

Lipid accumulation and membrane fluidity influence mycelial stability and riboflavin production by the riboflavinogenic fungus *Eremothecium ashbyii*

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Citation

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Abstract

The plant pathogenic filamentous hemiascomycete fungus *Eremothecium ashbyii* is a natural overproducer of riboflavin (vitamin B₂). Preceding riboflavin overproduction, microdroplets of lipid were observed in the hyphae of *E.ashbyii* as yellow fluorescent bodies on staining with the lipid specific dye Nile blue. Following this the fungus was grown on different substrates-olive oil, sunflower oil and glucose. Lipid accumulation was followed as a time course by gravimetry. The mycelial lipid was fractionated into triglycerides and phospholipids and relative proportions of constituent fatty acids in them was estimated by GC MS during growth and riboflavin overproduction on each substrate. Changes in mycelial morphology were followed as a time course. In parallel, mycelial growth on each medium was converted to protoplasts whose membrane fluidity was monitored by measuring the fluorescence anisotropy using 1,6 - diphenyl - 1,3,5 - hexatriene (DPH). Lipid accumulation and extracellular lipase activity was maximum at 48 h of growth on all growth substrates. Maximum lipase production coincided with maximum lipid accumulation preceding riboflavin overproduction. A GC-MS analysis of fatty acids during growth (48 h) and riboflavin overproduction (96 h) showed the presence of a large percentage of unsaturated fatty acids in triglycerides and phospholipids. Decrease in Octadecadienoic acid (C 18:2) in the triglycerides during the production phase correlated with riboflavin production while mycelial stability correlated to the Octadecenoic acid (C 18:1) content of the phospholipids. Olive oil-grown mycelia showed maximum lipid accumulation, riboflavin production, lipase activity, membrane fluidity, stability and least morphological changes. Maximum riboflavin was obtained on olive oil medium due to a greater decrease in Octadecadienoic acid content in the triglycerides and the stability of olive oil grown mycelia was attributed to the high content of Octadecenoic acid in its phospholipids.

INTRODUCTION

Riboflavin (vitamin B₂) is the precursor of the coenzymes Flavin mononucleotide (FMN) and Flavin adenine dinucleotide (FAD) which function as electron acceptors in various oxidation-reduction reactions in the cell. Riboflavin forms an important ingredient of animal feed supplements for monogastric animals such as poultry (Coopermann and Lopez, 1991) as well as multivitamin formulations. Though competitive chemical processes are used for the industrial production of riboflavin, much of the riboflavin required for animal feed purposes is derived by the biotechnical route on account of its cost effectiveness (Vandamme, 1992). Among the microorganisms used in the past and present for the commercial production of riboflavin, the hemiascomycete fungi *Eremothecium ashbyii* and *Ashbya gossypii* are important natural overproducers of the vitamin. Commercial

fermentations for riboflavin production using *E. ashbyii* and *A. gossypii* were first established in 1940 and 1946 respectively (Perkins, et al., 1999; Wickerham, et al., 1946). Much of the current knowledge about riboflavin production has been acquired with mutants of *E. ashbyii* and *A. gossypii*.

One previously reported interesting feature of riboflavin over production by *E.ashbyii* during growth on glucose medium is that it is preceded by an accumulation of lipids (Starka, 1957; Pujari and Chandra, 2001). More recently a large number of lipid bodies were reported in an overproducing mutant of *E.ashbyii* UV-18-57 while absence of lipid bodies was reported in a non flavinogenic mutant of *E.ashbyii* UV-85 (Pujari and Chandra, 2001). In the closely related *A. gossypii*, accumulation and degradation of lipid bodies has been shown to accompany riboflavin

overproduction (Stahmann et al., 1994). This points to a probable role for lipids in riboflavin overproduction. Another interesting observation regarding riboflavin overproduction by *E.ashbyii* is that the production and excretion of riboflavin is accompanied by changes in the mycelial morphology. A riboflavin overproducing UV mutant of *E.ashbyii* UV-18-57 showed large hyphae and bulbous mycelia whereas the wild type and a non flavinogenic mutant UV-85 showed thinner mycelia (Pujari and Chandra, 2001). Hence questions of fundamental interest are whether lipid accumulates during flavinogenesis by *E.ashbyii*, can lipid accumulation be correlated to flavinogenesis, how does the growth substrate affect lipid accumulation, mycelial morphology and riboflavin production and whether the bulbous mycelial forms will always be evident or is there some way of modulating it.

The present study was undertaken as a first step to understanding the accumulation of lipids in the hyphae, the influence of growth substrate on composition of stored lipid and correlating the composition of the membrane phospholipids, with the membrane fluidity and changes in mycelial morphology of *E. ashbyii* during exponential growth (48 h) and riboflavin overproduction (96 h). *E.ashbyii* was grown on different growth substrates (glucose, olive oil and sunflower oil) and the intracellular lipids were visualized by staining with Nile Blue. The total mycelial lipid was extracted and estimated gravimetrically in each case. The mycelial lipid was fractionated into different classes (triglycerides, phospholipids and glycolipids) and the fatty acid profile of the the triglyceride and phospholipid fraction during growth (48 h) and riboflavin overproduction (96 h) was analysed by GC-MS. The relative proportions of constituent fatty acids was calculated in each case. The observations were related to the changes in the mycelial morphology and lipase activity of the fungus during the course of riboflavin production. In parallel, membrane fluidity changes during growth and riboflavin overproduction were studied using the fluorescent membrane probe DPH to measure the fluorescence anisotropy of *E. ashbyii* membrane preparations obtained from protoplasts of the mycelia grown on the different substrates. Membrane fluidity and fluorescence anisotropy of the probe are inversely related, more fluid the membrane lesser the anisotropy and vice versa. The probe which was used in the present study, DPH is a trans-polyene (Suwalsky et al., 1990) with a rod like shape which aligns itself with phospholipid acyl chains to become evenly distributed

within the bilayer core (Suwalsky et al., 1990; Sklar et al., 1997) and detects changes that occur in the membrane interior. An attempt was made to understand whether the composition of fatty acids of the membrane phospholipids could be correlated to our observations on the membrane fluidity and mycelial morphology of *E. ashbyii*. Our study is the first of its kind and our results show that lipid accumulation correlates to riboflavin overproduction in *E.ashbyii* and Octadecadienoic acid plays a role in riboflavin production while Octadecenoic acid may play a role in influencing mycelial morphology and stabilizing the mycelia.

MATERIALS AND METHODS

ORGANISM AND CULTURE CONDITIONS

E.ashbyii NRRL 1363, was obtained from NCAUR, Illinois, U. S. A. The culture was maintained on PDA (Potato Dextrose Agar) slants (g/l: mashed Potato extract 200, glucose 20, Agar agar 20) at pH 6.5 and 30°C. Subculturing was done every four weeks onto fresh medium. For preparation of the preinoculum, the mycelial growth on one slant was transferred under aseptic conditions to 250 ml Erlenmeyer flasks containing 50 ml of preinoculum medium (Mitsuda et al., 1978). The inoculated flasks were incubated on a rotary shaker at 30 °C and 120 rpm for 24 h. For studies on lipid accumulation, membrane fluidity, morphological changes and riboflavin production; 1% of a 24 h old pre-inoculum was added to each of 500 ml Erlenmeyer flasks containing 100 ml of production medium (Mitsuda et al., 1978) which contained (in g/l) yeast extract 2, KH₂PO₄ 2, NaCl 1, MgSO₄.7H₂O 0.1 and one of the carbon sources as follows: glucose 30 g/l or 15 g/l of either olive oil or sunflower oil emulsion in 2% PVA. The flasks were incubated on a rotary shaker at 30 °C and 120 rpm. The total intracellular lipid, changes in mycelial morphology, lipase activity and riboflavin production was studied as a time course for five days. Mycelial growth from 6 Erlenmeyer flasks each containing 100 ml of the riboflavin production medium was used for each growth substrate (glucose, olive oil and sunflower oil) for each time point. For membrane fluidity studies a known weight of the mycelial growth obtained on each medium was converted to protoplasts whose membrane fluidity was monitored during the growth (48 h) and riboflavin overproduction (96 h) phases only.

ANALYTICAL

DETERMINATION OF MYCELIAL DRY WEIGHT

AND RESIDUAL SUBSTRATE

For determination of the mycelial dry weight, 100ml of the pooled culture broth was filtered through a preweighed Whatman filter paper No 1. The supernatant was used for the estimation of the residual substrate, lipase activity and extracellular riboflavin. The harvested mycelia were dried overnight on the preweighed Whatman filter paper No 1 at 60 °C till a constant weight was obtained.

Residual glucose in the culture supernatant was determined by the DNS method (Miller, 1959). Residual oil was determined by the gravimetric estimation of lipid extracted from the cell-free culture supernatant using hexane (Plummer, 1978).

RIBOFLAVIN ESTIMATION

Riboflavin was estimated fluorimetrically using the ISI standard procedure (IS 1374, 1979). From the cell free culture supernatant, 10 ml of a suitable dilution was taken in two tubes marked A and B, 1 ml of the riboflavin standard (1 µg/ml) was added to tube A and 1 ml of distilled water was added to tube B. The solutions were then acidified using 1 ml of glacial acetic acid followed by the addition of 0.5 ml of 4 % KMnO_4 to each tube in order to oxidize the impurities. After 2 minutes 0.5 ml of 3 % H_2O_2 was added to both the tubes in order to oxidize the residual KMnO_4 . The fluorescence of the solutions was measured using an ELICO Fluorometer Model CL-53 to give readings A (Standard + Sample) and B (Sample alone). Into tube B, 20 mg of Sodium dithionite was added and the fluorescence measured within 10 seconds (reading C). The riboflavin concentration in the original sample was calculated using the formula :

$$(B-C) \times 1 \times \text{Dilution factor} = \text{Riboflavin conc } (\mu\text{g/ml}).$$

$$(A-B) \times 10$$

Care was taken to ensure that the ratio (B-C) falls between 0.66 and 1.5.

$$(A-B)$$

Microscopic observation of lipid accumulation

The intracellular lipids were observed by fluorescence microscopy using a Leica fluorescence microscope after mixing 1ml of Nile blue solution (1mg/ml in Methanol) with 100 µl of the culture broth. Blue light was used for excitation.

Gravimetric estimation of intracellular lipid

Mycelial lipid was extracted using Hexane-Isopropanol mixture (3:2) (Hara and Radin, 1978). The mycelia were pooled and harvested as described above at intervals of 24, 48, 72, 96 and 120 h. The mycelia were given a quick wash with Hexane-Isopropanol mixture to remove adhering lipid and crushed in a pestle and mortar along with acid washed glass beads to obtain a homogenate. The total mycelial lipid was extracted by adding 10 ml Hexane-Isopropanol thrice to the homogenate. The pooled lipid extracts were filtered through cotton wool into a preweighed round bottom flask and the solvent was rotary evaporated. The residue was weighed to obtain the gravimetric weight of intracellular lipid. Membrane fluidity studies were done for the 48 h and 96 h old mycelia only. For this mycelia were harvested from 8 Erlenmeyer flasks each containing 100 ml of the riboflavin production medium was used for each growth substrate at each time point, a known weight of the harvested mycelium was used for gravimetric estimation of lipid and the remaining was used for membrane fluidity studies. The gravimetric estimation was done in triplicate as a time course for each medium and the standard deviation values were calculated.

FRACTIONATION OF INTRACELLULAR LIPIDS INTO DIFFERENT CLASSES

The fatty acid content of the triglycerides and phospholipids was analysed for glucose, olive oil and sunflower oil grown mycelia at 48 h and 96 h only, since these points denoted growth (maximum lipid accumulation) and riboflavin overproduction respectively. A known weight of lipid obtained on the three different media at 48 h and 96 h of growth were reconstituted in 10 ml. of Methanol-Dichloromethane (1:1) mixture and fractionated into polar lipids (phospholipids), glycolipids and triglycerides (non-polar lipids) using the Solid Phase Extraction (SPE) procedure as follows (Abraham, et al., 1998).

The total lipid extract was dried on a hot plate (45°C) under a gentle stream of nitrogen and re-suspended in 10 ml Dichloromethane (DCM). The sample (5 ml sample volume x 2) was applied to a pre-equilibrated Solid Phase Extraction glass column (SPE column) with a silica based sorbent (Insoluble silica 1g/6ml column reservoir, average pore size = 54 Å; specific surface area 521 m² g⁻¹; surface pH = 7). Samples and solvents were passed through the SPE column by vacuum pressure. Non-polar lipids (triglycerides) were eluted with 20 ml DCM and the fractions stored in stoppered vials, glycolipid fraction was eluted with 20 ml acetone from

the column and stored. Polar lipids (phospholipids) were eluted with 20 ml methanol and collected in stoppered vials. The solvent in the different lipid fractions was rotary evaporated and weight of each fraction was determined by gravimetry. The triglycerides and phospholipids were reconstituted using 10 ml of Methanol-Dichloromethane (1:1) mixture and subjected to methylation as detailed in the following section.

PREPARATION OF METHYL ESTERS FOR GC-MS ANALYSIS

The extract prepared as above for the triglycerides and phospholipids was dried under a stream of pure N₂ gas on a hot plate at 45 °C and saponified overnight at 4 °C by adding 0.05 ml of dichloromethane (DCM), 2 ml of methanol and 0.5 ml of 1M KOH. Next morning, 1 ml of DCM, 1 ml 0.1M Phosphate buffer (pH 7.0) and 0.18 ml 6N HCl was added to effect phase separation. The lower DCM phase containing the lipids was collected in a 4 ml sample vial. The extraction with DCM was repeated twice with 0.5 ml DCM. The DCM extracts were pooled together and dried under a stream of pure N₂ gas on a hot plate at 45 °C, 1.5 ml of the methylating reagent (Methanol:Dichloromethane:33%HCl::10:1:1) was added to the residue and incubated in a hot air oven at 105 °C for 1.5 h. For phase separation 1 ml of phosphate buffer pH 7.0 and 1 ml of DCM was added and vortexed for 20 seconds. The lower DCM phase was transferred to a clean GC vial with teflon liner. The procedure was repeated and the DCM extract containing the fatty acid methyl esters (FAME) was dried under a stream of pure N₂ gas at 45 °C. The sample of FAME was stored in 0.5 ml of Octane and kept frozen until GC analysis.

GC ANALYSIS OF FATTY ACID METHYL ESTERS

The fatty acid methyl esters were separated and identified by running a GC MS analysis using 6890 N system for GC (Agilent Technologies) and 5973 N MSD fitted with a 30 m x 25 µm BPX5 capillary column. Helium gas at 16 lbs pressure was used as carrier at a flow rate of 1 ml/min. A temperature programme of 80 °C to 280 °C and a split ratio of 1:10 was used. Individual fatty acid methyl esters were identified by comparison with standard FAMEs obtained from Promochem, GmBH and Sigma Chemical Company, U.S.A. The individual fatty acid concentration in each sample was computed by area normalization of peaks using the Chemstation software (Agilent Technologies) according to the formula:

$$\% \text{ free fatty acids} = \text{Peak area of Sample} \div 100$$

Sum of total peak areas

The concentration of each fatty acid so obtained was multiplied by the dilution factor and then divided by the weight of lipid to obtain the relative proportion of each fatty acid in the triglyceride and phospholipids fractions for each growth substrate. Samples were analyzed in duplicates and averages calculated.

SCREENING AND ASSAY FOR LIPASE ACTIVITY

Lipase activity was screened using a plate assay as well as by TLC of the products of Triolein utilization.

For the plate assay CaCl₂-Tween 80 plates were used (Sierra, 1957). The products of Triolein utilisation were visualised by TLC of the culture supernatant using precoated silica gel plates (Merck, Darmstadt, Germany). The solvent system used as the mobile phase consisted of Petroleum ether: Diethyl ether: Glacial acetic acid in the ratio of 16: 3: 1 and the reagent for development of plates consisted of Ceric sulphate / Ceric ammonium sulphate (1g) plus Ammonium molybdate (21g) dissolved in 31 ml concentrated H₂SO₄ in a total volume of 500 ml.

Lipase was assayed colorimetrically (Lakshmi, 2000). The reaction mixture consisting of 500 µl substrate emulsion (1mM p-nitrophenyl stearate in undecane), 500 µl culture supernatant and 500 µl Phosphate buffer (pH 7.2) was incubated at 30 °C in a gyratory shaker at 200 rpm for 20 minutes. From the aqueous layer 500 µl was transferred to 500 µl borate buffer (pH 10.6) and O.D. was measured at 410 nm. A standard graph of p-nitrophenol in the range 10 nmole to 100 nmole in Borate buffer was used.

One unit of Lipase activity is defined as the amount of enzyme necessary to produce 1nmole of p-nitrophenol/min under the assay conditions using p-nitrophenyl stearate as substrate.

PREPARATION OF CELL MEMBRANES FROM PROTOPLASTS OF

The cell wall of *E.ashbyii* is fragile and this facilitates rapid protoplasting upon being treated with lytic enzymes [10]. The membrane fluidity studies were done using protoplasts of *E.ashbyii* mycelia obtained on different media at 48 h and 96 h of growth only since these time points represented the growth and production phases respectively. A known weight

of the mycelia after the gravimetric estimation of mycelial weight at both these time points on each medium was suspended in 5 ml osmotic stabilizer (0.2 M phosphate buffer pH 5.8 containing 0.04 M ammonium sulphate), 1 ml of 2 mg / ml of Novozyme (in 0.2 M phosphate buffer) was added to it and incubated on a rotary shaker at 120 rpm and 30°C. Protoplast formation was observed under a fluorescence microscope at intervals of 30 minutes.

PURIFICATION OF PROTOPLASTS AND MEMBRANE PREPARATION FOR FLUORESCENCE ANISOTROPY

The protoplasts formed were sedimented by centrifugation at 2,500 rpm and 4°C at the end of the incubation period (2 h). The pellet obtained was suspended in 5ml Tris-HCl buffer (15 mM, pH 7.4) and allowed to lyse by incubation for 1 h at 4 °C. The mixture was centrifuged at 15,000 rpm for 30 minutes at 4 °C and the supernatant containing the intracellular organelles as well as solubles was decanted. Sediments of ghosts were re-suspended in buffer of same strength, washed thrice with Tris buffer and the supernatant fluid discarded. The membrane pellet was suspended in 15 ml of 0.1 M Tris-buffer pH 7.4 and used for anisotropy studies after measuring the optical density of the membrane suspensions at 540 nm.

FLUORESCENCE ANISOTROPY STUDIES USING DPH AS MEMBRANE PROBE

The absorption and emission spectra of DPH (10^{-5} M) in THF (Tetrahydrofuran) and emission spectrum of DPH in membrane were recorded. The absorption spectra of DPH revealed the absorption maxima to be 356 nm. Taking this to be the excitation wavelength the emission spectrum was scanned and maximum was found to be 458 nm. Hence anisotropy measurements were carried out at 458 nm. For the probe DPH to go and position itself into the membrane and thereby give reasonable fluorescence intensity in the range 60-100 during the anisotropy experiment, a molar ratio of DPH to lipid of 1:100 was maintained in the present study.

The weight of the membrane preparation was determined as follows: 1ml of the membrane suspension was dried overnight at 60°C in an oven and the total weight of the membrane obtained thereby was calculated. Amount of protein present in that volume of the membrane suspension was determined and hence amount of lipid present was calculated by subtracting the weight of protein from the total weight. From the GC-MS analysis, the fatty acid

components in the phospholipid of membranes obtained in each medium was determined and since the molecular weight of these lipids were known, the approximate molarity of the membrane suspension in terms of the lipids present was determined. A molar ratio of DPH to lipid as 1:100 was maintained accordingly for the three different media. Since the anisotropic measurements were done for membrane preparations from 48 h and 96 h old mycelia obtained on each medium, samples having equal weights of membranes and equal O.D. at 540 nm were taken for the two time points for each medium. The final reaction mixture consisted of 3ml of membrane preparation and 0.3 ml of DPH in THF such that the final concentration of DPH was 10^{-6} M. In order to deproteinise the membranes 0.1mg/ml proteinase (pepsin) was added and incubated for 1 hour. Fluorescence polarization was measured in an F-4500 Hitachi spectrofluorimeter at 356 nm excitation and 458 nm emission (slit width 5/5). Fluorescence anisotropy was measured over the temperature range 15 °C - 60 °C. All the experiments were done in triplicates using membrane preparations from mycelia grown on glucose, olive oil and sunflower oil media at both time points (48 h and 96 h) and sd values calculated. For calculating the anisotropy, both the horizontal and vertical polarizations were measured to obtain the correction factor G and the I_{\parallel} and I_{\perp} respectively. Fluorescence intensities were measured for the following positions of the polarizer (90, 90), (90, 0), (0, 0) and (0, 90). The first two values are for the horizontal polarization and the latter two are for the vertical polarization. Extent of fluorophore rotation can be quantified by anisotropy (r)

Figure 1

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\perp} + 2GI_{\perp}}$$

G is the correction factor for transmission efficiency.

Relationship between polarization (P) and anisotropy (r) can be given by the following expression (Lakowicz, 1983).

Figure 2

$$r = \frac{2P}{3-P} \quad \text{and} \quad P = \frac{3r}{2+r}$$

Protein estimation

Protein was estimated by the method of Lowry et al. (Lowry et al., 1951) using the Phenol reagent.

Chemicals

1,6 - Diphenyl - 1,3,5 - hexatriene (DPH) was obtained from Sigma Chemical Company, St.Louis, USA. All other chemicals used were of analytical grade from local sources.

RESULTS

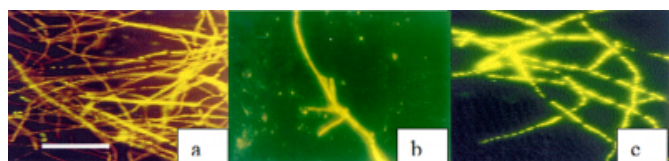
VISUALISATION OF INTRACELLULAR LIPIDS AND MYCELIAL STABILITY

E. ashbyii, formed septate hyphae with microdroplets on all three substrates. Staining with Nile blue, a hydrophobic lipid specific dye (C.I. 51180), resulted in an intense yellow fluorescence of the droplets when illuminated with ultraviolet light indicating intracellular lipid accumulation (Fig 1 a-i).

The stability of the hyphae, in terms of non-production of bulbous forms and resistance to lysis was greater on olive oil than on glucose and sunflower oil. No bulbous forms were observed even on the 5th day (Fig 1 a-c).

Figure 3

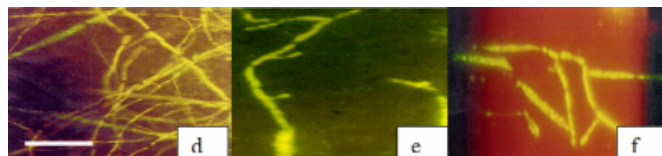
Figure 1 a-c : Olive oil-grown mycelia showing intense lipid accumulation and no bulbous forms stained with Nile blue (Bar, 100 μm).



Sporulation was not evident even on the 6th day. In the case of mycelia grown on sunflower oil medium, filamentous nature was observed up to the 4th day, sporulation and bulbous mycelial forms were seen on the 4th day with onset of lysis by the 5th day (Fig 1 d-f).

Figure 4

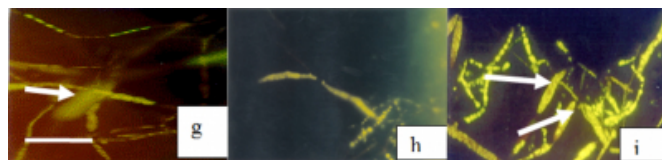
Figure 1 d-f: Sunflower oil-grown mycelia with less lipid accumulation and bulbous forms at 96 h stained with Nile blue (Bar, 100 μm).



The growth and maturation of mycelia was faster on glucose. Sporulation was observed by the 2nd day, formation of bulbous forms of mycelia with intracellular riboflavin accumulation was seen on the 3rd day, onset of lysis was observed on the 4th day and complete lysis was evident by the 5th day (Fig 1 g-i).

Figure 5

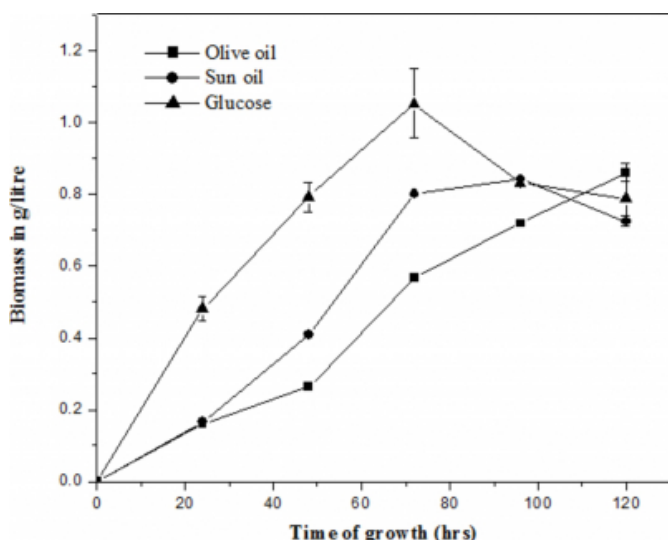
Figure 1 g-i: Glucose-grown mycelia with lowest lipid accumulation and bulbous forms at 48 h (Bar, 100 μm).



The microscopic observations corresponded well with the pattern of biomass changes. The biomass on glucose medium declined after 72 h of growth, on sunflower oil the decline in biomass started after 96 h of growth while the mycelial growth on olive oil was most stable and did not show any decline even after 96 h of growth (Fig 2).

Figure 6

Fig 2: Biomass production by during growth on glucose, sunflower oil and olive oil containing media.



LIPID ACCUMULATION AND RIBOFLAVIN PRODUCTION

On all three media the maximum lipid accumulation was observed at 48 h of growth (Table 1) and the riboflavin production started increasing after 48 h of growth (Figs 3-5). Lipids constituted 46.1 % of the weight of olive oil-grown mycelia, 15.3 % of the weight of sunflower oil-grown mycelia and 10.2 % of that of glucose-grown mycelia at the end of 48 h (Table 1) (Figs3-5). Maximum riboflavin was obtained on olive oil medium (282.03 µg/ml) followed by glucose (216.96 µg/ml) and sunflower oil (108.64 µg/ml) (Figs3-5).

Figure 7

Table 1: Ratio of different classes of lipids fractionated by SPE out of the total lipid extracted from mycelia during growth (48 h) and riboflavin production 96 h) on different growth substrates.

Medium	Total lipid content (%) of biomass (g)		Triglyceride (%) of total lipid		Phospholipid (%) of total lipid		Glycolipid (%) of total lipid	
	48 h	96 h	48 h	96 h	48 h	96 h	48 h	96 h
Glucose	10.2 ± 0.2	4.9 ± 0.1	19 ± 1	10 ± 0.7	64 ± 5	20 ± 3	17 ± 1	6 ± 0.5
Olive oil	46.1 ± 0.7	4.4 ± 0	68 ± 2	40 ± 3	10 ± 0	10 ± 0.2	5 ± 0.6	5 ± 0.2
Sunflower oil	15.3 ± 0.6	3.5 ± 0.1	50 ± 4	44 ± 3	15 ± 1	4 ± 0.1	18 ± 1	12 ± 1

Figure 8

Fig 3: Lipid accumulation, lipase activity, residual substrate and riboflavin production by during growth on olive oil.

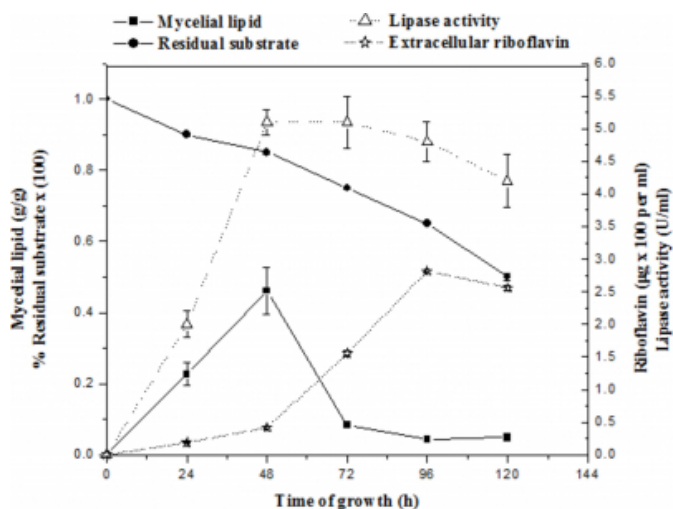


Figure 9

Fig 4: Lipid accumulation lipase activity, residual substrate and riboflavin production by during growth on sunflower oil.

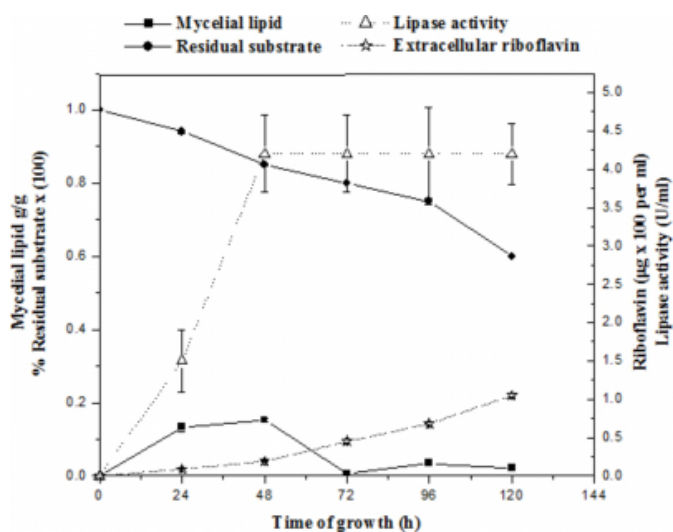
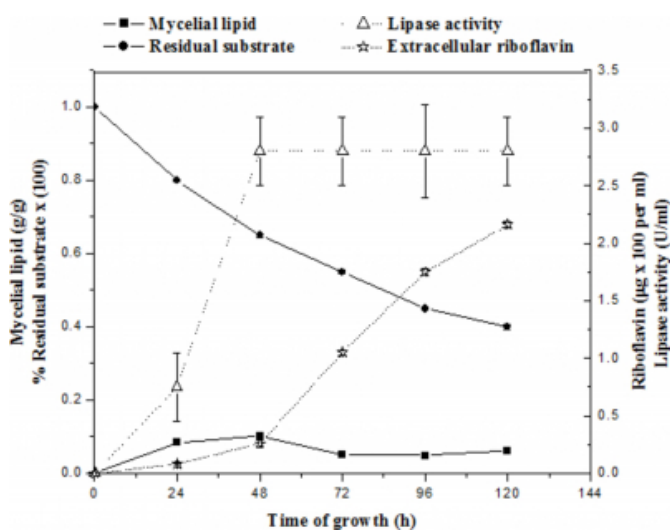


Figure 10

Fig 5: Lipid accumulation lipase activity, residual substrate and riboflavin production by during growth on glucose.



FATTY ACID PROFILE OF THE TRIGLYCERIDES AND MEMBRANE PHOSPHOLIPIDS

Irrespective of the growth substrate used, the 48 h and 96 h old mycelia obtained on glucose, olive oil and sunflower oil medium showed the presence of a large proportion of unsaturated fatty acids in the triglycerides and phospholipids (Tables 2 and 3). An observation was the decrease in the Octadecadienoic acid (C18:2; Linoleic acid) content in the triglycerides of glucose and olive oil grown mycelia at 96 h during riboflavin production (Table 2). In the case of glucose and olive oil-grown mycelia the decrease was very significant, and was about ten-fold or more while it was minimally reduced in sunflower oil-grown mycelia (Table 2) (Fig 6).

Figure 11

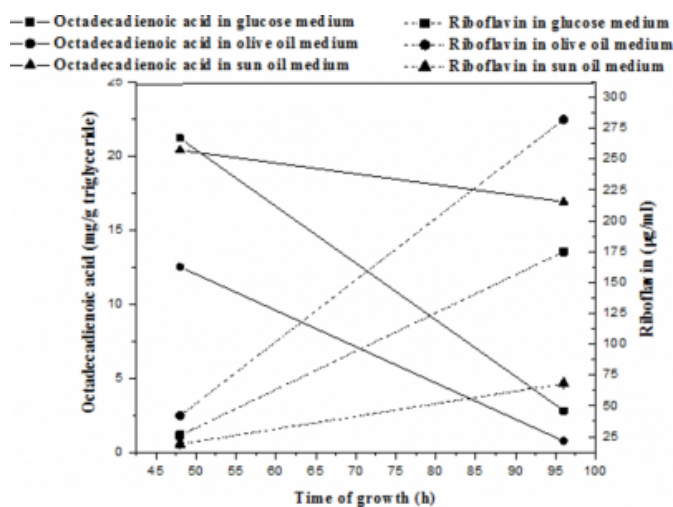
Table 2: GC–M S analysis of fatty acid composition of triglycerides during the growth phase (48 h) and riboflavin production phase (96 h) on different growth substrates

Name of the fatty acid	Glucose grown mycelia		Sunflower oil grown mycelia		Olive oil grown mycelia		Olive oil *	Sunflower oil *
	48 h	96 h	48 h	96 h	48 h	96 h		
1. Tetradecanoic acid (14:0)	3.5	3.4	0.84	0.66	0.78	0.84	0.2	ND
2. Hexadecenoic acid (16:1)	16.2	16.9	6.3	5.2	5.0	4.5	ND	ND
3. Hexadecanoic acid (16:0)	8.5	8.6	8.9	7.9	4.9	5.6	13.7	6.5
4. Cyclopropanoctic acid	1.3	ND	ND	ND	ND	ND	ND	ND
5. Octadecadienoic acid (18:2)	17.2	14.5	5.7	5.1	8.1	1.5	13.6	39.9
6. Octadecenoic acid (18:1)	24.6	28.6	57.9	43.8	25.7	38.9	49.4	30.7
7. Octadecanoic acid (18:0)	2.2	2.1	1.3	2.5	1.3	1.4	2.6	3.1

Note: Averages of two independent experiments are reported. The values are expressed per weight of triglyceride. ND: Not detected. *Composition of the oils used in the present study

Figure 12

Fig 6: A comparison of the extent of decrease in Octadecadienoic acid content of intracellular lipid in mycelia obtained with the increase in extracellular riboflavin production on different growth substrates.



Interestingly, Octadecenoic acid (C 18:1) was found to be present in the highest percentage in the phospholipids of the membrane preparations obtained on all the three media during growth (48 h) and riboflavin over production (96 h) (Table 3). In the case of membrane preparations from glucose and sunflower oil grown mycelia, a decrease in the content of Octadecenoic acid was observed during riboflavin over production (96 h) while in the case of membrane preparations from olive oil-grown mycelia, the Octadecenoic acid content increased in the 96 h old membrane preparations (Table 3).

Figure 13

Table 3: GC–M S analysis of fatty acid composition of phospholipids during the growth phase (48 h) and riboflavin production phase (96 h) on different growth substrates.

Name of the fatty acid	Glucose grown mycelia		Sunflower oil grown mycelia		Olive oil grown mycelia	
	48 h	96 h	48 h	96 h	48 h	96 h
1.Tetradecanoic acid (C14:0)	1.88	2.0	0.61	0.86	1.2	1.2
2.Hexadecenoic acid (C16:1)	11.2	15.0	5.9	8.2	4.8	4.4
3.Hexadecanoic acid (C16:0)	23.8	28.0	21.5	23.7	22.0	22.8
4. Octadecadienoic acid (C18:2)	19	16.5	16.1	16.1	19.0	19.2
5. Octadecenoic acid (C18:1)	40.4	32.5	47.9	44.9	48	52.9
6.Octadecanoic acid (C18:0)	1.9	2.0	2.9	1.3	2.9	ND

Note: Averages of two independent experiments are reported. The values are expressed per weight of phospholipid.

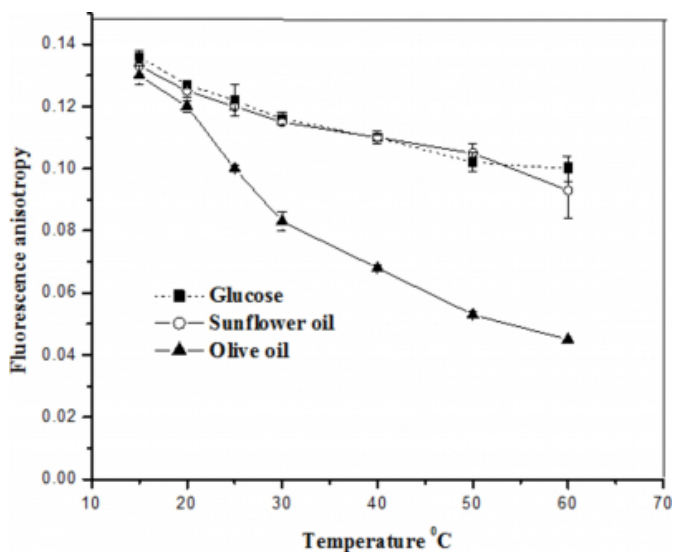
ND: Not detected.

FLUORESCENCE ANISOTROPY OF THE MEMBRANE PREPARATIONS

In general, the highest fluidity (low anisotropy) was observed in the case of membrane preparations obtained from olive oil grown mycelia both during riboflavin over production (96 h) and exponential growth (48 h) (Figs 7 and 8).

Figure 14

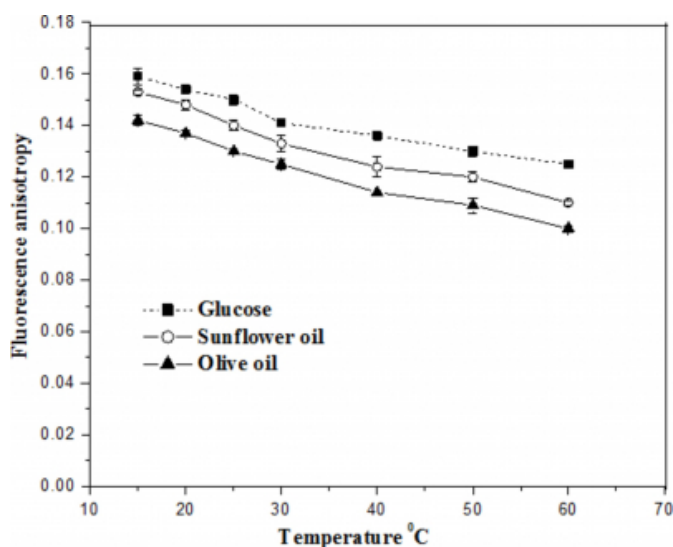
Figure 7: Fluorescence anisotropy of membrane preparations from mycelia grown on glucose, olive oil, sunflower oil media during the growth phase (48 h).



During growth the membrane preparations obtained from sunflower oil grown mycelia and those from mycelia obtained on glucose medium showed similar fluidity characteristics. While during riboflavin over production, membranes of sunflower oil grown mycelia showed intermediate fluidity and least fluidity was observed in the case of membrane preparations of glucose grown mycelia (Figs 7 and 8).

Figure 15

Figure 8: Fluorescence anisotropy of membrane preparations from mycelia grown on glucose, olive oil, sunflower oil media during the riboflavin production phase (96 h).

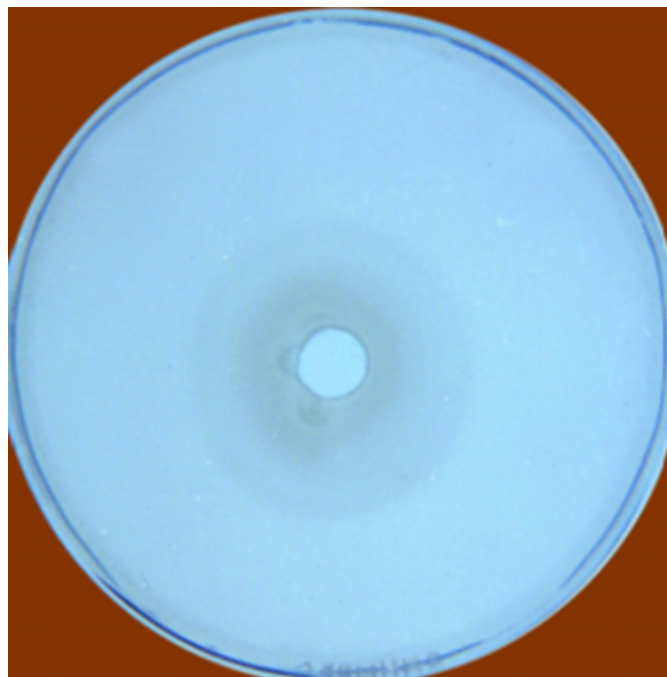


LIPASE ACTIVITY

Evidence of lipase activity was seen as a white precipitate of Ca-oleate on CaCl₂-Tween 80 plates upon addition of culture supernatants of olive oil, sunflower oil and glucose media (Fig 9).

Figure 16

Figure 9: Lipase activity seen as a white precipitate on CaCl-Tween 80 plates upon addition of cell free culture supernatant of olive oil medium.



TLC of the cell free culture supernatant of Triolein medium showed the appearance of Octadecenoic acid as one of the degradation products indicating an extracellular cleavage of this substrate via extracellular lipase activity (data not shown).

The lipase activity was quantified by a colorimetric assay. Maximum lipase production was observed at 48 h of growth on all the three media (Figs 3-5), which corresponded to the accumulation of maximum lipid prior to riboflavin over synthesis. Maximum lipase was produced on olive oil medium (5 U/ml) (Fig 3) followed by sunflower oil (4.25 U/ml) (Fig 4) and glucose medium (2.5 U/ml) (Fig 5). The increase in extracellular lipase activity corresponded to an increase in biomass and decrease in the carbon source in the culture supernatant (Figs 3-5).

A 1.5 fold increase in the lipase activity (7.5 U/ml, 6.5 U/ml and 4.2 U/ml on olive oil, sunflower oil and glucose respectively) was observed in all the three cases upon addition of Tween 80 to the medium. Tween 80 alone as sole carbon source did not support growth of *E.ashbyii*.

DISCUSSION

In the riboflavin over producer *E.ashbyii*, an accumulation of lipid was observed preceding flavinogenesis and the

decrease in Octadecenoic acid content of triglycerides influenced amount of riboflavin produced. The utilization of lipid substrates was due to the activity of an extracellular lipase secreted by *E.ashbyii*. We observed that mycelial morphology and membrane characteristics in *E. ashbyii* differ during growth (48 h) and riboflavin over production (96 h) and Octadecenoic acid content of the phospholipids may play a role in increasing the membrane fluidity and stabilizing the mycelia during riboflavin over production by *E. ashbyii*.

LIPID ACCUMULATION IN

In this study lipid accumulation during riboflavin over production by *E.ashbyii* was followed as a time course when the organism was grown on glucose, sunflower oil and olive oil as sole carbon source (Figs 1, 3, 4, 5). Maximum lipid accumulation was observed at 48 h of growth followed by the disappearance of accumulated lipids during the course of riboflavin production suggesting a role for lipids in providing precursors for flavinogenesis. An interesting finding here was that lipids accumulated on a non lipid growth substrate such as glucose prior to flavinogenesis (Fig 5). Hence based on the present study and earlier reports (Starka, 1957; Pujari and Chandra, 2001) it maybe concluded that *E.ashbyii* prefers a lipid metabolism to a carbohydrate one for riboflavin oversynthesis. Depending on the growth substrate up to 46 % of the mycelial dry weight in *E.ashbyii* consisted of lipid at 48 h (Table 1). This is higher than that observed in the closely related *A.gossypii* where lipid accumulation of up to 25% was reported prior to riboflavin over synthesis (Stahmann et al., 1994).

Our study shows for the first time that lipid accumulation and riboflavin over production in *E.ashbyii* correlate with each other. A better understanding of the lipid metabolism and the role it plays not only in the riboflavin overproduction but also in the physiology of *E.ashbyii* will emerge by studying the activities of the enzymes involved in the formation of lipid bodies such as ATP citrate lyase, Malic enzyme, Isocitrate dehydrogenase and Isocitrate lyase, as a time course during growth and riboflavin overproduction.

FATTY ACID PROFILE OF TRIGLYCERIDES AND RIBOFLAVIN PRODUCTION

A GC-MS analysis of the triglycerides of *E.ashbyii* showed the presence of unsaturated fatty acids as major constituents, irrespective of the growth substrate (Table 2). In particular,

amongst the unsaturated fatty acids Octadecenoic acid was the most abundant indicating a larger metabolic role for this fatty acid. However Octadecadienoic acid seemed to play a role in flavinogenesis. This conclusion is supported by the observation that the amount of riboflavin produced appeared to be dependent on the degree of decrease in Octadecadienoic acid content. Maximum riboflavin production and maximum decrease in Octadecadienoic acid content was observed in the triglycerides of olive oil-grown mycelia in the production phase (96 h) (Figs 3 and 6) (Table 2). A significant decrease in Octadecadienoic acid content along with high riboflavin production was observed in the case of glucose-grown mycelia also (Fig 5 and 6) (Table 2). The sunflower oil-grown mycelia did not show a significant decrease in Octadecadienoic acid content. Lesser amounts of riboflavin were produced by sunflower oil-grown mycelia in comparison to glucose and olive oil-grown mycelia (Fig 4 and 6) (Table 2). A clearer picture on this score will emerge by doing a flux analysis when the organism is grown on ¹³C labelled Glucose and ¹³C labelled fatty acids.

MYCELIAL STABILITY IS INFLUENCED BY GROWTH SUBSTRATE AND MEMBRANE FATTY ACIDS

In general, morphology and stability of *E.ashbyii* mycelia was found to be influenced by the growth substrate. Maximum stability in terms of absence of bulbous mycelial forms and subsequent mycelial lysis was observed for olive oil-grown mycelia (Figs 1 & 2). Sunflower oil-grown mycelia exhibited intermediate stability while the glucose-grown mycelia were least stable (Figs 1 & 2). The appearance of bulbous mycelial forms as well as sporulation was not seen prior to riboflavin secretion extracellularly in olive oil-grown mycelia, in spite of the fact that maximum amount of riboflavin was produced on olive oil medium. This is in contrast to the earlier reports of bulbous forms as well as sporulation in the mycelia of *E.ashbyii* prior to extracellular riboflavin secretion in the wild type and an overproducing mutant (Pujari and Chandra, 2001). Appearance of bulbous mycelial forms had been interpreted as the response to changes in the osmotic tension during riboflavin synthesis (Pujari and Chandra, 2001). and attributed to the presence of a thin cell wall in *E.ashbyii* (Lakshmi, 1996). It is quite possible that reduced morphological changes in olive oil-grown mycelia during riboflavin production can be related to the fatty acid composition of the membrane phospholipids as a mechanism to counter the build up of intracellular osmotic tension

rendering the mycelia resistant to swelling and lysis. In the present study we have observed that membrane preparations from 96 h old (riboflavin over production) olive oil-grown mycelia which produced the maximum amount of riboflavin showed greater fluidity (lower anisotropy) in comparison to the membrane preparations from glucose and sunflower oil-grown mycelia, which produced lesser amounts of riboflavin. We postulate that the fatty acid composition of membrane phospholipids could be rendering the olive oil grown mycelia more stable and this could explain our observations on the reason why the olive oil grown mycelia secrete riboflavin into the extracellular medium without showing either bulbous mycelial forms or mycelial lysis.

It has been reported that microorganisms respond to stresses such as changes in osmolarity, pH and presence of toxic chemicals by altering the permeability and fluidity of the membranes. In particular, alterations in the fatty acid composition of the membrane phospholipids have been reported. Fatty acid analyses of the plasma membrane of the salt tolerant yeast *Candida membranefaciens* showed a growth phase and dose-dependent increase in the level of Octadecatrienoic acid (C 18:3) in 1.35 M NaCl-stressed cells (Khaware et al., 1995). Halotolerance in the melanized yeast like fungi *Hortaea werneckii*, *Phaeotheca triangularis*, and *Aureobasidium pullulans* has been reported to be due to C₁₆ and C₁₈ chain fatty acids with a high percentage of Octadecadienoic acid (C 18:2) (Turk et al., 2004). Studies on the yeast *S. cerevisiae* suggest a role for acyl chain unsaturation in ethanol tolerance (Thomas et al., 1978; Mishra and Prasad, 1989). Heat and ethanol-tolerant cells of *S. cerevisiae* were reported to have membranes enriched with Octadecenoic acid (Swan and Wilson, 1999). In another study ethanol tolerance in *S. cerevisiae* was reported to be dependent on cellular Octadecenoic acid content (You et al., 2003). Our present study on *E.ashbyii* under different growth conditions has shown that the content of the different fatty acids in the mycelia of *E.ashbyii* grown on different substrates at 48 h and 96 h of growth (Tables 2 and 3), and membrane fluidity are of importance in countering mycelial swelling and subsequent lysis due to build up of intracellular osmotic tension. From our findings we postulate that Octadecenoic acid content of the membrane phospholipids may play a role in countering the build up of osmotic tension by increasing the membrane fluidity thereby making the cell more resistant to lysis upon intracellular accumulation of riboflavin. Therefore membrane preparations from olive oil grown mycelia exhibited maximum stability and higher

fluidity. Further validation of our reasoning relating mycelial stability to the membrane phospholipids can be obtained by studying the types of lipids in membranes of *E.ashbyii* mycelia obtained on the three growth substrates used in the current study, during growth and riboflavin production.

LIPASE ACTIVITY

The appearance of Octadecenoic acid as one of the products of Triolein degradation as well as the appearance of a white precipitate of Ca-oleate upon addition of the cell free culture supernatant to CaCl₂ Tween 80 plates, confirms the activity of an extracellular lipase which is involved in cleaving the lipid substrates. The observation of maximum lipase production at 48 h of growth on all the three media which corresponded to the accumulation of maximum lipid prior to riboflavin oversynthesis needs to be studied further for a probable role of the lipase in providing precursors for flavinogenesis (Figs 3-5).

Tweens, like triacylglycerol, contain an ester group that can be hydrolysed by lipase. This behaviour of Tweens as a lipase substrate could explain the inductive effect of Tween 80 on the production and release of lipase by *E. ashbyii*. This is consistent with the findings reported for the lipase from *Penicillium citrinum* (Maliszewska and Mastalerz, 1992) and *Mortierella vinacea* (Gapsar et al., 1999). Tween 80 probably has an ancillary role in enhancing lipase production as it did not support growth of *E.ashbyii*.

The production of lipase is mostly inducer-dependent, and in many cases, oils act as good inducers of the enzyme (Sharma et al., 2001). Lipase production by *Candida rugosa* grown in batch culture has been observed to be affected by the initial concentration of Octadecenoic acid—one of the major products of hydrolysis of the lipase inducers used (oils, Tween 80, etc.) (Gordillo et al., 1995). The addition of olive oil and Tweens to the medium increased the extracellular lipase production in the oleogenic fungus *M. vinacea* (Gaspar et al., 1999). An inductive influence of olive oil has been reported for *Aspergillus niger* lipase (Pokorny et al., 1994), *P. citrinum* (Maliszewska and Mastalerz, 1992) and *Rhizopus delemar* (Espinosa et al., 1990). The induction of maximum lipase in olive oil grown *E.ashbyii* maybe attributed to its high content of Octadecenoic acid.

CONCLUSIONS

In the present study we have shown intracellular lipid accumulation and changes in membrane fluidity during growth and flavinogenesis by *E.ashbyii* using three different

growth substrates - glucose, olive oil and sunflower oil. Maximum lipid accumulation was observed in olive oil-grown mycelia, followed by sunflower oil and glucose-grown mycelia whereas maximum riboflavin production was observed in olive oil-grown mycelia, followed by glucose and sunflower oil grown mycelia. Analysis of the fatty acid composition of the triglyceride and phospholipid fractions by GC MS showed the presence of a large percentage of unsaturated fatty acids and showed that riboflavin production could be correlated to the degree of decrease in Octadecadienoic acid content of triglycerides in the production phase. An extracellular lipase activity was shown which was involved in utilization of the lipid substrates. This is the first report of lipase activity in *E.ashbyii*.

Mycelial stability was the greatest on olive oil medium followed by sunflower oil, then glucose-grown mycelia. Membrane fluidity was correlated with the fatty acid composition of the membrane phospholipids and our observations on the mycelial stability in terms of non production of bulbous forms and resistance to lysis correlated with the Octadecenoic acid content of the membrane phospholipids. Maximum fluidity was observed in the case of membrane preparations of olive oil grown mycelia due to the above reason followed by those obtained on sunflower oil and the membranes obtained from mycelia grown on glucose exhibited least fluidity. Hence we postulate that the content of Octadecenoic acid in the membrane phospholipids may play a role in stabilising the mycelia by influencing the membrane fluidity.

Based on our results we postulate that the decrease in Octadecadienoic acid content in the triglycerides and the content of Octadecenoic acid in the phospholipids of *E. ashbyii* mycelia play important roles in contributing to riboflavin production and mycelial stability of *E.ashbyii* respectively. *E.ashbyii* uses a lipid metabolism rather than carbohydrate metabolism and the increased demand for FAD due to the increased activity of the enzymes of the β -oxidation pathway may have been the starting point for the riboflavin overproduction by this organism.

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