Use of Transition Studies in Continuous Cultures of *Lipomyces starkeyi*, an oleaginous yeast, to Investigate the Physiology of Lipid Accumulation

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The physiological changes that occurred in chemostat cultures of an oleaginous yeast, *Lipomyces starkeyi* CBS 1809 undergoing transitions from steady-state carbon-limiting to steady-state nitrogen-limiting conditions have been investigated. A sequence of events has been identified initiated by the presentation of excess glucose to the culture and culminating in the accumulation of substantial quantities of intracellular lipid. The intracellular concentrations of adenine nucleotides and citrate have been determined and their possible regulatory significance in the control of catabolic and anabolic pathways is discussed.

**INTRODUCTION**

An oleaginous micro-organism may be defined as one that has the potential to accumulate substantial lipid (typically 25 to 70% of the dried biomass) when cultivated under optimal conditions (Ratledge, 1982). These conditions are satisfied when the medium is limiting in a nutrient, which is usually nitrogenous, and when there is an excess of the principal carbon source, which is usually glucose.

As a result of previous work carried out examining oleaginous yeasts, an hypothesis has been advanced to explain the phenomenon (Botham & Ratledge, 1979). Briefly, this states that under conditions of nitrogen-limitation the excess glucose continues to be transported into the cell and is then dissimilated by the glycolytic and pentose phosphate pathways, eventually leading to the production of intramitochondrial acetyl-CoA. As a result of glucose catabolism, there is an increase in the intracellular adenylate energy charge with a concomitant decrease in the intracellular concentration of AMP.

Botham & Ratledge (1979) provided evidence that under these conditions the intramitochondrial NAD*-dependent isocitrate dehydrogenase of oleaginous yeasts would be inactivated, thereby placing a stricture on the oxidative role of the tricarboxylic acid cycle. Subsequent studies indicated that under conditions of nitrogen-limitation, citrate synthase would be fully active (Boulton & Ratledge, 1980), resulting in the accumulation of citrate within mitochondria via equilibration with aconitase. Citrate may then be transported into the cytosol in exchange for malate (Evans et al., 1983) and there be cleaved into acetyl-CoA and oxaloacetate by ATP: citrate lyase, thus providing precursor C₃ units for lipogenesis. This latter enzyme has been well-documented from mammalian sources (Srere, 1975), but in yeasts appears to be confined to those species that are potentially oleaginous (Boulton & Ratledge, 1981a). In addition, preliminary studies indicated that ATP: citrate lyase may catalyse the rate-determining step in fatty acid biosynthesis in oleaginous yeasts as well as fulfilling a regulatory role in controlling rates of lipogenesis in these organisms (Boulton & Ratledge, 1981b).

Our laboratory has been one of the few to investigate growth of oleaginous yeasts in continuous culture (Gill et al., 1977; Hall & Ratledge, 1977; Ratledge & Hall, 1979). These studies indicated that lipid concentrations could be achieved similar to those observed in batch cultures providing the medium had a high C:N ratio and a dilution rate of 0·02 to 0·06 h⁻¹ was used. However, the behaviour of oleaginous yeasts in chemostat culture has so far only been investigated after the establishment of steady-state conditions of carbon- or nitrogen-limitation.
This report describes the results of experiments in which various metabolic parameters were monitored when chemostat cultures of the typical oleaginous yeast, *Lipomyces starkeyi* CBS 1809, underwent transitions from conditions of carbon- to nitrogen-limitation. In so doing it was hoped that the sequence of events leading to accumulation of lipid could be identified following introduction of excess glucose into the growth vessel. In addition, it was considered that a study of the physiological changes occurring during the transitions would provide corroborative evidence for the validity of the hypothesis already outlined, since this was mainly based on results of experiments performed with isolated enzymes.

**METHODS**

**Yeast and medium.** The yeast used in this study was *Lipomyces starkeyi* CBS 1809. In the majority of experiments a glucose/salts medium was used as described by Botham & Ratledge (1979). Carbon-limiting medium contained 10 g glucose l⁻¹ and 1.5 g NH₄Cl l⁻¹. Transitions were initiated by replacing the inflowing medium with one identical in composition but containing 100 g glucose l⁻¹. In certain experiments, a defined medium was used of the following composition (g l⁻¹): KH₂PO₄, 7.0; Na₂HPO₄, 2.0; MgSO₄·7H₂O, 1.5; CaCl₂, 2H₂O, 0.1; FeCl₃·6H₂O, 0.008; ZnSO₄·7H₂O, 0.001; thiamine, 0.005; calcium pantothenate, 0.005; 4-aminobenzoic acid, 0.005; nicotinic acid, 0.005; riboflavin, 0.005 and pyridoxin. HCl, 0.005; with biotin at 5 μg l⁻¹.

**Cultural conditions.** Transition experiments were performed in a conventional 8 litre chemostat with an operating volume of 5.2 l. Yeasts were grown at 30°C and a dilution rate of 0.06 h⁻¹ and the culture was maintained at pH 5.5 by the automatic addition of NaOH. Air was delivered to the vessel at 5 l min⁻¹ and the culture was stirred at 600 r.p.m. Foaming was controlled by the timed addition of polyglycol P2000 antifoam (Bevaloid Ltd, Beverley, U.K.) to a final concentration of approximately 0.1% (v/v).

**Sampling procedures.** For citrate and adenine nucleotide analyses a rapid sampling device was fitted to the chemostat as described by Knowles (1977). This method gave a quenching time of less than 1 s (Knowles, 1977).

**Monitoring of growth.** Washed yeast samples were dried to constant weight in tared vials by treatment in a vacuum oven at 80°C.

**Determination of glucose.** The concentration of glucose in culture filtrates was determined using a commercially available kit (GOD-Perid, Boehringer-Mannheim GmbH, Mannheim, W. Germany).

**Determination of NH₄⁺.** Concentrations of NH₄⁺ in culture filtrates were determined by the method of Chaney & Marbach (1962).

**Determination of lipid concentration.** Total lipid content of yeast was determined by a method based on that of Folch et al. (1957) as described previously (Boulton & Ratledge, 1981a).

**Determination of intracellular adenine nucleotide concentrations.** Yeast was removed from the chemostat using a rapid sampling device and quenched in 1·4 M-H₂SO₄. Adenine nucleotides were determined in the quenched samples using the modified luciferin-luciferase procedure of Chapman et al. (1971) as described by Botham & Ratledge (1979).

**Determination of intracellular citrate concentration.** Yeast (18 ml) was removed from the chemostat using a rapid sampling device and quenched with 4 ml 1·4 M-H₂SO₄. After neutralizing the sample with 1·M-NaOH, precipitated protein and cell debris were removed by centrifugation and the supernatant retained for analysis. At the time of sampling, samples of spent medium were retained to determine extracellular citrate concentrations. Citrate was determined by the coupled citrate lyase-malate dehydrogenase spectrophotometric procedure of Williamson & Corkey (1969).

**Determination of ATP: citrate lyase activity.** The specific activity of ATP: citrate lyase (EC 4.1.3.8) was determined in crude French pressure cell extracts as described previously (Boulton & Ratledge, 1981a).

**RESULTS AND DISCUSSION**

In recent studies on the biochemistry of microbial lipid accumulation the oleaginous yeast, *Lipomyces starkeyi* CBS 1809, has been used (Boulton & Ratledge, 1981a, b). Boulton & Ratledge (1981a) confirmed that this yeast could accumulate substantial lipid when grown in continuous culture, and this organism was employed in the transition experiments described here.

Initial experiments (Fig. 1a) showed that under steady state carbon-limited conditions the biomass, of approximately 5 g l⁻¹, contained 4% (w/w) lipid. When the transition was initiated, the biomass increased as soon as excess glucose was introduced into the growth vessel and this was accompanied by a rapid decrease in the NH₄⁺ concentration in the medium. However,
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although the culture became nitrogen-limited from the third to fourth hour, as gauged by the disappearance of this nutrient from the medium, no appreciable lipid accumulation occurred until approximately 24 h after initiation of the transition. This was a reproducible occurrence (see Fig. 2) and similar results were obtained with the oleaginous yeast, Candida 107 (Boulton, 1982). These results indicated that there was not a simple causal relationship between the onset of nitrogen-limitation and the accumulation of lipid. It remained, therefore, to investigate the intervening physiological changes that must have occurred.

Fluctuations in intracellular adenine nucleotide concentrations during transitions from carbon- to nitrogen-limitation

Gill et al. (1977) demonstrated that the specific rate of lipid synthesis [expressed as g lipid synthesized (g lipid-free yeast)−1 h−1] in chemostat cultures of Candida 107 was of a similar order under both carbon- and nitrogen-limiting conditions. This has been confirmed for other oleaginous yeasts (Boulton & Ratledge, 1981 a; C. T. Evans, unpublished work). The suggestion has been made, therefore, that nitrogen-limitation does not induce higher lipogenic rates, but that other biosynthetic pathways dependent upon a supply of nitrogen decline such that lipogenesis eventually becomes the dominant feature of the metabolism of the yeast.

Botham & Ratledge (1979) considered that the primary mechanism responsible for controlling the flux of metabolism between lipogenic and other pathways, in oleaginous yeasts, was probably the variation in intracellular concentrations of adenine nucleotides. The validity of this concept was tested by following changes in adenine nucleotide content during a transition from carbon- to nitrogen-limitation as compared with the time course for lipid accumulation.
The results (Fig. 1 b) showed that there was an 
11-fold decrease in the content of AMP during the course of the transition accompanied by smaller increases in the levels of ADP and ATP. The computed energy charge \([\frac{\text{ATP} + \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}]\) increased during the transition from 0.13 to 0.57. Thus, the observed increase in energy charge was in most part due to the dramatic fall in intracellular AMP content. Furthermore, the rate of decrease in AMP content was most rapid in the initial part of the transition, a basal level being achieved approximately 7 h after the disappearance of nitrogen from the medium. This result supports the suggestion that the decrease in AMP content and concomitant increase in energy charge occurred as a direct consequence of nitrogen depletion, which itself was the primary signal to divert the bulk of the yeast's metabolism into a largely anabolic mode in order to accommodate the excess carbon source.

The adenine nucleotide contents and computed energy charge values were of a similar order to those determined for batch cultures of \textit{Candida} 107 (Botham & Ratledge, 1979). However, they were clearly not typical of the majority of biological systems so far examined. Thus, Atkinson (1977) reported that the energy charge values of all cells under all, or nearly all, conditions lie within the range 0.87 to 0.94. However, due attention was paid to the experimental procedures employed in this study such that reacidification of the cell debris after the first treatment with \(\text{H}_2\text{SO}_4\) did not yield further adenine nucleotides. Furthermore, the observed low energy charge values could not be explained by adenine nucleotide excretion and the sampling method was sufficiently rapid to prevent enzymic degradation of ATP and ADP. In addition, Botham (1978) demonstrated that the use of other quenching agents did not increase the observed yields of adenine nucleotides from yeast samples.

\textit{Fluctuations in the intra- and extracellular citrate concentration during transitions from carbon to nitrogen-limitation}

The mechanism by which variations in adenine nucleotide concentrations could lead to citrate accumulation, as postulated by Botham & Ratledge (1979), has already been discussed. Citrate may serve a dual role in oleaginous micro-organisms; firstly, in the supply of precursor acetyl units for lipogenesis via the intermediacy of ATP : citrate lyase (Boulton & Ratledge, 1981 a) and secondly, as a possible activator of acetyl-CoA carboxylase, an effect reported for numerous lipogenic systems (Volpe & Vagelos, 1976).

When \textit{Lipomyces starkeyi} CBS 1809 was cultivated under conditions of carbon-limitation, the extracellular citrate concentration was 12 \(\mu\text{M}\) and, during a transition this gradually increased to 38 \(\mu\text{M}\) when steady-state nitrogen-limiting conditions were achieved. The initial intracellular citrate content of 3.8 nmol (mg dry weight\(^{-1}\)) rapidly fell when the transition was initiated; however, when nitrogen became exhausted from the medium, the value rapidly increased to reach a plateau of 6.5 nmol (mg dry weight\(^{-1}\)) (Fig. 1 c). Knowles (1977) assumed that the intracellular water content of micro-organisms lies within the range 2 to 4 \(\mu\text{l (mg dry weight)}\)\(^{-1}\). From this it follows that the citrate content of carbon-limited yeast was 0.95 to 1.9 mM and this increased to 1.6 to 3.2 mM during the transition.

Our results may be interpreted in the following fashion. When the transition was initiated, the culture was limited by neither carbon nor nitrogen. Presentation of additional glucose would be reflected in an overall increase in the rate of metabolism of the yeast. Since at this stage there would be no stricture placed upon the operation of the tricarboxylic acid cycle, as AMP levels were relatively high, an increase in oxidative rates would account for the observed decrease in intracellular citrate content. As the transition progressed, nitrogen became exhausted from the medium between 3 and 4 h, and this was accompanied by a rapid fall in the intracellular AMP content. This latter metabolite reached its lowest value between 8 and 11 h and this was immediately followed (8 to 13 h) by a rapid increase in intracellular citrate levels. This provides powerful corroborative evidence for the proposal of Botham & Ratledge (1979) that the decline in AMP concentration is responsible for channelling carbon flow into citrate synthesis and, subsequently, for the accumulation of lipid.

In previous communications we provided evidence suggesting that ATP : citrate lyase catalyses the rate-limiting step in lipogenesis in \textit{L. starkeyi} (Boulton & Ratledge, 1981 \textit{a, b}). This
is contrary to the generally accepted premise that this position is occupied by acetyl-CoA carboxylase in the majority of biological systems so far studied (Volpe & Vagelos, 1976; Bloch & Vance, 1977). The results presented here support our contention in that the citrate content of the yeast increased when the organism entered a lipogenic phase. This would indicate that either the rate of citrate efflux from mitochondria or the rate of citrate cleavage was rate-limiting under these conditions, depending in which cellular compartment the citrate accumulated. When the transition was initiated, the extracellular citrate concentration immediately increased. This citrate must have been derived from a cytosolic citrate pool since citrate could not have left the mitochondrion without first passing through the cytosol. This argues therefore that ATP: citrate lyase must operate at a lower rate (i.e. is the rate-limiting step) than the rate of citrate efflux from the mitochondrion.

Variations in the specific activity of ATP: citrate lyase during transitions from carbon- to nitrogen-limitation

Evidence for the involvement of ATP: citrate lyase in many mammalian tissues has been provided by the observation that its activity positively correlates with altered lipogenic rates induced by nutritional or hormonal factors (Kornacker & Lowenstein, 1963, 1964, 1965a, b). This is an induction-repression phenomenon (Yen & Mack, 1980). We showed that ATP: citrate lyase occurs solely in oleaginous micro-organisms; however, there was no correlation between observed enzyme activity and lipid content (Boulton & Ratledge, 1981a).

This result was confirmed in that, when the activity of ATP: citrate lyase was monitored in a separate transition experiment, no significant variation was observed (Fig. 2). Therefore, clearly the regulatory role we have ascribed to ATP: citrate lyase must be effected at the enzyme rather than the gene level. Possible strictures placed upon the activity of the enzyme in vitro would not be detected in the artificial conditions of the in vitro enzyme assay mixture.

Although the evidence presented so far supports the hypothesis of Botham & Ratledge (1979), an explanation is still required as to why constant AMP and citrate concentrations were attained at 11 and 13 h, respectively, after the initiation of the transition, although appreciable lipid accumulation did not commence until approximately 26 h (Fig. 1). The situation is further complicated in that examination of the data presented in Fig. 1(a) shows that the bulk of the increase in biomass occurred after nitrogen disappeared from the medium. Clearly this could not have been due to growth, nor was it explained by utilization of the excess glucose to accumulate lipid. We interpret this increase in biomass as representing biosynthesis of a short-term storage product, which has been tentatively identified as largely trehalose (C. T. Evans, unpublished data). Thus, under steady-state carbon-limiting conditions, the metabolism of the yeast is geared to scavenge all available carbon. When an excess of carbon is made available to the culture, it is

![Graph](image-url)

Fig. 2. Variation in the specific activity of ATP: citrate lyase (○) compared with the biomass (△), lipid content (○) and pattern of utilization of NH₄⁺ (■) during a transition from carbon- to nitrogen-limitation.
immediately assimilated but, because it cannot be immediately metabolized presumably because key enzymes such as phosphofructokinase are not fully active, it is initially stored in a readily mobilized form. When carbon-excess conditions persist, the metabolism of the yeast adapts so that the carbon stored as carbohydrate, which may be regarded as an intermediate, is utilized to synthesize lipid, which may therefore be regarded as a long-term carbon store. It follows that there must be a mechanism which acts as a signal to mobilize carbohydrate and utilize the released carbon to synthesize lipid. This signal has not as yet been identified but is possibly also related to changes in energy charge. Examination of the data presented in Fig. 1 shows that when lipid was being synthesized most rapidly (24 to 28 h), this was accompanied by a second cycle of rapid increase in energy-charge. Possibly, variations in adenine nucleotide concentrations may control the flux of metabolism between gluconeogenesis and lipogenesis. However, it is equally possible that the observed variations in adenine nucleotide levels are themselves a consequence of the intracellular conversion of carbon between carbohydrate and lipid and as such of no regulatory significance.

REFERENCES


