

## Full Length Research Paper

# Biodegradation of petroleum oil by fungi isolated from *Treculia africana* (Dec'ne) seeds in Nigeria

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Petroleum crude oil biodegrading fungi were isolated from *Treculia africana* seeds in the presence and absence of petroleum fumes. An assessment of the relative ability of each fungus to biodegrade petroleum crude oil, kerosene, diesel, unspent engine oil, spent engine oil and extracted oil from *T. africana* seeds on minimal salt solution was investigated using changes in optical density read on a spectrophotometer and gas chromatographic analyses. Ten fungi were isolated from *T. africana* seeds in the presence and absence of petroleum fumes. These included one species each of *Mucor*, *Paecilomyces*, *Rhizopus* and *Syncephalastrum*, four species of *Aspergillus* and two species of *Penicillium*. It was evident that the fungi used in this research work were capable of biodegrading the petroleum and extracted *T. africana* seed oil hydrocarbon, though at different rates. *Rhizopus* had the highest degrading ability in kerosene, unspent engine oil, crude oil and the extracted oil from the seed, while *Penicillium pinophyllum* had the lowest ability to degrade the oil. The gas chromatogram (GC) showed that *Paecilomyces* biodegraded the hydrocarbons in the crude oil compared to the control (crude without fungi) using up some carbon atoms (C<sub>12</sub>-C<sub>24</sub>) after the 40 days of incubation, suggesting n-alkane biodegradation. Also the GC analysis of the seed oil of *T. africana*, after 40 days of incubation, showed a reduction in the seed oil hydrocarbons, removing C<sub>10</sub>-C<sub>15</sub>.

**Key words:** Hydrocarbon utilization, *Treculia Africana*, seeds, petroleum crude oil, fungi.

## INTRODUCTION

Various microorganisms have been reported to possess the capability for utilizing hydrocarbons as their source of carbon and energy (Atlas, 1981). The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera. A diverse group of bacteria and fungi have been shown to have this ability. In a review made by Zobell (1946), more than 100 species representing 30 microbial

genera have been shown to be capable of utilizing hydrocarbons. Recent studies continue to expand the list of microbial species, which have been demonstrated to be capable of degrading petroleum hydrocarbons (Nwachukwu, 2000). Microorganisms available for bioremediation include a range of bacteria like *Arthrobacter*, *Pseudomonas*, *Flavobacterium* and fungi such as *Penicillium*, *Cladosporium*,

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*Aspergillus* etc, isolated from water (freshwater, brackish and marine) or soil (Atlas and Bartha, 1972).

*Treculia africana* (Dec'ne) belongs to the family Moraceae. It contains many seeds, which are buried in the spongy pulp of the fruit. *T. africana* is a riverine fruit tree of the tropical African rain forest. The seeds are extracted after macerating the fruit in water. Analysis of the hexane extracts of *T. africana* seed indicate that it contains a stearine solid fat fraction, resembling that of palm-kernel oil, and an oleine fraction with a composition similar to that of cotton seed oil (Burkill, 1997).

Microorganisms used for biodegradation have been isolated from the soil or water (Amund et al, 1978; and Salminen et al., 2004). No report has been on the use of fungi isolated from *T. africana* seeds in the biodegradation of crude petroleum. The aim of this paper was to isolate pathogenic fungi from *T. africana* seeds. These pathogenic fungi were then used to biodegrade petroleum hydrocarbon to assess the potential ability of the fungi to biodegrade the hydrocarbons using optical density and gas chromatographic methods.

## MATERIALS AND METHODS

### Collection of seeds

African bread fruit (*T. africana*) seeds were collected from three different markets, Agege and Oyingbo markets in Lagos state, and Ogere market in Ogun state, Nigeria (Long 8° N Lat 4°E). Diseased seeds were separated from the healthy seeds. The visually diseased seeds were used in this experiment. The seeds were sampled from the markets monthly for 9 months. About 1000 seeds were collected from each market at every sampling period. The various oil used here were: crude oil, spent engine oil, unspent engine oil, kerosene, diesel and extracted oil from the seeds of *T. africana*.

### Isolation, identification and screening for hydrocarbon degrading fungi

Sixty diseased seeds of *T. africana* were surface sterilized by soaking them in a solution of common bleach (sodium hypochloride) and sterilized distilled water in a ratio of 3:2, for 2 min and then rinsed with three changes of sterilized distilled water. To isolate fungi from the seeds under petroleum crude oil fumes, the modified methods of Amund et al. (1987) was adopted. Sixteen filter papers (Whatman No. 1001125) were sterilized in the oven at 40°C for 15 min. Eight of the dried filter paper was dipped in 250 ml petroleum crude oil contained in a 500 ml beaker for about 15 s with the help of a sterile forcep, and drained. The petroleum crude oil was obtained from Shell Escravos Port Harcourt, Nigeria. Each of the eight crude oil treated filter papers was placed on the cover of 8 Petri dishes containing solidified potato dextrose agar (PDA) and sterilized diseased seeds (seven seeds per plate) under sterile conditions. The aim of the petroleum fumes was to supply the fungi with hydrocarbons through vapour transfer from petroleum fumes. Another set of eight Petri dishes with the PDA and *T. africana* surface sterilized seeds, were without the 'oiled' filter papers, but contained the 8 non-oil treated filter papers and they served as control. All the plates were incubated at room temperature (28 -31°C) in the incubator, and observed daily for fungal growth. This process was carried out monthly for the 9 months sampling period. The developing fungal colonies were sub cultured aseptically into fresh PDA plates to get pure culture of isolates. A part of each pure culture was then

aseptically transferred into sterilized PDA slants, which was previously prepared in the 14 ml McCartney bottles, and served as stock culture.

To identify the fungi, light microscopic examination was carried out and cultural characteristics such as colour of the fungal colony, number of days taken for the fungi to reach maximum growth or diameter (9 cm) of the Petri dish, and texture of the fungal growth were noted. The morphological and cultural features of each fungus were compared with descriptions given by Talbot (1971); Deacon (1980); Domschet et al. (1980) and Bryce (1992) for identification. Some mycologist (Prof N.U Uma) within the department of Botany and Microbiology, University of Lagos, was consulted for confirmatory identification of the fungi.

### Extraction of oil

The method of extraction of oil of *T. africana* seeds was adopted from the oil extraction methods of Egan et al. (1981). The visually healthy seeds were ground using a ceramic pestle and mortar before blending in an electric blender. An amount of 200 g of the ground seed was packed into the extraction thimble before covering with a small ball of cotton wool. The thimble was inserted in a quick fit plain body soxhlet extractor. Petroleum ether in the quantity of 200 ml (60 - 80°C) was poured in a 250 ml round-bottom flask of known weight, which was connected to the extractor and refluxed on an electric thermal heater for 5 h. The other was then collected in the plain body extractor and then separated from the flask that contained the oil. The flask containing the oil was then heated in an oven at 103°C for 30 min. It was cooled and weighed to get the final weight. The percentage oil content of the sample was calculated using the ratio of the amount of oil produced to the weight of sample used expressed as a percentage. The procedure was repeated until at least 250 ml of the oil was extracted from the seed.

### Confirmatory test for hydrogen utilization potential of the fungi

The estimation of hydrocarbon utilizers were obtained using enrichment procedure as described by Nwachukwu (2000). A minimal salt solution (MSS) containing 2.0 g of Na<sub>2</sub>HPO<sub>4</sub>, 0.17 g of K<sub>2</sub>SO<sub>4</sub>, and 0.10 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 4 g of NH<sub>4</sub>NO<sub>3</sub> and 0.53 g K<sub>2</sub>SO<sub>4</sub> dissolved in 1000 ml distilled water and sterilized in an autoclave. Fifty-six test tubes were sterilized, plugged with cotton wrapped in aluminum foil, and placed on test-tube racks. There were 7 test-tube racks, containing eight test-tube each. In each test-tubes 10 ml of the minimal salt solution (MSS) was added and each of 6 racks had either 2 ml of petroleum crude oil, diesel, kerosene, spent engine oil, unspent engine oil or extracted oil from *T. africana* seed. The seventh rack served as control, it had only the MSS in its test-tubes. Six fungi (choice was based on their growth from seed in the petroleum fumes), were inoculated in each test-tube in the rack. The last tube (seventh tube) on each rack served as a second control, and was not inoculated with any fungus. Each of the test tubes was plugged with sterilized cotton wool wrapped with aluminum foil to ensure maximum aeration and prevent cross contamination. All the test tubes were then inoculated at the room temperature in an incubator for 40 days. Constant shaking of the test-tubes was ensured to facilitate oil/cell phase contact. The ability to degrade petroleum crude, diesel, kerosene, spent engine oil, unspent engine oil and extracted oil from *T. africana* seed (based on growth of the organism on the MSS medium) were measured every 5 days using the visual method based on the turbidity of the MSS. The turbidity was measured using the spectrophotometer at a wavelength of 530 and 620 nm. This experiment was repeated twice. Results were statistically analyzed using T-test, Anova (F-test) and Duncan multiple range test as described by Parker (1979). The percentage contribution of each fungus in biodegrading the various oil

**Table 1.** Fungi Isolated from diseased seeds of *Treculia africana* in the presence and absence of petroleum fumes.

<i>Treculia africana</i> seeds without petroleum fumes	<i>Treculia africana</i> seeds with petroleum fumes
<i>Aspergillus flavus</i>	<i>Aspergillus flavus</i>
<i>Aspergillus niger</i>	<i>Aspergillus niger</i>
<i>Aspergillus japonicus</i>	<i>Aspergillus wentii</i>
<i>Aspergillus wentii</i>	<i>Paecilomyces</i> sp
<i>Mucor</i> sp	<i>Penicillium chrysogenum</i>
<i>Paecilomyces</i> sp	<i>Penicillium pinophyllum</i>
<i>Penicillium chrysogenum</i>	<i>Rhizopus</i> sp
<i>Penicillium pinophyllum</i>	
<i>Rhizopus</i> sp	
<i>Syncephalastrum</i> sp	

(kerosene, crude oil, diesel, unspent engine oil, spent engine oil and extracted oil from seed) after the 40th day of incubation was calculated. To determine the percentage contribution of each fungus in each oil, after the 40th day, the optical density (OD) of each fungus was divided by the total OD of the various fungi in particular oil and expressed as a percentage.

#### Gas chromatographic (GC) analysis of some oil samples

Gas chromatographic analysis were carried out to assess and further confirm the ability of each fungi, *Paecilomyces* and *Aspergillus niger* isolated from *T. africana* seeds to biodegrade the hydrocarbons. After incubating for 40 days, as done above, the degraded hydrocarbons were extracted based on the physical changes and OD that were observed in the test-tubes after the 40 days incubation. These tubes were: (a) MSS + crude oil; (b) MSS + crude oil + *Paecilomyces*; (c) MSS + extracted oil and (d) MSS + extracted oil + *A. niger*.

The method of Song and Bartha (1990), Kampfler and Steoif (1991) and Salminen et al. (2004) was used for extraction of samples and GC analysis. For the extraction process about 20 ml hexane was used. Each sample was poured into a separating funnel and 20 ml of hexane was added and shaken well and the different oil collected. Column was prepared to get pure extracts. This was done using a 150 ml burette; the base of the burette was blocked with cotton wool to provide a base for the silica gel. About 20 ml of hexane was poured into a beaker and about 5 g of  $\text{Na}_2\text{CO}_3$  was added, which was the drying agent. The drying agent was poured into the burette. The silica gel helped to remove impurities.

The extract was poured into the burette and 20 ml hexane was added. The extract diffused down the column and was collected in a 14 ml McCartney bottle. All traces of hexane in the extract were allowed to evaporate by leaving the McCartney bottle opened, and the final extract was used subsequently. Gas chromatographic analysis was carried out using Perkin Elmer Auto-system GC equipped with flame ionization detector. A 30 m- fused capillary column with internal diameter of 0.25 mm and 0.25 mm thickness was used, and the peak areas were analyzed with a SRT model 203 peak simple chromatography Data system. About 1-2 ml of extracted sample was injected. The column temperature was 60°C for 2 min to 300°C programmed at a rate increase of 120°C/min. Nitrogen was used as carrier gas with pressure of 30 ml. Hydrogen and air flow rates were 30 ml/min respectively.

## RESULTS AND DISCUSSION

Table 1 shows the fungal species isolated from diseased

seeds of *T. africana* in the presence and absence of petroleum fumes. Ten fungal species were isolated which included one species each of *Mucor*, *Paecilomyces*, *Rhizopus* and *Syncephalastrum*, four species of *Aspergillus* and two species of *Penicillium*. More fungal species were isolated from the diseased seeds incubated without petroleum fumes. The growth pattern of fungi in the MSS and oil shows that the growth of each fungus had different maximum growth peaks (Figures 1 and 2), providing a fluctuation in the growth pattern of the fungi in the oil media. The growth pattern thus shows that the utilization of the different hydrocarbon used varied widely among the fungi. *Rhizopus* had the highest percentage contribution in the biodegradation of 33.13% in unspent engine oil while *P. pinophyllum* had the least of 6.80% in kerosene (Table 2). Generally, *Rhizopus* ranked highest in the degradation of oil in all the oil used except diesel and spent engine oil (Table 3). The least in ranking in the biodegradation was *P. pinophyllum* for all the oil used except in the spent engine oil as shown in Table 3.

The results of this work indicate that many of the fungal species isolated from *T. africana* seeds in the presence and absence of petroleum fumes were capable of biodegrading petroleum hydrocarbon. This *T. africana* seeds may be added to the known sources of hydrocarbon degrading fungi. The results further prove that fungi could also play a role in surviving in hydrocarbon rich environment, supporting the reports of Plante-Cunney (1993). An interesting observation in this study was the growth of each fungus in the presence of oil compared to when oil was absent. This probably means the fungi used the oil for its growth. Shaw (1995) found that microorganism breakdown hydrocarbons and use the energy to synthesize cellular components. After being completely broken down the reaction releases  $\text{CO}_2$ ,  $\text{H}_2\text{O}$  and energy used to create cellular biomass (Keeler, 1996). It is evident from the results obtained that the fungi were more active in the extracted oil than in other oil (Figure 2). This may be due to the fact that the fungi were isolated from the *T. africana* seed where the oil was extracted, and these fungi have being adapted to using the seed oil hydrocarbon as their source of carbon. Also the

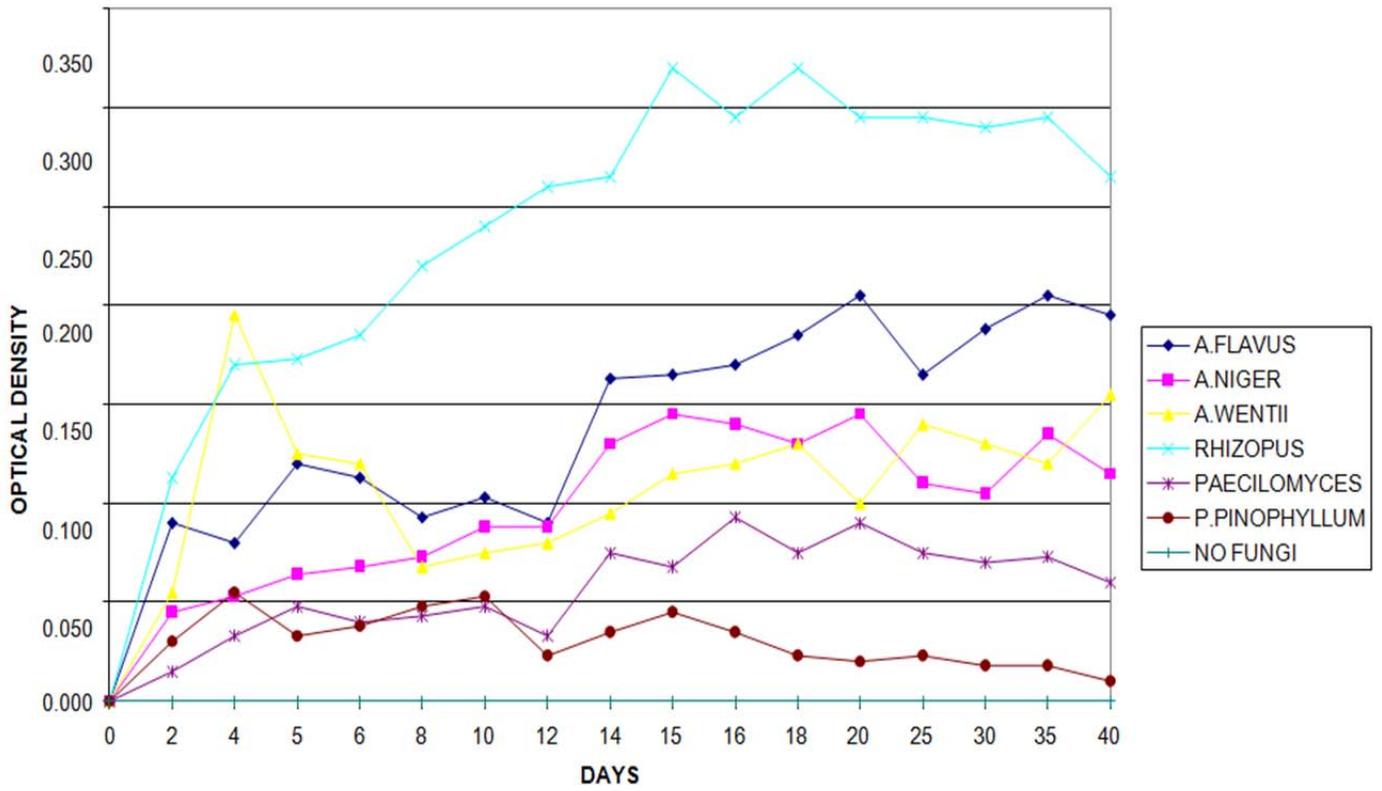


Figure 1. The growth pattern of fungi in minimal salt solution and unspent engine oil using 530 nm.

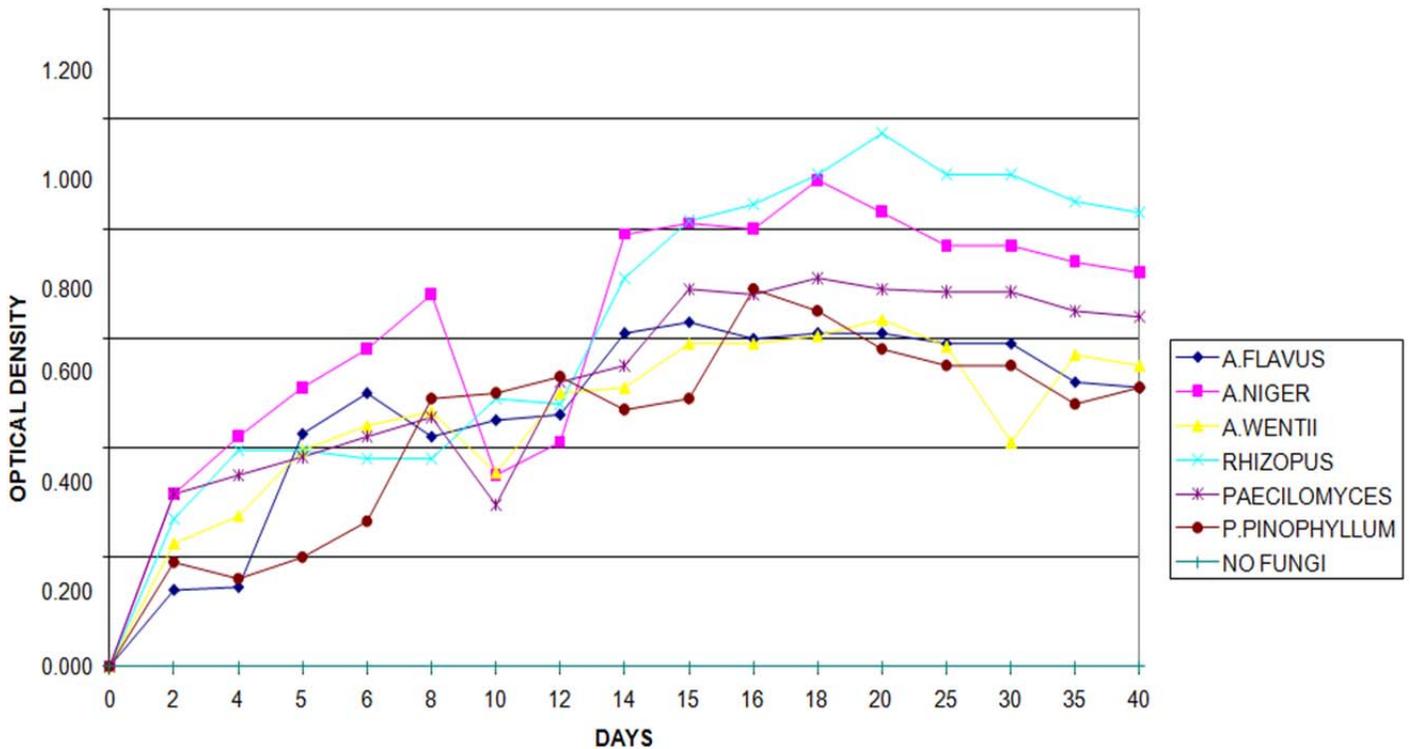


Figure 2. The growth pattern of fungi in minimal salt solution and extracted oil from *Treculia africana* seed using 530. nm

**Table 2.** Percentage contribution of Biodegradation of each fungus in the different oil after 40 days incubation.

Oil	Percentage contribution of biodegradation of each fungus (%)					
	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Aspergillus wentii</i>	<i>Rhizoipus</i>	<i>Paecilomyces</i>	<i>Penicillium pinophyllum</i>
Kerosene	20.87	14.08	16.51	27.19	14.56	6.80
Diesel	17.83	20.93	11.63	18.61	20.61	10.85
Unspent engine oil	24.38	14.38	19.38	33.31	7.50	1.25
Spent engine oil	14.63	18.58	21.74	19.37	11.46	14.23
Crude oil	17.65	18.70	17.85	19.26	14.16	12.45
Extracted oil from the <i>Treculia africana</i> seed	13.56	19.15	14.63	22.08	17.02	13.56

**Table 3.** The order of biodegradation of the various fungi on the different oil after 40 days of incubation.

Oil	<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Aspergillus wentii</i>	<i>Paecilomyces</i> sp	<i>Penicillium pinophyllum</i>	<i>Rhizoipus</i> sp.
Kerosene	2*	5	3	4	6	1
Diesel	4	1	5	2	6	3
Unspent engine oil	2	4	3	5	6	1
Spent engine oil	4	3	1	6	5	2
Crude oil	4	2	3	5	6	1
Extracted oil from the <i>T. africana</i> seed	5	4	4	3	6	1

\* Ranking 1 to 6 represents the order of biodegradation from the highest to the lowest.

various fungi were less active in kerosene compared to other oil, which is probably due to the hydrocarbon present in kerosene. Amanchukwu et al. (1989) observed that most microorganisms find it difficult biodegrading kerosene, attributing this to its type of hydrocarbon chain. Increased turbidity and emulsification of the oil was observed during the course of these investigations. Emulsification is a known part of hydrocarbon degradation (Geyer, 1980), and might be a probable indication of hydrocarbon utilization by the fungi.

The chromatogram of the MSS and crude oil (control), shows the detection of C<sub>12</sub> - C<sub>24</sub>; the peaks for these carbons were obvious (Figure 3). The growth of *Paecilomyces* in MSS and crude oil indicates a complete disappearance of the peaks in C<sub>12</sub> - C<sub>24</sub> after 40 days incubation (Figure 4). It was only C<sub>12</sub> peak that was not completely absent but reduced in the chromatogram (Figure 4). Also the chromatogram for the MSS and extracted oil from seed (control), C<sub>12</sub> - C<sub>24</sub> were detected and their peaks were obvious (Figure 5). The chromatogram of the growth of *Aspergillus niger* in MSS and the extracted oil from *T. africana* seed after 40 days incubation showed that the peaks of C<sub>10</sub>-C<sub>16</sub> were absent (Figure 6). The peak system chromatogram could

not detect some of the carbons in the oil artificially inoculated with *A. niger*, and *Paecilomyces* probably due to the biodegradation of the oil by these fungi suggesting n-alkane biodegradation. This observation is similar to the work of Salminen et al. (2004) who worked on the potential for aerobic biodegradation of petroleum hydrocarbons in boreal subsurface, suggesting an n-alkane degradation due to the removal of C<sub>n</sub>-C<sub>15</sub> in their study.

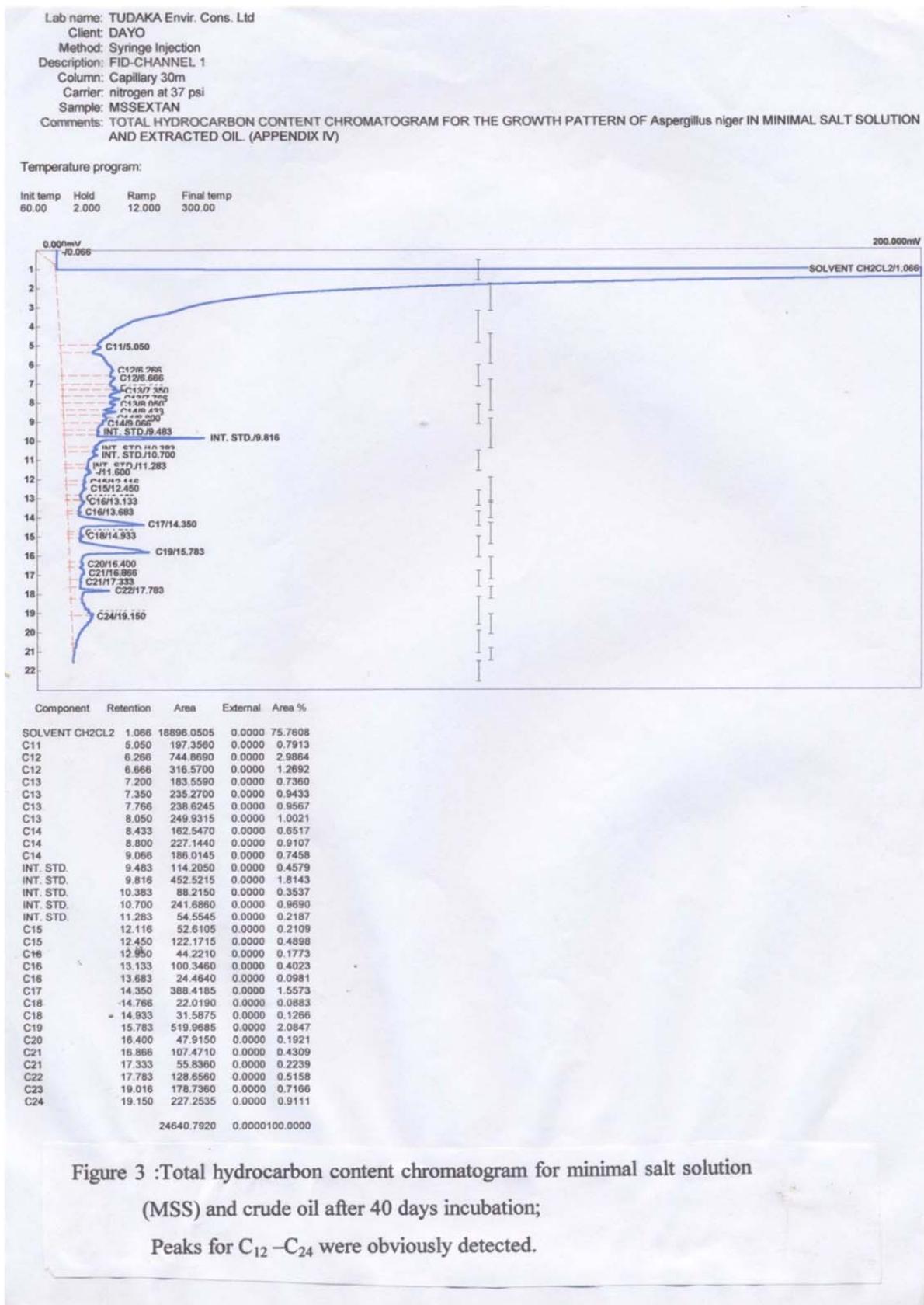
In conclusion, it is evident from the results in this work that all the fungi isolated from *T. africana* seed have the potential to biodegrade petroleum crude oil and petroleum products.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

### ACKNOWLEDGEMENT

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**Figure 3.** Total hydrocarbon content chromatogram for minimal salt solution (MSS) and crude oil after 40 days incubation; peaks for C<sub>12</sub>–C<sub>24</sub> were obviously detected.

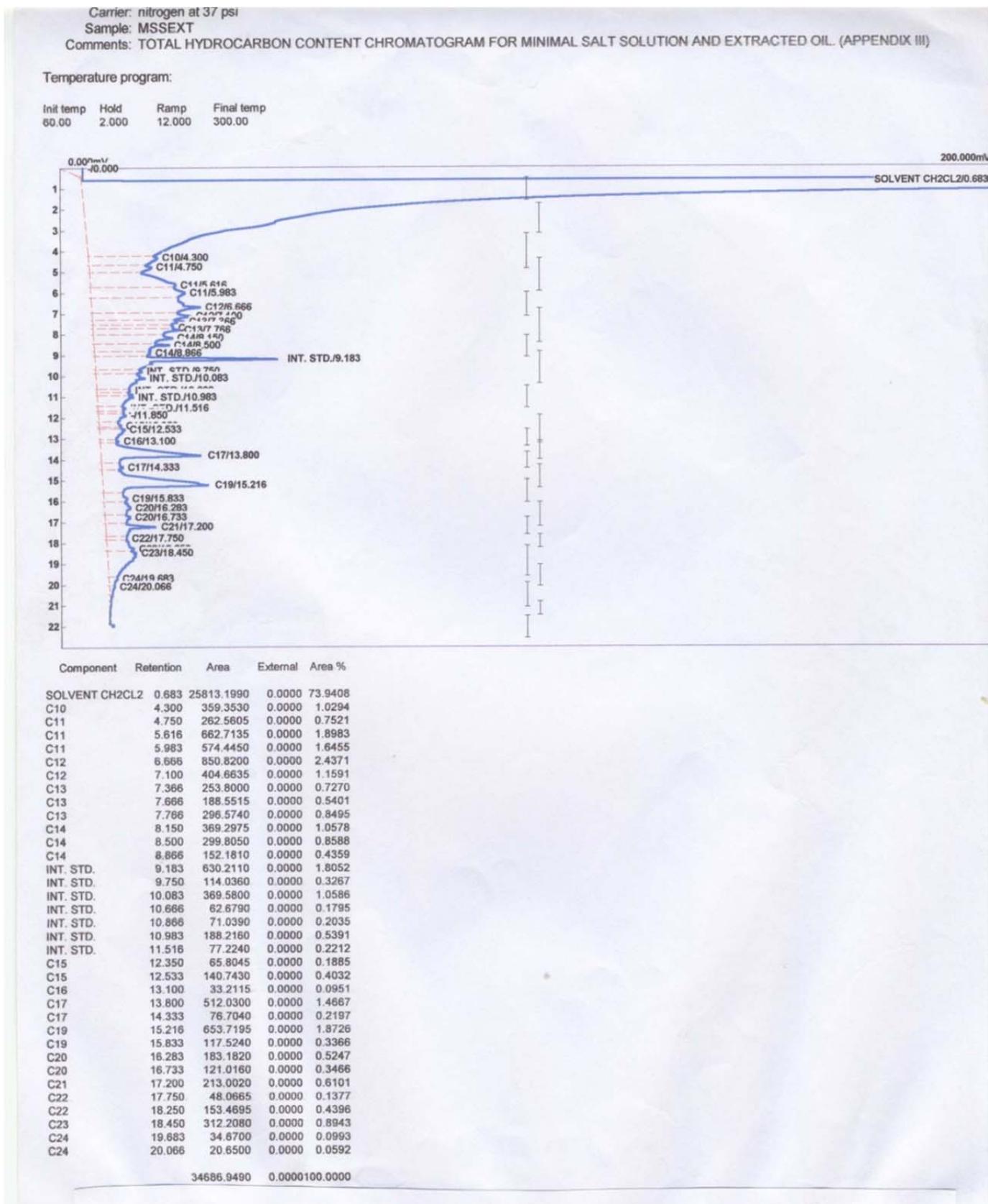
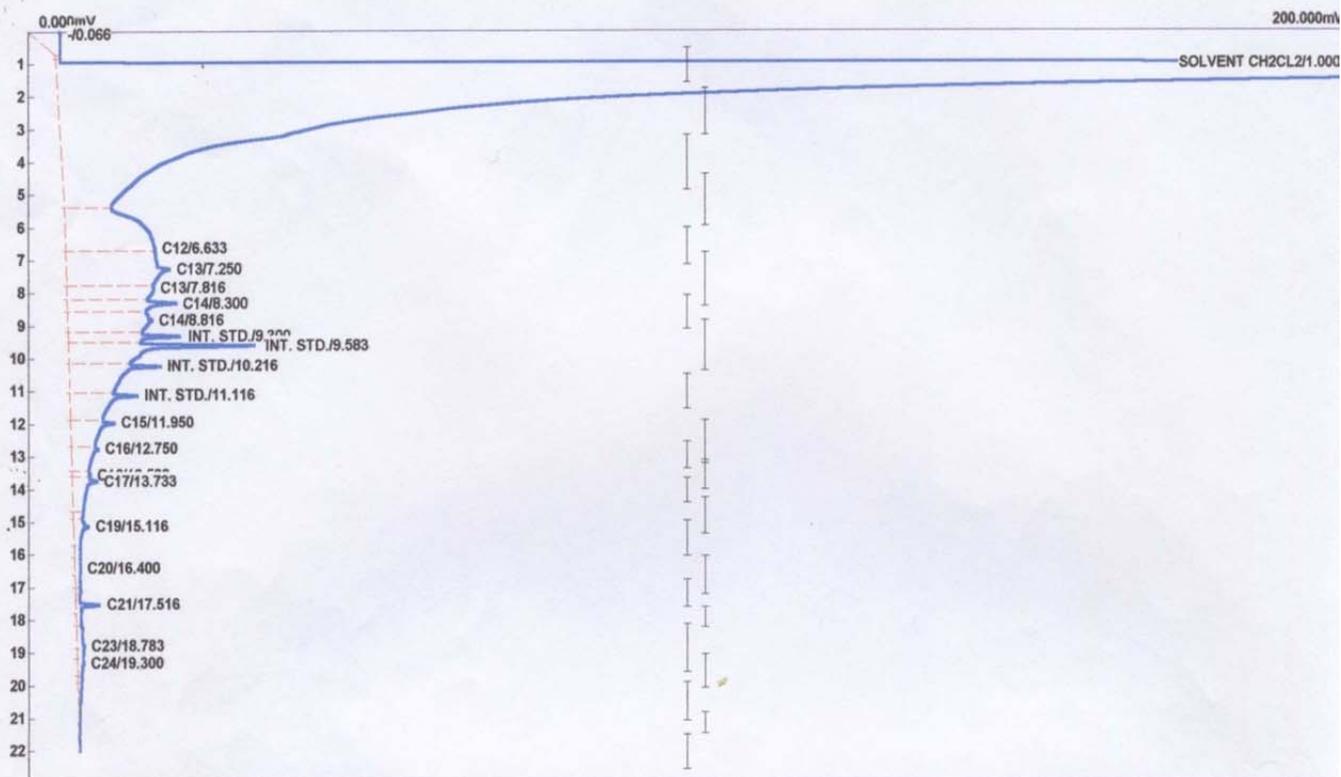


Figure 4. Total hydrocarbon content chromatogram for the growth of paecilomyces in MSS and crude oil after 40 days incubation; peaks for C<sub>12</sub>-C<sub>24</sub> were completely absent except C<sub>21</sub> peak that was only reduced.

Lab name: TUDAKA Envir. Cons. Ltd.  
 Client: DAYO  
 Method: Syringe Injection  
 Description: FID-CHANNEL 1  
 Column: Capillary 30m  
 Carrier: nitrogen at 37 psi  
 Sample: MSSCR  
 Comments: TOTAL HYDROCARBON CONTENT CHROMATOGRAM FOR MINIMAL SALT SOLUTION AND CRUDE OIL. (APPENDIX I)

Temperature program:

Init temp 60.00 Hold 2.000 Ramp 12.000 Final temp 300.00



Component	Retention	Area	External	Area %
SOLVENT CH2CL2	1.000	20451.4510	0.0000	79.7118
C12	6.633	921.0840	0.0000	3.5900
C13	7.250	880.8705	0.0000	3.4333
C13	7.816	335.3110	0.0000	1.3069
C14	8.300	293.6030	0.0000	1.1444
C14	8.816	459.5920	0.0000	1.7913
INT. STD.	9.300	239.5140	0.0000	0.9335
INT. STD.	9.583	496.1410	0.0000	1.9338
INT. STD.	10.216	452.0780	0.0000	1.7620
INT. STD.	11.116	308.7980	0.0000	1.2036
C15	11.950	217.9295	0.0000	0.8494
C16	12.750	140.0580	0.0000	0.5459
C16	13.533	26.5730	0.0000	0.1036
C17	13.733	136.7620	0.0000	0.5330
C19	15.116	66.6020	0.0000	0.2596
C20	16.400	22.0500	0.0000	0.0859
C21	17.516	32.3470	0.0000	0.1261
C23	18.783	28.6540	0.0000	0.1117
C24	19.300	24.8020	0.0000	0.0967
		25534.2200	0.0000	100.0000

Figure 5. Total hydrocarbon content chromatogram for minimal salt solution (MSS) and extracted oil after 40 days incubation; peaks for C10-C24 were obviously detected.

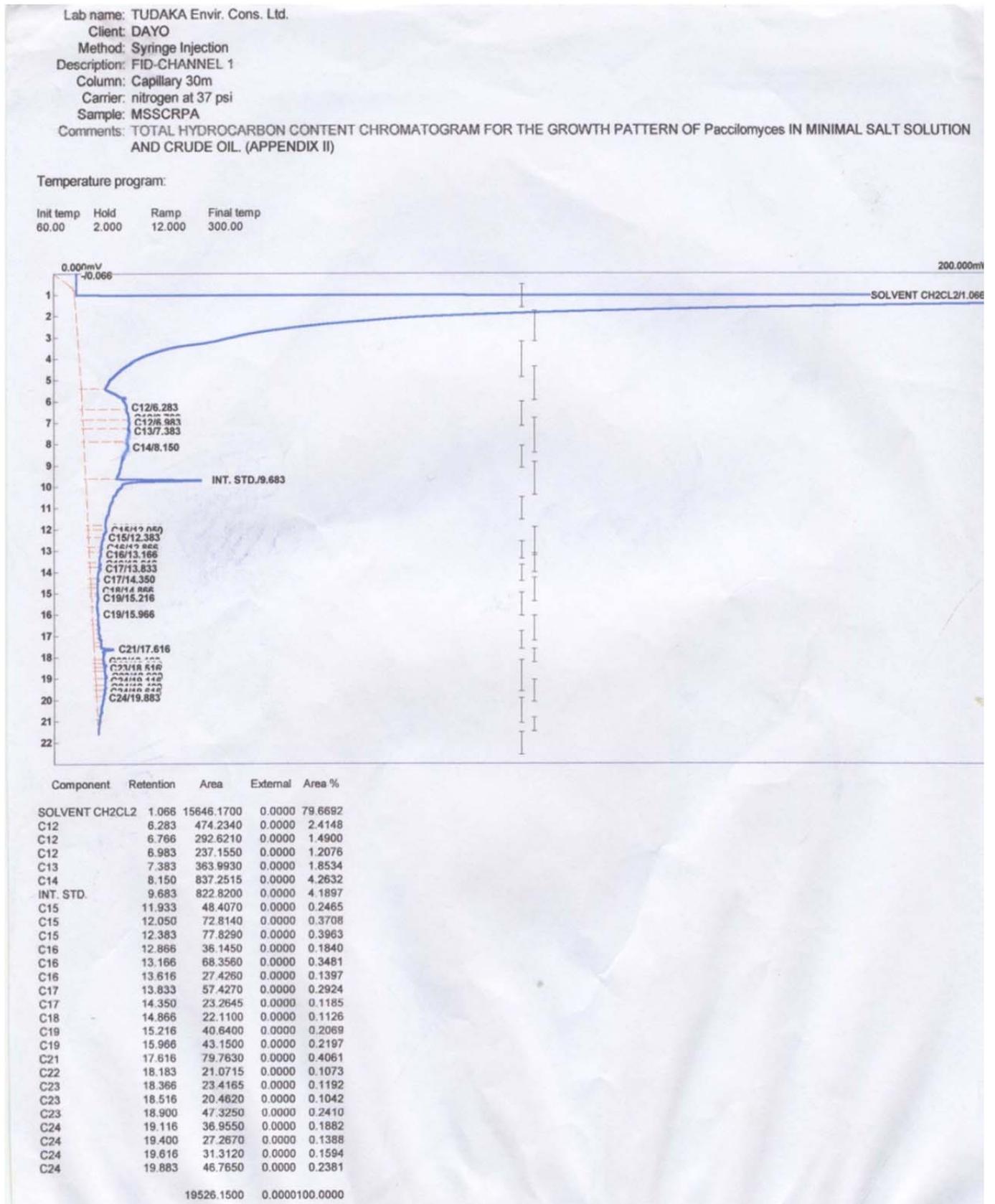


Figure 6. Total hydrocarbon content chromatogram for the growth of *Aspergillus niger* in minimal salt solution (MSS) and extracted oil after 40 days incubation; peaks for C10- C16 were absent or reduced detected.

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