

**OPTIMIZATION OF OIL PRODUCTION BY *Lipomyces tetrasporus*
ISOLATED FROM SOIL IN THE NORTHERN PROVINCE.**

BY

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Submitted in fulfilment of the requirements for the degree of

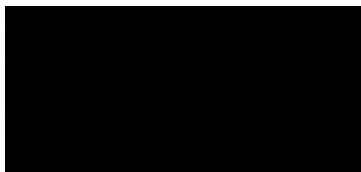
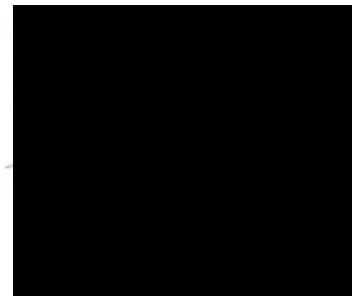
MASTER OF SCIENCE

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March 1999

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DECLARATION

I declare that the dissertation hereby submitted to the University of the North for the degree of Master of Science has not previously been submitted by me for a degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledged.

Signed: VRibok .

Date: 03/03/99

ACKNOWLEDGEMENTS

THIS PROJECT IS DEDICATED TO MY FAMILY.

I would like to thank the following:

THE ALMIGHTY GOD for the strength that kept me going and making things possible for me.

Dr. Emil K. Abotsi for his appealing managerial and leadership roles and always willing to listen.

Members of staff in the Departments of Biochemistry (especially Mr Tloti) and Microbiology for their encouragements, invaluable criticism and interest shown in research.

My fellow post-graduate students (classes of 1997-1999) for their unchallengeable warmth and encouragements.

Prof. J. L. S. and Ms E. Pretorius (University of Orange Free State) for providing the yeasts cultures, sharing their skills and always willing to assist.

Dr. du Plessis (Division of Food Science and Technology at the CSIR) for his skill in Gas Chromatography and assistance.

Foundation for Research and Development (FRD) for their continued financial support through the study.

My family especially my grandmothers, Granny (Magaret) and Mama (Mirriam) for their support, patience, undying love, trust and belief in me and my uncle (Christopher) for the financial support.

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ABSTRACT

The Lipomycetaceae are of commercial interest as many have been analysed for their ability to accumulate large quantities of lipids when grown in a medium with excess of carbon and a deficit of nitrogen (C/N= 50:1). It has, moreover, been suggested that *L.starkeyi* may have biotechnological value since it can accumulate lipids similar to palm oil up to 62.5% of the cell biomass. Accumulation of lipids by oleaginous yeasts, *Lipomyces starkeyi*, *Lipomyces tetrasporus* and *Lipomyces sp.* was studied when grown in BASAL medium at different concentrations of glucose and industrial waste media (SAB and PASSI) in batch and fed-batch cultures. The fatty acid profiles were also determined by gas chromatography.

It is reported in this study that based on the specific rates of lipid production CBS 2516 T was the most productive strain for lipid production in BASAL medium in batch culture and UOFS-Y-2082 performed better than CBS 5910 T. The three yeasts showed different degrees of tolerance to glucose when cultured in media with different concentrations of glucose. Maximum accumulation of lipids by UOFS-Y-2082 and CBS 2516 T occurred in 1 % glucose medium but in 2 % by CBS 5910 T. The industrial waste media were most efficiently utilized for both biomass and lipid production in all yeasts studied. In fed-batch cultures, the total lipid content increased when UOFS-Y-2082 was fed at 1 % and 2 % glucose levels but decreased in CBS 5910 T when fed at all glucose concentration levels. The fatty acid composition of the lipids produced by all the three strains were similar in composition, but not identical to that of palm oil. These results showed that the industrial waste media could be considered for the production of oils similar to palm oil.

CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

The Lipomycetaceae is a well defined yeast family with diverse phenotypic and ecological characteristics (Van der Walt *et al*, 1987) and exhibits a diversity of carbohydrate patterns and lipid composition (Jansen van Rensburg *et al*, 1992). A few members of the Lipomycetaceae (i.e. *L. anomalis*, *L. kononenkoae*, *L. lipofer*, *L. starkeyi* and *L. tetrasporus*) were analysed for their abilities to accumulate large quantities of lipids when grown in a medium with low nitrogen content (carbon:nitrogen ratio in the order of 50:1) (Jansen van Rensburg *et al*, 1992). When grown with an excess of carbon and a deficit of nitrogen, in both batch and continuous cultures, protein and nucleic acid synthesis become curtailed but lipid synthesis continues at an undiminished rate and thus lipids build up with respect to the remaining biomass (Ratray, 1988).

The Lipomycetaceae are of commercial interest as many members are oleaginous and therefore capable of producing large amounts of lipids (Ratray, 1988). It has, moreover, been suggested (Ratray, 1988) that *L. starkeyi* may have biotechnological value since it can accumulate lipids similar to palm oil up to 62.5 % of the cell biomass (Suzuki and Hasegawa, 1974). The accumulation of lipids is triggered by exhaustion of a growth nutrient other than carbon, thus preventing cell proliferation but still allowing conversion of the carbon substrate.

1.2. LIPOMYCETACEOUS YEASTS

The members of the Lipomycetaceae comprise a natural family, which is easily distinguished from other saccharomycetalean (endomycetalean) families by (i) the production of extracellular amyloid material that gives rise to slimy or vitreous colonies

on solid media, (ii) sexual reproduction which is usually associated with the formation of attached, multispored asci, (iii) the absence of fermentation and (iv) the ability to utilize certain heterocyclic compounds (such as imidazole and thymine) as sole source of nitrogen (Van der Walt, 1992).

The diversity in terms of habitat specificity in the Lipomycetaceae is evident with *Babjevia* and *Lipomyces* which are soil associated whereas *Dipodascopsis* and *Zygozoma* are usually associated with plants and insects (Van der Walt, 1992). The anamorphic genus *Myxozyma* seems to be ecologically diverse, occurring in plant, soil and insect sources (Van der Walt, 1992). Vegetative reproduction within the family occurs by budding and by the formation of septate hyphae (Van der Walt, 1992). Several other phenotypic characters have been investigated in the Lipomycetaceae. The coenzyme Q composition of the family ranges from CoQ8 to 10 (Van der Walt, 1992) and Weijman and Van der Walt (1989) distinguished between the genera of the Lipomycetaceae using whole cell monosaccharide composition. While the soil associated genus *Lipomyces* (inclusive of *Waltomyces*) is found to contain galactose with lesser amounts of glucuronic acid, the opposite pattern is observed for the insect-associated genera *Dipodascopsis* and *Zygozoma*. The anamorphs in *Myxozyma* are also distinguished from the teleomorphs on the basis that the former contain more mannose than glucose in their cells (Weijman and Van der Walt, 1989).

1.3. LIPIDS IN YEASTS

The lipid content of many yeasts has been reported to be highly variable depending not only on the individual species but also the growth conditions. In addition, the lipids of mycelium and yeast cells, reproductive structures as well as those of cells in different stages of development vary. In general, phospholipids, sterols (and their esters), triacylglycerols and fatty acids are the major components.

Although certain species of yeasts, such as *Saccharomyces cerevisiae* (ATCC 7754), have

a very high lipid content, the average value in the fungi is about 17 % lipid. Several genera of the fungi seem to be capable of producing high amounts of lipids. These include *Claviceps*, *Penicillium*, *Aspergillus*, *Mucor*, *Fusarium* and *Phycomyces* (Woodbine, 1959). The lipids can represent up to 64 % of the dry weight of *Penicillium spinulosum* (Losel, 1988). The fluctuating proportions of total cellular lipid present in membranes correspond closely to the amount of these structures as seen in electron micrograms. The relative proportions of these and of storage lipids also vary considerably with growth conditions and development stage (Losel, 1988). In comparison, the average lipid content of the yeasts is 5-15 % (w/w) but for oleaginous yeasts it is 30 % or more lipid, except the *Saccharomyces* spp. which produce less (Hunter and Rose, 1971; Rattray *et al.*, 1975).

Ratledge and Wilkinson (1988a) defined lipids as compounds that are more soluble in organic solvents (chloroform, ethers, alcohols, etc.) than in water. They divided the lipids into two groups i.e. the fatty acid associated lipids and lipids containing isoprene units such as terpenoid lipids. Fatty acids are commonly found in living organisms. In yeast, fatty acids generally consist of a chain of even numbered carbon atoms (i.e. 16 and 18) and a terminal carboxyl group. The fatty acids are saturated or unsaturated where one or more double bonds are produced and present in the cis- configuration. The predominant fatty acids found in yeast are palmitic acid (16:0) and stearic acid (18:0) representing the saturated fatty acids with the unsaturated fatty acids such as palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). When comparing the fatty acid composition in yeasts, several factors should be taken into account since oxygen availability, culture age, pH, growth rate, temperature and the composition of the growth medium can alter the fatty acid composition (Rattray, 1988; Viljoen *et al.*, 1986).

In yeasts, fatty acids are rarely found in their free form and are as a rule esterified to a glycerol backbone to form among others, phospho-, glyco- and neutral lipids. The phospholipids are associated with membranes of the cell and consist of the two esterified fatty acids and a phosphate group with an attached functional group like serine to form

phosphatidylserine . The glycolipids are associated with membranes and cell walls and consist of the two esterified fatty acids and one or more sugar residues attached to the third carbon of the glycerol backbone. Characteristic of the neutral lipids is that they are usually observed as oil droplets in the cell and function as storage material (Ratledge and Wilkinson, 1988b). The compounds also consist of the glycerol backbone and three esterified fatty acids. Diacylglycerol and monoacylglycerol are formed by the hydrolysis of triacylglycerol by lipases. The accumulation of large amounts of these oil droplets (which are similar to plant oils) have been noted in several oleaginous yeasts (Ratledge, 1991; Suzuki and Hasegawa, 1974) such as *Lipomyces starkeyi*.

1.4. LIPID ACCUMULATION

The ability of microorganisms to accumulate lipid is confined to a relatively small number of species. The accumulation of plant-like triacylglycerol oils occurs principally in the eukaryotic organisms; bacteria are not ready accumulators of triacylglycerols and instead may produce specialized lipids such as the polyester, poly-beta-hydroxybutylated, or wax esters.

Lipids accumulate in oleaginous microorganisms when there is still carbon available to the cells but there is the absence of another nutrient to enable the cells to proliferate. Usually, the limiting nutrient is nitrogen, but it can also be P, K, Mg, S or Fe. In the absence of new cell production, the existing cells continue to assimilate the carbon available to them (reviewed in Ratledge, 1989).

The usual course of lipid accumulation during a batch culture is found to be a two-stage process: stage 1 being cell proliferation and balanced growth culminating with nitrogen exhaustion from the medium, and stage 2 being that of lipid accumulation. From the physiological point of view, however, lipid accumulation is not caused by nitrogen depletion which induce a much higher rate of lipid synthesis (Kessell, 1968) but by the synthesis of other cell constituents such as protein and nucleic acids, which require nitrogen depletion. It is the rate of lipid synthesis relative to the rate of synthesis of other

cell components that determines whether lipids accumulate in microorganisms.

The process of lipid accumulation can also be achieved in single-stage continuous culture if the growth rate of the organism is kept considerably less than the maximum, and under such conditions lipid accumulation may reach the same concentration as that achieved in batch culture. Continuous cultivation of a number of oleaginous yeasts has been reported in *Candida* sp. 107 (Fukui and Takana, 1979), *Candida curvata* = *Apiotrichum curvatum*, *Cryptococcus albidus*, *Lipomyces starkeyi* and *Trichosporon pullulans* (reviewed in Ratledge, 1989). Essentially, the organisms must still be grown with a surfeit of carbon to nitrogen so that the supply of nitrogen to the culture becomes the rate-limiting step to growth. With a long residence time in the fermenter vessel (the chemostat) the cells have sufficient time to convert the excess glucose into lipid. If the residence time is decreased, even with the same medium composition, the cells have insufficient time to accumulate high amounts of lipid before becoming washed out of the vessel (Ratledge, 1989). Lipid accumulation is, therefore, not different from the observed accumulation of various storage polymers in bacteria growing in continuous culture and is attainable with most other oleaginous organisms if grown in continuous culture (Ratledge, 1989).

A continuous culture system has many advantages over a batch production system, not least of which is a production of a product that has an unvarying composition. In a batch culture, the fatty acid composition of a microbial oil can vary between quite wide limits (reviewed in Ratledge, 1989; Kessell, 1968), but in continuous culture Gill *et al.* (1977) have shown that under steady-state conditions the lipid composition, as reflected by its fatty acid composition, remains almost unchanged over many weeks of operation. This clearly is an essential prerequisite for any commercial development of such a process. Thus, a desirable fatty acid composition, once attained, could be maintained indefinitely, and a product of known and predictable composition could be produced for as long as the steady-state conditions could be maintained.

Although continuous culture techniques are more advantageous than the batch culture for studying the control of lipid accumulation, the commercial use of continuous culture in biotechnological processes is much less evident due to the high capital costs which are involved and the need for stringent precautions to ensure absence of contaminating organisms (Ratledge, 1989).

1.5. BIOCHEMISTRY OF LIPID ACCUMULATION

Organisms, principally eukaryotic ones, which can accumulate 20 % or more of their biomass as lipid have been known for many years. Such organisms have been termed 'oleaginous' (reviewed in Ratledge, 1989) in keeping with oil-bearing plants which are similarly named. The amount of lipid which has to be accumulated within a cell for it to be designated as oleaginous has no fixed lower limit, but for practical purposes of production and extraction it is usually taken as being about 20 %. However, as a result of biochemical studies on the mechanism of lipid accumulation, a biochemical definition was suggested based on the possession of one of the key enzymes for lipogenesis, namely ATP:citrate lyase (reviewed in Ratledge, 1989). Eukaryotic organisms without this enzyme appear unable to achieve the same degree of lipid accumulation as those which possess it. This cleavage of citrate, therefore, precedes the route for fatty acid biosynthesis which begins with acetyl-CoA.

An understanding of the basis of oleaginicinity is important as this provides a rationale for either metabolic or genetic manipulation of the organism. Too frequently, improvements to industrial fermentations are attempted without the necessary information about basic pathways. Thus the following account of the biochemistry of oleaginicinity should be seen as a first step to providing some of this information which, until recently, has been lacking.

When an oleaginous microorganism is grown in order to accumulate lipid, a nitrogen-limiting medium is usually used. When nitrogen becomes exhausted from the medium,

or reaches a very low concentration in continuous cultures, the cells are faced with a surfeit of carbon. The carbon source continues to be assimilated and, as it continues to be metabolized, the intracellular concentrations of various key intermediates change. Of principal significance is the rapid decrease in AMP concentration immediately following nitrogen exhaustion. This is due to activation of AMP deaminase which converts AMP into IMP and NH_3 (reviewed in Ratledge, 1989).

The biochemical mechanisms of the process of lipid accumulation in yeast has been investigated and the following propositions were made as possible contributing factors (Botham and Ratledge, 1979).

1. Glucose uptake may continue unchecked into cells when nitrogen limits growth. The subsequent metabolism of glucose obliges the organism to find a suitable means of accommodating the surplus carbon as well as the energy, generated by respiration, and NADPH arising from the pentose phosphate cycle.
2. Acetyl-CoA carboxylase, the probable regulatory enzyme of fatty acid biosynthesis (Gill *et al.*, 1977), may be either hyper-active or not repressed or not subject to feedback inhibition when the organism enters a lipid- accumulating condition.
3. Lipid turnover may be negligible in an oleaginous organism so that what is synthesized is kept, whereas in a non-oleaginous yeast lipid turnover may occur at all times.
4. Intermediary metabolism may be regulated by the prevailing concentrations of adenine nucleotides within the cell (i.e. energy charge) which must change according to the prevailing growth conditions. In an oleaginous organism, this change would lead, in someway, to an undiminished or even increased flow of carbon from glucose into lipid. In a non-oleaginous organism, the energy charge of cells entering a carbon-excess growth situation would lead to a repression of the flow of carbon into lipid.

The studies on the above propositions led Botham and Ratledge (1979) put forth the following hypotheses:

Lipid accumulation depends upon several factors but is not necessarily caused by them.

For example, neither glucose transport nor fatty acid biosynthesis must be repressed or inhibited; nor must the lipid which is synthesized be degraded. Therefore the reason for lipid accumulation must lie elsewhere. It is believed that the key to oleagenicity lies in the build-up of ATP and the depletion of AMP in cells whose growth is limited by the supply of nitrogen (or presumably any other nutrient besides carbon). It is also assumed that the concentrations of nucleotides within the mitochondria are similar to, or even more than those measured in the total intracellular contents. The depletion of AMP in nitrogen-limited cells leads to an inactivation of mitochondrial NAD⁺-dependent isocitrate dehydrogenase. The isocitrate which accumulates then equilibrates to citrate via aconitase which is unaffected by changes in energy charge. The citrate is then transported out of the mitochondrion and cleaved by ATP:citrate lyase into oxaloacetate and acetyl-CoA. The acetyl-CoA becomes available for fatty acid biosynthesis, with acetyl-CoA carboxylase, the regulatory enzyme of this pathway, being stimulated by citrate.

Botham and Ratledge (1979) further suggested that, neither ATP:citrate lyase nor acetyl-CoA carboxylase will be depleted of ATP as a co-substrate when the energy charge is high. The oxaloacetate arising from citrate cleavage is probably converted to malate and then decarboxylated to pyruvate. The pyruvate then enters the mitochondrion to be dissimilated into acetyl-CoA and oxaloacetate; the subsequent synthesis of citrate, via citrate synthase, is not unduly affected by a high energy charge. Lipid accumulation, therefore, results from the concerted action of several enzymes and metabolic activities.

1.6. CONTROL OF FATTY ACID BIOSYNTHESIS

The fatty acid biosynthesis of microbial cells is, qualitatively, extremely flexible. Even in non-lipid-requiring microorganisms it is often dramatically modified in response to environmental parameters such as media composition, supply of external fatty acids, pH, incubation temperature, aeration, period of incubation or illumination. Thus, the ratios of saturated to unsaturated, branched- to straight-chain, unsaturated to cyclopropane, medium- to long-chain fatty acids are quite variable, and probably depend on distinct

control mechanisms (Ratledge and Evans, 1989).

Like biosynthetic processes in general, fatty acid biosynthesis is principally subject to two types of control; long-term adaptive changes of enzyme concentration, due to enzyme synthesis or degradation, and short-term regulation of enzyme activities by small molecular allosteric ligands. In microorganisms, fatty acid synthesis proceeds mainly for the purpose of cellular membrane formation. Therefore, a close co-ordination of this process with other macromolecular syntheses involved in cell proliferation appears meaningful. Such a correlation is possibly indicated by the finding of highly purified acetyl-CoA carboxylase from *E. coli* (Ratledge and Evans, 1989).

1.7. INFLUENCE OF CULTURE CONDITION ON LIPID CONTENTS

1.7.1. Nutrient limitation

Over the last two decades there has been a resurgence in yeast lipid accumulation research and much information is now available on the effects of different culture conditions on the total lipid contents of yeasts (Ratledge, 1978). The fundamental requirement for lipid accumulation by yeasts is a deficiency of a particular nutrient and an excess of carbon-containing nutrients. Thus, in batch culture, cell proliferation ceases when the limiting nutrient becomes exhausted, while the excess carbon source continues to be assimilated by the cell and channelled into lipid (Kessell, 1968). This typical two-stage pattern is more pronounced with oleaginous yeasts. A nitrogen source is the usual limiting nutrient and C/N ratio of 50:1 is frequently used. Other limiting nutrients such as iron, phosphate, sulphate and magnesium have also been used (Gill *et al.*, 1977). In the lipid-accumulating phase there is no increase in the rate of lipid synthesis, although lipid accumulates because synthesis of other cellular components (e.g. nucleic acids and proteins) cease due to the specific nutrient limitation (Kessell, 1968). Some yeasts respond differently to the specific nutrient limitations and so each particular yeast needs to be evaluated separately.

1.7.2. Carbon substrates

The majority of reports concerning the effects of growth conditions on lipid accumulation have dealt mainly with the composition of the extracted lipid rather than the total amount of lipid synthesized. However, some of the early workers in this field have studied the effects of substrate concentration, pH value, dissolved oxygen tension and temperature on the total lipid content of yeast cells (Ratray *et al.*, 1975). As expected, increasing the substrate (glucose) concentration results in higher cellular lipid contents of yeasts such as *Candida 107* (Gill *et al.*, 1977) and *Lipomyces starkeyi* (Boulton and Ratledge, 1981). All these yeasts are typical of Crabtree-negative yeasts which do not suffer a repression of their oxidative metabolism by an increase in the concentration of glucose. On the other hand, yeasts like *Saccharomyces cerevisiae* are Crabtree-positive and so suffer from a repression in the presence of high concentration of glucose.

The type of carbon substrate used for growth can also markedly affect the final lipid content of cells. Variations in lipid content of 30 %, 33 %, 40 % and 49 % have been observed with *C. curvata* grown on ethanol, glucose, lactose and xylose as carbon sources, respectively (Evans and Ratledge, 1983). Polysaccharides such as starch are not widely attacked by oleaginous yeasts. Ethanol appears to be a potentially useful substrate for lipid production and the yields are substantially higher on ethanol than on carbohydrates; over 30 % (w/w) conversion of substrate into lipid having been achieved compared to a maximum of about 22 % with glucose (Ratledge, 1982). The increased yields from ethanol are due to its more reduced nature, and hence higher intrinsic energy content, compared with glucose. A number of waste materials have been considered for lipid production including whey from cheese and dairy production, as a useful way of upgrading it to more valuable materials.

Considerable variations in total lipid content as well as fatty-acyl composition occur when yeasts are grown on hydrocarbon or fatty acids (Ratledge and Evans, 1989). With these substrates, the fatty-acyl residues of the yeast lipid reflect the chain length and, to a

certain extent, the degree of unsaturation of the original starting material. Fatty acids, or their esters, have been used as substrates or co-substrates with oleaginous yeasts in an attempt to increase the specific content of a particular fatty-acyl residue.

1.7.3. Nitrogen sources

As already mentioned, the concentration of nitrogen source in the growth medium has the greatest influence on the lipid content of most yeast cells (Ratledge, 1982). When the concentration of carbon source is fixed, an increase in the concentration of nitrogen supply results in a corresponding decrease in the final lipid content of cells. In some studies, it was reported that higher lipid contents could be achieved when an organic, rather than an inorganic, nitrogen source was used for growth (Woodbine, 1959). It has been shown that certain yeasts, belonging predominantly to the genus *Rhodospiridium*, can increase their lipid contents from less than 20 % to over 50 % when glutamate or urea is used instead of NH_4^+ ions as nitrogen source (Evans and Ratledge, 1984).

1.7.4. Vitamins and salts

The vitamin content of the growth medium has also been reported to affect lipid accumulation of yeasts. Inositol deficiency has been found to increase the lipid content of some strains of *S. cerevisiae* to as high as 48 % but more usually to about 13 % of the biomass (Ratledge, 1978). These values are achieved by severely curtailed cell proliferation but without impeding lipid biosynthesis. The absence of thiamin and pyridoxin has been shown to give decreased amounts of lipid in *S. cerevisiae*. It has also been reported that increasing concentrations of NaCl (0-10 %) in a growth medium results in elevated lipid contents of *Candida albicans* and *S. cerevisiae* (reviewed in Ratledge and Evans, 1989), though, like many of the other effects, the increase in lipid content appears to have been brought about by curtailment of cell growth and division.

1.7.5. Anions and cations

Since phospholipids are probably the most important cellular lipids, a medium deficiency

of inorganic phosphate should predictably affect microbial lipid composition. Increasing the phosphate content of the medium above that normally supplied does not alter the phospholipid composition of *Saccharomyces pombe* although it changes the fatty acid and polar lipid composition of *Candida utilis* (reviewed in Rose, 1989). The data reported by Ramsay and Douglas (reviewed in Rose, 1989) merit special attention since they were obtained in a chemostat, in which *Saccharomyces cerevisiae* was grown under conditions of phosphate limitation. Compared with cells grown under phosphate sufficiency, those grown in phosphate-limited cultures contained 40 % less triacylglycerol, 67 % less sterol ester, 46% less phosphatidylserine, 46 % more phosphatidylcholine and 38 % more phosphatidylethanolamine. A report on the effect of phosphate deficiency in *Pseudomonas diminuta* on the lipid composition of this bacterium deserves attention. When this bacterium was grown under phosphate deficiency, cells were found by Minnikin *et al.* (reviewed in Rose, 1989) to contain an appreciable proportion of an acidic glycolipid which presumably takes over any essential membrane functions that phosphorus-containing lipids carry out in phosphate sufficient bacteria.

Limitations of several other anions and cations such as P, K, Mg, S and Fe (reviewed in Rose, 1989) have been reported not to affect microbial lipid composition. Interestingly, though, *Candida* sp. No. 107, when grown under magnesium-limited conditions, accumulated residues of myristic acid (C₁₄) (Gill *et al.*, 1977).

1.7.6. pH, temperature and aeration

Very little has been reported on the significant changes in total lipid content for cells cultivated under different pH conditions. Using growth medium with pH values varying from 3.0 to 7.5, very little variation in lipid content was observed with either *Rh. grasilis* (Kessell, 1968) or *Candida 107* (Gill, *et al.*, 1977).

Growth temperature is known to affect significantly the total lipid content of yeasts (Ratray *et al.*, 1975; Ratledge, 1982). The general tendency is for the lipid content to

increase as the temperature is dropped. A decrease from 25°C to 10°C with a strain of *C. Lipolytica* increased the lipid content from 6.6 % to 8.5 % (reviewed in Ratledge and Evans, 1989); an increase from 12.5 % to 14.4 % was observed with *Sacch. cerevisiae* when the growth temperature was dropped from 30°C to 15°C (Hunter and Rose, 1971). With the oleaginous yeast, *Candida 107*, the lipid content, in continuous culture, varied from 17.9 % to 21.5 % to 14 % at temperatures of 19, 30 and 33°C respectively (Ratledge and Hall, 1979). Temperature generally influences the degree of unsaturation in yeasts (reviewed in Ratledge and Evans, 1989). In cultures in Erlenmeyer flasks, the effect of temperature is complex. In addition to the direct effect on fatty acid synthesis and desaturation systems, temperature modifies the growth rate and the dissolved gas (O₂) content. However, according to Rose (1989), the direct effect of temperature on fatty acid desaturation has been shown to be probably the main effect in a batch culture.

There have been numerous studies on the effects of oxygen supply on lipid accumulation in yeasts but, unfortunately, many have been carried out in batch cultures where the growth rate is continuously changing and is directly influenced by aeration rate. It has been shown that the lipid content of the chemostat-grown *Candida 107* increases from 10.4 % to 19.7 % to 23 % when the aeration rate is raised from 0.05 to 0.1 to 0.5 (vvm), respectively (Ratledge and Hall, 1979). Further aeration of this culture results in a small decrease in lipid content. In other studies, it is reported that the total lipid content of Crabtree-positive yeasts is lower under anaerobic conditions than in aerobic culture (Ratray *et al.*, 1975). However, oxygen deficiency in such yeasts leads to an essential requirement for lipid itself, principally as sterols and unsaturated fatty acids.

1.7.7. Growth rate

The major determinant of the lipid content of a yeast is the rate at which the organism grows. Observations made in batch culture on the changes in either total lipid content or of components of the lipid must take account of any alteration which may have occurred to the growth rate of the organism as well as the fact that the growth rate itself changes

continuously during batch growth. Thus changes in pH value, dissolved oxygen tension, temperature, addition or subtraction of a nutrient, will inevitably have an effect on growth rate of the yeast.

As has been shown by many workers using chemostat cultures of yeasts, changes in growth rate can cause substantial changes in the lipid content of cell, particularly if the yeasts are grown without carbon limitation. Alterations in relative proportions of lipid contents are especially susceptible to changes in growth rate and, even during batch culture, can undergo considerable changes (Evans and Ratledge, 1989).

Minor changes in the growth rate would probably have the greatest influence on the constancy of fatty acid composition, but these changes are unlikely to be excessive. However, the variations in fatty acid composition of *Candida* 107 with varying growth rates did show distinct difference, depending on which nutrient was made limiting (Gill *et al.*, 1977). With carbon limiting, fatty acids in all three lipid fractions become more unsaturated with decreasing growth rate, whereas with limitations of all other nutrients including P and Mg, the reverse tend to occur. Variations in the growth rate of carbon-limited cells appear to produce the greatest variations in both lipid and fatty acid composition (Gill *et al.*, 1977).

1.8. YEAST, AN ALTERNATIVE LIPID SOURCE

Yeasts produce beneficial products such as alcohol, beverages single cell protein (SCP), and many biochemicals used in the food and pharmaceutical industries. As noted earlier, some yeasts also produce beneficial lipids and lipid containing emulsifying compounds (Jacob 1989). The advantages of using yeasts as lipid producers are that (1) they produce lipids similar to vegetable oils and fats, (2) they can be grown reasonably well on cheap agroindustrial and food industrial wastes, (3) their lipids can be produced at a faster rate in large capacity reactors than the usual time consuming agricultural practices, and (4) most of the potential lipid producers and their products seem to be relatively non-toxic

to humans. (Jacob, 1993).

Plants have been a source of oil since the beginning of recorded history. Large amounts of oil are extracted from the seeds of different plant species (e.g. olive oil and palm oil). However, attempts have been made since the seventies to explore the use of oleaginous yeasts and fungi as alternative lipid source, particularly the oils and fats similar to those already extracted from plants. For example, three *L. starkeyi* strains have been isolated from South African soils which accumulate more than 30% neutral lipids and produce free fatty acids similar to those of palm oil (Van Rensburg *et al*, 1995). Recently a total of 27 lipomycetaceous yeasts were isolated from 200 different soils in the Northern Province of South Africa and two of the isolates representing the species *L. starkeyi* and *L. tetrasporus* were found to produce as much as 47.0% and 48.5% lipids respectively (Mothibeli *et al*, 1996). These lipids, on basis of their fatty acid content, were found to be similar to that of palm oil.

The large accumulation of lipids which are similar to palm oil in these oleaginous yeasts have made these yeasts to be considered to be of biotechnological value. Although, palm oil equivalent produced from a microbial source may not contain all components of the one produced from a plant source, it is important to note that the comparable fatty acid composition is worth considering since the monounsaturated fatty acids are beneficial in protection against coronary heart disease and the essential fatty acids, namely linoleic and oleic acid are required for normal healthy growth (MOPOPC, 1996). It is, therefore, possible that the palm oil equivalent produced from microbial sources can be supplemented to make it have the acceptable properties of a natural palm oil. Hence, it is important to explore the possibility of optimising the production of the palm oil equivalents and other oils from the oleaginous yeasts isolated from soil in the Northern Province.

1.9. IMPORTANCE OF PALM OIL

Palm oil is a vegetable oil produced from the fruit of the *Elaeis guineensis* tree that originated in West Guinea. Palm oil is semi-solid at room temperature and can be fractionated into solid and liquid fractions known as stearins and oleins respectively. Ninety percent of palm oil is used for a variety of food products. The wealth of data now available show that palm oil is nutritionally wholesome (MPOPC, 1996). Although the percentage of palm oil in non-food uses is small in relation to that used for food, it is important since most of the palm oil products are further processed to products of higher value.

1.9.1. Food uses

Palm oil is used in such products as bread and rolls, pancakes, waffles, crackers, ready to eat cereals, chips, popcorn, toaster tarts, cookies, cakes, pies, canned puddings, candies, coffee whitener, non-dairy toppings, infant formulas, cocoa mixes, fried potatoes and onions, soups, gravies and frozen and dry mix entrees. It is also especially useful in the manufacture of margarines since it imparts natural coloring and a high glyceride content which gives a desired consistency without hydrogenation, high resistance to oxidation, a good shelf life, make it suitable for hot climates and possesses properties that are desirable in cakes and other bakery products. As a frying fat, palm oil has a high resistance to oxidation and does not leave an unpleasant room odor, due to the absence of linolenic acid in its composition.

1.9.2. Medical uses

Palm oil and palm oil products are naturally occurring sources of the antioxidant vitamin E constituents, tocopherols and these natural antioxidants act as scavengers of damaging oxygen free radicals and are hypothesised to play a protective role in cellular aging, atherosclerosis and cancer. Like other fats, palm oil, helps to maintain healthy skin and hair, ensures proper growth, and enables the body to absorb vitamins. In addition, palm oil has a relatively high concentrations of monounsaturated fat, in the form of oleic acid.

Studies have shown that diet high in monounsaturated fatty acids helps lower low density lipoproteins (LDL) cholesterol, as do polyunsaturated fatty acids. In addition, monounsaturates also help in maintaining HDL cholesterol levels in the blood which is beneficial in protecting against coronary heart disease.

Palm oil is an excellent dietary energy source, cholesterol free and does not contain trans fatty acids. It is also rich in beta-carotene and a precursor of vitamin A and tocotrienols, all of which have antioxidant properties. Fats serve distinct roles in the diet and in the body. In the diet, fats provide essential fatty acids and energy and are a carrier for the fat soluble vitamins. Those vitamins are A, D, K and E. Among the functions of vitamin A are promoting good night vision, the health of mucous membranes and skin, the growth of bone and reproduction. The principal role of vitamin E in the body appears to be an anti-oxidant. It prevents oxidation of unsaturated fatty acids, phospholipids, and vitamin A. It may also help maintain cell membrane stability and be essential for normal neurological function. In the body, the functions for fat include energy reserve, thermal insulation, organ protection, tissue membrane structure and cell metabolism.

1.9.3. Other uses

Vegetable oils were used as a fuel by Rudolf Diesel in 1900 when demonstrating the compression engines. Since then publications referring to similar usage of cracked products of oils and fats have been used. Recent research has demonstrated that crude palm oil can be used directly as a fuel to run cars fitted with suitably modified (Elsbett) engines. The exhaust fumes from crude palm oil were found to be cleaner than those from diesel engines, with essentially no sulphur or nitrogen oxides (MPOPC, 1996). It is also economical and safer to transport crude palm oil than diesel because of the high flash point (crude palm oil: 240°C, vs. diesel 52°C).

Palm oil, besides containing no aromatic compounds and being non-toxic, was found to have flash and aniline points greater than 65°C, making it suitable as a base in drilling

muds. Mud made with palm oil was found to have better emulsion stability and rheological properties and to give better control of fluid loss.

The incorporation of both C16-C18 and C12-C14 fatty acids in soaps is important as they provide the cleaning, solubility and foaming properties required. Tallow and coconut oil, respectively, have been the traditional sources of these fatty acids. A comparison between the fatty acids of palm oil, palm stearin, tallow, palm kernel oil, palm kernel olein and coconut oil indicates that the first three are rich in C16-C18 fatty acids while palm kernel and coconut oils are rich in C12-C14 fatty acids. However, for palm oils to establish a niche in the market as raw materials, soaps manufacturers have to be convinced that apart from price competitiveness, they will yield soaps with properties and performance comparable if not superior to those of soaps from tallow and coconut oil (MPOPC,1996).

1.10. OBJECTIVES

In recent years, nutritional studies have shown the potential benefits of including long-chain polyunsaturated fatty acids in the diet. The most easily available sources of polyunsaturated fatty acids are from vegetable oils such as palm oil, corn oil, soybean oil, coconut oil and sunflower seed oil.

Industrially, vegetable oils are incorporated into fat blends for the manufacture of a variety of food products and also for production of healthy skin lotions and soaps. Thus, the importance of vegetable oils in the diet and industry cannot be overemphasised, and they will continue to play a very important role in our daily lives.

The very high rate of population growth, especially, sets food producers a particularly difficult task. It is just in this respect that a microbial contribution might be most valuable. With the current awareness of environmental issues and the increasing preference for environmentally-friendly products, a constant search for alternative sources for the production of vegetable oils have intensified. Because of the ready availability of raw

materials, technology, capital and market demand, the non-food applications of palm oil and palm oil products as mentioned earlier is expected to have a bright future. Furthermore, microorganisms being simpler than plants, easier to grow and not affected by adverse climatic conditions will be an alternative choice for the production of edible oils (MPOPC, 1996).

It is against this background that attempts are made in this study to optimise conditions for the production of edible oils and other industrially important oils from lipomycetaceous yeasts isolated from soil in the Northern Province, South Africa. However, for the production to be economical, it is important to explore low cost substrates such as agricultural waste and effluents from the brewery, dairy and fruit industries for microbial oil production.

Most of the work on microorganisms has been carried out in either stationary or shake cultures, although some studies on the factors influencing lipid formation have used stirred fermenters, hence, the use of different culture systems (batch and fed-batch) and their influence on growth and production of oils by the two yeasts, namely *Lipomyces sp.* and *L. tetrasporus* are investigated. Furthermore, the effects of different concentrations of the carbon source (glucose) are studied as well as the composition of the oil extracted.

CHAPTER 2

MATERIALS AND METHODS

2.1. Yeast strains

Three yeast strains, *Lipomyces starkeyi* (CBS 2516T), *Lipomyces tetrasporus* (UOFS-Y-2082) and *Lipomyces sp* (CBS 5910T) used throughout this study, were obtained from the University of Orange Free State (UOFS), South Africa. Stock cultures were maintained on yeast extract-malt (YM) slants (Wieckerham, 1951) containing (in grams per litre of distilled water): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; and 2 % agar at 4°C and the stock cultures were transferred monthly. For storage, the cultures were kept at -80°C until required for revival.

2.2. Growth media

The BASAL medium contained the following constituents (in grams per litre of distilled water): yeast extract, 10; peptone, 20; and glucose, 20 (C/N = 50:1) (Van Rensburg *et al.*, 1995). The pH was adjusted to 5.5 and the medium sterilized in an autoclave at 121°C for 15 min.

2.3. Shake flask culture

A loopful of the yeasts from the stock cultures was inoculated into 10 ml of the BASAL growth medium in a test tube and incubated at 30°C with shaking at 200 rpm in a Controlled Environment Incubator shaker (New Brunswick, New Jersey, USA). When the cultures reached the exponential growth phase after 48 h, 8 ml was transferred into 500 ml Erlenmeyer flasks containing 200 ml of the growth medium of the same composition as the primary culture medium. After 48, 72, 96 and 120 h, 1 ml of the cell suspension was harvested (in duplicate) from each flask and the absorbance read at 570 nm with Spectronic Genesys 5 spectrophotometer (Milton Roy).

2.4. Dry cell weight measurement

Ten millilitre samples (in duplicate) of each of the cultures (described above) were harvested after 48, 72, 96 and 120 h and transferred into preweighed 15 ml sterile centrifuge tubes and centrifuged at 8,000 xg in a GS-6R centrifuge (Meck) for 10 min at 4°C. The supernatants were collected and stored at 4°C for the determination of residual glucose or reducing sugar. The pellets were washed twice with 10 ml of distilled water and freeze dried at -10°C for 4-6 h depending on the amount of the biomass. The freeze-dried samples were weighed and expressed in g biomass / l.

2.5. Residual glucose analysis

The supernatants collected during the dry weight determination were analysed for residual glucose concentration using the Glucose-Trident Kit (Sigma). Five microlitres of the supernatant was added to 1 ml of the Glucose-Trident reagent in Eppendorf tubes and incubated for 18 min at room temperature (25°C) and the absorbance read at 505 nm. D-glucose was used as a reference sugar and the glucose concentration was calculated as described in the protocol.

2.6. Extraction of lipids

Freeze-dried samples of the different yeast strains, cultivated under appropriate conditions were weighed and transferred into extraction thimbles. The lipid fractions were extracted with the appropriate solvents (or solvent mixtures) in two steps. During extraction, butylated hydroxytoluene (0.05% w/v) was added to the solvent/ solvent mixtures to prevent lipid oxidation. Neutral lipids were extracted in the first step for 5 h with hexane in a Soxhlet apparatus. In the second extraction step, hexane was replaced by chloroform-methanol mixture (2:1 v/v) to extract the polar lipids for 5 h. After each extraction step the solvent was evaporated at 80°C and the flasks dried in a dessicator containing calcium chloride. The weights of the lipid fractions were determined from the difference between the preweighed flasks and the weight after extraction and evaporation. Each lipid fraction was dissolved in 1 ml chloroform and stored in amber bottles under nitrogen and kept at

-20°C for Thin Layer Chromatography (TLC) and Gas Chromatographic (GC) analysis.

2.7. Effect of substrate (glucose) concentration on lipids production

To determine the effect of glucose concentration on lipid production, the culture conditions were the same as those described in section 2.3 and the media ingredients were the same as those in section 2.2 except that the glucose concentrations used were 1 %, 2 % and 4 % (i.e different C/N ratios). At various intervals (72, 96 and 120 h) the cells were harvested and growth, residual glucose and yields of lipids were determined as described above.

2.8. Lipid production in fed-batch culture

To determine the effect of fed-batch culture on lipid production, the culture conditions were the same as those described in section 2.3 and the media ingredients were the same as those in section 2.2. except that the glucose concentrations were 1 %, 2 % and 4 % and the cultures used were only CBS 5910 T and UOFS-Y-2082. After 72 h the residual glucose was determined as described in section 2.5 and the cultures fed with glucose such that the glucose concentrations were the same as the initial concentrations. For the control cultures distilled water was used as the replacement media. Media replacement was repeated after every 48 h. The cells were harvested each time before feeding. Growth, residual glucose and yields of lipids were determined at each point as described above.

2.9. Effect of industrial wastes on lipids production

2.9.1. Media

The effluent (SAB) was collected from the South African Breweries, Pietersburg and the waste (PASSI) was an orange waste collected from the Granor Passi Fruit Juice Processing Factory, Pietersburg. The SAB medium was boiled for 1 h and centrifuged at 8000 xg for 10 min to remove denatured proteins. The pH of the medium was adjusted to 5.4. The PASSI medium homogenized in a waring blender for 1 min and centrifuged

at 10 000 rpm for 10 min. The supernatant was then boiled for 1 h and centrifuged at 8 000 xg for 10 min. The pH was adjusted to 5.4. Both media were sterilized in an autoclave at 121°C for 15 min. The total reducing sugar concentration in each medium was determined using the Glucose- Trident Kit (Sigma) and the total soluble protein concentration was determined with the BIORAD reagent.

2.9.2. Culture conditions

The culture conditions were the same as those described in section 2.3 except that the media used in these experiment were the industrial wastes prepared in section 2.9.1. At various intervals (72, 96 and 120 h) the cells were harvested for the determination of growth, dry weights, residual glucose, reducing sugars, total protein content and yields of lipids.

2.9.3. Determination of total reducing sugars

The supernatants collected during the dry weight determination described in section 2.4. were analysed for residual sugar using the dinitrosalicylic acid (DNS) method (Summer and Summer, 1929).

The DNS reagent was prepared as follows:

One gram of DNS acid was moistened with a few drops of distilled water in a 250 ml conical flask. Twenty millilitres of 1 M NaOH were added slowly to the solution with constant shaking and finally, 50 ml of distilled water was added. When the DNS acid had dissolved completely, 30 g of potassium sodium tartrate was added to the solution. The solution was then made up to 100 ml with distilled water.

For reducing sugar determination, one millilitre of the DNS reagent was mixed with 1 ml of the sample in a test tube and to prepare a blank the sample was replaced with 1 ml of distilled water. One hundred microlitres of 0.1 M NaOH was added to each tube to make it alkaline since neutral or acidic media with high buffering capacity can adversely influence the results. The tubes were incubated in boiling water for 5 min, then cooled

under running water for 1 min. Ten millilitres of distilled water was added into each tube and the absorbance read at 540 nm. The sugar concentrations of the samples were extrapolated from the calibration curve constructed with the absorbance readings obtained from D-glucose which was used as a reference sugar.

2.9.4. Determination of total protein content

The BIO-RAD protein assay method was used to determine the total protein content in both effluents. Two millilitres of the BIO-RAD dye was added into 800 μ l samples of concentrations : 5, 10, 15, 20 and 25 μ g/ml. To prepare a blank 800 μ l of the sample was replaced with distilled water. The absorbance was read at 595 nm. The protein concentration was extrapolated from the calibration curve constructed using absorbance readings obtained and bovine serum albumin (BSA) was used as a reference protein.

2.10. Thin layer chromatography analysis

Plates (10x20 cm.0.25 mm) of silica gel 60 F (Merck,Darmstadt,Germany) were activated at 60°C for 15 min. Aliquots of the neutral lipid extracts of each organism were applied to plates and transferred into a tank saturated with hexane: ethyl ether: acetic acid (75:25:1 v/v) which was used as a mobile phase. After migration of the solvents to a suitable point, the plates were dried and developed for 1-2 min in a tank saturated with iodine vapour at 60°C for visualization. The lipid spots could be observed in daylight but they were also visualized under UV light for spots that could not be easily observed. The chromatogram was finally examined and the spots identified by comparing the R_f values with those of authentic triacylglycerol standards (Sigma).

2.11. Gas chromatography analysis

The GC analysis of the lipids was carried out by the Division of Food Science and Technology at the CSIR (Pretoria, South Africa). The neutral and the polar lipid extracts were methylated using the boron trifluoride reagent. Methyl esters were dissolved in hexane and 1 to 2 μ l aliquots injected into the column [Omegawax 320 (30 mm x 0.32

mm)]. A Helwett Packard 5890 gas chromatograph, equipped with flame ionisation detector, was used and the helium flow rate about 2 ml per minute. Peak areas and total peak area were used to calculate the fatty acid composition. Results were expressed as g fatty acid per 100 g lipids.

CHAPTER 3

RESULTS

In this study, growth, substrate utilization and lipid production by three species of oleaginous yeasts have been investigated. The three oleaginous yeasts were *Lipomyces starkeyi* (CBS 2516 T), *Lipomyces tetrasporus* (UOFS-Y-2082) and *Lipomyces sp.* (CBS 5910 T). Growth, lipid production and substrate utilization by the yeast strains were determined when cultivated in a batch culture in normal BASAL medium, BASAL medium of different carbon: nitrogen ratios, in effluents collected from the South African Breweries (SAB) and orange juice waste (PASSI) from Granor Passi and in BASAL media of different carbon: nitrogen ratios in a fed- batch culture.

3.1. Growth

To compare the growth of the three oleaginous yeasts, the growth patterns of the yeasts were determined spectrophotometrically by reading the absorbancies at 570 nm after cultivation for 48, 72, 96 and 120 h in a batch culture. Fig. 3.1. shows the growth curves of the three yeast strains in the BASAL medium (C/N = 50:1). Maximum growth was reached between 96 and 120 h by the three strains. The fastest growth rate was observed with *Lipomyces starkeyi* (CBS 2516 T) and the lowest with the *Lipomyces tetrasporus* (UOFS-Y-2082) although at the initial stages of growth (up to 48 h) UOFS-Y-2082 grew faster than CBS 5910 T. The dry cell weights also followed a similar pattern (Tables 3.1.1 - 3.1.3.).

3.1.2. Lipid production

Lipid accumulation was determined by the Soxhlet extraction method. The neutral lipids were extracted with hexane and thereafter replaced by chloroform- methanol mixture (2:1 v/v) to extract the polar lipids. The total lipid was considered as the sum of the extracted

neutral and polar lipids and expressed in gram lipids per 100 g biomass. Since the biomass concentration was low up to 48 h, the samples were not sufficient for lipid extraction, hence the lipid content was only determined after 72 h.

3.1.2.1. Neutral lipids

Fig. 3.2.1 shows the accumulation of neutral lipids by the three yeast strains in a batch culture. The highest amount of neutral lipids produced was 45 % by CBS 5910 T at 72 h followed by UOFS-Y-2082 which produced 30 % after 96 h. The least amount of neutral lipids (25 %) was produced by CBS 2516 T after 72 h of cultivation (Fig. 3.2.1). However, the production of neutral lipids in CBS 5910 T, CBS 2516 T and UOFS-Y-2082 decreased with time to 6 %, 19 % and 22 % respectively after 120 h.

3.1.2.2. Polar lipids

Generally, the accumulation of polar lipids bear a reciprocal relationship with the neutral lipids. This relationship was observed particularly between 72 and 96 h. For example, at 72 h, CBS 2516 T and CBS 5910 T produced 41.75 % and 19.5 % neutral lipids and 24.75 % and 24.75 % of polar lipids, respectively. After 72 h, the highest amount (44 %) of polar lipids was produced by CBS 2516 T and the least (10 %) by UOFS-Y-2082 at 96 h but increased after 120 h (Fig. 3.2.2.). High polar lipid content also seemed to be related to the growth of the cells. For example, when the growth of CBS 2516 T was higher than UOFS-Y-2082 between 72 and 96 h, the polar lipid content seemed to correlate with growth, since more polar lipids (41.80 %) were observed in the CBS 2516 T than the UOFS-Y2082 (24.8 %) during the same period.

3.1.2.3. Total lipids

In the batch culture, CBS 5910 T and CBS 2516 T accumulated more lipids than UOFS-Y2082 after 72 h. The highest lipid content (72 %) was observed in the CBS 5910 T and the least (44 %) in UOFS-Y-2082. Whereas the total lipids increased in the UOFS-Y2082 from 44 % to 59 % after 120 h, those in CBS 5910 T (from 72 to 27.80 %) and CBS 2516

T (66.5-33.32 %) decreased within the same period. The data suggest that CBS 5910 T and CBS 2516 T are better producers of lipids than UOFS-Y-2082 in a BASAL medium (Fig. 3.2.3.).

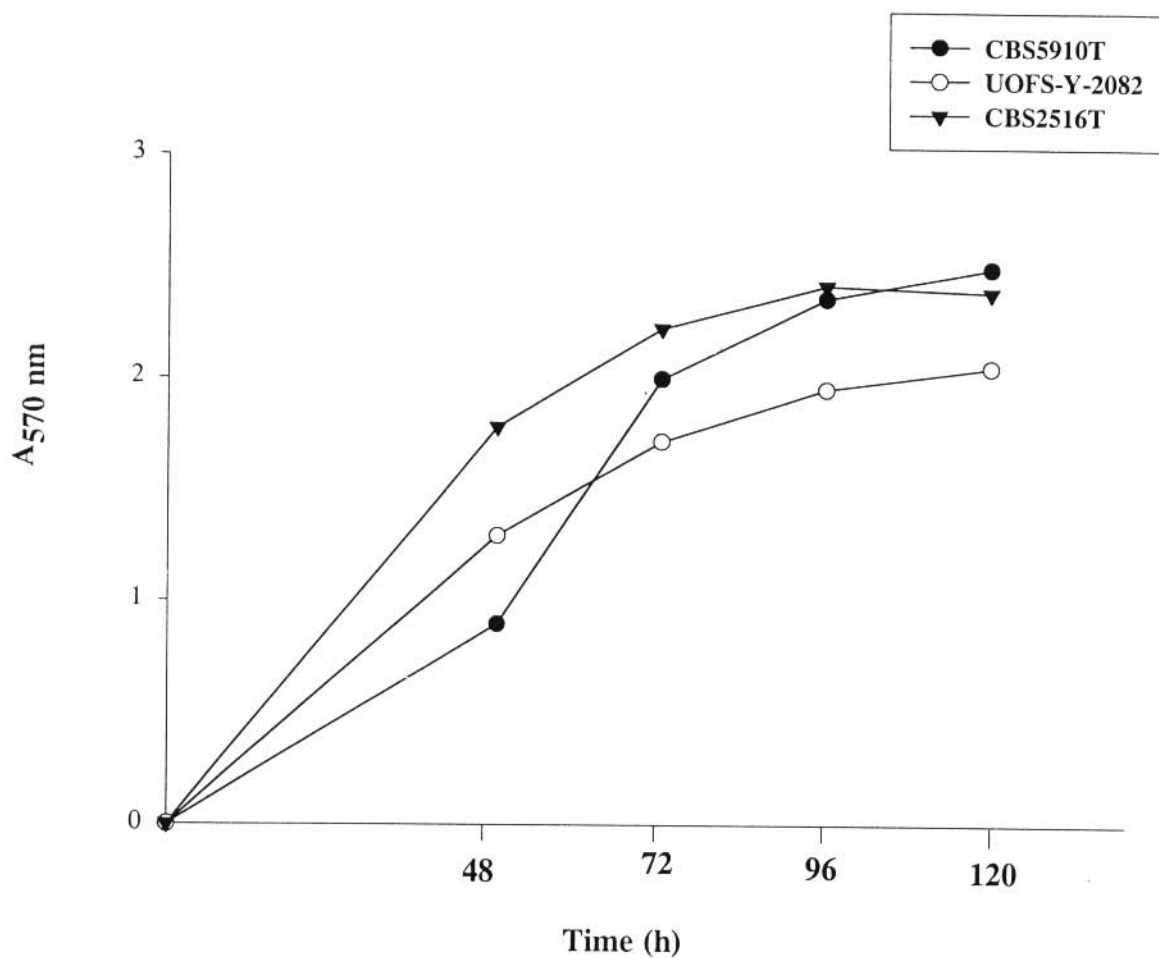


Fig. 3.1. Growth curves of different strains of oleaginous yeasts in BASAL medium (C/N= 50:1) at various cultivation times in a batch culture. Each point represent the mean of two separate experiments each determined in duplicate. The culture conditions are as described in the Materials and Methods.

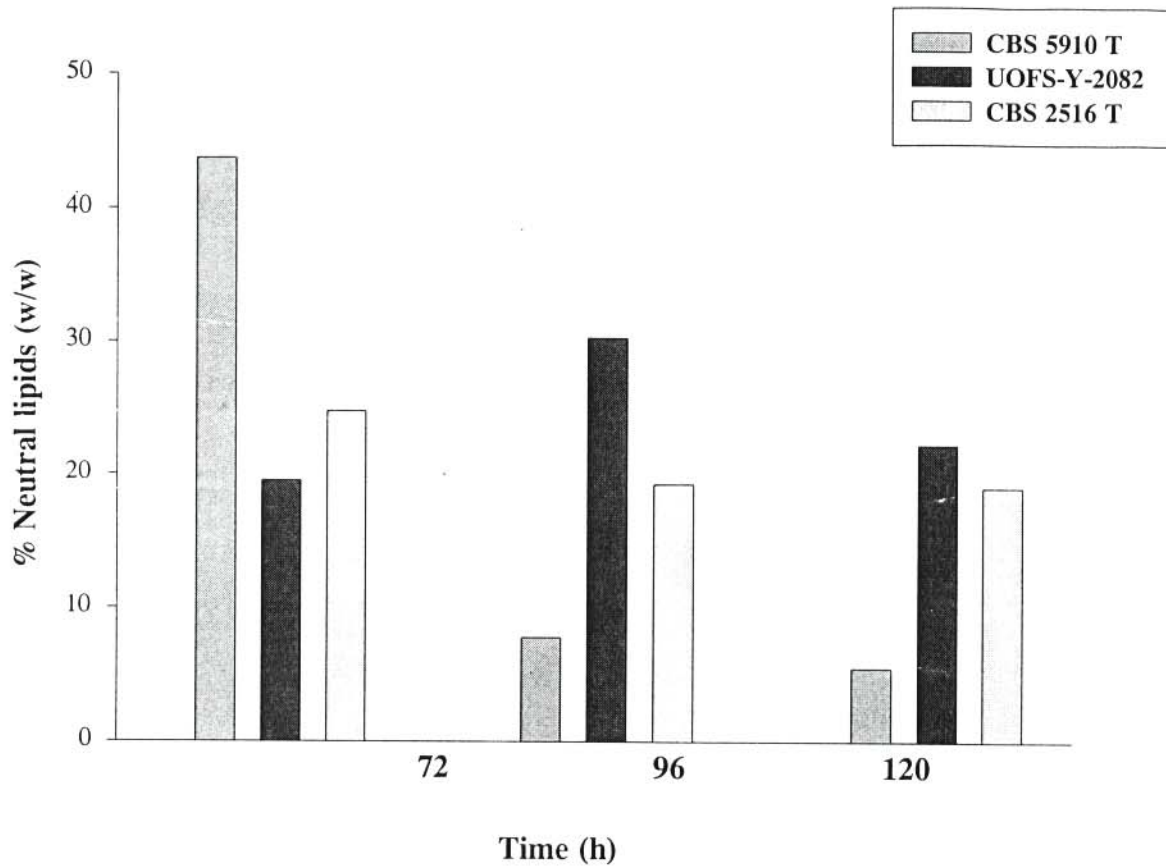


Fig. 3.2.1. Accumulation of neutral lipids extracted from the three oleaginous yeasts cultivated in BASAL medium in a batch culture at various cultivation times (72, 96 and 120 h). The neutral lipid content is expressed as g neutral lipids / 100 g biomass.

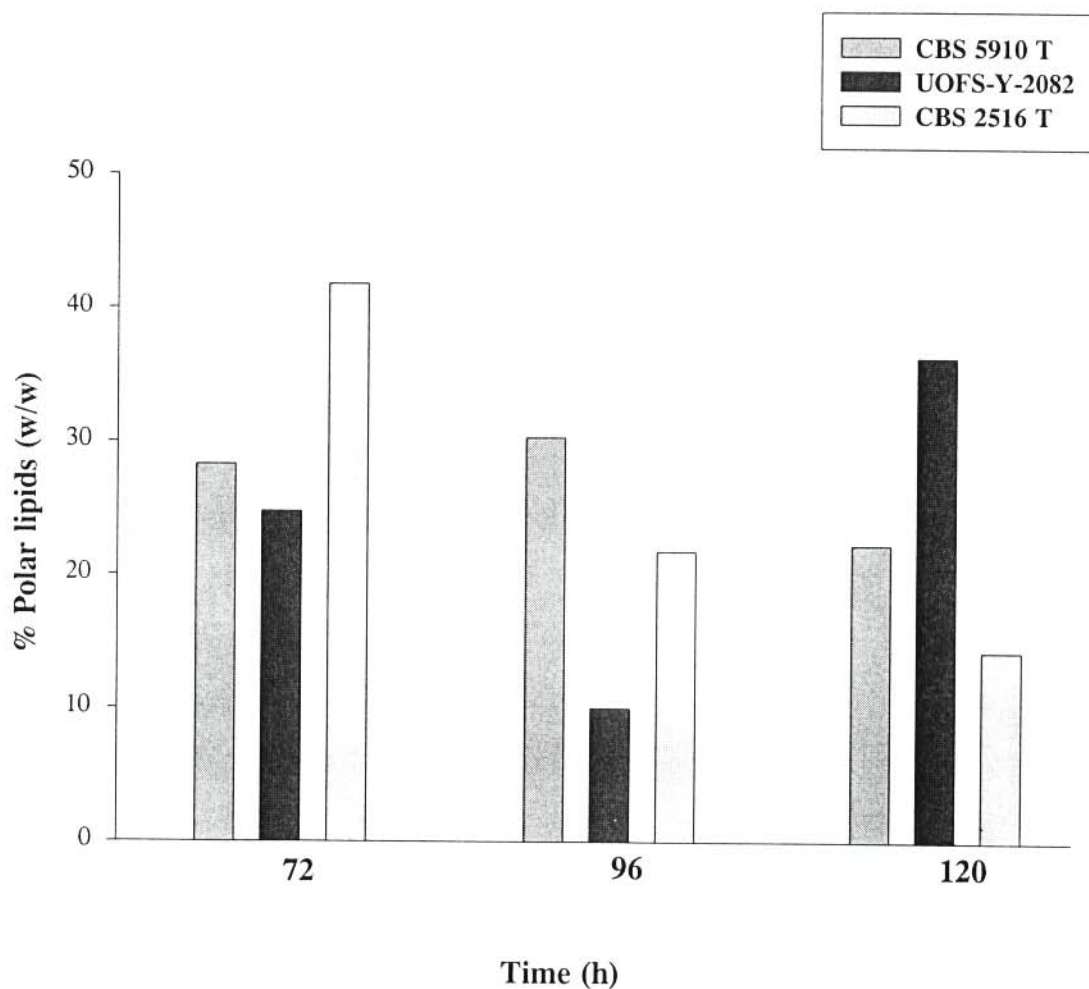


Fig. 3.2.2. Accumulation of polar lipids extracted from the three oleaginous yeasts cultivated in BASAL medium in a batch culture at various cultivation times (72, 96 and 120 h). The polar lipid content is expressed as g polar lipids / 100 g biomass.

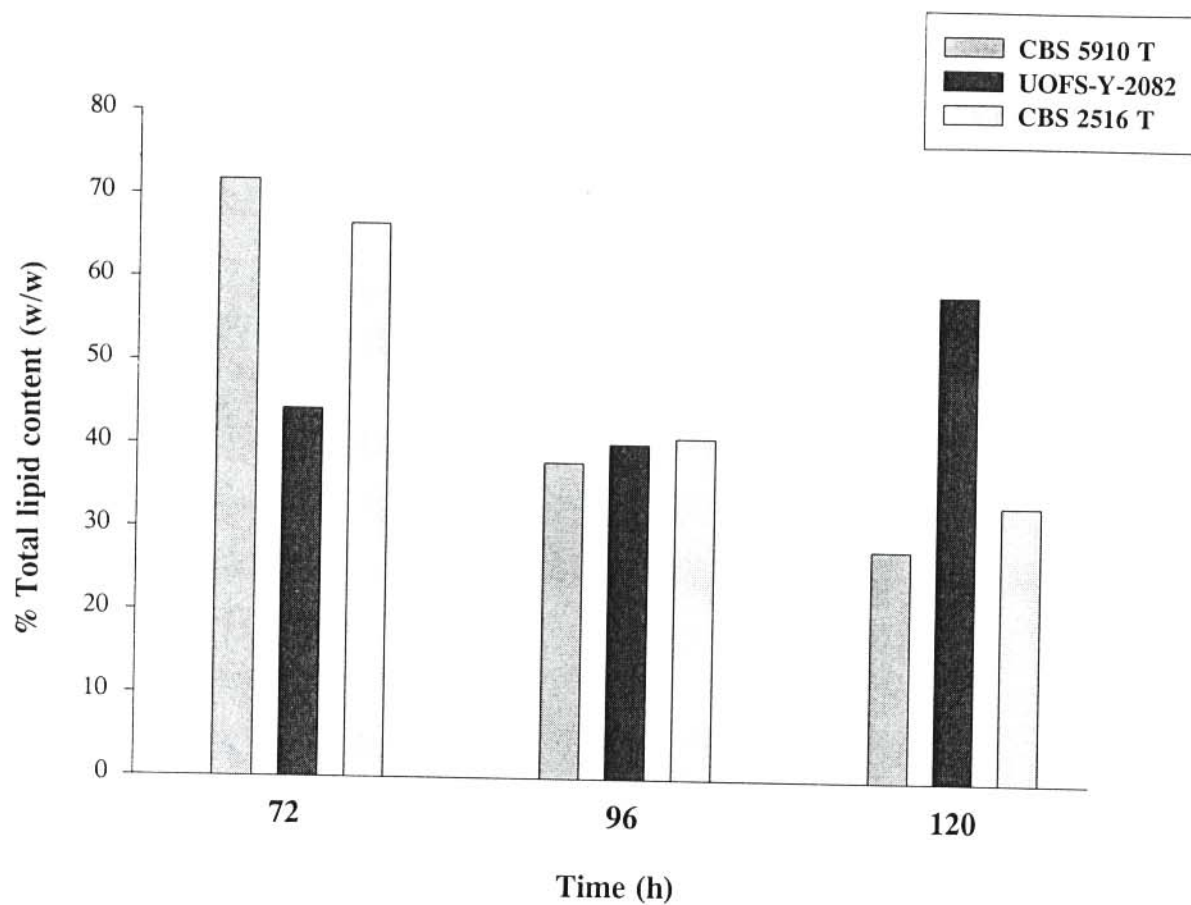


Fig. 3.2.3. Total lipid accumulation in the three oleaginous yeasts cultivated in BASAL medium in a batch culture at various cultivation times (72, 96 and 120 h). The total lipid content is expressed as g total lipids per 100 g biomass.

3.2. EFFECT OF SUBSTRATE (GLUCOSE) CONCENTRATION ON LIPID PRODUCTION

In order to find the optimum concentration which could support growth and lipid production by the three oleaginous yeasts, different concentrations (1 %, 2 % and 4 %) of glucose were used in the culture media. Growth and lipid production were followed until 120 h.

3.2.1. Growth

Maximum growth of CBS 2516 T and UOFS-Y-2082 was observed in cultures cultivated in 1 % medium and the lowest in 4% and 2 %, respectively, after 72 h (Fig. 3.3.1 and 3.3.2). But with CBS 5910 T, maximum growth was observed in 4 % and the lowest in 1 % (Fig. 3.3.3). However, after 72 h the growth pattern changed in CBS 2516 T and UOFS-Y-2082 when the growth in 1 % medium was slow and decreased below the levels attained in 2 % and 4 % cultures.

3.2.2 Total lipids

The total lipids accumulated in CBS 5910 T and UOFS-Y2082 at 72 h decreased as the concentration of glucose was increased in the media (Fig. 3.4.1 and 3.4.2). However, with CBS 2516 T there were fluctuations in lipid production as the concentration of glucose in the media increased (Fig. 3.4.3). These preliminary data suggest that lipid accumulation could best be achieved when CBS 5910 T and UOFS-Y-2082 are cultured in media containing 1 % glucose for 72 h but CBS 2516 T accumulated the highest amount of lipid when cultured in 2 % glucose for 96 h.

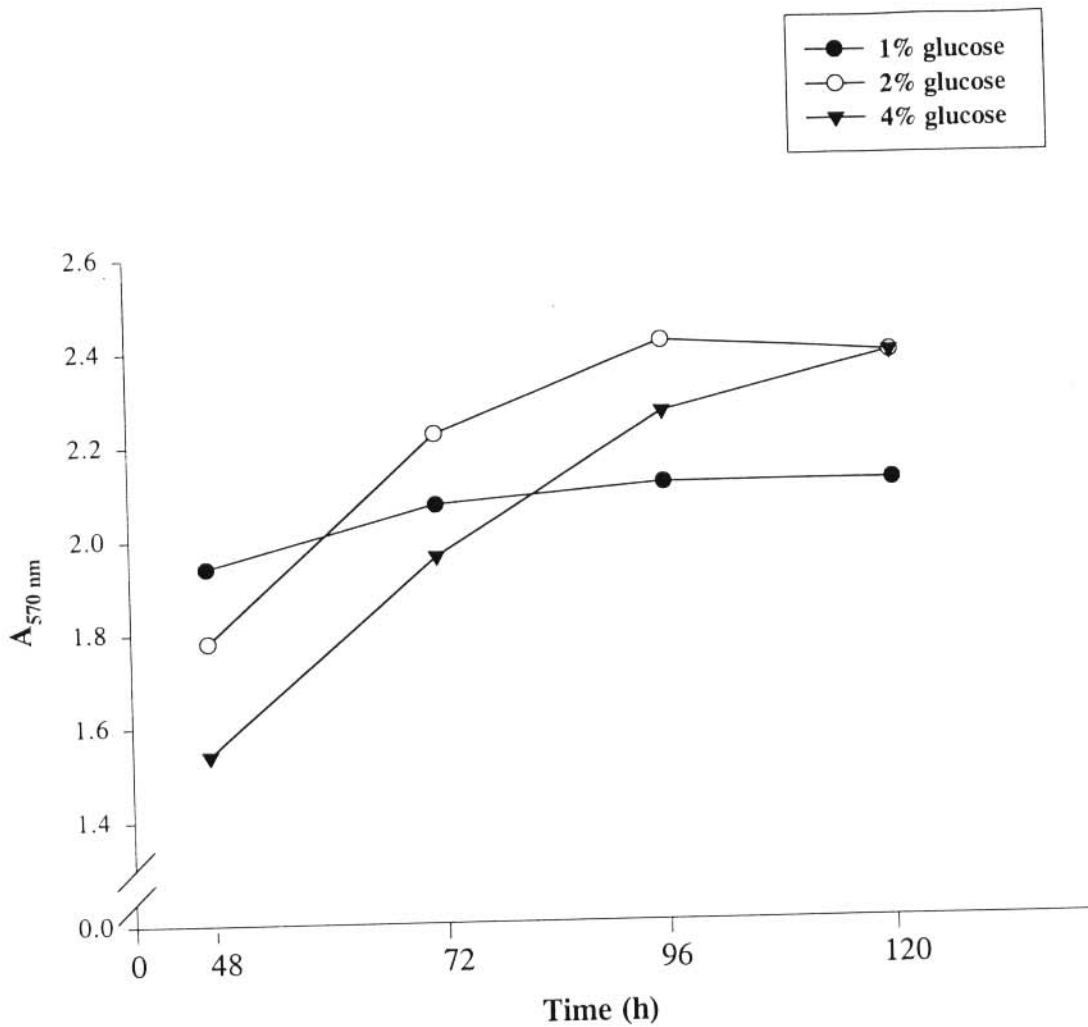


Fig. 3.3.1. Growth curves of CBS 2516 T cultures in BASAL medium (C/N= 50:1) of different glucose concentrations at various cultivation times in a batch culture. The culture conditions are as described in the Materials and Methods.

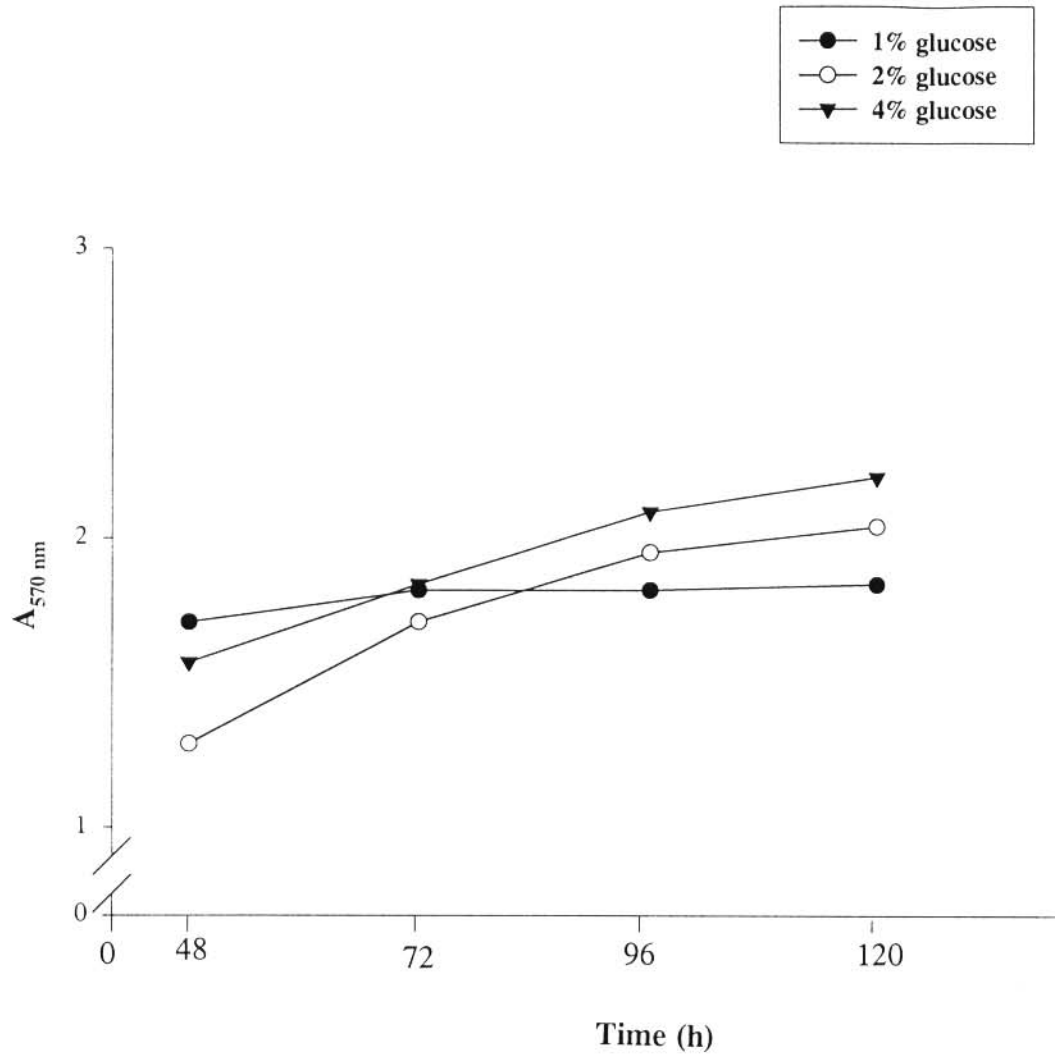


Fig. 3.3.2. Growth curves of UOFS-Y-2082 cultures in BASAL medium (C/N= 50:1) of different glucose concentrations at various cultivation times in a batch culture. The culture conditions are as described in the Materials and Methods.

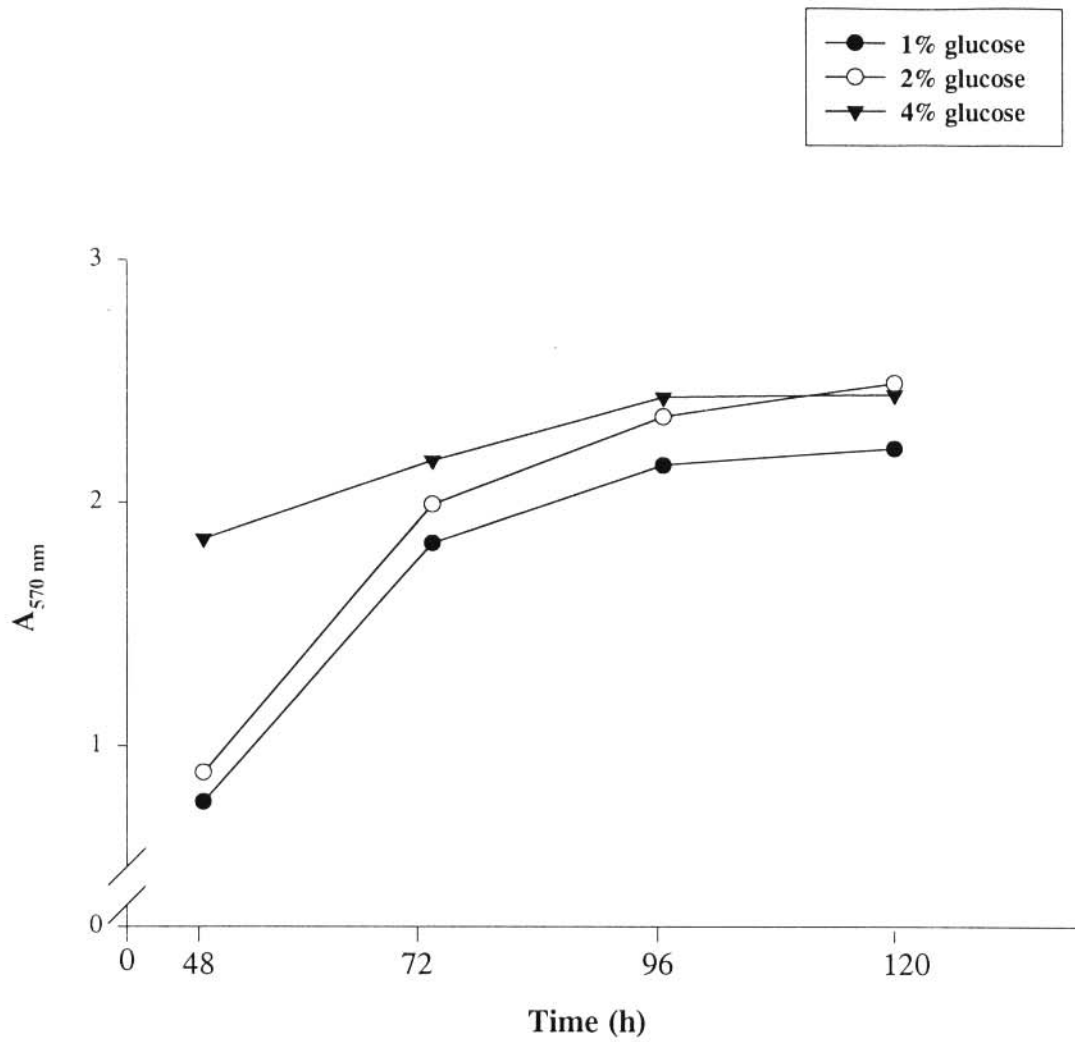


Fig. 3.3.3. Growth curves of CBS 5910 T cultures in BASAL medium (C/N= 50:1) of different glucose concentrations at various cultivation times in a batch culture. The culture conditions are as described in the Materials and Methods.

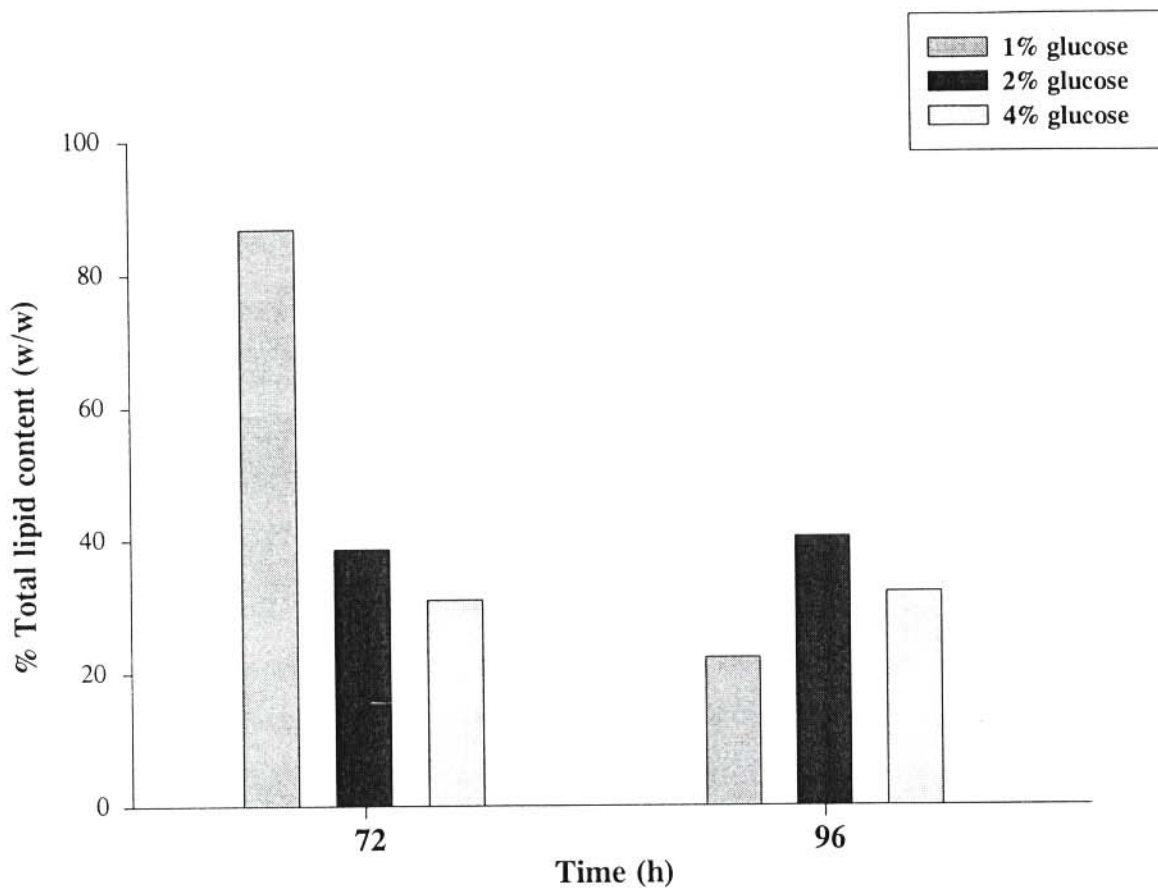


Fig. 3.4.1. Total lipid accumulation in CBS 5910 T cultivated in BASAL medium of different glucose concentrations in a batch culture at various cultivation times (72, 96 and 120 h). The total lipid content is expressed as g total lipids per 100 g biomass.

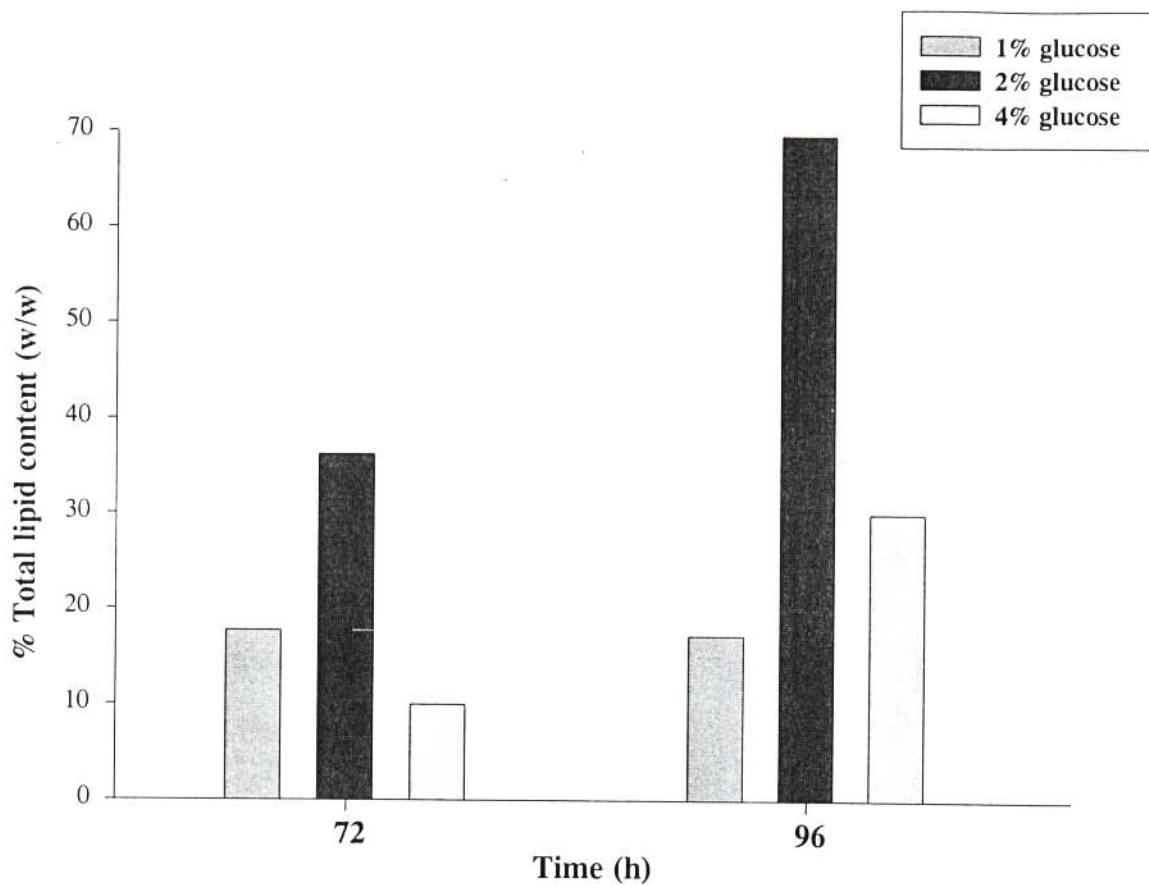


Fig. 3.4.2. Total lipid accumulation in CBS 2516 T cultivated in BASAL medium of different glucose concentrations in a batch culture at various cultivation times (72, 96 and 120 h). The total lipid content is expressed as g total lipids per 100 g biomass.

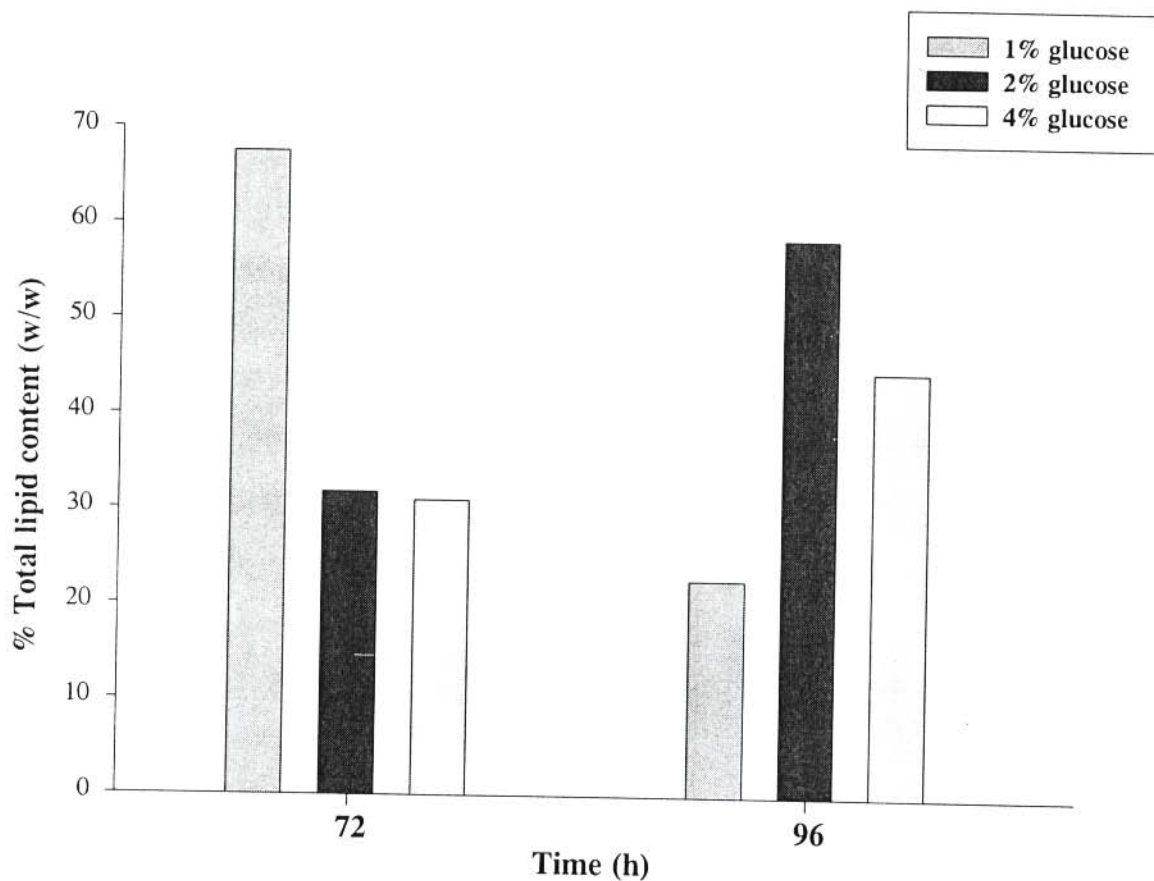


Fig. 3.4.3. Total lipid accumulation in UOFS-Y-2082 cultivated in BASAL medium of different glucose concentrations in a batch culture at various cultivation times (72, 96 and 120 h). The total lipid content is expressed as g total lipids per 100 g biomass.

3.3. EFFECT OF MEDIUM ON LIPID PRODUCTION

To compare lipid production by the three yeast strains in different media (BASAL, SAB and PASSI), the yeast strains were cultured and lipid production was determined until 120 h.

3.3.1. Growth

Maximum growth rate was observed with CBS 5910 T and the lowest with CBS 2516 T in both SAB and PASSI media (Fig. 3.5.1. and 3.5.2.). In PASSI medium both CBS 5910 T and CBS 2516 T reached their maximum growth at 72 h, but UOFS-Y-2082 only reached its maximum at 120 h. In the SAB medium CBS 5910 T grew the fastest and UOFS-Y-2082 showed the slowest growth. All the three yeast strains never reached their maximum growth, but still continued growing. The SAB medium might have contained a carbon source other than a reducing sugar which was utilized for growth after the reducing sugar had almost got depleted in the medium. This proposition is supported by the reducing sugar levels shown in Figs 3.9.1- 3.9.3. The residual reducing sugar reached its lowest level after 96 h, but increased thereafter from 2.4 to 2.8 g/l at 120 h (Tables 3.3.1- 3.3.3).

3.3.2. Lipid production

3.3.2.1. Total lipids

Maximum production of lipids was observed at different times depending on the yeast strain and the medium used. The maximum lipid content was observed with CBS 5910 T and CBS 2516 T after 72 h in all media (Tables 3.1.2 and 3.1.3) whereas with UOFS-Y-2082, the maximum production occurred after 120 h in BASAL and SAB but at 96 h in PASSI medium (Table 3.1.1). However, the maximum lipid output (i.e. the efficiency with which lipid is synthesized) expressed as gram lipid per litre per hour, occurred at 72 h in all the media by all the yeast strains (Tables 3.1.1 - 3.1.3). This occurred when there

was still enough residual carbohydrate in the media. This observation also corresponds with the lipid yield (expressed in grams lipid per litre of substrate utilized).

In terms of the efficiency of the conversion of the substrate into lipids, UOFS-Y-2082 was most efficient when grown in PASSI medium and harvested after 72 h. But when the specific rate of lipid production is considered, CBS 2516 T seemed superior when cultured in PASSI medium. The specific rates of lipid production by CBS 2516 T were, 0.012, 0.009 and 0.007 g of lipid/ g of yeast/ h in PASSI, BASAL and SAB, respectively (Tables 3.1.1 - 3.1.3) and these rates corresponded with the lipid contents of yeasts in their respective media.

Considering UOFS-Y-2082 which is the strain isolated in the Northern Province, South Africa, its efficiency in lipid production in SAB medium compared favourably with CBS 2516 T. The specific rates of lipid production by UOFS-Y-2082 were 0.009, 0.007 and 0.006 g lipid/ g yeast/ h in PASSI, SAB and BASAL media, respectively (Table 3.1.1). CBS 5910 T performed better in SAB medium than in PASSI and BASAL media (0.01, 0.007 and 0.003 g/ g/ h, respectively).

The results suggest that both PASSI and SAB media could be used for lipid production by the three yeasts in a batch culture.

3.3.2.2. Neutral and polar lipids

With respect to the ratio of neutral to polar lipids, the highest value was observed when CBS 5910 T was cultivated for 72 h in the BASAL medium. The value obtained for UOFS-Y-2082 when grown in SAB medium compared favourably with that of CBS 5910 T (1.472 and 1.549 for UOFS-Y-2082 and CBS 5910 T respectively). In PASSI medium, all the yeast strains seemed to produce more polar lipids than neutral lipids (Tables 3.1.1-3.1.3).

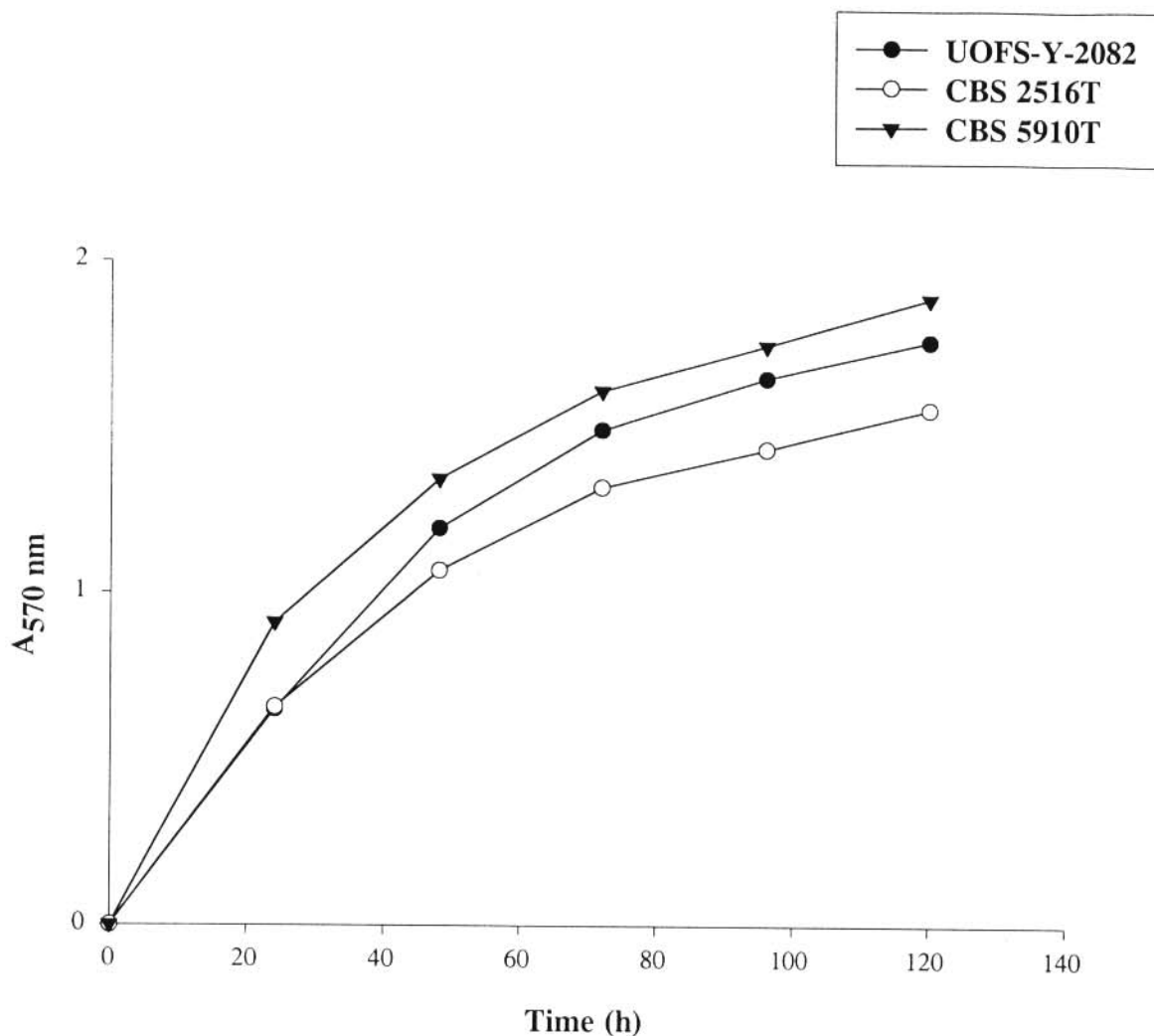


Fig. 3.5.1. Growth curves of different strains of oleaginous yeasts cultivated in SAB medium. Growth was determined by reading the absorbance at 570 nm at various cultivation times (24, 48, 72, 96 and 120 h). Each point represent the mean of two separate experiments each determined in duplicate.

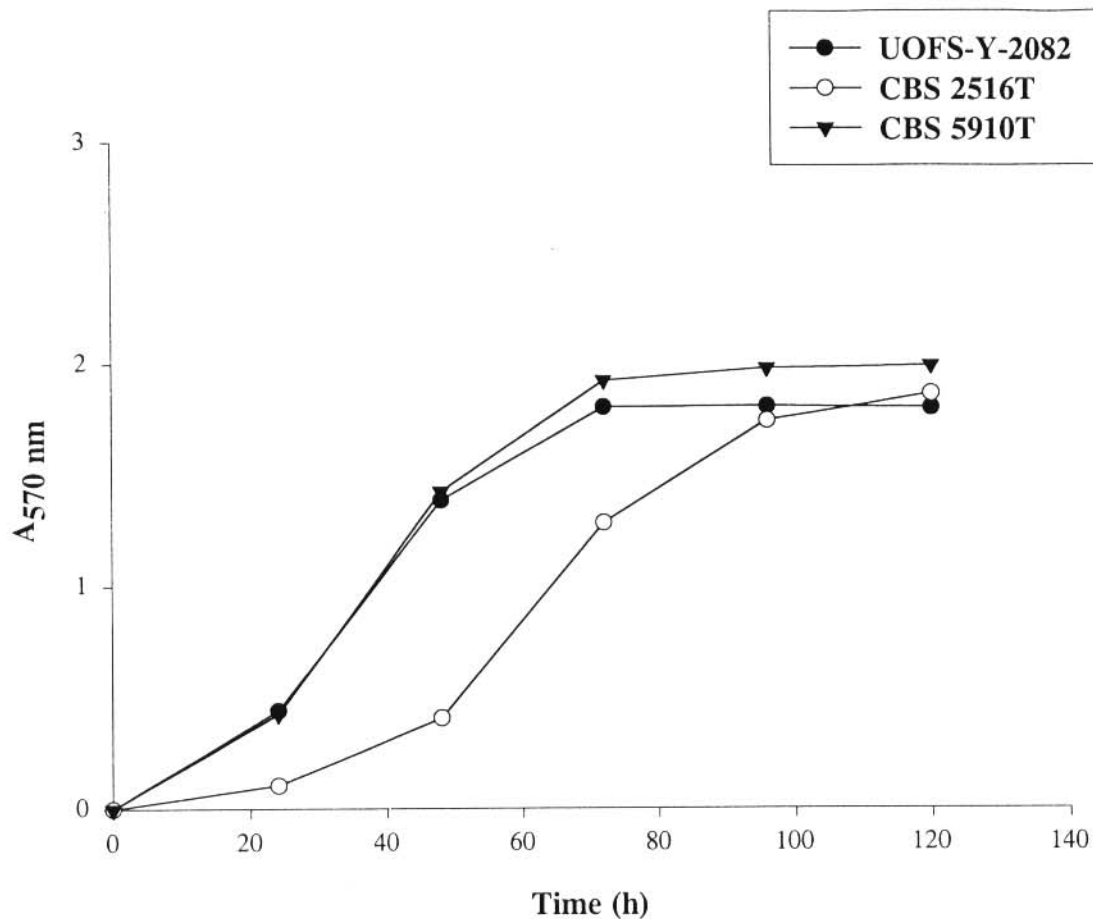


Fig. 3.5.2. Growth curves of different strains of oleaginous yeasts cultivated in PASSI medium. Growth was determined by reading the absorbance at 570 nm at various cultivation times (24, 48, 72, 96 and 120 h). Each point represent the mean of two separate experiments each determined in duplicate.

Table 3.1.1. Lipid production by UOFS-Y-2082 cultivated in different media at various cultivation times in a batch culture.

COMPONENTS	BASAL			SAB			PASSI		
	72 h	96 h	120 h	72 h	96 h	120 h	72 h	96 h	120 h
Biomass (g/dl)	0.27	0.28	0.45	0.08	0.09	0.11	0.17	0.235	0.26
% Total lipid content (w/w)	44.25	40.25	58.5	50.38	53.08	82.31	67.69	87.2	45.38
% Neutral lipid (w/w)	19.5	30.25	22.25	30.0	31.54	12.31	11.51	11.55	15.38
% Polar lipid (w/w)	24.75	10.00	36.25	20.28	21.54	70.0	56.54	75.65	30.0
Residual substrate (g/dl)	0.881	0.604	0.096	0.125	0.008	0.24	0.38	0.185	0.135
Substrate utilized (g/dl)	1.119	1.396	1.904	0.06	0.085	0.21	0.18	0.375	0.425
Biomass yield (g biomass/100 g substrate)	24.129	20.057	23.634	133	105.8	114.3	94.4	62.7	61.2
Total lipid produced (g/dl)	0.11	0.11	0.18	0.31	0.35	0.43	0.67	0.80	0.10
Lipid yield (g lipid/100 g substrate)	9.47	7.87	9.29	51.66	41.18	20.48	37.22	21.24	24.0
Ratio (neutral: polar)	0.788	3.025	0.614	1.472	1.464	0.177	0.204	0.153	0.513
Lipid output (g lipid/ l/ h)	0.015	0.011	0.015	0.043	0.036	0.036	0.093	0.083	0.008
Specific rate of lipid produced (g lipid/ g yeast/ h)	0.006	0.004	0.005	0.007	0.005	0.007	0.009	0.009	0.004

Table 3.1.2. Lipid production by CBS 5910 T cultivated in different media at various cultivation times in a batch culture.

COMPONENTS	BASAL			SAB			PASSI		
	72 h	96 h	120 h	72 h	96 h	120 h	72 h	96 h	120 h
Biomass (mg/dl)	0.65	0.875	0.855	0.08	0.13	0.15	0.20	0.27	0.32
% Total lipid content (w/w)	71.75	38.0	27.75	76.53	44.23	75.77	84.76	61.54	29.62
% Neutral lipid (w/w)	43.75	7.75	5.5	21.15	6.15	15.0	19.23	22.69	6.54
% Polar lipid (w/w)	28.25	30.25	22.25	55.38	38.08	60.77	65.38	38.85	23.08
Residual substrate (g/dl)	0.915	0.498	0.012	0.165	0.11	0.28	0.41	0.22	0.165
Substrate utilized (g/dl)	1.09	1.502	1.988	0.045	0.10	0.21	0.15	0.34	0.395
Biomass yield (g biomass/100 g substrate)	59.63	58.26	43.01	177.8	130	71.4	35.71	79.41	81.01
Total lipid produced (g/dl)	0.26	0.34	0.34	0.031	0.051	0.059	0.079	0.106	0.126
Lipid yield (g lipid/100 g substrate)	23.44	22.90	16.9	68.89	51.0	28.09	52.66	31.18	31.90
Ratio (neutral: polar)	1.549	0.256	0.247	0.38	0.16	0.25	0.29	0.59	0.28
Lipid output (g lipid/ l/ h)	0.036	0.035	0.028	0.004	0.005	0.005	0.010	0.011	0.010
Specific rate of lipid production (g lipid/ g yeast/ h)	0.003	0.002	0.002	0.010	0.005	0.002	0.007	0.003	0.003

Table 3.1.3. Lipid production by CBS 2516 T cultivated in different media at various cultivation times in a batch culture.

COMPONENTS	BASAL			SAB			PASSI		
	72 h	96 h	120 h	72 h	96 h	120 h	72 h	96 h	120 h
Biomass (g/dl)	0.61	0.23	0.37	0.10	0.10	0.13	0.24	0.35	0.42
% Total lipid content (w/w)	66.5	41.0	33.25	51.15	46.07	83.08	84.58	53.07	49.99
% Neutral lipid (w/w)	24.75	19.25	19.0	16.15	17.30	20.0	23.08	16.15	11.92
% Polar lipid (w/w)	41.75	21.75	14.25	35.0	28.77	63.08	61.50	36.92	38.07
Residual substrate (g/dl)	0.862	0.151	0.022	0.128	0.0095	0.235	0.415	0.24	0.18
Substrate utilized (g/dl)	1.138	1.849	1.978	0.082	0.201	0.21	0.145	0.32	0.38
Biomass yield (g biomass/100 g substrate)	53.60	12.44	18.71	121.95	49.75	61.90	165.52	109.4	110.5
Total lipid produced (g/dl)	0.24	0.90	0.15	0.39	0.39	0.51	0.94	0.14	0.17
Lipid yield (g lipid/100 g substrate)	21.06	4.89	7.35	47.93	19.55	24.33	65.03	43.0	43.47
Ratio (neutral: polar)	0.593	0.885	1.333	0.46	0.59	0.32	0.37	0.44	0.31
Lipid output (g lipid/ l/ h)	0.033	0.094	0.013	0.054	0.041	0.043	0.131	0.015	0.014
Specific rate of lipid production (g lipid/ g yeast/ h)	0.009	0.004	0.003	0.007	0.005	0.007	0.012	0.006	0.004

3.4. LIPID PRODUCTION IN FED-BATCH CULTURES

To compare growth and lipid production by the oleaginous yeasts being investigated, CBS 5910 T and UOFS-Y-2082 were selected because from the previous results in the batch cultures (Tables 3.1.1 and 3.1.2), the highest lipid output was given by CBS 5910 T in the BASAL medium and UOFS-Y-2082 is the strain recently isolated by Mothibeli *et al.* (1996) from the soil in the Northern Province.

3.4.1. Growth

Figs. 3.6.1., 3.7.1, 3.6.2 and 3.7.2 show the growth curves of CBS 5910 T and UOFS-Y-2082 cultures in batch and fed-batch culture respectively. Maximum growth was reached between 120 and 144 h by CBS 5910 T cultures in both control and fed-batch cultures. However, with UOFS-Y-2082 control cultures, only the 1 % glucose culture reached its maximum growth at 144 h but at higher concentrations growth still continued. In the fed-batch cultures the growth continued until 168 h in all concentrations. It was also observed that when the cultures were fed at 72 h and 120 h, the UOFS-Y-2082 continued to grow but the CBS 5910 T did not show any substantial increase in growth over the controls. Probably, the UOFS-Y-2082 is more glucose tolerant than CBS 5910 T.

3.4.2. Lipid production

When the UOFS-Y-2082 cultures were fed after 72 h, there were increases in the total lipid content in 1% and 2 % glucose media but in 4 % cultures an increase was only observed at 144 h after a second feeding at 120 h (Table 3.2). For example, in the 2 % glucose media the lipid content increased from 39.70 % (w/w) to 47.75 % (w/w) at 120 h, but in the 4 % media the lipid content decreased from 51.5 % at 72 h to 50.51 at 120 h after which it increased to 52.6 % at 144 h. These observations showed that there were no substantial increases or decreases in lipid content in 4 % glucose. A similar

observation was made in 1 %.

On the contrary, after feeding the CBS 5910 T cultures at 72 h the lipid content decreased at all concentrations after 120 h (Table 3.2). This seems to confirm the previous suggestion that CBS 5910 T is less glucose tolerant. It was also generally observed that when the cultures were fed with fresh medium at the various concentrations, there were reductions in the lipid content after the first 24 h before any increases were noticed. Probably the yeasts needed a period of readjustment to the media conditions.

Interestingly, the CBS 5910 T seemed to perform better in the fed-batch than the batch cultures at the various concentrations, but more lipid was produced by UOFS-Y-2082 in batch than fed-batch cultures, particularly in 1 % and 2 % glucose. For example, the lipid content of UOFS-Y-2082 in 1 % and 2 % batch cultures were 56.76 and 58.5 % at 120 h, respectively. Whereas in fed-batch cultures the values were 42.5 and 47.75 % in 1 % and 2 % media, respectively (Table 3.2), in 4 % cultures, the lipid content fluctuated both in the batch and fed-batch cultures after feeding and refeeding.

It was also important to compare the lipid composition in both batch and fed-batch cultures. Table 3.2 shows that more neutral lipids were produced by UOFS-Y-2082 in batch than fed-batch cultures in 1 % and 2 % glucose. In 4 % medium, however, more neutral lipids were produced in fed-batch than batch cultures up to 120 h. In the CBS 5910 T cultures more neutral lipids were produced in the fed-batch than batch cultures. These results again show that the performance of the yeast strains and the lipid composition depend on the culture conditions such as the concentration of the carbon substrate.

Since one of the objectives of this study was to produce lipids which are similar in composition to palm oil (palm oil equivalent) and palm oil contains predominantly neutral lipids, it was necessary to evaluate the ratios of neutral to polar lipids produced by the

different strains under different conditions. Generally, for CBS 5910 T the ratios were higher in fed-batch than batch cultures in 1 % and 2 % media when cultivated up to 120 - 144 h. With UOFS-Y-2082 the ratios were higher in batch than fed-batch cultures, although the ratios fluctuated considerably in both culture systems.

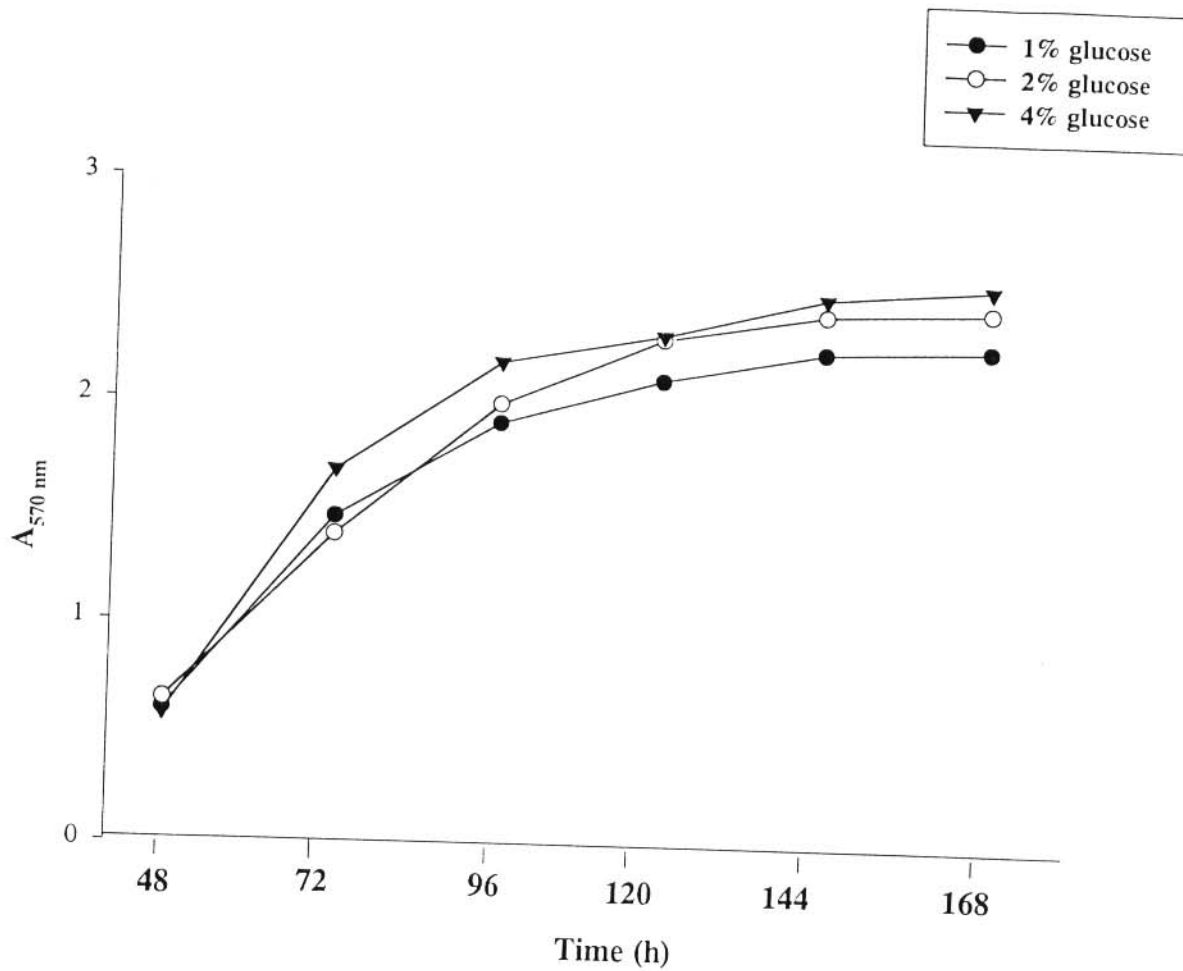


Fig. 3.6.1. Growth curves of CBS 5910 T grown in BASAL medium containing different concentrations of glucose at various cultivation times in a batch culture (control for fed-batch). The culture conditions are as described in the Materials and Methods.

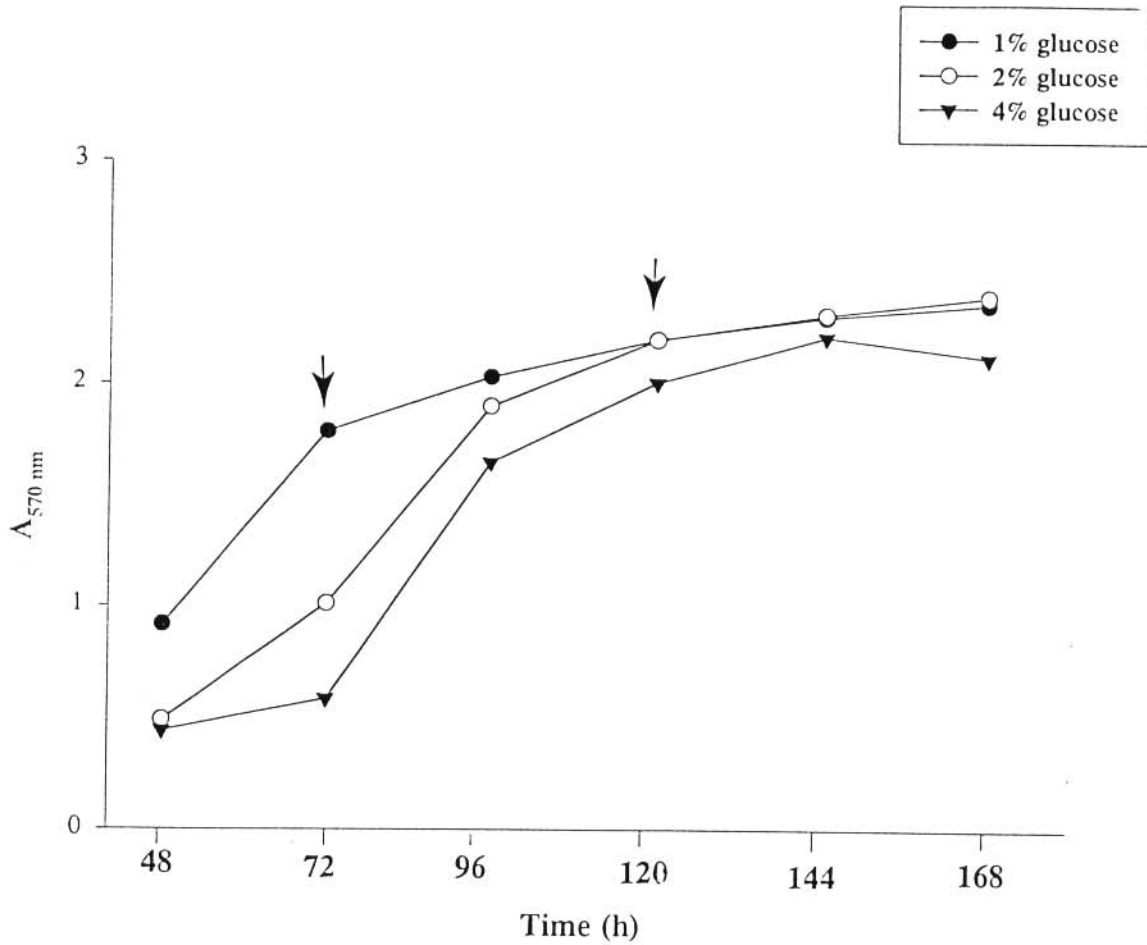


Fig. 3.6.2. Growth curves of CBS 5910 T grown in BASAL medium containing different concentrations of glucose at various cultivation times in a fed-batch culture. The culture conditions are as described in the Materials and Methods. The arrows indicate feeding points.

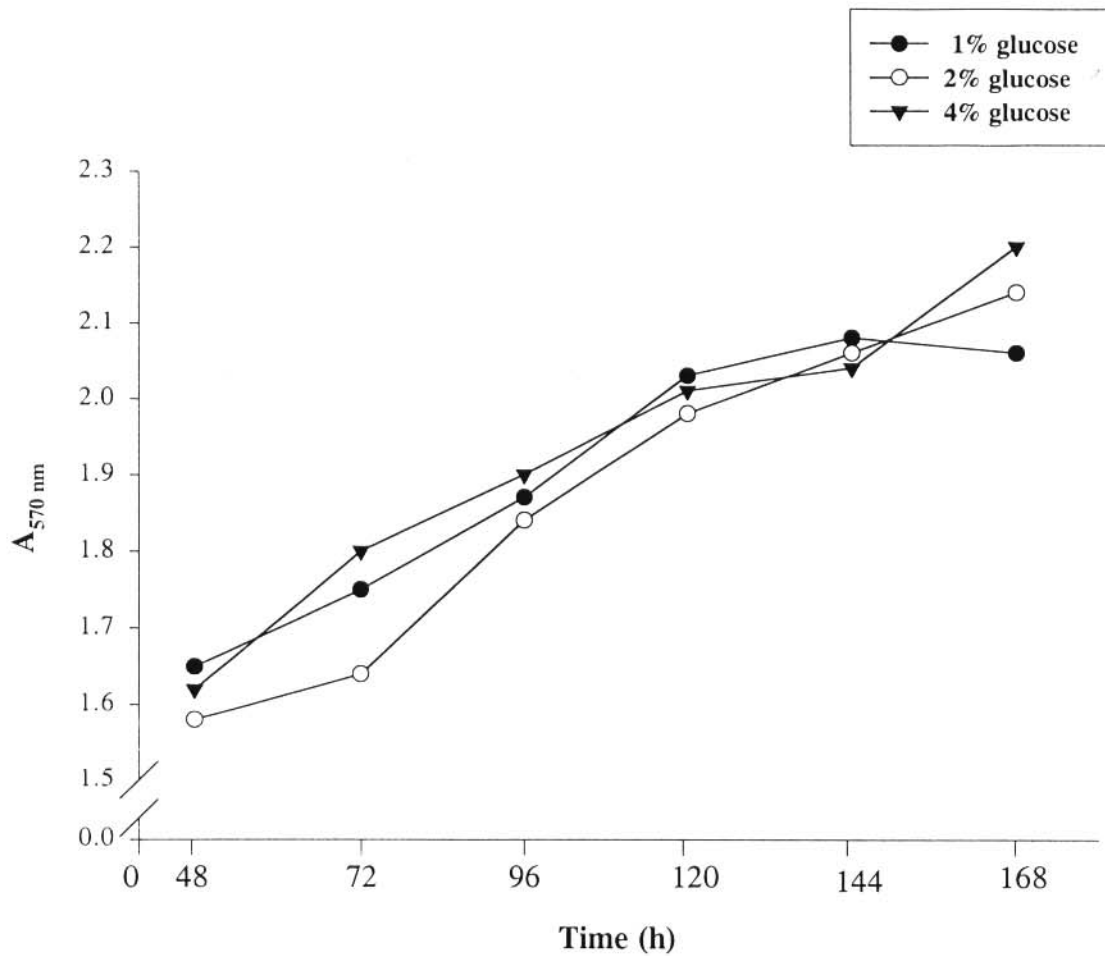


Fig. 3.7.1. Growth curves of UOFS-Y-2082 grown in BASAL medium containing different concentrations of glucose at various cultivation times in a batch culture (control for fed-batch). The culture conditions are as described in the Materials and Methods.

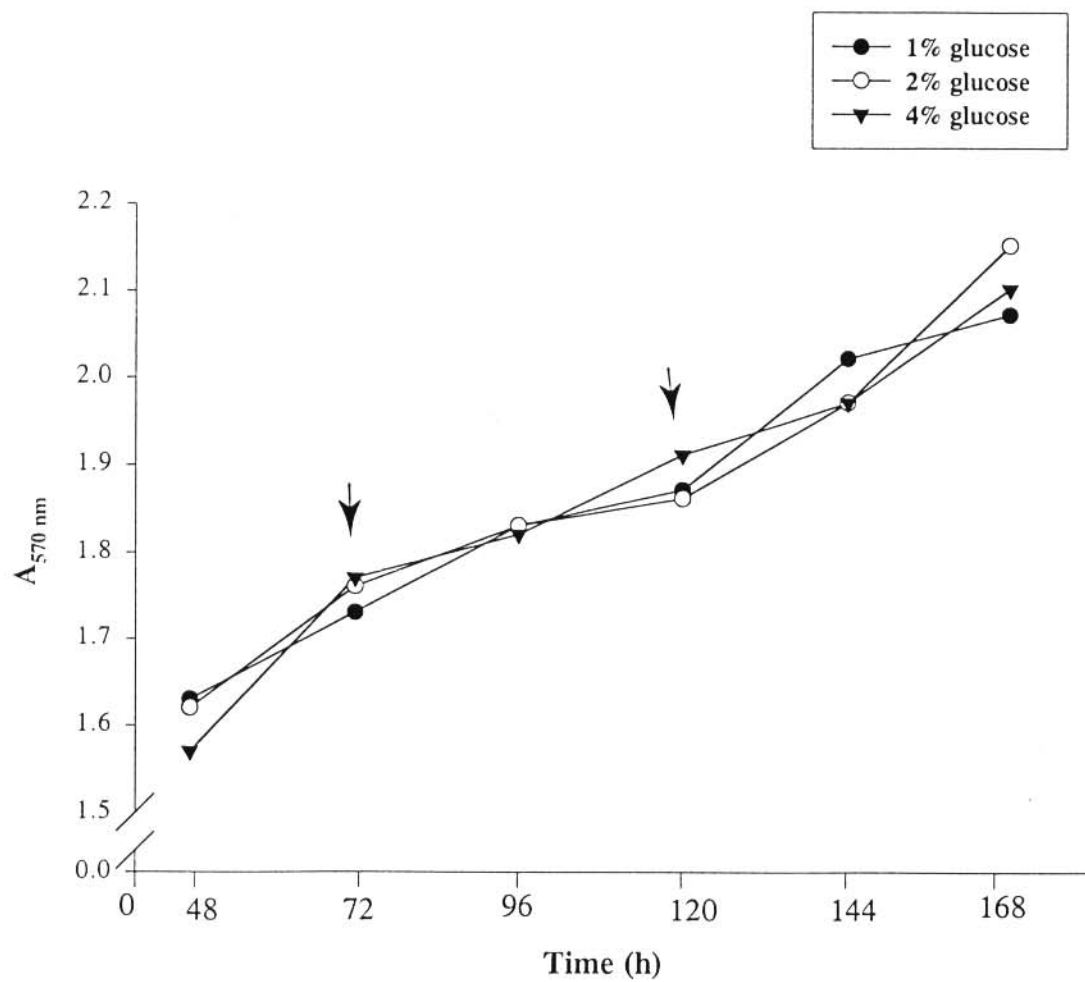


Fig. 3.7.2. Growth curves of UOFS-Y-2082 grown in BASAL medium containing different concentrations of glucose at various cultivation times in a fed-batch culture. The culture conditions are as described in the Materials and Methods. The arrows indicate feeding points.

TABLE: 3.2 Lipid content of oleaginous yeasts cultivated in BASAL medium of 1, 2 and 4% glucose in batch and fed-batch culture

Glucose conc.	Cultivation time (h)	Control						Fed-batch					
		UOFS-Y-2082			CBS 5910 T			UOFS-Y-2082			CBS 5910 T		
		N	P	T	N	P	T	N	P	T	N	P	T
1 %	72	20.60	25.00	45.60	44.86	28.86	73.72	19.33	22.36	41.69	43.78	27.05	70.83
	96	20.83	18.33	39.16	16.07	26.88	42.95	18.92	21.15	40.07	45.23	22.79	68.02
	120	18.92	37.84	56.76	14.55	16.27	30.82	15.56	26.94	42.50	25.00	15.38	43.52
	144	21.62	16.22	37.84	12.09	15.75	27.84	17.92	24.22	42.14	16.13	25.81	41.94
	168	11.11	22.22	33.33	8.77	12.56	21.33	8.90	25.86	34.76	5.48	20.55	26.03
2 %	72	19.50	24.75	44.25	43.75	28.25	71.75	17.50	22.20	39.70	42.50	26.30	68.80
	96	30.25	10.00	40.25	7.75	30.25	38.00	17.17	20.50	37.67	15.60	34.38	49.38
	120	22.25	36.25	58.50	5.50	22.25	27.75	21.05	26.70	47.75	12.52	25.93	38.45
	144	11.32	62.26	73.58	7.00	25.00	32.00	12.20	30.72	42.92	8.67	27.92	36.59
4 %	168	30.30	18.18	48.48	9.30	25.30	34.60	4.00	35.50	39.50	5.00	24.90	29.90
	72	16.67	30.00	46.67	28.98	39.10	68.08	17.50	34.20	51.70	25.00	37.50	62.50
	96	10.00	23.33	33.33	15.54	26.41	41.95	15.00	35.45	50.45	22.45	31.95	54.40
	120	14.71	28.60	43.37	12.24	14.17	26.41	32.17	27.34	50.51	16.08	22.64	38.72
	144	23.26	30.30	53.56	11.81	16.54	28.35	22.34	30.26	52.60	14.03	25.35	39.38
	168	12.92	21.31	34.23	14.89	19.15	34.04	9.17	41.62	50.79	11.43	27.00	38.43

NOTE: N, % neutral lipids (w/w); P, % polar lipids (w/w) and T, % total lipid content (w/w)

3.5. CHANGES IN GROWTH, RESIDUAL CARBON SOURCE AND LIPID ACCUMULATION.

The residual glucose and growth patterns of the three oleaginous yeasts in BASAL media (batch culture) were followed spectrophotometrically by reading the absorbance at 505 and 570 nm, respectively, at various cultivation times (48, 72, 96 and 120 h). The total lipid produced was determined and expressed as the sum of the percentages of the neutral and polar lipids as described in section 2.6. of the Materials and Methods. In CBS 5910 T and CBS 2516 T cultures, the production of total lipids peaked at 72 h when there was still enough residual carbohydrate in the media (Fig. 3.8.1. and 3.8.2.), but in the UOFS-Y-2082, the maximum production of lipids occurred at 120 h when the residual glucose was almost depleted (Fig. 3.8.3.).

In the fed-batch cultures, lipid production improved only when the UOFS-Y-2082 was fed with 2 % glucose at 72 h and cultured up to 120 h, but the CBS 5910 T cultures rather showed a decrease. When the lipid contents were compared at 120 h with the controls, the lipid production improved in the CBS 5910 T cultures but decreased in the UOFS-Y-2082 cultures. There were also decreases in the lipid production when the UOFS-Y-2082 cultures were fed with 1 % and 4 % glucose. The reason for these observations are not clear. Probably, the various strains have different abilities to tolerate glucose under different culture conditions (Figs 3.11.1a-3.11.3b and 3.12.1a-3.12.3b).

A similar trend was observed when the yeasts were grown in the industrial waste media. In PASSI medium CBS 5910 T and CBS 2516 T reached their maximum lipid production levels at 72 h, but the production by UOFS-Y-2082 peaked only after 96 h. However, in the SAB medium, the three yeasts produced the maximum lipids at 120 h. Interestingly, CBS 5910 T showed an earlier peak at 72 h. Also all the yeasts seemed to reach their maximum production levels when the residual carbon source was almost depleted in SAB, but in the PASSI medium, high lipid production was observed when there was still

enough residual carbon source (Figs 3.9.1a-3.9.3b and 3.10.1a-3.10.3b).

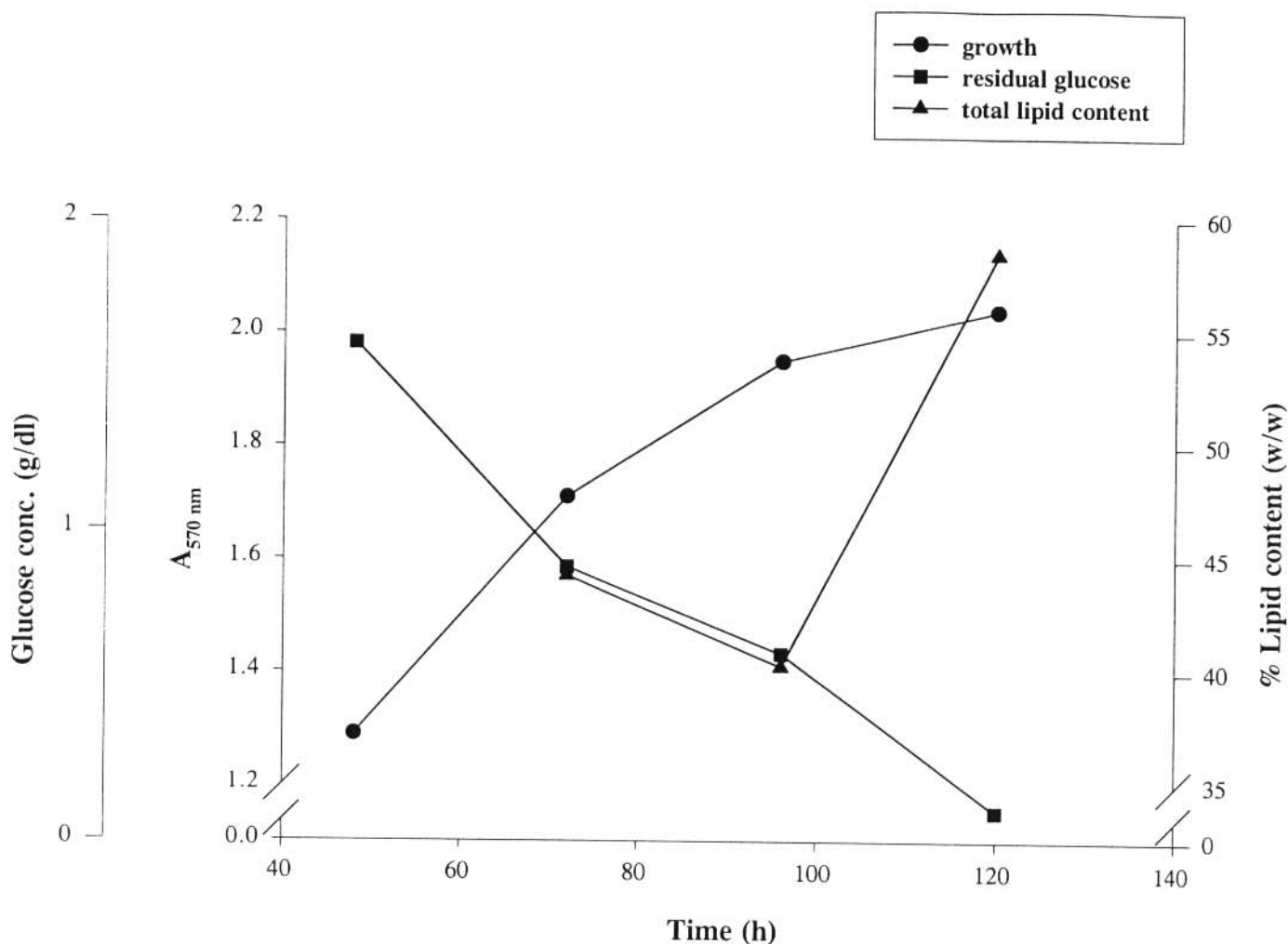


Fig. 3.8.1. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a BASAL medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

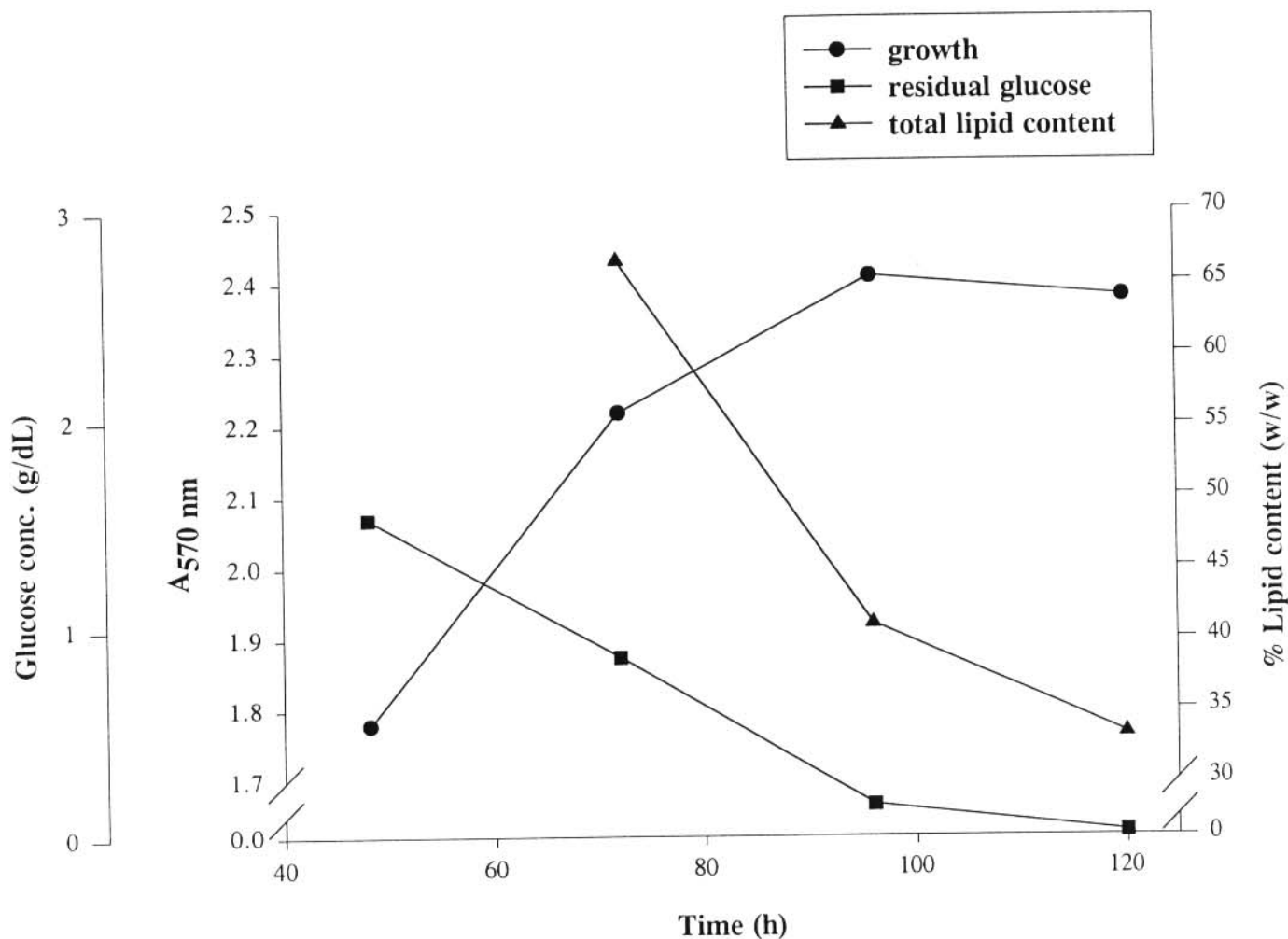


Fig. 3.8.2. Changes in growth, residual glucose and total lipid production in CBS 2516 T cultivated in a BASAL medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

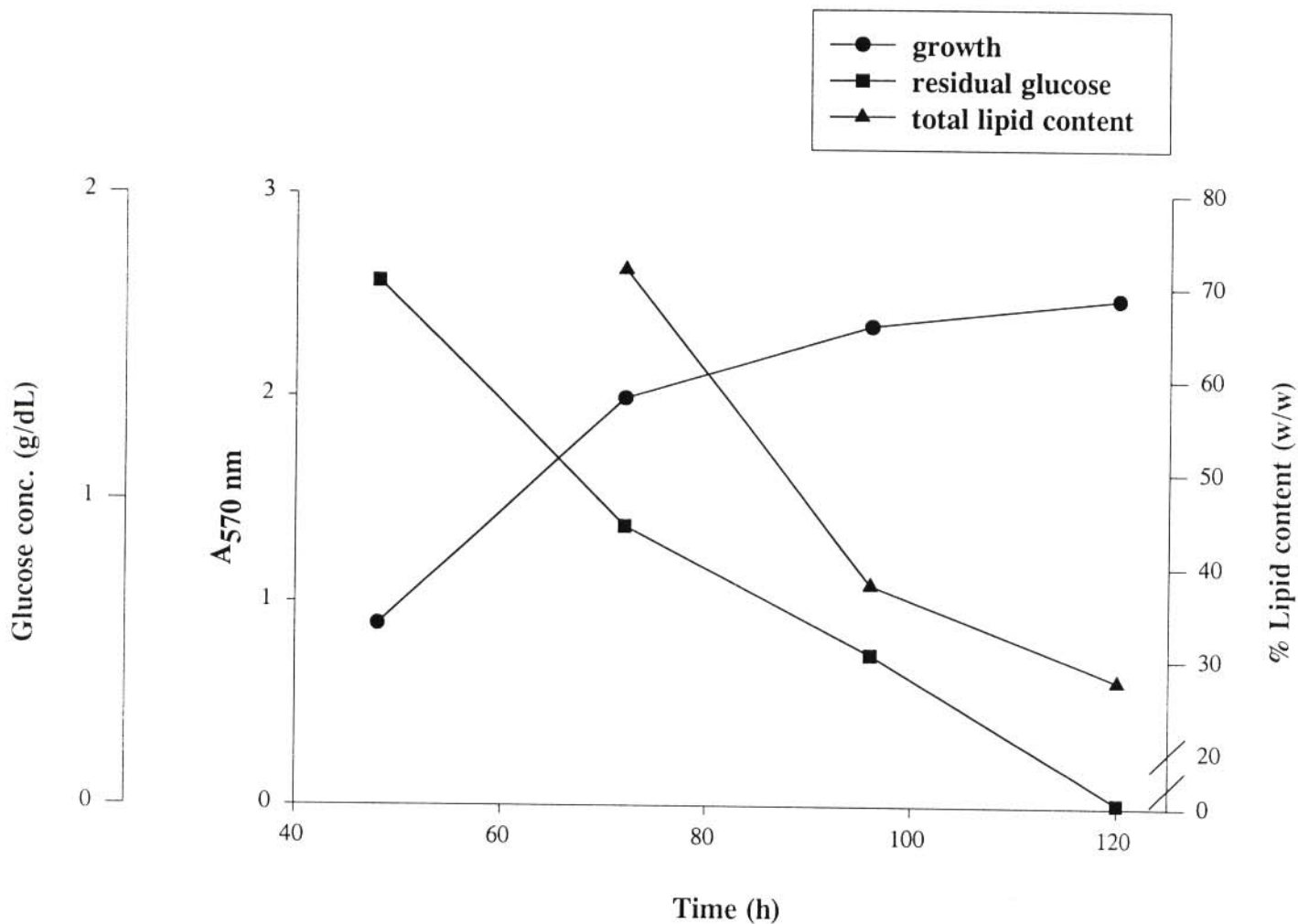


Fig. 3.8.3. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a BASAL medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

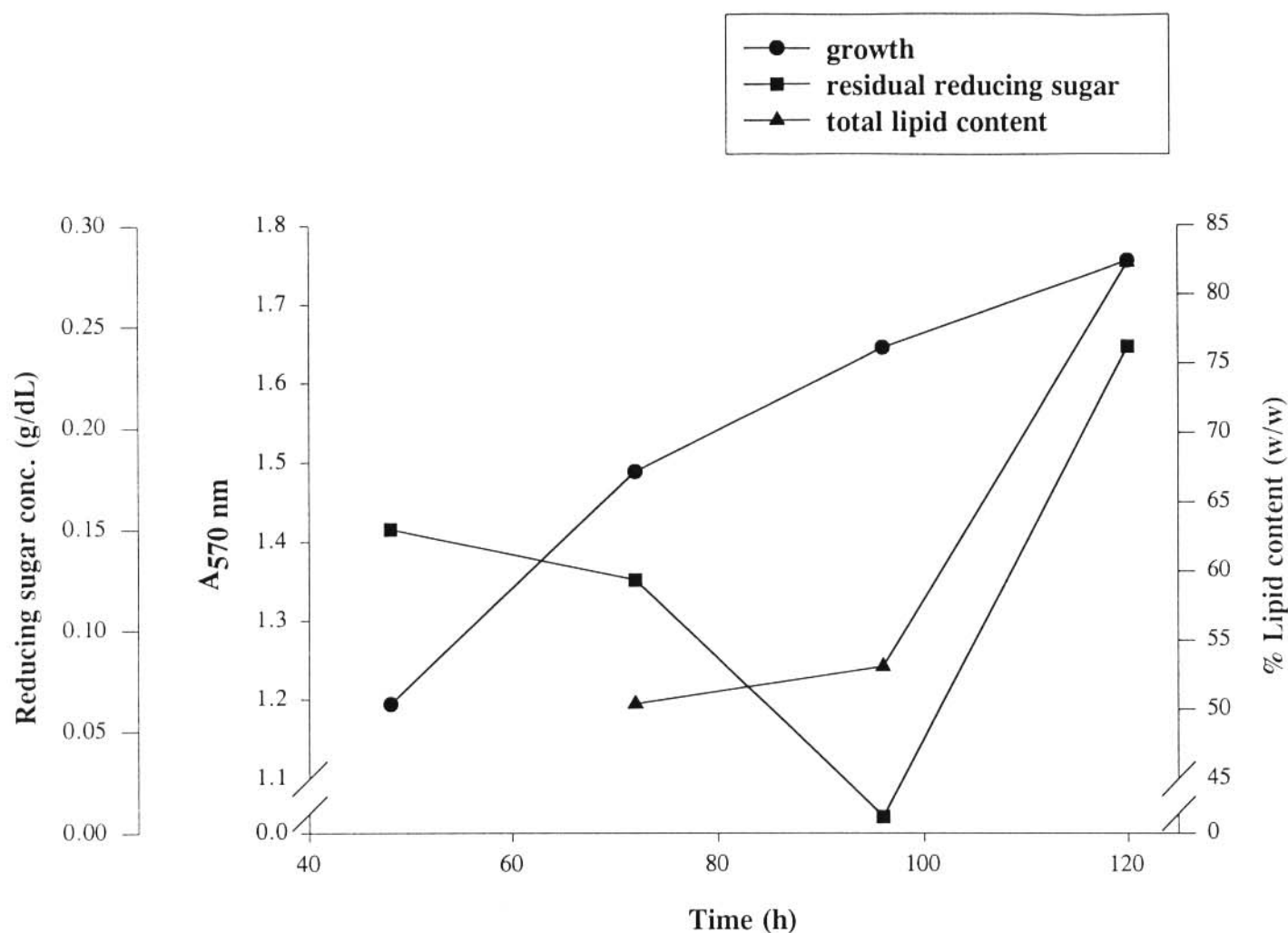


Fig. 3.9.1. Changes in growth, reducing sugars and total lipid production in UOFS-Y-2082 cultivated in the SAB medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

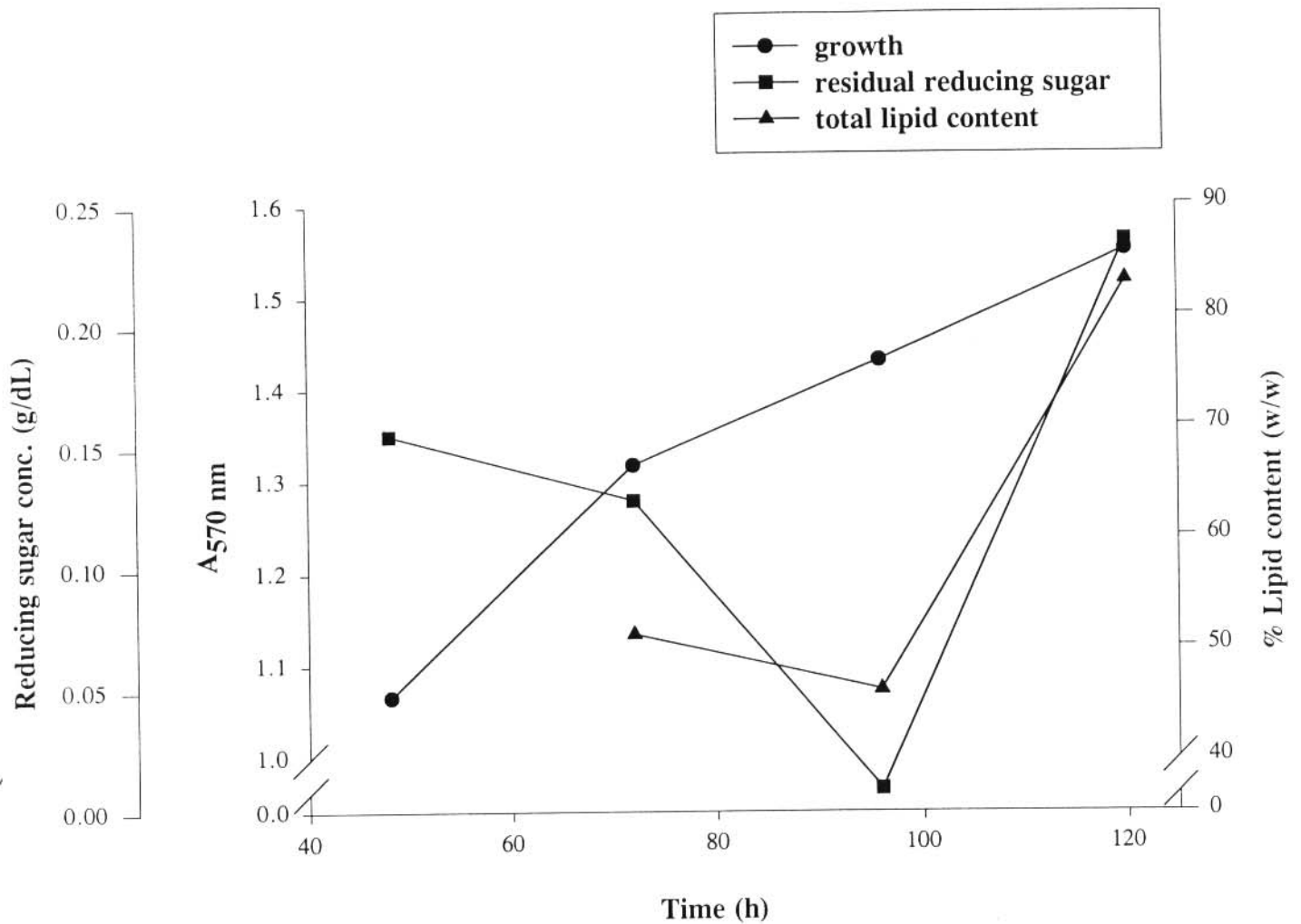


Fig. 3.9.2. Changes in growth, reducing sugars and total lipid production in CBS 2516 T cultivated in the SAB medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

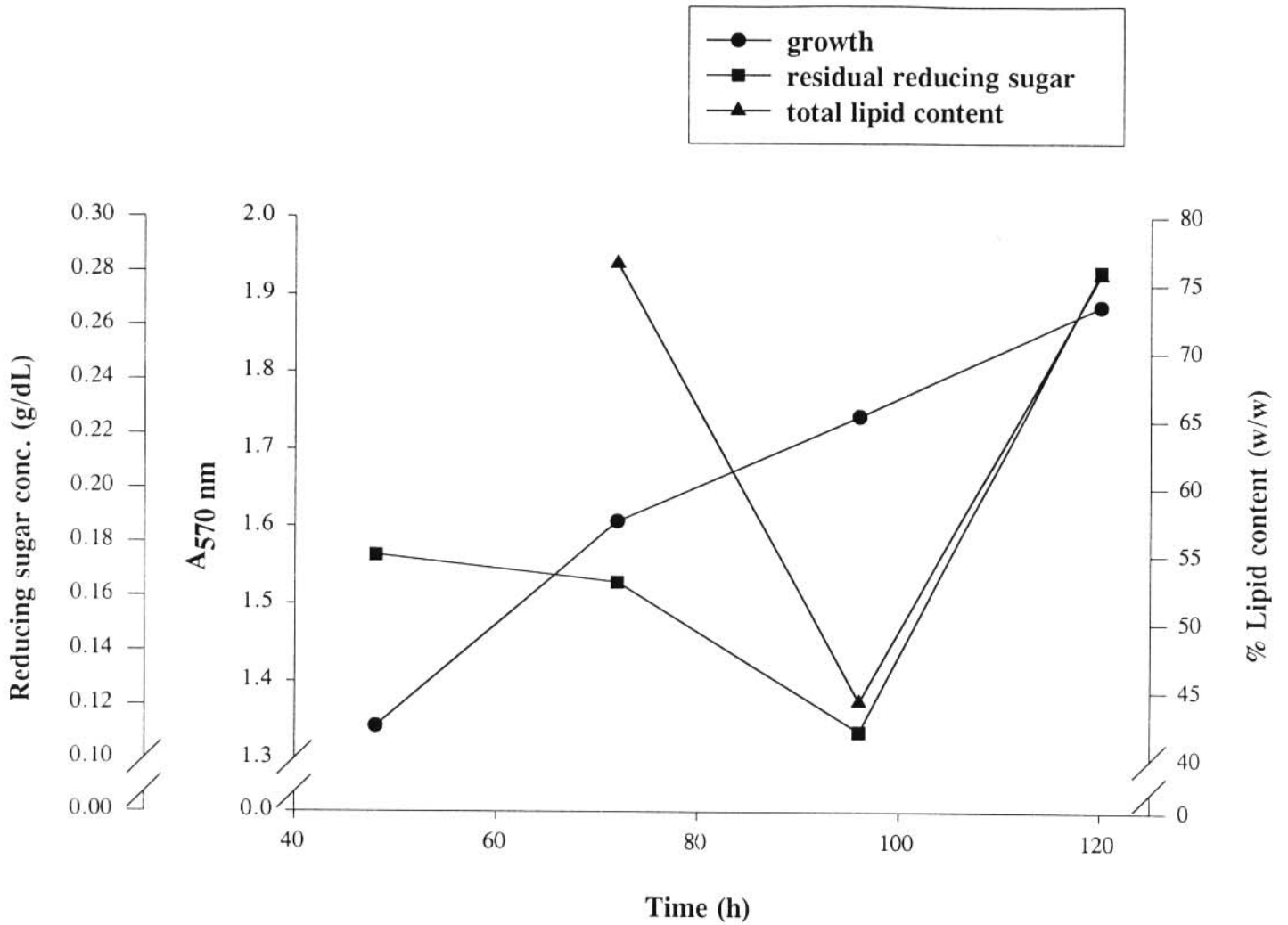


Fig. 3.9.3. Changes in growth, reducing sugars and total lipid production in CBS 5910 T cultivated in the SAB medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

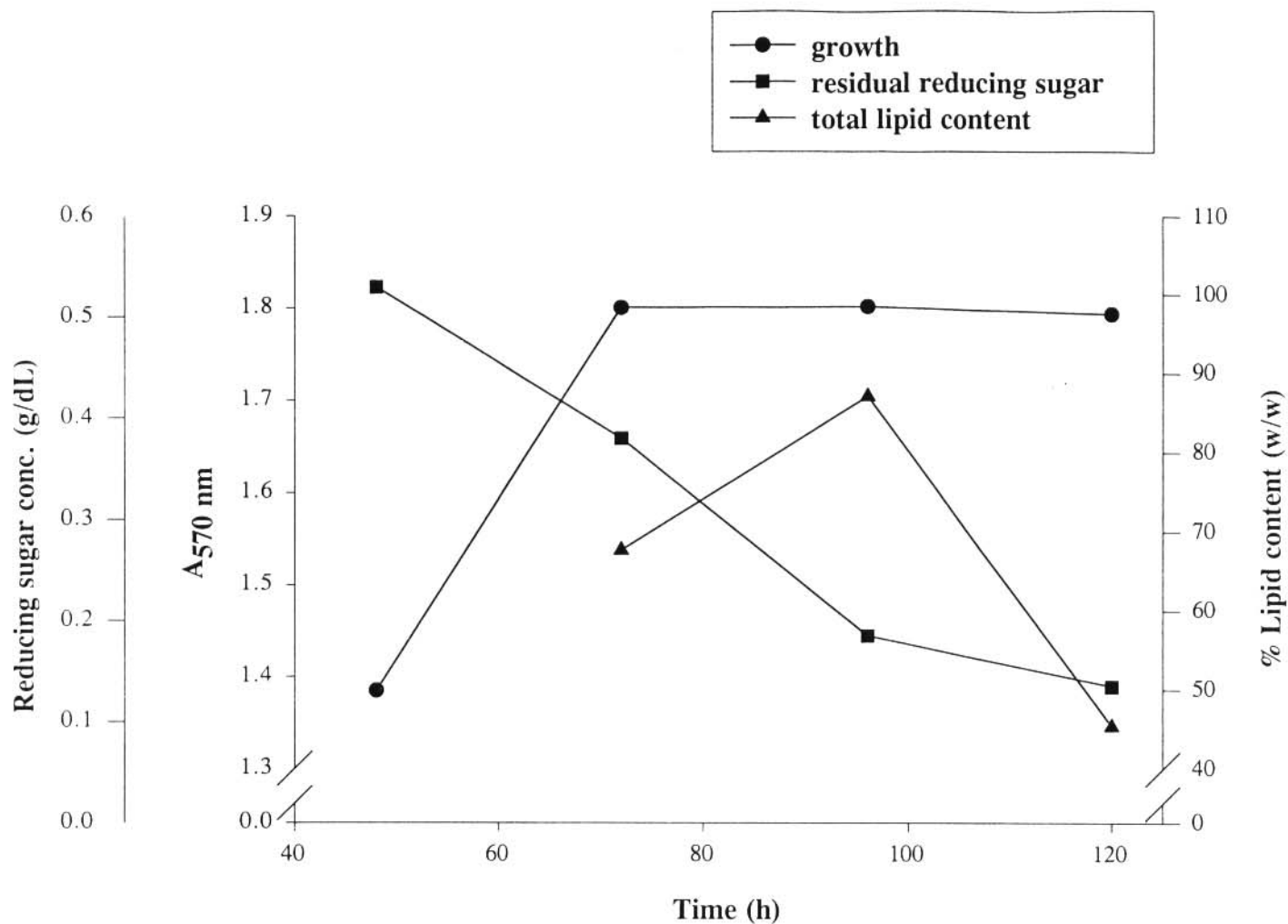


Fig. 3.10.1. Changes in growth, reducing sugars and total lipid production in UOFS-Y-2082 cultivated in the PASSI medium in a batch culture at various cultivation times (48, 72, 96 and 120 h). Growth and reducing sugars were determined by reading the absorbance at 570 and 540 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

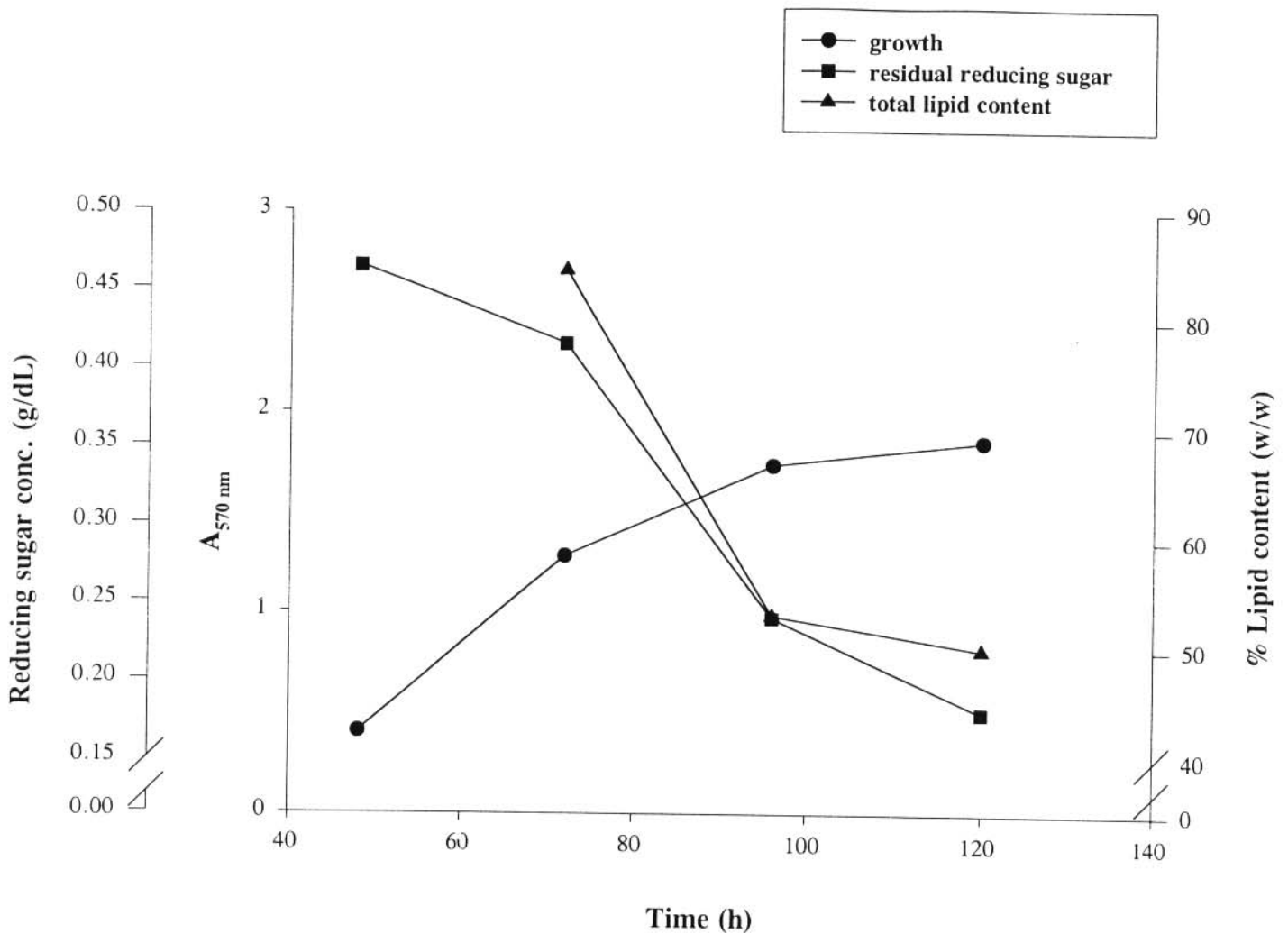


Fig. 3.10.2. Changes in growth, residual and total lipid production in CBS 2516 T cultivated in the PASSI medium in a batch culture at various cultivation times (48, 72, 96 and 120 h) Growth and reducing sugars were determined by reading the absorbance at 570 and 540 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

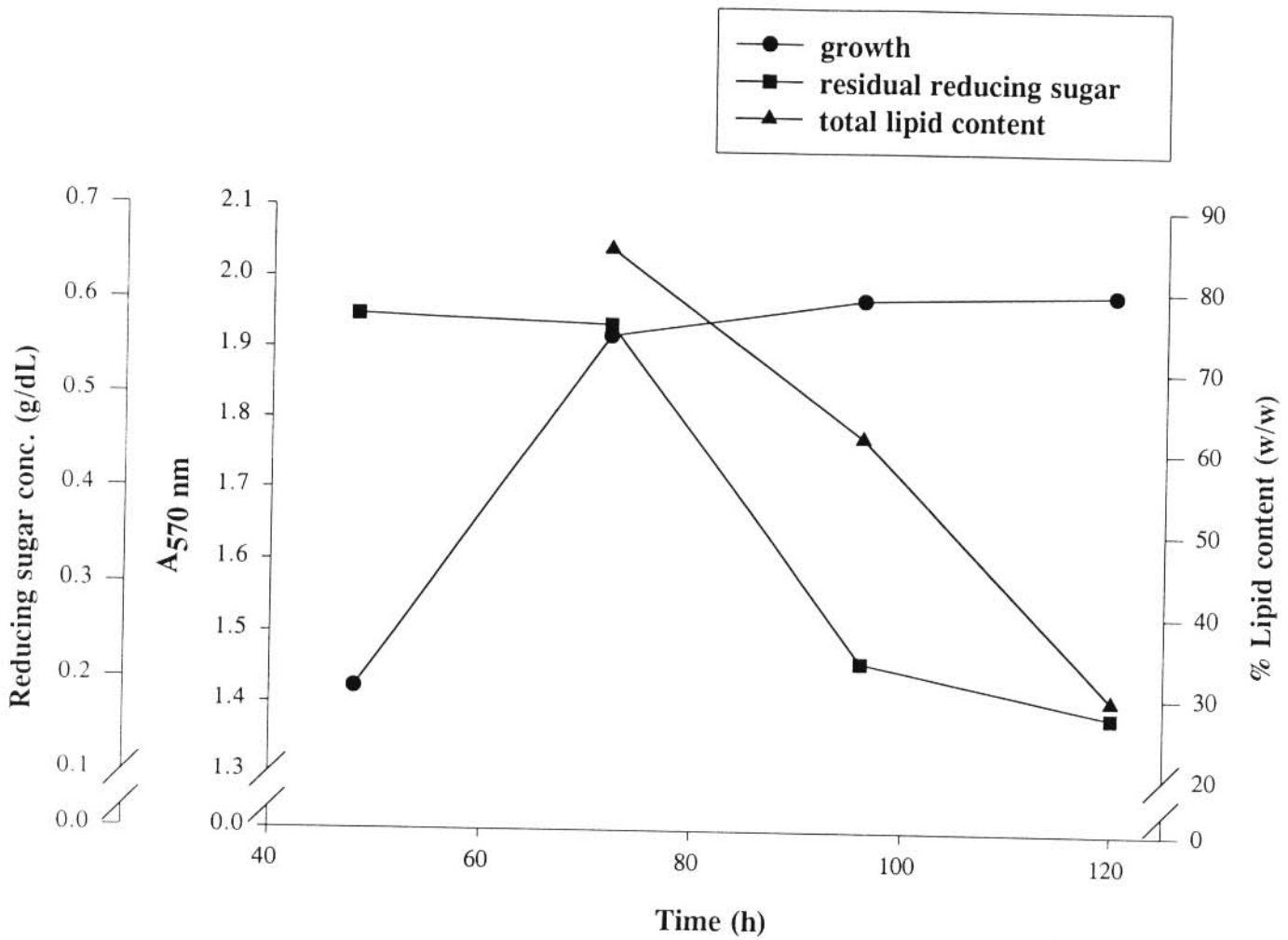


Fig. 3.10.3. Changes in growth, residual and total lipid production in CBS 5910 T cultivated in the PASSI medium in a batch culture at various cultivation times (48, 72, 96 and 120 h) Growth and reducing sugars were determined by reading the absorbance at 570 and 540 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

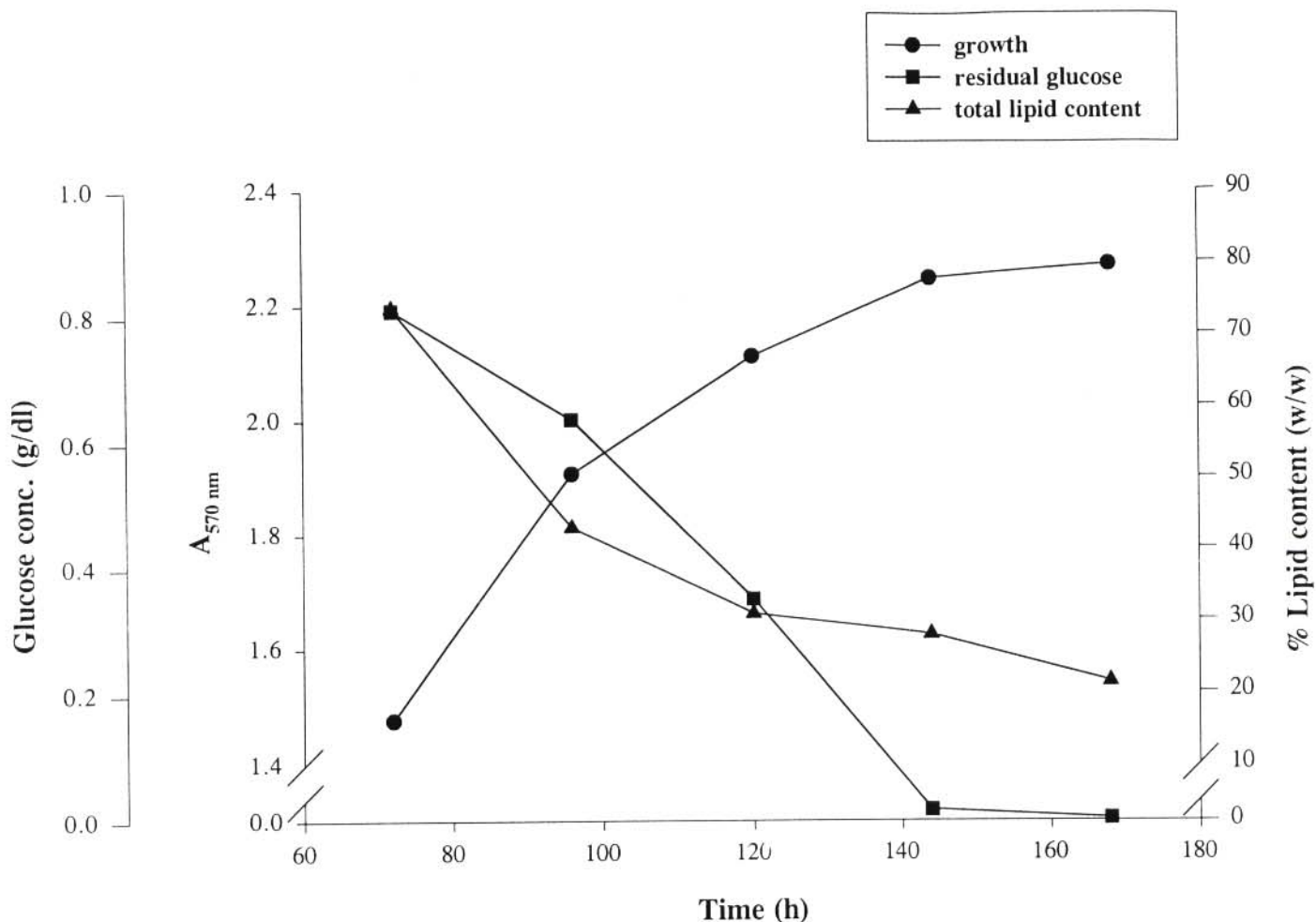


Fig. 3.11.1a. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a 1 % BASAL medium in a batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

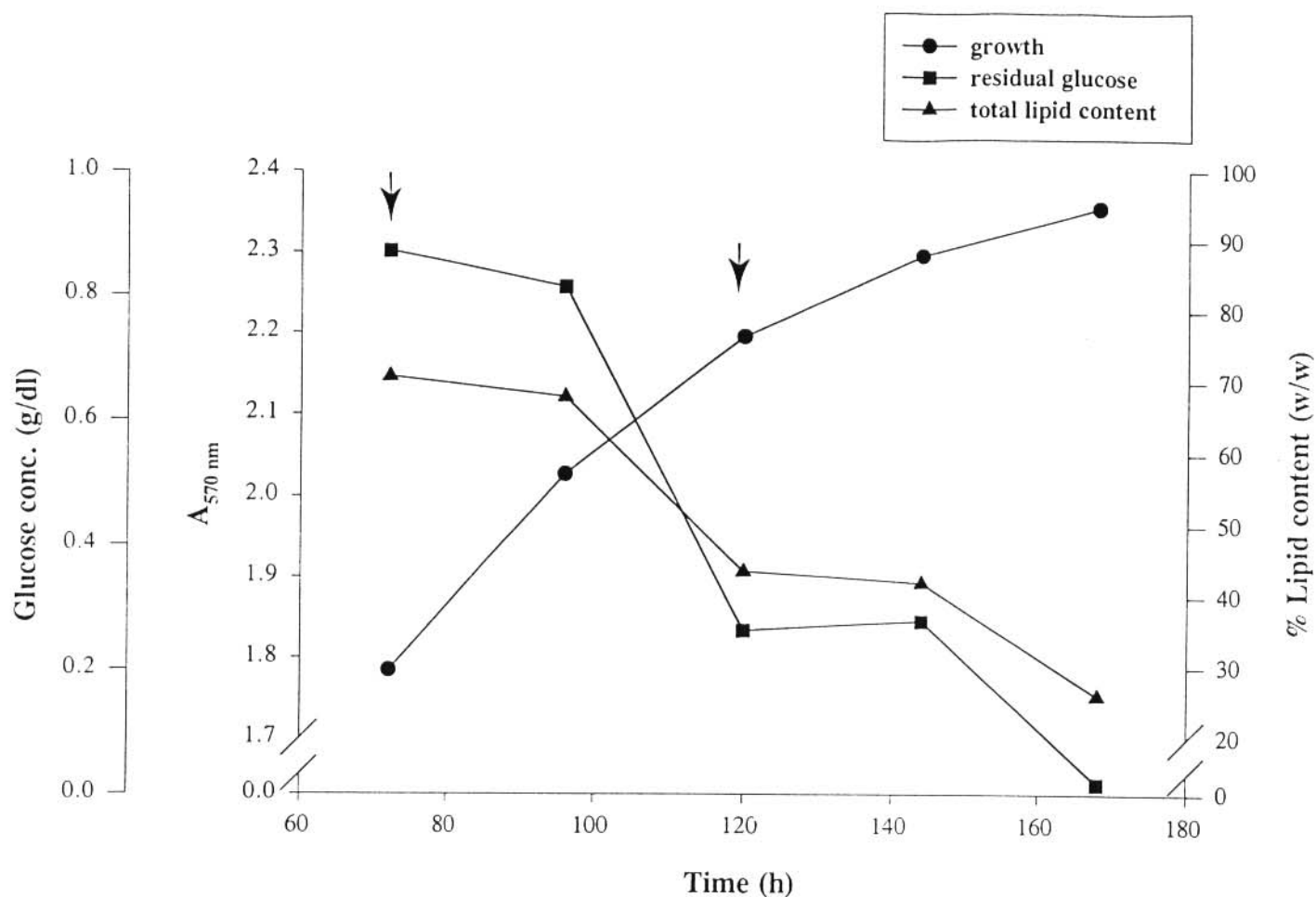


Fig. 3.11.1b. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a 1 % BASAL medium in a fed-batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods. The arrows indicate feeding points.

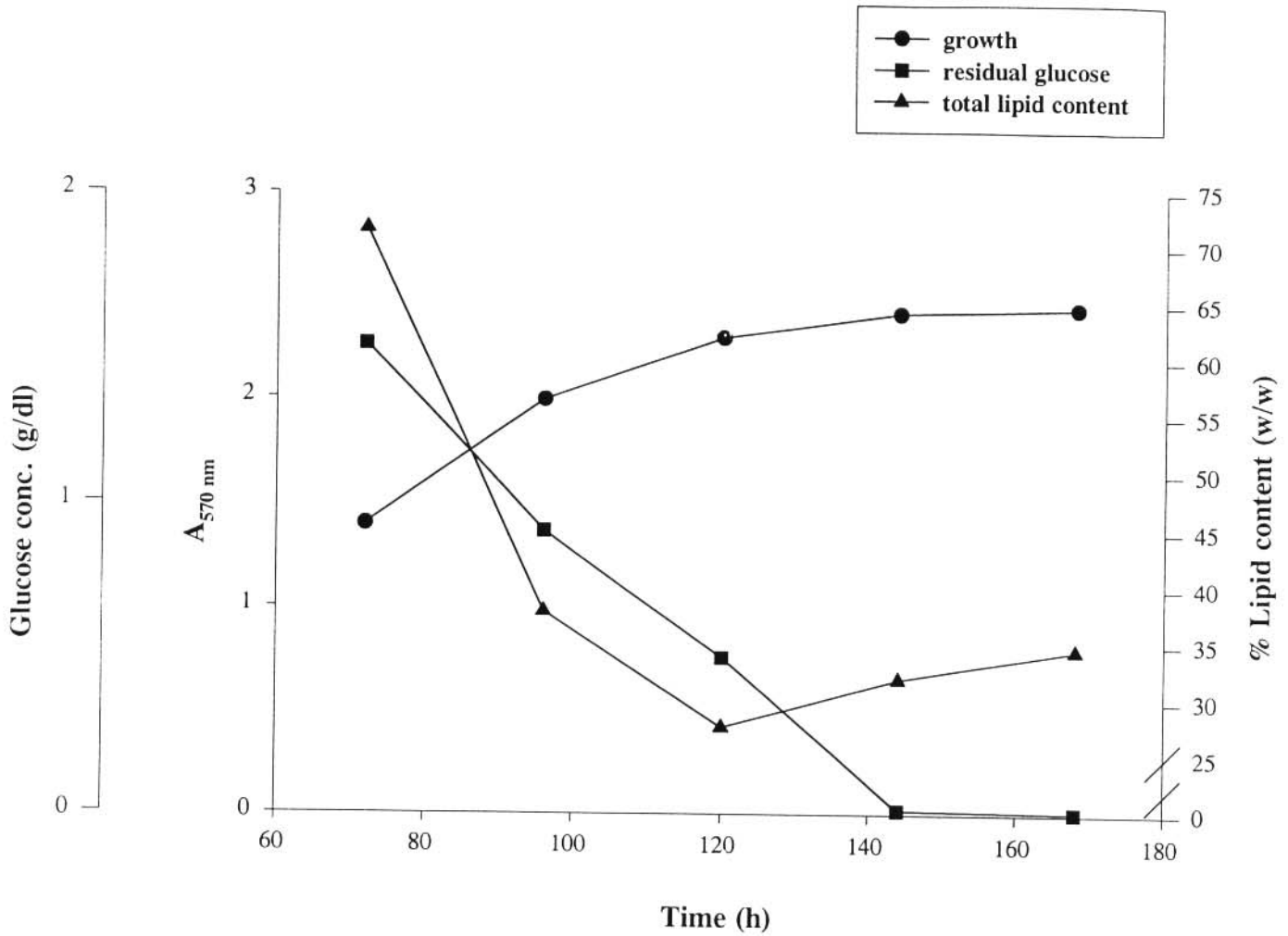


Fig. 3.11.2a. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a 2 % BASAL medium in a batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

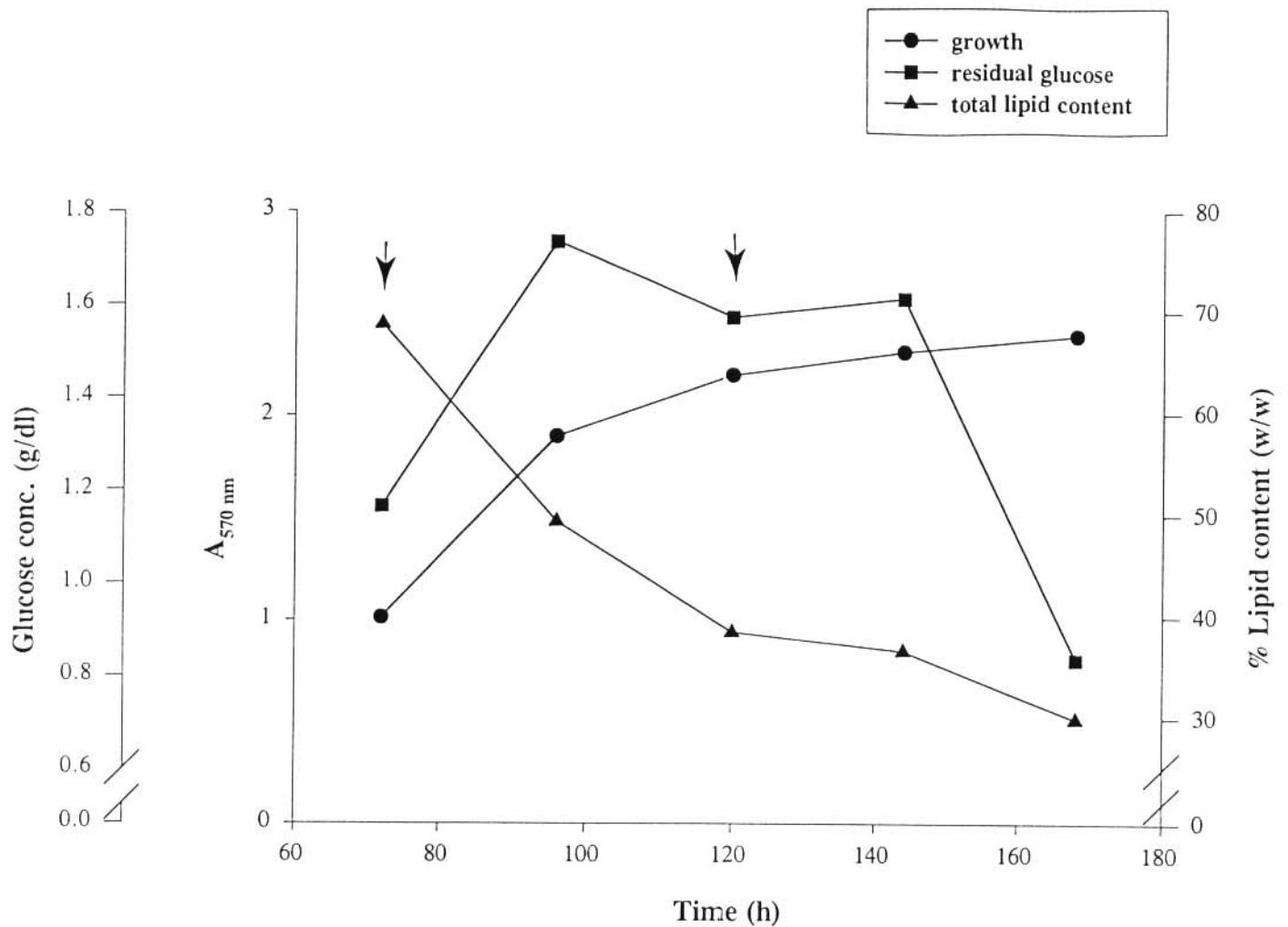


Fig. 3.11.2b. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a 2 % BASAL medium in a fed-batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods. The arrows indicate feeding points.

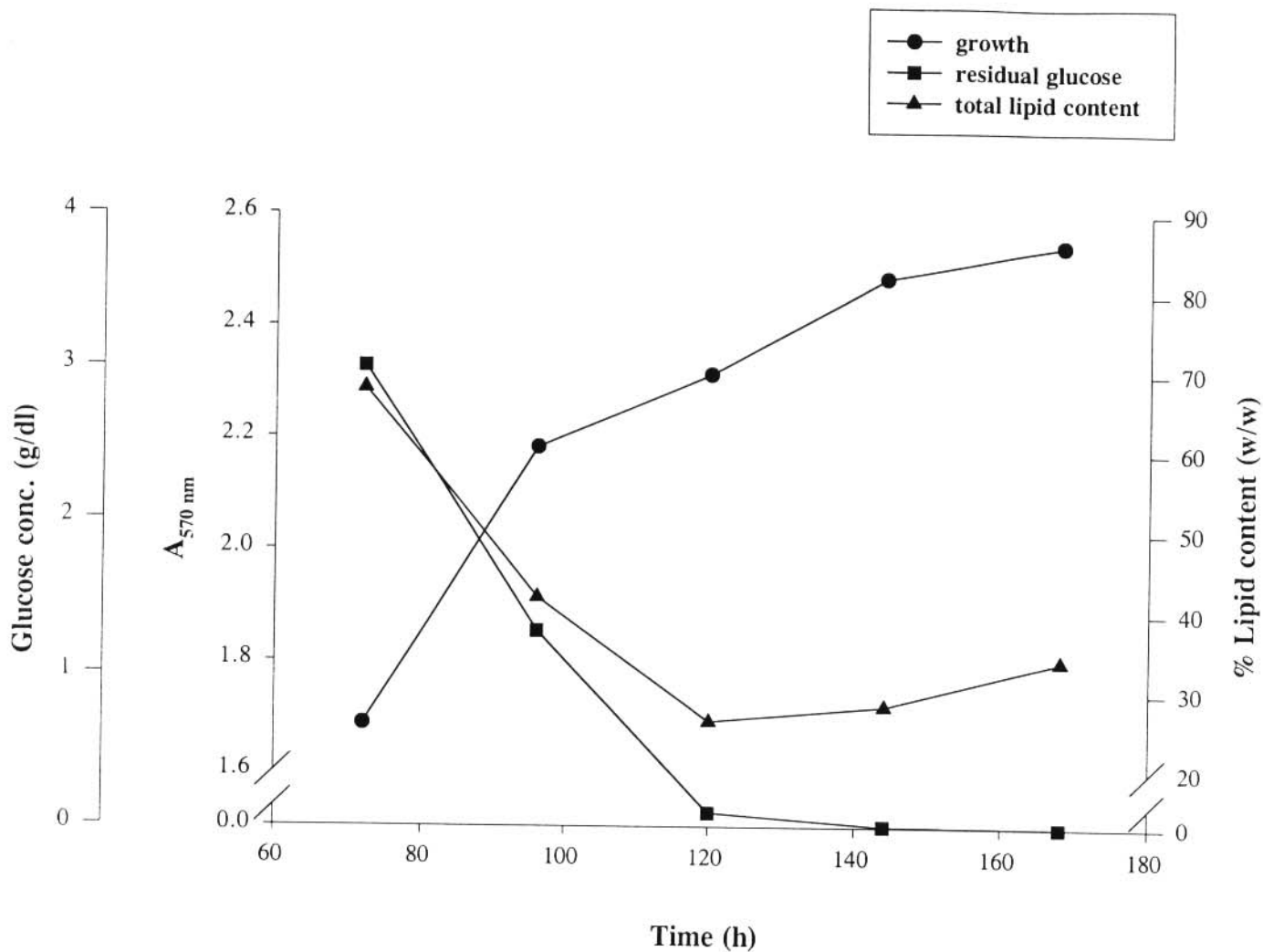


Fig. 3.11.3a. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a 4 % BASAL medium in a batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

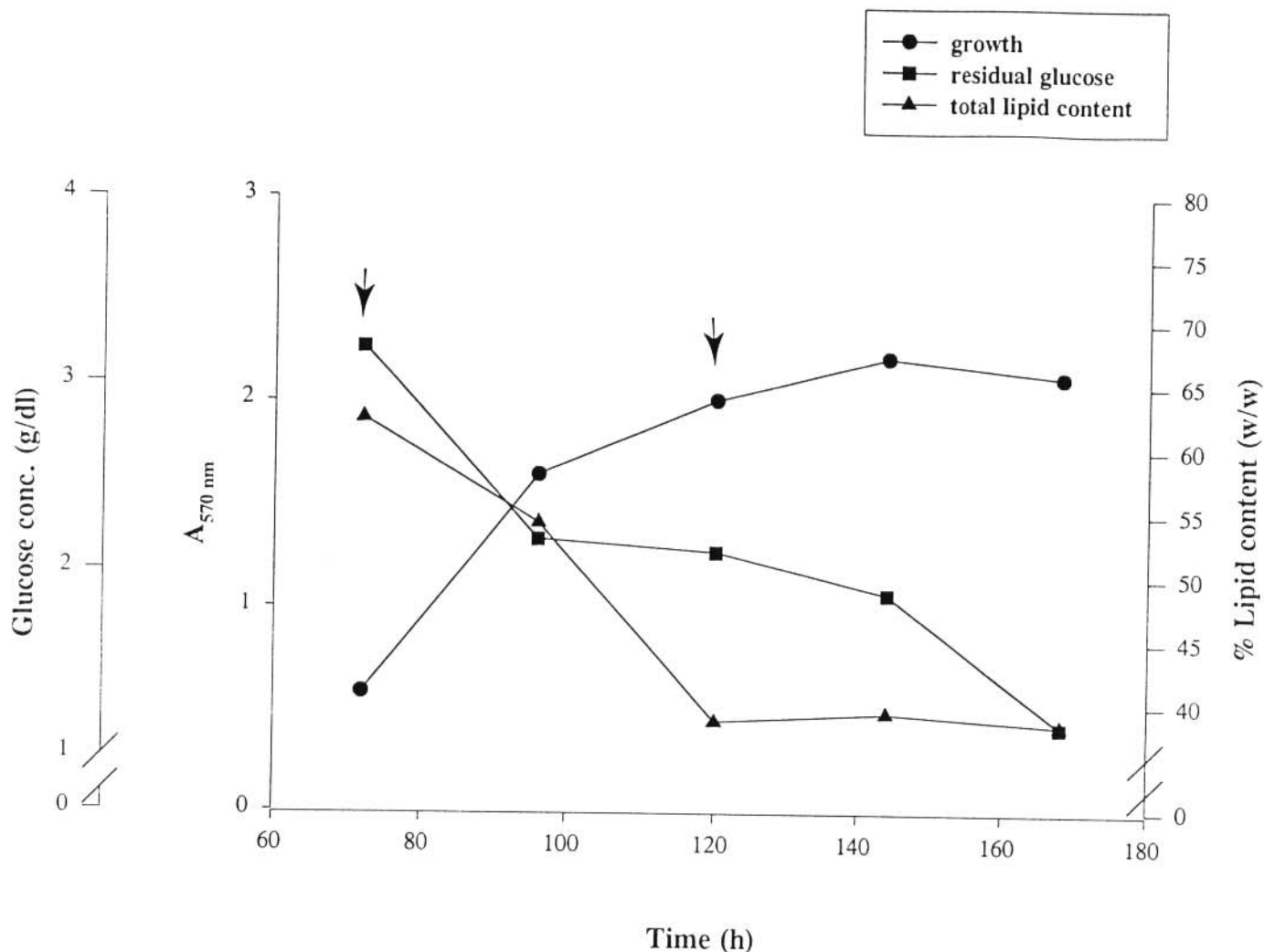


Fig. 3.11.3b. Changes in growth, residual glucose and total lipid production in CBS 5910 T cultivated in a 4 % BASAL medium in a fed-batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods. The arrows indicate feeding points.

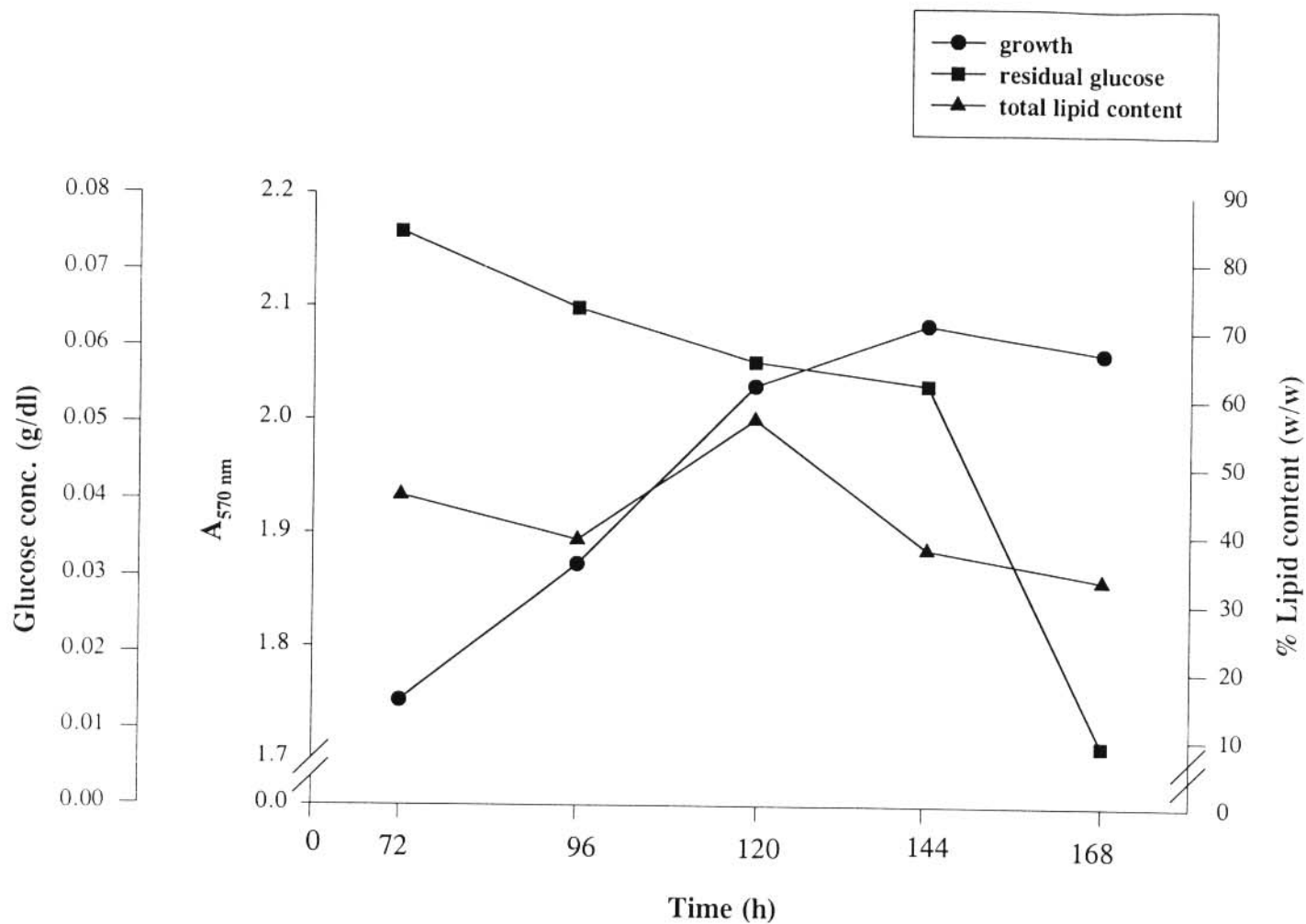


Fig. 3.12.1a. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a 1 % glucose BASAL medium in a batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

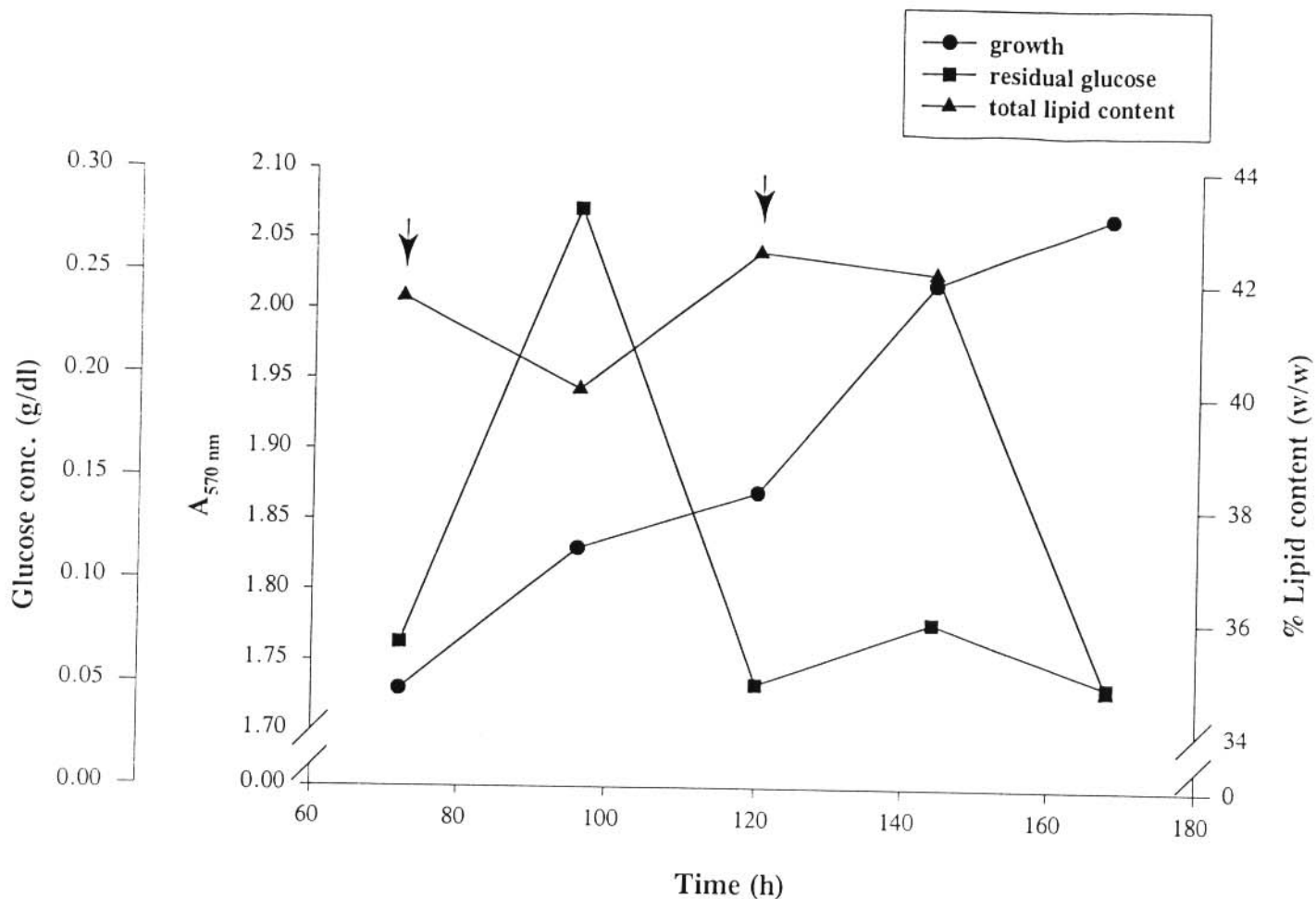


Fig. 3.12.1b. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a 1 % glucose BASAL medium in a fed-batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods. The arrows indicate feeding points.

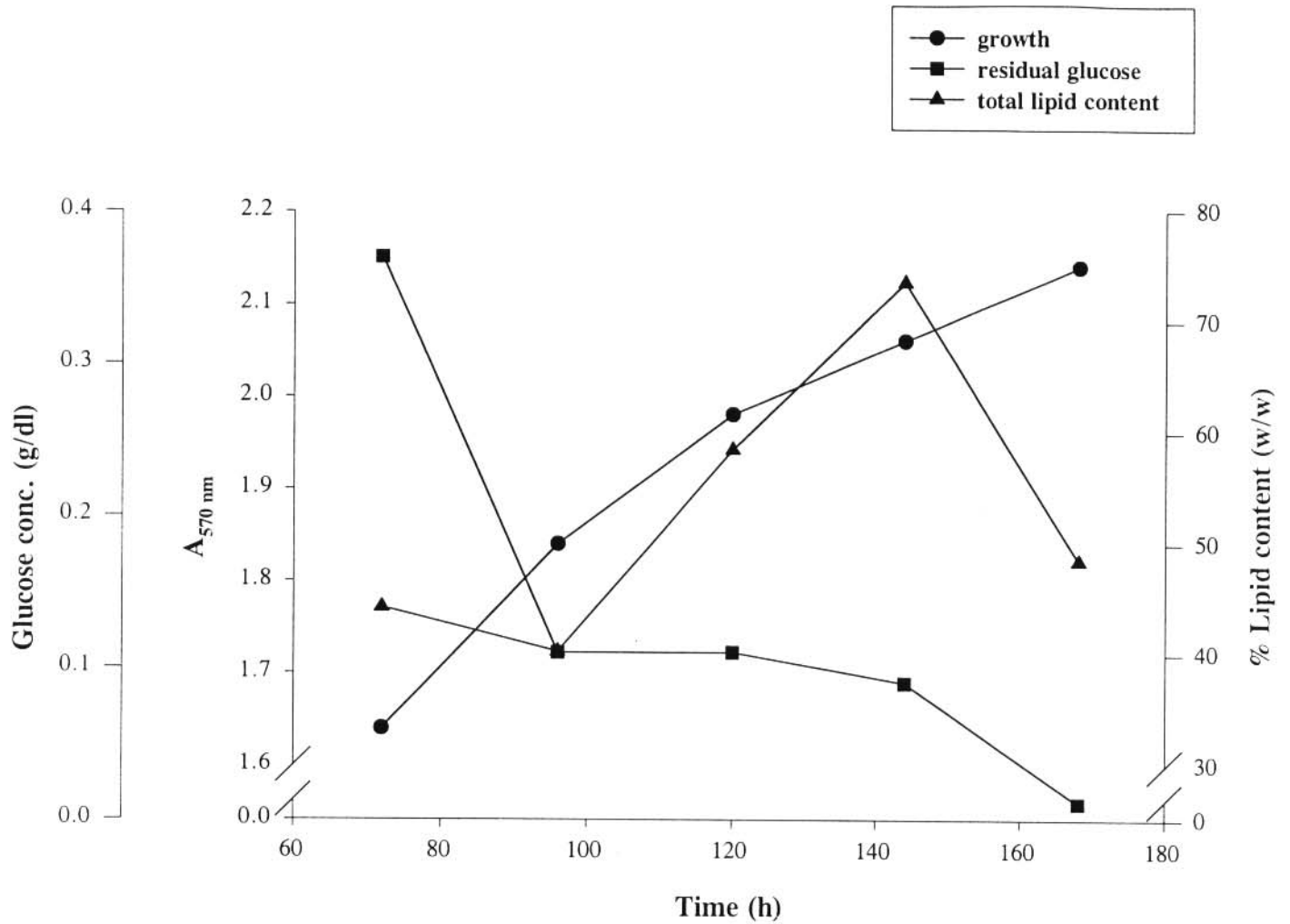


Fig. 3.12.2a. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a 2 % glucose BASAL medium in a batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

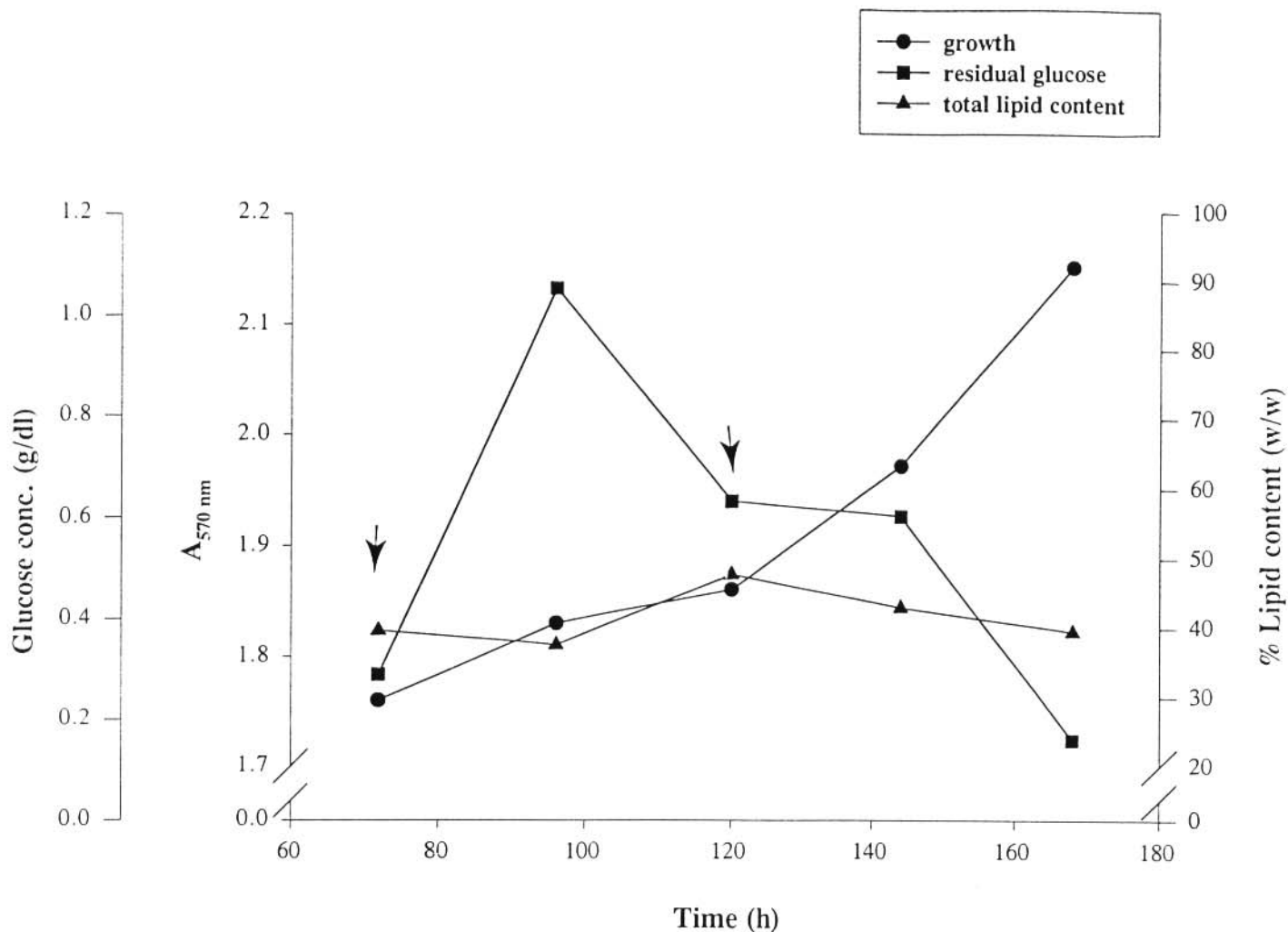


Fig. 3.12.2b. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a 2 % glucose BASAL medium in a fed-batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods. The arrows indicate feeding points.

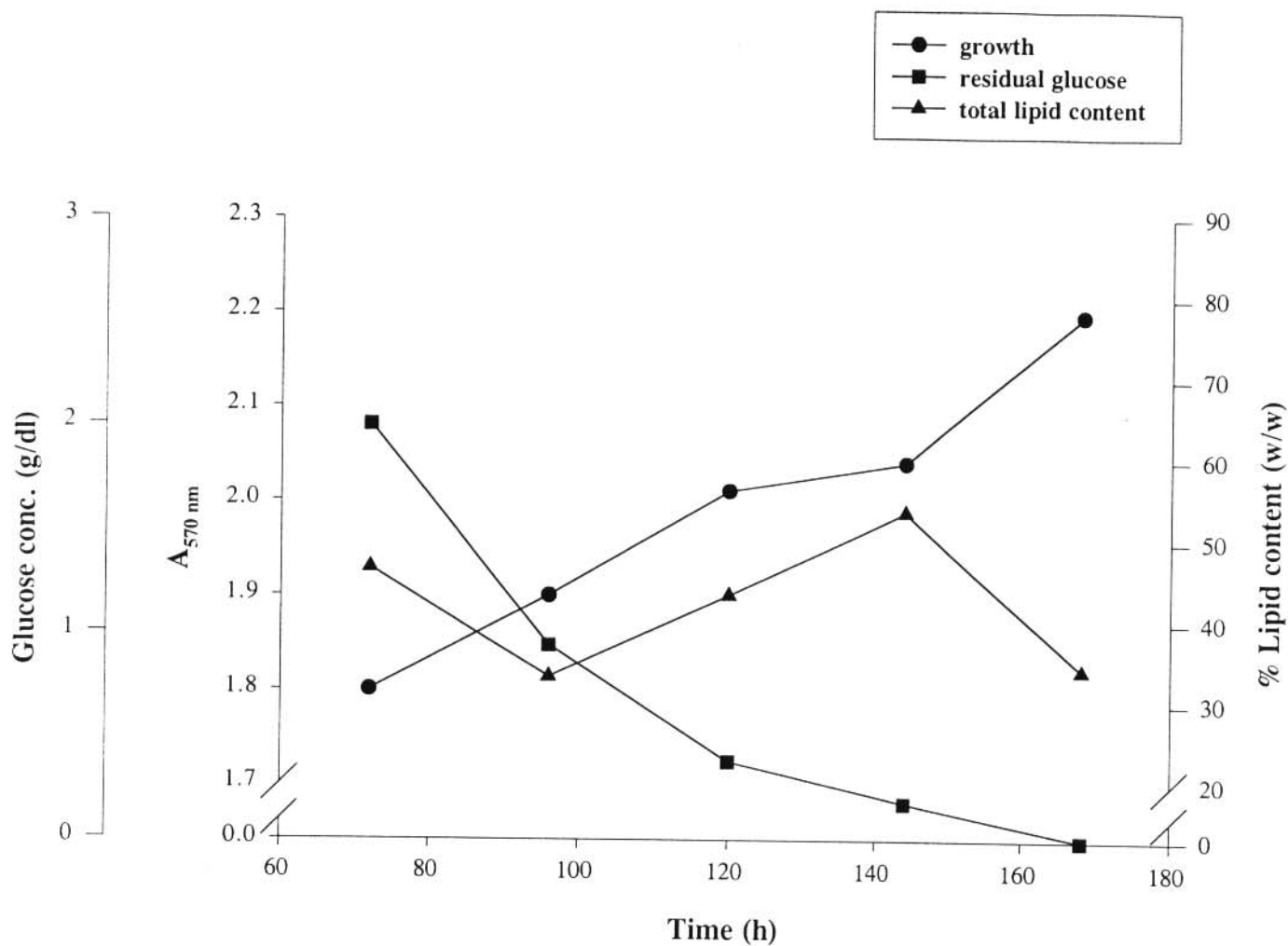


Fig. 3.12.3a. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a 4 % glucose BASAL medium in a batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods.

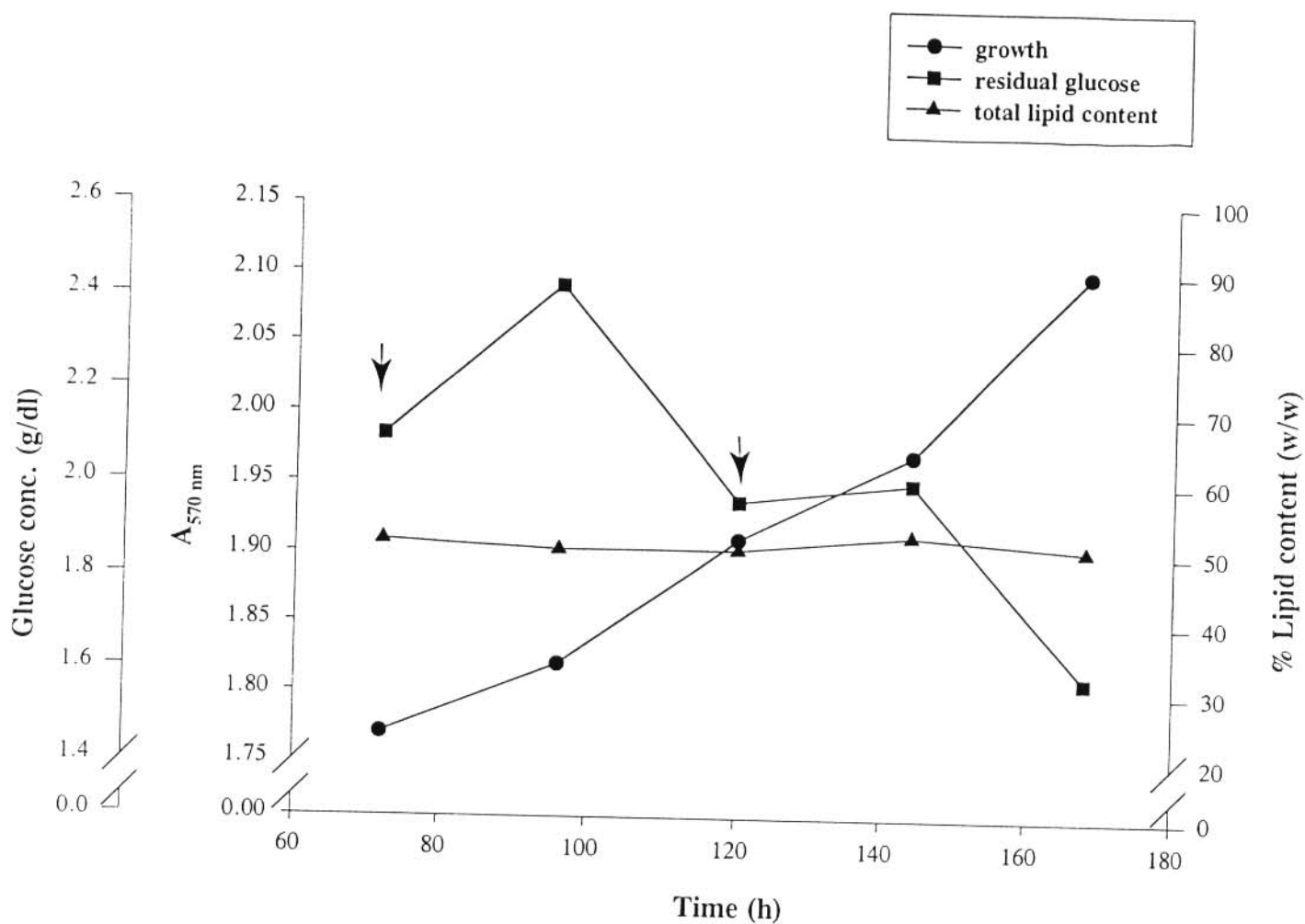


Fig. 3.12.3b. Changes in growth, residual glucose and total lipid production in UOFS-Y-2082 cultivated in a 4 % glucose BASAL medium in a fed-batch culture at various cultivation times. Growth and residual glucose were determined by reading the absorbance at 570 and 505 nm, respectively. Total lipid production was determined and expressed as the sum of neutral and polar fractions extracted as described in Materials and Methods. The arrows indicate feeding points.

3.6. FATTY ACID COMPOSITION

Thin Layer Chromatography (TLC) and Gas Chromatography (GC) were used for the analysis of the composition of the lipid and fatty acid fractions, respectively, extracted from the three oleaginous yeasts grown in the BASAL, SAB and PASSI media. Typical thin layer chromatograms for the neutral lipids of cultures cultivated in BASAL, SAB and PASSI media are shown in Figures 3.13.1, 3.13.2 and 3.13.3. The lipid profile observed on the TLC plates for all three media were the similar for these oleaginous yeasts in all the media. From the R_f values of the standards used for the TLC it was found that the neutral lipid fractions extracted from the three oleaginous yeasts were triolein, trilinolein, tristearin and tripalmitin. Three spots with low R_f (x_1 , x_2 , x_3) and one with high R_f value (x_4) could not be identified because their R_f s did not correspond with any values of the lipid standards used.

The fatty acid composition of the neutral and polar lipids were analysed by Gas Chromatography. The neutral and polar lipid composition of the three oleaginous yeasts are presented in Tables 3.3.1 and 3.3.2. All the yeast strains contained similar fatty acids but in varying quantities. The major fatty acids identified were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (18:1) and linoleic acid (C18:2). Other fatty acids which occurred in trace amounts were lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0), margaric acid (C17:0), linolenic acid (C18:3), arachidonic acid (C20:0), gadoleic acid (20:1), behemic acid (C22:0), lignoceric acid (C24:0) and C24:1. There was one particular fatty acid with a retention time of 2.696 which appeared on the chromatogram (Fig 3.14) between C12:0 and C14:0 could not be identified by GC although the amount ranged from 4 to 97 % and 5 to 86 % in the neutral and polar lipids, respectively.

For CBS 2516 T and UOFS-Y-2082, the five major fatty acids were highest in PASSI cultures but for CBS 5910 T, the values were highest in the SAB cultures. Comparing the

fatty acid composition of the three organisms, CBS 2516 T seemed to produce the highest amounts of the major fatty acids in the neutral lipid fraction. The highest major polar fatty acid components were produced in the industrial wastes by CBS 5910 T and UOFS-Y-2082 but the CBS 2516 T produced most of these in BASAL media. Comparing the organisms, UOFS-Y-2082 produced the highest amounts of the major fatty acids in the industrial waste media. In addition, it was observed that both neutral and polar fatty acids increased with time in all the cultures.

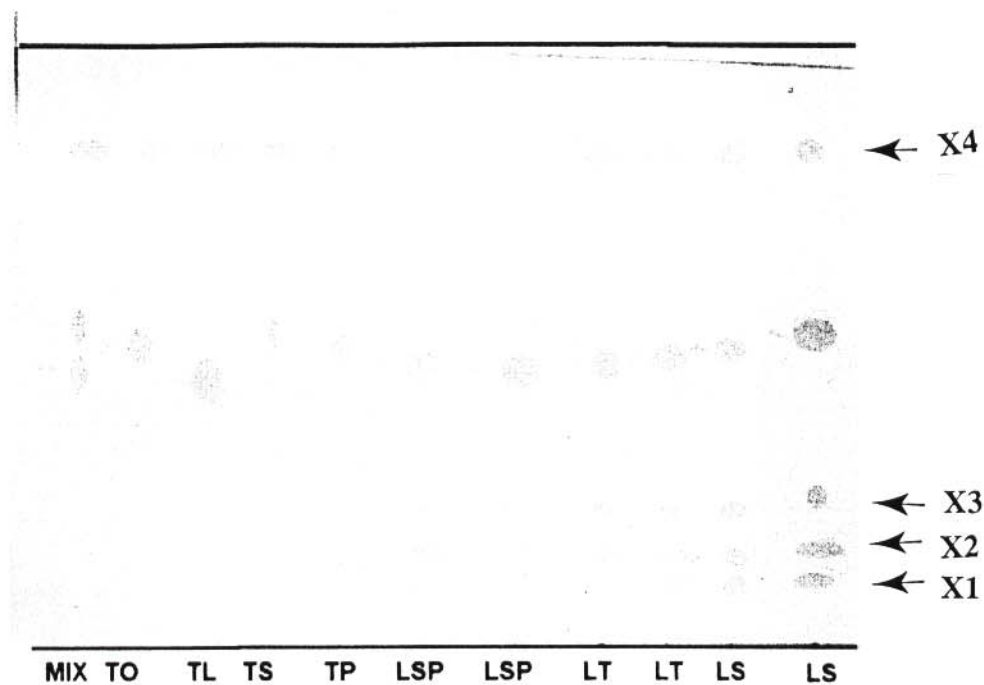


Fig. 3.13.1. Thin layer chromatogram of neutral lipids of the three oleaginous yeasts cultivated in the BASAL medium (C/N=50:1) in a batch culture. Neutral lipids were chromatographed from the silica gel glass plates as described in the Materials and Methods. The composition of the neutral lipids from the three organisms were identified by comparing the R_f values with authentic triacylglycerol standards. Abbreviations for standards: TO, triolein; TL, trilinolein; TS, tristearin; TP, tripalmitin and MIX, mixture of all the standards. Abbreviations for neutral lipid fractions from different oleaginous yeasts: LSP, *Lipomyces sp*; LT, *Lipomyces tetrasporus* and LS, *Lipomyces starkeyi*.

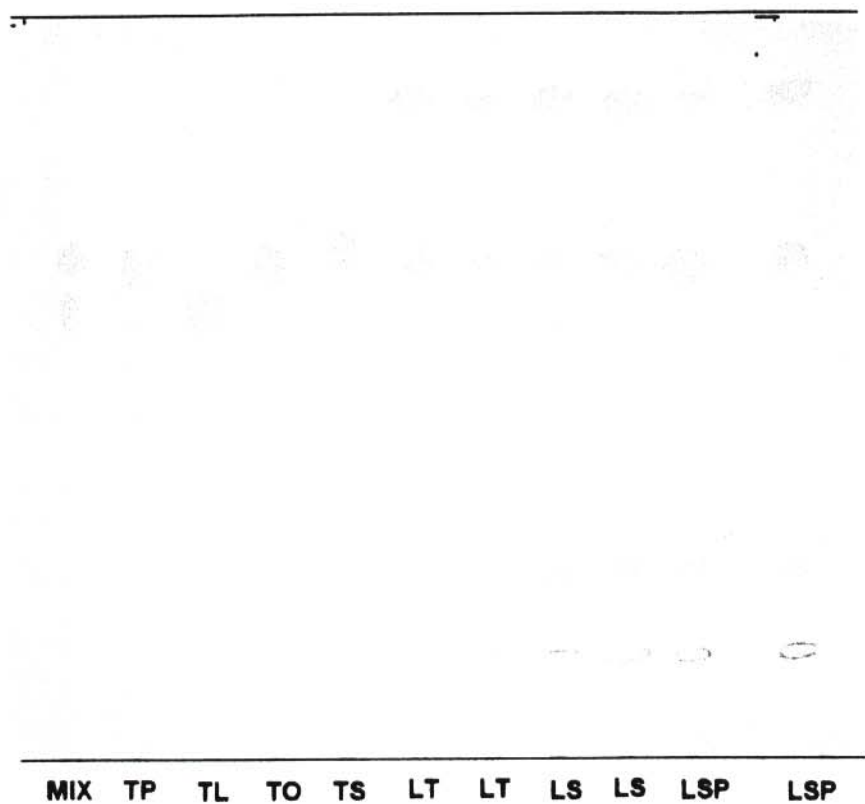


Fig. 3.13.2. Thin layer chromatogram of neutral lipids of the three oleaginous yeasts cultivated in the SAB medium in a batch culture. The composition of the neutral lipids from the three organisms were identified by comparing the R_f values with authentic triacylglycerol standards. Abbreviations for standards: TO, triolein; TL, trilinolein; TS, tristearin; TP, tripalmitin and MIX, mixture of all the standards. Abbreviations for neutral lipid fractions from different oleaginous yeasts: LSP, *Lipomyces sp*; LT, *Lipomyces tetrasporus* and LS, *Lipomyces starkeyi*.

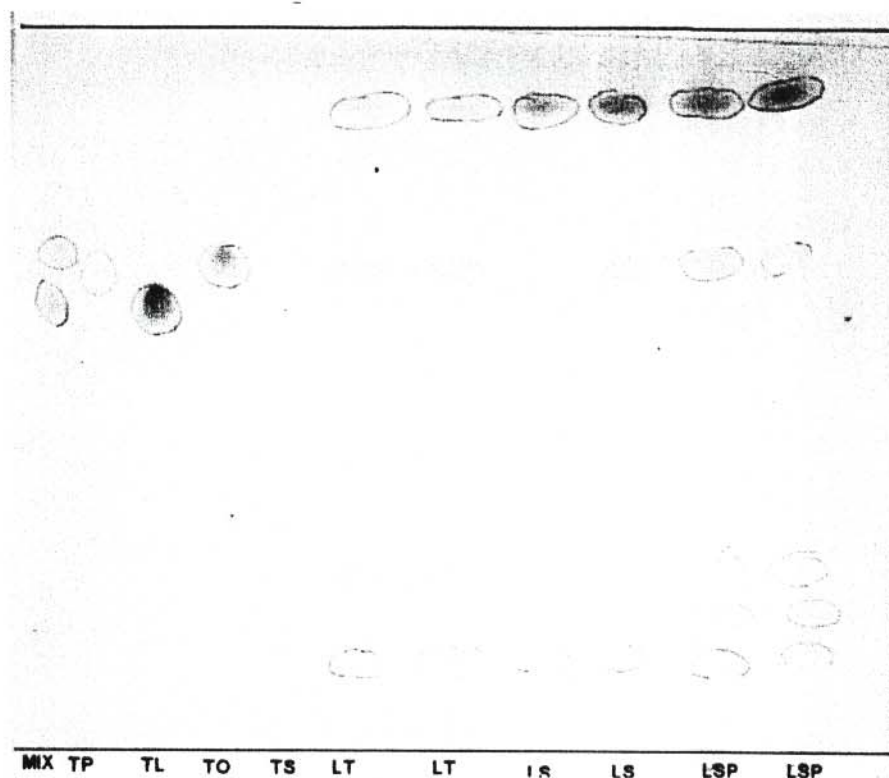


Fig. 3.13.3. Thin layer chromatogram of neutral lipids of the three oleaginous yeasts cultivated in the PASSI medium in a batch culture. The composition of the neutral lipids from the three organisms were identified by comparing the R_f values with authentic triglycerol standards. Abbreviations for standards: TO, triolein; TL, trilinolein; TS, tristearin; TP, tripalmitin and MIX, mixture of all the standards. Abbreviations for neutral lipid fractions from different oleaginous yeasts: LSP, *Lipomyces sp*; LT, *Lipomyces tetrasporus* and LS, *Lipomyces starkeyi*.

Table: 3.3.1. The amount and fatty acid composition of neutral lipid fractions in CBS 5910 T, CBS 2516 T and UOFS-Y-2082

organism	12:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	24:0	24:1	und
S 5910 T (B- 72 h)	t	t	0.11	6.02	0.98	0.24	0.48	1.74	14.0	1.35	<0.08	0.11	t	0.44	0.34	t	71.96
S 5910 T (B- 96 h)	t	0.32	0.2	21.81	4.13	0.38	1.05	5.42	54.91	3.86	t	0.47	0.14	0.56	1.22	0.23	3.76
S 5910 T (PASSI)	t	0.44	t	9.00	1.17	0.63	t	3.04	7.05	1.91	t	t	t	0.72	t	t	74.67
S 5910 T (SAB)	t	0.27	t	11.92	1.17	0.40	0.20	3.81	17.21	1.02	t	0.23	t	0.64	1.03	t	61.84
UOFS-Y-2082 (B- 72 h)	t	t	t	1.05	t	t	t	0.15	1.15	0.26	t	t	t	t	t	t	97.16
UOFS-Y-2082 (B- 96 h)	t	t	t	0.65	0.16	0.08	0.07	0.38	1.22	0.10	t	t	t	t	t	t	96.57
UOFS-Y-2082 (PASSI)	0.53	0.85	t	26.60	3.20	1.79	t	10.82	29.67	2.35	t	t	1.26	4.39	2.38	t	9.76
UOFS-Y-2082 (SAB)	t	0.2	t	8.61	0.71	0.26	t	2.71	9.20	0.35	t	t	t	0.41	0.38	t	76.48
S 2516 T (B- 72 h)	t	0.07	0.11	2.86	0.86	0.10	0.20	0.63	3.88	0.32	t	t	t	0.07	t	t	90.16
S 2516 T (B- 96 h)	t	0.21	t	11.83	1.28	0.63	0.65	3.26	13.97	0.55	t	0.16	t	0.17	0.25	t	66.69
S 2516 T (PASSI)	t	1.05	t	36.75	3.21	2.44	t	13.12	26.99	4.86	t	t	1.36	4.29	2.47	t	nd
S 2516 T (SAB)	t	0.22	t	8.74	0.68	0.23	t	2.60	8.18	0.66	t	t	t	0.29	0.58	t	76.41

Note: B= BASAL medium;t= trace (<0.05 g/ 100 g fatty acid). und=unidentified, nd= not determined; palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), lauric acid (12:0), myristic acid (14:0), pentadecanoic acid (15:0), margaric acid (17:0), isomargaric acid (17:1), linolenic acid (18:3), arachidonic acid (20:0), gadoleic acid (20:1), behemic acid (22:0), lignoceric acid (24:0) and C24:1. All PASSI and SAB cultures were cultivated for 72 h.

Table: 3.3.2. The amount and fatty acid composition of polar lipid fractions in CBS 5910 T, CBS 2516 T and UOFS-Y-2082

Organism	12:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	24:0	24:1	und
SS 5910 T (B- 72 h)	t	0.16	0.14	11.26	2.07	0.36	0.85	2.78	25.84	3.50	0.09	0.18	t	0.25	0.51	t	51.77
SS 5910 T (B- 96 h)	t	0.23	0.16	16.75	3.50	0.27	0.84	3.45	39.50	3.84	t	0.27	t	0.30	0.68	t	30.19
SS 5910 T (PASSI)	t	t	t	24.06	2.73	1.23	t	7.29	23.40	7.07	t	t	t	2.09	t	t	25.72
SS 5910 T (SAB)	t	0.46	0.37	22.18	8.85	0.86	0.82	3.75	32.31	9.38	t	t	t	t	t	t	19.28
DFS-Y-2082 (B- 72 h)	0.08	0.16	0.18	3.99	0.56	0.26	0.24	1.63	5.2	0.51	t	0.09	t	0.22	0.24	t	86.32
DFS-Y-2082 (B- 96 h)	t	0.25	0.28	17.89	2.60	1.70	1.60	7.32	26.28	1.71	t	0.29	t	0.43	0.68	t	38.75
DFS-Y-2082 (PASSI)	t	0.41	0.40	25.07	4.17	0.43	0.64	5.63	48.72	3.68	t	0.44	0.14	0.72	1.30	t	7.72
DFS-Y-2082 (SAB)	t	0.30	0.13	28.33	4.15	0.38	0.41	9.37	44.94	4.11	t	0.45	0.12	0.65	1.43	t	5.13
S 2516 T (B- 72 h)	0.07	0.44	0.25	21.90	2.21	0.74	0.65	6.40	26.24	1.12	t	0.27	0.05	0.34	0.56	t	38.23
S 2516 T (B- 96 h)	t	0.54	0.41	27.84	3.21	1.41	1.59	6.85	30.86	1.54	t	0.32	t	0.33	0.48	t	23.95
S 2516 T (PASSI)	t	0.53	4.88	14.55	1.95	0.83	0.23	4.60	14.11	3.49	t	0.45	t	1.53	0.79	t	41.07
S 2516 T (SAB)	t	0.46	t	16.50	2.43	t	t	4.34	24.31	5.55	t	t	t	1.21	1.97	t	13.33

Note: B = BASAL medium; t = trace (<0.05 g/ 100 g fatty acid), und = unidentified, nd = not determined; palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), lauric acid (12:0), myristic acid (14:0), pentadecanoic acid (15:0), margaric acid (17:0), isomargaric acid (17:1), linolenic acid (18:3), arachidonic acid (20:0), gadoleic acid (20:1), behenic acid (22:0), lignoceric acid (24:0) and C24:1. All PASSI and SAB cultures were cultivated for 72 h.

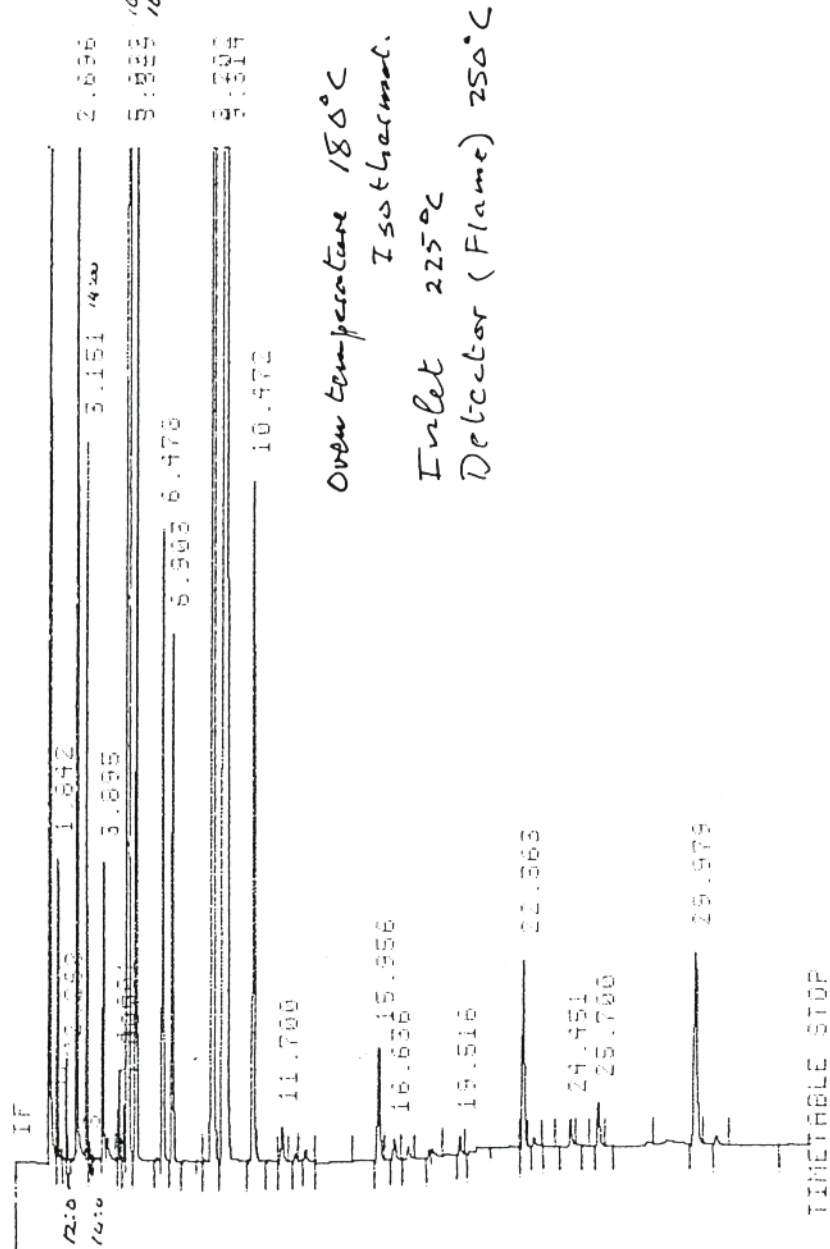


Fig. 3.14. A typical gas chromatogram of the polar lipid fractions extracted from CBS 2516 T cultivated in BASAL medium after 72 h of cultivation. Gas chromatographic analysis were carried out as outlined in the Materials and Methods. GC conditions: oven temperature, 180 °C; inlet temperature, 225 °C and detector (flame) temperature, 250 °C.

CHAPTER 4

DISCUSSION

Lipid accumulation by microorganisms has been known for many years (Woodbine, 1959) and their ability to accumulate lipids is confined to a relatively small number of species. Those microorganisms that can accumulate more than 20 % of lipid in their biomass are referred to as oleaginous species (Ratledge, 1989). Lipids accumulate in oleaginous microorganisms when there is still carbon available to the cells but there is an absence of another nutrient, usually nitrogen, to enable the cells to proliferate. In the absence of new cell production, the existing cells continue to assimilate the carbon available to them and this is then converted into lipid (Ratray, 1988).

In the present study, lipid production by a novel Lipomycetaceous yeast, *L. tetrasporus* (UOFS-Y-2082) isolated from soil in the Northern Province, South Africa by Mothibeli, *et al.*, (1996) was compared with two other oleaginous yeasts namely *L. starkeyi* (CBS2516 T) and *Lipomyces sp.* (CBS 5910T) in different media, including an industrial effluent and a waste in batch and fed-batch cultures.

All the three species accumulated more than 20 % (w/w) lipid in BASAL medium in a batch culture after 72 h, hence they can truly be referred to as oleaginous yeasts as defined by Ratledge, (1989). The total lipid contents of *Lipomyces sp.* (CBS 5910 T), *L. starkeyi* (CBS 2516 T) and *L. tetrasporus* (UOFS-Y-2082) were 72, 67 and 44 %, respectively, after 72 h in batch culture when the carbon source was 2 % glucose in the BASAL medium. The total lipid content of UOFS-Y-2082 was comparable with that reported (48 %) by Mothibeli, *et al.*, (1996) for the same species when grown in glucose as a carbon source without stating when the cells were harvested.

However, when the culture period was prolonged to 120 h, the total lipid content of UOFS-Y-2082 increased to 50 % but decreased to 28 % and 33 % in CBS 5910 T and CBS 2516 T respectively, under the same conditions. On the other hand, when the lipid yields (g lipid/ 100 g substrate) were considered at 72 h, the highest value was observed with CBS 5910 T and the least with UOFS-Y-2082, but at 120 h a different pattern emerged. The yield was highest with CBS 5910 T and least with CBS 2516 T. These observations suggest that there is no direct relationship between the total lipid content and the yield. According to Gill, *et al.*, (1977), the specific rates of lipid production (expressed as g lipid/ g yeast per h) seem to offer a better basis for comparison. The specific rates of the three strains did not necessarily coincide with the trend shown by lipid contents since neither the lipid output nor lipid yield (dp/ds) takes into account the biomass concentration. For the yeast strains studied, the maximum specific rates were 0.009 for CBS 2516 T, 0.006 for UOFS-Y-2082 and 0.003 for CBS 5910 T at 72 h and 0.005, 0.003, 0.002 for UOFS-Y-2082, CBS 2516 T and CBS 5910 T at 120 h respectively. These results suggest that CBS 2516 T was the most productive strain for lipid production in batch culture in BASAL medium and UOFS-Y-2082 performed better than CBS 5910 T.

The efficiency with which lipid is synthesized from a substrate is expressed as lipid yield (Gill, *et al.*, 1977) or fat coefficient (Woodbine, 1959); this value is important when the cost of substrate is to be evaluated for a microbial process. From the results, CBS 5910 T was the most efficient in converting the substrate (glucose) into lipids. It is almost two times more than UOFS-Y-2082.

An idealized pattern of lipid accumulation in oleaginous microorganisms (Gill, *et al.*, 1977; Kessel, 1968) was observed with the oleaginous yeasts cultivated in the BASAL medium in a batch culture (Figs. 3.3.1- 3.3.3). This pattern is thought of as a two-stage process: stage 1 being cell proliferation and balanced growth culminating with nitrogen exhaustion from the medium, and stage 2 being that of lipid accumulation. In all

oleaginous yeasts studied, the maximum lipid accumulation occurred after 72 h of cultivation after the exponential phase of growth when cell proliferation has ceased.

In order to optimize production of oil in these oleaginous yeasts, accumulation of lipids in yeasts cultivated in different concentrations of glucose (1 %, 2 % and 4 %) was investigated. The three yeast strains showed different degrees of tolerance to glucose. Only CBS 5910 T showed a concentration dependent growth. UOFS-Y-2082 and CBS 2516 T reached their maximum growth by 72 h when cultured in 1 % glucose media. Maximum lipid accumulation of 87 % and 68 % occurred in CBS 5910 T and UOFS-Y-2082 respectively, at 72 h when cultured in BASAL medium containing 1 % glucose but CBS 2516 T only achieved its maximum lipid production level after 96 h in 2 % glucose media. These data suggest that conditions for optimization of lipid production by these yeasts may be different and need to be evaluated separately. However, it is important to note that although the total lipid content of the newly isolated yeast strain (UOFS-Y-2082) was slightly lower than CBS 5910 T, it appeared to be a better producer than CBS 2516 T. It needs to be pointed out here also (as stated earlier in the discussion) that a better basis of comparison would be the specific rates of lipid production (which were not determined in these experiments). The reasons for these observations are not clear. Probably, at higher carbon: nitrogen (C:N) ratios (2 % and 4 % glucose) the cells were still proliferating and the lipid synthetic phase was delayed or high C:N ratios above a certain optimum would inhibit lipid production. These results seem to support the findings of other workers (reviewed in Rose, 1989) that high C:N ratios in medium tend to support good cell proliferation and little lipid production because for a considerable lipid yield to be achieved during the fattening phase, the carbon nitrogen ratio has to be low.

Although yeasts can be considered for lipid production, Gogolewski, *et al.*, (1996) considered high cost of production as one of the factors that militates against the use of yeast as a potential source for lipids. However, the production costs of oleaginous yeasts can be reduced by culturing them on industrial wastes, which are often ecologically

harmful. To make this study economically viable, an industrial effluent from South African Breweries (SAB) and orange juice waste from Granor Passi (PASSI) were used for media preparation and lipid production in these media were compared with a synthetic (BASAL) medium to assess their suitability.

The accumulation of lipids by oleaginous yeasts cultivated in the industrial media (SAB and PASSI) in batch cultures followed the same pattern observed in the BASAL medium (described above). From the data, the maximum biomass yield was obtained at 72 h in all media with UOFS-Y-2082 and CBS 2516 T but *Lipomyces sp.* (CBS 5910 T) reached its maximum biomass production at 120 h in PASSI medium. Furthermore, it was observed that the medium which was most efficiently converted into biomass and lipid was SAB by UOFS-Y-2082 and CBS 5910 T and PASSI by CBS 2516 T. For lipid production, the two industrial wastes seemed to be better than the BASAL medium. For example, the highest specific rate of lipid production in PASSI medium was 0.009 by UOFS-Y-2082 and 0.01 by CBS 5910 T in SAB medium, after 72 h. The BASAL medium was the least efficiently utilized for both biomass and lipid production in all oleaginous yeasts studied.

In a report by Evans and Ratledge, (1983) it was shown that biomass production by oleaginous yeasts is affected by the media composition. SAB effluent and the orange juice waste (PASSI) vary in composition with regard to total reducing sugar and nitrogen present. PASSI contained a higher concentration of reducing sugar (5.6 g/l) than SAB (2.1 g/l) whereas PASSI contained 0.045 g/l nitrogen compared with SAB 0.071 g/l. Interestingly, the BASAL medium which contained the highest concentration of substrate (20 g glucose/ l) did not seem to support much growth. This indicates that the SAB effluent and the orange juice waste (PASSI) might contain some other nutrient(s) which supports microbial growth. The lipid content of *L. tetrasporus* (UOFS-Y-2082) in SAB was 50- 53 % and 46- 67 % in PASSI compared favourably with that of 48.5 % reported by Mothibeli *et al.*, (1996).

It was also important to determine the proportions of neutral to polar lipids under the various culture conditions by the three yeast strains, because from the literature (Ratray, 1988; Ratledge and Evans, 1989; Gogolewski, *et al.*, 1996b) production of neutral lipids or polar lipids depends on the media composition and age of cultures. In this study the highest ratio of neutral to polar lipids was observed when CBS 5910 T was cultivated in BASAL medium and the value obtained by UOFS-Y-2082 in SAB medium was comparable to that of CBS 5910 T. In PASSI medium all the yeast strains seemed to produce more polar lipids than neutral lipids. However, when the cultivation time was prolonged, Gogolewski, *et al.*, (1996b) observed that the participation of triacylglycerols in the lipid fraction increased and that of phospholipids, decreased. In this study this observation varied with each organism and growth media. The data therefore suggest that for the production of neutral lipids the BASAL and SAB media should be considered.

According to Evans and Ratledge, (1983) lipid yield by oleaginous yeasts is expressed as the total amount of lipid obtained from cells grown per 100 g substrate. The lipid yields observed in SAB and PASSI media showed that these media were more efficiently converted into lipids when compared with the BASAL medium at 72 h. This clearly shows that the SAB effluent and fruit juice waste (PASSI) could be used for biomass and oil production by the yeast strains studied. It must be noted that prolonged growth resulted in low efficiency of substrate conversion into lipids.

Most studies on the evaluation of the potential of yeasts for lipid accumulation were established using batch cultures (Woodbine, 1978; Evans and Ratledge, 1983). In these studies, however, high productivities for both cell mass and lipid have not been obtained because of the limitations of batch culture (Yamauchi, *et al.*, 1983). An alternative culture system used for lipid production by yeasts is the continuous culture (Gill, *et al.*, 1977; Evans and Ratledge, 1983) because besides being a more efficient and cost-effective means of cultivating yeast on large scale, it is able to give close control over the physiological state of the organism. Moreover, under the steady-state conditions of

continuous culture, a constant composition of the cell is produced; this includes not only the total amount of lipid within the cells, but also the fatty acyl moieties of the lipid (Gill, *et al.*, 1977). The application of continuous culture to lipid production has been examined with respect to *Candida* 107 (Gill, *et al.*, 1977; Hall and Ratledge, 1977) *Rhodotorula gracilis* (Ratledge and Hall, 1979) and *Lipomyces starkeyi* (Boulton and Ratledge, 1981). Information on the use of fed-batch culture for lipid production is scanty. In 1983, Yamauchi *et al.* attempted lipid production by *L. starkeyi* using a micro-computer aided (DO-stat system) fed-batch culture. They claimed success since they obtained total lipids which were about twenty-fold higher than the value observed in the previous batch culture.

Data from this study showed that in all the strains used, most lipid production occurred when the cells had almost reached their stationary phase but there was still a residual carbohydrate source (Figs 3.11.1a-3.11.3b and Figs 3.12.1a-3.12.3b). It was, therefore, necessary to investigate the effect of prolonging the productive phase by feeding the cells with fresh carbon source. The feeding strategy adopted was to bring the glucose concentrations to their initial concentrations after the cells had been cultured for 72 h and the feeding was repeated at 120 h. Only two of yeast strains were used for comparison. The choice was based on the fact that, *L. tetrasporus* (UOFS-Y-2082) is the newly isolated strain and *Lipomyces sp* (CBS 5910 T) is one of the established yeast strains which seemed to have produced the highest amount of lipid in the BASAL medium. The results showed that by feeding and refeeding the cells, they were maintained in active state condition in the culture for a longer period. The growth patterns of the two yeast strains did not change substantially when the fed-batch cultures were compared with the batch cultures. Interestingly, at low concentration (1 % glucose) growth of CBS 5910 T seemed stimulated but at the highest concentration (4 % glucose) used, growth was inhibited in the fed-batch cultures and the cultures reached the maximum growth between 120 and 144 h. But the UOFS-Y-2082 cells continued to grow at all the glucose concentrations used in the fed-batch cultures. These observations seem to suggest that

CBS 5910 T may be less tolerant to high glucose concentrations. Some yeasts have been reported to suffer a repression of their oxidative metabolism by an increase in the concentration of glucose (Ratledge and Evans, 1989).

The total lipid content of the UOFS-Y-2082 increased in fed-batch cultures when fed after 72 h at 1 % and 2 % glucose levels but at 4 % level of feeding the increase was only observed after the second feeding at 120 h (Table 3.2). On the contrary after the first feeding (at 72 h) of the CBS 5910 T cultures, the lipid content decreased at all levels of glucose concentration. This observation seems to confirm the previous suggestion that CBS 5910 T is less glucose tolerant.

Since one of the major objectives of this study was to produce lipids which are similar to palm oil (palm equivalent) and palm oil contains predominantly neutral lipids, it was interesting to observe that the ratios of neutral to polar lipids were higher in 1 % and 2 % CBS 5910 T and UOFS-Y-2082 cultivated in fed-batch and batch cultures, respectively. The variations in the lipid composition of these oleaginous yeasts in media of different glucose concentrations showed that the performance of the yeast strains and the lipid composition depended on the culture conditions such as the composition of the carbon substrate. Therefore it could be agreed that the lipid content of different oleaginous yeasts vary depending on the substrate composition as well as on the conditions and prolongation of culture time (Gogolowski, *et al.* 1996b).

When comparing the fatty acid composition in yeasts, several factors should be taken into account since oxygen availability, culture age, pH, growth rate, temperature and the composition of the growth medium can alter the fatty acid composition (Ratray, 1988; Viljoen *et al.*, 1986). Composition of total lipids, fatty acids and acylglycerol fractions quoted in the literature (Ratledge, 1989) differ considerably qualitatively as well as quantitatively. The same observation was made in this study with the three oleaginous yeasts cultivated in different media (Tables 3.2.1. and 3.2.2.).

Since one of the major objectives of this study was to optimize the production of lipids which are similar in composition to palm oil (palm oil equivalents), and palm oil predominantly contains neutral lipids, it was also important to analyse the lipid as well as the fatty acid compositions of the lipids produced under various conditions. In addition, conditions which would favour production of more neutral than polar lipids (high neutral to polar lipid ratios) needed to be optimized. It was interesting to observe that higher ratios of neutral to polar lipids were accumulated by CBS 5910 T when cultivated in media containing low (1 %-2 %) than at a higher concentration (4 %) of glucose. With UOFS-Y-2082, the ratio was higher in fed-batch than batch cultures. The results suggest that substrate concentration, method of culture as well as the yeast strain may affect the lipid composition. Several workers (Gill *et al.*, 1977; Botham and Ratledge, 1979; Yamauchi *et al.*, 1983; Viljoen, *et al.*, 1986; Rattray, 1988; Gogolewski, 1996) have shown that the composition of medium, type of organism and culture conditions affect the yield of lipids as well as its composition.

The major lipids produced by the three yeasts and analysed species by TLC were tripalmitin, tristearin, triolein and trilinolein. Three spots with low *R_f* values and one spot with high *R_f* value could not be identified by TLC because of lack of appropriate lipid standards. However, the lipid profiles of the yeasts are similar to what has been identified in many other oleaginous yeast (MOPOPC, 1996).

To further characterise the oils produced by the yeasts in various media, the fatty acid compositions were analysed by GC. The results (Table 3.1) showed that the neutral fatty acid composition in all media produced by the different yeast strains were similar but not identical to those reported for palm oil (MPOPC, 1996). According to Rattray, (1988) the major fatty acids in palm oil are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). The major fatty acids identified and quantified in this study were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Other fatty acids lauric acid (12:0), myristic acid (14:0),

pentadecanoic acid (15:0), margaric acid (17:0), isomargaric acid (17:1), linolenic acid (18:3), arachidonic acid (20:0), gadoleic acid (20:1), behemic acid (22:0), lignoceric acid (24:0) and C24:1, appeared in traces. One fatty acid with a retention time of 2.696 which appeared on the chromatogram (Fig 3.14) between C12:0 and C14:0 could not be identified by GC although the amount ranged from 4 to 97 % and 5 to 86 % in the neutral and polar lipids, respectively.

Reports by Viljoen, *et al.*, (1986) showed that a high degree of variation in relative concentrations as well as composition, of fatty acids occurred during the exponential and early stationary growth phases when *Debaryomyces vanriijiae*, *Saccharomyces ludwigii*, *Endomyces fibuliger* and *Metchnikowia reukaufii* were used. Greater reproducibility and a relatively stable fatty acid composition were obtained with cells harvested in the late stationary phase. In this study the fatty acid composition was determined in the early stationary phase but no further analyses were carried out beyond this point. However, the percentage fatty acid increased at 96 h when compared with those at 72 h in BASAL medium. Although there were increases in the fatty acid levels in BASAL medium when the cultures were prolonged from 72 h to 96 h, the values were still less than the amounts obtained when UOFS-Y-2082 and CBS 2516 T were grown in SAB and PASSI media for 72 h. The data suggest that the industrial waste media were better than the BASAL medium for the production of oil which were similar in composition to palm oil.

The fatty acyl groups of lipids (in order of abundance) reported in yeasts are: oleate > palmitate > linoleate > stearate Ratledge, (1988). In this study, the order of abundance seems to vary with the yeast strain and the medium composition. Some of the fatty acids were observed in abundance when the yeasts were grown in one medium and in traces when cultivated in the others. Van Rensburg, *et al.*, (1995) observed the same variations when the yeasts were cultivated in different carbon: nitrogen ratios and suggested that this discrepancy may be due to the possibility that the carbon: nitrogen ratio may enhance activities of enzymes responsible for the synthesis of those fatty acids.

In conclusion, this study has shown that the yeast strain, *Lipomyces tetrasporus* (UOFS-Y-2082), isolated from the soil in Northern Province, South Africa and the two other yeast strains *Lipomyces sp* (CBS 5910 T) and *Lipomyces starkeyi* (CBS 2516 T) are oleaginous. Based on the specific rates of lipid production CBS 2516 T was the most productive strain in BASAL medium in batch culture and UOFS-Y-2082 performed better than CBS 5910 T under the same conditions. In addition, the industrial waste media were found to be suitable for lipid production by all the strains since they compared favourably with the BASAL medium. Considering the total lipid content of the two yeast strains (CBS 5910 T and UOFS-Y-2082) the UOFS-Y-2082 performed better in the fed-batch cultures at low concentrations (1 % and 2 %) of glucose than in batch, but the opposite was observed with the CBS 5910 T at all glucose concentration. The major fatty acid composition of all the yeast strains were similar to that of palm oil. Hence, it is proposed that other industrial wastes should be explored for oil production by oleaginous yeasts.

Thus by comparison it seems that microbial oils and fats might be able to achieve economical parity with conventional sources of oils and fats, if cheaper sources of carbon other than synthetic medium were to be available in suitable quantities.

CHAPTER 5

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