PRODUCTION OF LIPIDS BY FERMENTATION PRELIMINARY REPORT

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SUMMARY: Forty seven species of soil fungi were obtained from Fermentation Laboratory, Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi.

The presence of lipids/fats in these fungi was investigated. The fungi were cultured by surface culture method using two different media, one reported by Preuss (1984), the other medium was modified during the present study in which sodium nitrate was used instead of ammonium nitrate and the amount of glucose was also doubled.

The presence of lipids/fats was confirmed in twelve species. The amount of lipid produced was also calculated. The capability of fungal species to produce lipids was : Aspergillus oxysporum 76.60%, A. solani 45.80%, A. ustus 38.00% and A. semitectum 37.80%.

From the results it is concluded that the local species of fungi has the potential to produce lipids in high quantity.

It is concluded further that a medium containing ammonium nitrate as nitrogen source and glucose as a carbon is the best for lipid synthesis.

Key Words: Aspergillus oxysporum, A. solani, A. ustus and A. semitectum.

INTRODUCTION

Over the past two decades, research on biosynthesis of lipids has, after a sluggish start, proceeded at an astonishing pace with the result that pathways leading to synthesis of the commoner lipids are now well charted.

The term "LIPID" was traditionally used to describe a wide variety of natural products including fatty acids and their derivatives; steroids, terpenes, carotenoids and bile acids which are readily soluble in organic solvents such as diethyl ether, benzene, hexane, chloroform or methanol. However, now-a-days the term is restricted to fatty acids and their naturally occurring derivatives such as esters or amides and to compounds closely related biosynthetically to fatty acids.

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Lipids may be subdivided into two broad classes 'simple', which can be hydrolysed to give one or two different types of products per molecule and 'complex' which contain three or more hydrolysis products per molecule. The terms 'natural' and 'polar' respectively are used more frequently to define these classes, but are less precise and may occasionally be ambiguous.

The common fatty acids of plant and animal origin contain even number of carbon atoms (4-24) in straight chains with terminal carboxyl groups and may be fully saturated or contain one, two or more (upto six) double bonds. Fatty acids of animal origin are comparatively simple structures, whereas plant fatty acids may be more complex and may contain a variety of functional groups. Bacterial fatty acids usually consist of simpler saturated and monoenoic components. However, very complex high molecular weight acids, the mycolic acids have also been reported in certain bacterial species. Although numerous analyses have been made of the overall lipid composition of fungi, only one organism, namely Saccharomyces cerevisiae has been analysed for lipid composition of it is plasma membrane. The plasma membrane in this yeast contains two main classes of lipids: glycerophospholipids and sterols (14).

During the past hundred years extensive studies have been carried out to determine the potential application of microorganisms for the production of fat. The expansion of world population and it is an important nutritional problem which has been the subject of several assessments. The food and agricultural organization has emphasized that the rate of increase in world population remains at about 1.5 percent per annum and some two-thirds of the present population are now underfed. The very high rate of population growth in the Far East and Latin America, especially, set food producers a particularly difficult task. Increasing industrialization, as part of a general social advance triggered increased efficiency of agriculture and so to a greater food supply. It is just in this respect that a microbial contribution might be most valuable. Abundant literature has been accumulated on fat synthesis by fungi (1-16). But no work has been done in this part of the world.

Table 1: Results of lipid screening of fungal species.

S. No	Name of organisms	Results
1	*Arachria terrutric*	-
2	*Aspergillus carbonarius*	-
3	*A. Clavatus*	-
4	*A. candidus*	+
5	*A.flavus*	-
6	*A. fumigatus*	-
7	*A. giganteus*	+
8	*A. nidulans*	+
9	*A. niger*	+
10	*A. ochraceus*	-
11	*A oryzae*	-
12	*A.sydowi*	+
13	*A. terreus*	-
14	*A. ustus*	+
15	*A. versicolar*	-
16	*Blastoeladiella emersonii*	-
17	*Candida albicans*	-
18	*Cladosporium clamporilae*	-
19	*Circinella museae*	-
20	*Curvularia clavata*	-
21	*C. lunata*	-
22	*C. ovoidea*	-
23	*C. tuberculata*	-
24	*C. versiculosa*	-
25	*Drechslera hawaiiensis*	-
26	*D. papendorfii*	-
27	*D. rostrata*	-
28	*Epicoculum nigricans*	-
29	*Fusarium acuminatum*	+
30	*F. moniliforme*	-
31	*F. oxysporum*	+
32	*F. semitectum*	+
33	*F. solani*	+
34	*Helminthosporium nodulosum*	-
35	*H. tetramea*	-
36	*Monilia stophila*	-
37	*Penicillium aurantio-brunneum*	-
38	*P. chrysogneum*	-
39	*P. crustorum*	-
40	*P. cyaneum*	-
41	*P. expansum*	-
42	*P. funiculosum*	+
43	*P. javanicum*	-
44	*P. oxalicum*	-
45	*P. oxysporum*	+
46	*P. vinaceum*	-
47	*Rhodotorula glutinis*	-

+ Positive result

- Negative result

EXPERIMENTAL

ORGANISMS USED:

Forty seven different fungal species examined for the presence of lipid in the present study. The fungal species are listed in Table 1. The fungal species used in the study were obtained from the Fermentation Research Laboratories, Department of Pharmaceutics, Faculty of Pharmacy, University of Karachi. The pure and identified organisms were maintained on PDA agar slants.

MAINTENANCE OF STOCK CULTURE:

The stock culture of the fungal species were maintained on Potato Dextrose Agar (PDA) slants and were stored in refrigerator till further use. Potato Dextrose Agar slants contained the following:

Potato	200 g
Dextrose	200 g
Agar	200 g
Distilled water	1000 ml

PREPARATION OF MEDIA:

Potatoes were washed rapidly in running tap water, cut in sliced form and then cooked in 500 ml boiling water for 30 minutes. At the same time agar was dissolved by heating in the remaining 500 ml water. The potatoes juice was strained and poured into agar solution and to this dextrose was added and stirred till dissolved completely. The volume of the medium was made up to one litre. The pH of the medium was adjusted to 6.0 with the help of diluted HCI. The medium was properly agitated while tubing to ensure that each tube had an equal proportion of solid matter. The test tubes containing medium were sterilized at 1 p.s.i. at 121°C for 30 minutes.

CULTIVATION OF FUNGI:

The fungal species were grown in two different media.

(a) Medium 1 (17) had the following composition:

Commercial glucose	20 g/litre
Ammonium nitrate	1.0 g/litre
Ferric chloride-6H_2O_2	0.016 g/litre
Magnesium sulphate-7H_2O	0.50 g/litre
Potassium dihydrogen phosphate	0.68 g/litre
Zinc sulphate-7-H_2O	0.005 g/litre

(b) Medium II (modified medium of Pruess *et al.* (17)) had the following composition:

Commercial glucose	40 g/litre
Sodium nitrate	1.0 g/litre
Ferric chloride-6-H_2O	0.016 g/litre
Magnesium sulphate-7-H_2O	0.50 g/litre
Potassium dihydrogen phosphate	0.68 g/litre
Zinc sulphate-7-H_2O	0.005 g/litre

The ingredients were dissolved in 1000 ml tap water. From this solution, aliquots of 100 ml were transferred into each Roux bottle and sterilized at 15 p.s.i. (121°C) for 15 minutes. When cooled, each bottle was inoculated with one ml spore suspension. The spore suspension was prepared from the stock culture of organisms maintained in PDA, by scrapping the spore of each tube with 5 ml of sterile water. The bottles were incubated at 28°C ± 2°C for 12 days by surface culture technique. At the end of incubation period, mycelium of each fungi was separated, washed and freeze-dried.

Table 2 : The R*f* values of lipids fraction produced by fungal species.

S. No.	Name of organisms	Spots	R*f* value
1	*Aspergillus candidus*	Black	0.58
2	*A. giganteus*	Black	0.28
3	*A. niger*	Black	0.43
4	*A. nidulans*	Black	0.37
5	*A. sydowi*	Black	0.61
6	*A. ustus*	Black	0.34
7	*Fusarium acuminatum*	Black	0.78
8	*F. oxysporum*	Black	0.12
9	*F. semitectum*	Black	0.43
10	*F. solani*	Black	0.66
11	*Penicillium funiculosum*	Black	0.23
12	*P. oxysporum*	Black	0.36

Table 3: Total Lipids Produced By Dry Mycelium Of The Fungi On Preuss Medium Containing Ammonium Nitrate As Nitrogen Source.

Fungal species	Dried mycelial weight (mg)	Total lipids (mg)	% of total lipids
Aspergillus candidus	770	26.42	3.43
A. giganteus	506	136.25	26.93
A. niger	605	148.30	24.51
A. nidulans	888	180.80	20.36
A. sydowi	1188	388.80	32.73
A. ustus	856	318.00	37.15
Fusarium acuminatum	668	222.50	33.30
F. oxysporum	568	278.00	48.94
F. semitectum	815	305.20	37.45
F. solani	128	62.00	48.43
Penicillium funiculosum	799	230.40	28.84
P. oxysporum	802	210.30	26.22

EXTRACTION OF FUNGAL LIPIDS:

Preliminary extraction of fungal lipid was carried out by the method reported by Pederson (1962) and purified according to the method of Folch *et al.* (11).

The freeze-dried mycelia were homogenized and refluxed for about three hours with a sufficient amount of chloroform: methanol mixtures (2:1, v/v) in an atmosphere of carbon dioxide gas. This extract was separated by centrifugation and the residue refluxed again for another one hour with sufficient fresh mixture of solvents. These extracts were collected and then evaporated under reduced pressure in carbon dioxide atmosphere at a temperature not exceeding 30°C.

IDENTIFICATION OF THE LIPID:

The process of identification was carried out on TLC plates. For this purpose TLC glass plates 20x20 cm coated with 0.25 mm gel G (E. Merck) were used. The lipid samples dissolved in chloroform:methanol:ether mixture (1:1:1, v/v), were spotted at the bottom of the plate. The control samples corresponding to different lipid classes were also spotted on the same plate. Chloroform:methanol:acetic acid: water (70:20:2:2, v/v) was used as solvent system. The plates were removed after the solvent front reached 10 cm length and immediately placed, without drying in the second solvent n-hexane:diethyl ether:acetic acid (70:30:1, v/v) and allowed the solvent to reach a front of 20 cm.

After the development, the plates were left for few minutes in a tank of iodine vapour and then the plates were sprayed with a solution of 50% sulphuric acid and the lipids made visible as a black deposit of carbon by heating the plates at 180°C for an hour.

TOTAL LIPID CONTENT:

The crude lipids were dissolved in a mixture of chloroform:methanol (2:1, v/v). They were mixed throughly with one-fifth of their volume of 0.003N magnesium chloride solution and separated by centrifugation. The walls of the centrifuge tubes and the remaining upper phase were washed twice with 1:5 portions of the solvent mixture. The lipid samples and washings were collected and dried under reduced pressure to obtain total lipids.

RESULTS AND DISCUSSION

Forty seven fungal species belonging to fourteen different genera were evaluated for identification and quantification of lipids.

Fungal species used in the study are listed in Table

Table 4: Total Lipids Produced By Dry Mycelium Of The Fungi On Preuss Medium Containing Ammonium Nitrate As Nitrogen Source.

Fungal species	Dried mycelial weight (mg)	Total lipids (mg)	% of total lipids
Aspergillus candidus	768.4	120.4	15.67
A. giganteus	561.8	237.4	42.26
A. niger	596.3	215.1	36.07
A. nidulans	698.1	63.2	9.05
A. sydowi	805.0	202.3	25.13
A. ustus	599.4	37.4	6.24
Fusarium acuminatum	526.8	23.0	4.36
F. oxysporum	738.4	332.0	44.96
F. semitectum	691.6	9.0	1.30
F. solani	755.0	27.0	3.58
Penicillium funiculosum	696.8	19.0	2.72
P. oxysporum	755.0	27.0	3.58

1. These include *Arachria, Aspergillus* (14 species), *Blastoeladiella, Candida, Circinella, Cladosporium, Curvularia* (5 species), *Drechslera* (3 species), *Epicoclum, Fusarium* (5 species), *Helminthosporium* (2 species), *Monilia, Penicillium* (10 species) and *Rhodotorula.*

The stock cultures were maintained on PDA slants and stored in refrigerator. Cultivation of fungal species, surface culture technique was used, utilizing Roux bottle 1L capacity. Organisms were grown in two different liquid media marked I and II for 12 days at 28°C ± 2°C.

Medium I represents the original medium of Pruess *et al.* (17), while medium II was the modified from of Number I. Medium II was designed by replacing ammonium nitrate by sodium nitrate from medium I and increasing glucose concentration from 20 g/L to 40 g/L.

Results of the lipid screening of fungal species in medium I and II were found similar and are presented in Table 1. The R*f* values of lipid spots are given in Table 2. Out of 47 fungal species tested, twelve species, *Aspergillus candidus, A. giganteus, A. nidulans, A. niger, A. sydowi, A. ustus, Fusarium acuminatum, F. oxysporum, F. semitectum, F. solani, Penicillium funiculosum* and *P. oxysporum* indicated positive results.

The total lipid content of twelve (12) fungal species was determined in both media to observe the difference of nitrogen source and sugar concentration.

Tables 3 and 4 indicate the overall results of dry mycelial weight, total lipids and % of total lipids obtained from medium I and II respectively.

Maximum lipid content was noted in *Fusarium oxysporum* i.e. 48.94% and 44.96% when grown in medium I and II respectively. Overall lipid content of fungal species was recorded more comparative in medium I and medium II. Only *Aspergillus candidus* showed a poor response in medium.

Preuss *et al.* (17) cultivated 24 molds on two different types of media: (1) a glucose-inorganic salts medium containing calcium carbonate in excess and (2) a glucose-malt-sprouts medium. The lipid content of the mycelia varied from 1.1 to 19.9 per cent, with an average of 6.0 per cent, when the molds were grown on the glucose-inorganic salts (synthetic) medium. The average lipid content of all the moulds was over 46 per cent greater when grown on the inorganic medium than when grown on the synthetic medium. However, some

moulds, for example, A. nidulans and *Paecilomyces variata*, yielded more lipid on the latter medium than on the former medium.

The conditions under which a mold is grown exert an influence on its fat content. The kind of sugar makes a large difference. Glucose is superior to sucrose for the production of fat from *Aspergillus nidulans, Penicillium spinulosum* and *Penicillium javanicum* (12). Xylose is also good carbon source for these molds (13). Maltose, arabinose, galactose, lactose, and starch are inferior source of carbonhydrates.

Nitrogen likewise exerts a strong influence on the amount of fat formed. Garrido and Walker (13) studied ammonium nitrate, sodium nitrate, urea, ammonium sulphate, and ammonium chloride as nitrogen source for the same three molds. Ammonium nitrate was the best source for all three molds, followed by sodium nitrate (for *A. nidulans* only).

In case of medium II, highest lipid production, i.e. 44.96% was also noted in *Fusarium oxysporum* followed by *Aspergillus giganteus* (42.26%), *Aspergillus niger* (36.07%) and *Aspergillus sydowi* (25.13%).

From the results of the present studies, it is concluded that the locally isolated species of fungi have the potential to produce lipids in high quantity, specially *Fusarium oxysporum* and other members of *Fusarium* and also the genera *Aspergillus* are potent lipid producers. In addition it is also concluded that a medium containing ammonium nitrate as nitrogen source and glucose as carbon is the best for lipid synthesis.

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