Regulation of the metabolism of methanol, dihydroxyacetone and glycerol in the yeast Hansenula polymorpha.
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In recent years there has been an increasing interest in the possible application of microorganisms for the production of fine chemicals. A thorough understanding of the metabolic pathways in microorganisms leading to the desired products, and their in vivo functioning, generally will be required for the selection of the most suitable (mutant) production strains and process conditions. This thesis reports the results of studies in which various physiological and biochemical aspects of yeast, and a firm basis for further studies aiming to optimize strain properties and physiological conditions for DHA production up to industrially interesting levels.

In methylotrophic yeasts methanol is oxidized to formaldehyde by the enzyme alcohol oxidase. Energy generation occurs by oxidation of formaldehyde to CO2 via a linear pathway. Synthesis of cell material is initiated by assimilation of formaldehyde via the xylose 5-phosphate (Xu5P) pathway (Fig. 1). The condensation of formaldehyde and Xu5P is catalyzed by DHA synthase, a transketolase type of enzyme, which results in glyceraldehyde 3-phosphate and DHA formation. The latter compound is subsequently phosphorylated by DHA kinase, the second enzyme of the assimilation pathway.

The enzymes involved in the metabolism of methanol in H. polymorpha are regulated both by catabolite repression and induction by methanol (Chapter 2). Although all enzymes followed a comparable pattern during growth on a number of substrates in batch cultures, variations in the degree of induction and/or repression occurred to some extent, indicating the involvement of additional, independent, regulatory mechanisms. The essential role of DHA synthase and DHA kinase, two enzymes proposed to fulfill a key role in the methanol assimilatory pathway, was confirmed by the observation that mutants lacking either of these enzymes failed to grow on methanol as the sole carbon source. Both mutants, however, were still able to grow, and to oxidize methanol, when incubated with mixtures of methanol and an auxiliary substrate (i.e. xylose). Mutational inactivation of DHA kinase resulted in accumulation of DHA from methanol (resting cell suspensions), and mixtures of methanol and xylose (resting cell suspensions and growing cells). Both mutants were investigated in more detail. The elucidation of glycerol metabolism in H. polymorpha is described in Chapter 3. The available literature suggested that glycerol metabolism in methylotrophic yeasts involved the so-called oxidative pathway (glycerol dehydrogenase and DHA kinase, see Fig. 1). Contrary to expectation, the DHA kinase-negative mutant (strain 17B) was not affected in its ability to utilize glycerol. Furthermore, strain 17B was still able to grow on DHA, albeit slowly and only when this substrate was supplied at relatively high concentrations. This was not due to leakiness of the DHA kinase-mutation, but the result of a slow conversion of DHA into glycerol via the reverse reaction of the NAD-dependent glycerol dehydrogenase (DHA reductase). Further studies revealed that in H. polymorpha glycerol in fact is metabolized via the phosphorylative pathway, involving glycerol kinase. Important differences between the glycerol kinase from H. polymorpha and that of most other organisms are its pH optimum (approx. 7.5 and 9 or higher, respectively) and its sensitivity to inhibition by hydrazine, a compound normally used in the assay for glycerol kinase activity. The partially purified enzyme from H. polymorpha was specific for glycerol, no activity with any other substrate was detected, but the affinity for glycerol and ATP was relatively low (Km values of 1.0 mM and 0.5 mM, respectively). The glycerol 3-phosphate formed from glycerol is further metabolized via a membrane-bound, NAD(P)-independent glycerol 3-phosphate dehydrogenase. The essential role of glycerol kinase and glycerol 3-phosphate dehydrogenase in glycerol metabolism was confirmed by the isolation and characterization of mutants of strain 17B unable to grow on glycerol because of mutational inactivation of either of these enzymes. The initial glycerol-negative mutants also lacked DHA kinase and were now completely unable to grow on DHA. Reintroduction of DHA kinase activity, by genetic crossing with a wild type strain, only resulted in very low growth rates on glycerol. This indicates that, even when glycerol dehydrogenase and DHA kinase are present at significant levels, the oxidative pathway (via DHA) is only of minor importance during growth on glycerol.

The physiological responses of H. polymorpha wild type and mutant strains 17B (DHA kinase-negative), 17BGS1 (DHA kinase- and glycerol kinase-negative) and 70M (DHA synthase-negative) to growth on mixtures of xylose and methanol in
carbon-limited continuous cultures are described in Chapters 4 and 5. No differences in enzyme levels or cell densities were observed during growth of the wild type strain and these mutants on xylose alone. With each strain, increasing methanol concentrations (0 - 30 mM) in the feed resulted in an increased induction of the enzymes of methanol metabolism and complete utilization of both substrates at steady state. With the wild type strain the cell densities on mixtures of xylose and methanol increased. It could be calculated that the additional amounts of cell material synthesized were approximately the same as expected during growth on the same amounts of methanol alone, i.e. growth yields were additive. This utilization of methanol did not significantly alter the cell
densities in cultures with strain 17B or 17BG51 but instead resulted in excretion of DHA. The distribution of methanol over dissimilatory and assimilatory pathways in *H. polymorpha* is controlled accurately. With strain 17BG51 (completely unable to metabolize DHA) the molar ratio between DHA excreted and methanol consumed remained approximately equal to the value observed for the wild type strain (40 % of the methanol consumed is assimilated). The data thus show that in mutant strain 17BG51 the *in vivo* functioning of DHA synthase has not changed by inactivation of DHA kinase (and loss of ability to convert methanol carbon into cell material).

Residual methanol started to accumulate in cultures of the DHA kinase-negative mutants when the methanol concentration in the feed exceeded a certain threshold value (60 mM for strain 17B). Concomitantly, the activities of alcohol oxidase and the rate of DHA accumulation strongly decreased, whereas the activities of other methanol enzymes were not significantly affected. Further investigations, involving transitions between steady states with different methanol concentrations in the feed and of gradient cultures, revealed that the decrease in alcohol oxidase followed the wash-out curve (dilution rate = 0.05 h⁻¹). This, and the absence of significant amounts of inactive alcohol oxidase protein in cell-free extracts, strongly suggested that synthesis of the enzyme had become repressed. Increasing DHA accumulation (from formaldehyde and Xu5P), with increasing methanol concentrations in the feed of the mutant cultures, will cause a progressive reduction in the availability of the limiting carbon source. Eventually, this will result in a reduction of the growth rate below the dilution rate employed, and wash-out of the cultures. The latter, however, was not observed and instead methanol metabolism was switched off and the mutant strains resumed growth on xylose alone.

To our initial surprise, a mutant blocked in DHA synthase activity (strain 70M) behaved exactly like the wild type strain when grown in continuous cultures on mixtures of xylose and methanol. A mutant blocked in both DHA synthase and DHA kinase (strain 17B70M) showed essentially the same responses as observed with strain 17B, both with respect to enzyme activities (except for DHA synthase), cell densities and DHA excretion. This revealed that a DHA synthase type of enzyme was still present in strain 70M. When using an assay with enhanced sensitivity, low but reproducible DHA synthase activities could be detected in cell-free extracts. Following hydroxylapatite chromatography, this activity was shown to be associated with classical transketolase and not with the DHA synthase protein peak, still present in this mutant. The *H. polymorpha* transketolase was purified (525-fold) to homogeneity. The native enzyme was dimeric, as has been reported for other transketolases, with a subunit molecular weight of 74 kD. This enzyme catalyzed the reaction between formaldehyde and Xu5P only slowly (3 % of the classical transketolase activity with ribose 5-phosphate and Xu5P). Kinetic studies with the purified transketolase of *H. polymorpha* showed that the enzyme possesses a poor affinity for (hydrated) formaldehyde (Kₐ = 5 mM). Possible explanations for the *in vivo* functioning of transketolase in formaldehyde fixation in strain 70M are discussed (Chapters 5 and 7).

Accumulation of DHA and glycerol from methanol and auxiliary substrates was studied in more detail with resting cell suspensions of strain 17BG51 (Chapter 6). The ratio between the glycerol and DHA produced varied depending on a number of experimental conditions. The data suggest that *in vivo* the conversion of DHA into glycerol by NADH-dependent DHA reductase is determined mainly by the intracellular NADH/NAD⁺ ratio (or intracellular NADH concentration). Highest glycerol levels were obtained when supplying the cell suspensions with relatively low oxygen tensions. Further support for this hypothesis was obtained when studying the effect of an inhibitor of the electron transport chain on the methanol-dependent conversion of DHA into glycerol. Whereas with strain 17B production of DHA already occurred from methanol alone, with strain 17BG51 the presence of a second substrate, to replenish the Xu5P (acceptor molecule for the DHA synthase reaction), was required. The nature of the second substrate influenced the amount of DHA and glycerol produced to a limited extent, C₅-sugars being the most effective ones. The levels of these C₅-compounds increased when higher xylose and methanol concentrations were supplied, either by their repeated addition or by starting out with higher initial substrate concentrations. Both approaches resulted in a strong shift towards glycerol production. Repeated addition of the substrates resulted in a maximum accumulation of 0.75 M glycerol (1 g.g cells⁻¹.day⁻¹) and it could be calculated that 50 % of the xylose and 20 % of the methanol supplied was recovered as glycerol.

85