THE UNIVERSITY OF HULL

Studies on Lipid Accumulation and Genetics of *Rhodosporidium toruloides*

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by

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My thoughts on the life cycle of *Rhodosporidium toruloides*

"I never asked you to be perfect - did I?"

Sassoon.

The Imperfect Lover.

STUDIES ON LIPID ACCUMULATION AND GENETICS OF RHODOSPORIDIUM TORULOIDES.

INTRODUCTION.

MATERIALS AND METHODS.

RESULTS.

INTRODUCTION

1. General Background.

For thousands of years micro-organisms have been used in the production of foodstuffs. In the past 40 years, while baking and brewing have remained important industries, new industries have grown up around new products. These include single cell protein, antibiotics, vitamins, pesticides and biopolymers. One area which has not yet been fully exploited is the ability of some micro-organisms to accumulate high concentrations of intracellular lipids. In certain micro-organisms, when growth ceases if a nutrient (other than carbon) in the growth medium has been exhausted, the carbon source still continues to be consumed. The excess carbon is channelled into the synthesis of lipid which appears as droplets inside the cell.

Of the total world production of oils and fats, about 80% are used for human consumption with the remaining 20% becoming incorporated into products such as soaps, paints, detergents, waxes and polishes. The majority of these oils and fats are derived from plant oilseeds, with animal and marine sources making up only 20% of the total (Ratledge, 1982 and 1986). Few of the oil producing plants are grown in Europe, so that the United Kingdom must import much of the oil consumed in this country. Even with the expansion of rapeseed oil production, which now supplies 20 to 25% of our needs, the UK is still heavily dependent on imports and therefore susceptible to change in market prices. The development of an alternative source of oils and fats in Europe is clearly desirable, and so

the possibility of using oleaginous micro-organisms to produce single cell oil must be examined.

At present it would seem that single cell oil has an advantage over other production methods only when a high value commodity is being produced. The only single cell oil process now in use is the production of a fungal oil rich in gamma linolenic acid by J & E Sturge Ltd., at Selby in N. Yorkshire. However future changes in world market forces coupled with improvements in production methods may make the production of medium and low value commodities by this process economically viable.

The technology for the large scale continuous cultivation of micro-organisms has already been successfully developed by companies interested in producing single cell protein. Continuous culture is preferable to batch culture in an industrial process because of the low running costs and constancy of the product. The growth rate of the micro-organism can be adjusted as required, and continuous culture also provides micro-organisms 'on-stream' with the concomittant production advantages of an open system, i.e. no downtime, increased production, scaled down plant size, ease of control and automation (Dawson, 1984).

The apparently biphasic process of cell growth followed by lipid accumulation would not seem particularly well suited for development as a single stage continuous culture process. However work carried out in this laboratory showed that by using a low dilution rate (0.02 to 0.05 per hour) and a high C:N ratio,

lipid accumulation could be achieved in a single stage continuous culture. (Gill et al. 1977, Hall and Ratledge, 1977, Ratledge et al., 1983). Thus the technology to produce single cell oil already exists; what is needed is a suitable organism to produce lipid commercially.

The term 'oleaginous' has been used to describe micro-organisms which produce 25% or more of their biomass as lipid (Ratledge, 1982). This is an arbitrary decision, but was adopted as 25% is probably the lowest limit for any micro-organism to be of use commercially. Oleaginous micro-organisms may be algae, yeasts or fungi. It seems unlikely that algae will be the organisms chosen to produce single cell oil as the production costs would be prohibitively high (Milner, 1951). Yeasts would appear to be the most promising candidates as their ovoid form and high growth rates make it possible to produce large quantities of biomass in a short time.

2. The Biochemistry Of Lipid Accumulation In Oleaginous Yeasts.

The biochemical processes leading to lipid accumulation were first investigated in this laboratory (Botham and Ratledge, 1979). It was already known that when certain species of yeast were grown with an excess of carbon and a deficit of nitrogen, protein and nucleic acid synthesis cease but lipid synthesis continues, leading to a build up of lipid within the cell. Four mechanisms which could account for this process were proposed. These were:

1. Glucose uptake continues unchecked after the nitrogen supply

is exhausted. The organism is therefore obliged to find a suitable means of accommodating the surplus carbon and energy, as well as the NADPH generated by the pentose phosphate cycle.

2.Acetyl CoA carboxylase, the suggested regulatory enzyme of fatty acid biosynthesis (White and Klein, 1965, White and Klein, 1966, Volpe and Vagelos, 1976, Gill and Ratledge, 1973) is hyper-active, or not repressed, or not subject to feedback inhibition in oleaginous yeasts.

3. Lipid turnover may be high in a non-oleaginous yeast, negligible in an oleaginous yeast.

4. Intermediary metabolism of oleaginous micro-organisms is regulated so that there is an uninterrupted flow of substrate to the formation of acetyl CoA, which then fuels lipid synthesis.

In 1979 Botham and Ratledge showed that the rate of glucose uptake did not limit the growth of yeast cells and thus could not be regulating lipid accumulation. Lipid turnover, both in the oleaginous Candida 107 and non-oleaginous C. utilis, was negligible, and neither could oleaginicity be attributed to differences in acetyl CoA carboxylase regulation. Further investigations led to the conclusion that the most important factor was the build up of ATP and depletion of AMP which occurs when growth is limited by supply of a nutrient but carbon is present. It was shown that in oleaginous yeasts, mitochondrial NAD+ dependent isocitrate dehydrogenase (NAD+:ICDH) has an absolute requirement for AMP. As the concentration of AMP declines the activity of the enzyme ceases, leading to a build up of the substrate of the enzyme, isocitrate. Aconitase

facilitates the equilibration of isocitrate to citrate so that citrate accumulates in the mitochondrion.

NAD+:ICDH from other micro-organisms has been found to be sensitive to the concentration of adenine nucleotides in the cell (Hathaway and Atkinson, 1963, Mitsushima et al., 1978, Atkinson, 1970). In a cell in a nitrogen limited, carbon excess environment, the 'energy charge' (Atkinson, 1969) of the cell is high and the concentration of AMP is low. Boulton (1982) found that in cells undergoing transformation from carbon limited to nitrogen limited conditions, a decrease in AMP concentration was followed by an increase in intracellular citrate and lipid, thus confirming the role of mitochondrial NAD+:ICDH in lipid accumulation.

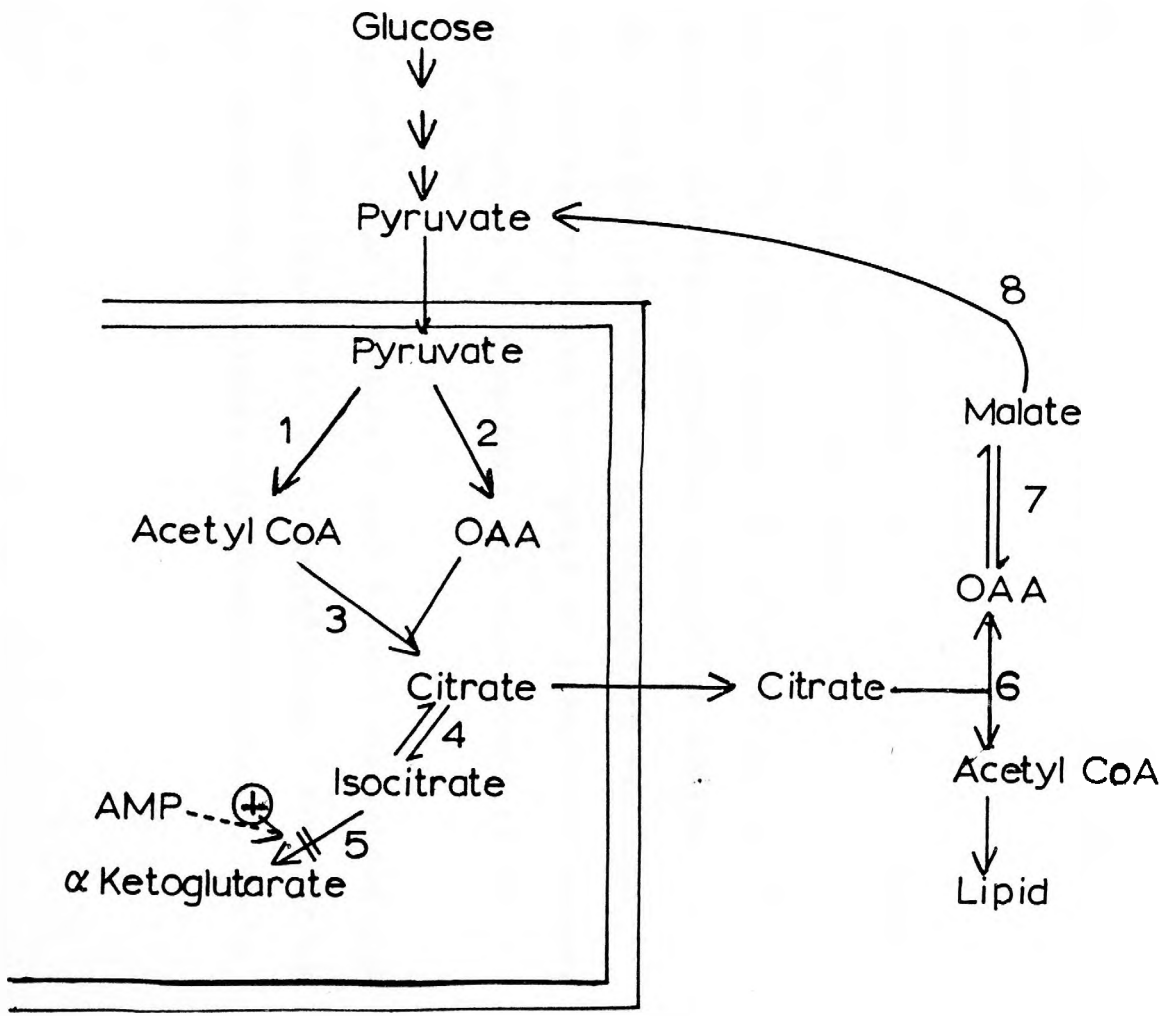
The second difference between oleaginous and non-oleaginous yeasts was found to be the presence of the enzyme ATP:citrate lyase in oleaginous yeasts. Citrate which has accumulated due to the inhibition of NAD+:ICDH can cross the inner mitochondrial membrane and is cleaved by ATP:citrate lyase to give oxaloacetate and acetyl-CoA. The acetyl-CoA is then available for fatty acid biosynthesis leading to lipid accumulation.

As the enzyme ATP:citrate lyase would appear to have a key role in the accumulation of lipid in oleaginous yeasts the distribution of the enzyme among oleaginous and non-oleaginous yeasts was investigated (Boulton and Ratledge, 1981). The enzyme was detected in 13 yeasts capable of accumulating lipid to 20% (w/w) or more of their biomass, but not in 10 yeasts, which do

not accumulate lipid. These results confirmed the importance of ATP:citrate lyase in lipid accumulation. The processes leading to lipid accumulation in oleaginous yeasts are summarised in Fig.1.

As ATP:citrate lyase (ACL) is the key enzyme of lipid accumulation and is found in all oleaginous yeasts, any attempt to investigate the genetics of oleaginicity will include, for example, isolating ACL-less mutants of an oleaginous strain to examine the effect that this has on the biochemistry of the cell, and possibly re-introducing the ACL gene by protoplast fusion or transformation with a plasmid. If several different mutations in the ACL gene could be identified, in vitrocomplementationstudies could provide information about the structure of the enzyme. This method has been used to reconstruct active fatty acid synthetase from the subunits of inactive mutant enzymes in S. cerevisiae (Wieland et al., 1979, Werkmeister et al., 1981). The enzyme isolated from Lipomyces starkeyi has a very high molecular weight (510 000) similar to that reported for rat hepatic ACL (Boulton, 1983). The mammalian enzyme is known to be a tetramer, but it is not clear whether the subunits are identical (i.e. $\alpha 4$ configuration) or different (e. g. $\alpha 2\beta 2$ configuration) (Linn and Srere, 1979). In vitrocomplementationof subunits from ACL-less mutants could provide information about the enzyme stucture. However it is possible that the loss of ACL in oleaginous yeasts could be a lethal mutation. ACL is known to be active under

Fig.1. The metabolism of glucose into lipid. From Botham and Ratledge 1979.



OAA = oxaloacetate

1. Pyruvate dehydrogenase
2. Pyruvate carboxylase
3. Citrate synthase
4. Aconitase
5. NAD^+ isocitrate dehydrogenase
6. ATP: citrate lyase
7. Malate dehydrogenase
8. Malic enzyme

5 is only active in the presence of AMP. When the concentration of AMP falls, isocitrate equilibrates to citrate.

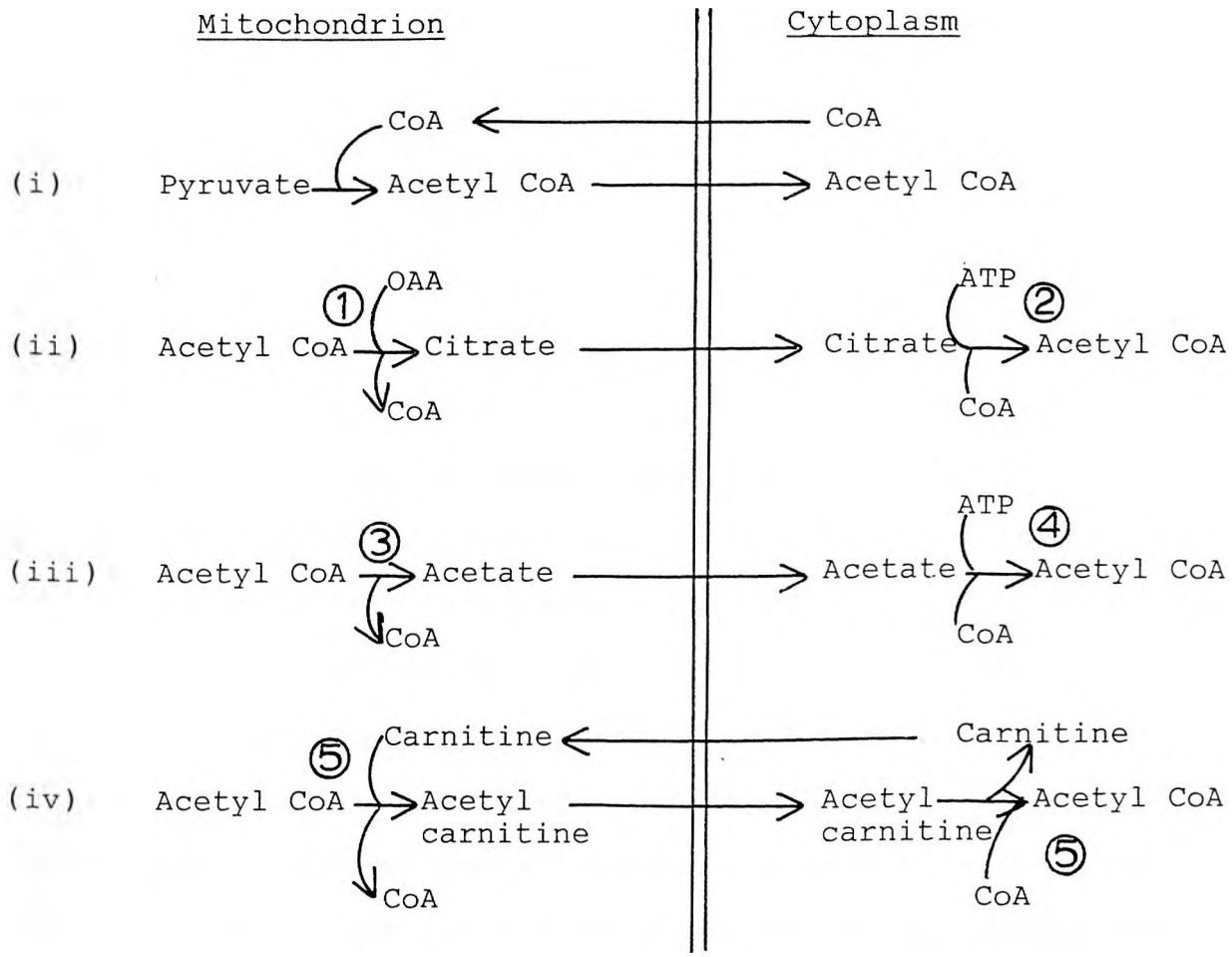
carbon limited as well as nitrogen limited conditions (Boulton and Ratledge, 1981) and may be required to supply acetyl groups to the cytoplasm for all intermediary metabolism, and not just for lipid biosynthesis. At the time of commencing the research project the supply of acetyl groups to the cytoplasm had not been investigated in oleaginous yeasts. In Saccharomyces cerevisiae four possible pathways for the transport of acetyl CoA across the mitochondrial membrane have been discussed and investigated (Kohlhaw and Tan-Wilson, 1977). See Fig.2. These were:

1. Direct transfer of acetyl-CoA. This was considered inoperative due to the size of the CoA moiety.
2. Transfer as citrate, involving citrate synthase in the mitochondrion and ATP:citrate lyase in the cytoplasm. ATP:citrate lyase is not active in S. cerevisiae and so this route was also eliminated.
3. Transfer as acetate, involving acetyl-CoA hydrolase and acetyl-CoA synthetase. As the activity of acetyl-CoA hydrolase was found to be very low, it is unlikely that this route contributes significantly to the supply of cytosolic acetyl-CoA.
4. Transfer as acetyl carnitine involving carnitine acetyl transferase (CAT). As CAT activity in S. cerevisiae was high enough to account for the required transport of acetyl-CoA across the mitochondrial membrane, this pathway was considered to be operative.

In oleaginous yeasts, routes 1 and 3 would be considered inoperative for the same reasons as in S. cerevisiae. However as ATP:citrate lyase is active in oleaginous yeast, pathway 2 could

Fig. 2

Possible Mechanisms Of Acetyl Group Transfer From Mitochondrion To Cytoplasm.



1. Citrate synthase E. C. 4.1.3.7
2. ATP:citrate lyase E. C. 4.1.3.8
3. Acetyl CoA hydrolase E. C. 3.1.2.1
4. Acetyl CoA synthetase E. C. 6.2.1.1
5. Carnitine acetyl transferase E. C. 2.3.1.7

From: Srere, 1965.

be operative either in addition to, or instead of, pathway 4. It was not known whether CAT is present in oleaginous yeasts and so part of the research project was to discover whether CAT is active in these yeasts, and if so, to determine the relative importance of the two pathways in the supply of cytosolic acetyl-CoA.

Although the biochemistry of lipid accumulation has been examined in some detail, the genetics of oleaginicity has not been investigated. Two pieces of evidence, however, suggest that a study of the genetics of lipid accumulation is needed before single cell oil production can be optimised.

1. It has been noted (Ratledge, 1982) that some oleaginous species have apparently lost the ability to accumulate much lipid when re-examined at a later date. For example in 1962 Pedersen reported lipid contents of 50 to 60% in Cryptococcus terricolus (now renamed Cryptococcus albidus var. albidus, Lodder, 1970). However attempts to repeat these results (Hall, 1978) were unsuccessful. Various media formulations were tried but the highest lipid content found with any strain of C. albidus was 1.9%. Of three strains of C. terricolus examined at a later date (Boulton and Ratledge, 1984) one was found to be capable of accumulating 35 - 40% lipid in batch culture. The lipid content of Rhodotorula gracilis (now R. glutinis NCYC 59) was found to be 42% in 1968 (Kessell) and 18% in 1978 (Hall). This loss of oleaginicity cannot be attributed to changes in growth medium or conditions, and it would seem to be due to a genetic change in the yeast.

2. In 1961 Cullimore and Woodbine reported that lipid accumulation in single spore isolates of oleaginous Lipomyces strains had been studied. Although the parent strain had accumulated only 7.7% (w/w) lipid under a particular set of growth conditions, the lipid contents of cultures grown from single spore isolates ranged from 11 to 31%. It was stated that by the use of single spore isolates from Lipomyces starkeyi improvement in fat synthesising ability might be possible, but the work does not appear to have been continued as no further results have been published.

3. Choice of Rhodosporidium toruloides as a suitable species for genetic studies.

The list of oleaginous yeasts includes Candida curvata (now considered to be Trichosporon cutaneum, pers. comm of Mrs. B Kirsop to C. Ratledge), Cryptococcus albidus, Hansenula saturnus, Lipomyces lipofer, L. starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, R. gracilis, R. graminis, Trichosporon cutaneum and Yarrowia lipolytica. From these it was necessary to choose a suitable species in order to study the genetics of oleaginicity. Although Y. lipolytica (syn. Candida lipolytica, Endomycopsis lipolytica, Saccharomycopsis lipolytica) may have a lipid content of 17 to 47% lipid this only occurs when it is grown on hydrocarbons (Ratledge, 1980) or fats (Tan and Gill, 1984). When grown on glucose its lipid content is only 9% and citrate is usually produced in increased quantities (Evans, 1983). The presence or absence of ATP:citrate lyase in this

yeast has not been determined but as the accumulation of lipids only occurs during growth on hydrocarbons or fats, this yeast is something of a special case and did not appear to be the best candidate for study. For this project it would be preferable to choose a species which has already been the subject of genetical studies. However most studies of yeast genetics have been of S. cerevisiae or Schizosaccharomyces pombe and not the yeasts listed above. Two exceptions to this are Hansenula and Rhodosporidium. Wickerham and Burton (1954) described procedures for demonstrating which of the Hansenula species are heterothallic and which homothallic, and for the isolation of single ascospores. More recently protoplast fusions between strains carrying auxotrophic markers (Savchenko and Kapul'tsevich 1980) have been found to produce diploid cells which gave rise to mitotic segregants both spontaneously and after induction by haploidising agents (Freeman and Peberdy, 1983). H. saturnus may contain up to 21% lipid (Hopton and Woodbine, 1960), and the activity of ATP:citrate lyase in H. saturnus and H. ciferrii has been measured (Boulton and Ratledge, 1981). ATP:citrate lyase activity was found in H. saturnus, but not H. ciferii. H. saturnus may be a suitable candidate for study, but the maximum lipid contents reported are lower than the level considered by Ratledge (1982) to be of use commercially, and it would be more profitable to study a yeast capable of accumulating much higher concentrations of lipid.

The work that has been done with R. toruloides indicates that this species is also amenable to

genetic manipulation. Auxotrophic mutants of Rhodosporidium toruloides have been isolated after exposure to ultraviolet light or nitrosoguanidine (Bottcher and Samsonova, 1977, 1978). Protoplast fusions between auxotrophic parents gave rise to genetically stable prototrophic hybrids (Becher and Bottcher, 1980).

The biochemistry of lipid accumulation was investigated chiefly in Candida, Lipomyces and Rhodosporidium species (Botham and Ratledge, 1979, Boulton and Ratledge, 1983, Evans et al., 1983, Evans, 1983). In order that the investigation of the genetics of lipid accumulation will be complementary to the study of the biochemistry of the process, a yeast from one of these three genera would seem to be the best candidate for genetical studies. The two disciplines of genetics and biochemistry are closely and inseparably linked. If detailed information about the biochemistry of a particular process is available this will greatly assist the study of the genetics of that process.

The oleaginous Candida curvata (now considered to be T. cutaneum) reproduces asexually and does not sporulate so that classical genetic experiments which require the crossing of two parents and isolation of the progeny would not be possible.

When oleaginous Lipomyces species are grown on agar plates, colonies become surrounded by a thick mucous capsule, making the isolation of single colonies extremely difficult. It would in theory be possible to isolate mutants lacking the capsule but this may cause an imbalance in the carbon metabolism of the cell and indirectly influence lipid accumulation.

Furthermore, attempts to repeat the work of Cullimore and Woodbine (1962) with single spore isolates have not been successful as the Lipomyces cultures now held in culture collections do not sporulate (Ratledge and Curson, unpubl, Hugh Browne, personal communication).

Rhodosporidium species do not secrete a polysaccharide capsule, and appear to be amenable to genetic manipulation as shown by the published data on the isolation of auxotrophic mutants and fusion of protoplasts (see above). Lipid contents as high as 84% w/w have been reported (Evans, 1983) in one strain, R. toruloides CBS 14. This is the highest lipid content ever reported for any micro-organism. It would be advantageous for the species chosen for the study of the genetics of oleaginicity to have a very high maximum lipid content as this allows for a great deal of measurable variation in lipid contents among mutants or genetically manipulated strains. R. toruloides was therefore chosen as a suitable species for an investigation of the genetics of oleaginicity in yeast.

4. Rhodosporidium toruloides.

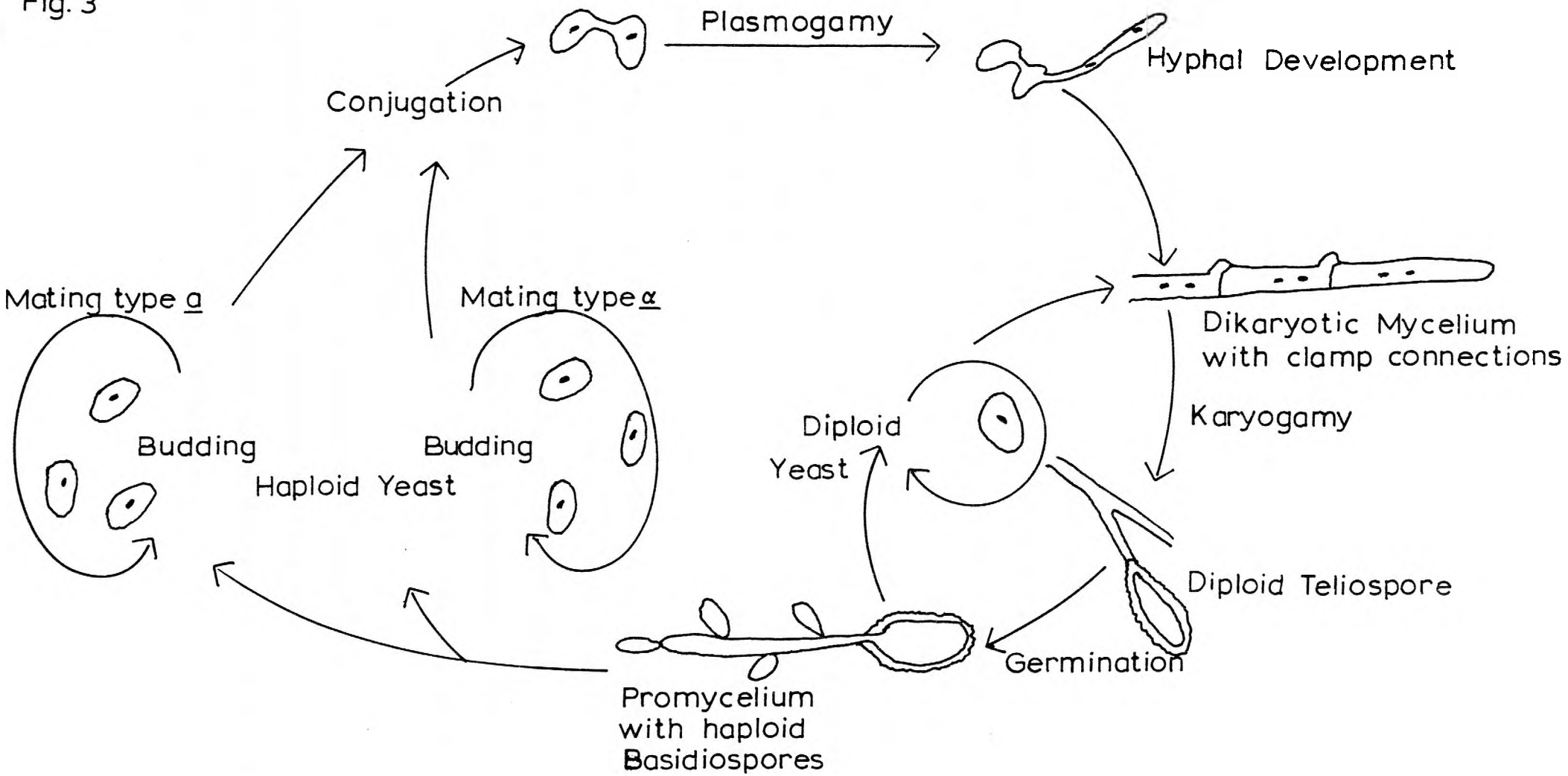
The genus Rhodosporidium was proposed when it was discovered that some strains of Rhodotorula glutinis were capable of sexual interaction (Banno, 1963, Banno, 1967, Lodder, 1970). Rhodotorula yeasts had until this time been regarded as members of the Fungi Imperfecti as no perfect stage was known. Banno (1967) discovered that a prototrophic mycelial stage formed after conjugation and plasmogamy between two auxotrophic strains of Rhodotorula that were considered haploid. The mycelium was

dikaryotic with clamp connections, which are small hooks positioned next to the septa. They are formed as the result of a mechanism that allows one nucleus of each type to be present in each hyphal cell after division, and are characteristic of dikaryons. It was also found that this mycelial stage produces thick-walled, resting spores which could germinate to produce a promycelium. Basidiospores bud off the promycelium and divide to form yeast cells with two mating types, so completing the life cycle. Banno's representation of the life cycle of R. toruloides is shown in Fig.3. The two mating types were originally designated A and a by Banno (1967). Other publications have referred to them as A1 and A2 (Lodder, 1970) or α and a (National Collection of Yeast Cultures catalogue). I have chosen to use the terms α and a (where A = α) as this leads to less confusion during verbal discussion than the use of A and a, and is the form used by the culture collections NCYC and Centraalbureau voor Schimmelcultures (CBS).

The events which occur during the mating process of Rhodosporidium were investigated by Abe et al., (1975). Mating type (mt) α cells constitutively produce the mating pheromone α-factor. In response to this mt a cells produce mating tubes and secrete the pheromone a-factor. This induces α cells to form mating tubes. The mating tubes elongate towards the mating partner. The tips of the tubes recognize the partner and fuse. Mycelial growth then commences.

Banno also observed that in some cases yeasts were capable of producing mycelium without first mating with another

Fig. 3



Life Cycle of Rhodosporidium toruloides. From Banno 1967.

strain of opposite mating type. Measurements of the DNA content per cell suggested that those yeasts were diploid, whereas those that only produced mycelium after mating were haploid. It was suggested that the diploid yeasts arose after failure of meiosis in the germinating teliospore, and that the mycelium produced from them was dikaryotic and indistinguishable from that produced after mating between two haploid yeasts. However more recently Samsonova et al. (1980a) reported that this was not the case, and that mycelium formed from single R. toruloides yeast cells is mononuclear, whereas mycelium formed after conjugation is binuclear. It would seem that a diploid yeast produces a diploid mycelium, not a dikaryon. It was also found that in protoplast fusions between cells of the same or opposite mating type, mycelium-forming cells were only produced after fusion of protoplasts of opposite mating type. To form mycelium a cell must not only be diploid but heterozygous for mating type alleles.

Measurements of the DNA content of R. toruloides isolated from the environment showed that some strains appeared to be aneuploid, and that this did not affect their viability (Bottcher et al., 1980, Bottcher and Samsonova, 1980a). This contradicted the view held by Banno (1967), who found only haploid or diploid yeasts.

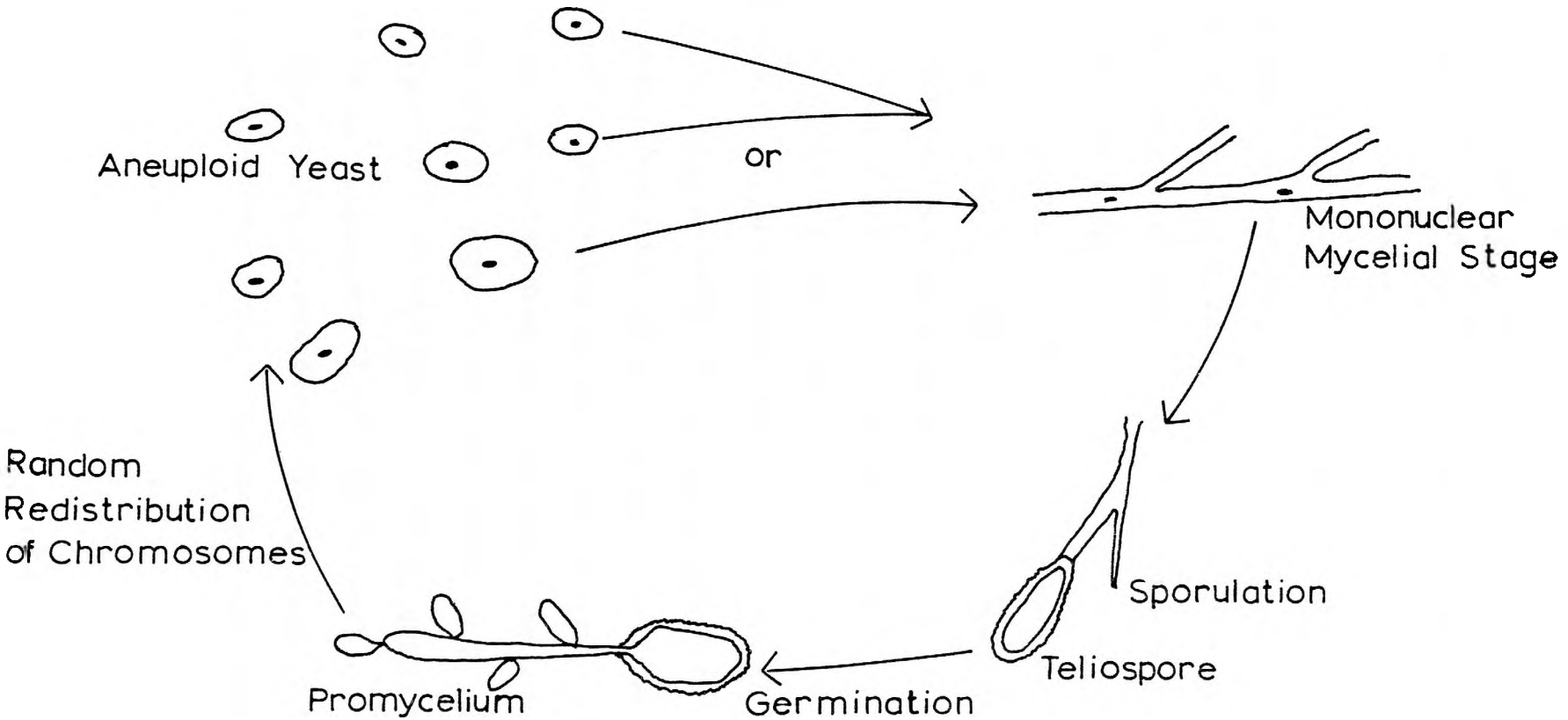
Attempts to isolate auxotrophic mutants from strains of R. toruloides (Bottcher and Samsonova, 1980 a&b) showed that the frequency of isolation of auxotrophic mutants was much lower in some strains than in others, supporting the view that some

strains are aneuploid. A study of the mating behaviour of the progeny isolated after crossing two auxotrophic parent strains (Samsonova et al., 1980b) found that in addition to strains with mating type a and mating type α, some strains showed bisexual (i.e. mating with both a and α strains) or neutral (no mating observed) mating behaviour. The conclusion drawn from these investigations was that spores produced on the promycelium of R. toruloides are not haploid meiosis products, but are formed after an irregular distribution of chromosomes during germination of the teliospores. In contrast to Banno's conclusions it was stated that "it is possible that R. toruloides represents a primitive eukaryotic organism which is not in possession of an apparatus for meiosis, although it can perform karyogamy leading to an unstable synkaryon" (Samsonova and Bottcher, 1980).

Thus at the time of commencing the research project two opposing views on the life cycle of R. toruloides were held. Banno's claims of a well ordered life cycle with haploid, dikaryotic and diploid stages were contradicted by the views of Bottcher et al who reported that aneuploid yeasts form an unstable synkaryon which is resolved by the random redistribution of chromosomes after germination of the resistant spore stage. This version of the life cycle of R. toruloides is shown in Fig.4.

Strains of R. toruloides which were considered haploid were chosen for a study of phenylalanine ammonia lyase (PAL) production (Gilbert et al., 1983, Tully and Gilbert, 1985). This enzyme, which is produced in considerable amounts by R.

Fig 4



Alternative View of the Life Cycle of R. toruloides. From: Samsonova and Böttcher, 1980.

toruloides both in batch and continuous culture, is of potential therapeutic (Hoskins *et al* , 1980) and industrial importance (Yamada *et al*, 1981). It has been shown (Gilbert and Tully, 1982) that production of PAL in *R. toruloides* is induced by L-phenylalanine and that induction is brought about by *de novo* synthesis of the enzyme rather than activation of a proenzyme. The control of synthesis of PAL mRNA has been studied, and the results obtained suggest that alterations in the amount of PAL in cells of *R. toruloides* result primarily from alterations in the amount of functional mRNA present in the cell (Gilbert *et al.*, 1983). The PAL gene was identified from a genomic library of *R. toruloides* in bacteriophage λ1059 using labelled cDNA from partially purified PAL mRNA, which was prepared from total cellular mRNA by sucrose gradient centrifugation and agarose gel electrophoresis. Analysis has shown the size of the PAL mRNA to be 2.5 kb (Gilbert *et al*, 1985). A plasmid (constructed from pBR322, pUC8 and 2μ plasmid) bearing the PAL gene has been used to transform *pal*$^-$ mutants of *R. toruloides*. In most cases *pal*$^+$ transformants contained an active PAL gene on an unstable autonomously replicating plasmid, but in a few transformants the PAL gene became integrated into the host chromosome. It was also shown that the PAL gene is not expressed in *S. cerevisiae* and that the 2μ origin of replication from *S. cerevisiae* does not function in *R. toruloides* (Tully and Gilbert, 1985).

The mating pheromones of *R. toruloides* have also been the subject of recent study. Unlike *S. cerevisiae* which produces

simple peptide mating factors, the pheromones of R. toruloides and other basidiomycete yeasts are lipopeptides. Miyakawa et al (1982) identified a trypsin type endopeptidase which is found only in mt a cells and is highly specific for α-factor. This enzyme appears to be membrane bound and, as in S. cerevisiae, metabolism of the pheromone mediates the biological effect of the pheromone on the target cell. This effect is enhanced by reducing reagents but strongly inhibited by sulfhydryl blocking reagents (Miyakawa et al, 1985). A model for the mode of action of α-factor on mt a cells has been proposed. This involves the transmembrane coupling of pheromone hydrolysis with phosphorylation of membrane proteins, leading to cellular differentiation. The mechanism for the induction of a-factor production by α-factor has not yet been elucidated.

5. Aims Of Research.

The aim of the research project was to discover as much as posible about the genetics of lipid accumulation in R. toruloides. In order to achieve this the following areas were studied:

1. the lipid content of a large number of strains of R. toruloides.
2. the activity of ATP:citrate lyase and the dependency of the cytosolic acetyl-CoA pool on this activity.
3. the use of fast screening methods to detect the lipid content of yeast.
4. the isolation of ACL-less mutants of R. toruloides.

5. the DNA content and ploidy of *R. toruloides*.
6. the isolation of basidiospores from *R. toruloides*.
7. the variation in lipid content and DNA content of *R. toruloides* during continuous culture.
8. the life cycle of *R. toruloides*.

MATERIALS AND METHODS.

1. Source Of Yeast Strains Used.

Yeasts were obtained from the following sources:

Prefix to strain number.	Culture Collection.
NCYC	National Collection of Yeast Cultures, Food Research Institute, Colney Lane, Norwich.
CBS	Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.
ML	Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Florida.
IFO	Institute for Fermentation, Osaka, Japan, except for IFO 0559 and IFO 0880 which were received fom Dr. M. Tully, Centre for Applied Microbiology and Research, Porton Down, Salisbury.

See Table 1 for a complete list of the strains of *R. toruloides* used.

Candida curvata strains R and D were obtained from Professor E. G. Hammond, Iowa State University, USA.

2. Maintenance Of Cultures.

Yeasts were stored in plastic straws in liquid nitrogen refrigerators using glycerol (5% final concentration) as a cryoprotectant (Kirsop, 1984). No loss of viability was detected using this method. For short term storage yeast was stored on

Table 1

Strain Numbers Of All Strains Of Rhodosporidium toruloides Included In This Study.

IFO	0559	CBS	14
	0871		315
	0880		350
	1236		2370
	8766		5490
	10075		6450
	10076		6681
ML	2573	NCYC	921
	2589		979
	2590		
	2921	ATCC	26217

The strains NCYC 921, CBS 14 and IFO 0559 are equivalent strains from different culture collections (NCYC catalogue).

YMG agar slopes (see section 3.1) at 4^{o} C.

3. Media.

3.1 YMG.

This consisted of (g/l) yeast extract, 5.0; malt extract, 5.0; glucose, 5.0. The pH was adjusted to 5.5 with HCl. For solid medium, Lab M agar was added at a concentration of 15 g/l.

3.2 NLM.

Nitrogen Limited Medium consisted of (g/l) glucose, 30; Sodium L-glutamate, 1.25; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2.2\ H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7\ H_2O$, 0.0001. The pH of the medium was adjusted to 5.5 with HCl. In some experiments the use of an inorganic nitrogen source was required for batch culture of the yeast. In this case, ammonium tartrate was substituted for sodium glutamate.

For the culture of high lipid content colonies on agar plates, the concentrations of sodium glutamate and yeast extract were varied (see Results section 4.2). Lab M agar was added at a concentration of 15 g/l to form solid medium.

3.3 Triolein Medium.

This was a modified version of NLM in which glucose and glutamate were omitted and triolylglycerol (15.0 ml/l) and NH_4Cl (3.0 g/l) were added.

3.4 MM-N.

Minimal medium without nitrogen source consisted of (g/l) glucose, 20.0; KH_2PO_4, 2.0; $MgSO_4$, 0.5; $CaCl_2.2H_2O$,

0.1; $FeCl_3.6H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.00007; $CuSO_4.5H_2O$, 0.00001. The pH was adjusted to 5.5 with NaOH.

3.5 MM.

Minimal medium consisted of MM-N with the addition of 1.0 g/l $(NH_4)_2SO_4$. 15 g/l of Lab M agar were added for solid medium.

3.6 Enrichment medium .

Medium for the enrichment of mutants lacking the enzyme ATP:citrate lyase in a culture that has been exposed to a mutagen consisted of glycerol, 12.3 ml/l; fluoroacetate, 50μM; plus (g/l) $MgSO_4.7H_2O$, 0.5; $FeCl_3.6H_2O$, 0.002; $ZnSO_4.7H_2O$, 0.00007; $CuSO_4.5H_2O$, 0.00001; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 2.0, $CaCl_2.2H_2O$, 0.1; inositol, 0.005. The pH was adjusted to 5.5 with HCl.

3.7 Indicator medium.

Medium to indicate the presence of colonies that accumulate acid during growth consisted of glycerol, 12.3 ml/l; bromocresol green, 5 ml/l of a 0.1% w/v solution in ethanol; plus (g/l) sodium L-glutamate, 1.25; KH_2PO_4, 0.31; Na_2HPO_4, 0.09; $MgSO_4.7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2.2H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001; inositol, 0.005, Lab M agar, 15.0. The pH was adjusted to 5.5 with HCl.
All the media described in sections 3.1 to 3.6 were sterilised by autoclaving at 121^{o} C for 15 minutes.

3.8 CLM.

Carbon limited medium consisted of (g/l) glucose, 12.0; NH_4Cl, 3.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7$ H2O, 1.5; yeast

extract, 1.5; $CaCl_2.2\ H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7\ H_2O$, 0.0001 $MnSO_4.4H_2O$, 0.001; $CuSO_4.5H_2O$, 0.0005. The pH of the medium was adjusted to 5.5 with HCl. CLM was sterilised using Sartorius pressure filtration equipment fitted with 0.2 μ pore size filters, and stored in sterile 20 litre aspirators.

4. Assay Of ATP:citrate lyase Activity.

4.1 Growth of yeasts.

Yeasts were grown on an orbital shaker at 190 rpm, 30° C, in 250 ml conical flasks containing 100 ml NLM.

4.2 Preparation of Cell Free Extract.

Cells were harvested after 3 days growth, centrifuged at 10 000 g for 20 minutes and washed once in 50 mM Tris/HCl buffer pH 7.5 containing 1 mM $MgCl_2$ and 0.5 mM dithiothreitol. The cells were resuspended in 20 ml extraction buffer and disrupted by a single passage through a pre-cooled French press at 35 MPa. The extraction buffer consisted of 50 mM Tris/HCl pH 7.5 containing 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 20 mM tripotassium citrate, all added immediately prior to use.

Whole cells and debris were removed from the extract by centrifugation at 45 000 g for 30 minutes at 4° C. The supernatant was filtered through Whatman No. 1 filter paper to remove solidified lipid and retained on ice for assay.

4.3 Determination of Protein Content Of Cell Free Extract.

The protein content was determined by the Biuret method (Robison and Hodgson, 1940): 0.5 ml of the cell free extract was placed

in a boiling tube, 1 ml of 3 M NaOH was added and the contents treated in a boiling water bath for 5 minutes. After cooling, 1 ml of $CuSO_4.5H_2O$ (2.5% w/v) was added and the contents mixed thoroughly. After 10 minutes the mixture was centrifuged at 10 000 g for 3 minutes and the A_{555} of the super natant was read against a reagent blank. A calibration curve was constructed using bovine serum albumen (0 - 5.0 mg/l); this was linear up to a concentration of 5.0 mg/ml.

4.4 ATP:citrate lyase Assay.

E.C.4.1.3.8., ATP:citrate oxaloacetate lyase was assayed at 30^{o} C by the coupled procedure of Srere and Lipmann (1953) in which the oxaloacetate product is reduced to malate by the action of malate dehydrogenase, with the concommitant decrease in A340 due to the oxidation of NADH.

The reaction mixture contained in 1.0 ml: Tris/HCl buffer, pH 8.3, 250 mM; $MgCl_2$, 10 mM; dithiothreitol, 1 mM; ATP, 10 mM; tripotassium citrate, 20 mM; coenzyme A, 0.2 mM; malate dehydrogenase (pigeon breast muscle, Sigma), 10 units and extract. Reactions were initiated by the addition of coenzyme A.

5. Determination Of The Total Lipid Content Of Yeast.

After 7 days' growth on NLM as in section 4.1, cells were harvested by centrifugation at 10 000 g for 20 minutes and washed once in 50 mM Tris/HCl buffer pH 7.5. The cells were resuspended in a small volume of distilled water and transferred to a round-bottomed glass vessel for lyophilisation.

Total lipid content was determined according to Folch et al. (1957). Lipid was extracted from approximately 500 mg of accurately weighed lyophilised yeast by overnight immersion in 150 ml chloroform/methanol (2:1 v/v). Cell debris was removed by filtration through Whatman No. 1 filter paper and the extract was washed successively with 25 ml NaCl (0.9% w/v) and 2 x 25 ml distilled water. The washed extract was dried with anhydrous $MgSO_4$. After filtration to remove solids the extract was evaporated to dryness. The lipid residue was dissolved in diethyl ether and transferred to a tared vial. The solvent was allowed to evaporate, then solvent residue was removed by drying in a vacuum oven at 50^{o} C. The vial was weighed after cooling in a desiccated vessel.

6. Assay For The Activity Of Carnitine Acetyl Transferase.

6.1 Growth Of Yeasts.

Yeasts were grown on NLM or triolein medium on an orbital shaker as described in section 4.1.

6.2 Preparation of Cell Free Extract.

The method of Tracy and Kohlhaw (1975) in which protease activity is reduced to a minimum was used. Cells were harvested by centrifugation at 10 000 g and washed once in 50 mM phosphate buffer pH 7.5. The cells were resuspended in 15 ml of the same buffer containing 1.5 mM phenyl methyl sulfonyl fluoride, 1.6 M $(NH_4)_2SO_4$, and disrupted by a single passage through a pre-cooled French press (35 MPa). The extract was stirred for 30 minutes at 4^{o} C, then centrifuged at 45 000 g for 30 minutes to

remove whole cells and debris. The extract was filtered through Whatman No. 1 filter paper to remove solidified lipid, then dialysed overnight at 4° C using narrow gauge dialysis tubing, against 250 x volume of 50 mM phosphate buffer pH 7.5. The protein content was determined as described in section 4.3 and the extract retained on ice for assay.

6.3 Assay of Carnitine Acetyl Transferase.

E.C.2.3.1.7., Carnitine acetyl transferase (CAT) was assayed at 30° C by the method of Kohlhaw and Tan-Wilson (1977), in which the formation of free CoA from acetyl CoA is continuously monitored by following the change in colour of 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) at 412 nm.

The reaction mixture contained in 1 ml: Tris/HCl buffer pH 7.5, 50 mM; DTNB, 0.4 mM; acetyl CoA, 0.1 mM; L-carnitine, 1.25 mM. Reactions were initiated by the addition of carnitine.

7. Protoplast Formation and Cell Wall Regeneration.

The method of Dickinson and Isenberg (1982), with some modifications, was used to make protoplasts from the strain IFO 0880.

7.1 Protoplast Formation From Strain IFO 0880.

The yeast was grown overnight in YMG medium on an orbital shaker at 190 rpm, 30° C. The culture was centrifuged and resuspended in fresh medium so that OD_{640}=0.8 (approximately 10^7 cells/ml). Incubation was continued as before for three hours to ensure the cells were in the exponential phase of growth. A 10 ml sample was then centrifuged and washed twice in 10 ml 0.6 M

sorbitol, 20 mM MES (2(N-Morpholino)ethanesulfonic acid) buffer pH 6.0. The cells were then resuspended in 1 ml 100 mM EDTA, 100 mM MES buffer pH 6.0, 5 mM dithiothreitol (added just before use) and incubated for a further 90 minutes. The cells were then centrifuged and washed once in 0.6 M sorbitol, 20 mM MES pH 6.0, and resuspended in 1 ml of protoplasting buffer containing 20 mg/ml Novozym 234 (Novo Enzyme Products Ltd.). The protoplasting buffer consisted of 0.6 M KCl, 0.01 M citric acid/Na_2HPO_4 buffer pH 5.5, 5 mM dithiothreitol (added just before use). The cell suspension was incubated on an orbital shaker at 190 rpm, 30^{o} C, for one hour.

The formation of protoplasts was checked by microscopic examination. Two loopfuls of the cell suspension were placed on a microscope slide. Protoplasting buffer (200 μl) was added to one and 200 μl of distilled water to the other. After mixing, the two preparations were examined and compared using a microscope. Protoplasting was assumed to be complete when all the cells in the distilled water lysed and those in the protoplasting buffer were spherical in appearance.

7.2 Regeneration Of The Cell Wall Around The Protoplasts.

The protoplasts were encapsulated in alginate beads to allow regeneration of the cell wall (Vidoli et al., 1982). The protoplasts were washed three times in 1.2 M sorbitol. Care was taken to centrifuge at low speed and not to form a vortex when mixing to prevent breakage of the cell membranes. The protoplasts were resuspended in 0.25 ml 1.2 M sorbitol and 2 ml 2% alginic acid was added. In a laminar flow cabinet the

protoplast suspension was dropped into 50 mM $CaCl_2$, from a sterile Pasteur pipette, to form alginate beads. The $CaCl_2$ was poured off and the beads were added to 100 ml YMG medium in a 250 ml conical flask. This was incubated on an orbital shaker for 5 days. The beads and medium were poured over sterile gauze to collect the beads, which were then washed twice in 0.6 M KCl. They were placed in a 35 ml bottle and 3 ml 0.1 M citrate buffer pH 6.0 was added. This was shaken at 30° C for 10 minutes to disrupt the beads. The cells were then diluted in 0.9% NaCl and plated on YMG plates.

8. Staining Of Intracellular Lipids.

8.1 Experiments with fluorochrome staining of intracellular lipids.

These experiments were carried out using the fluorochrome 1,8 naphthoylene-1',2'-benzimidazole (Pomoshchnikova et al., 1981).

Using a 3 day culture of R. toruloides IFO 0880 grown on NLM, a calibration curve was constructed relating OD_{640} to dry weight. A sample of the yeast culture was centrifuged and washed twice in 0.9% NaCl, then resuspended in 50 mM phosphate buffer pH 7.5 such that OD_{640} = 1.0. Two 10 ml portions of this cell suspension were put into 35 ml bottles. One of these was heated in a boiling water bath for 10 minutes, the other was left untreated, then 30 μl of a 1 mg/ml solution of the fluorochrome in 96% ethanol was added to each. Both suspensions were incubated in a shaking water bath at 30° C for 15 minutes, then centrifuged and washed three times in 10 ml buffer, and finally

resuspended in buffer. The OD_{640} of the suspensions was measured, the suspensions were diluted in buffer as necessary, and the fluorescence of the samples was measured. For use as the standard solution, 30 μl of a 1 mg/ml solution of the fluorochrome in 96% ethanol was added to 10 ml buffer.

The fluorescence of the stained suspensions was measured in a fluorimeter at the following wavelengths: absorption 425 nm, emission 495 nm. A graph was drawn relating yeast content of the suspension to fluorescence. See Results Section 4.1. Then in order to relate lipid content of the yeast to fluorescence the procedure was repeated using six different cultures of R. toruloides with different lipid contents. A sample of each culture was stained with fluorochrome as described above and the remainder of the culture was harvested and the lipid content determined as described in section 5.

8.2 Staining Of Replica Prints Of Yeast Colonies.

Yeast was grown on liquid NLM initially, then the culture was diluted and spread on NLM agar plates to give approximately 50 colonies per plate. The plates were incubated at 30° C until the colonies were 2 - 3 mm in diameter (three to four days). A replica plate was made using a block and sterile velvet pad. A filter paper (9 cm diameter Whatman No. 1) was then pressed onto the original plate to take a 'print' of the colonies. The filter paper was dried in a vacuum oven (20 min, 50° C in vacuo). The filter paper was added to a glass petri dish containing Sudan Black B (0.08% w/v in 95% ethanol) and left to stain for 20 minutes. The filter was removed using forceps and excess stain

washed off in a glass petri dish containing 95% ethanol by swirling gently for 3 minutes. The filter was then transferred to another dish containing 95% ethanol and left to destain for 5 minutes, removed and allowed to dry.

The lipid content of the cells grown on NLM plates was determined by spreading 0.2 ml of the liquid culture on agar plates. After 3 days incubation at 30^{o} C cells were harvested by adding 2 - 3 ml of distilled water and scraping the cells from the surface of the agar. The cell suspension was transferred to round-bottomed flasks and lyophilised, then the lipid content was determined as in section 5.

9. Mutagenesis.

8.1 Use Of Ultra Violet Light As A Mutagen.

Yeast was grown overnight in a 100 ml conical flask containg 20 ml YMG medium, on an orbital shaker at 190 rpm, 30^{o} C. Fresh medium was added to adjust the cell density of the culture to 5×10^{5} cells/ml. Two ml of this culture was spread over an empty, sterile plastic petri dish and placed under an ultra-violet lamp (λ=254 nm) at a distance of 25 cm from the light source. Samples (0.1 ml) were removed at 1 minute intervals, serially diluted in sterile 0.9% NaCl and spread on YMG plates. After 2 days incubation at 30^{o} C, the colonies on the plates were counted and a killing curve was constructed. See Results Section 6.1.

8.2 Use Of EMS As A Mutagen.

Yeast was grown for 2 days in a 250 ml conical flask containing

100 ml YMG, on an orbital shaker at 190 rpm, 30° C. The cell density of the culture was approximately 1 x 10^8 cells/ml. Five ml of the culture was centrifuged at 4000 rpm in a bench centrifuge, and washed twice in 10 ml 0.2 M phosphate buffer pH 8.0. The cells were then resuspended in 9.8 ml of this buffer pre-warmed to 30° C. A sample of 0.1 ml was removed for the control, and 0.3 ml of methane sulphonic acid, ethyl ester (EMS) was added. The suspension was incubated in a shaking water bath at 30° C and samples were removed at 5 or 10 minute intervals for up to 110 minutes. The samples were diluted as follows: 0.1 ml was added to 10 ml sodium thiosulphate, mixed, and held at room temperature for 10 minutes to inactivate the EMS. The sample was then serially diluted in 0.9% NaCl and plated on YMG plates. The plates were incubated at 30° C for 2 days, then the colonies were counted and a killing curve was constructed.

For the isolation of mutants from a culture, after the culture had been exposed to the mutagen for the desired length of time 10 ml of 12% sodium thiosulphate was added to the cell suspension (to give a final concentration of 6%). The suspension was held at room temperature for 10 minutes to inactivate the mutagen and then centrifuged and washed once in 6% sodium thiosulphate, then twice in 0.9% NaCl. The required procedure for the isolation of mutants was then followed (see section 10.1 and 12).

9.3 Use Of NTG As A Mutagen.

The method for the use of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was the same as that for EMS, except that the buffer used

for washing and resuspending the yeast was 0.2 M citrate buffer pH 5.0. NTG was added as a solid to the washed cell suspension to give a final concentration of 0.1% w/v.

10. Nystatin Enrichment For Auxotrophs.

10.1 Enrichment Method.

The method of Snow (1966) was used. The mutagenised culture was divided into 1 ml portions and each was added to 9 ml YMG in a 25 ml conical flask. The cultures were incubated on an orbital shaker for 2 days at 190 rpm, 30^{o} C. The cultures were harvested by centrifugation and washed twice with sterile 0.9% NaCl, then resuspended in 10 ml MM-N and transferred to a 25 ml conical flask. These cultures were incubated overnight, then centrifuged and resuspended in 9 ml MM in a 25 ml conical flask. Nystatin solution was prepared by dissolving nystatin in 95% ethanol at a concentration of 1 mg/ml. One ml of this solution was added to 9 ml sterile distilled water and after 6 hours incubation in MM, 1 ml of nystatin solution was added to each culture to give a final nystatin concentration of 10 μg/ml. Incubation was continued for 20 to 60 minutes, then the cultures were centrifuged, washed twice in 0.9% NaCl and plated on YMG plates. Throughout the procedure the cell density was kept below 1×10^{8} cells/ml to prevent cross-feeding.

10.2 Use Of Nystatin To Kill Growing Cells Selectively.

A three day old yeast culture grown in YMG was harvested by centrifugation, washed twice in MM-N and resuspended in 10 ml volumes of MM-N to give a cell density of approximately 1×10^{7}

cells/ml. The cultures were incubated in a shaking water bath overnight, centrifuged and resuspended in 0.9% NaCl. One ml of the suspension was added to either 8 ml MM-N or 8 ml MM. The cultures were incubated for a further 6 hours, then nystatin was added as described in section 9.1. Incubation was continued and samples were removed after 0, 20, 40 and 60 minutes. Samples were washed and diluted in 0.9% NaCl and plated on YMG plates. These were incubated at 30^{o} C for 3 days before counting the colonies. Throughout the experiment the cell density was kept below 1 x 10^{8} cells/ml to prevent cross-feeding.

11. Isolation Of Auxotrophs.

Yeast was exposed to 3% EMS or 0.1% NTG as described in sections 8.2 and 8.3 until 90% of the cells were killed, as determined previously from the results of the killing curves. After the required exposure time and washing to remove the mutagen, nystatin enrichment for auxotrophs was carried out as described in section 10.1. After plating on YMG plates and incubating for 2 days at 30^{o} C, the colonies were replica plated to MM plates. Any colonies failing to grow on the MM plates were identified on the original YMG plates and transferred to fresh YMG plates. After overnight incubation these colonies were then replica plated to a series of nine pool plates (Cold Spring Harbor Laboratory Manual - Methods in Yeast Genetics.) to determine the growth requirement of the auxotrophs. The pool plates were prepared by adding supplements to MM agar, as shown in Table 2. Reversion of auxotrophs to prototrophy was measured by growing a single colony of the auxotroph in supplemented minimal medium (40 ml in 100 ml conical flask, incubated with shaking at 30 C, 48h). The cells were washed, diluted and plated on MM and supplemented MM plates. The reversion frequency was calculated from the proportion of the population capable of growth on MM.

Table 2

Composition Of Pool Plates For Auxanography.

Pool No.	Supplement	Final Concn. mg/l	Pool No.	Supplement	Final Concn. mg/l
1	adenine sulphate	20	6	adenine sulphate	20
	L-histidine HCl	20		guanine HCl	20
	L-phenylalanine	50		L-cysteine	20
	L-glutamic acid	100		L-methionine	20
				uracil	20
2	guanine HCl	20			
	L-leucine	30	7	L-histidine HCl	20
	L-tyrosine	30		L-leucine	30
	L-serine	375		L-isoleucine	30
				L-valine	150
3	L-cysteine	20		L-lysine HCl	30
	L-isoleucine	30			
	L-tryptophan	20	8	L-phenylalanine	50
	L-alanine	20		L-tyrosine	30
				L-tryptophan	20
4	L-methionine	20		*L-threonine	200
	L-valine	150		L-proline	20
	*L-threonine	200			
	*L-aspartic acid	100	9	L-glutamic acid	100
				L-serine	375
5	uracil	20		L-alanine	20
	L-lysine HCl	30		*L-aspartic acid	100
	L-proline	20		L-arginine HCl	20
	L-arginine HCl	20			

Stock solutions of the above solutions were added to MM to give the final concentrations shown above, then sterilised by autoclaving. Those marked * were filter sterilised and added aseptically after autoclaving.

12. Attempted Isolation Of ACL-less Mutants.

The following procedures were used in an attempt to isolate mutants of R. toruloides strain 7203 without active ATP:citrate lyase. Strain 7203 is an very stable inositol requiring mutant of IFO 0880, which was the kind gift of Dr. M Tully, CAMR, Porton Down. This strain was used as it was felt that the presence of an additional genetic marker in an ACL-less strain would be of use in further genetic experiments, and so a strain already carrying a stably inherited genetic marker was chosen as the parent strain.

12.1 Determination of Citrate.

The method of Williamson and Corkey (1969) was used to determine the concentration of citrate in the growth medium of acid accumulating strains. The citrate is cleaved by citrate lyase (Sigma, from Enterobacter aerogenes) producing oxaloacetate which is reduced to malate by the action of malic dehydrogenase. The increase in A_{340} due to the oxidation of NADH is measured. The reaction mixture contained in 1 ml; triethanolamine HCl buffer pH 7.4, 0.05 M, containing 10 mM MgSO4 and 5 mM EDTA; NADH, 0.1 mM; citrate lyase, 0.5 units; malic dehydrogenase, 5 units.

Reactions were initiated with citrate lyase and allowed to go to completion. A calibration curve was constructed using 0 - 0.8 mM tripotassium citrate.

12.2 Procedure For The Isolation Of ACL-less Mutants.

A 2 day culture of strain 7203 was exposed to EMS for 100 minutes as described in section 9.2. After inactivation of the mutagen

and washing, the cells were resuspended in 2.0 ml YMG, divided between 20 25 ml conical flasks each containing 10 ml YMG and incubated in a shaking water bath at 30° C for 2 days. The 20 cultures were each spun down, washed in 0.9% NaCl twice and resuspended in 1 ml enrichment medium; 0.1 ml of each suspension was then used to inoculate 20 volumes of 10 ml enrichment medium (which contains fluoroacetate) in 25 ml conical flasks. These cultures were incubated with shaking at 30° C for 5 days, then diluted in 0.9% NaCl and plated on indicator plates. Cultures of 7203 and Yarrowia lipolytica NCYC 825 (a citrate accumulator) were plated as controls. After 3 days incubation at 30° C the plates were examined for a colour change and any colonies which had changed the colour of the agar from blue to yellow by lowering the pH of the medium were patched onto YMG plates and replica plated to MM, MM + inositol, indicator and YMG plates. Those that grew on MM + inositol, YMG, and changed the colour of the indicator plates were grown in NLM for 2 days and the citrate concentration of the medium was determined as described in section 12.1. Any isolates which had caused a build up of citrate in the growth medium were then assayed for ATP:citrate lyase activity.

13. Determination Of DNA Content.

13.1 Calibration Curve.

A calibration curve for the diphenylamine assay was prepared as follows. E. coli DNA (Sigma) was dissolved in 5 mM NaOH at a concentration of approximately 0.3 mg/ml. A standard solution of

DNA was prepared by mixing this solution with an equal volume of 1.0 M $HClO_4$ and heating to 70° C for 15 minutes. The DNA content was determined spectrophotometrically. The standard solution of DNA was diluted in 0.5 M $HClO_4$ and 1 ml was added to 2 ml 2% diphenylamine in glacial acetic acid (prepared just prior to use). 0.1 ml 54 mM acetaldehyde was added with thorough mixing and the samples were incubated at 30° C overnight. The optical density at 595 nm and 700 nm was then determined (Burton, 1955, Burton, 1968, De Ley, 1971, Stuart-Riggsby et al., 1982,).

13.2 Cell Counting.

To determine the DNA content per yeast cell the strain to be tested was grown in YMG for three days, then centrifuged and washed three times in distilled water. The cells were resuspended in distilled water and samples were removed for counting. Sodium dodecyl sulphate (SDS), 0.001%, was included to prevent clumping of the cells, and samples of the cells diluted in distilled water and mixed well were counted in a haemocytometer. At least 50 squares were counted and this was repeated three times for each sample. The yeast suspension was diluted to contain 1.25 to 5.0 x 10^8 cells/ml. 1 ml samples were dispensed and stored at -20° C in sealed vials until required (Sarachek, Rhoads and Schwarzhoff, 1981, Rhoads and Sarachek, 1984).

13.3 Dry Weight Determination.

To determine the DNA content per unit dry weight of yeast, 10 ml of the washed yeast suspension was centrifuged in a dried,

weighed centrifuge tube. The supernatant was decanted and the tubes were dried in a 50° C oven for 24 hours. The tubes were cooled in a desiccator and then weighed. Two tubes were used for each sample. The washed yeast suspension was dispensed into 1 ml volumes and stored at -20° C until required.

13.4 Extraction of DNA.

To extract the DNA from the yeast cells, (Bostock, 1970) 1 ml 1 M $HClO_4$ was added to the 1 ml yeast samples, and incubated at 0° C for 30 minutes to remove cold acid soluble material. The sample was then centrifuged in an Eppendorf bench centrifuge and washed twice in cold 0.5 M $HClO_4$. The pellet was resuspended in 1 ml 0.5 M $HClO_4$ and heated to 70° C for 20 minutes, then centrifuged and the supernatant decanted and retained. The pellet was resuspended in 1 ml 0.5 M $HClO_4$ and heated to 70° C for 20 minutes, centrifuged and the supernatants from the two extractions were pooled. 1 ml of the pooled supernatants was used for the diphenylamine assay as described in section 13.1. As slight turbidity of the samples may give incorrect results, the optical density at 595 nm and 700 nm was determined and $A_{595} - A_{700}$ was used to calculate the DNA content of the sample. 1 ml of 0.5 M $HClO_4$ incubated with diphenylamine and acetaldehyde was used as a control.

14. Isolation Of Basidiospores.

Rhodosporidium toruloides IFO 10076 produces colonies with a rough surface when grown on agar plates. When examined under the microscope these colonies are seen to consist of yeast cells

which develop into mycelium after 2 days incubation. The mycelium grows out from the colony and down into the agar to form a ring around the colony. After further incubation, teliospores are produced on the mycelium and cause the appearance of a dark brown ring around the colony. Teliospores from this strain were used to isolate single basidiospores. Using a 10 day old plate culture of IFO 10076, free yeast cells were scraped from the surface of the agar. The agar containing the mycelium and teliospores, which grow down into the agar, was then collected by cutting 1 cm squares with a sterile blade. Two squares were suspended in 10 ml sterile H_2O. This preparation was then homogenised to produce a suspension of agar, yeast cells, clumps of mycelium with teliospores attached and free teliospores.

Sucrose gradients were prepared by holding a centrifuge tube vertically in a test tube rack and adding 35 ml sterile 20% sucrose solution. This was frozen to -70^{o} C, then allowed to thaw at 2^{o} C. (University of Leicester, Basic Cloning Techniques Course Manual). Three ml of the suspension containing teliospores was cooled to 2^{o} C, then layered onto the sucrose gradient and centrifuged in a bench centrifuge with a swing-out rotor, 3000 rpm for 3 minutes at 2^{o} C. Pellets from two such gradients containing clumps of mycelium with attached teliospores but very little agar or other material were combined, washed in H_2O to remove sucrose and again centrifuged on a sucrose gradient. The pellet was washed and dried at 52^{o} C overnight to kill yeast cells and mycelium. This preparation was then resuspended in distilled water and retained at 4^{o} C overnight.

This rehydration of the spores was found to lead to quicker and more uniform germination. The preparation was then incubated in 10 ml YMG medium on a shaking incubator at 30° C, 190 rpm. Samples were removed at intervals for microscopic examination. After 10 hours the first basidiospores were produced. The suspension was then sonicated for 30 seconds to free the basidiospores from the promycelia. One ml of the suspension was layered onto a 12 ml sucrose gradient. This was centrifuged for 2 minutes at 2000 rpm, 2° C, in a bench centrifuge with a swing-out rotor. Fractions, 0.5 ml, were removed from the top of the gradient and examined for the presence of basidiospores. The top six fractions containing single basidiospores were plated on YMG agar. After 2 days incubation at 30° C, small smooth colonies were formed. See Fig 5.

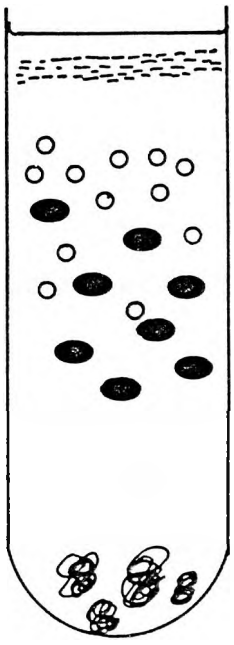
15. Continuous Culture of Rhodosporidium toruloides.

Three different chemostats were used for the continuous culture of R. toruloides, with working volumes of 1.5, 2.1 and 3.5 litres. Constant volume was maintained by an overflow device in the 2.1 litre chemostat and by pumped constant level devices in the 1.5 and 3.5 litre chemostats. In all other respects the three chemostats were identical. Stirring was achieved using flat bladed impellers at 400 rpm. The air supply was passed through two sterile 0.2 μ PTFE membrane filters connected in series. The aeration rate was maintained at 1 vol air/vol medium/minute. Antifoam addition was maintained at preset intervals using a timing device and pump. The pH was maintained

FIGURE 5

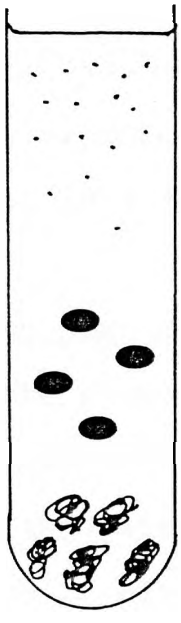
Separation of teliospores and basidiospores on sucrose gradients.

a). Results of first spin.
5 minutes at 3000 rpm.
The pellets from two tubes were washed, combined and centrifuged again on an identical sucrose gradient.



After washing and drying the pellet from the second spin, the teliospores were allowed to germinate, then sonicated to free the basidiospores from the promycelia.

b). Results of third spin.
2 minutes at 2000 rpm.



at 5.5 by the automatic addition of KOH, and the pH of samples taken from the chemostat was checked against a standard buffer solution daily. Temperature was maintained at 30° C by pumping water from a thermostatically controlled water bath around stainless steel tubing inside the chemostat pot. The assembled apparatus was sterilised by autoclaving at 121° C for 45 minutes. The sterile chemostat was then filled with CLM, which had been stored in 20 litre aspirators for several days before use to ensure its sterility.

Samples from the chemostat were removed through an air lock device into sterile screw-top bottles. The inoculum was prepared by incubating the required strain of yeast in 100 ml CLM in a 250 ml conical flask with a bottom outlet in a shaking incubator at 30° C. To ensure that the chemostat was free from contamination with any other micro-organism the following checks were made. After filling the chemostat with medium it was allowed to run for 24 hours at the appropriate temperature, aeration rate and pH to ensure its sterility before inoculation, After inoculation, samples were removed daily, examined microscopically and streaked on YMG agar plates which were incubated at 30° C for 7 days and examined at intervals for the presence of contaminants.

15.1 Determination Of The DNA Content Of Chemostat Cultures.

To determine the DNA content of cells from the chemostat cell population a sample of 100 µl was used to inoculate 100 ml YMG medium in a 250 ml conical flask. This was incubated at 30° C in a shaking incubator for 4 days, and the cells were then

harvested and the DNA content determined as in sections 12.2 to 12.4

15.2 Lipid Accumulation In Cells From Chemostat Cultures.

To determine the ability of the chemostat cell population to accumulate lipid, a sample of 8 ml was used to inoculate 800 ml NLM in a 1 litre vortex aerated vessel which was incubated at 30° C for 7 days. Cells were then harvested and the lipid content determined as in section 5.

15.3 Biomass Determination.

For routine measurements growth was monitored by measuring the A_{640} of cell suspensions diluted in distilled H_2O using distilled H_2O as a blank. The result was then compared to a calibration curve of A_{640} against dry weight of the yeast.

16. Electron Microscopy Of The Mycelial Stage.

The mycelial form of R. toruloides IFO 10076 was grown on YMG agar plates incubated at 30° C until a ring of brown mycelium surrounded the colonies. The agar was then flooded with 4% glutaraldehyde fixative in 0.1M sodium cacodylate buffer at pH 7.0. After one hour, pieces of agar less than 1 mm^3 were cut from the plate. The samples were then rinsed twice (one hour each) in 0.1 M sodium cacodylate buffer pH 7.2 before post-fixing in 1% osmium tetroxide in the same buffer for 12 hours at 4° C. After a brief rinse in buffer, dehydration was carried out in increasing concentrations of ethanol. The specimens were embedded in Araldite resin (Glauert and Glauert, 1958) and

polymerised for 2 days at 60° C.

Ultrathin sections (90 - 150 nm) were cut on glass knives using a Huxley Mark II ultramicrotome, and mounted on 150 mesh Formvar coated copper grids. Sections were stained with 2% aqueous uranyl acetate for 30 minutes and lead citrate for 10 minutes (Reynolds, 1963).

A Jeol 100c transmission electron microscope at 80 KV was used to examine the sections.

I am extremely grateful to Mrs. J. Mundy of the Department of Zoology, Hull University, for carrying out the specimen preparation and electron microscopy of *R. toruloides*.

RESULTS.

1. ATP:citrate Lyase Activity And Lipid Content In Rhodosporidium toruloides.

The enzyme ATP:citrate lyase (ACL) is regarded as the key enzyme of oleaginicity and has been found in all oleaginous yeasts and moulds examined to date. Many strains of R. toruloides are available from culture collections, but the lipid content and ACL activity have been measured in relatively few strains. For this study the activity of ACL and lipid content of 21 strains of R. toruloides grown on NLM in batch culture were measured. It had been noted (Evans, 1983) that the lipid content of R. toruloides was increased if the nitrogen source in NLM was sodium L-glutamate instead of NH_4^+. Both of these nitrogen sources were used in this study to see if this is the case with all strains of R. toruloides. The results are given in Table 3.

The highest lipid content recorded was 30% of dry weight, and the lowest 3.8%, with the remainder distributed more or less evenly between these two points. In all except three cases the lipid content was higher when the nitrogen source was glutamate instead of NH_4^+. The ACL activities also varied widely, and no correlation between ACL activity and lipid content was found. One strain, CBS 315, had no ACL activity, and the lipid content of this strain was very low.

2. Carnitine Acetyl Transferase Activity.

In yeasts that do not have the the enzyme ACL, transport of

Table 3

Lipid content and ATP:citrate lyase activity in 21 strains of R. toruloides grown on NLM, with either sodium glutamate or ammonium tartrate as the nitrogen source.

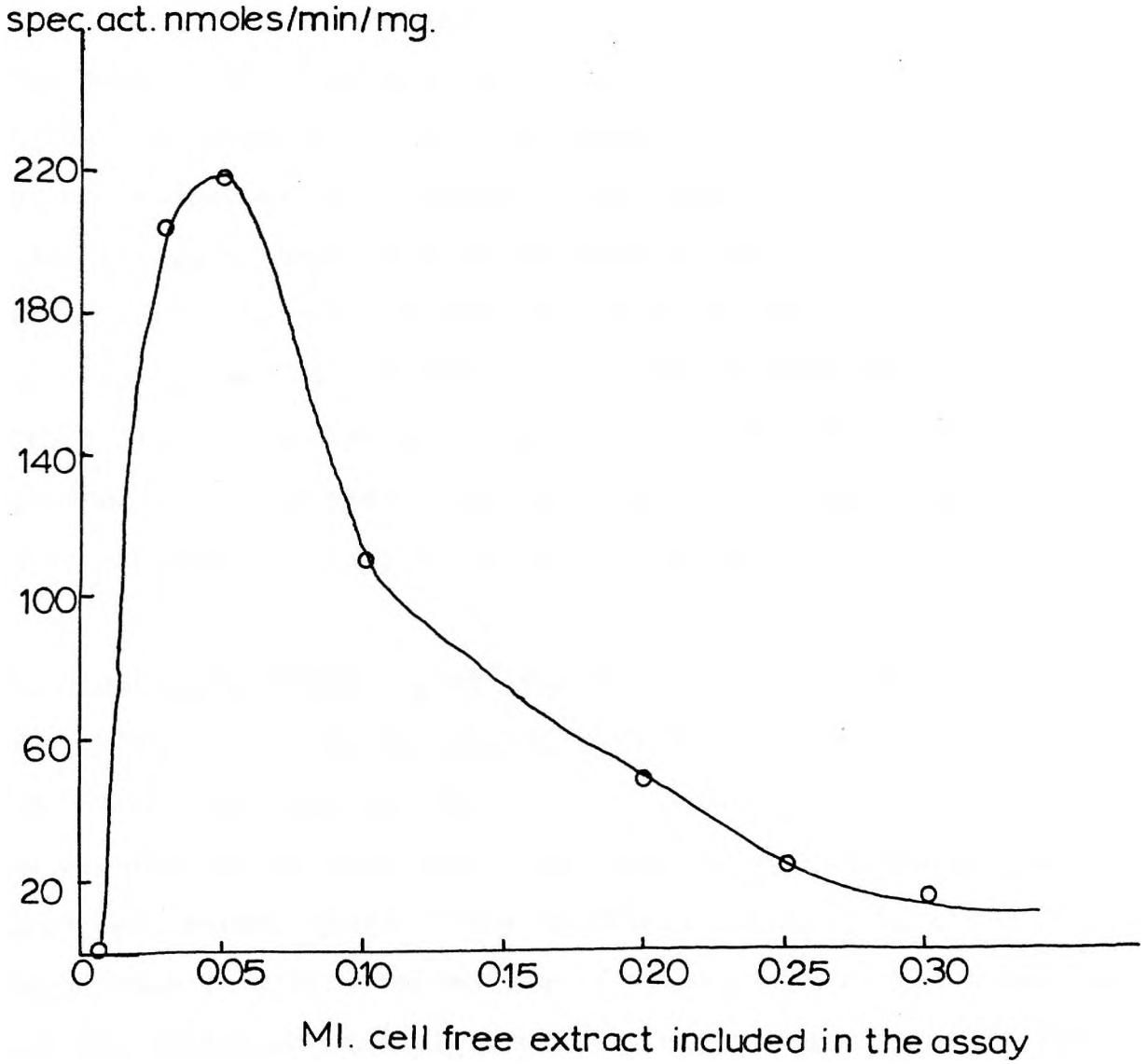
	GLUTAMATE		NH_4^+	
Strain	Lipid %	ACL*	Lipid %	ACL*
IFO 8766	30	56	14	57
NCYC 921	28	34	11	11
ATCC 26217	25	86	13	12
CBS 6681	25	32	14	40
CBS 2370	20	36	9	14
IFO 1236	19	24	4	16
IFO 10075	18	54	5	15
CBS 14	18	41	17	87
IFO 0871	18	36	2	13
ML 2589	17	57	6	22
CBS 5490	17	59	8	13
IFO 0880	15	31	20	29
IFO 0559	15	31	20	29
IFO 10076	14	55	8	33
NCYC 979	14	7	4	8
CBS 350	14	28	24	30
CBS 6450	9	8	7	13
ML 2573	6	80	2	84
ML 2921	5	52	4	11
ML 2590	4	45	2	16
CBS 315	4	0	4	0

* Specific activity of ACL in nmoles/min/mg protein.

acetyl groups out of the mitochondrion for fatty acid biosynthesis in the cytoplasm is mediated by the activity of carnitine acetyl transferase (CAT). In oleaginous yeasts an alternative route is provided by the action of citrate synthase in the mitochondrion converting acetyl CoA and oxaloacetate to citrate, which then crosses the mitochondrial membrane and is cleaved to acetyl CoA and oxaloacetate by ACL in the cytoplasm. This route is known to be operative in oleaginous yeasts, but it is not known whether this occurs instead of, or as well as, transport via CAT. The CAT activity in 7 strains of R. toruloides, 5 other strains of oleaginous yeast and 2 non-oleaginous yeasts was measured.

Initial comparisons of CAT activity in cell free extracts of Candida utilis NCYC 359 and R. toruloides IFO 0880 showed variable, generally low specific activities. Further investigation, carrying out the enzyme assay with a cell free extract of C. utilis, showed that the specific activity recorded depended on the amount of cell free extract included in the assay system (See Fig 6). This suggested the presence of an inhibitor in the cell free extract which was then dialysed for 16 h against 500 ml 50 mM phosphate buffer, pH 7.5 at 4^{o} C. The assay was repeated with the result that the specific activity was found to be independent of the volume of cell free extract included in the assay, and was slightly higher than the highest value previously recorded (250 nmoles/min/mg protein as opposed to a maximum of 218 nmoles/min/mg protein before dialysis). All subsequent assays were therefore carried out with dialysed cell

Fig. 6



Carnitine acetyl transferase activity in non-dialysed cell-free extract of Candida utilis NCYC 359

free extracts. The results are shown in Table 4.

It had been observed that CAT activity in Candida tropicalis was considerably increased when alkanes were used as the sole carbon source in the growth medium (Ueda et al., 1982). A 20-fold increase in activity compared to that found with cells grown on glucose was observed. The yeasts listed in Table 4 will not utilize alkanes as a carbon source, but most will grow on fatty acids or triacylglycerols. Four of the yeasts were grown on triolein medium and CAT activity was determined as before (See Table 5). In all cases a considerable increase in activity occurred, with one yeast, Candida curvata D, showing a 30-fold increase over activity in glucose grown cells.

3. Protoplast Formation And Cell Wall Regeneration.

The life cycle of R. toruloides consists of two haploid yeasts mating to form mycelium which produces diploid resting spores. On germination meiosis occurs and haploid basidiospores are produced (Banno, 1967). Thus in theory clssical genetic techniques involving the mating of two strains carrying markers, and the isolation of haploid progeny may be used to study the genetics of R. toruloides. However in practice this is not the case. It would seem that some strains are aneuploid (Bottcher et al., 1980), and when these strains sporulate, not all the basidiospores are viable (Samsonova and Bottcher, 1980). I have found that if two haploid strains are mated, mycelial growth is slow and teliospore production does not occur for several weeks. It is difficult to separate the mycelium from the parent yeasts,

Table 4

Specific activities of CAT and ACL, and lipid contents of various yeasts grown on glucose.

All yeasts were grown on glucose/glutamate NLM.

Species	Strain	CAT activity (nmol/min/mg protein).	ACL activity (nmol/min/mg protein).	Lipid %
R. toruloides	NCYC 921	7	34	28
	IFO 8766	10	50	30
	CBS 315	80	0	4
	ML 2590	53	45	4
	ML 2921	47	52	5
	ATCC 26217	104	85	25
	IFO 0880	30	40	15
L. starkeyi	CBS 1809	13	46	40
	CBS 6047	12	27	41
C. curvata	R	43	5	40
	D	56	7	34
T. cutaneum	40	39	10	15
C.utilis	NCYC 359	250	0	6
S.cerevisiae	NCYC 817	193	0	3

Table 5

CAT activities in four yeasts grown on triolein.

Yeast	CAT activity on triolein medium (nmol/min/mg protein).	Fold increase over activity in glucose-grown cells.
R. toruloides CBS 14	477	5.0
T. cutaneum 40	162	4.2
C. curvata D	1638	29.3
R. toruloides ATCC 26217	928	8.9

as the mycelium grows over the surface of the agar extremely slowly and its progress tends to be followed by the yeast, so that only a small area of mycelium is free from yeast cells. Cutting very small sections of mycelium from the plate to transfer to another plate usually results in the death of the mycelium. Teliospore production is sparse, and in order to isolate progeny from these the teliospores must be separated from the mycelium and allowed to germinate. The only procedure available for the isolation of basidiospores is micro-manipulation, which results in low viability of the isolated basidiospores, since the separation of the spores from the promycelia often results in cell damage.

Random spore analysis may be carried out instead of isolating basidiospores from teliospores. For this to be done, the teliospores are allowed to germinate on agar plates and the micro-colonies that form from basidiospores are picked off and grown separately before determining their phenotype. The disadvantage of this method is that because teliospores produce 2 a and 2 α basidiospores, spores (and yeast cells that bud from them) of opposite mating type are growing in extremely close proximity on an agar plate, providing ideal conditions for mating. In theory, if mating does take place this should lead to further mycelial growth, but Banno (1967) reports the existence of some diploid yeasts. The micro-colonies may, therefore, be the haploid yeast grown from a single basidiospore, or diploid yeast produced by the mating of two haploid cells. Even if no mating takes place, micro-colonies growing from basidiospores

that were produced by the same teliospore are extremely close together and it would be very difficult to be certain that any colony picked was the product of one basidiospore, and not the result of several micro-colonies joining together.

Recently new techniques of genetic manipulation and analysis have been developed. Protoplast fusion and genetic engineering are both methods that may be used to study the genetics of R. toruloides and indeed have already been used with this yeast (Samsonova et al., 1980, Tully and Gilbert, 1985). Both of these techniques require that the cell wall of the yeast is removed and later allowed to regenerate. As it was felt that transformation with a plasmid or protoplast fusion may be used in this research project to study the genetics of lipid accumulation in R. toruloides, experiments to find a successful method of removing and regenerating the cell wall of R. toruloides were carried out. These experiments are described below.

3.1 Protoplast Formation.

Protoplasts of strain IFO 0880 were formed using the method described in Materials and Methods Section 7. Samples were taken and examined at intervals for the formation of protoplasts. After 40 minutes incubation, approximately 80 % of the cells appeared to have formed protoplasts; that is they were spherical in shape and osmotically sensitive. After 60 minutes, 100% of the cells has formed protoplasts.

The experiment described above was carried out using the protoplasting enzyme preparation Novozym 234 at a

concentration of 20 mg/ml. Novozym 234 contains three enzymes. The main activity is 1,3 α glucanase, but 1,3 β glucanase and 1,6 β glucanase are also present. The Novozym 234 was used immediately after it was obtained from the suppliers, Novo Enzyme Products Ltd. When the experiment was repeated some nine months later, no protoplasts were formed, even when the concentration of Novozym 234 was increased to 50 mg/ml. However it was found that protoplasts were formed as before if Lyticase (Sigma, 1,3 β glucanase) was included in the protoplasting mixture at a concentration of 1 mg/ml as well as 20 mg/ml Novozym 234. Lyticase alone had little effect on the cells, causing approximately 10% of the cells to become osmotically sensitive after 2 hours incubation. If β-glucuronidase (Sigma, 1,6 β glucanase) was included at a concentration of 1 mg/ml with Novozym 234 at 20 mg/ml, approximately 80% of the cells became osmotically sensitive after an incubation period of one hour.

3.2 Cell Wall Regeneration.

Protoplasts were encapsulated in alginate beads to allow cell wall regeneration. See Materials and Methods Section 7.2. After 5 days incubation in alginate beads susupended in YMG medium, the beads were disrupted and the cells were plated on YMG plates.

An experiment was carried out to determine the proportion of viable cells formed after protoplast formation and cell wall regeneration. A culture of IFO 0880 was divided into two portions after the pre-incubation stage. Novozym 234 (recently obtained from the suppliers) was added to one of the

two suspensions and the procedure was continued as before. After one hour's incubation, 100% of the cells to which Novozym 234 had been added had formed protoplasts. Both preparations were carefully washed in 1.2 M sorbitol, and encapsulated in alginate beads. After incubation, disruption of the beads and inoculation of YMG plates, the number of colonies formed from each preparation was counted. The results showed 3.79×10^9 colony forming units/ml in the disrupted beads from the control culture (no Novozym 234 added) and 6.87×10^8 c.f.u./ml in the culture which had been incubated with Novozym 234. Therefore 18% of the population were viable after removing the cell walls, washing and allowing the cell walls to regenerate.

4. Staining Of Intracellular Lipids.

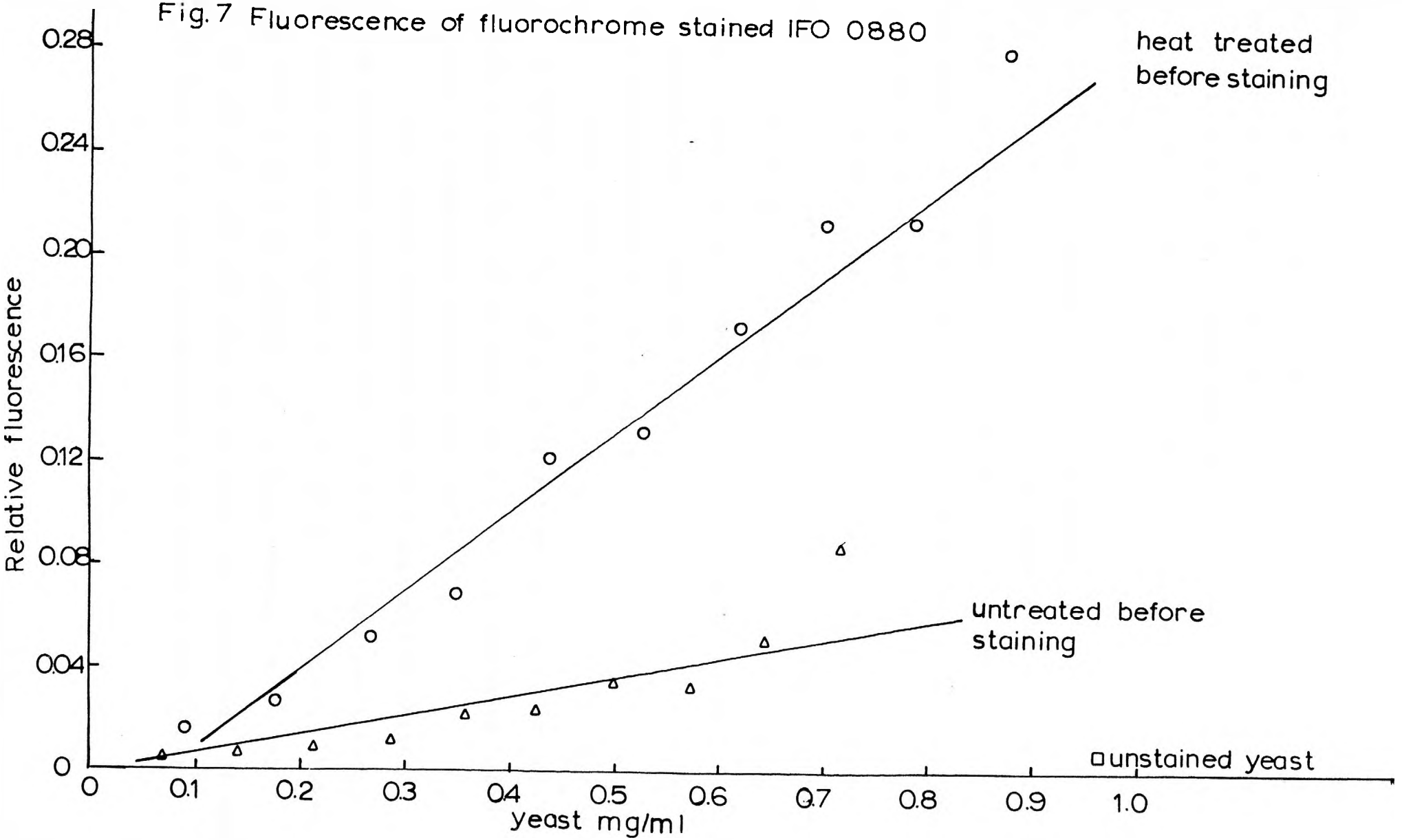
The method for determining the lipid content of yeast described in Materials & Methods section 5 gives accurate repeatable results and because the lipid is extracted from the yeast in order to measure the amount produced, the lipid is then available for further analysis. However this method has the disadvantage that the yeast must first be freeze dried, then extracted with chloroform and methanol overnight, and then the extract must be washed several times. After each wash it may take several hours for the aqueous and non-aqueous phases to completely separate, and after the solvent has finally been evaporated leaving behind the lipid, the lipid must be redissolved and dried. Thus the results are not available until at least two days after the cells were harvested. Also the cultures harvested to determine the

lipid content of the cells must contain at least 500 mg dry weight of yeast. In order to study the genetics of lipid accumulation, or to identify potential lipid accumulators among a group of isolates, a fast screening method is desirable. Ideally the results should be available within a few hours, and require only a small volume of liquid culture, or a colony growing on an agar plate as the test material. It may be possible to speed up or miniaturise the lipid extraction procedure to some extent, but a method which measures lipid inside the cell, using a stain to identify the lipid, will clearly be faster. However for such a method to be succesful, the stain must reach all the intracellular lipid and not only the more accessible cell membrane lipids.

4.1 Staining Of Liquid Cultures With Fluorochrome.

The method of Pomoshchnikova *et al.* (1981) was examined as a potential fast screening method. This method involves use of the fluorochrome 1'8 naphthoylene-1,'2'-benzimidazole to stain intracellular lipids in washed yeast suspensions. In preliminary experiments, *R. toruloides* IFO 0880 grown on NLM was stained with the fluorochrome and the fluorescence of the stained yeast suspension was measured (See Fig 7). As a correlation between the amount of lipid-containing yeast present and fluorescence was shown, the staining procedure was repeated with yeast cells having lipid contents between 1.1 and 16.7% of the biomass. Pomoshchnikova *et al.* had shown a correlation between lipid content and fluorescence of stained yeasts with a lipid content of up to 8%, using *Candida tropicalis* and *Saccharomyces*

Fig. 7 Fluorescence of fluorochrome stained IFO 0880



cerevisiae. However in this present study with R. toruloides, no such correlation was found, either with untreated or heat treated cells (See Table 6). Varying the concentrations of yeast between 1.0 and 10 mg/ml or fluorochrome (0.003 to 0.06 mg/ml), or the staining conditions (longer periods of incubation, higher incubation temperature) had little effect on the results.

4.2 Staining Of Replica Prints Of Colonies.

The detection of 'high lipid' colonies among a background of low lipid' colonies growing on an agar plate, for instance after exposure to a mutagen, would allow large numbers of individual colonies to be screened rapidly.

In this case there is an additional difficulty to be overcome. Colonies growing on a plate must have a lipid content indicative of that which would be produced if grown in large scale liquid culture. That is, cells which would accumulate high concentrations of lipid in liquid culture must also accumulate high concentrations of lipid when growing on a plate.

Yeast grown on NLM agar has a maximum lipid content of approximately 5%. The use of different media with lower sodium glutamate contents and no yeast extract led to increased lipid contents in oleaginous strains (See Table 7).

Even higher concentrations were achieved by omitting sodium glutamate completely and including 0.8 g/l yeast extract. The yeast was first grown in liquid medium on an orbital shaker, then plated on solid medium (of the same composition as the liquid medium, with the addition of agar) and incubated. It was found that high lipid contents could be produced after only 2

TABLE 6

Fluorescence of stained yeast suspensions with different lipid contents.

Untreated yeast.

Strain	Days Growth	Yeast mg/ml	Lipid cont.%	Lipid mg/ml	Fluores-cence	Fluores-cence: lipid ratio
IFO 0880	7	5.2	1.2	0.0624	0.050	0.80
IFO 0880	3	4.8	1.1	0.0528	0.042	0.79
CBS 14	7	3.0	3.9	0.1170	0.029	0.25
CBS 2370	7	1.1	16.7	0.1837	0.020	0.11
IFO 8766	7	2.4	11.5	0.2760	0·031	0.11
ML 2573	7	1.4	1.2	0.0168	0.016	0.95

Heat treated yeast.

Strain	Days Growth	Yeast mg/ml	Lipid cont.%	Lipid mg/ml	Fluores-cence	Fluores-cence: lipid ratio
IFO 0880	7	4.1	1.2	0.0492	0.058	1.18
IFO 0880	3	3.8	1.1	0.0418	0.041	0.98
CBS 14	7	4.6	3.9	0.1794	0.019	0.11
CBS 2370	7	2.7	16.7	0.4509	0.041	0.09
IFO 8766	7	4.6	11.5	0.5290	0.086	0.16
ML 2573	7	2.1	1.2	0.0252	0.039	1.55

Fluorescence of standard solution of fluorochrome = 0.20.

TABLE 7

Lipid content of cells grown on agar plates with varying concentrations of sodium L-glutamate (no yeast extract included).

Sodium L-glutamate g/l	Total dry wt of cells from 10 plates g	Lipid content of biomass %
0.00	0.084	8.8
0.01	0.137	11.0
0.10	0.460	18.4
1.00	1.564	14.7

Preparation of plates.

Samples (0.2 ml) of a 3 day culture of IFO 0880 grown on NLM were spread on each plate. After incubation at 30 ^{o}C for 3 days, the cells were harvested by adding 2 - 3 ml distilled H_2O and scraping the cells from the surface of the agar.

days growth in liquid medium followed by 3 days on solid medium (See Table 8).

It had been reported (C. T. Evans, personal communication), that high and low lipid contents could be detected in yeast colonies by the staining of replica prints with Sudan Black B in ethanol. Non-oleaginous yeast colonies took up little or no stain, whereas those with a high lipid content stained deep blue or black. The method was said to work satisfactorily with Candida curvata, Trichosporon cutaneum and Lipomyces starkeyi, but had not been used with R. toruloides. Using this staining method, although it was possible to repeat the reported results with Candida and Lipomyces species, it was not possible to detect differences between Rhodosporidium colonies containing 5% and 35% lipid. The lipid contents were determined using cells recovered from the plates, and not cultures grown in liquid medium, so that there is no doubt that the lipid contents of cells grown on high nitrogen and low nitrogen agar were substantially different and that this difference should have been detected by the staining procedure.

The problem did not appear to be that the red pigment of R. toruloides was masking the stain. When the stain was applied to the colony prints, the colonies appeared purple or black instead of red. However on destaining, both high lipid and low lipid colonies destained to the same extent. Different concentrations of ethanol for destaining (25 to 95%), and different solvents for the stain (isopropanol, butanol, octanol) were tried. However under any given set of conditions, the

TABLE 8

Lipid content of yeast after growth on high N and low N media.

Yeast	Medium	Incubation Period days	Lipid content % of biomass
C. curvata D	high N liquid	7	6.7
	low N liquid	7	40.6
R. toruloides IFO 0880	high N liquid	7	2.9
	low N liquid	7	47.7
	low N liquid followed by low N solid	2 3	44.1 46.3

Low N medium is NLM except no sodium glutamate included, 0.8 g/l yeast extract.

High N medium is Low N with 3.0 g/l NH_4Cl added.

appearance of replicas prints from high and low lipid colonies were virtually identical.

5. Use Of Nystatin To Kill Growing Cells Selectively.

Nystatin selection has been successfully used with S. cerevisiae to increase the proportion of auxotrophic cells in the population (Snow, 1966). The method is based on the fact that the antimycotic nystatin selectively kills growing cells and has little effect on non-growing cells. A test was carried out to see if nystatin has the same effect on R. toruloides. Using a culture of CBS 315 grown in YMG medium, growth was stopped by washing the cells in distilled water to remove all the original growth medium, and incubating overnight in MM-N, a minimal medium with no nitrogen source. The culture was then centrifuged and the supernatant discarded. The cells were resuspended in either fresh MM-N, or MM (minimal medium with NH_4^+ as the nitrogen source). Incubation was continued so that growth would re-start in the culture to which nitrogen had been added. Nystatin solution was then added to both cultures, incubation continued and samples removed at intervals to determine the viability of growing and non-growing cells in the presence of nystatin. The results are shown in Table 9. This clearly shows that within 20 minutes, nystatin has a much greater effect on the viability of growing cells than non-growing cells. In the nystatin enrichment procedure, after growth has been stopped by removal of the nitrogen source, growth of prototrophs is re-started by transferring the cells to MM. However auxotrophs will not grow

TABLE 9

The affect of nystatin on growing and non-growing cells.

Exposure time minutes	% survival in culture with no added N	% survival in culture with added N
0	100	100
20	50	0.24
40	37	0.11
60	32	0.16

The growth of a culture of IFO 0880 was stopped by overnight incubation in a medium with no nitrogen source. The culture was then divided into two portions, and NH_4Cl added to one of them. After a further 6 hours incubation, nystatin was added and samples taken to determine the viability of the cells.

in MM and so when nystatin is added the only prototrophs are killed. The results of this experiment show that this procedure can be used with R. toruloides with as much success as with S. cerevisiae.

6. Mutagenesis And Isolation Of Auxotrophs.

There were three reasons for attempting to isolate auxotrophs from R. toruloides. These reasons are outlined below.

a). To produce strains with stable genetic markers that could then be used in subsequent experiments e.g. mating, protoplast fusion.

b). To determine mutagenesis conditions that led to the generation of a large number of mutants, so that the same conditions could be used to generate mutants without active ATP:citrate lyase. There is no simple selection procedure for such mutants and so it is desirable to start with a culture in which many mutants have been induced. Too little exposure to the mutagen, for example, will result in very few mutations in the population, whereas too much will kill almost all the cells, leaving only those which are resistant to the mutagen.

c). To provide information about the range of auxotrophs which can be isolated from different strains. Bottcher and Samsonova (1980a) found that in some strains of R. toruloides, only a limited number of auxotrophs could be isolated, and concluded that this is due largely to aneuploidy of these strains.

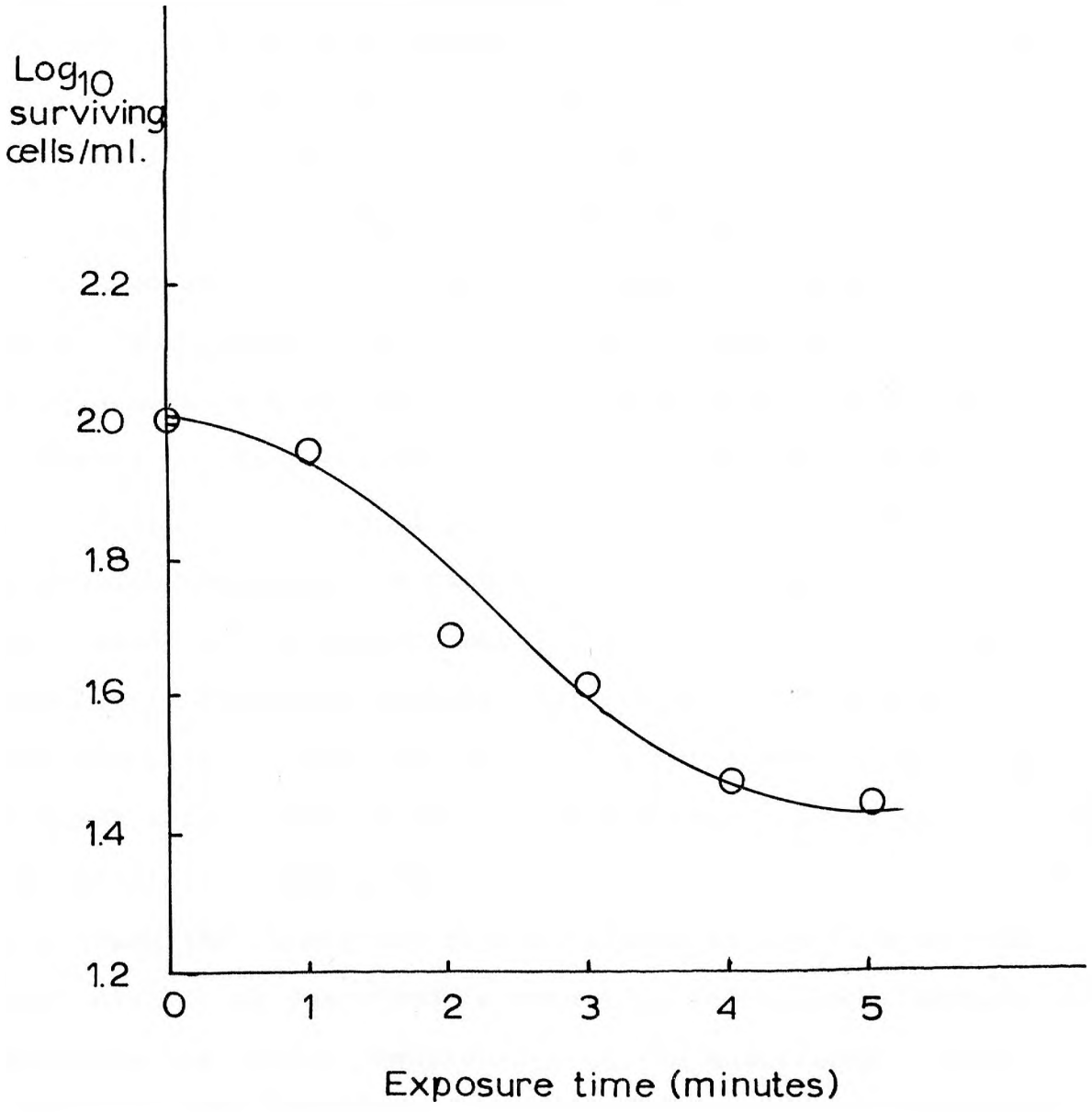
Three different mutagens (UV light, EMS and NTG) were used to isolate auxotrophs from R. toruloides. The results

obtained with them are given below.

6.1 Use Of Ultra-Violet Light As A Mutagen.

The killing curve of R. toruloides CBS 14 exposed to UV light (λ =254nm) is shown in Figure 8. To isolate auxotrophs from this strain a culture was exposed to UV light for 5 minutes. Nystatin enrichment was used to increase the proportion of auxotrophs in the population. Samples of the culture were then spread on YMG plates and after incubation the colonies were replica plated to MM and YMG plates. Any colonies that grew on YMG but not MM were patched onto fresh YMG plates using sterile matchsticks, incubated until the colonies had grown up and then replica plated to MM, MM+adenine, MM+methionine and YMG plates in that order. This would identify any auxotrophs requiring adenine or methionine for growth. Two colonies that grew on MM+ade but not MM, and four colonies that grew on MM+met but not MM were identified in this way. The auxotrophs were again replica plated to MM, MM+ade, YMG or MM, MM+met, YMG and also streaked out on each type of plate. After the second replica plating, there was some growth of each colony on the MM plates, and on all the plates streaked with the isolates. Colonies were again taken from the original YMG plates (i.e. the plates that the cultures were spread on after nystatin selection), patched onto fresh YMG plates and replica plated to MM, MM+ade, YMG or MM, MM+met, YMG. Reversion to prototrophy had occurred in all cases and all colonies grew on all the plates.

Fig. 8



Killing of Rhodosporidium toruloides CBS 14 with ultra violet light.

6.2 Use Of EMS And NTG As Mutagens.

The sensitivity of six strains of R. toruloides to EMS was investigated. See Figures 9 & 10. There was much variation in the sensitivities of different strains to this mutagen. The sensitivity of IFO 0880 and CBS 315 to NTG was also investigated. See Figure 9. From the killing curve of IFO 0880 exposed to EMS the exposure time needed to kill 90% of the population was calculated as 75 minutes, and this exposure time was used to isolate auxotrophs from IFO 0880. Auxotrophs were identified using a screening method that detects mutants with one of 20 different growth requirements. After mutagenesis, the culture was divided into 20 equal portions. This was done so that independent mutations causing the same phenotype could be identified. If the culture was not divided it would not be possible to determine whether isolates with the same phenotype were genetically identical or not. After nystatin selection, cultures were plated on YMG plates and then replica plated to MM and YMG plates. Any colonies growing on YMG only were patched onto fresh YMG plates and replica plated to a series of nine 'pool plates' as described in Materials and Methods Section 11 to determine the growth requirements of the auxotrophs. Using strain IFO 0880, auxotrophs with the following growth requirement were isolated from 20 flasks:-

lysine (4 independent mutations), adenine (2),
proline (1), arginine (1).

Auxanography was repeated to confirm the identification, with the same results. The reversion frequencies of these auxotrophs

Fig.9

Killing curves of two strains of R. toruloides exposed to 3% EMS or 0.1% NTG

Abscissa: Minutes exposure to EMS or NTG

Ordinate: Log_{10} surviving cells/ml.

○ IFO 0880 exposed to EMS

● IFO 0880 exposed to NTG

□ CBS 315 exposed to EMS

■ CBS 315 exposed to NTG

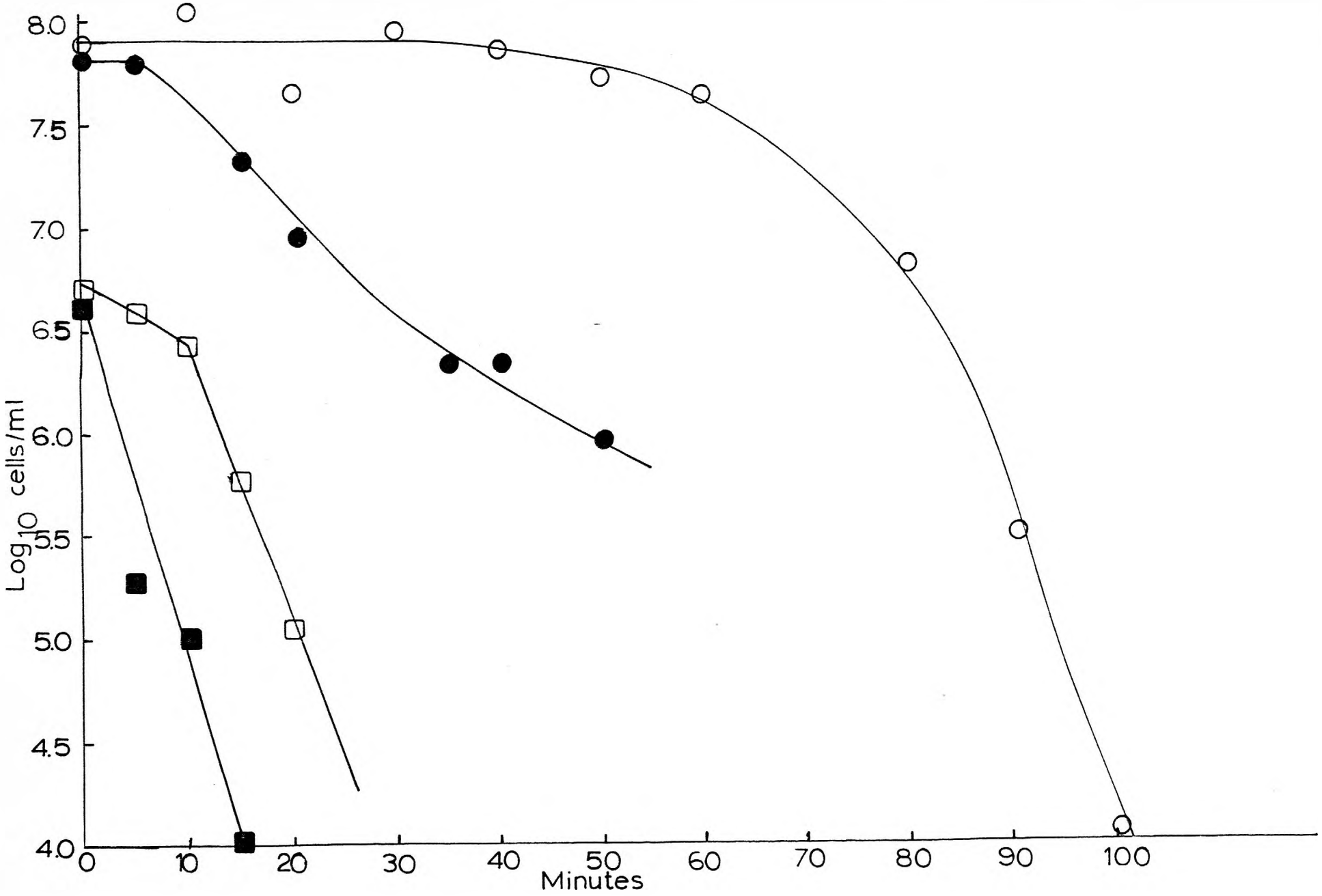

8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Log10 cells/ml
0
10
20
30
40
50
60
70
80
90
100
Minutes

Fig.10

Killing curves of four strains of R. toruloides exposed to 3% EMS

Abscissa: Minutes exposure to EMS

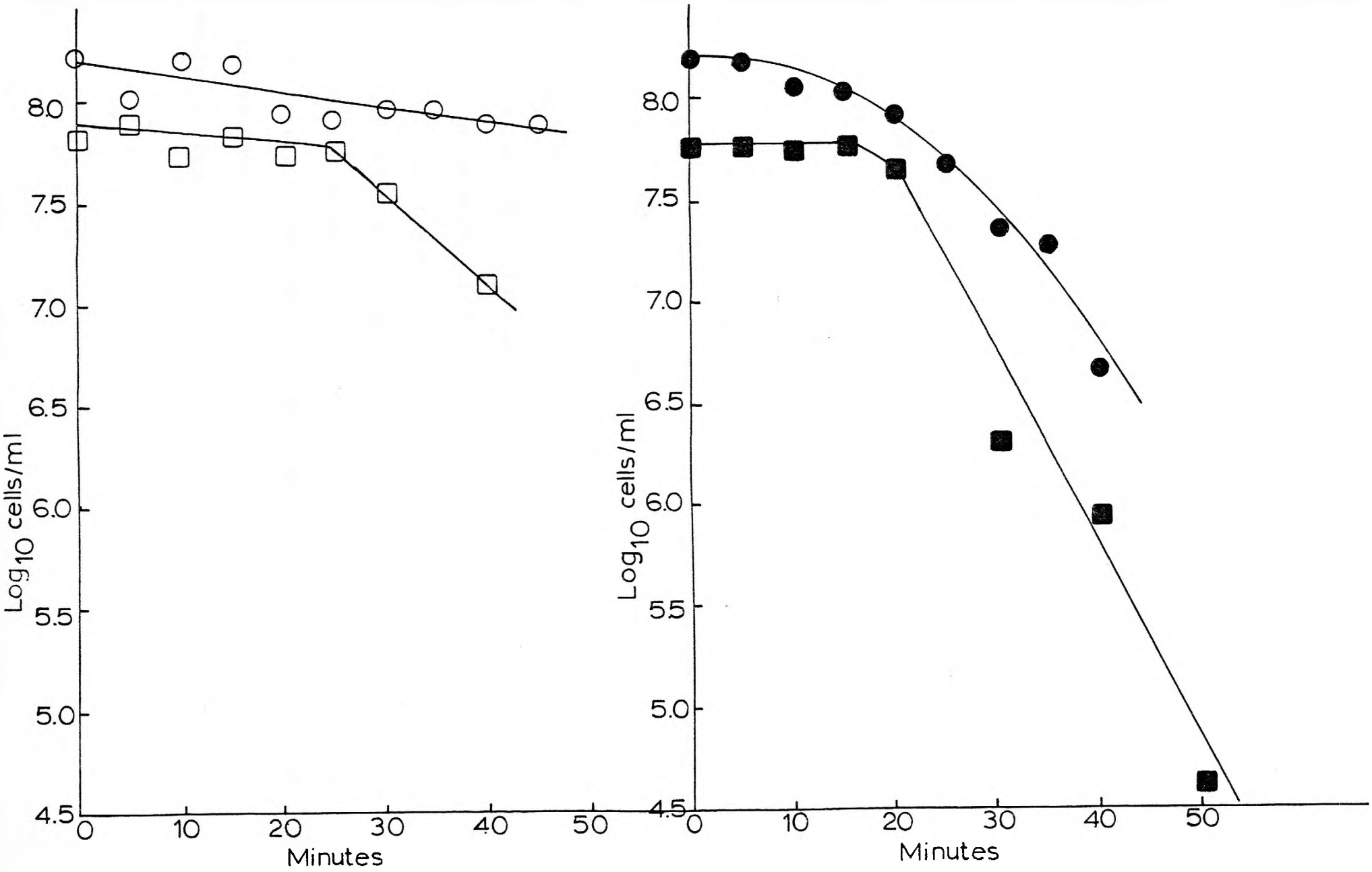
Ordinate: Log_{10} surviving cells/ml

○ IFO 10076

□ IFO 0559

● ML 2589

■ CBS 6681


8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
Log10 cells/ml
0
10
20
30
40
50
Minutes
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
Log10 cells/ml
0
10
20
30
40
50
Minutes

were calculated and are shown in Table 10.

Mutagenesis, nystatin selection and auxanography were repeated with a fresh culture of IFO 0880, and this time the following auxotrophs were isolated:-

lysine (3), adenine (1), plus a class of auxotroph requiring either cysteine or methionine for growth (3).

Mutagenesis using NTG (90% kill) followed by nystatin selection led to the isolation of the following auxotrophs:-

arginine (3), lysine (1), alanine (1), cysteine or methionine (7).

If the strain IFO 0880 is haploid (Banno, 1967, Abe and Sasakuma, 1986), it should be possible to isolate auxotrophs requiring any of the supplements included in the auxanography testing. However as shown by the above results, the same few classes of auxotrophs are repeatedly isolated. This may occur because the method used (recovery in YMG after mutagenesis, nystatin selection and auxanography) favours the growth of certain classes of mutation which are then isolated more frequently than any others. In order to isolate mutants auxotrophic for tryptophan (as an example of an amino acid for which no auxotrophs had been isolated, even though it offers several possible sites for the inactivation of an enzyme), a culture was divided into 20 portions and grown in MM+tryptophan after mutagenesis. After nystatin enrichment the cells were again grown in MM+trp and nystatin enrichment was repeated. Samples were then spread on MM+try plates, incubated and replica plated to MM plates.

TABLE 10

Reversion frequencies of IFO 0880 auxotrophs isolated after exposure to EMS.

Growth Requirement	Isolate Number	Reversion Frequency
proline	1	4.4×10^{-6}
lysine	2	$< 7.1 \times 10^{-9}$
lysine	3	1.9×10^{-5}
lysine	4	$< 7.7 \times 10^{-9}$
lysine	5	1.3×10^{-7}
adenine	6	$< 4.9 \times 10^{-6}$
adenine	7	$< 7.2 \times 10^{-6}$
arginine	8	3.8×10^{-7}

Isolates 6 and 7 have the same growth requirement but were isolated from different portions of the culture after mutagenesis, and so do not represent the same mutation isolated twice. This also applies to 2, 3, 4 and 5.

Tryptophan was chosen as it has a long biosynthetic pathway and there are many steps at which a mutation could cause a requiremnt for tryptophan. However despite screening more than five thousand colonies after two rounds of nystatin selection, no tryptophan requiring auxotrophs were found.

Using strain CBS 315 exposed to EMS (90% kill), the nystatin enrichment procedure was followed and auxotrophs were identified using the same series of pool plates as were described for the identification of auxotrophs of IFO 0880. The following auxotrophs were isolated:

arginine (9), adenine (3), methionine (1),
isoleucine (1), uracil (1).

Using the *ilv*$^-$ strain, a culture was exposed to EMS a second time and then allowed to grow in MM+isoleucine+adenine. After nystatin selection, auxotrophs requiring both adenine and isoleucine were isolated.

A culture of CBS 315 was exposed to EMS (90% kill) and then grown out in either MM+tryptophan, MM+methionine and MM+valine. Tryptophan was used for the same reasons as for IFO 0880, methionine was used because it had already been shown to be possible to isolate *met*$^-$ auxotrophs, so that if no *trp*$^-$ or *met*$^-$ auxotrophs were found during this experiment, it may be due to a fault in the procedure. Valine was used because no *val*$^-$ auxotrophs had been isolated and its biosynthetic pathway is separate from that of tryptophan thus increasing the number of sites at which a mutation would lead to an auxotroph (either *trp*$^-$ or *val*$^-$) being formed. Nystatin enrichment was carried out twice

with each of the cultures, the cells were plated on the same medium that they had been grown in after mutagenesis and replica plated to MM plates. Six independently arising methionine auxotrophs were found, but no auxotrophs requiring tryptophan or valine were isolated.

Three attempts were made to isolate auxotrophs from the strain CBS 6681 following exposure to EMS or NTG, nystatin enrichment and auxanography to detect any of 20 different growth requirements. No auxotrophs were detected. The procedure was repeated with IFO 10076, but no auxotrophs were isolated with this strain either.

7. Attempted Isolation Of ACL-less mutants.

ACL is the key enzyme of lipid accumulation and in order to study the effect of removing this enzyme from an oleaginous yeast, it was necessary to isolate mutants lacking this enzyme. If this could be achieved, it may be possible to re-introduce the enzyme by transformation with a plasmid carrying the ACL gene (from a gene library of *R. toruloides* in a suitable vector, Gilbert *et al.*, 1985) and compare the effect of this with the effect of introducing the ACL gene into a citrate accumulating yeast, or a non-oleaginous, non-citrate accumulating yeast. If several ACL less strains were isolated, *in vitro* complementation of the enzyme sub-units may provide information about the structure of ACL (Wieland *et al.*, 1979, Werkmeister *et al.*, 1981).

Unfortunately, although four ACL-less isolates were

found, these reverted to ACL+ strains within a few days of being identified, and subsequent experiments failed to yield any more ACL-less strains. The procedure for the isolation of ACL-less mutants, as described in Materials and Methods Section 12 was carried out with the following results. A culture of strain 7203 was exposed to EMS to induce mutations, then grown in medium containing fluoroacetate. Fluroacetate is taken up by cells and metabolised to fluorocitrate, which is a potent inhibitor of aconitase and so causes cell death. However cells in which ACL is not active will accumulate citrate when grown in medium with a high C:N ratio. This will have the effect of diluting the fluorocitrate in these cells and so ACL-less mutants will be less sensitive to fluoroacetate than the wild-type cells. After this enrichment process, the cultures were spread on plates containing a pH indicator so that any acid-accumulating mutants may be identified. After three days incubation at 30^{o}C, the indicator plates spread with strain 7203 were unchanged in colour (i.e. blue). Those spread with Yarrowia lipolytica (a citrate accumulator) were completely yellow in colour indicating that citrate production by the yeast had occurred. Four colonies out of approximately three thousand from the mutagenised culture of 7203 had a yellow 'halo' around the outside of the colony. These colonies were replica plated to MM, MM+inositol, indicator and YMG plates. All four were found to require inositol for growth (parent strain 7203 requires inositol) and changed the colour of the indicator plates. Three of the four colonies (H1, H2, H3) had been isolated from the same

portion of the mutagenised culture and so were probably genetically identical. The fourth isolate, J, originated from a separate portion of the culture.

The four acid-producing isolates were grown in NLM for 2 days and the citrate concentration in the growth medium was then determined. The results are given in Table 11.

The activity of ACL in the four isolates was then determined, and the assay was also repeated four days after the first assay.

When re-streaked on indicator plates after the second assay, no colour change was observed. No citrate could be detected in the growth medium and all attempts to re-isolate ACL-less mutants from the original set of indicator plates were unsuccessful. The procedure was repeated with strain 7203, and then twice with strain IFO 0880, but no more ACL-less mutants were detected.

At this stage of the research project it was felt that instead of continuing to try to isolate mutants defective in lipid accumulation, it was necessary to find out more about the ploidy and life cycle of *R. toruloides*. As discussed in the Introduction, it is not clear whether the yeast phase of *R. toruloides* is always haploid, sometimes diploid or occasionally aneuploid. If IFO 0880 (and its derivative, 7203) is not actually haploid, as is always claimed when this strain is used for research (e. g. Banno, 1967, Abe *et al.*, 1978, Abe and Sasakuma, 1986, M. Tully, personal communication) then it is not an ideal choice as a strain used to study the genetics of lipid

TABLE 11

Citrate production and ACL activity in acid-producing isolates.

Isolate	Citrate conc^{n} in growth medium mM	ACL spec act a	ACL spec act b
7203	0.00	7.9	8.0
Y. lipolytica	0.12	nd	nd
H1	0.12	0.0	7.9
H2	0.12	9.2	6.2
H3	0.14	5.9	6.8
J	0.08	0.0	7.8

nd - not determined.

specific activity of ACL expressed in nmoles/min/mg.

assay b was carried out 4 days after assay a.

accumulation. It was therefore decided to determine the DNA content of several strains of R. toruloides to gain more information about the ploidy of these strains and their suitability for genetic studies.

8. Determination Of DNA Content Per Cell.

The DNA contents published by Banno in 1967 supported the view that the yeast phase of the life cycle is haploid, but that some diploid yeasts are occasionally found. However other publications (Bottcher et al., 1980, Bottcher and Samsonova, 1980a) claimed that many strains of R. toruloides are aneuploid. The strain IFO 0880 is thought to be haploid but the shape of the killing curve for IFO 0880 exposed to EMS (See Section 6.2) and the fact that only a limited number of classes of auxotrophs can be isolated from it suggest that it may actually be aneuploid. In order to determine the ploidy of this and other strains, the DNA content per cell was measured.

The DNA content per cell in 8 strains of R. toruloides was determined using the method given in Materials and Methods Section 13. The procedure was repeated three times for each strain and the results obtained are shown in Table 12. Values range from 21 to 59 fg/cell. If the lowest figure, 21 fg/cell is assumed to be the haploid level, then there is one haploid strain (CBS 315), two diploid (CBS 14 and IFO 0559), three hyper-haploid aneuploid (IFO 0880, ML 2589, ML 2590) and two hyper-diploid aneuploid (CBS 6681 and IFO 10076).

TABLE 12

DNA content of 8 strains of R. toruloides.

Strain	a	b	c	Mean
CBS 315	20	20	23	21
ML 2589	26	24	24	25
IFO 0880	29	29	29	29
ML 2590	31	36	30	32
CBS 14	47	43	41	44
IFO 0559	43	46	45	45
CBS 6681	50	58	50	53
IFO 10076	53	54	70	59

The DNA content was measured after growth in YMG to stationary phase and is expressed in fg/cell.
Three determinations were made for each strain. The three values (a, b and c) and the mean are given for each strain.

Although the DNA content of strains of R. toruloides has been measured before, it is not known whether the DNA content of any one strain remains constant over a long period, either in continuous culture or during storage with repeated sub-culture. For this reason, the DNA content of R. toruloides during continuous culture was investigated.

9. Continuous Culture Of Strain CBS 315.

This strain has the lowest DNA content of the 8 strains in which DNA/cell was measured. A culture grown from a single colony of strain CBS 315 was used to inoculate a chemostat maintained at a dilution rate of 0.02 h^{-1}. Samples were taken from the chemostat and grown to stationary phase in batch culture (YMG medium). The DNA content per cell and per unit dry weight of the batch culture were measured. DNA content cannot be determined in cells taken directly from the chemostat as they would be in different stages of the cell cycle, and each cell may contain up to twice its basal level of DNA as it synthesises DNA in preparation for cell division. Therefore samples of cells from the chemostat were grown to stationary phase in batch culture before determining the DNA content.

After 30 days continuous culture the dilution rate was increased to 0.1h^{-1} and the experiment was continued for a further 7 days. The results are shown in Figure 11. The morphology of the cells in the chemostat or colonies grown on agar plates did not change during the course of the experiment.

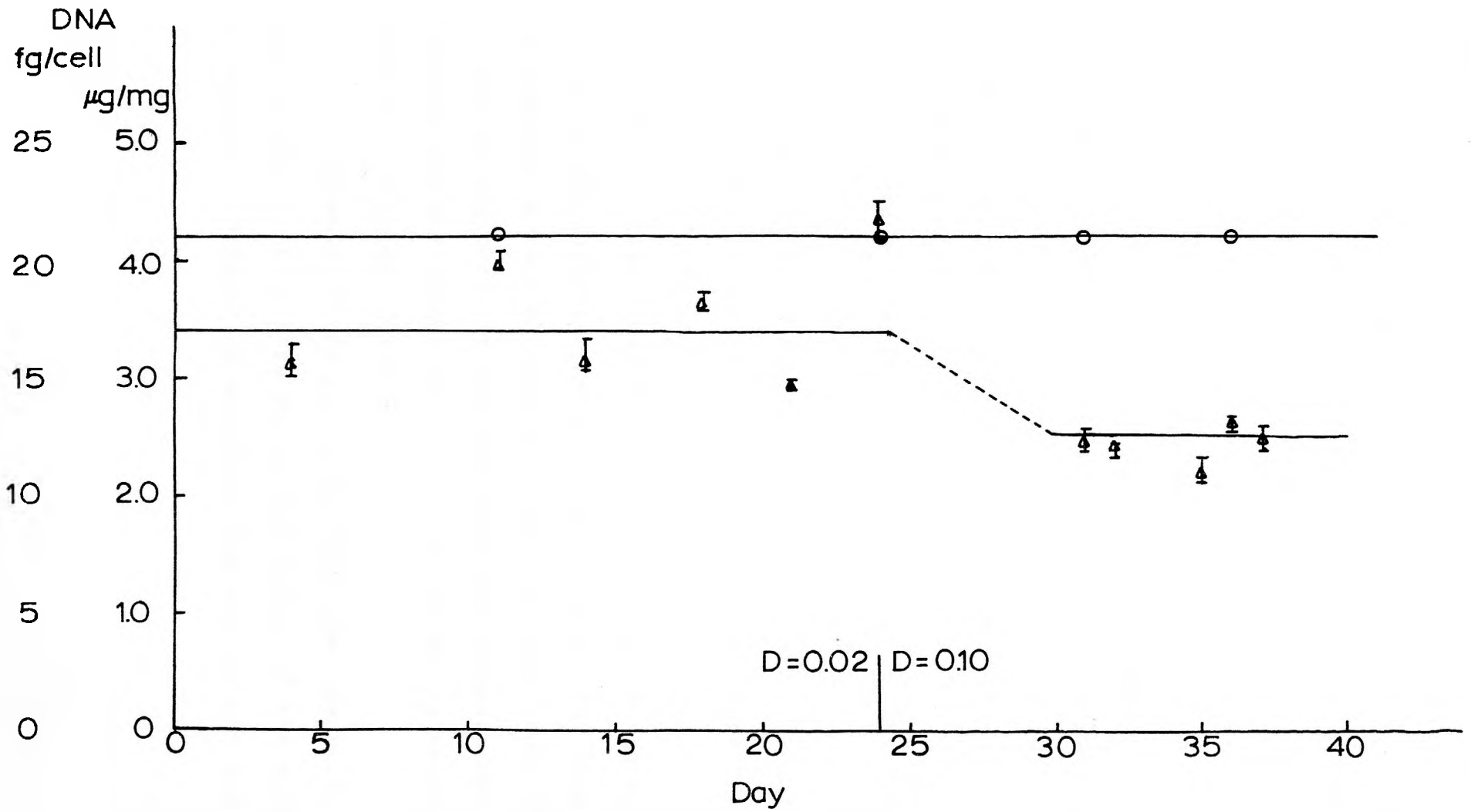
Fig. 11

DNA content of CBS 315 in continuous culture

o DNA fg/cell

Δ DNA μg/mg

Three determinations were made in each case. The highest and lowest values and the mean are plotted. Samples were grown to stationary phase in YMG, then harvested to determine the DNA content.


DNA
fg/cell
μg/mg
25
5.0
20
4.0
15
3.0
10
2.0
5
1.0
0
0
0
5
10
15
20
25
30
35
40
Day
D=0.02
D=0.10

10. Continuous Culture Of Strain ML 2589.

A chemostat was inoculated with a culture grown from a single colony of strain ML 2589. The dilution rate was maintained at $0.02h^{-1}$ for 15 days. Samples were taken to measure the DNA content per cell, and number of cells per unit dry weight as with strain CBS 315 (Section 8). The results are shown in Table 13.

11. Continuous Culture Of Strain CBS 14.

This strain has been reported as having an extremely high lipid content (Evans, 1983) but subsequent attempts to repeat those results have failed. Lipid contents of only 30 to 50% of the dry weight were found instead of the original 80% or more (J. Curson, personal communication).

In order to discover if the change in lipid content is related to a change in DNA content, a chemostat was inoculated with a culture of CBS 14 grown from a single colony. The dilution rate was maintained at $0.1h^{-1}$. Samples were taken and grown in batch culture to stationary phase in YMG to determine DNA content, and also grown in NLM to determine the potential of the cells in the chemostat to accumulate lipid. Two alternative carbon sources in NLM, glucose and fructose, were used. The morphology of the cells in the chemostat and colonies grown on agar plates did not change during the course of the experiment. The results are shown in Figure 12.

The experiment was repeated, this time gradually increasing the dilution rate during the course of the experiment. Again samples were taken to determine the DNA content and lipid

TABLE 13

DNA content and cell dry weight during continuous culture of ML 2589.

Day	DNA fg/cell	No. of cells/ mg dry wt.	dry wt./cell pg.
2	20.8	1.46×10^8	6.9
4	20.7	2.13×10^8	4.7
8	41.2	7.36×10^7	13.5
15	43.8	8.03×10^7	12.5

Cells grown in continuous culture in CLM at a dilution rate of 0.02 h^{-1}. Samples were grown to stationary phase in YMG to determine the DNA content.

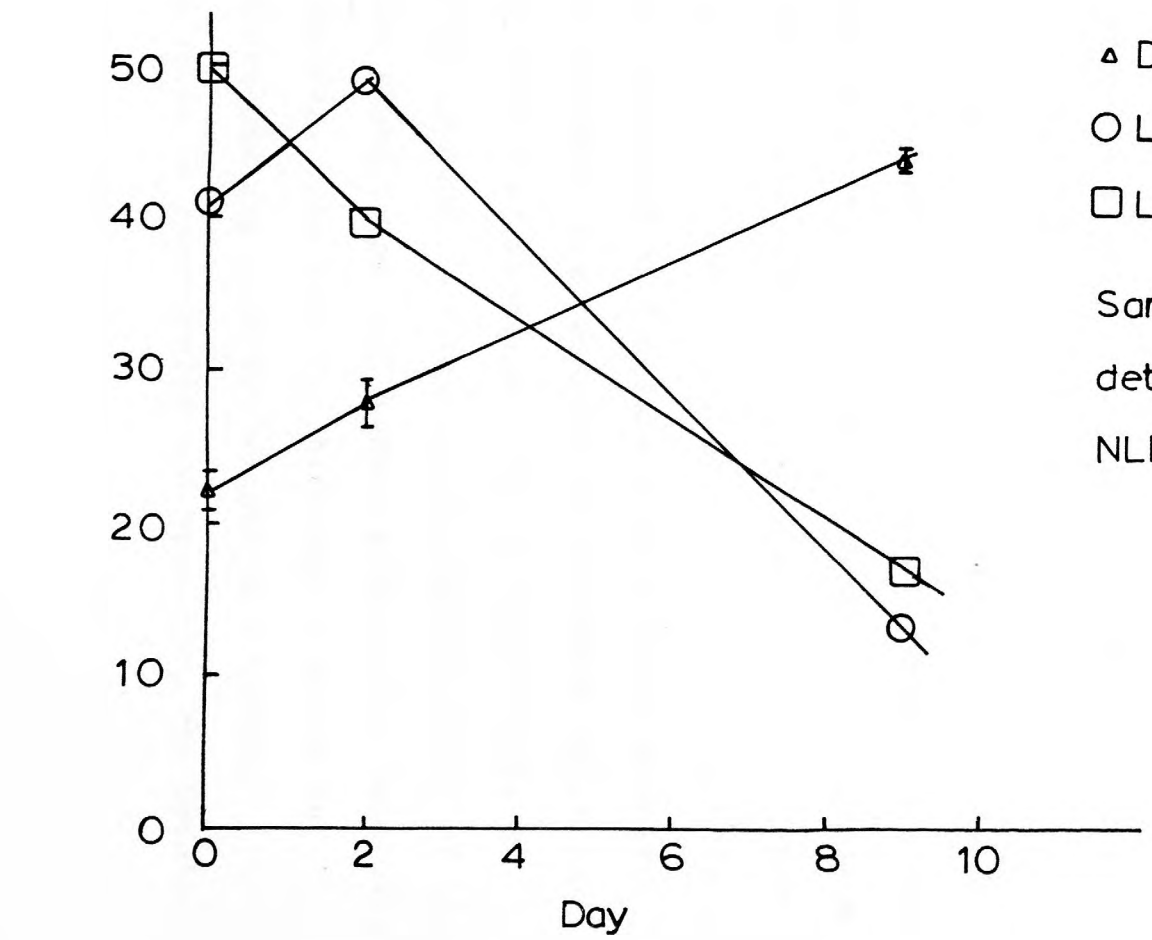


Fig. 12

DNA and lipid content of CBS 14 during continuous culture.

△ DNA fg/cell

○ Lipid (C source = glucose)

□ Lipid (C source = fructose)

Samples were grown in YMG to determine the DNA content and NLM to determine the lipid content.

content. The results are shown in Figure 13. In samples from the chemostat examined under the microscope the morphology of the cells did not change until day 21, when several cells with extensions resembling mating tubes were seen. The colony morphology of samples taken from the chemostat and grown on agar plates did not alter.

12. Continuous Culture Of Strain IFO 10076.

This strain has the highest DNA content/cell of those strains which were examined. A chemostat was inoculated with a culture grown from a single colony of IFO 10076. The dilution rate was initially $0.02h^{-1}$, and was increased to $0.1h^{-1}$ on day 53 of the experiment. After seven days continuous culture, some of the yeast had formed mycelia, making it impossible to calculate DNA per cell. Even when less then 1% of the population consists of mycelium it is not possible to calculate the DNA content per cell as the cells cannot be counted accurately. Very little mycelium may be present and it would seem possible to assign values of 1, 2 or 3 yeast cells to short lengths of mycelium, but when this is done results from replicates of the same sample differ widely. This is due to two factors: the tendency of the yeast cells to congregate around lengths of mycelium forming dense clumps of cells that cannot be counted, and the resulting distortion of the counting chamber as these large cell masses push up the coverslip over the haemocytometer. See Fig 17. Although it would be possible to calculate DNA per unit dry weight of a sample consisting of a mixture of yeast cells and mycelium, the results

Fig. 13

DNA and lipid content of CBS 14 during continuous culture.

a: Lipid content of cells grown on NLM with the carbon sources

glucose ○

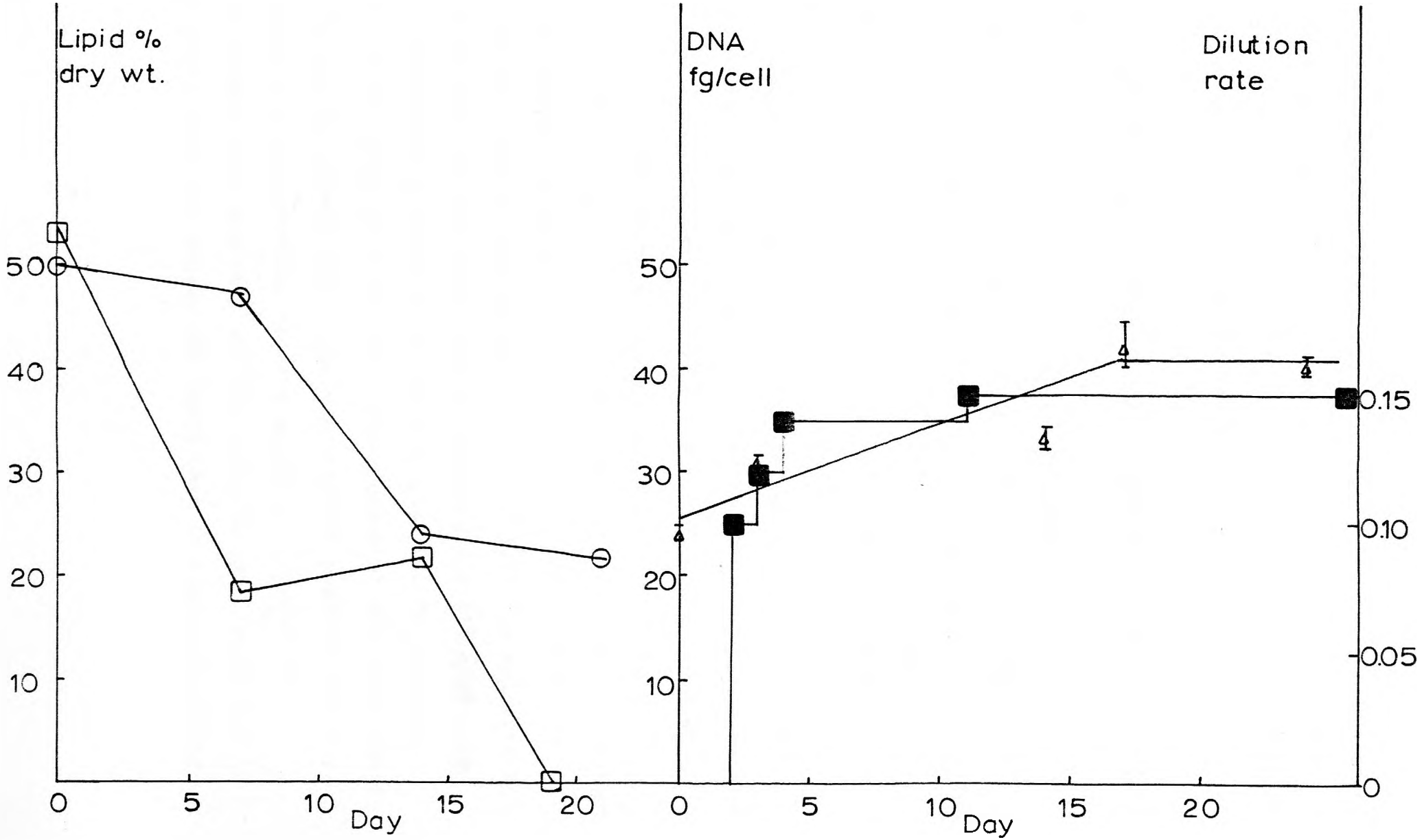
fructose □

b: Dilution rate ■

DNA content fg/cell ▵

The graph of DNA content shows the result of three determinations. The upper and lower values and the mean are plotted.

Samples were grown in YMG to determine the DNA content and NLM to determine the lipid content.


Lipid %
dry wt.
50
40
30
20
10
0
5
10
15
20
Day
DNA
fg/cell
50
40
30
20
10
0
5
10
15
20
Day
Dilution
rate
0.15
0.10
0.05
0

would be meaningless as the size of yeast cells and proportion of yeast cells to mycelium in the population can both vary. The changes in morphology of cells in the chemostat during the course of the experiment are shown in Table 14.

13. Isolation Of Basidiospores And Crosses Of Basidiospores With Strain CBS 315.

Twenty four colonies grown from single basidiospores were isolated using the method described in Materials and Methods Section 14. The colonies resembled those formed by the strain CBS 315 (small, regular, smooth and shiny) rather than those usually formed by the parent strain IFO 10076 (large, irregular, rough, eventually forming a brown ring of mycelium with teliospores around the edge of the colony). These differences are shown on photographs of the two strains CBS 315 and IFO 10076. An attempt was made to mate yeast grown from the basidiospore isolates with CBS 315 carrying the markers *ade*$^-$ *ilv*$^-$ (i.e. requiring both adenine and isoleucine for growth). CBS 315 has a low DNA content which remains stable in continuous culture, but does not accumulate lipid as the enzyme ATP:citrate lyase is not active in this strain. It was hoped that by crossing CBS 315 *ade*$^-$ *ilv*$^-$ with basidiospore isolates it would be possible to introduce ATP:citrate lyase into CBS 315. The progeny of the cross would be tested for growth requirements by replica plating, and any progeny with non-parental markers would be screened for ATP:citrate lyase activity and DNA content. Any isolates with a low DNA content and active ACL would then be examined for the

TABLE 14

Morphological changes seen during continuous culture of IFO 10076.

Day	Diln. rate h^{-1}	Morphology
0	inoculum	Only budding cells seen.
5	0.02	Some cells with extensions resembling mating tubes.
7	0.02	No hyphae seen in sample, but large clump of mycelium in pot.
12	0.02	Mycelium in pot no longer present.
22	0.02	No hyphae seen in sample
26	0.02	Large clumps of mycelium in pot. Smaller clumps with teliospores seen in sample.
29	0.02	Long, loosely packed hyphae and cells with hyphae growing out seen in sample.
46	0.02	Germinating teliospores seen in sample.
49	0.02	Many teliospores produced on agar plates.
53	0.10	More germinating teliospores seen in sample.
56	0.10	Mycelium present in pot, only budding cells seen in sample.
60	0.10	Cells with extensions resembling mating tubes seen in sample.
63	0.10	Mycelium still present in pot, cells with extensions seen in sample.

During the course of the experiment dry weight determinations showed that a 'steady state' was never reached. The dry weight per ml. fluctuated, increasing when large quantities of mycelium were visible in the chemostat, and decreasing when the amount of mycelium visible had decreased.

stability of DNA content and lipid production in continuous culture.

When crossing strains of *R. toruloides* of different mating type, mycelium production is not always seen if the two cultures are streaked across one another at right angles on an agar plate as described by Samsonova *et al.* (1980). Mycelium production is seen much more clearly if the two cultures are both streaked over the agar together, using a sterile loop to thoroughly mix the cultures. In order to confirm that production of mycelium was due to mating of the two strains, and not self-fertilisation of the IFO 10076 isolates, crosses were made in the following manner. One agar plate was used for each IFO 10076 isolate. Three parallel streaks were made on each plate:-

1. IFO 10076 basidiospore isolate.
2. CBS 315 *ade*$^-$ *ilv*$^-$.
3. Both cultures mixed together.

The plates were then incubated at 30^{o}C and examined at intervals for the production of mycelium. It was expected that 50% of the IFO 10076 isolates would be of opposite mating type to CBS 315 (which is mt α) as teliospores produce two *a* and two α basidiospores (Banno, 1967). In cases where the basidiospore isolate is mt *a*, mycelium should be produced around the mixed streak, but not around the pure streaks of CBS 315 or IFO 10076.

Two days after inoculating the plates mycelium was produced by 12 of the IFO 10076 isolates, both in the pure streak and the mixed streak. The remaining plates showed no mycelium formation from any of the streaks. After another 24 hours

incubation two more IFO 10076 isolates were producing mycelium from both the pure and the mixed streak. After a total of 13 days incubation all IFO 10076 isolates had produced mycelium (and teliospores) from both the pure and the mixed streaks. The pure streaks of CBS 315 ade- ilv- never produced mycelium. The experiment was repeated twice with similar results each time, the only difference being the number of days incubation before mycelium production was seen from the basidiospore isolates. In crosses of CBS 315 ade- ilv- with IFO 0880 mt a (5 replicates), mycelium was produced from the mixed streak only, never from the two pure streaks. Mycelium production from the mixed streak was seen after four days incubation in all five replicates.

14. Continuous Culture Of Basidiospore Isolates.

Seven of the 24 basidiospore isolates were grown in continuous culture in a chemostat. Samples were taken as before (Sections 9 to 12) to measure DNA content per cell, and samples were also streaked on YMG plates daily, and examined after three to four days incubation.

In the first experiment the DNA content of the inoculum was 20 fg/cell. This gradually increased to 42 fg/cell by day 10 of continuous culture. On day 11, microscopic examination showed some hyphae present in the culture. Samples of the culture were streaked on YMG agar plates each day and examined after incubation. Up to day 12, all the colonies formed were smooth and showed no sign of mycelial growth. On day 13, some colonies with a wrinkled appearance characteristic of mycelial growth were

observed. On examination these colonies proved to consist of free yeast cells on the surface of the colony, with hyphae growing into the agar underneath, and were thus identical to colonies of IFO 10076 from which the basidiospores were isolated. The percentage of mycelial colonies on the plates rose to 50% by day 17 (See Figure 14). Accurate measurements of DNA content per cell were not possible after day 11, as the presence of hyphae in the population, even at a low level, distorts the results obtained for cell counts.

In the second experiment, mycelial growth was found in all the samples taken to estimate DNA content per cell, so that it was not possible to determine this. Samples plated out showed an increase in the proportion of mycelial colonies during the first eight days. On days 9, 10, 11 the percentage of non-mycelial colonies remained at 3%. The experiment could not be continued past day 11 as mycelial growth in the chemostat blocked the outlet tube. See Figure 15.

In the remaining five experiments, with different basidiospore isolates, although the inoculum was grown from a smooth, non-mycelial colony, samples of the inoculum gave rise to more than 90% mycelial colonies, which increased to 100% by the second day of continuous culture. In all cases mycelial growth was visible inside the chemostat, forming large mould-like growths around the internal structure of the chemostat.

Fig. 14

DNA content and percentage of mycelial colonies formed during continuous culture of a basidiospore isolate of IFO 10076.

% mycelial colonies ○

DNA content fg/cell △

Three determinations were made. The upper and lower values and the mean are plotted. Samples were grown to stationary phase in YMG before determining the DNA content.

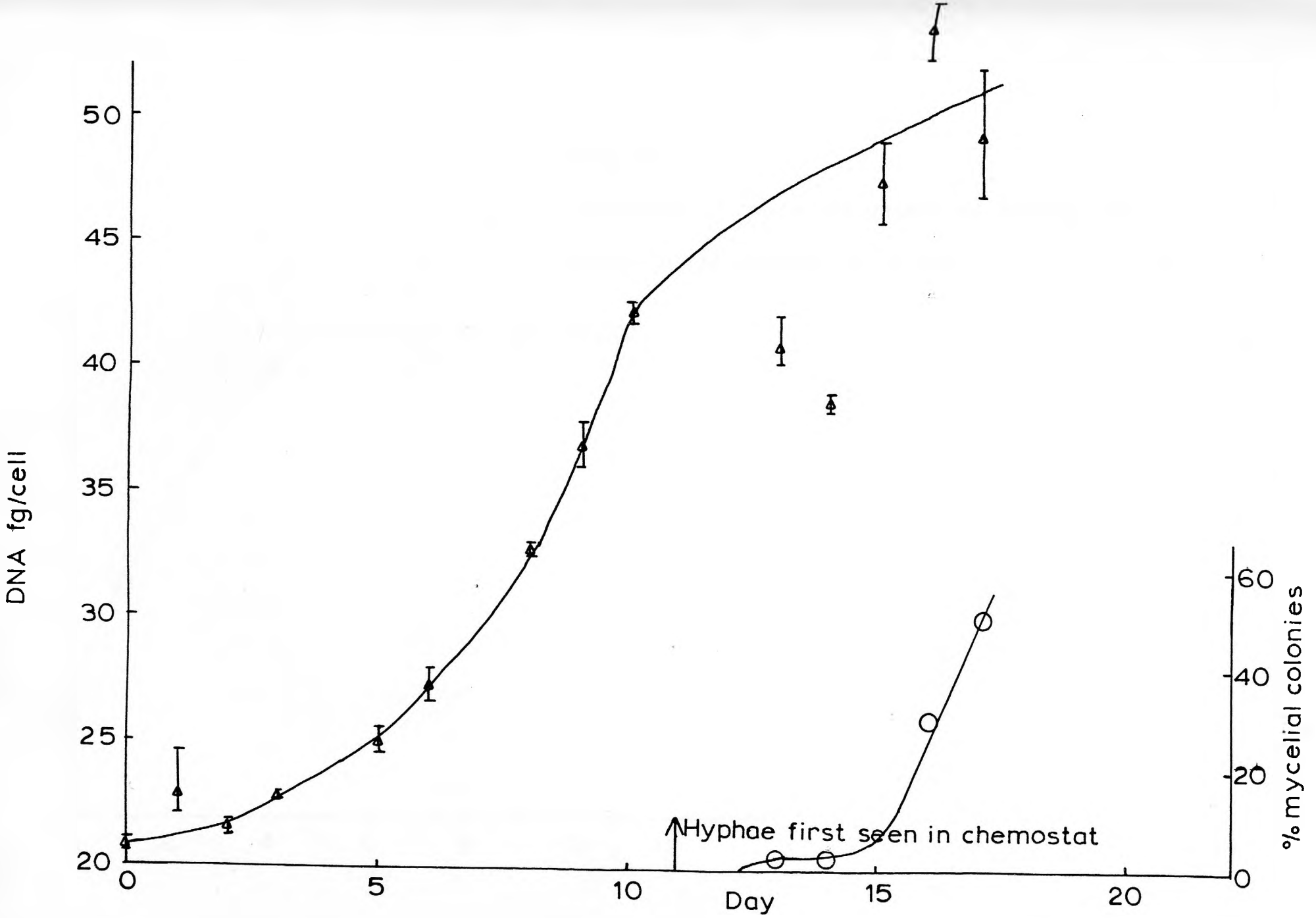

DNA fg/cell
50
45
40
35
30
25
20
0
5
10
15
20
Day
Hyphae first seen in chemostat
60
40
20
0
% mycelial colonies

Fig. 15

Increase in mycelial colonies during the continuous culture of a basidiospore isolate.

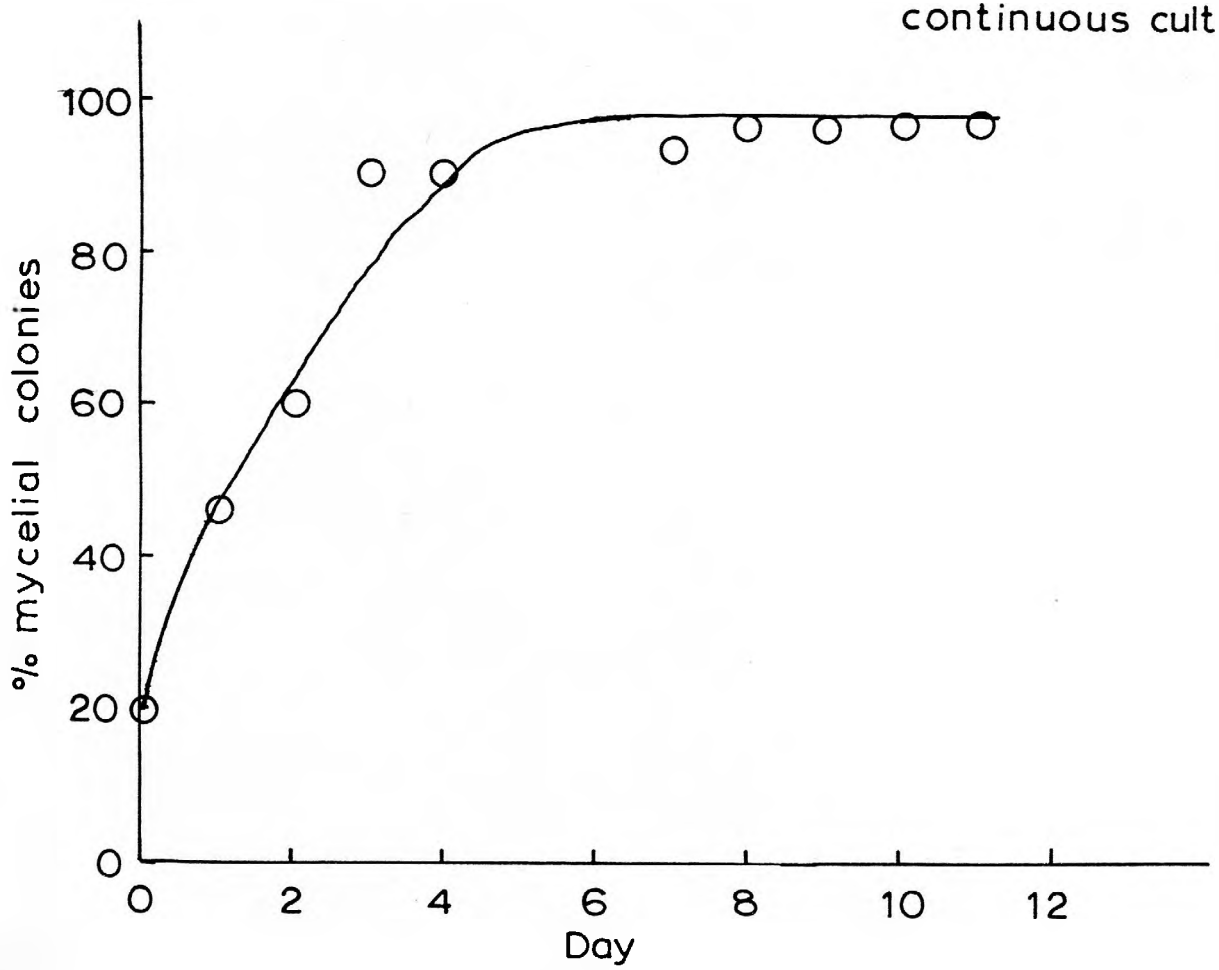
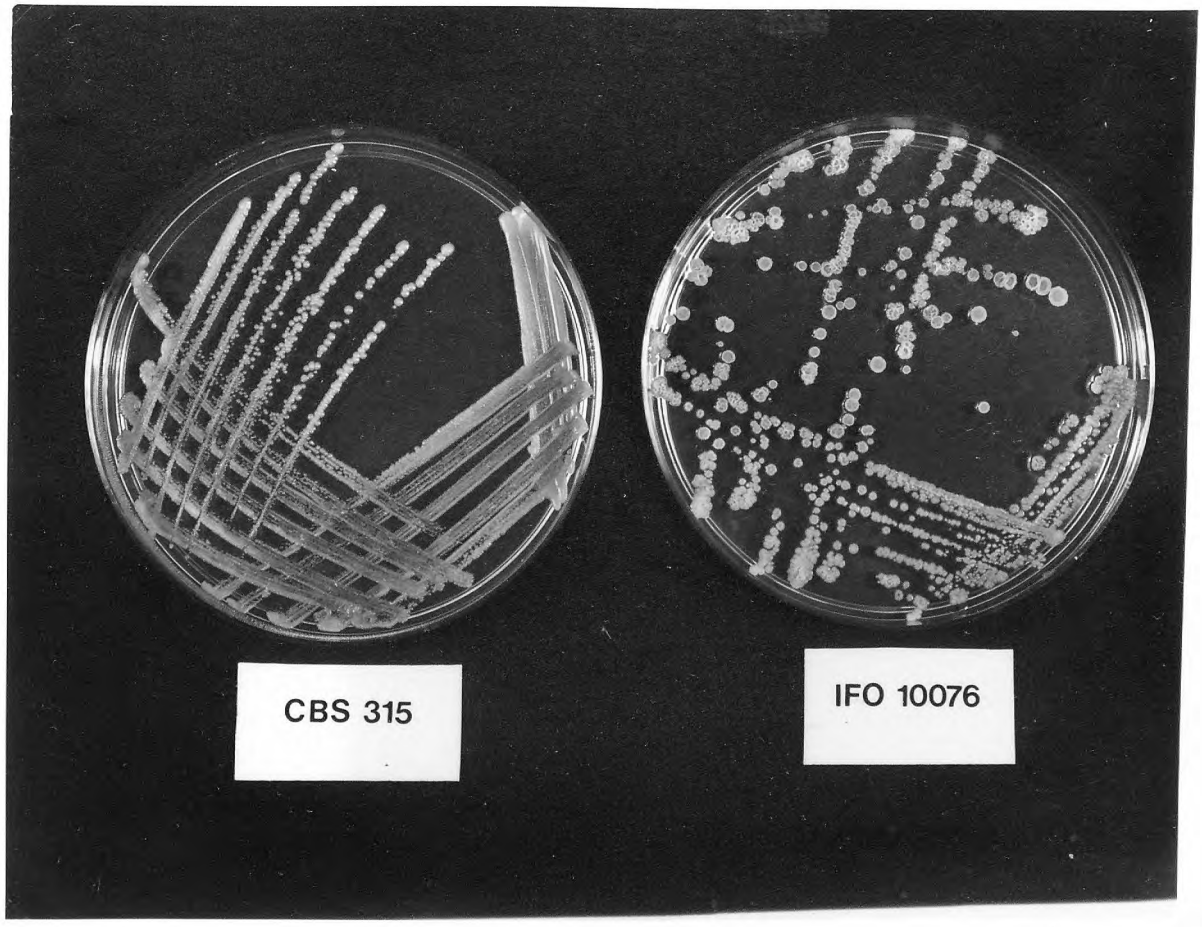
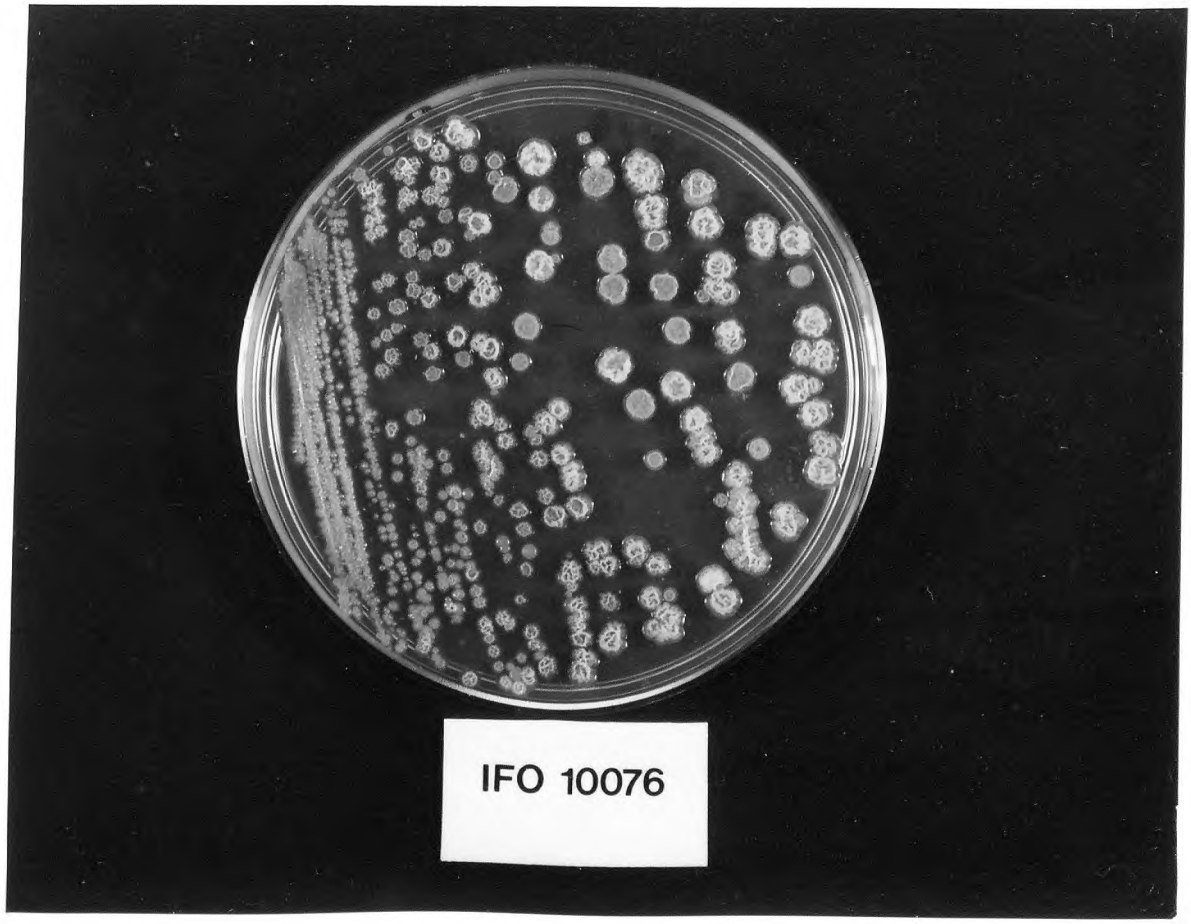


Figure 16

a) Morphology of CBS 315 and IFO 10076 colonies after 3 days incubation on YMG plates at 30 ^{o}C. CBS 315 forms smooth, shiny, regular colonies. IFO 10076 forms rough, irregular mycelial colonies, with a few small smooth shiny colonies.

b) IFO 10076 colonies after 7 days incubation on YMG plates at 30 ^{o}C. Teliospores are now present around the outside of the colony.


CBS 315
IFO 10076


IFO 10076

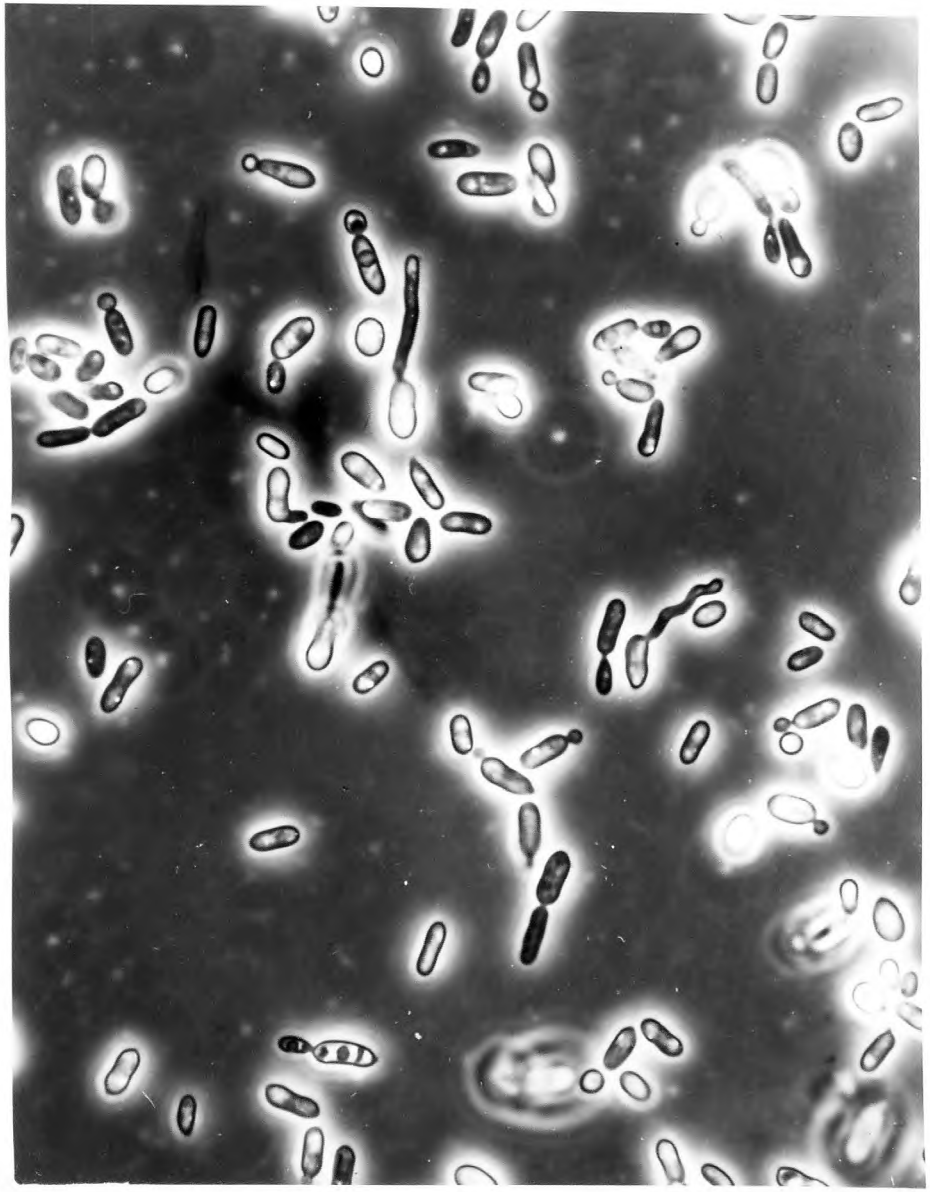
c) Cross between CBS 315 and IFO 0880, after 3 days incubation on YMG plates at 30 °C. The streaks of the single strains are smooth and non-mycelial, the mixture of the two strains of opposite mating type produces mycelial growth.

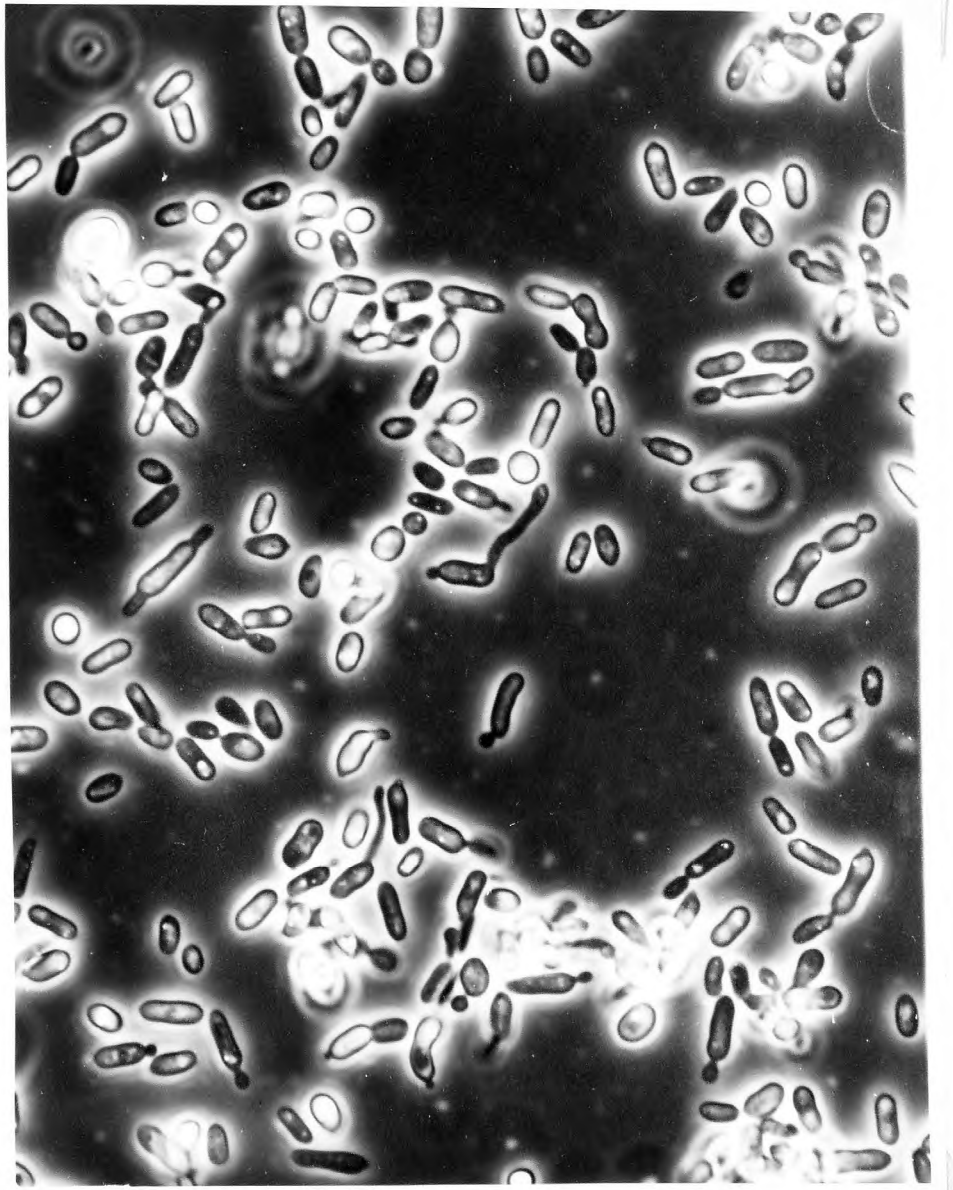

CBS
315
MIX
IFO
0880

Figure 17 Mycelium formation in IFO 10076.

a) and b) Cells commencing mycelial growth.

Magnification x 4000.



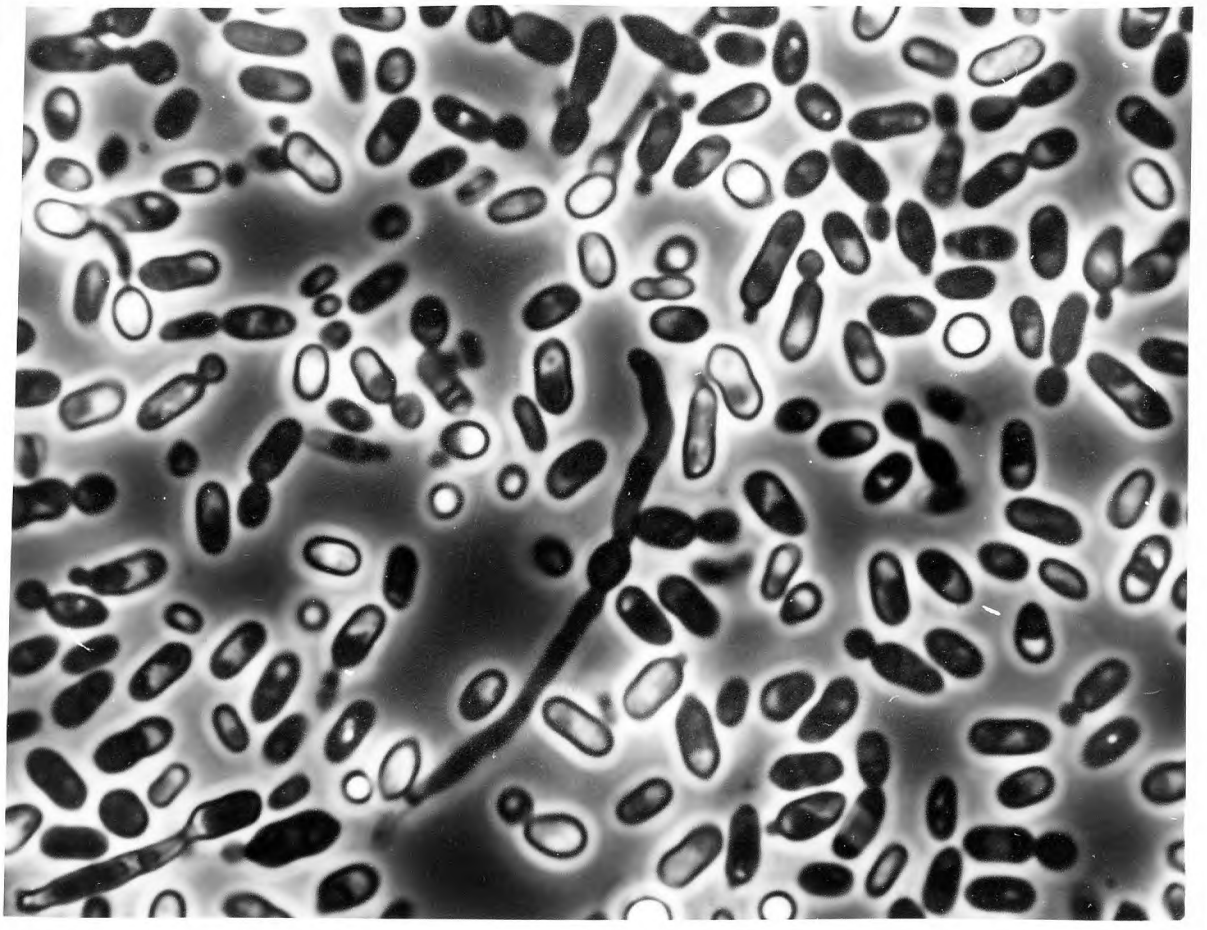


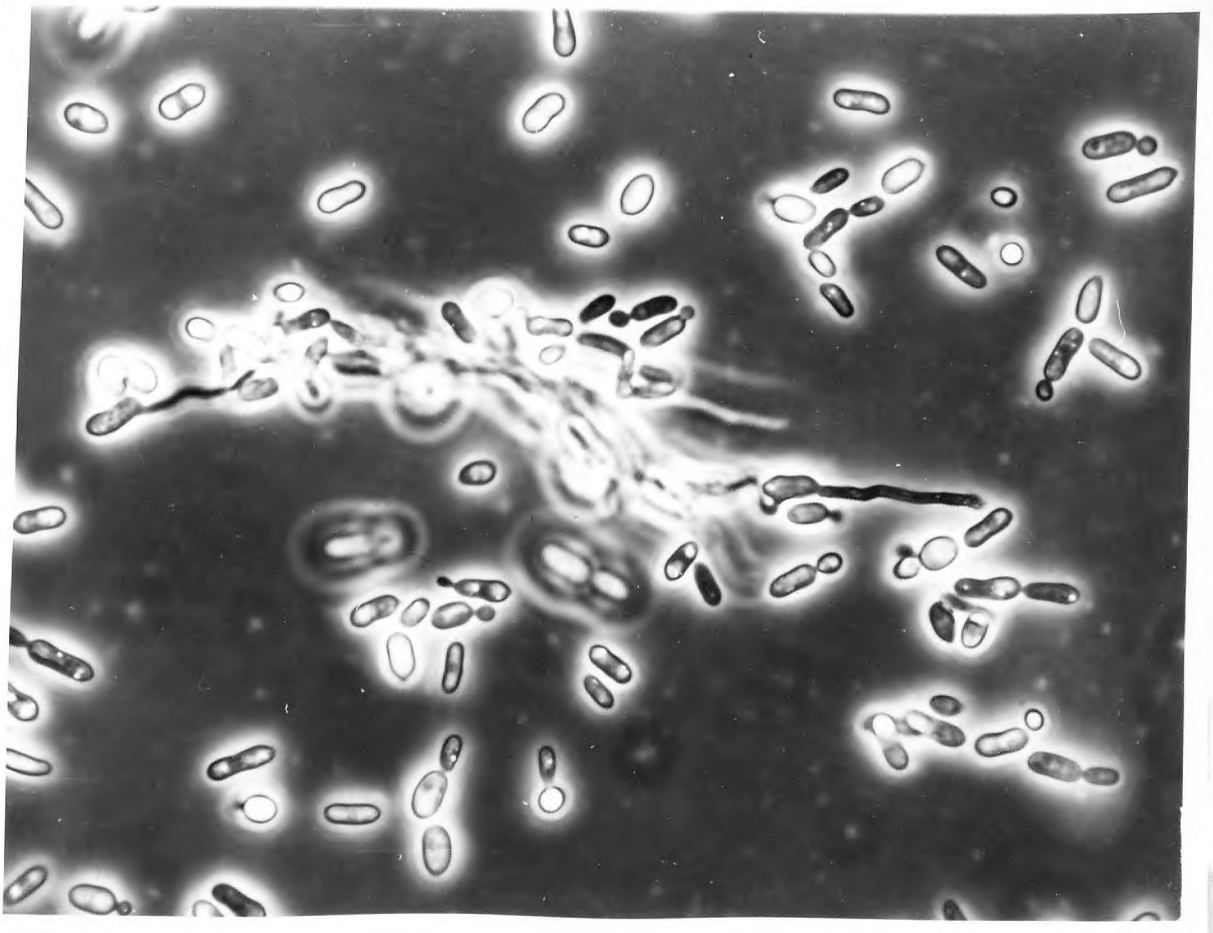
c) Cells commencing mycelial growth.

Magnification x 6000.

d) Hyphal strand with yeast cells surrounding it.

Magnification x 4000.



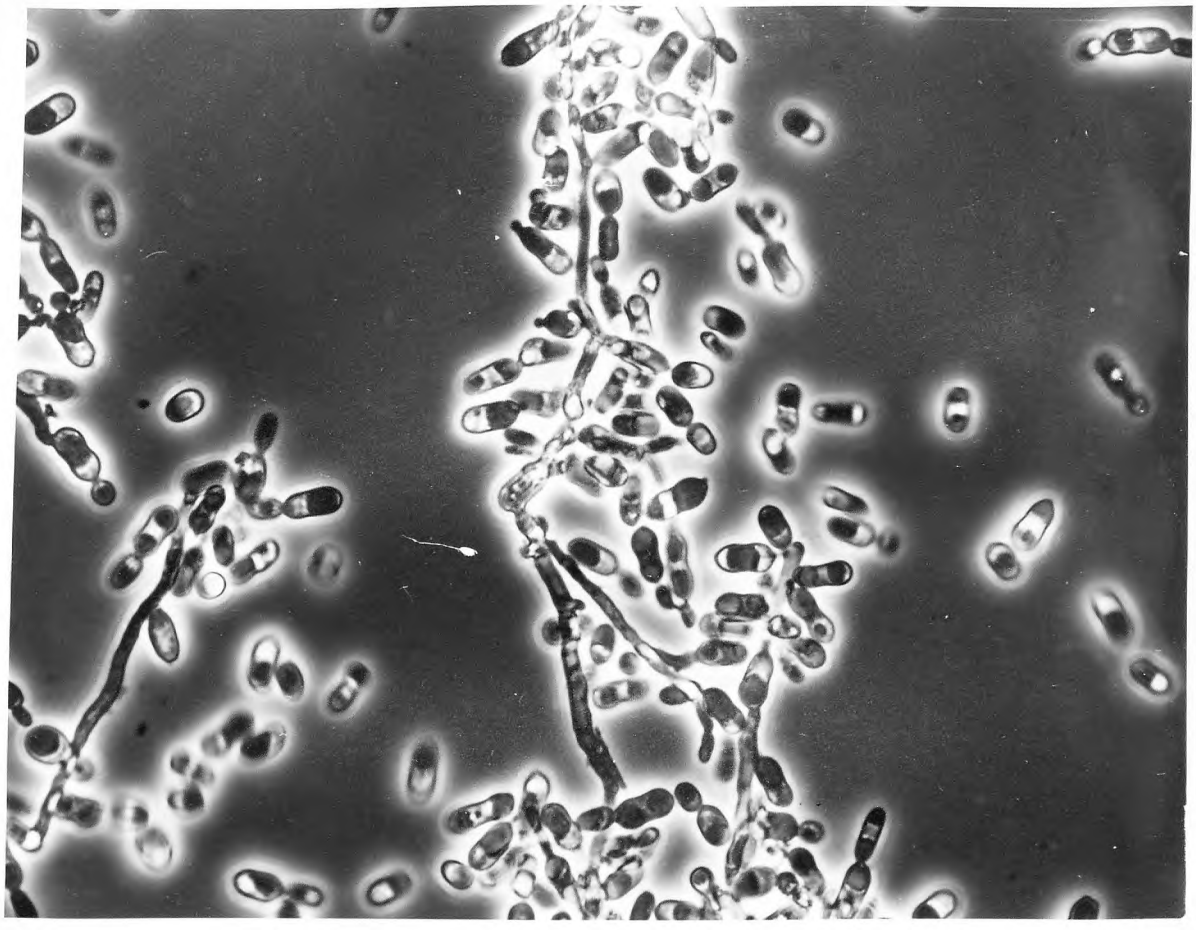


e) Mycelium with yeast cells attached.

Magnification x 4000.

f) Large cluster of yeast cells around mycelium.

Magnification x 4000.



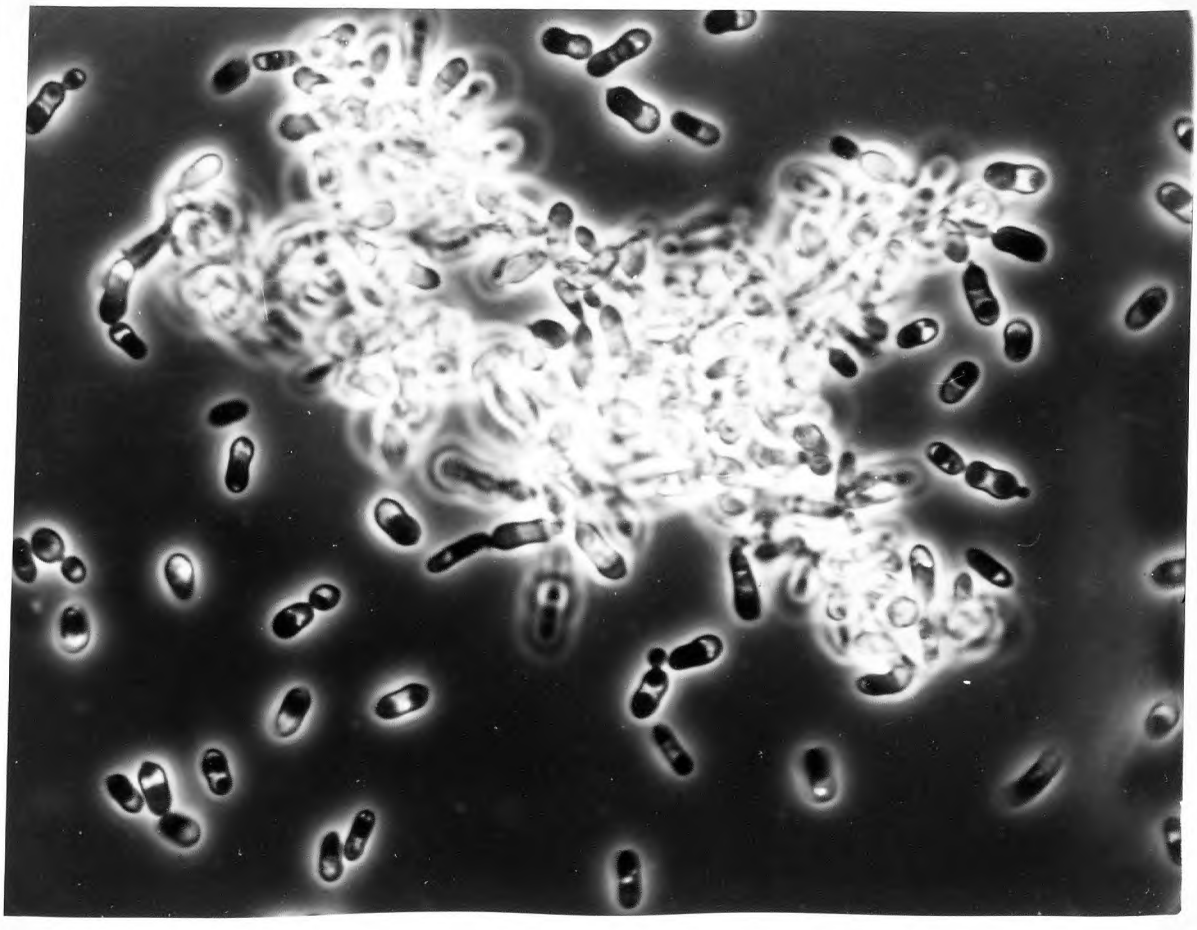
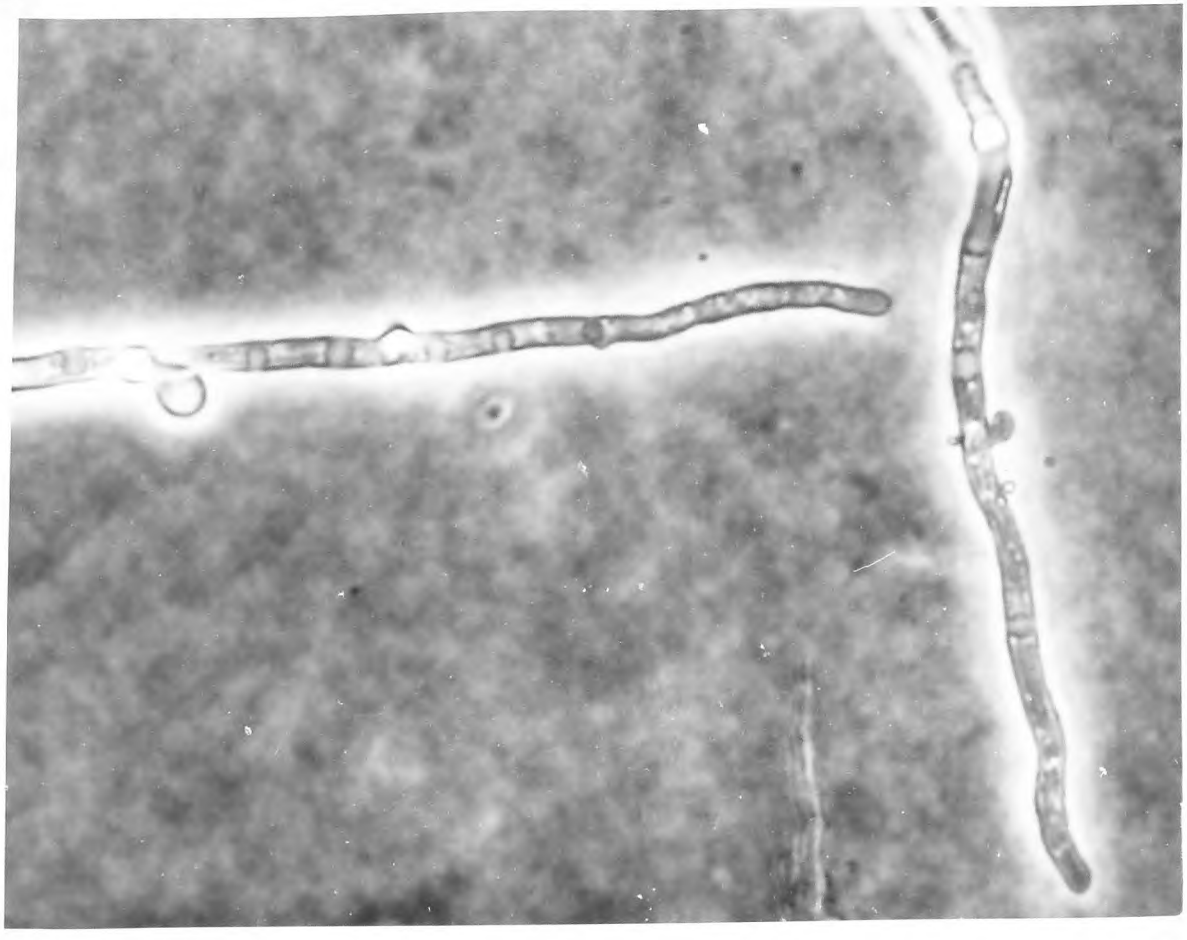
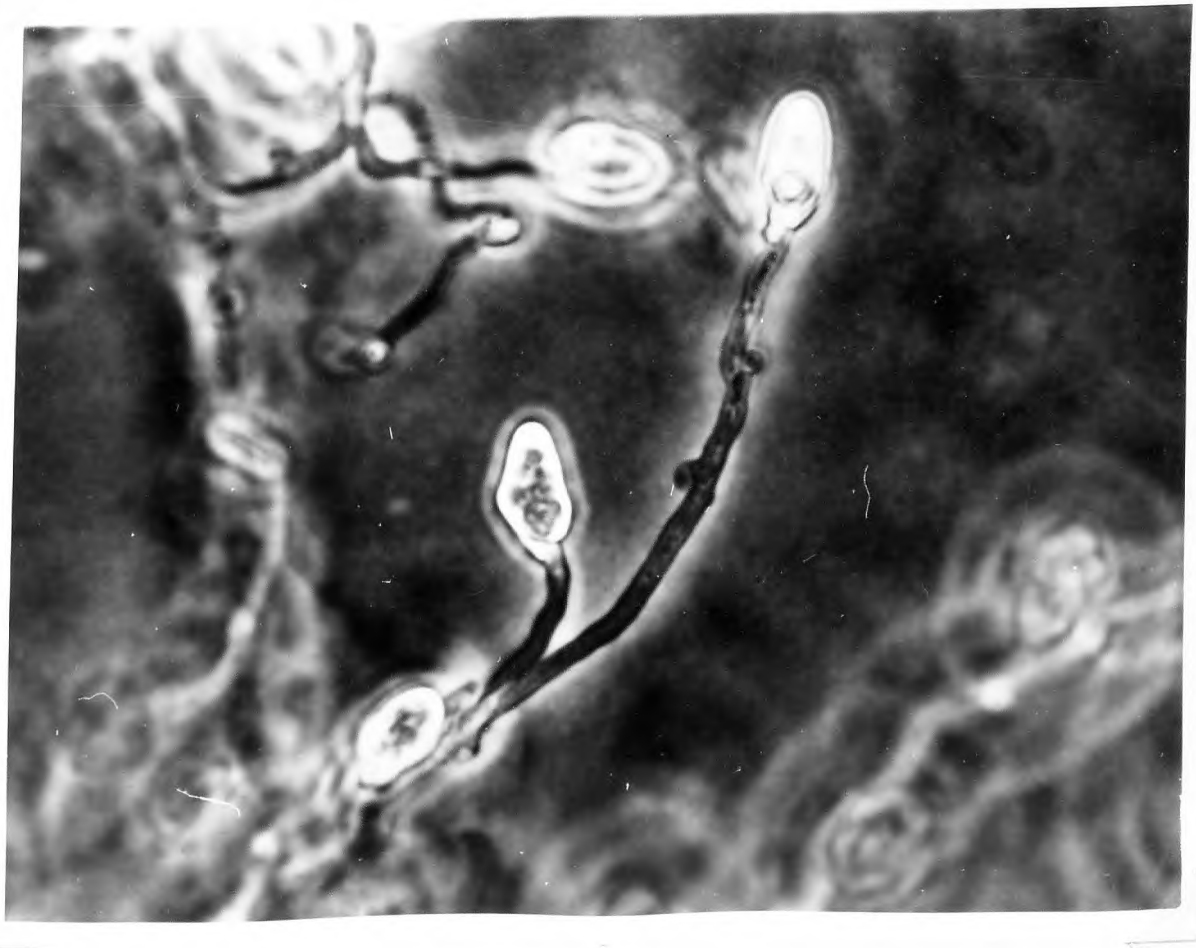


Figure 18

a) IFO 10076 mycelium with septa and pseudoclamps.
Magnification x 6000.

b) IFO 10076 mycelium with teliospores and pseudoclamps.
Magnification x 6000.





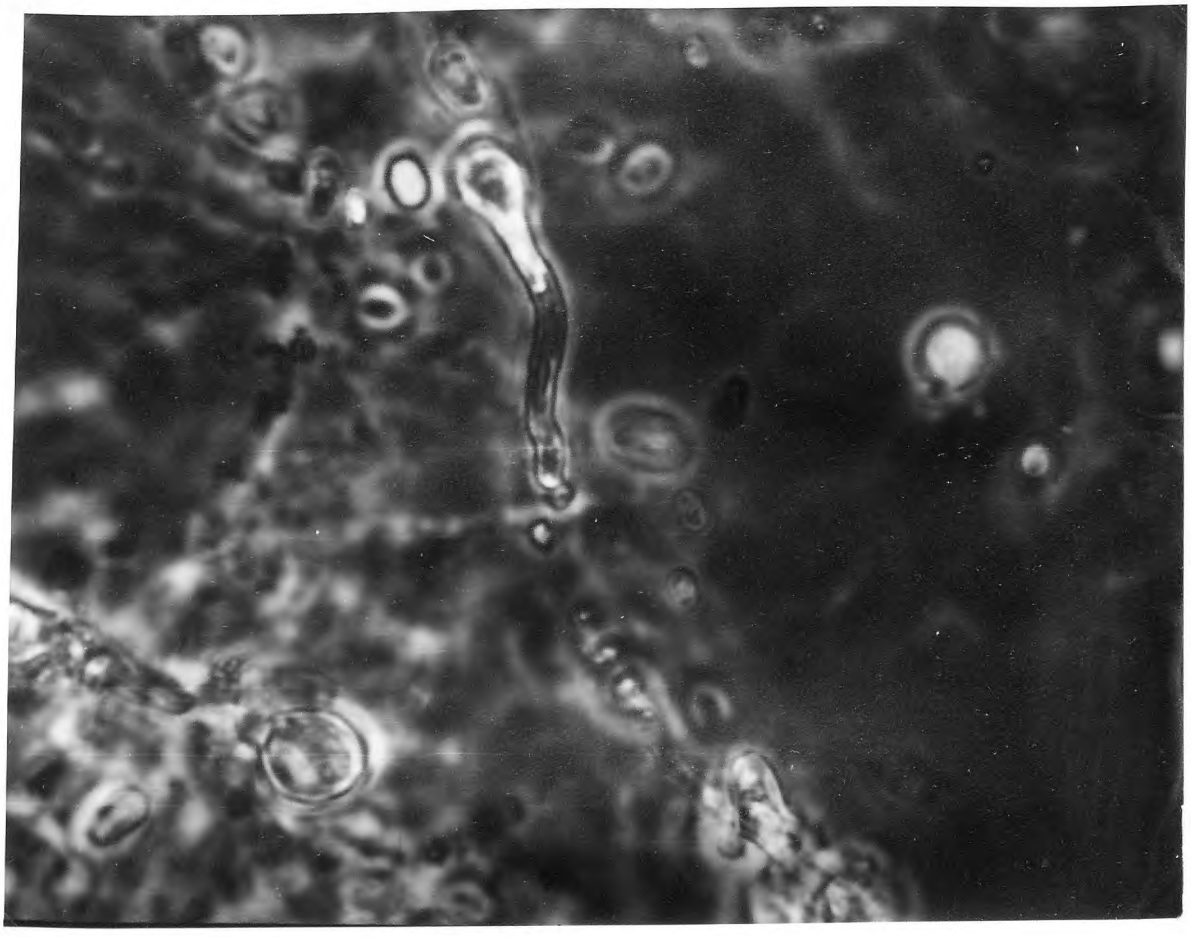
c) Complete promycelium.

Magnification x 6000.

d) First basidiospore produced at the tip of the promycelium.

Magnification x 4000.

Basidiospores were parted from the promycelium by sonication and separated on a sucrose gradient at this stage.



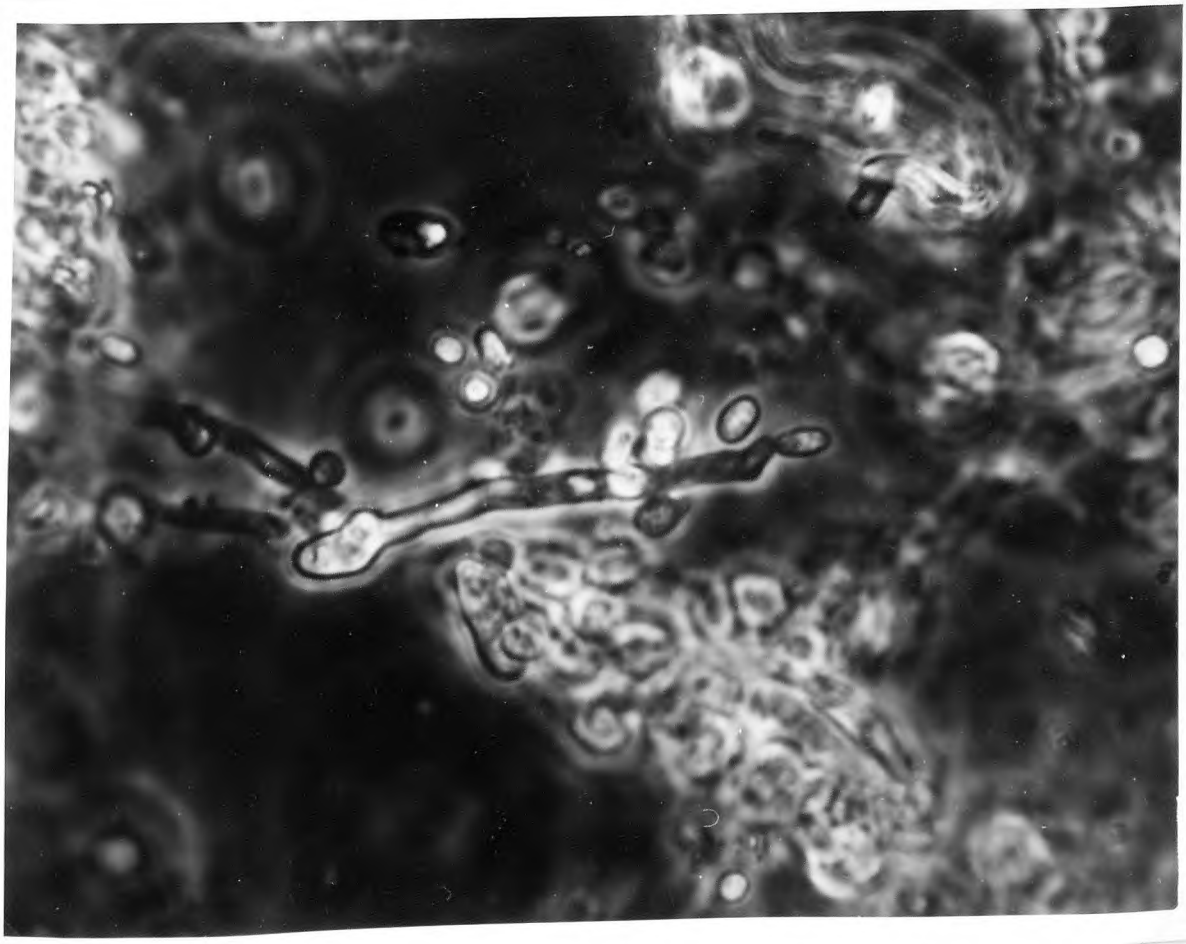


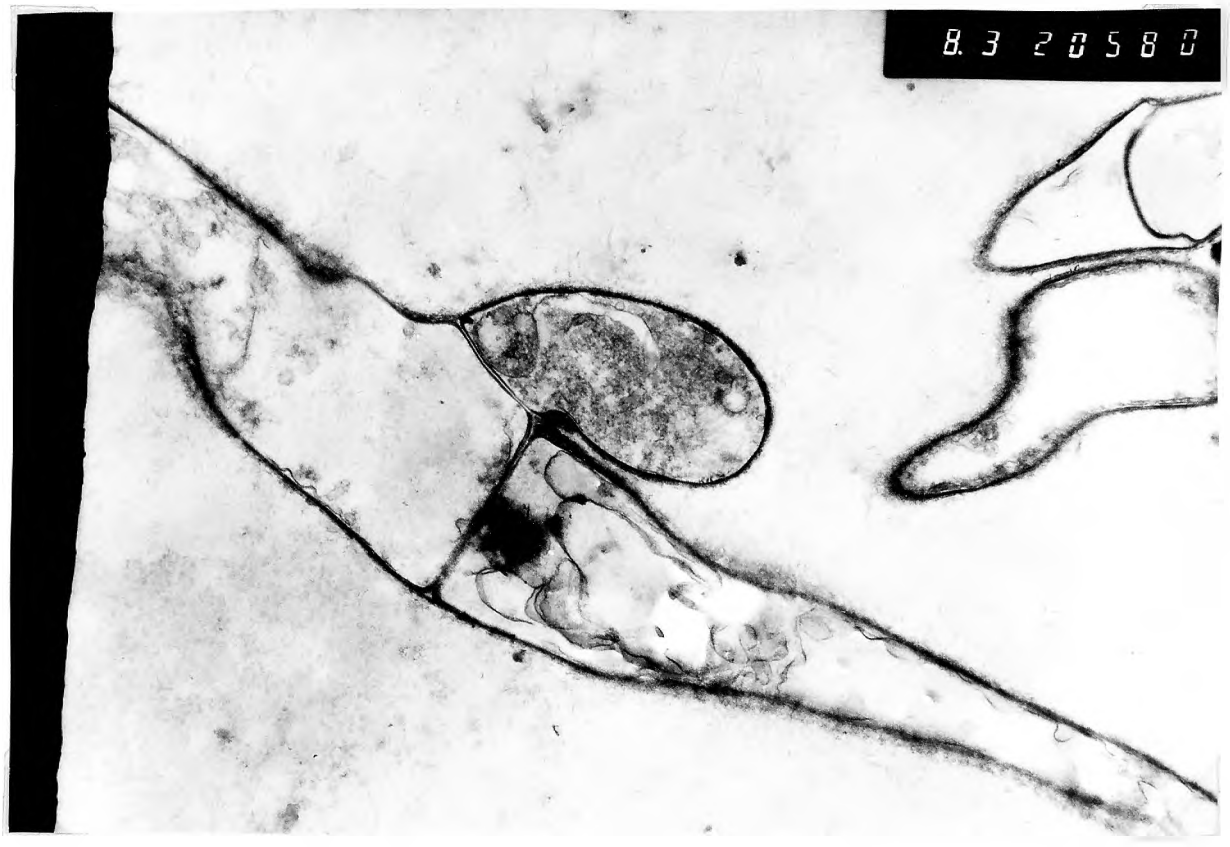
Figure 20 Electron Micrographs of Pseudoclamps of IFO 10076

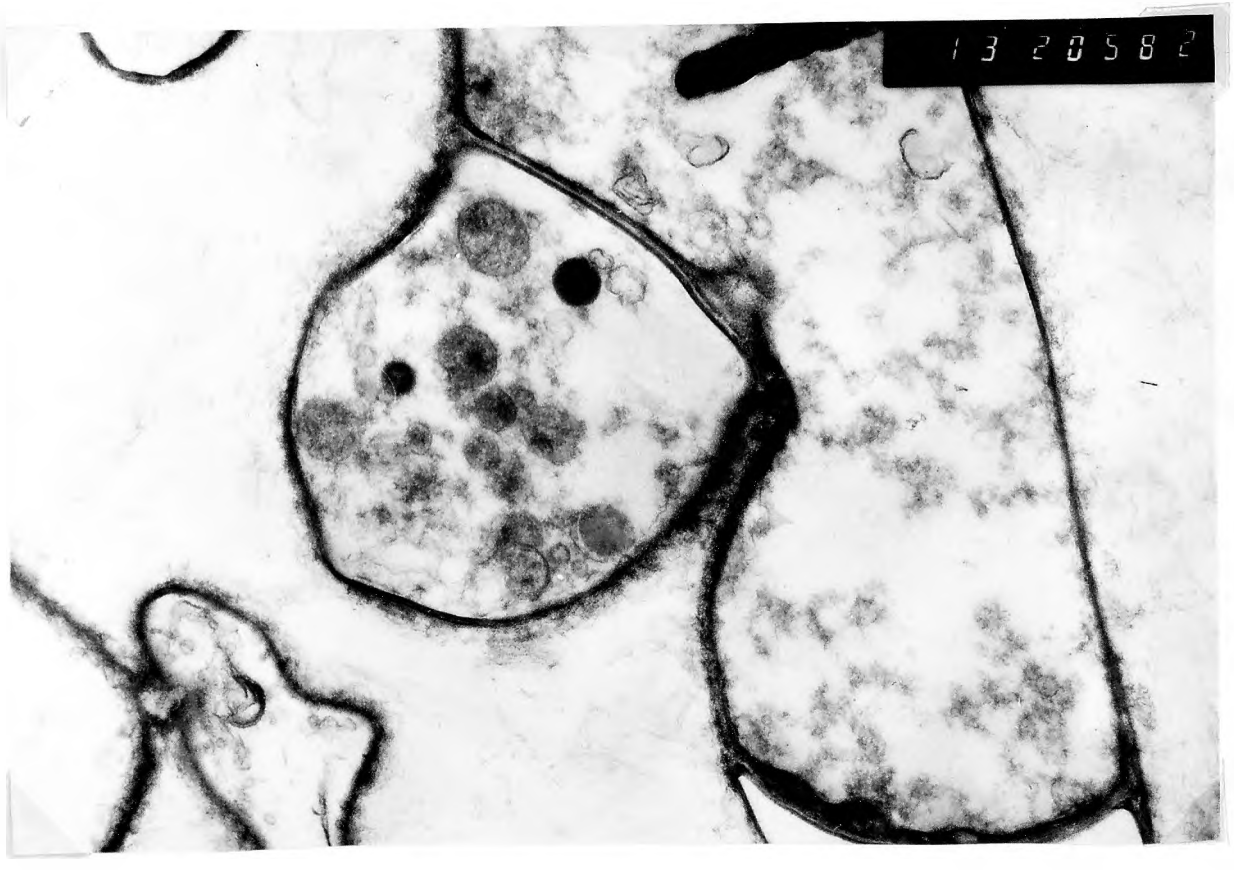
a) Pseudoclamp near septum.

Magnification x 16 000

b) Interseptal peudoclamp.

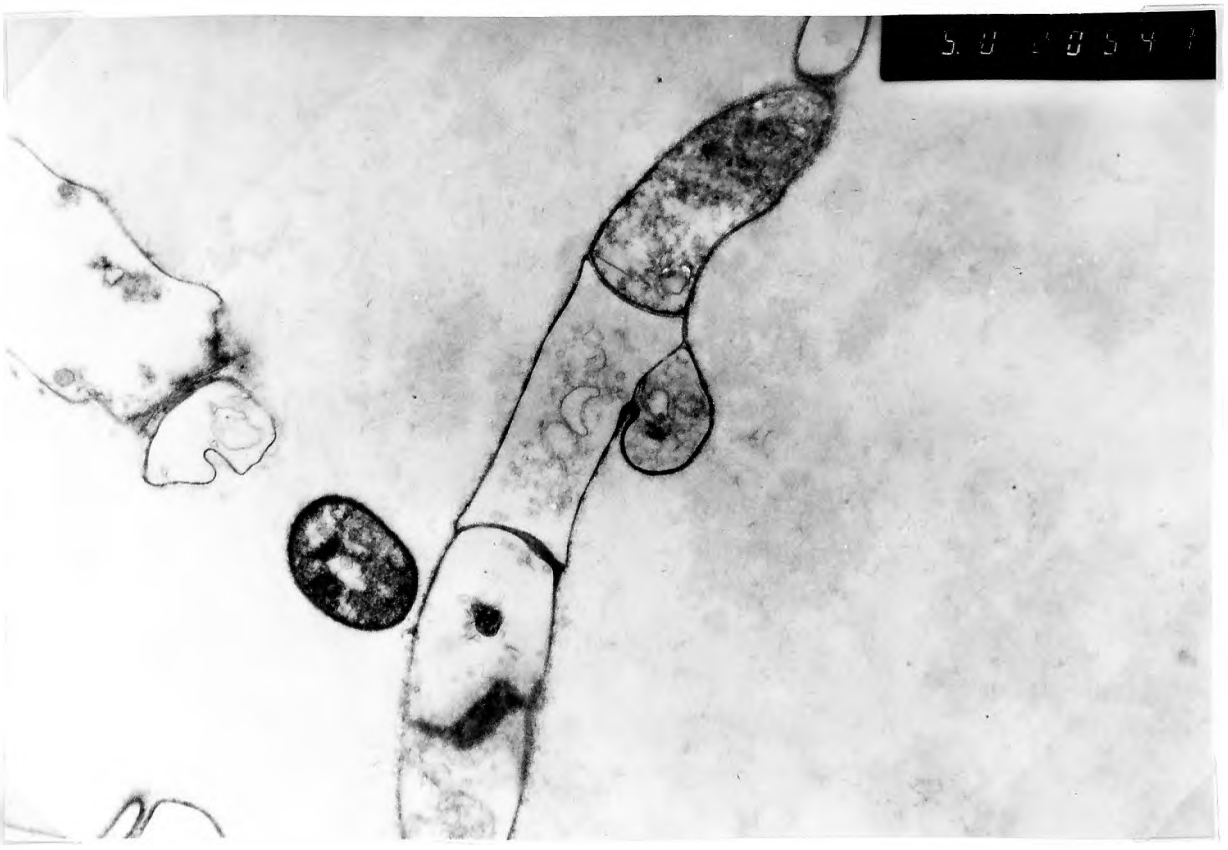
Magnification x 26 000


8.3 20580


13 20582

c) Interseptal pseudoclamp

Magnification x 10 000



DISCUSSION

1. ATP:citrate Lyase Activity And Lipid Content In Rhodosporidium toruloides.

Of the 21 strains of Rhodosporidium toruloides investigated, all showed ATP:citrate lyase activity except for one strain, CBS 315. In most cases the ACL activity was higher when the nitrogen source was sodium glutamate instead of ammonium tartrate. The lipid content of cells grown on medium containing glutamate was also generally higher, although there was no correlation between ACL activity and lipid content. The biochemical explanation for this increased lipid content was provided by Evans (1983), who found that it is the change in activity of glutamate dehydrogenases (GDH) that brings about the increased lipid content of glutamate grown cells. The growth of cells on glutamate leads to an increase in the activity of NAD^+:GDH, which converts glutamate to NH_4^+ and 2-oxoglutarate resulting in a high intracellular concentration of both these metabolites. The elevated intracellular NH_4^+ pool and decreased glucose 6-phosphate pool also found in glutamate grown cells of R. toruloides prevent the diversion of carbon into polysaccharide synthesis (Trevelyan and Harrison, 1955, Dicks and Tempest, 1967, Quain and Haslam, 1979). When cells are grown on NH_4^+, the activity of $NADP^+$:GDH increases. This enzyme synthesizes glutamate from NH_4^+ and 2-oxoglutarate, so that the intracellular NH_4^+ concentration does not build up as it does in glutamate grown cells (Evans and Ratledge, 1984).

In oleaginous yeasts, ACL activity is similar under carbon-limited and nitrogen-limited conditions and the specific activity of ACL does not correlate with the lipid content of the cell (Boulton and Ratledge, 1981). In the 20 strains of R. toruloides in Table 3 with ATP:citrate lyase, the ACL activity does not correlate with lipid content. In some cases when a very high ACL activity was recorded, the lipid content was extremely low (e. g. ML 2573, ACL spec. act. 80 nmoles/min/mg, lipid content 6%). It would seem that although ACL is an absolute requirement for oleaginicity, other factors must also be optimised before the cell can accumulate high concentrations of lipid. This has implications for the genetic manipulation of non-oleaginous yeasts. Clearly a cell cannot be induced to accumulate lipid simply by introducing the ACL gene and growing the yeast under nitrogen-limiting conditions.

As expected, the strain CBS 315 which does not possess active ACL has a low lipid content, similar to that of Saccharomyces cerevisiae or Candida utilis grown under the same conditions. Microscopic examination of this strain after growth on NLM confirms the absence of intracellular lipid droplets.

2. Carnitine Acetyl Transferase Activity.

Published values of CAT activity in glucose grown S. cerevisiae vary from 4 to 15 nmoles/min/mg protein (Claus et al., 1983) to 1167 nmoles/min/mg protein (Kohlhaw and Tan-Wilson, 1977). My own results, showing a specific activity of around 200 nmoles/min/mg protein (See Table 4) fall between these two

values. It is not clear why these values should vary to such a great extent. The low values recorded by Claus et al. (1983) may be due to the presence of an inhibitor in the assay system. CAT is known to be inhibited by divalent cations and reagents specific for protein thiol groups (Chase, 1969) and I found it necessary to dialyse the cell free extracts to remove inhibitors before reproducible results could be obtained. It may be argued that overnight dialysis could lead to a significant decrease in specific activity; however the cell free extract was prepared exactly as described by Tracy and Kohlhaw (1975) to minimise proteolytic activity, and under these conditions enzyme activity was found to decrease by less than 10% over a two week period (Kohlhaw and Tan-Wilson, 1977).

CAT activity was found to vary widely among the yeasts listed in Table 2. In non-oleaginous yeasts, provision of cytosolic acetyl CoA depends entirely on this enzyme, so that it is to be expected that CAT activity should be highest in these yeasts, whereas in oleaginous yeasts, it had been anticipated that CAT activity may be low or even non-existent, as ACL also supplies acetyl CoA to the cytoplasm. However among the oleaginous yeasts, both ACL and CAT activity vary considerably. For example, NCYC 921 has a low CAT activity, but ATCC 26217 has high activities of both CAT and ACL. Clearly both CAT and ACL are important in the supply of cytosolic acetyl CoA in oleaginous yeasts, but the relative importance of the two routes is different even among different strains of the same species. In C. curvata species R and D and T. cutaneum, the observed activity

of ACL has always been low (Boulton and Ratledge, 1981) despite the high lipid contents which can be achieved. In these yeasts, CAT must provide much of the acetyl CoA for lipid synthesis.

In yeasts grown on alkanes, the activity of carnitine acetyl transferase is greatly elevated (Ueda et al., 1985). This is due to the 'carnitine shuttle' which transports acetyl units from peroxisomes, where alkanes are metabolised, to the mitochondria. Although none of the oleaginous yeasts included in this study will grow on alkanes, they will grow on fatty acids or triacylglycerols. The CAT activity of 4 yeasts grown on triolein increased considerably over the activities of glucose-grown cells. The greatest rise was seen with C. curvata D which showed a 30-fold increase. It would seem that in oleaginous yeasts CAT can transport acetyl groups into the mitochondria when metabolising fatty acids, or out of mitochondria when synthesising fatty acids.

The presence of CAT activity in oleaginous yeasts means that ACL is not the only enzyme that supplies cytosolic acetyl CoA in these yeasts. Thus the deletion or alteration of ACL in an oleaginous yeast would not be expected to be a lethal mutation, and so it would seem possible to follow this approach to studying the genetics of oleaginicity in R. toruloides (See Section 6 below).

3. Protoplast Formation.

The use of snail digestive juice to form protoplasts of R. toruloides has been described (von Hedenstrom and Hofer, 1974,

Sipiczki and Ferenczy, 1977, Becher and Bottcher, 1980, Bottcher et al., 1980). However the maximum yield of protoplasts formed using βglucuronidase/arylsulphatase was 90%. Some of the procedures that could be used to study the genetics of R. toruloides require the formation of protoplasts (e. g. protoplast fusion, transformation). As other enzyme preparations for the formation of protoplasts from yeast are now commercially available, the use of one of these, Novozym 234, was examined to see if the yield of protoplasts could be improved.

It was found that using the method of Dickinson and Isenberg (1982) the yield of protoplasts was 100% after an incubation period of one hour. It was also shown that protoplasts formed by this method were capable of regenerating a cell wall and re-commencing yeast-like growth. The manufacturers of Novozym 234 (Novo Enzyme Products Ltd.) claim that when stored at temperatures below 5 ^{o}C, the product remains active for at least one year. However I found that the activity had significantly decreased after 9 months storage at 4 ^{o}C. Novozym 234 contains a mixture of enzyme activities, and by adding other commercially available enzymes to the protoplasting mixture it was possible to demonstrate that the 1,3 βglucanase activity was lacking in the 9 month old Novozym 234. This may be a disadvantage in the use of Novozym 234 for the formation of protoplasts from R. toruloides, but in any laboratory where protoplasts were routinely required, fresh supplies of the enzyme preparation could be ordered at short intervals to minimise the loss of activity seen during long term storage.

4. Evaluation Of Fast Screening Methods To Detect Lipid Content Of Yeast.

Any scheme to study the genetics of lipid accumulation in oleaginous yeasts requires the screening of large numbers of cells, liquid cultures or colonies to detect lipid content, for instance after mutagenesis, mating, protoplast fusion or the introduction of foreign genes. The conventional method used for determining total lipid content by solvent extraction, although accurate, is extremely slow, and requires about 0.5 g of washed, freeze-dried yeast. Microscopic examination to observe lipid droplets in cells can be carried out immediately, but is still time consuming if large numbers of cultures have to be examined, and it is possible only to distinguish between 'high', 'medium' and 'low' lipid cells. Two novel methods for the rapid measurement of lipid in yeast were examined. The first of these two methods had already been published, the second had been partially developed by C. T. Evans and passed on for further examination and assessment.

4.1 Fluorochrome staining.

This method (Pomoshchnikova et al., 1981 and 1983) involves the use of the fluorochrome 1'8 naphthoylene-1,'2'-benzimidazole to stain the intracellular lipids of yeast cells in suspension. It has the advantages that the results are available within a few hours and large numbers of cultures can be treated simultaneously. Preliminary trials showed a correlation between the amount of stained yeast in a suspension and the fluoresence

of that suspension. The method was then tested with yeasts of different lipid contents to look for a correlation between lipid content and fluoresence. The published data available only show the fluorescence of yeasts with a low lipid content (up to 8% w/w). My results using the same method showed no relationship between the lipid content of a yeast and the fluorescence intensity of the stained suspension, regardless of whether the suspension was heat-treated before staining, and it would seem that this method will not be of use as a fast screening method to detect lipid content of yeast. The initial results showing a relationship between yeast concentration and fluoresence were probably due to the staining of cell membrane lipids only, so that increased yeast concentration results in increased fluorescence, but increased intracellular lipid concentration at any given yeast concentration does not affect the fluoresence.

4.2 Staining Of Colony Prints.

This method has the advantage that the approximate lipid content of individual colonies can be determined, so identifying, for example, a single high lipid colony against a background of low lipid colonies. The initial problem to be overcome was to find a solid growth medium that would induce potentially oleaginous cells to accumulate lipid as they would do in liquid NLM. After testing various media, a satisfactory formulation was arrived at. The replica printing and staining method (described in Materials and Methods Section 8.2) when used with C. curvata did not stain colonies with a lipid content of less than 10%, with 14 to 22% lipid colonies stained pale blue and with 35% or more lipid

colonies stained dark blue or purple. It was also possible to distinguish between colonies of C. curvata and S. cerevisiae growing on the same low nitrogen agar plate by the colour of the colony prints (Evans et al., 1985). Similar results were obtained with T. cutaneum and L. starkeyi. The yeasts listed above are all white or cream coloured and the replica prints of the colonies contain no pigment which might interfere with or obscure the results of a colour-based test such as this one. However the red pigment (rhodotorulin) of R. toruloides obviously presented a problem. In fact, the method proved unsuitable for distinguising between low and high lipid colonies of R. toruloides, but close visual inspection of the replica prints suggested that this was because of the failure of the stain to penetrate the lipid droplets of the yeast sufficiently, and not simply a problem of one coloured compound masking another. Stained prints of all colonies were purple or black, but the stain appeared to be present only on the surface of the print, and on destaining high and low lipid colonies destained to the same extent. Treatments to permeabilise the yeast cell wall and allow the stain to reach the lipid were unsuccessful as disruption of the yeast cell allows both lipid and stain to leak out during destaining, leaving an unstained print. Other solvents for the stain were found to give the same results as ethanol. Thus although this method works well with other oleaginous yeasts, it appears to be of no use for distinguishing between high and low lipid colonies of R. toruloides, and a much more complex identification method had to be devised to identify

ACL-less mutants (see Materials and Methods Section 12). It seems that both lipid staining methods failed to work with R. toruloides for the same reason: that the cellular structure of this basidiomycete yeast does not allow stains to penetrate to the lipid droplets as it can with ascomycete yeasts.

5. Isolation Of Auxotrophs.

5.1 Nystatin Enrichment.

Nystatin enrichment was routinely used in the isolation of auxotrophs from R. toruloides after the effectiveness of the method had been demonstrated (See Results Section 5). During the nystatin enrichment procedure, the growth of the cells is stopped, then the growth of prototrophs is re-started. When nystatin is added, the actively metabolising cells are killed by it. The results show that after 40 minutes exposure to nystatin, on average some 330 actively growing cells are killed for each non-growing cell, thus bringing about a considerable enrichment of the proportion of auxotrophs in the population.

5.2 Isolation Of Auxotrophs After Mutagenesis.

Although the use of ultra-violet light as a mutagen led to the isolation of several different auxotrophs, all were found to revert to prototrophy shortly after their growth requirements had been identified (See Results Section 6.1). As the use of other mutagens, namely EMS and NTG led to the isolation of mutants with low reversion frequencies, the use of UV light was not continued.

The range of auxotrophic mutants that can be isolated

from strains of R. toruloides has been discussed previously (Bottcher and Samsonova, 1978 and 1980a). These workers found that with the strain they studied most extensively, Rg1, certain auxotrophs were isolated more frequently than others. The most commonly isolated auxotrophs required methionine, cysteine, leucine, arginine or adenine. Auxotrophs requiring tryptophan, tyrosine, threonine, isoleucine, serine, glycine or alanine were never isolated. When other strains were examined, the frequencies for isolation of certain auxotrophs were slightly different, but mutants requiring tryptophan, tyrosine, threonine, serine or glycine were never found. I found a similar pattern with the strains I examined, isolating auxotrophs requiring lysine, adenine, arginine, cysteine or methionine, proline and alanine from the strain IFO 0880, and auxotrophs requiring arginine, adenine, methionine, isoleucine and uracil from CBS 315. Auxotrophs requiring tryptophan could not be isolated from either strain, nor could mutants requiring valine be isolated from CBS 315. There are several possible causes for the mutant frequencies described.

1. It may be that, for instance, tryptophan was not present in sufficiently large quantities in YMG, the recovery medium used after mutagenesis, to allow the growth of trp^- auxotrophs. However in subsequent experiments MM+tryptophan was used as the recovery medium, and this still did not lead to the isolation of trp^- auxotrophs.

2. It may be that tryptophan is not transported into the cell, and so all try^- cells die after mutagenesis, however this

is not only unlikely but was ruled out by Bottcher and Samsonova (1978) demonstrating that tryptophan was taken up by R. toruloides.

3. Another possible explanation is the aneuploidy of Rhodosporidium cells. If there are, for example, two copies of the tryptophan biosynthesis genes in a cell but only one copy of the adenine biosynthesis genes, then ade$^-$ auxotrophs will clearly be isolated much more frequently than trp$^-$ auxotrophs. This was considered by Bottcher and Samsonova (1980a) to explain the differences in mutant frequencies, and also the slight differences seen between strains. If the degree of aneuploidy differs between strains, then the number of different types of auxotrophs that can be isolated will also differ. However I intend to show, when discussing the results obtained from continuous culture of R. toruloides, that the results obtained for the DNA contents of strains of R. toruloides may be misleading, and the conclusion that many strains are aneuploid, to varying degrees, is incorrect.

4. Another possible explanation for the difference in mutant frequencies is that alternative biosynthetic pathways exist for many essential metabolites (Jensen, 1976). Due to the broad substrate specificity of many enzymes, if one enzyme is inactivated, the end-point of the pathway may be reached by a different route. Thus it may be necessary to induce mutations in several different pathways before the synthesis of an essential amino acid becomes impossible, greatly reducing the chance of isolating certain auxotrophs. However there is no evidence to

suggest that R. toruloides has alternative biosynthetic pathways for tryptophan, valine, tyrosine and all the other amino acids for which auxotrophs have never been isolated. None of these four possible explanations satisfactorily accounts for the failure of independent workers to isolate certain classes of auxotroph, and it seems that further investigation will be necessary before this phenomenon can be explained.

Another factor influencing the isolation of mutants from R. toruloides is that the mutation rate of some strains can be very low. Bottcher and Samsonova (1980b) found that in one strain, IFO 0559, the frequency of isolation of auxotrophic mutants was extremely low. To investigate this phenomenon, they investigated the frequency of 'back mutation' from $nic2^-$ to prototrophy. The reversion rate was measured instead of the forward mutation rate as reversion to prototrophy is not greatly affected by the number of copies of the gene present in the cell, so that the presumed aneuploidy of this strain would not interfere with the results. The yeast was exposed to UV or NTG and the number of revertants was calculated. The reversion frequency after treatment with either mutagen was approximately 20 times lower in IFO 0559 than in another strain, Rg1. However this experiment measured the reversion rate of two different mutations, one in each strain, and the reversion rates of the two mutations would be expected to be different. The low forward mutation rate of IFO 0559 is probably due to the high average DNA content compared to Rg1.

Using strains IFO 10076 and CBS 6681, I was not able to

isolate any auxotrophic mutants. This may be due to the defective mutagenesis described by Bottcher and Samsonova (1980b); however both CBS 6681 and IFO 10076 have a very high DNA content and failure to isolate auxotrophs may be due to the high DNA content, which indicates diploidy, rather than insensitivity of the DNA to mutagens.

6. The Isolation Of ACL-less Mutants.

The reasons for isolating ACL-less mutants and the procedure used have been discussed in Results (Section 7) and Materials and Methods (Section 12). To summarise: ACL-less mutants may produce ACL sub-units which have alterations or small deletions causing the loss of activity of ACL. If the altered ACL was partially purified using procedures developed for the partial purification of ACL from Lipomyces species (Boulton and Ratledge, 1983) then sub-units from different mutants could be used for in vitro complementation studies (Wieland et al., 1979, Werkmeister et al., 1981). ACL has a molecular weight of about 510 000 and is thought to be composed of four sub-units, but it is not clear how many different types of sub-units are involved. If, for example, sub-units from mutant A and mutant B could be reconstituted to form active ACL, then there must be two different types of sub-units, one of which is inactivated in mutant A, and the other in mutant B. It would also be possible to investigate ways of re-introducing the wild-type ACL gene into the ACL-less mutant (transformation, protoplast fusion, mating) so that the feasibility of introducing this gene into non-oleaginous

organisms (e. g. CBS 315, Yarrowia lipolytica, Saccharomyces cerevisiae) could be determined.

No simple isolation technique for these mutants was available. The loss of ACL activity would be expected to cause the loss of oleaginicity, but techniques for rapidly determining the lipid content of yeast cells are not applicable to R. toruloides (see Section 4 above). The method that was devised to isolate ACL-less mutants would detect cells accumulating and secreting citrate, which would occur if the cells were grown in N-limiting medium, when ACL was no longer active. Other mutations may also cause the accumulation of citrate; for instance mutations affecting the activity of aconitase or NAD^+dependent isocitrate dehydrogenase. Total inactivation of these enzymes would be lethal, but a reduction in their activity may lead to a build-up of citrate inside the cell. However if ACL was still active in these cells, the citrate would be cleaved by ACL and would not be secreted by the cell as in ACL-less mutants. The citrate-detecting method was clearly successful in identifying four citrate-accumulating isolates, two of which showed no ACL activity. Three of the citrate accumulators, H1, H2 and H3, had been isolated from the same recovery flask after mutagenesis and, as citrate accumulators were found in only two groups out of twenty examined, it seems probable that H1, H2 and H3 were derived from the same mother cell and were genetically identical. However H2 and H3 showed ACL activity while H1 did not. When the assay for ACL was repeated four days later, all four isolates now showed ACL

activity, and subsequent assays showed that citrate no longer accumulated in the growth medium. It appears that H2 and H3 had reverted from ACL^- to ACL^+ during the period of growth in preparation for the first assay, and H1 and J reverted before the second assay was carried out. No other ACL-less mutants were isolated.

The reasons for the reversion of the ACL-less mutants are not clear. An alternative route for acetyl groups crossing the mitochondrial membrane is provided by CAT in IFO 0880 (See Section 2 above) so that while the cell is actively growing, lipid synthesis to provide cell membrane components etc. may still be carried out. However there would seem to be a very strong selection pressure against ACL-less mutants. It may be that the accumulation of citrate has an adverse effect on the cell, although many yeasts accumulate citrate without being poisoned by it. Obviously if a stable ACL-less mutant was isolated, the effect of the loss of this enzyme on the metabolism of the cell could have been investigated, but as a stable mutant was not isolated it has not been possible to discover why a stable mutant cannot be isolated.

7. The DNA Content Of R. toruloides

As it does not appear to be possible to isolate stable ACL-less mutants by exposure to a mutagen, another means will have to be found if this approach to studying the genetics of oleaginicity is to be continued. However, at this stage there were some doubts about the suitability of IFO 0880 for this type of study.

This strain was supplied by Dr. M. Tully of the Centre for Applied Microbiology and Research, Porton Down, with the information that it was haploid, although other strains of R. toruloides, notably IFO 0559 which was supplied at the same time, were aneuploid. The view that this strain is haploid is supported by Banno (1967) and Abe et al. (1978, 1986). Bottcher et al. (1980), reporting the DNA contents of 6 strains of R. toruloides, found that IFO 0880 had the lowest DNA content of these 6 strains, and that the figure obtained for the DNA content of IFO 0880 was exactly half that of the highest figure obtained, again showing that IFO 0880 is haploid. The figures given by these workers for the DNA content of four of the strains were as follows: IFO 0880, 31 fg/cell; Rg1, 35 fg/cell; IFO 0559, 51 fg/cell; CCY-20-2-16, 53 fg/cell. However in another publication (Becher and Bottcher, 1980) the figures given for the DNA content of these strains are IFO 0880, 67 fg/cell; Rg1, 65 fg/cell; IFO 0559, 97 fg/cell; CCY-20-2-16, 95 fg/cell. The values quoted for the DNA content of micro-organisms depend to some extent on the exact method used to determine them, and results obtained by different laboratories may vary. Although D. Becher and F. Bottcher are authors of both papers, it must be assumed that the figures quoted for DNA contents in Bottcher et al. (1980) were obtained by one of the other authors of that paper. What is important, though, is that the relative DNA content of the strains is different in the two sets of figures. If the lowest value of each group is assigned the value 1.00, then the results read: IFO 0880, 1.00; Rg1, 1.13; IFO 0559, 1.65; CCY-20-2-26,

1.71 (Bottcher et al., 1980) or Rg1, 1.00; IFO 0880, 1.03; CCY-20-2-16, 1.46; IFO 0559, 1.49 (Becher and Bottcher, 1980). In the first set of figures IFO 0880 has a value 13% lower than Rg1, whereas in the second set it is 3% higher, showing that IFO 0880 may actually be aneuploid and that the degree of aneuploidy of the strains IFO 0559 and CCY-20-2-16 is variable.

The shape of the killing curves of IFO 0880 exposed to EMS and NTG (See Figure 9) also suggest that the culture of IFO 0880 in my possession is not haploid. The 'shoulder' on the curve, which is not seen with the strain CBS 315, implies that a lethal mutation in one copy of a gene does not kill the cell, and so there must be two copies of at least some essential genes.

These doubts about the ploidy of IFO 0880 and the stability of the DNA content of other strains resulted in my determining the DNA content of 8 strains in my own culture collection. The results showed that IFO 0880 is not haploid, with a relative DNA content of 1.38 (if CBS 315 is assigned the value 1.00). Two other strains, ML 2589 and ML 2590 also appear to be aneuploid, and the strains CBS 14 and IFO 0559 would seem to be diploid on this basis. Two strains, CBS 6681 and IFO 10076, have a relative DNA content greater than 2.00. Further investigation of these two strains revealed that both form mycelium when grown in liquid YMG. A small proportion (less than 0.1%) of the cells in the culture may switch from yeast-like to mycelial growth, forming short lengths of hyphae. As the frequency of the occurrence of this phenomenon is so low, it is not always observed when counting cells in order to determine the

DNA content per cell, but as discussed in Results (Section 12), a very small proportion of these hyphae in a liquid culture can lead to an under-estimate of the cell number, and hence an over-estimate of the DNA content per cell.

The DNA content of CBS 315 corresponds to 18580 kb, and is similar to that of haploid strains of S. cerevisiae which contains 13800 kb (Mortimer and Schild, 1981).

To examine the stability of the DNA content of several of these strains, the DNA content of yeasts grown in continuous culture was determined. In each case, the inoculum was grown from a single colony of the strain to be investigated, so that all results refer to the progeny of a single cell, and are not concerned with the effects of the selection of different genotypes present in the inoculum.

The DNA content per cell of strain CBS 315 remains constant at 21 fg/cell during 40 days of continuous culture at a dilution rate of $0.02h^{-1}$ for 24 days, and then $0.10h^{-1}$. However the DNA content per unit dry weight of cells dropped when the dilution rate was increased. In any continuous culture experiment, the fastest growing cells will always become predominant in the culture. The proportion of cells that produce a greater number of progeny in a given time increases at the expense of more slowly growing cells. In yeast cells, budding occurs when the cell reaches a critical size. Thus a cell which is larger than the average will reach this critical size more rapidly than the average cell and bud more frequently, resulting in the selection of large cells in the chemostat culture. In CBS

315, grown at a low dilution rate, this advantage held by large cells had little effect on the composition of the culture. The low dilution rate and slow supply of the limiting nutrient (carbon) resulted in a low growth rate for the whole population, thus minimising the selective advantage of large cells. However when the dilution rate was increased, the effect of this selective advantage becomes apparent as the number of cells (and hence DNA content) per unit dry weight fell. Large cells had been selected from the random variation in cell size which had arisen during the early part of the experiment.

With the strain ML 2589, the average cell size was found to double approximately in 15 days at a dilution rate of $0.02h^{-1}$, and the DNA content per cell also increased from the haploid to the diploid level. Clearly this is a different phenomenon from that seen with CBS 315. Not only has the average cell size increased during continuous culture at a low dilution rate, but the average DNA content per cell has also increased.

With the strain CBS 14, the DNA content per cell increased from 22 to 44 fg/cell during one experiment ($D=0.1h^{-1}$, held constant over 10 days) and from 24 to 41 fg/cell in a second experiment (dilution rate increasing from $0.1h^{-1}$ to $0.15h^{-1}$, 24 days). In both cases, the lipid content of samples taken from the chemostat culture and grown as a batch culture in NLM, fell during the course of the experiment. In the PhD. thesis submitted by C. T. Evans, 1983, the lipid content of CBS 14 is reported to be up to 88% when grown on a modified version of NLM. Since this thesis was submitted, these

results have never been repeated (C. Ratledge and J. M. Curson, personal communication), with the highest lipid contents recorded at around 65%. My results show that the amount of lipid accumulated by this strain is related to its DNA content. If the DNA content of the culture used by Evans was low, then the chance combination of low DNA content and optimal growth conditions (media formulation, aeration etc.) may have produced this startling result of 88% lipid. However when the yeast was sub-cultured, and the experiment repeated, the DNA content may have risen (the repeated sub-culture mimicking the effect of continuous culture in selecting the larger cells in the population), and so the lipid contents recorded subsequently fell to the less remarkable levels of 30 to 50%.

The continuous culture of IFO 10076 demonstrates another phenomenon sometimes seen in R. toruloides. Diploid, self-sporulating strains of Rhodosporidium have been reported (Banno, 1967, Sipiczki and Ferenczy, 1977, Abe et al., 1986), in which uninucleate diploid yeast cells form mycelium and then sporulate without mating with another yeast cell. The culture of IFO 10076 from which the chemostat inoculum was prepared behaves in this way. On day 5 of the experiment, cells with extensions resembling mating tubes were seen. This occurrence marked the switch from yeast-like to mycelial growth (Abe et al., 1975, Abe and Sasakuma, 1986). Large areas of mould-like mycelial growth were seen inside the chemostat, and later teliospore formation and germination were also seen, thus demonstrating that the complete life cycle can take place when a self-sporulating strain

forms mycelium, and that teliospore and basidiospore formation occur in the same way as they do after the mating of two haploid yeasts. The difference between the two types of mycelium formation (mating or self-sporulating) is that mycelium formed from self-sporulating yeasts bears pseudoclamp connections, and is diploid, whereas that formed after mating between two haploid strains bears true clamp connections and is dikaryotic. The pseudoclamp connections of IFO 10076 are shown in Figure 20. Photographs of colonies of IFO 10076 (Figure 16) show the rough, irregular mycelial colonies, with a darker ring of teliospores around the edge of the colony. Smaller, smooth colonies are also present, making up 1 to 2% of the population.

8. Growth of Basidiospore Isolates.

The method that I developed for the isolation of basidiospores requires large numbers of teliospores initially. The strain IFO 10076 readily sporulates, and so this strain was used as the parent strain for the isolation of basidiospores. The colonies that grew from the basidiospores resembled those of haploid yeasts at first, but after several sub-cultures the morphology of the colonies changed to that of the parent strain. The strain CBS 315 has been shown to have a low DNA content which is stable in continuous culture (See Results Section 9), but does not accumulate lipid as the enzyme ATP:citrate lyase is not present in this strain. Results from the continuous culture of CBS 14 (Results Section 11) show that when the DNA content of cells is low, the amount of lipid accumulated is high. Thus attempts were

made to introduce the ACL gene into CBS 315 by crossing a genetically marked derivative of CBS 315 with basidiospores isolated from IFO 10076 in order to produce a strain with a stable, low DNA content and ACL activity. Basidiospores are known to be haploid (Banno 1967), whereas vegetative cells of R. toruloides appear to be aneuploid or diploid. Basidiospores isolated from IFO 10076 also have the potential to produce large numbers of teliospores which can then be separated from the parent yeasts and allowed to germinate to produce the haploid progeny of the cross. When crosses are made between, for example, CBS 315 and IFO 0880, mycelium production readily occurs but teliospore production is sparse, and can take many weeks to occur. The first teliospores produced begin to germinate before there are enough teliospores present to separate them from the parent yeasts, and the isolation of the progeny of this cross would be much more difficult than the isolation of the progeny of a cross made with IFO 10076, which produces large numbers of teliospores. If the characteristic of production of large numbers of teliospores could be introduced into CBS 315 along with the ACL gene, this would be of great assistance in making further crosses.

However when CBS 315 ade$^-$ ilv$^-$ was mixed with 24 basidiospore isolates, self-sporulation of the basidiospores occurred in all cases, and mating between the two strains did not take place. It may have been the case that CBS 315 ade$^-$ ilv$^-$ was defective in mating as the result of a mutation induced at the same time as the auxotrophic markers were induced. To test

this possibility, CBS 315 ade⁻ ilv⁻ was crossed with IFO 0880, and normal mating behaviour was seen in each of five replicates, showing that the mating ability of CBS 315 ade⁻ ilv⁻ had not been altered. Alternatively, it may be that Rhodosporidium has a tetrapolar mating system as in basidiomycete fungi (Raper, 1966) although there is no evidence of this from other published sources. If this were the case, the diploid teliospores of IFO 10076 would then contain all four alleles of a tetrapolar mating type system, e. g. a α b β . Whether CBS 315 is α b or α β, basidiospores of the mating type required to mate with CBS 315 (a β or a b) will be produced by IFO 10076, and so this would seem an extremely unlikely explanation for the failure of IFO 10076 basidiospores to mate with CBS 315.

The continuous culture of basidiospore isolates of IFO 10076 showed that the switch from yeast-like to mycelial growth occurred in all seven cases, although not always at the same rate. In one case it was possible to demonstrate that the average DNA content per yeast cell rose from the haploid to the diploid level before the production of mycelium was seen, and that the number of mycelial colonies on plates inoculated with samples of the chemostat culture then rose. In other cases mycelium production occurred shortly after the inoculation of the chemostat and the DNA content could not be calculated.

9. Diploidisation And Mating Type Switching In R. toruloides.

I now propose to put forward a hypothesis to account for the apparent aneuploidy of R. toruloides, the rise in DNA content per

cell during continuous culture, the occurrence of diploid, self-sporulating yeasts and the failure of IFO 10076 basidiospore cultures to mate with CBS 315.

The DNA contents published for strains of R. toruloides are based on the measurement of the amount of DNA that can be extracted from a known number of cells, and as such are average values of a large number of cells. On the basis of these measurements, some strains are considered to be haploid, some diploid and some aneuploid. However the figures suggesting anueploidy in some strains could also be arrived at if the cell populations in which the DNA content was being measured contained a mixture of haploid and diploid cells. Of 10 yeast cells, if 6 have a relative DNA content of 1.0 and 4 have a relative DNA content (rdc) of 2.0, the average value will be determined as 1.4 per cell and the population appears to be aneuploid. Diploid yeast cells are larger than haploid yeast cells. During continuous culture the larger cells are selected for by virtue of their more frequent budding, and the population becomes, for instance, 4 haploid, 6 diploid (average rdc = 1.6) then 2 haploid, 8 diploid (average rdc = 1.8) and so the rdc rises from the haploid to the diploid level during the continuous culture experiment.

If this is the explanation for the rise in DNA content seen with R. toruloides, then a mixture of haploid and diploid cells must arise from a single cell, which forms a colony on an agar plate, and then the inoculum for a chemostat. This could occur in the following way. The phenomenon of mating type

switching in S. cerevisiae is well known (Herskowitz and Oshima, 1981, Nasmyth, 1982). Mating type switching may be described as homothallic, in which cells change their mating type at almost every cell division, or heterothallic in which switching occurs at a much lower frequency. In the heterothallic strains, the occasional switching of mating type leads to the formation of a few m.t. a cells among a population of m.t. α cells, or vice versa. Mating can take place so that diploids are formed. Diploid cells can sporulate but show no mating ability. In S. cerevisiae, mating type is determined by a 'cassette' system (Nasmyth, 1982) in which cassettes of genetic information encoding both mating types are present, but not expressed, in all cells. A copy of one of the cassettes is also present at the 'playback' locus, MAT, and it is the copy at this locus that is expressed and determines the mating type. In homothallic cells, the HO gene encodes an endonuclease which cuts both strands of DNA at one end of the MAT locus, leading to the excision of the existing cassette and its replacement with a copy of the opposite mating type cassette, thus switching mating type. In heterothallic cells, which do not contain the HO endonuclease, switching occurs as a result of intrachromosomal recombination between mating type genes. Mating type switching has also been observed in Kluveromyces lactis and Schizosaccaromyces pombe (Herskowitz and Oshima, 1981) but is not as well characterised as in S. cerevisiae.

The occurrence of mating type switching in R. toruloides would explain the formation of a mixture of haploid and diploid

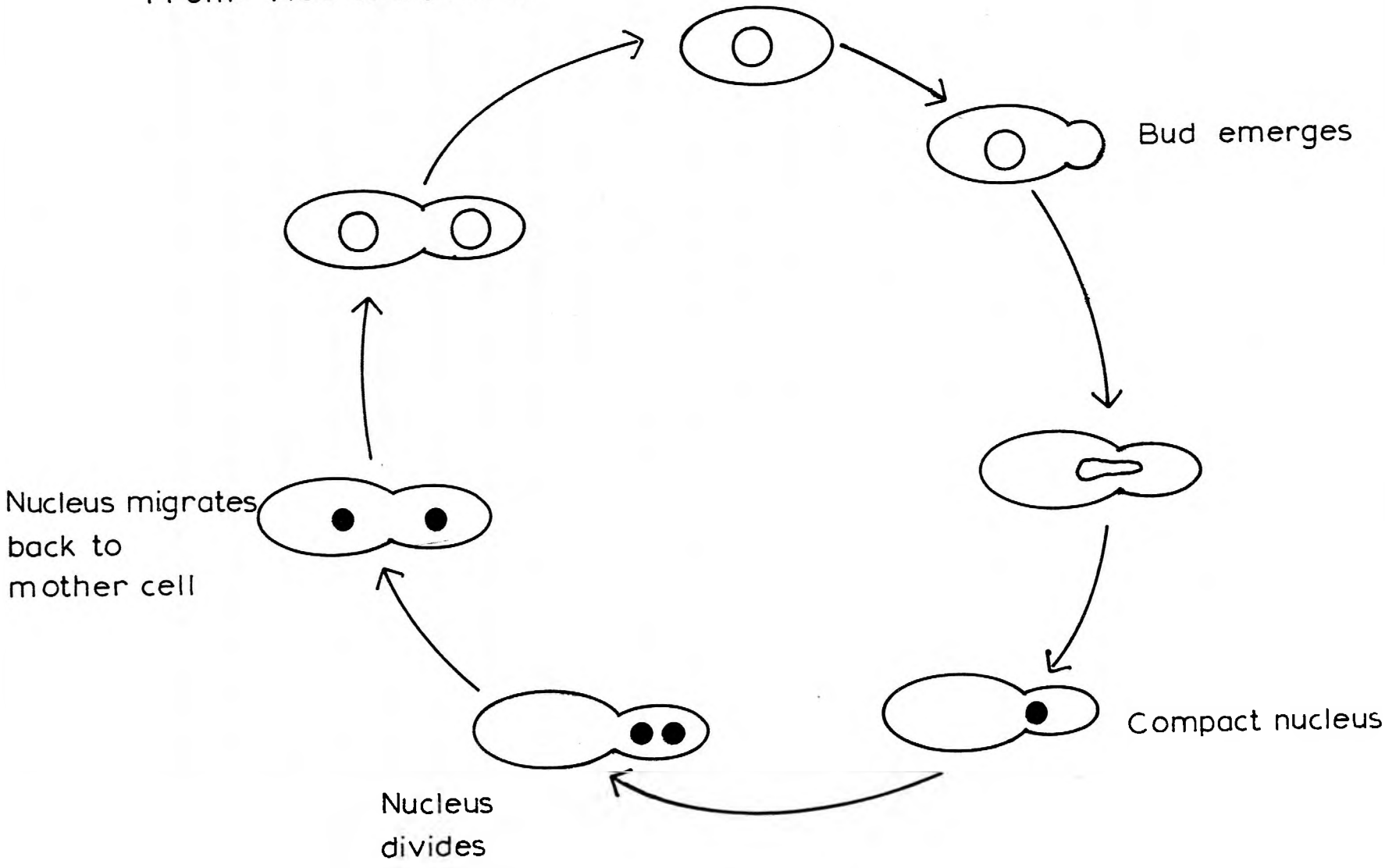
cells arising from a single (haploid) cell. During the early stages of a continuous culture experiment, one, or possibly a few cells, may switch mating type. This would be a very infrequent event, possibly similar to the heterothallic switching of S. cerevisiae. The cells that had switched would continue to bud and divide, but some would mate with cells of the original mating type to form diploids. The diploid cells would gradually become predominant in the culture due to their increased size and frequent budding, and so the average DNA content of the population would rise.

What this hypothesis does not explain is why some diploid yeast cells should continue to grow as yeast, and why others should switch to mycelial growth. Protoplast fusion experiments with Rhodosporidium (Becher and Bottcher, 1980) have shown that for a diploid cell to form mycelium both mating type alleles must be present and that a/a or α/α diploids will not form mycelium. In diploids formed after mating, both mating type alleles are present, so why do the cells not commence mycelial growth immediately in the same way as cells of opposite mating type streaked together on a plate do?

The answer may lie in the nuclear behaviour of R. toruloides at cell division. Abe et al. (1977) described the process of cell division in haploid R. toruloides cells. Normally the nucleus becomes compact and moves into the bud in preparation for cell division. The nucleus divides and one nucleus moves back into the mother cell, the nuclei expand and the bud is released. This sequence is shown in Fig. 21. However

Fig. 21 Position of the nucleus during cell division.

From: Abe et al., 1984.



in some cases (3 to 30% of samples examined) budding cells have been observed with two nuclei in the mother cell, and none in the bud (Abe et al., 1977). If, as a rare event, one of the nuclei does not move to the bud after nuclear division, the mother cell may be left with two nuclei, of the same mating type, which could then fuse to form an a/a or α/α cell. The anucleate bud could be re-absorbed by the mother cell or separated from it, forming a non-viable, anucleate cell. The mother cell is now diploid and can continue to bud and divide, but will not form mycelium. If, at a later time, the mating type at one m.t. locus switches, this will lead to the production of a diploid a/α cell which then forms diploid mycelium. In a study of diploid, self-sporulating strains (Abe and Sasakuma, 1986), binucleate cells and budding cells with three to four compact nuclei were sometimes observed. These are presumably cells in which diploidisation has occurred, but fusion of the two nuclei has not (yet) taken place.

The idea of diploidisation followed by mating type switching explains why, in the continuous culture experiments described earlier, the average DNA content of the population rises, and mycelium production may then follow. If haploid cells were to switch mating type the diploid cells formed by mating would immediately form mycelium instead of persisting as diploid yeasts, and the rise in DNA content could not be measured.

The diploid, self-sporulating yeasts are cells in which diploidisation has taken place. When the cells are streaked on an agar plate, a yeast colony of diploid cells is formed. Mating type switching then occurs in some of the cells, leading to

mycelial growth and teliospore formation. All the cells in the colony are capable of switching, but not all of them do so at the same time. When free yeast cells are taken from the top of the colony and re-streaked the phenomenon is repeated. Each colony grows from a diploid yeast cell, and during the growth of the colony mating type switching occurs in some of the cells. Occasionally, switching does not take place in any of the cells in the colony and a smooth colony is formed, but if cells from the smooth colony are re-streaked, the vast majority of the colonies formed are mycelial. This is, in fact, exactly what happens when IFO 10076 is grown on agar plates.

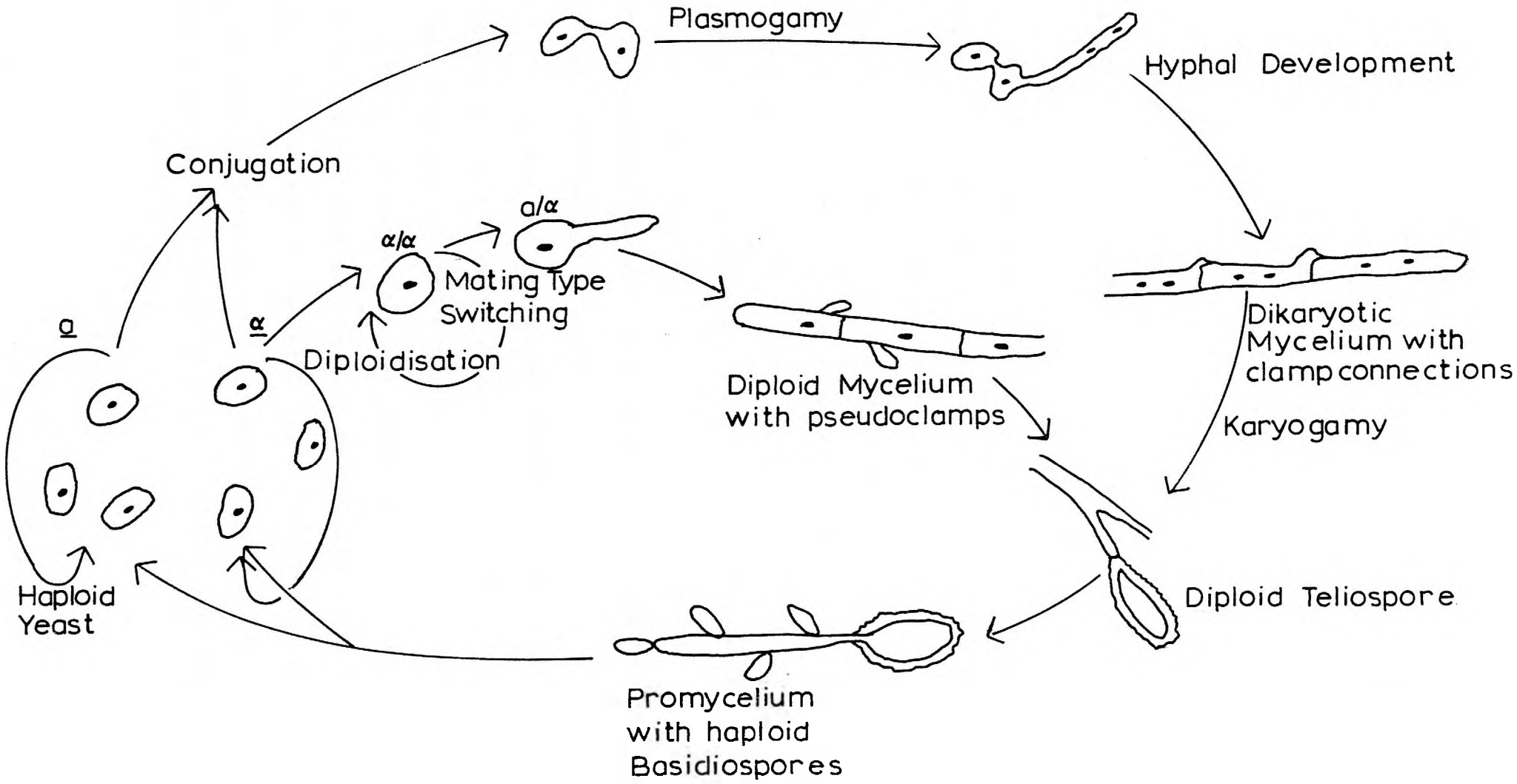
It can be predicted from the proposed hypothesis that basidiospores isolated from germinated teliospores of IFO 10076 will bud and divide to produce haploid yeast cells that form smooth colonies. Eventually, through a random process, diploidisation occurs producing diploid yeasts, and then mating type switching leads to mycelium formation, thus re-creating the diploid, self-sporulating yeasts of the parent strain. Although it was not possible to determine the DNA content of all the basidiospore isolates, in one continuous culture experiment it was shown that the average DNA content of the culture rose from the haploid to the diploid level, and mycelium production then occurred. In all other cases, the smooth colonies that were grown from the isolated basidiospores became mycelial colonies after one or more sub-cultures, thus behaving as predicted by the hypothesis. The cultures that were crossed with CBS 315 ade^- ilv^- still showed smooth yeast-like growth, but were probably

diploid, and mating type switching then occurred to produce a/α diploids which do not mate (Herskowitz and Oshima, 1981). In all cases, eventually the diploid yeasts formed mycelium, but none mated with CBS 315.

Clearly, the frequency at which the two random events, diploidisation and mating type switching, take place are different in different strains of R. toruloides. In CBS 315 diploidisation never occurs. In ML 2589, and probably IFO 0880 (judging by its DNA content alone, as no continuous culture experiments have been carried out with this strain) diploidisation occurs rarely, but often enough to increase the DNA content of batch cultures to greater than the haploid level. In CBS 14 and IFO 0559, diploidisation obviously takes place but mating type switching is a very rare event, although some mycelium production was seen after 21 days continuous culture of CBS 14. In IFO 10076, both diploidisation and mating type switching occur more frequently than in other strains, leading to the production of teliospores from basidiospore isolates in the space of a few weeks. I suggest that both processes are random, and are probably the consequence of 'accidents' at nuclear division, but that a particular genotype may confer a greater propensity towards these abnormal occurrences.

Figure 22 shows the proposed life cycle for R. toruloides, demonstrating how diploidisation and mating type switching fit into the life cycle proposed by Banno (1967).

Fig 22



Diploidisation and Mating Type Switching in the Life Cycle of R toruloides.

10. Alternative Hypotheses.

At this stage it is appropriate to consider alternative explanations for the observations that have been made and examine ways in which the hypothesis that haploid yeasts become diploid yeasts which then switch mating type at one m. t. locus and form mycelium may be tested.

The most obvious alternative hypothesis is that some strains of Rhodosporidium toruloides actually are aneuploid. A study of diploid, self-sporulating strains by Abe and Sasakuma (1986) included the measurement of the DNA content of individual nuclei by microphotometry. Haploid, aneuploid and diploid cells were found. In the case of haploid and diploid cells, the results quoted show a standard error of ± 13 to 15 % of the fluorescence intensity. The figures quoted for the aneuploid cells show a standard error of ± 30%. All the supposed aneuploid cells are within one standard error of the haploid DNA content. Unless some improvement in the techniques used can produce less variable results, the existence of aneuploid yeasts cannot be proved or disproved by this method.

Bottcher and Samsonova (1980a) claimed that the failure to isolate certain classes of auxotrophs is due to aneuploidy, and that when one copy of a gene is present in a cell, mutations in this gene are expressed, but when two copies are present, mutations in one copy of the gene do not alter the phenotype. However the failure to isolate certain classes of mutants is also seen in haploid strains (CBS 315 and Rg1), so that this is not solely a feature of aneuploidy. Strains that appear to be

aneuploid will have a lower mutation rate than haploid strains because the rate of isolation of mutants from diploid cells in the culture will decrease the average mutation rate, but this will not affect the rate of isolation of specific auxotrophs.

If the inoculum for chemostat consisted of aneuploid cells, then the rise in DNA content must be due to the random segregation of chromosomes at mitosis, leading to constant change of the number of chromosomes in a cell. This would tend to cause an increase in the DNA content of cells as, for example, if a cell with a chromosome number of n+3 (where n is the haploid chromosome number) divided to produce one cell with n+6 chromosomes and one cell with fewer than n chromosomes, the cell with fewer than n chromosomes would be non-viable, leaving a cell with n+6 chromosomes to replace a cell with n+3 chromosomes. If, instead, the n+3 cell divided to produce n+2 and n+4 cells, this would lead to no change in the average DNA content of the population. Although this explanation for the rise in DNA content of aneuploid cells is theoretically possible, it is quite unlike the chromosomal behaviour of any other organism. Also, this hypothesis does not explain why the DNA content should always proceed from the haploid or near-haploid level to the diploid level. If the rise in DNA content was due to the random re-assortment of chromosomes, the DNA content could continue to rise until a theoretical maximum was reached, and would not cease to rise when the diploid DNA content was reached.

The use of flow cytometry to measure the distribution of DNA contents in S. cerevisiae cells has been demonstrated

(Alberghina, 1986). If this method was used to study the DNA content of R. toruloides cells during continuous culture it would be possible to determine whether the population consisted of a mixture of haploid and diploid cells or aneuploid cells. The changes in the distribution of DNA contents during continuous culture could also be examined, demonstrating how the rise in the average DNA content per cell is brought about.

The mechanism of diploidisation in R. toruloides has not been demonstrated. What has been shown is that the nuclear behaviour of dividing cells does not always follow exactly the same pattern (Abe et al., 1977), and it is possible to speculate about the exact sequence of events that occasionally leads to the formation of a diploid cell. As diploidisation is known to be a rare event, it will clearly be difficult to observe, but it may be possible to observe diploidisation in cultures grown from basidiospores of IFO 10076. A comparison of the nuclear behaviour of CBS 315 (no diploidisation) and IFO 10076 (frequent diploidisation) should provide clues as to the nature of the mechanism.

Mating type switching in diploid cells is also a rare event. That it has occurred may be demonstrated in the following manner. The mating type of a basidiospore isolate may be determined by crossing with haploid a and α strains as soon as possible after isolation of the basidiospores, and before diploidisation has occurred. When the mating type is known, the culture is allowed to grow on agar plates, sub-culturing until teliospores are produced. The teliospores are then allowed to

germinate and the mating type of the basidiospores again determined. This experiment would show that basidiospores of both mating types are produced from one basidiospore of known mating type. Mating type switching must have occurred to produce the second mating type.

Mating type switching in S. cerevisiae is now well characterised (Herskowitz and Oshima, 1981, Nasmyth, 1982). This has been possible because of the nature of the life cycle of S. cerevisiae and the large and rapidly increasing amount of knowledge of the genetics of this yeast. In R. toruloides, with its complicated life cycle and little known genetic systems, it is unlikely that the exact mechanism of mating type switching will be elucidated in the near future.

11. The Genetics Of Lipid Accumulation In R. toruloides.

The aim at the start of this research project was to discover how lipid accumulation is genetically controlled in R. toruloides. What the project has actually demonstrated is that it will be extremely difficult to achieve this aim. Diploid cells accumulate less lipid than haploid cells, for reasons that have not been discovered. The strain that had previously accumulated the highest concentration of lipid ever recorded in an oleaginous micro-organism does not remain haploid when sub-cultured or grown in continuous culture. The only strain that has been found to remain haploid does not accumulate lipid, as the key enyzme of oleaginicity, ACL, is not found in this strain. Although it is possible to isolate ACL-less mutants of an oleaginous strain in

preparation for a study of ACL in R. toruloides, the mutants are not stable and quickly revert to the wild type. The most important conclusions that can be drawn at the end of the research project relate to the life cycle of the yeast, and unfortunately show that this species is far from ideal for studying the genetics of lipid accumulation in an oleaginous yeast. The most fruitful line of research in future is likely to be to determine the DNA content of as many strains as possible, in the hope of finding another strain that behaves like CBS 315 in that it always remains haploid, but also has the enzyme ATP:citrate lyase, and so always accumulates the same amount of lipid. This strain may then be used to study the genetics of lipid accumulation in R. toruloides.

SUMMARY

Lipid accumulation in the basidiomycete yeast Rhodosporidium toruloides was investigated. The ATP:citrate lyase activity and lipid content of 21 strains of Rhodosporidium toruloides in batch culture were measured, using either an organic or an inorganic nitrogen source. The activity of carnitine acetyl transferase was also measured, and it was shown that this enzyme provides an alternative source of cytoplasmic acetyl-CoA for lipid biosynthesis. Two novel methods for the estimation of the lipid content of yeast cells were evaluated. The staining of intracellular lipids with fluorescent dye was found to be unsatisfactory. The staining of replica prints of yeast colonies with Sudan Black B was found to give good results with other oleaginous yeasts, but was not suitable for use with R. toruloides. The sensitivity of several strains of R. toruloides to the mutagens EMS and NTG was determined, and stable auxotrophic mutants were isolated from two of these strains. A method for the isolation of mutants without the enzyme ATP:citrate lyase was devised, but the mutants isolated reverted to the wild type shortly after it had been demonstrated that the enzyme was not active in these isolates. The DNA content of strains of R. toruloides was determined, and the change in DNA content during continuous culture was followed. It was shown that the average DNA content per cell remains constant during continuous culture of the strain CBS 315, but rises during the continuous culture of the strains ML 2589 and CBS 14.

Morphological changes during the growth of a diploid, self-sporulating strain were followed, and basidiospores were isolated from this strain. A hypothesis to explain the apparent aneuploidy of R. toruloides, the change in DNA content during continuous culture and the behaviour of diploid, self-sporulating strains was proposed.

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