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Composition and enzyme activities in *Aspergillus flavipes* grown on crude petroleum oil and glucose

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Aspects of the growth, cellular composition and enzymology of *Aspergillus flavipes* grown on a medium with crude oil or glucose as the sole source of carbon are reported. When grown on crude oil, the total lipid content of *A. flavipes* was considerably greater than on glucose medium. A large number of fatty acids, particularly saturated fatty acids, were observed when grown on crude oil and more lipid was excreted into the growth medium. The total mycelial carbohydrate and glycogen contents were considerably less in crude oil medium, whereas the mycelial protein content was similar in both media. The activity of some enzymes like NAD^+-alcohol dehydrogenase, $NADP^+$-aldehyde dehydrogenase, iso-citrate lyase and catalase was greater when crude oil was the carbon source. Conversely, the activity of succinate dehydrogenase was greater after growth on glucose medium.

THE subject of degradation and utilization of hydrocarbons by fungi has received considerable attention[1–3] but much more remains to be done[4]. A strain of *Aspergillus flavipes* isolated from the effluents of the Madras Oil Refineries that can degrade hydrocarbons in crude petroleum oil was reported earlier[5]. Work on utilization of hydrocarbons has mainly been carried out on a few selected bacteria, and some fungi, notably species of *Candida* and the kerosene fungus *Cladosporium resinae*[6–8]. Much of this work has used specific hydrocarbons such as alkanes of known chain length. In the present study, however, we have examined the potential of *A. flavipes* isolated from the effluents of the Madras Oil Refineries to degrade crude petroleum oil, which often forms around 2% (v/v) or more of the effluent from the refinery. The emphasis in the study has been on the utilization of crude oil *per se* rather than particular components of the oil.

We have examined the amount of total protein, total carbohydrate, glycogen, total lipids, total sterol, fatty acids (saturated and unsaturated) in *A. flavipes* grown with either crude oil or glucose as the sole source of carbon. As a preliminary to understanding the physiology of utilization of crude oil, some data are given on the enzymology of crude oil and glucose utilization.

Materials and methods

Organism. A single spore isolate of *A. flavipes* isolated from the effluents of the Madras Oil Refineries Ltd., Manali, Madras, was used throughout the study. It was maintained on potato dextrose agar (PDA).

Culture techniques. Standard microbiological techniques were used in the preparation of media, sterilization etc.[9]. The fungus was grown in a mineral salts solution of Bushnell and Haas[10] supplemented with 0.1% (w/v) yeast extract, adjusted to pH 6.0. Glucose (10 g l^{-1}) was added to mineral salts solution before the autoclaving. Crude oil was filter-sterilized (pore size, 0.41 μm) and added to sterile mineral salts solution to give 10 g l^{-1}. Flasks (250 ml) containing 100 ml medium were inocu-

lated with 1 ml of a spore suspension (1×10^6 spores ml^{-1}) and incubated at $28 \pm 2°C$ under static conditions. Spores were harvested in sterile distilled water from PDA plates overlaid with cellophane.

Determination of fungal growth. The mycelial mat was harvested by filtering through a predried and weighed Whatman no. 1 filter paper. The mat was washed three times with chloroform and then with distilled water, and dried at 100°C for 25 h; the dry weight of the mycelium was then determined.

Protein estimation. Total protein was estimated by the method of Lowry *et al.*[11] using bovine serum albumin as standard.

Determination of total sugar and glycogen. Total sugar and glycogen were estimated according to Roe[12] and Morales *et al.*[13], respectively.

Estimation of total lipid. Total lipid was extracted from 10 g fresh weight of mycelium as described by Folch *et al.*[14] and estimated gravimetrically.

Separation and estimation of neutral, glycolipid and phospholipid by column chromatography. The total lipid was fractionated into its constituent lipid classes by silicic acid column chromatography[15]. Neutral lipid was eluted with eight column volumes of chloroform, glycolipid with one column volume of 99% (v/v) acetone and phospholipid with eight column volumes of methanol. Solvents were evaporated and the lipid fractions were estimated gravimetrically. The percentage of lipid fractions in total lipid was then calculated.

Extraction of extracellular lipid. Extracellular lipid was extracted from the culture medium by the method of Siporin and Cooney[16]. The mycelial mat was removed from the medium by filtering the culture through two layers of Whatman no. 1 filter paper. In cultures grown on crude oil a thin layer of residual oil was present on the surface of the medium. This layer was removed by shaking 100 ml of medium with 10 ml petroleum ether and removing the upper solvent phase. The aqueous phase was adjusted to pH 2 with HCl and extracted three times with two volumes of chloroform : methanol (2 : 1 v/v).

Extraction of total extracellular lipid by thin layer chromatography (tlc). The total extracellular lipid was separated into free fatty acid and phospholipid fractions by tlc using chloroform : acetone : methanol : acetic acid : water (8 : 1 : 5 : 25 : 0.25 v/v) as solvent[16]. Lipids were visualized by iodine vapour. Spots corresponding to standard free fatty acid and phospholipid were eluted with hexane, dried in nitrogen atmosphere and stored at –4°C. The amount of total free fatty acid[17] and phospholipid[18] was estimated spectrophotometrically.

Fatty acid analysis. Fatty acids were converted to their methyl esters using borontrifluoride : methanol[19] and separated by gas chromatography using a polar column (10% w/w, diethylene glycol succinate on chromosorb/Aw) operated at 190°C. The relative proportions of the different fatty acids were calculated by manual measurement.

Measurement of enzyme activity. Activities of the NAD^+-alcohol dehydrogenase, $NADP^+$-alcohol dehydrogenase and $NAD(P)^+$-aldehyde dehydrogenase were measured spectrophotometrically at 340 nm following the procedures of Bonnichsen[20], Demoss[21] and Seegmiller[22], respectively. Succinate dehydrogenase was assayed[23] at 428 nm. Isocitrate lyase was measured[24] at 324 nm. Catalase was measured as described by Beers and Sizer[25].

Statistical analysis. All experiments were carried out in duplicate and each experiment repeated three times, except the cellular and extracellular fatty acid experiments, which were repeated twice with no replicates. Data obtained were analysed statistically[26]. Student's *t* test was used to calculate the degree of significance between glucose and crude oil.

Results

Growth and composition (cellular) of A. flavipes. When grown in static culture on glucose the stationary phase was reached after 6 days, but on crude oil the growth was not complete until the twelfth day. To determine the cellular composition, the fungus was harvested at four sample times during its growth period (Figure 1).

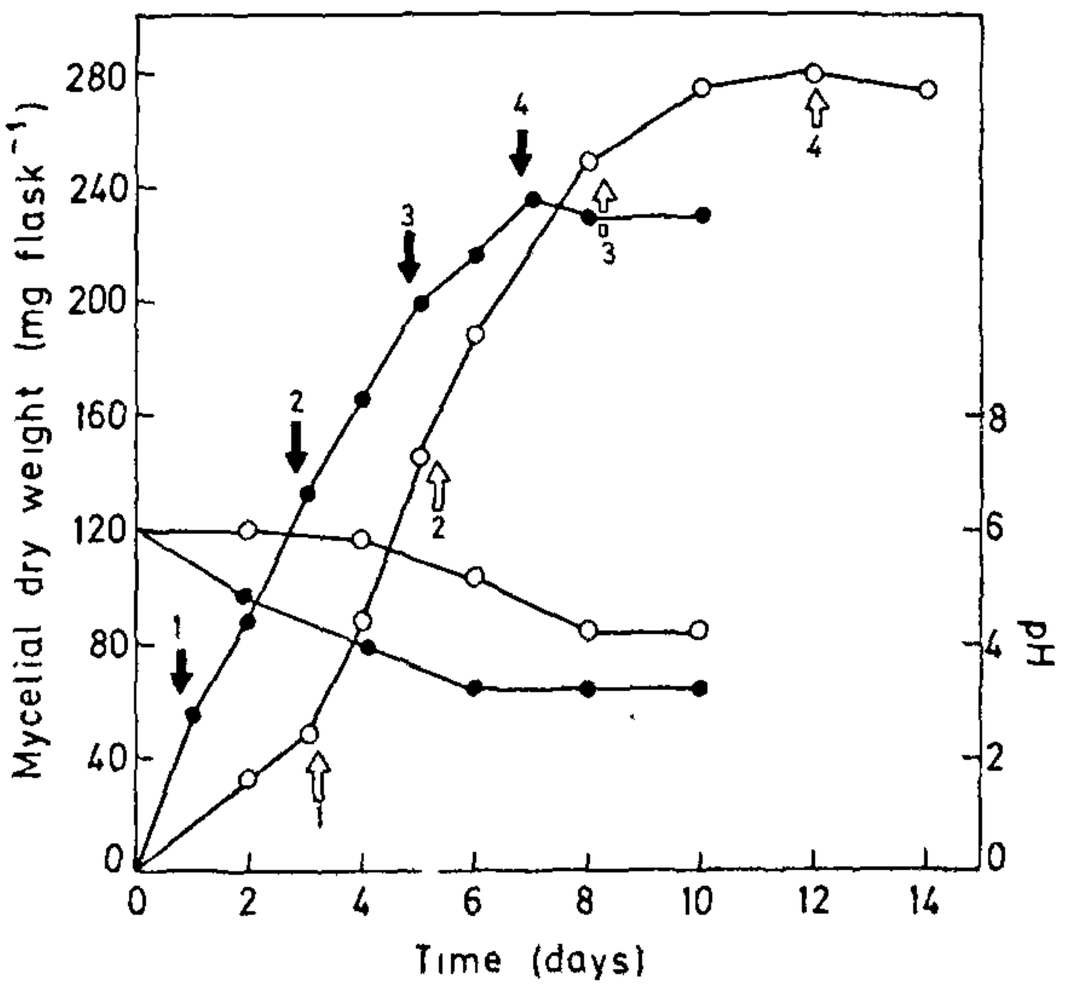

Figure 1. Growth of *A. flavipes* grown on medium with glucose and crude petroleum oil as the sole source of carbon ● glucose; O crude oil. Arrows indicate sample times The experiment was carried out in triplicate.

Variations in cellular composition of *A. flavipes* were observed after growth on crude oil or glucose media. Total carbohydrate and glycogen contents were approximately twofold greater in mycelia grown on glucose although protein content was not significantly different between the two media (Table 1). Considerable increases in total lipid and sterol contents were observed for the crude-oil-grown cultures (Table 1). In cultures grown on crude oil, neutral lipid was the major fraction followed by glycolipid, whereas in glucose-grown cultures glycolipid was the major fraction, followed by neutral lipid. However, on both media phospholipid was the minor fraction (Table 2). The lipid of crude-oil-grown cultures contained a variety of fatty acids compared to that of glucose-grown cultures. Also, the crude-oil-grown cultures showed an increase in the percentage of total unsaturated fatty acid with time (Table 3).

Extracellular lipid. In the presence of crude oil

Table 1. Cellular composition of *A. flavipes* grown on medium with glucose and crude oil as the sole source of carbon

Sample time	Total protein[a]		Total carbohydrate[a]		Total glycogen[a]		Total lipid[a]		Total sterol[a]	
	Glucose	Crude oil	Glucose	Crude oil	Glucose	Crude oil	Glucose	Crude oil	Glucose	Crude oil
1	180 ± 5	190 ± 9	191 ± 6***	76 ± 5	76 ± 3	32 ± 2	26 ± 2	76 ± 5***	14 ± 1	52 ± 3***
2	100 ± 6	106 ± 6	285 ± 15***	105 ± 9	163 ± 8	67 ± 5	41 ± 3	264 ± 10***	24 ± 2	104 ± 5***
3	86 ± 5	91 ± 4	309 ± 15***	143 ± 11	242 ± 18	90 ± 5	37 ± 3	219 ± 6***	28 ± 1	114 ± 6***
4	143 ± 7	147 ± 12	278 ± 13***	152 ± 9	233 ± 15	99 ± 4	22 ± 2	142 ± 2***	18 ± 1	92 ± 5***

Each value represents the mean ± SEM of six estimations.
[a]Values expressed as µg/mg dry weight.
***$P < 0.001$.

Table 2. Composition of total cellular lipids of *A. flavipes* grown on medium with glucose and crude oil as the sole source of carbon

Sample time	Total lipid					
	Neutral lipid[†]		Glycolipid[†]		Phospholipid[†]	
	Glucose	Crude oil	Glucose	Crude oil	Glucose	Crude oil
1	28 ± 3	77 ± 5**	60 ± 2**	18 ± 0.9	12 ± 0.8**	3 ± 0.01
2	30 ± 1	83 ± 3**	67 ± 0.8**	13 ± 0.4	4 ± 0.1	2 ± 0.1
3	45 ± 3	75 ± 1**	52 ± 3**	22 ± 1	5 ± 0 08	4 ± 0 1
4	41 ± 2	72 ± 2**	52 ± 3**	25 ± 0.2	2 ± 0 2	4 ± 0.2

Each value represents the mean ± SEM of six estimations.
[†]Percentage of total lipid.
**$P < 0.01$.

Table 3. Fatty acid composition of total cellular lipid of *A. flavipes* grown on medium with glucose and crude oil as the sole source of carbon

Carbon source	Sample time	Fatty acid*											
		Saturated									Unsaturated		
		10 0	12 0	13 0	14.0	15.0	16:0	17:0	18 0	Total[†]	16:1	18.1	Total[‡]
Glucose	1	0	0	0	tr	0	37.5	0	0	37.5	0	62 4	62 4
	2	0	0	0	tr	0	33 9	0	0	33 9	0	66 0	66 0
	3	0	tr	0	tr	0	0	0	45.5	45.4	0	54.5	54 5
	4	0	tr	0	tr	0	0	0	33.3	33.3	0	66.6	66.6
Crude oil	1	1 6	2.3	9 6	8 4	7.5	3.4	tr	23.9	56.8	26.6	16.5	43.1
	2	1.6	9.1	0	6.7	12.7	6.1	tr	6.9	43 3	18 0	38.6	56 6
	3	0	0	0	0	5 1	4 6	tr	4.6	14.9	21.5	63.8	85.4
	4	0	0	0	0	1.4	3.3	tr	4 3	9.1	12.2	78.5	90 8

*Recorded as percentage of total fatty acid: tr – trace.
[†]Total saturated fatty acid; [‡]Total unsaturated fatty acid.
10 0, Decanoic acid; 12.0, dodecanoic acid; 13 0, tridecanoic acid; 14.0, tetradecanoic acid; 15.0, pentadecanoic acid; 16 0, hexadecanoic acid, 17:0, heptadecanoic acid; 16 1, *cis*-Δ9-hexadecanoic acid; 18.1 *cis*-9-octadecanoic acid.

Table 4.

a, Total extracellular lipid† in medium with glucose and crude oil as the sole source of carbon following growth of *A. flavipes*

Sample time	Glucose	Crude oil
1	0	24 ± 0 9***
2	25 ± 0.4	71 ± 2 3***
3	28 ± 0 9	86 ± 4.5***
4	27 ± 1.1	104 ± 6 9***

†Values expressed as mg/l of medium.
***$P < 0\,001$.

b, Composition of extracellular lipid† in medium with glucose and crude oil as the sole source of carbon following growth of *A. flavipes*

	Free fatty acid		Phospholipid	
Sample time	Glucose	Crude oil	Glucose	Crude oil
1	0	66 ± 2***	0	14 ± 0.2
2	40 ± 2	77 ± 4**	19 ± 0.7	12 ± 0.1
3	57 ± 1	79 ± 4**	17 ± 0.3	11 ± 0 3
4	56 ± 2	79 ± 4**	17 ± 0 4	9 ± 0.2

Each value represents the mean ± SEM of six estimations.
†Recorded as percentage of total lipid.
***$P < 0.001$,
**$P < 0.01$.

A. flavipes excreted three to four times more lipid into the growth medium than when grown on glucose (Table 4 *a*). When the total lipid was fractionated by column chromatography, it failed to separate into definite fractions such as neutral lipid or glycolipid. However, after tlc free fatty acids were found to be the major lipid fraction. The free fatty acid content was nearly double in the medium with crude oil than in the medium with glucose (Table 4 *b*). The level of phospholipid was significantly higher in glucose medium than in crude oil medium. Methyl esters prepared from free fatty acid fractions of lipid extracted from medium with glucose or crude oil gave both saturated and unsaturated even-chain fatty acids; no odd-chain fatty acids were detected (Table 5).

Enzymology. In cultures grown on crude oil, the activities of NAD^+-alcohol dehydrogenase and $NADP^+$-aldehyde dehydrogenase were nearly three and two times more (respectively) than that of glucose-grown cultures, whereas $NADP^+$-alcohol dehydrogenase and NAD^+-aldehyde dehydrogenase did not show any significant difference in their activity on the two media (Table 6). Succinate dehydrogenase activity was considerably lower in the crude-oil-grown cultures, whereas isocitrate lyase and catalase activities were considerably higher in the crude-oil-grown cultures (Table 6).

Table 5. Fatty acid composition† of extracellular lipid excreted by *A. flavipes* in medium with glucose and crude oil as the sole source of carbon

Carbon source	Sample time	Saturated fatty acid 12.0	Saturated fatty acid 16.0	Saturated fatty acid 18.0	Total saturated fatty acid	Unsaturated fatty acid 18 1
Glucose	1	tr	tr	tr	tr	tr
	2	tr	24.1	35.8	60.0	40.0
	3	tr	26.2	39.3	66.1	33 8
	4	tr	32.0	28.1	60.1	25.0
Crude oil	1	19 0	33.2	tr	52.2	47.7
	2	31 4	33.4	tr	64 8	35.1
	3	9 1	27.2	30.0	66.3	33 6
	4	25.1	14 8	8.6	48 6	51.3

tr – trace.
†Each value recorded as percentage of total lipid.
12 0, Dodecanoic acid, 16.0, hexadecanoic acid; 18 0, octadecanoic acid, 18 1, *cis*-Δ9-octadecanoic acid.

Table 6 Activity† of some enzymes of *A. flavipes* grown on medium with glucose and crude oil as the sole source of carbon

Sample time	NAD^+-alcohol dehydrogenase: Glucose	NAD^+-alcohol dehydrogenase: Crude oil	$NADP^+$-alcohol dehydrogenase: Glucose	$NADP^+$-alcohol dehydrogenase: Crude oil	NAD^+-aldehyde dehydrogenase: Glucose	NAD^+-aldehyde dehydrogenase: Crude oil	$NADP^+$-aldehyde dehydrogenase: Glucose	$NADP^+$-aldehyde dehydrogenase: Crude oil	Succinic dehydrogenase: Glucose	Succinic dehydrogenase: Crude oil	*Iso*-Citrate lyase: Glucose	*Iso*-Citrate lyase: Crude oil	Catalase: Glucose	Catalase: Crude oil
1	21 6±1 4	32 5±2 9*	15 3±0 6	18 1±0 4	22 1±0 2	20 2±0 1	40 2±1.1	41 7±2 1**	22 0±1 2	14 0±1 4	8 1±0 7	23 7±1 4***	22 1±2 2	140 4±6 7***
2	19 3±0 6	51 3±1 8***	17 2±0 7	17 2±0 2	25 5±0 1	21 7±0 5	35 8±3 2	61 2±4 3**	41 4±3 2	21 0±1 4	17 4±1 3	48 6±2 8***	70 0±2 6	293 6±4 3***
3	20 0±0 9	61 0±1 9***	16 2±0 5	18 8±0 8	27 2±0 5	29 5±0 4	30 8±1 5	62 5±5 8**	54 8±4 3	13 8±0 9	12 2±1 7	56 5±4 0***	53 2±1 8	244 7±10 0***
4	16 4±1 5	52 2±3 9***	19 7±0 2	16 1±0 2	30 2±0 8	27 2±0 8	23 8±1 7	53 7±3.0**	40 2±2 3	10 2±0 6	18 4±0 8	40 6±2 0***	21 2±0 5	120 4±6 2***

†Activity expressed as nmol/min/mg protein.
***$P < 0\,001$.
**$P < 0\,01$.
*$P < 0\,05$
Each value represents the mean ± SEM of six estimations

Discussion

It is interesting to note that the lipid content of *A. flavipes* was considerably greater (sixfold) when grown on crude oil as the sole carbon source compared to glucose. *A. flavipes* accumulates lipid more efficiently than either *Candida lipolytica*[29] or *Cladosporium resinae*[30] where two to threefold increases in lipid content of cultures grown on *n*-hexadecane have been reported. Growth of *A. flavipes* on crude oil resulted in the occurrence of a greater number of fatty acids, particularly saturated fatty acids (Table 3). The fatty acids are oxidation products of *n*-alkanes in the crude oil which are then incorporated into the cellular lipids. The content of saturated fatty acids decreases progressively in cultures grown on crude oil. This may be due to some of the short-chain saturated fatty acids being converted into long-chain fatty acids, especially unsaturated fatty acids, which can be used to produce storage lipids[4].

The function of extracellular free fatty acids, which increase during growth on crude oil (Table 4 *b*), is possibly to emulsify the hydrocarbons to facilitate their uptake by the fungus[26].

Induction of NAD^+-alcohol dehydrogenase and $NADP^+$-aldehyde dehydrogenase in crude-oil-grown *A. flavipes* suggests their involvement in the oxidation of alkanes in crude oil. Iso-citrate lyase is the key enzyme of the glyoxylate cycle which plays an important role in the biosynthesis of cellular components such as malate or succinate. Activity of isocitrate lyase was significantly greater when *A. flavipes* was grown on crude oil. Conversely, the activity of succinate dehydrogenase was greater in glucose medium (Table 6). From these results, it is possible to infer that the intermediates of tricarboxylic acid cycle are synthesized from acetyl COA derived from the degradation of fatty acids via glyoxylate cycle[29]. Peroxisomes are thought to contain several enzymes involved in fatty acid metabolism such as catalase, iso-citrate lyase, etc. The increase in these enzymes observed in crude-oil-grown *A. flavipes* suggests an increase in the number of peroxisomes although we have no data to support this supposition. However, this finding is in agreement with those of other workers, who report a similar increase in catalase and iso-citrate lyase activities and increase in the number of peroxisome for *C. resinae*[4] and *Candida tropicalis*[30].

The work described here is the first report on the composition and enzyme activities in a fungus grown on crude petroleum oil and glucose. The most significant aspect of the study lies in the fact that an attempt has been made here to elucidate the metabolism and enzymology of *A. flavipes* when grown on crude petroleum oil, which is a natural component of industrial wastes and effluents.

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