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MICROBIOLOGICAL STUDIES ON PSYCHROTOLERANT FUNGUS ISOLATED FROM DEEP FREEZE

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ABSTRACT

This study was conducted to investigate the possible effects of some cultural and physical factors affecting the growth of psychrotolerant fungi isolated from deep- freeze, and to identify the best environmental conditions for growth of isolates and their biodegradation capability. A total of 100 samples of food stuffs were collected from twenty household deep-freezer (five samples from each freezer of randomly selected houses) in Riyadh, Saudi Arabia. Samples were cultured on Saboraud's agar plates at 25 – 27°C for 5 days. A considerable number of fungal isolates were recovered, purified by repeated subculturing, and maintained on Saboraud's agar slants for subsequent expriments. Fungi were identified macro and microscopically and by CABI Bioscience Identification Services, UK. Isolated and identified fungi were tested mycelial growth responses to changes in incubation temperature, pH and oxygen level (aeration). The loss of the oil and its fractions was determined gravimetrically. The fraction of saturates was resolved by GC/MS analysis to determine the loss of each component of the n-alkanes and iso-alkanes present. From all the samples obtained from deep freeze in this study, only one fungal isolate was identified, *Aspergillus niger*. The experimental strain of A.niger exhibited its optimal growth at temperature range between 25°C and 35°C and at aerobic conditions. *Aspergillus niger* biodegraded crude oil at a mean of 0.86 ml ± 2.6 at 7th harvest day, 0.6 ml ± 2.0 at 14th harvest day and 0.40 ml ± 4.0 at 21st harvest day. Fungi isolated from colder environments labeled as psychrotolerant survive and grow at varied environmental conditions including pHoxygenation, temperature and *Aspergillus niger* can be of importance in biodegradation of oil.

Key words: Psychrotolrant, *Aspergillus niger, petrolum degradation, environment, deep-freezer.*

INTRODUCTION

Fungi, like other microorganisms, are capable of surviving the full range of temperature normally experienced in environments in which they live. Activ growth, however, will often be possible for only part of the year. In some seasons water may be unfavourable either as a result of freezing or of drought, and the other times suitable substrate may be lacking. Hence a great many fungi have a maximum temperature of growth of 30 - 40°C and a minimum of a few degrees above the freeznig point of water. Fungi that can grow near freezing point or even a little below are termed psychrotolerant and if incapable of growth above 20°C, psychrophilic **[Edwards (1990)]**. Fungi surviving in warm habitat are termed thermotoleran if they are capable of growth at 50°C or more, and thermophilic if incapable of growth below 20°C **[Carlile & Watkinson (1997)]**.

Psychrophilic and psychrotrophic microorganisms have the ability to grow at 0°C. Psychrotrophic microorganisms have a maximum temperature for growth above 20°C and are widespread in natural environments and in foods. Psychrotrophic microorganisms have a maximum temperature for growth at 20°C or below and are restricted to permanently cold habitates **[Gounot (1986)]**. Psychrophilic fungi are known from among the Mucorales, Ascomycetes and Deuteromycetes, many cold- tolerant yeasts are known and some of them including Basidiomycetes yeast, are psycrophilic. The vast majority of fungi that are neither psychrophilic nor thermophilic are sometimes termed mesophiles, favouring intermediate temperatures most filamentous fungi are mesophilic, growing at temperatures within the range of 15 -35°C. Some mesophiles are psychrotolerant or thermotolerant **[Carlile & Watkinson (1997)]**.

The effects of temperature on fungi depend on many factors, including the genus, species and strain of the fungus, the amount of available water, kind of nutrients, and many other environmental factors. Of course, temperature is also a crucial factor. Most of the research on temperature relationships for the fungi has been done in the food industry where heat is commonly used to prevent fungal growth. Much of this research involves wet heat, which is more effective than dry heat **[Farrell & Rose (1967)]**.

The growth requirements for fungi may vary from strain to strain, although culture of some species and genera tend to grow best on similar

media. However, some fungi deteriorate when kept on the same medium for prolonged periods. Most laboratories prefer not to keep a large stock of different media and the majority of isolates can be maintained on a relatively small range depending on the specialization of the collection, e.g. medical fungi grow well on Saboraud's medium. Many species grow well in the dark, but others prefer daylight. Nearly all fungi are aerobic and, when grown in tubes or bottles, obtain sufficient oxygen through cotton wool plugs or loose bottle caps. Most common fungi (*Aspergillus* and *Penicillium*) grow well over the range of pH 3 to 7, although some can grow at pH 2 and below (Smith and Onions 1994)

This study aims at the isolatation and identification of fungi from household deepfreezers in Riyadh, Saudi Arabia. The possible effects of temperature, pH. and aeration on their growth and biodegradation capability of the recovered fungal isolate(s) were investigated.

MATERIALS AND METHODS

Isolation and identification of psychrotolerant fungi.

A total of 100 food samples were collected from twenty household deepfreezers (5 samples from each deepfreezer) that have been randomly selected from houses in Riyadh, Saudi Arabia. Samples were cultured on Saboraud's agar medium for fungal isolation. Four replicate sets of plates were made for each sample. Plates were incubated at 25 – 27°C for 5 days. All media used were prepared according to Atlas (1993). Fungi were identified on the basis of macro and microscopical features according to Raper and Thom (1949), Gilman (1957), Raper and Fennell (1965) and Moubasher (1993). The identification was checked to the species level by CABI Bioscience Identification Services, UK.

Effect of temperature, pH and oxygen on fungal growth.

The effect of different degrees of temperature, pH oh the medium and oxygen level on fungal growth, expressed as myceliai dry weight, were studied. The fungal isolates were grown in 250 ml - capacity Erlenmeyer flasks containing 100 ml of dextrose - salts liquid medium (composed of (g / l) : dextrose, 10; NH_4NO_3, 0.60 ; $NaNO_3$, 1.40 ; KH_2PO_4, 0.45 ; K_2HPO_4, 0.15 ; Na_2HPO_4, 0.45 ; NaH_2PO_4, 0.15 ; $MgSO_4.7H20$, 0.50 ; $ZnSO_4$, 0.001; $CuSO_4$, 0.001 ; $MnSO_4$, 0.001 and $FeSO_4$, 0.001) the treatment was conducted in triplicates. Sterilized

media were inoculated with 5 mm agar disk from 7-days old culture from the margin of the fungal growth. The inoculated flasks were incubated at 4, 25, 30 and 40°C for 21 days. In order to determine the effect of pH on fungal growth, culture media were adjusted to different pH values (4, 7 and 9) and incubated at 4, 25, 30 and 40°C for 21 days. Cultures also were tested aerobically and anaerobically to determine the effect of aeration on fungal growth. Harvesting of cultures were carried out on the 7th, 14th and 21st days of icubation , the culture were filtered and the mycelia mats were collected in previously weihed Whatman No.1 filter paper, dried at 70°C for 48 hours and the myclial dry weights were determined.

Evaluation of oil - degrading activity of fungal species isolated.

And iso-alkaneTheexperimental fungal species (Aspergillus niger) aws cultivated in 250 ml Erlenmeyer flasks containing 100 ml **medium** of Fedorak and Westlake (1981) supplemented with 1% (w/v) of crude petroleum oil. The oil was sterilized separately by passing through a membrane filter (0.450 um), then adding to the autoclaved basal medium. The culture flasks were inoculated, each, with 5 disds (0.5 cm diameter) and incubated at 30°C on a rotary shaker operated at 100 rpm for 21 days. Harvesting of cultures was carried out on the 7th, 14th and 21st days of icubation: four flassks were harvested at each period.The same methods were used for the control samples as described by Odu (1972). The loss of the oil and its fractions was determined gravimetrically. The fraction of saturates was resolved by GC/MS analysis to determine the loss of each component of the n-alkanes s present.

Gas Chromatography- Mass Spectrometry (GC/MS).

Gas chromatography-mass spectrometry (GC/MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC/MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification.

In this study, oil samples were diluted with dichloromethane to get clear liquids and then injected into GC/MS. Components were

resolved over a 60 meter HP-5 capillary column with a mass range of 35 – 450 amu. Percent of biodegradation of oil was measured at 7, 14 and 21 days.

RESULTS

From all the samples obtained from deep freeze in this study, only one fungal isolate was identified, *Aspergillus niger*. When this fungus was tested for its growth response to changes in incubation temperature, pH of the medium and avaialable oxygen level, the following results were obtained:

Temperature – On the 7th harvest day, growth was observed at 4 to 40°C, however, optimal growth was seen between 25 and 35°C. Similarly, harvest done on the 14th and 21st days, optimal growth was seen at temperatures between 25 and 35°C. (Table 1 and Fig 1)

pH – Growth of *Aspergillus niger* was optimal at pH 7 on the 7th and 14th harvest days. At 21st harvest days, the growth was somewhat higher than that at pH 7, meanwhile, the magnitudes of dry biomass weight of the 14-day old cultures were greater than that obtained from cultures harvested after 7 or 21 days of incubation (Table 1 and Fig 2).

Oxygen – growth of *Aspergillus niger* was optimal at aerobic condition. The growth differences between aerobic and anaerobic conditions were significantly higher under aerobic condition at 14th and 21st harvest days where mycelial growth measured by mycelia weight was more than doubled (Table 1 and Fig 3).

Active oil biodegradation – compared to the control, *Aspergillus niger* biodegraded crude oil at a mean of 0.86 ± 2.6 ml at 7th harvest day, 0.6 ± 2.0 ml at 14th harvest day and 0.40 ± 4.04 ml at 21st harvest day. (Table 2 and Fig 4 - 5).

Table (1): Effect of temperature, pH and oxygen on fungal growth (Aspergillus niger).

	Temperatures				pH			Oxygen	
	4°	25°	30°	40°	4	7	9	aerobic	Anaerobic
7	0.121	0.573	0.592	0.127	0.658	0.809	0.638	0.573	0.438
14	0.235	1.621	1.599	0.198	0.950	1.325	0.952	1.621	0.417
21	0.155	0.951	1.176	0.164	0.697	0.752	0.776	0.951	0.366

Note: Values are reported as mycelium dry weights in mg.

Table (2): Biodegradation of crude oil by Aspergillus niger.

	Biodegradation (%)			
	Control		*Aspergillus niger*	
	Degraded crude oil (1 ml)	%	Degraded crude oil (1 ml)	%
4	1 ± 0.000	1	0.86 ± 2.6	86
14	1 ± 0.000	1	0.6 ± 2.0	60
21	1 ± 0.000	1	0.41 ± 4.0	40.7

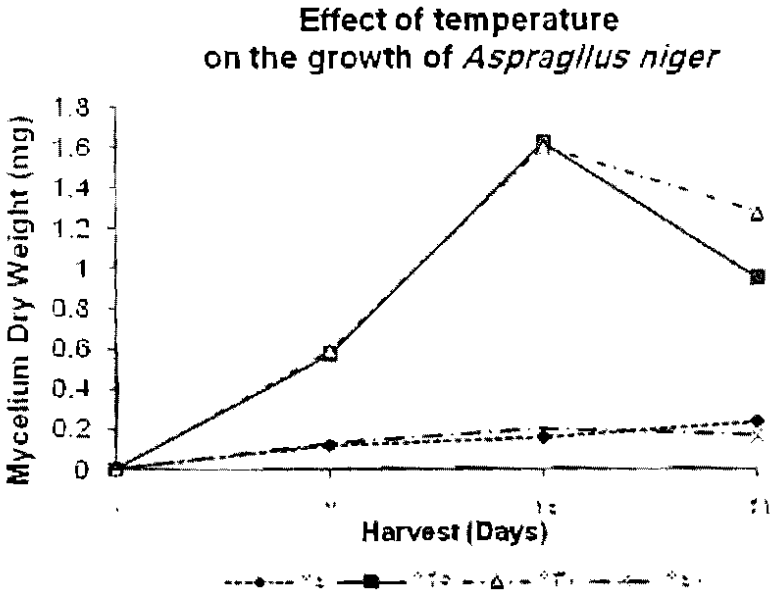

Fig. (1): Effect of temperature on the growth of Aspergillus niger.

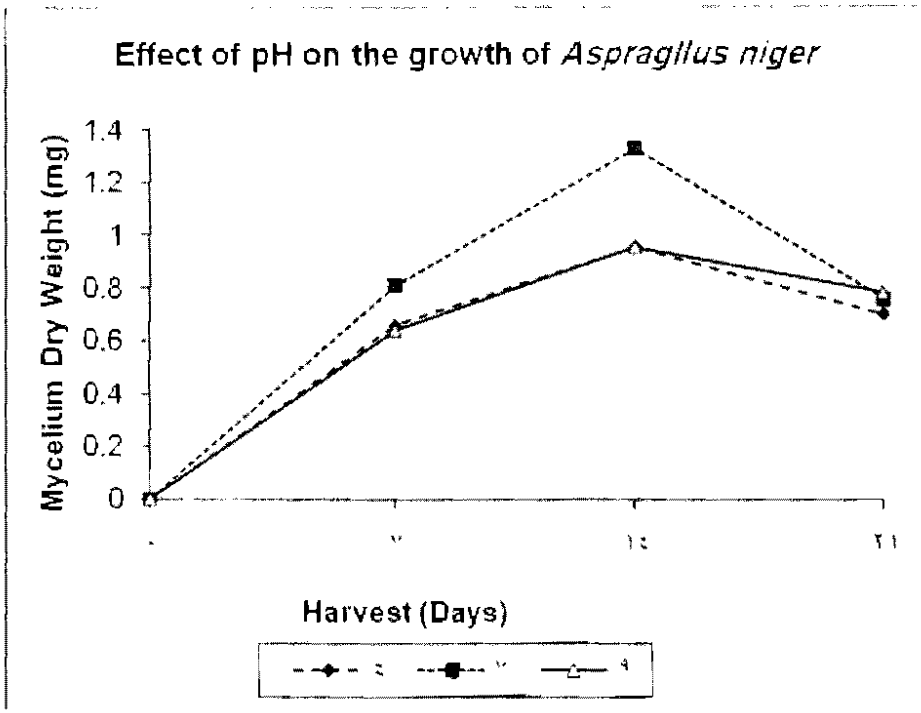

Fig. (2): Effect of pH on the growth of Aspergillus niger.

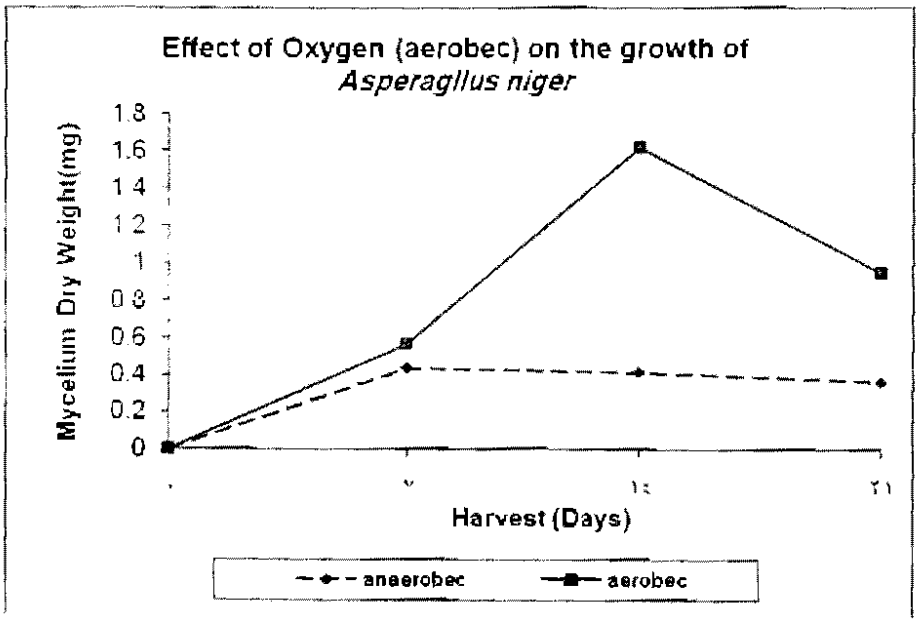

Fig. (3): Effect of oxygen on the growth of Aspergillus niger.

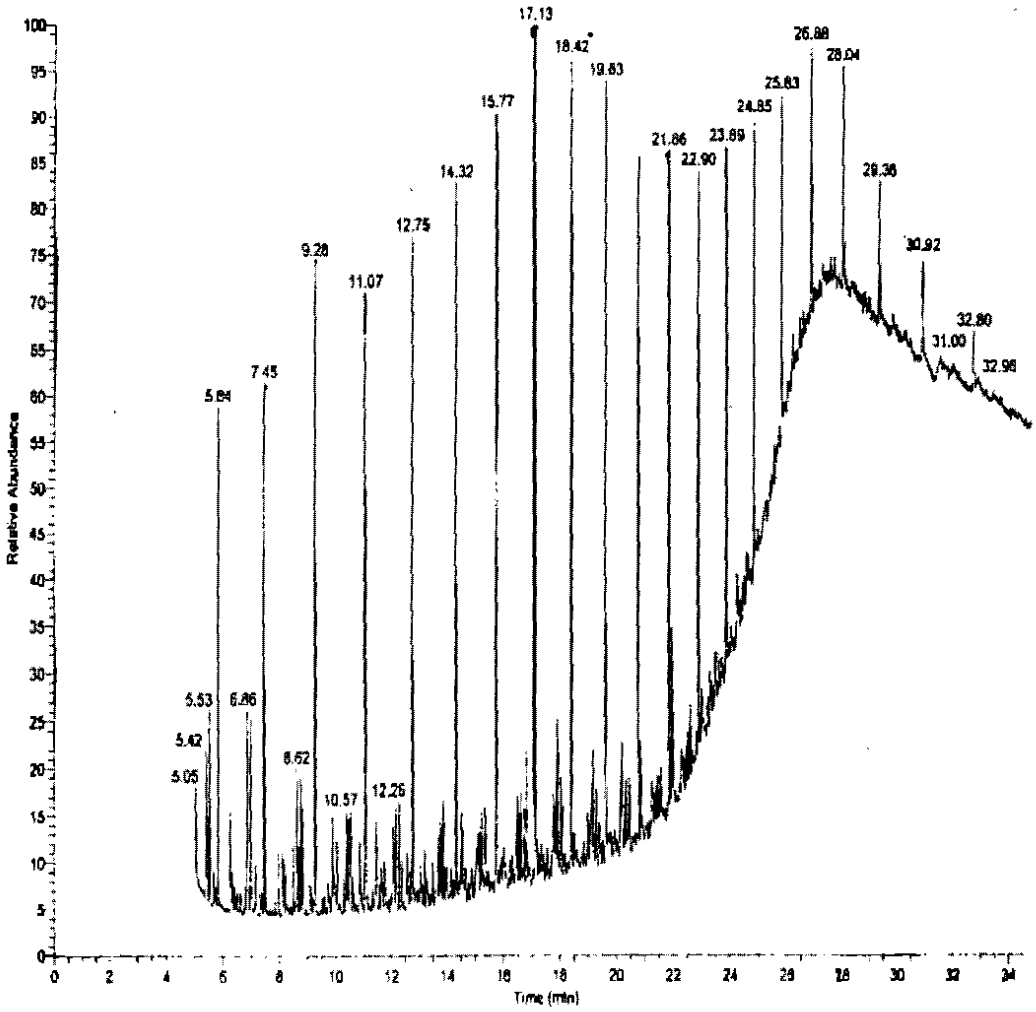

Fig. (4): Biodegradation of crude oil by Control.

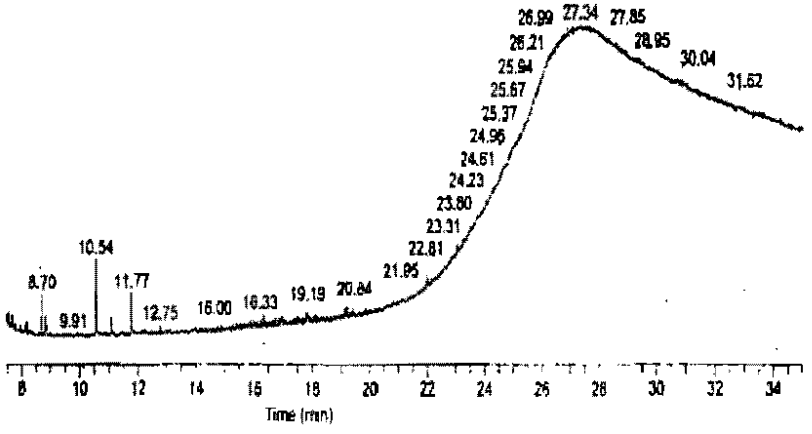

Fig. (5): Biodegradation of crude oil by Aspergillus niger.

DISCUSSION

This study presents some informations about the environmental diversity of fungal growth. Such that, the isolated fungal strains of *Aspergillus niger*, a known fungus with mesophilic characteristic. They are extremely drought-tolerant, being able to grow in substrates so dry with water activities as low as 0.62, that almost nothing else can compete with them. And while some *Aspergilli* are thermotolerant, they cover the full spectrum of temperatures at which life can thrive **[Pitt (1981)]**. The ability of this fungi to survive and thrive at changing temperatures, pH and oxygenation was thought to be due to mycotoxin production. Some strains of *Aspergillus niger* in fact have been reported to produce ochratoxins **[Abarca (1994)]** but other investigators disagree; claiming that the report was based upon misidentification of the fungal species. Recent evidence suggests that some true *Aspergillus niger* strains do produce ochratoxin A. **[Samson et al., (2004)]**.

The ability of microorganisms to grow at low temperature may be correlated with a lower temperature characteristic than that of mesophiles, an increasing proportion of unsaturated fatty acids in the lipid phase of the cell membrane, and a protein conformation functional at low temperature **[Gounot (1986)]**.

In exploring the feasibility of the use of *Aspergillus niger* for the facilitated biodegradation of waste oils, they may show affinity for hydrocarbon substrates and decreases the napthalene and biphenyl content of a synthetic crude concomitantly with utilization of alkanes. The use of yeasts in mixed culture systems for facilitating the biodegradation of spent oil in confined systems is recommended **[Ahearn & Berner (1978)]**. Therefore, the study of the petroleum hydrocarbon degradation was accomplished by filamentous fungi to degrade petroleum, added as the only carbon and energy source to a mineral medium **[Lemos (2001) and April (2000)]**.

CONCLUSION

Fungi isolated from colder environments labeled as psychrotolerant survive and grow at varied environmental conditions including pH, temperature and oxygenation. *Aspergillus niger* proved to be capable of growing optimally at neutral pH, temperature range of 25 - 35^{o}C and at aerobic condition and can be of importance in biodegradation of oil.

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دراسات ميكروبيولوجية على الفطريات المقاومة للبرودة المعزولة من المجمدات المنزلية

كوثر فؤاد عابد

قسم النبات – كلية التربية – الرياض – المملكة العربية السعودية

يهدف هذا البحث نحو عزل الفطريات التي تحتمل البرودة الشديدة ودراسة خصائصها التزريعية وتحديد أفضل الظروف البيئية لنموها. وقد تم جمع ١٠٠ عينة من الموادالغذائية والأطعمة المحفوظة بالمجمدات (الديبفريزر) المنزلية من عشرين منزل تم اختيارها بطريقة عشوائية من الأحياء السكنية بمدينة الرياض بالمملكة العربية السعودية (خمس عينات من كل مجمد بكل منزل) وتم زراعتها على الوسط الغذائي " سابورود الأجار" عند درجة حرارة ٢٥ – ٢٧ مئوية لمدة خمسة أيام، وتم عزل عدد كبير من السلالات الفطرية وتم تنقيتها وفحصها مجهريا ودرست صفاتها المظهرية والشكلية الدقيقة (المورفولوجية) وتنقيتها وتعريفها علي أنها سلالات تابعة لفطر " أسبيرجيللس نيجر"، وقد تم تأكيد التعريف بمعهد "كابي" بالمملكة المتحدة. تم دراسة تأثير التغير في درجة حرارة التحضي ودرجة الحموضة (ال بي اتش) ومستوى الأوكسيجين (التهوية) على نمو السلالة الفطرية المختارة على أساس الوزن الجاف للحصيرة الفطرية (الغزل الفطرى) في المزارع السائلة المهزوزة على مدى ٢١ يوم، وتم تقديرالنمو تحت مختلف الظروف عند اليوم السابع، واليوم الرابع عشر، واليوم الحادي والعشرين .هذا وقد تم دراسة قدرة هذه السلالة الفطرية على تكسير وهضم زيت البترول الخام من خلال زراعته في المزارع المهزوزة المحتوية على زيت البترول كمصدر وحيد للكربون لمدة ٢١ يوم عند درجة ٢٥ –٢٧ مئوية وأجريت عملية الحصاد عند اليوم السابع، واليوم الرابع عشر، واليوم الحادى والعشرين، وتم قياس نواتج تكسير أو هضم الزيت بواسطة فطر أسبيرجيللس نيجر عند كل من تلك الفترات الزمنية المذكورة باستخدام تقنية الكروماتوغرافيا الغازية وطيف الكتلة *Gas Chromatography- Mass Spectrometry (GC/MS).*

وقدأسفرت هذه الدراسة عن نتائج مبشرة بأهمية هذا الفطر وامكانية استخدامه فى التخلص من الملوثات البترولية .