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Production of Lipid and Fatty Acids during Growth of *Aspergillus terreus* on Hydrocarbon Substrates

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Abstract An *Aspergillus terreus*, isolated from oil contaminated soil, could degrade a wide range of petroleum hydrocarbons including the immediate oxidation products of hydrocarbons, like alkanols and alkanals. Among all the linear chain carbon substrates, highest growth of 39.1±3.8 g l^{-1} (wet weight) was observed when *n*-hexadecane was used as the sole source of carbon. The growth of the fungus on this highly hydrophobic substrate was associated with the morphological change of the hyphae and increase production of lipid in the cells. The lipid production in the hydrocarbon (*n*-hexadecane) grown cells was sevenfold higher than the corresponding glucose grown cells. The fatty acid profile of the lipid content formed in the hydrocarbon grown cells was significantly different from the glucose grown cells and was composed of fatty acids with chain length C_{14} to C_{33} as revealed from the liquid chromatography electrospray ionization mass spectrometry analyses. Among the ranges, the fatty acids with chain lengths C_{14} to C_{18} were predominant in the profile. Considering the fatty acid profile and the high level of lipid production, this *A. terreus* mediated production of lipid is envisaged to have potential application in the oleochemical industries including the production of biodiesel.

Keywords Filamentous fungi · *Aspergillus terreus* · Lipid · Fatty acid · Hydrocarbon

Introduction

The microbial degradation of petroleum hydrocarbon is an area that has been continuously stimulating interest of researchers since last several decades due to their scope for wide applications, such as bioremediation [1–3], production of secondary metabolites [4, 5], enzymes [6–8], biotransformation [9], etc. The importance of fungal degradation of petroleum hydrocarbons is growingly recognized and attracts attention due to the widespread and abundance of fungi in terrestrial ecosystem [10, 11]. Among the various fungi, *Aspergillus* species has been identified as one of the potential microbial agents for

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the biodegradation process [9, 12]. The biodegradation of petroleum hydrocarbons by *Aspergillus* species has been largely studied from the environmental perspective, whereas the metabolic consequence and production of other important metabolites during the degradation have not yet been adequately addressed so far. We report in this study the isolation of a broad spectrum hydrocarbon degrading *Aspergillus* strain and increase in induction of lipid during growth of the fungus on hydrocarbon substrates. In addition, the fatty acid profile of the lipids formed during the growth of the fungus on hydrocarbon substrates was investigated and reported in this study.

Materials and Methods

Sample Collection and Sampling

Petroleum contaminated soil samples from oil fields located near Digboi and Duliajan of Assam, India were collected in a sterile container. These were labeled and sealed and then transported to the laboratory for analysis. A total of 25 soil samples were collected for the present study.

Isolation and Cultivation of Fungi

The soil samples were transferred to test tubes containing 5.0 ml of sterilized distilled water. The test tubes were vigorously shaken, for approximately 60 s, to release the fungal spores. Several serial dilutions were made, and 1 ml portion from each dilution was pipetted on the surface of fungal agar medium and spread evenly throughout. The plates were kept at room temperature (±28°C) until the development of colonies (3–4 days). The fungi were purified initially on the fungal agar plates. The composition of fungal agar (Himedia, India) contains (in grams per liter) the following: papain digest of soya bean meal, 10; dextrose, 10; and agar, 15. The pure colonies formed in the plates were transferred to fungal agar slants and then incubated further at 28°C. The organism was maintained on fungal agar slants with periodic transfer to a new slant after every 15 d. For long-term storage, the fungal agar slants were sealed with paraffin and stored at 4°C. For growth studies on different carbon substrates, the pure colonies were transferred to 500-ml Erlenmeyer flasks containing 2% (*v*/*v* for liquid or *w*/*v* for solid) carbon substrates in 50 ml of basal medium [13] of the following compositions (grams per liter): $MgSO_4$ $7H_2O$, 0.2; NH_4NO_3, 1.0; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $FeCl_3$, 0.002; and yeast extract, 1.0 as growth inducer. At this concentration, yeast extract alone supported negligible growth. The pH of the medium was adjusted to 5.8, and the flasks were incubated at 28°C under static condition. The media containing the substrate was sterilized at 15 psi for 15 min before inoculating the organism. A 2% (*v*/*v*) *n*-hexadecane substrate (with a C/N ratio of 40/1) was found to be optimum for attaining maximum wet mass of the cells under the standard conditions of cultivation used in this investigation.

Identification of Filamentous Fungus

The identification of the filamentous fungal species was carried out through macroscopic observation of the colonies and examination of the microstructural characteristics and a comparative analysis with parameters established in the conventional taxonomy, by referring some of the books [14, 15]. The strain was then submitted to Microbial Type Culture Collection (MTCC), Chandigarh for species identification and accession number.

After identification of the organism, the colonies were transferred to test tubes containing fungal agar medium for further study.

Microscopic Analysis of the Fungus

The morphological characteristics of the hyphae were studied using scanning electron microscopy (SEM) following the general protocol for dehydration of biological samples [16]. Since the samples were non-conductive, gold coating of the dried samples were done in a Polaron Sputter coater unit (model SC 7620) after mounting on aluminum stubs fixed with double-coated carbon tape for 90 s. The vacuum chamber was maintained at 10^{-2} Torr and current of 1 mA at 1 kV voltage and then analyzed through SEM using the following setting conditions: 10–15 kV EHT, 50 μm aperture, and ×500 magnifications.

Lipid Analysis of the Cells Grown on Different Carbon Substrates

The harvested cells were washed on a filter paper (Whatman) thoroughly with 50 mM Tris–HCl buffer, pH 8.0 to remove the mineral salts of the medium and then briefly washed with *n*-hexane to remove the cell bound *n*-hexadecane, and finally washed with the ice-cold Tris buffer for several times to remove the hexane. The organic solvent washing step was omitted for glucose

Table 1 Growth of *A. terreus* in different carbon source.

Substrate	Wet weight[a] (g l^{-1})	Substrate	Wet weight[a] (g l^{-1})
n-Octane	12.8±1.9	Isoamyl alcohol	5.6±1.1
n-Dodecane	15.4±1.3	3-Butanol	3.0±1.5
n-Hexadecane	39.1±3.8	3-Octanol	3.2±1.9
n-Octadecane	24.9±1.2	2-Dodecanol	24.1±2.2
n-Docosane	15.7±1.6	2-Docosanol	21.8±1.3
n-Tetracosane	8.1±1.3	Cholesterol	10.2±1.9
Methanol	14.8±0.9	1-Dodecanal	15.9±1.3
Ethanol	15.4±1.5	1-Hexadecanal	17.9±2.1
n-Butanol	15.8±2.1	Pristane	5.3±2.9
n-Heptanol	5.2±0.8	Benzene	8.1±3.1
n-Octanol	6.0±1.4	Toluene	6.8±2.2
n-Decanol	8.6±1.3	Phenol	4.1±2.7
n-Undecanol	21.9±3.0	Naphthalene	2.6±1.5
n-Dodecanol	25.5±2.9	Testosterone	3.2±1.1
n-Tetradecanol	24.7±3.1	12-Hydoxydodecanoicacid	16.8±1.6
n-Hexadecanol	23.3±2.8	1,12-Dichlorododecane	12.7±2.4
n-Octadecanol	23.8±1.2	1,16-Hexadecadiol	10.9±1.2
n-Tetracosanol	17.9±1.9	Petrol sludge	12.5±1.8
n-Hexadecanol	15.4±1.6	Kerosene	8.1±2.0
n-Eicosanol	12.3±0.4	Crude oil	13.4±1.7
n-Docosanol	13.4±1.8	Yeast extract	10.1±2.9
Isopropanol	16.7±1.1	Glucose	40.3±2.1

[a] Maximum harvesting time of the cells was 96 h; each value represents the mean of four replicates±standard error ($P < 0.05$).

grown cells. Total lipids from the wet cells were extracted following the standard chloroform–methanol (2:1) method [17]. Fatty acids were isolated by hydrolyzing the lipid with ethanolic KOH (6% KOH in 95% ethanol) at 70°C for 3 h and 150 rpm. The pH of the reaction medium was then reduced to 2 by adding 1 N HCl and then kept standing overnight for phase separation. The fatty acid layer formed was then extracted with chloroform. The solvent was evaporated by rotavapor, and the extracted fatty acids were stored at −20°C until analysis.

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) (Q-Tof Premier, Waters) analysis was used to identify the fatty acids. Before analysis, the fatty acids were dissolved in 5 mM ammonium acetate in methanol. Identification of fatty acids was done with an ESI probe source in negative mode. ESI-MS was operated at spray voltage 2.5 kV, sample cone 60, extraction cone 4, and the heated capillary temperature at 250°C.

Results and Discussion

Different hydrocarbon degrading fungal strains were isolated from the oil contaminated soil samples collected from different locations of Digboi and Duliajan oil fields of Assam.

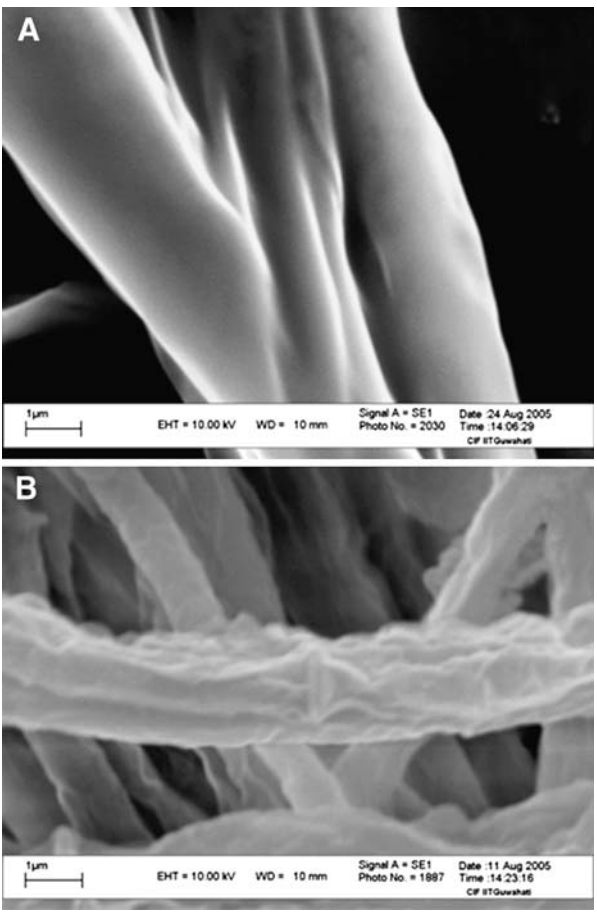

Fig. 1 SEM analysis of *A. terreus*. The filamentous fungal hyphae grown on **A** glucose and **B** *n*-hexadecane

Among these, the fastest growing species was considered for the present investigation. Based on the morphological characteristics, the organism was provisionally identified as *Aspergillus terreus* and subsequently confirmed its identity by using the identification service of the MTCC, Chandigarh. The organism was deposited in the MTCC with accession number MTCC 6324.

A. terreus MTCC 6324 could degrade a wide range of petroleum hydrocarbons, alcohol, and long-chain primary alkanols, secondary alcohols including cholesterol, and chlorinated aliphatic hydrocarbons as shown in Table 1. There are many reports on the degradation of specific classes of hydrocarbon substrates by microorganisms [11, 12, 18–23]. However, specific microorganisms capable of degrading a broad range of hydrocarbon substrates are rare and not adequately studied among filamentous fungi. Among all the studied complex carbon sources, maximum cell growth was observed in *n*-hexadecane with a wet weight cell mass 39.1±3.8 g l^{-1}, which is closer to cell mass of 40.3±2.1 g l^{-1} obtained by using glucose as the growth substrate. No surface activity of the culture broth measured by analyzing the surface tension of the culture supernatant using Auto Tensiometer (model DCAT 11EC, Dataphysics Instruments GmbH, Germany) was observed during growth of *A. terreus* on *n*-hexadecane or other similar hydrophobic hydrocarbon substrates used in this investigation. Hence, assimilation of such highly hydrophobic aliphatic hydrocarbon substrates by this filamentous fungus probably occurred only through a sorption mechanism, which is supported by the fact that the fungal hyphae were largely associated with the water insoluble top hydrocarbon layer during growth of the fungi on such hydrophobic hydrocarbon substrates. Notably, many microorganisms are known to produce surface active agents to facilitate degradation of water insoluble hydrocarbon substrates through emulsification that increases oil to water surface area for enhanced assimilation/

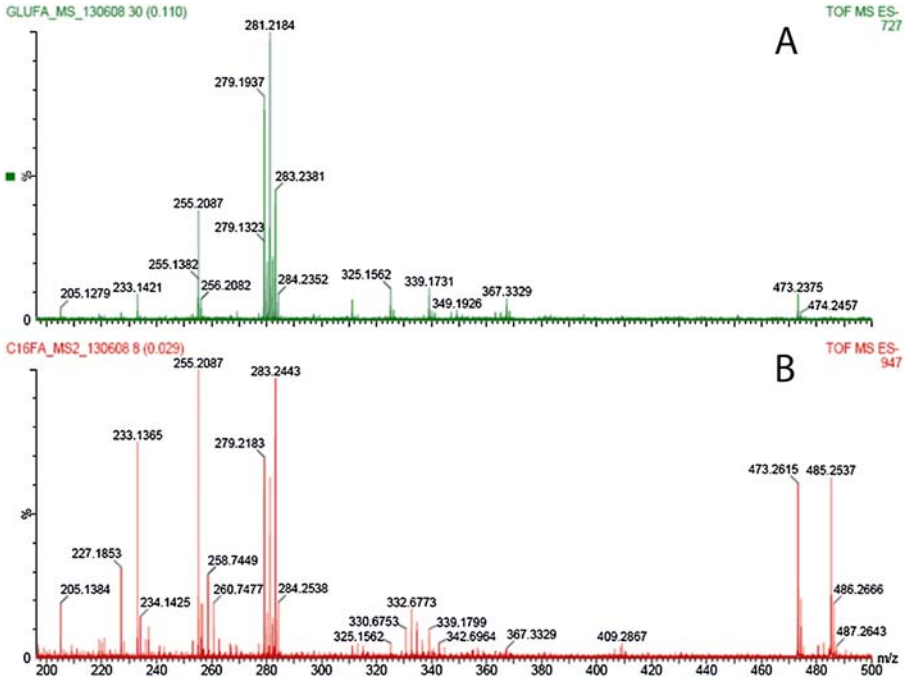

Fig. 2 LC-ESI/MS fatty acid profile of **A** glucose and **B** *n*-hexadecane cells

transport through the cell wall [24–26]. Degradation of such highly hydrophobic substrates through sorption is expected to occur through special modification of the hyphal cell wall. Scanning electron microscopic studies of the cell hyphae obtained from the growth of the organism on *n*-hexadecane and glucose separately were conducted, and we found that there was a marked difference between the cell wall morphology of the hydrocarbon grown cells and the glucose grown cells as shown in Fig 1. In glucose grown cells, the hyphae were smooth, whereas the cell surface of the *n*-hexadecane cells was uneven. The metabolic consequence of assimilation of these high energy hydrophobic substrates by the fungus was investigated by analyzing the cellular lipid content. The lipid production (*w*/*w* wet biomass) in *n*-hexadecane- and glucose-grown cells were 4.43% and 0.62%, respectively, thus showing sevenfold higher lipid content in *n*-hexadecane grown cells than the glucose-grown cells. Similar high level of lipid production was observed when crude oil and petroleum refinery waste sludge were utilized as the sole source of carbon for the growth of the fungus. No significant increase of lipid production was observed in the cells grown on short-chain alcohols, aromatic hydrocarbons, and yeast extract. To study the induction

Table 2 The cellular fatty acid profile of *A. terreus* in *n*-hexadecane- and glucose-grown cells.

Sl No	Name of fatty acid	Short cut name	m/z (M^+-H)	Relative intensity (%)		
				Hexadecane cells[a]	Glucose cells[b]	Ratio[c]
1	Myristic acid	14:0	227.1	30	NA	1:0
2	Palmitic acid	16:0	255.2	100	40	1:0.4
3	Stearic acid	18:0	283.2	97	47	1:0.5
4	Arachidic acid	20:0	311.5	5	4	1:0.8
5	Henicosanoic acid	21:0	325.1	5	7	1:1.4
6	Docosanoic acid	22:0	339.1	15	7	1:0.46
7	Tetracosanoic acid	24:0	367.3	2	5	1:2.5
8	Heptacosanoic acid	27:0	409.2	5	NA	1:0
9	Oleic acid	18:1	281.2	62	100	1:1.6
10	Linoleic acid	18:2	279.2	70	80	1:1.4
11	Tridecatetraenoic acid	13:4	205.1	20	5	1:0.25
12	Pentadecatetraenoic acid	15:4	233.1	75	10	1:0.13
13	Heptadecatetraenoic acid	17:4	260.7	20	NA	1:0
14	Nonadecahexaenoic acid	19:6	284.2	20	NA	1:0
15	Henicosaheptaenoic acid	21:7	311.1	2	7	1:3.5
16	Docosaheptaenoic acid	22:7	325.1	5	12	1:2.4
17	Docosatetraenoic acid	22:4	332.6	20	NA	1:0
18	Dotriacontatrienoic acid	32:3	473.2	60	5	1:0.08
19	Tritriacontatetraenoic acid	33:4	485.2	62	NA	1:0

NA fatty acids were not produced in this carbon source

[a] The relative intensities of the fatty acids were calculated considering palmitic acid for *n*-hexadecane grown cells as 100%

[b] The relative intensities of the fatty acids were calculated considering oleic acid for glucose-grown cells as 100%

[c] Ratio of relative intensity of fatty acids between hexadecane- and glucose-grown cells. All the data presented in this study were the mean of three or more experiments with a variation within 10%

levels of different fatty acids in these lipids, the cellular fatty acid profile was analyzed separately in *n*-hexadecane and glucose substrates (Fig. 2). We observed that the level of induction of saturated fatty acids in *n*-hexadecane grown cells was 4.8% higher than the glucose grown cells. Palmitic acid was the predominant fatty acid detected in *n*-hexadecane grown cells. Unlike *n*-hexadecane-grown cell, unsaturated fatty acids were predominant in glucose-grown cells. Oleic acid and linoleic acids were the predominant fatty acids in glucose-grown cells. The number of different fatty acids in *n*-hexadecane-grown cells were higher than the corresponding glucose grown cells as shown in Table 2. Moreover, high amount of unsaturated fatty acids (~9% *w*/*w* of total fatty acid content) with chain length C_{32} and C_{33} were present in *n*-hexadecane grown cells. These fatty acids were however either negligible or absent in the corresponding glucose grown cells as shown in Table 2. Although reports on long-chain fatty acids from microbial strains are available [27–29], the presence of these fatty acids in *Aspergillus* strains has not yet been reported in the literature, and the metabolic roles are not known in this strain.

Induction of very high amount of palmitic acid ($C_{16:0}$) in *n*-hexadecane grown cells is intriguing. Probably, palmitate is the product of terminal oxidation of *n*-hexadecane substrate catalyzed by the cell membrane associated enzymes, namely, Cyt P450, alcohol oxidase, and aldehyde dehydrogenase. We have recently reported the presence of Cyt P450 and alcohol oxidase in the cells of this fungus [8, 30, 31]. The myristic acid that is absent in glucose grown cells is suggested to be produced from the palmitic acid by β-oxidation pathway. These long-chain fatty acids directly derived from the substrate are thus stored as lipid in the cells. Although direct cellular transformation of aliphatic hydrocarbon substrate to the corresponding fatty acids has been described [18, 20], a report on the cellular accumulation of such high level of lipid content with these fatty acids is not yet known in the filamentous fungi. Among the different fatty acids, those with carbon chain lengths C_{14} to C_{18} were predominant in the lipid content of the hydrocarbon grown cells (Fig. 2). Considering the importance of these fatty acids in the production of various industrially useful products, the application of this *A. terreus* mediated production of lipid using waste hydrocarbon substrates is envisaged to have potential applications in those industrial processes where lipid is one of the primary feedstock such as lipid for biodiesel production.

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