture	Shape	Gram reaction
Ub	Creamy + dry	Ovoid	+ve
UDSM	Creamy-white + dry	Round	+ve
ML	Creamy + mucoid	Rods	-ve





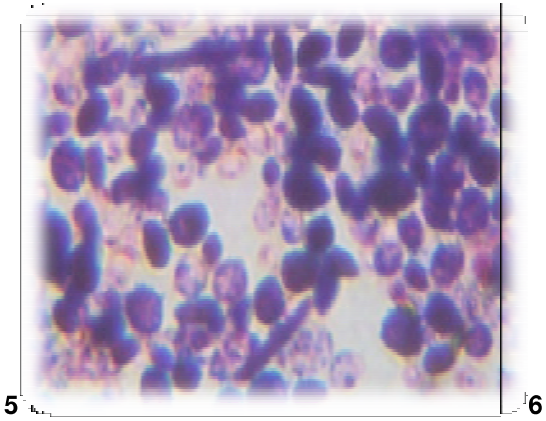
Figs. 2 and 3. Ub and UDSM colonies on malt extract agar plate

All three isolates produced genomic DNA bands of about 23000bp (Fig. 8). The size of the bacterial rRNA gene products obtained was about 595bp - 600bp. However, the size of the ITS regions amplified by ITS primers was about 600bp.

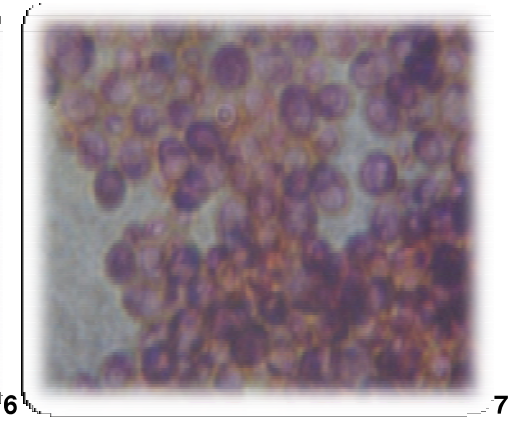


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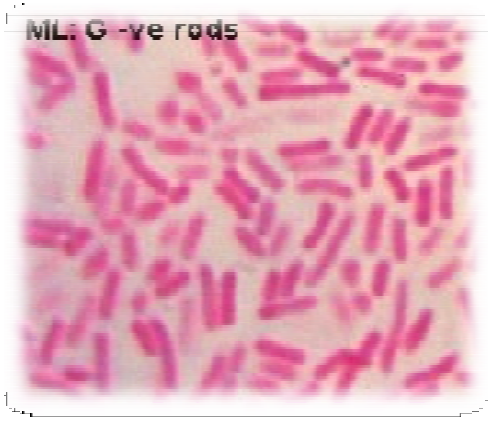
Fig. 4. ML colonies on nutrient agar plate



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Figs. 5-7. Micrographs of a gram reaction (100X): 5-UDSM isolate; 6-Ub isolate and 7-ML isolate

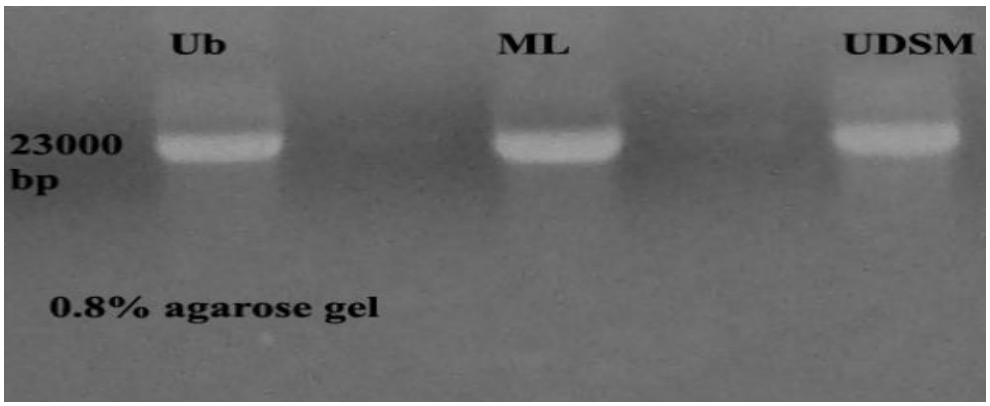


Fig. 8. Results of genomic DNA isolation of Ub, UDSM and ML isolates

3.2 The nucleotide sequences and phylogenetic trees

Table 2. ML isolate sequencing results

	Accession number	Max score	Total score	Query cover %	E Value	Max Ident. %
ML (1129 bp)	KF770979	1914	1914	98	0.0	99
16S rRNA gene	JQ398851	1907	1907	98	0.0	98
	JX310741	1905	1905	98	0.0	98
	FJ958193	1905	1905	98	0.0	98
	KC853306	1902	1902	98	0.0	98
	EF522822	1900	1900	98	0.0	98
	FJ655784	1898	1898	98	0.0	98
	KC455423	1896	1896	98	0.0	98
	KF841705	1894	1894	98	0.0	98
	EU360121	1894	1894	98	0.0	98

4. DISCUSSION

The primary goal of this study was to isolate and identify microorganisms from the oil contaminated soils. In this study, isolation of microorganisms was carried out using a traditional microbiological technique. The same isolation techniques were reported by [17] whereby; seven species (*Micrococcus, Pseudomonas, Flavobacterium*, *Serratia, Moraxella, Bacillus, Klebsiella*) of bacteria were isolated from oil-contaminated sites. Other researchers have also reported the isolation methods of this study from other parts of the world [8,18,19].

The growth media (Malt Extract broth/agar and Nutrient broth/agar) employed in this study, evidently facilitated in producing three morphologically different microorganisms. Two fungi and a bacterium were isolated in this study. These outcome safford additional proof that, these media are highly effective for the isolation of fungi and bacterium from oil contaminated soils as it was also observed in previous studies [1,4,18,19]. In addition, the isolated colonies in this study showed differences in morphology in terms of colour and shape of colonies (Table 1 and Figs 1-7). This is an indication that, despite the modest number of isolates obtained, the soils contaminated with crude oil might be considered as

rich sources of microorganisms. This opinion is supported by Samaei et al. [4] report which found several families of *Proteobacteria* and *Actinobacteria* from petroleum-contaminated Arctic soils using a [^{15}N] DNA - based stable isotope probing and pyrosequencing. Furthermore, Ruiz-Garcia et al. [20] isolated a number of microorganismsfrom petroleum and diesel oil-contaminated soils using enrichments process. But also, some more previous studies isolated yeasts and bacteria from soils contaminated with oil [8,15,19].

The use of bacterial 16S rRNA gene sequences in this study has been victorious owing to high specificity shown by specific primers (16F27 and 16R1488). Using the same primers, Van der Walt and Yarrow [7], Shumin et al. [21] observed the same high specificity. In additional, the Internal Transcribed Spacer regions (ITS) are the essentially universal molecular markers in fungal phylogenetic studies and their execution has shown to be prize-winning [16]. Furthermore, due to the lower level of conservation, the ITS region has been recommended to be more accurate for classification of the fungi [6]. In this regard then, this study employed the ITS1 + ITS 4 primers to identify the fungal isolates.

Upon using the sequence results of this study, the ML isolate which had all features of a bacterium (Table 1 and Fig 4 + 7) could be assigned to the Genus *Klebsiella*. According to [11], a 16S rRNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of <97% represents a new species. In this case then, ML isolate sequence had 98 - 99% maximum identity with the *Klebsiella* sp. (Table 2). These findings suggest that, the ML strain can be assigned to *Klebsiella* sp. as it is not separated from the type strains phylogenetic neighbors by sequence similarities well below those found between closely related to *Klebsiella* sp. (Table 2 above) and (Fig. 11). Moreover, reports of Bell et al. [5] and Santhini et al. [17] who also reported on the identification of *Klebsiella* sp. support the findings obtained from the conserved sequence of the 16S rRNA in this study.

The results of this study revealed further that, the Ub and UDSM isolates (Tables 1, 3 and 4, Figs. 2, 3, 5, 6, 9 + 10) could be assigned to the *genus Trichosporon* and *Candida,* respectively. But also, the Bootstrap values of 1000 (maximum) were displayed (Fig. 9 + 10), indicating that the higher the bootstrap value, the more reliable the phylogenetic analysis was. On the other hand, the difference in nucleotide substitution did not exceed 1% after Ub and UDSM sequences were subjected to the online nucleotide-nucleotide BLAST search tool algorithm [2] at the National Centre for Biotechnology Information (NCBI). Taking all these into account, these findings are in line with a report of [12] which emphasized that, the strains with less than 1% are considered con-specific and therefore do not represent distinct biological species which are separated by more than 1% nucleotide substitutions.

Identification of microorganisms by molecular analyses methods to gene level has been established to be more trustworthy [12,14,16,22]. In this study, the internal transcribed spacer region was suggested because of the popular molecular markers in phylogenetic studies of fungi. Furthermore, Gardes and Bruns [23] also revealed similar observation. In addition, owing to the lower level of conservation, Brookman et al. [22] proved the ITS region to be more precise for classification of the fungi compared with the 18S rRNA gene, which is highly conserved. In this study then, the phylogenetic analysis on ITS sequences of the two isolated fungi (by neighbour-joining analyses) could successfully resolve two genera (*Trichosporon* and *Candida*). These results are in line with the observations of previous reports whereby, higher resolution in taxonomic identification was attained after targeting the ITS region [12,23].

It is evident from earlier studies Samaei et al. [4], Ruiz-Garcia. [20] and the findings of this study that microorganisms are regular in soils contaminated with crude oils. This entails a great capability for these species to endure in such an environment and therefore indicates the strains could hold a capability of degrading petroleum hydrocarbons from the soils they inhabit. However, the few isolates of this work that included *Klebsiella*, *Trichosporon* and *Candida* in Dar es Salaam city, have given an indication to the identity and phylogeny of microorganisms in Tanzanian soil contaminated with crude oil. But also, it is a first report to be made available in Tanzania, which forms a base line for more bio-prospecting.

Table 3. UDSM isolate sequencing results

	Accession number	Max score	Total score	Query cover %	E Value	Max Ident. %
UDSM (1100 bp) ITS regions	JN989499	825	1223	79	0.0	99
	JQ585709	803	1180	79	0.0	99
	FJ515170	800	1174	85	0.0	99
	KF313193	773	1156	75	0.0	99
	JQ585709	803	1180	79	0.0	99
	KC966725	760	1355	74	0.0	98
	JN989535	834	1310	79	0.0	94
	JN997459	830	1295	79	0.0	94
	JN989534	830	1302	79	0.0	94

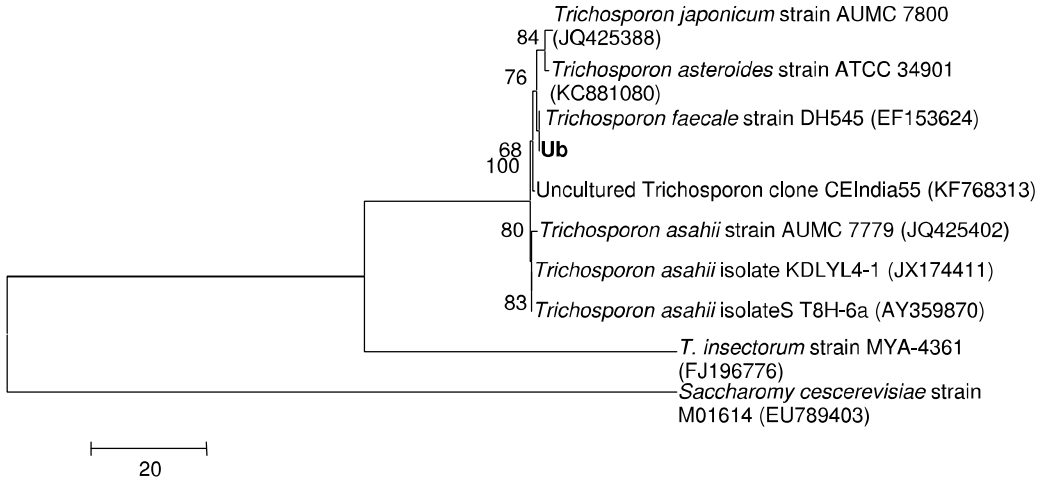


Fig. 9. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 10 Nucleotide sequences. *Saccharomyces cerevisiae* strain M01614 (EU789403) was used as an out group. Evolutionary analyses were conducted in MEGA5

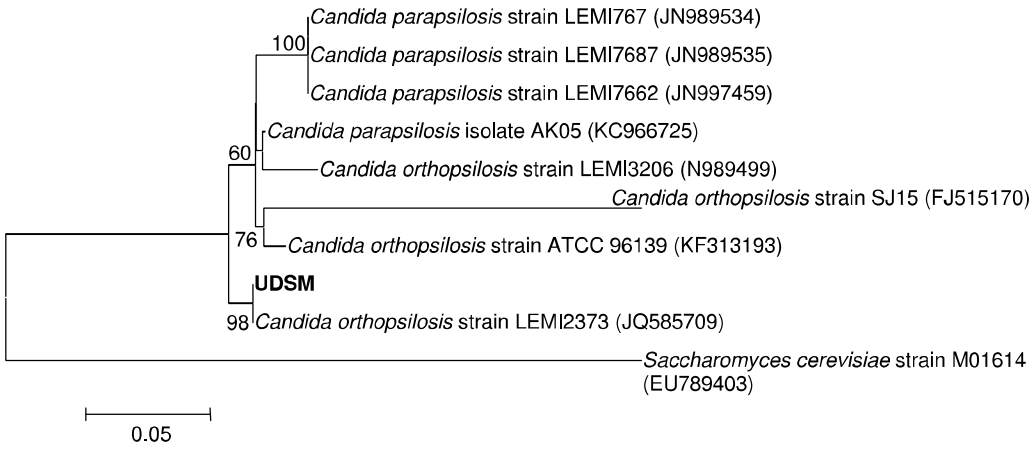


Fig. 10. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 10 Nucleotide sequences. *Saccharomyces cerevisiae* strain M01614 (EU789403) was used as an out group. Evolutionary analyses were conducted in MEGA5

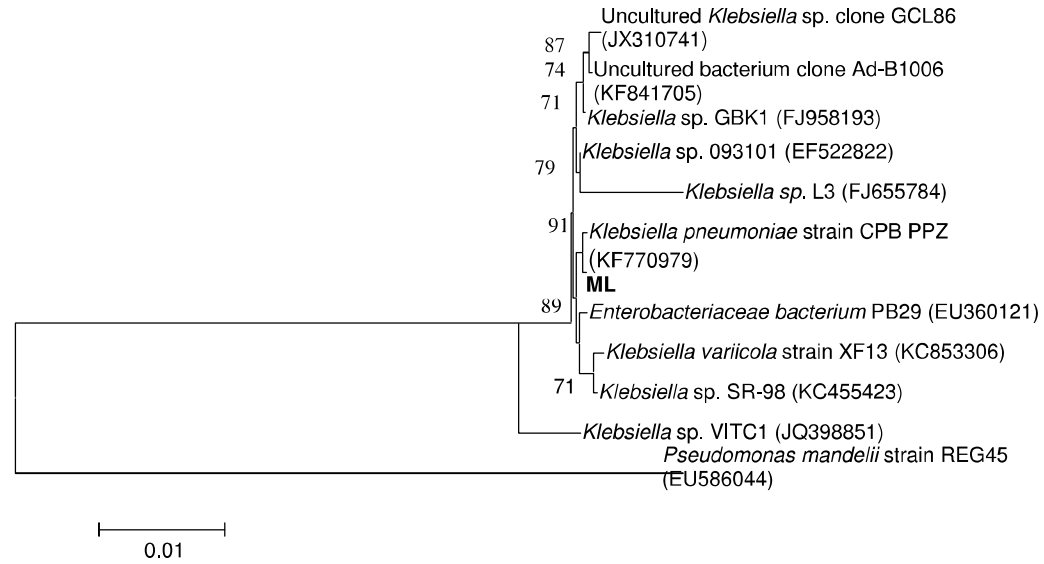


Fig. 11. The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 12 nucleotide sequences. *Pseudomonas mandelii* strain REG45 (EU586044) was used as an out-group. Evolutionary analyses were conducted in MEGA5

Table 4. Ub isolate sequencing results

	Accession number	Max score	Total score	Query cover %	E Value	Max Ident. %
Ub(1117bp) ITS regions	JQ425402	896	1252	80	0.0	99
	JX174411	886	1232	80	0.0	99
	JQ425388	886	1219	80	0.0	99
	AY359870	886	1232	80	0.0	99
	KF768313	884	1228	80	0.0	99
	FJ196776	879	1219	80	0.0	99
	EF153624	879	1219	80	0.0	99
	KC881080	870	1294	90	0.0	99

5. CONCLUSION

In conclusion, the results of this study confirmed presence of microorganisms in soils contaminated with crude oil in Dar es Salaam. Both traditional and molecular technique results demonstrated bacteria and fungi composed the soils studied. The information from the techniques employed in this study showed that there could be more novel bacterial and fungal species that could be recovered in future studies by using recently developed pyrosequencing technique that allows quick and detailed analysis of the microbial diversity in different samples in a single run. However, this particular-study provides base line data for further understanding of the soil contaminated with crude oil of Tanzania. Additionally, studies on the identified microorganisms to further investigate their potentials in oil degradation are recommended.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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