How overproduction of foreign proteins affects physiology of the recombinant strains of *Hansenula polymorpha*

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Changes in the activity of key enzymes of the methanol utilization pathway of the recombinant strains of methylotrophic yeast *Hansenula polymorpha* R22-2B and LAC-56 were studied at different rates of chemostat growth on methanol containing mineral media. It was shown that the strain R22-2B, initially having a 10-fold increased activity of dihydroxyacetone kinase (DHAK, a key enzyme of formaldehyde assimilation) acquired increased activity of formaldehyde dehydrogenase (FADH, a key enzyme of formaldehyde dissimilation) which resulted in the enhanced oxidation of formaldehyde to CO_2. Strain LAC-56, overproducing *Escherichia coli* β-galactosidase, acquired the decreased intracellular concentration of ATP which resulted in the decrease of the efficiency of formaldehyde assimilation catalyzed by DHAK and resulted in accumulation of toxic formaldehyde. As a consequence some biochemical responses occurred in cells that were directed to a diminishing of the toxic effect of accumulated formaldehyde, namely, the decreasing of methanol oxidase activity (to reduce the rate of formaldehyde synthesis), and the increasing of FADH activity (to increase the rate of formaldehyde oxidation).

1. Introduction

The wide use of recombinant strains in biotechnology requires concrete knowledge about the molecular mechanisms affecting the physiological behaviour of such strains. Introduction of a DNA vector into bacteria cells for purposes of cloned gene expression can perturb native cell functions at many levels. The presence of foreign DNA can alter regulation of chromosomal DNA replication, regulation of transcription of chromosomal genes, ribosome functions and RNA turnover, activities of regulatory proteins, chaperone and protease levels, membrane energetics and protein post-translational processing, as well as energy and intermediary metabolism of the cell. The combined effects of these interactions on the metabolic characteristics of the host-vector system have major implications for yields of cloned biotechnological products and interactions of genetically engineered organisms with their environment (see review Bailey 1993). Using known pathway energetics, maximum theoretical yield factors based on ATP, glucose, and O_2 have been estimated for recombinant *Escherichia coli* and compared with corresponding estimates for host cells alone, indicating major changes in carbon and energetic stoichiometry in recombinant cells in cases of high cloned gene expression (Bailey *et al* 1986). According to theoretical estimations recombinant cells of *E. coli* require more ATP and the most crucial consequence of the overproduction of foreign proteins could be the deficit of ATP (Da Silva and Bailey *et al* 1986). In the experimental study it was shown that *E. coli* recombinant cell growth rate declines monotonically as plasmid content increases and as does the efficiency of plasmid gene expression. Also the interrelationships among number of plasmids per cell, induction of expression of a plasmid gene, and recombinant population growth rate have been experimentally delineated for *Saccharomyces cerevisiae* containing plasmids. Specific growth rate of these recombinant yeasts exhibits a maximum as a function of plasmid content, an effect which was attributed to the

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Abbreviations used: MO, methanol oxidase; FADH, formaldehyde dehydrogenase; DHAK, dihydroxyacetone kinase; *Yx/s*, the yield of biomass; *D*, dilution rate; V_{max}, maximum growth rate; RQ, respiratory coefficient

interplay between beneficial effects of the plasmid in selective medium and parasitic effects on metabolism at larger plasmid content or with more plasmid gene expression activity (Bailey *et al* 1986).

It seems that oversynthesis of foreign proteins will decrease the pools of certain intracellular compounds, and this in turn, will lead to competition between intrinsic and foreign proteins synthesis for the cell's metabolic potential. Will certain key enzymes whose activity is regulated predominantly by the cell energy status will be the first targets affected by this competition? The experimental model used to answer this question was two overproducing recombinant strains of methylotrophic yeasts of *Hansenula polymorpha*.

The methylotrophic yeasts *H. polymorpha*, *Pichia pastoris* and *Candida boidinii* are rapidly becoming the systems of choice for the expression of recombinant proteins in yeast (see reviews Faber *et al* 1995; Sudbery 1996; Hollenberg and Gellissen *et al* 1997). Overproducers constructed on the basis of these strains are extremely efficient, for example, a process was developed for the production of phytases using *H. polymorpha* which resulted in exceptionally high concentrations of active enzyme (up to 13·5 g/L) representing over 97% of the total accumulated protein. These levels greatly exceed those reported so far for any yeast-based expression system (Mayer *et al* 1999). Methylotrophic yeasts have well developed biochemistry (see reviews Trotsenko *et al* 1987; Harder 1990; van der Klei *et al* 1991), genetics (see reviews Gleeson and Sudbery *et al* 1988; Sibirny *et al* 1988) and physiology (see reviews Hopkins and Muller *et al* 1987; Brinkmann *et al* 1990).

The objectives of this work were to elucidate how overproduction of the foreign proteins challenge the pathway of methanol utilization and, in turn, physiology of the recombinant strains of *H. polymorpha*.

In brief, the pathway of methanol utilization is as follows (Hopkins and Muller *et al* 1987; Trotsenko *et al* 1987; Harder 1990; Brinkmann *et al* 1990). The first reaction catalyzed by methanol oxidase (MO) is the oxidation of methanol to formaldehyde. Then, the flux of formaldehyde is subdivided between two metabolic branches: (i) dissimilation (or direct oxidation of formaldehyde to CO_2) that produces two molecules of NADH upon oxidation of one molecule of formaldehyde (the key enzyme is FADH), and (ii) assimilation in which formaldehyde is converted into cell biosynthesis (the key enzyme DHAK catalyzes formation of dihydroxyacetone phosphate through ATP hydrolysis).

The amounts of formaldehyde subdivided between assimilation and dissimilation are determined by the ATP/ADP ratio. Excess of ATP stimulates DHAK (assimilation) and inhibits FADH (dissimilation) and the excess of ADP limits the activity of DHAK and stimulates FADH. If formaldehyde is not utilized efficiently in assimilation and dissimilation, its increased concentrations decelerate the growth of the yeast; at a relatively high concentration formaldehyde becomes toxic. The special regulatory mechanism helps to overcome the toxicity; the excess of formaldehyde binds to MO and decreases the activity of the enzyme (catalyzing its own synthesis), thereby reducing the toxicity (Kato *et al* 1976).

The synthesis of the enzymes of methanol metabolism is induced by methanol and the corresponding activities are not detected during the growth in glucose or in glycerol (Hopkins and Muller 1987; Trotsenko *et al* 1987; Harder 1990; Brinkmann *et al* 1990).

These experiments were performed to answer the following questions:

(i) how overproduction of the foreign protein (β-galactosidase from *E. coli*) would challenge the pathways of methanol utilization?
(ii) will a 10-fold increase of the activity of DHAK (key enzyme of assimilation) enhance the efficiency of methanol utilization?
(iii) how such increase of DHAK will affect the functioning of methanol utilization pathway?
(iv) will a 10-fold increase in the activity of DHAK enhance the yield of the recombinant strain?

The strains used in the study were (i) *H. polymorpha* LAC-56 producing *E. coli* β-galactosidase in amounts consisting 12% of total cell proteins, (ii) *H. polymorpha* R22-2B having 10-fold increased activity of homologous DHAK and (iii) the control wild type (plasmid free) strain of *H. polymorpha* DL-1. These strains were cultivated in chemostat in conditions which mimics different degrees of substrate availability, from deep substrate limitation ($D = 0{\cdot}02$–$0{\cdot}08\ h^{-1}$) to methanol excess ($D = 0{\cdot}12$–$0{\cdot}16\ h^{-1}$). During chemostat growth essential physiological and biochemical parameters of the cells were measured, namely (i) the values of *Yx/s* (reflecting the efficiency of methanol conversion into biomass); (ii) the V_{max} – values of maximum growth rates; (iii) the values of extracellular concentrations of formaldehyde (reflecting the efficiency of its utilization in the assimilation and dissimilation pathways); (iv) activity of MO (reflecting the efficiency of methanol consumption and formaldehyde synthesis); (v) activity of DHAK (the key enzyme of assimilation); (vi) activity of FADH (the key enzyme of dissimilation); (vii) the values of intracellular concentration of ATP (reflecting the cell's energy status), (viii) the values of respiratory coefficient (RQ) reflecting the efficiency of O_2 consumption and CO_2 production. The preliminary results of the study were published earlier (Velkov *et al* 1996).

2. Materials and methods

2.1 *Microorganisms*

The recombinant strain of *H. polymorpha* R22-2B was obtained by integrative transformation of the mutant of *H.*

polymorpha leu2-356 dak16 which is auxotrophic for leucine and has no DHAK activity (Tikhomirova *et al* 1988; Velkov *et al* 1996) with the recombinant plasmid pR22-2B containing a cloned DNA fragment from *H. polymorpha* with the gene coding DHAK, the marker gene *leu2* from *S. cerevisiae* plasmid YEP13 (Ikonomova *et al* 1987; Tikhomorova *et al* 1988). The strain *H. polymorpha* R22-2B contained 10–12 copies of DHAK gene integrated in the genome (Velkov *et al* 1996). The recombinant strain of *H. polymorpha* LAC-56 was obtained by transforming of the mutant of *H. polymorpha leu2-356* (which is auxotrophic for leucine) with the recombinant plasmid pLAC-56 containing *LacZ* gene from *E. coli* encoding β-galactosidase under the control of MO promoter and the *leu2* marker gene from *S. cerevisiae* (Velkov *et al* 1996).

All mutant and recombinant strains used were derivatives of the wild strain DL-1. The wild-type strain of *H. polymorpha* DL-1 (VKM-Y 2447) was used as the control. Transformation was performed as described (Tikhomirova *et al* 1988).

2.2 *Media and cultivation*

YEPD, a rich medium containing 1% yeast extract, 2% peptone, and 2% glucose was used for determining the replicative stability of recombinant strains. The chemostat cultivation was performed in a minimal synthetic medium containing KH_2PO_4–1·0 g/l; $MgSO_4 \times 7H_2O$–0·5 g/l; NH_4Cl –0·8 g/l; $FeSO_4$–16·0 mg/l; $CaCl_2 \times 2H_2O$–0·66 mg/l; $ZnSO_4 \times 6H_2O$–0·18 mg/l; $CuSO_4 \times 5H_2O$–0·16 mg/l; $CoCl_2 \times 6H_2O$ –0·18 mg/l; $MnSO_4 \times 4H_2O$–0·15 mg/l; methanol (1% v/v) was used as the single source of carbon and energy (Velkov *et al* 1996). The strains were cultivated in a chemostat under methanol-limited conditions (and without leucine) in ANKUM–2 M fermenter (Biopribor, Russia) at pH 4·5, stirring – 500 rpm. The concentration of dissolved oxygen was maintained at 50% of saturation, the working volume – 2 l. Cultivation was performed at various dilution rates (D = 0·02–0·16 h^{-1}). The cells were considered in a steady state after a four–five-fold change of the culture liquid; pH was maintained constant by adding 10% KOH. Each strain was cultivated two times, the results of the corresponding measure during independent parallel chemostat runs were very similar (the variations of data points were 2–5%).

2.3 *Enzyme assays*

The activities of key enzymes of methanol metabolism were determined in cell-free extracts prepared as follows. Fresh biomass (0·2–0·3 g) was suspended in 0·3 ml of cold (0°C) 0·05 M Tris–HCl buffer containing 0·5 mM EDTA, pH 8.0). The cells were disrupted at 4°C by shaking with glass beads (0·5–0·6 mm in diameter) in polypropylene tubes for 3 min with 1 min intervals after each minute of shaking. The disrupted cell suspension was supplemented with 0·3 ml of the same buffer and centrifuged in an Eppendorf 5414 centrifuge (Germany) for 1–2 min at 20,000 *g*. The supernatant was collected, diluted and then used for enzyme assays. All the procedures were performed at 0–4°C. Enzyme activities were determined by spectrophotometry in a cuvette maintained at 30°C. MO (EC 1.1.3.13) was assayed as described (Kato *et al* 1976), FADH (EC 1.2.1.1) was assayed as described (Kato *et al* 1972), DHAK (EC 2.7.1.3.) was assayed as described (Kato *et al* 1988) and β-galactosidase (EC 3.2.1.27) was assayed as described (Miller 1982). The specific activity was measured as the amount of the enzyme required for conversion of 1 M of substrate per min per mg protein at 30°C (Miller 1982). Protein was determined as described (Bradford 1976)

2.4 *Determination of ATP*

Special precautions were taken to avoid anaerobic conditions during the transfer of samples from chemostat to the quenching solution. Nucleotides were extracted from 5 ml of cell suspension in tubes containing 12% $HCLO_4$ and 31 mM Na_2SO_4 as described (Bakker and Mangerich 1983). Extraction was performed for 1 h on a shaker at 0°C. The extract was centrifuged for 15 min at 15,000 *g* and neutralized with 2 M KOH containing 0·1 M MOPS. ATP was measured by luceferin-luceferase reaction as described (Kimmich *et al* 1975). The intracellular ATP pool was calculated as the amount of ATP divided by biomass dry weight.

2.5 *Determination of biomass yield*

Yx/s was calculated as a ratio of biomass dry weight to the amount of consumed methanol.

2.6 *Maximum growth rate*

V_{max} was determined by the dilution rate at which the culture was washed out. The concentration of residual methanol in the culture liquid was measured by gas-liquid chromatography on a Pye Unicum chromatograph (USA). Formaldehyde was determined as described (Nash 1953).

2.7 *Determination of RQ*

Concentrations of CO_2 and O_2 in the gas outleting from the fermentor were determined on gas-analysers Infralit-4 and Permolit-3 (VEB Junkalor, Germany). The values of RQ were calculated as the ratio of formed CO_2 to consumed O_2.

3. Results and discussion

The strain LAC-56, containing multiple copies of *LacZ* gene from *E. coli* integrated into a chromosome upon induction of the MO gene promoter (growth in methanol containing medium), demonstrated β-galactosidase activity consisting approximately 12% of the total cell protein. The strain R22-2B contained 10–12 copies of the DHAK gene integrated in the yeast genome and had a 10-fold increased activity of the enzyme (Velkov *et al* 1996), which, it seems, represent a small fraction of total cells protein (Hopkins and Muller *et al* 1987; Trotsenko *et al* 1987; Harder 1990; Brinkmann *et al* 1990). During all chemostat runs samples of the corresponding cultural fluids were tested for the possible presence of plasmid free segregants. The samples were plated onto rich YEPD medium and 200–300 of the appeared colonies were toothpicked onto mineral medium without leucine. No leucine negative segregants were found, which confirmed that the recombinant strains were stable.

3.1 *How does overproduction of DHAK and β-galactosidase change the physiology of the recombinant strains?*

Figure 1a shows the dynamics of *Yx/s* in R22-2B, LAC-56 and DL-1 strains at various dilution rates. R22-2B and LAC-56 displayed lower *Yx/s* than DL-1 at D ranging from 0·02 to 0·06 h^{-1} (deep substrate limitation). However, both recombinant strains revealed approximately equal values of *Yx/s* at D ranging from 0·08 to 0·10 h^{-1} (substrate excess). The residual methanol in the cultural fluids of all strains was not detected at all dilution rates in all chemostat runs. The data indicates that a 10-fold increase in the DHAK activity did not increase the efficiency of methanol conversion into biomass. LAC-56 strain had lower *Yx/s* than DL-1 and R22-2B at D = 0·02–0·08 h^{-1}. The most profound decrease of *Yx/s* values of LAC-56 (by 30%) was observed at D = 0·02 h^{-1} (deep substrate limitation), but there was no difference in *Yx/s* between LAC-56 and DL-1 at D = 0·10 h^{-1} (substrate excess).

According to the washout experiments, strain R22-2B had the value of V_{max} by 10% lower than that of DL-1; strain LAC-56 by 23% lower than that of DL-1; V_{max} of DL-1 was 0·22 h^{-1}.

Figure 1b shows the dynamics of accumulation of formaldehyde in the cultural fluids of the strains R22-2B, LAC-56 and DL-1. The strain R22-2B accumulated formaldehyde in the cultural liquid at higher dilution rates than DL-1. Formaldehyde was detectable at D = 0·1 h^{-1} in DL-1 and only at 0·14 h^{-1} in R22-2B. At high values of D, the concentration of non-utilized formaldehyde was two-fold higher in LAC-56 than in DL-1. As mentioned above, in all cases and at all dilution rates methanol was consumed completely. It seems (figure 1a, b) that although the increased DHAK activity in R22-2B did not increased the values of *Yx/s*, it increased the efficiency of formaldehyde utilization by the strain. The strain LAC-56 had considerably lower efficiency of formaldehyde utilization than that of control strain DL-1.

3.2 *How does overproduction of DHAK and β-galactosidase change the activities of the key enzymes of substrate utilization?*

Figure 2a shows the dynamics of DHAK activity in R22-2B, LAC-56 and DL-1 strains. Indeed, R22-2B had a 10-fold increased activity of DHAK that only slightly decreased with the increasing of dilution rates. In DL-1 and LAC-56 strains the activity of DHAK was independent of the values of D and of the increasing of the concentrations of formaldehyde in the culture liquids

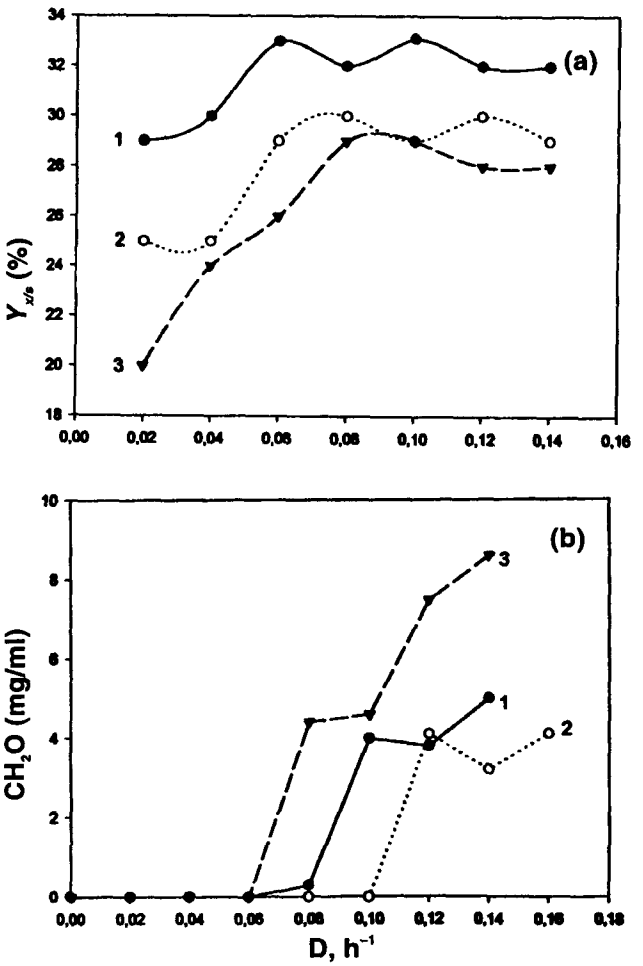

Figure 1. Dynamics of (**a**) biomass yield and (**b**) value of the concentrations of extracellular formaldehyde in *H. polymorpha* strains. (1) DL-1, (2) R22-2B and (3) LAC-56 cultivated in chemostat at different growth rates.

(figures 2a and 1b). This is in good agreement with the view, that activity of this enzyme is not regulated at the level of its synthesis, but at the level of the modulation of its activity by the ATP/ADP ratio (Hopkins and Muller 1987; Trotsenko *et al* 1987; Harder 1990; Brinkmann *et al* 1990).

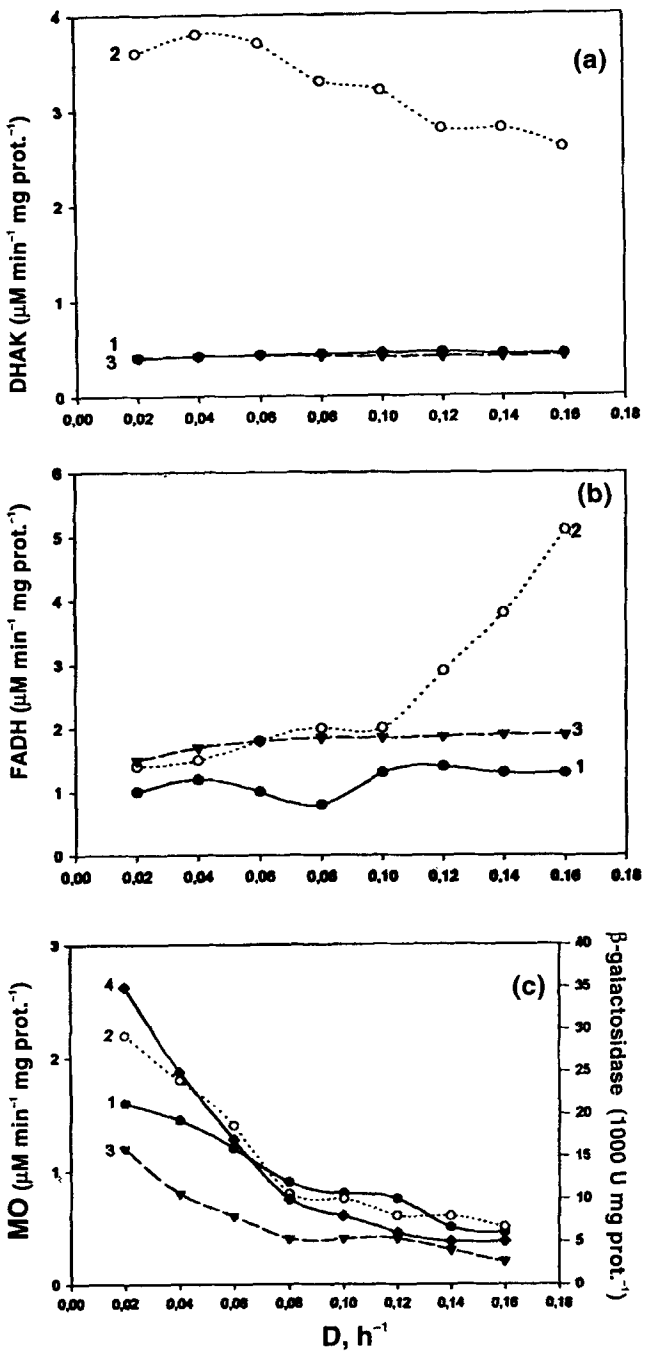

Figure 2. Dynamics of activities of the key enzymes of methanol utilization. (a) DHAK; (b) FADH; (c) MO in *H. polymorpha* strains. (1) DL-1, (2) R22-2B and (3) LAC-56 cultivated in chemostat at different growth rates; (4) activity of β-galactosidase in LAC-56.

Strain LAC-56 revealed the DHAK activities at all *D* similar to that of DL-1 (figure 2a) in spite of increased extracellular concentrations of formaldehyde (figure 1b). The question was what is the mechanism of the decrease of formaldehyde utilization in LAC-56? A decrease of the efficiency of its assimilation or dissimilation? Or both? Note, that in LAC-56 there was no detectable decrease in the DHAK activity measured *in vitro* (figure 2a).

Another key enzyme of formaldehyde utilization is FADH. Figure 2b shows that indeed, in LAC-56 the activity of FADH was enhanced at all *D* and increased with the increasing of *D* (from 30% at 0·02 h^{-1} to 80% at 0·1 h^{-1}). Strain R22-2B had two–three-fold higher activity of FADH (figure 2b).

It seems that the enhancing of DHAK activity in R22-2B (figure 2a) increased the rate of formaldehyde dissimilation (figure 1b) but not its assimilation into the biomass (figure 1a). The increased activity of FADH in LAC-56 (figure 2b) indicated that the decrease in the efficiency of formaldehyde utilization (figure 1b) was not due to decrease in its dissimilation rate. It is known that detoxification of an excess of formaldehyde could be provided by the dissimilation pathway (Jónes and Bellion 1991; Maidan *et al* 1997). It seems that intensive accumulation of formaldehyde in LAC-56 stimulated activity of FADH – the key enzyme of formaldehyde dissimilation (and detoxification). It was obvious that the efficiencies of formaldehyde utilization were changed in both recombinant strains, in R22-2 B the efficiency of formaldehyde utilization increased, but in LAC-56 decreased. The enzyme catalyzing the synthesis of formaldehyde is MO.

Figure 2c shows that at $D = 0{\cdot}02–0{\cdot}06\ h^{-1}$ the strain R22-2 B had the MO activity which was 30% higher than that of DL-1. It is known, that MO is the bottleneck (pacemaker) enzyme of methanol utilization (Brinkmann *et al* 1990). The fact of coordinated stimulation of FADH, which oxidases formaldehyde, and MO, which synthesizes it, is unclear. In LAC-56 the activity of MO decreased two-three-fold at all *D* (figure 2c). This decrease occurred in parallel to that of DL-1. The decrease in the MO activity with the increase of a growth rate of *H. polymorpha* is well known (van Dijken *et al* 1976). The dynamics of activity of β-galactosidase cloned under the control of the MO promoter was parallel to that of activity of MO (figure 2c) because the expression of these enzymes was controlled by the same promoters.

As mentioned above, high concentrations of formaldehyde cause its binding to MO, thus decreasing the enzyme activity and the rate of formaldehyde synthesis (Kato *et al* 1976). This mechanism additionally protects the cell from intoxication by formaldehyde. It seems that FADH stimulation and MO inhibition in LAC-56 could represent the response to high levels of formaldehyde which is directed to the decreasing of its concentration.

3.3 *How does overproduction of DHAK and β-galactosidase change the energy status of the recombinant strains?*

Figure 3 shows the dynamics of the pool of intracellular ATP in R22-2B, LAC-56 and DL-1 strains. R22-2B had nearly normal level of ATP, whereas in LAC-56 it was approximately 50% lower at all dilution rates. It seems that an ATP deficit in LAC-56 is deep enough to stimulate the activity of energy generating FADH and to decrease the efficiency of assimilation of formaldehyde by limiting the activity of DHAK; as was mentioned, DHAK phosporylates dihydroxyacetone through ATP hydrolysis (Harder 1990; Brinkmann *et al* 1990).

3.4 *How does overproduction of DHAK and β-galactosidase change the values of RQ?*

Figure 4 shows the dynamics of the RQ values during the cultivation of R22-2B, LAC-56 and DL-1. The strains DL-1 and LAC-56 displayed similar values of RQ at all D. But the strain R22-2B at $D = 0{\cdot}08$–$0{\cdot}14$ h^{-1} demonstrated the values of RQ approximately two-fold higher than that of DL-1 and LAC-56. The increase of RQ of R22-2B is due to the increase in the rate of formaldehyde oxidation to CO_2. This assumption is in good agreement with three-fold increase of the activity of FADH (figure 2b) and low concentration of formaldehyde in the cultural fluid of the strain (figure 1b).

3.5 *What is the logic of biochemical response of the recombinant strains on overproduction?*

In the R22-2B strain the 10-fold increase of the activity of DHAK resulted in the increase of the activities of MO and FADH (figure 2b, c) which in turn resulted in the increasing of the rate of formaldehyde dissimilation to CO_2 (figure 4). The level of ATP playing the central regulatory role in the activation/inhibition of the assimilation/dissimilation pathways, was not affected. Does this unexpected response of the cells means that there is an another mechanism of regulation of the assimilation/dissimilation pathways which is not modulated by the ATP level? Does this mean that this mechanism is coordinating the activities of MO and FADH with the increasing of DHAK in same directions (activities of all three enzymes were increased)? How will the cell respond to for example, 10-fold increase of FADH? By the parallel increase of DHAK and MO? Further studies could be helpful to answer these questions.

In case of LAC-56 strain in the beginning of the study it was assumed, that the cells would increase the efficiency of methanol consumption and utilization with the aim to compensate the expenditures for oversynthesis of the foreign protein. In reality, the situation was clearly opposite. Instead of increasing of the efficiency of substrate utilization, the strain accumulated formaldehyde, the toxic product of the incomplete utilization of methanol. This was probably due to a decrease in the intracellular ATP concentration, which reduces *in vivo* the activity of DHAK. In turn, this decreased the rate of formaldehyde assimilation. The regulatory machinery of the recombinant cell "interprets" the "unnatural" situation of overproduction as the lowering of its energy status because of the substrate limitations (although the extracellular substrates are non-limiting) and reduce the activity of ATP hydrolyzing enzyme(s) which leads to the accumulation of the toxic product(s) of incomplete substrate utilization.

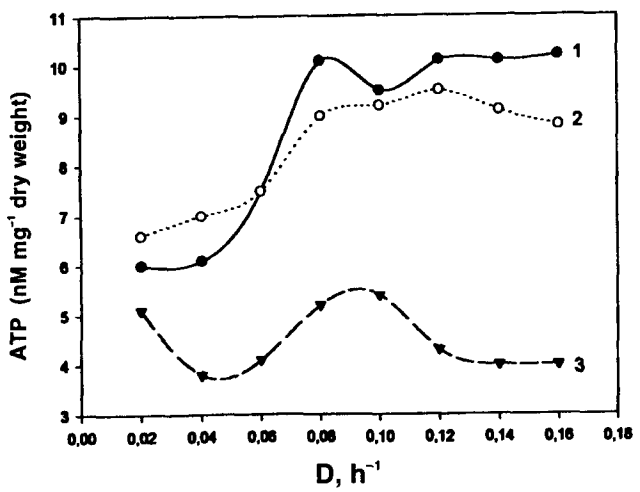

Figure 3. Dynamics of intracellular ATP concentration in *H. polymorpha* strains. (1) DL-1, (2) R22-2B and (3) LAC-56 cultivated in chemostat at different growth rates.

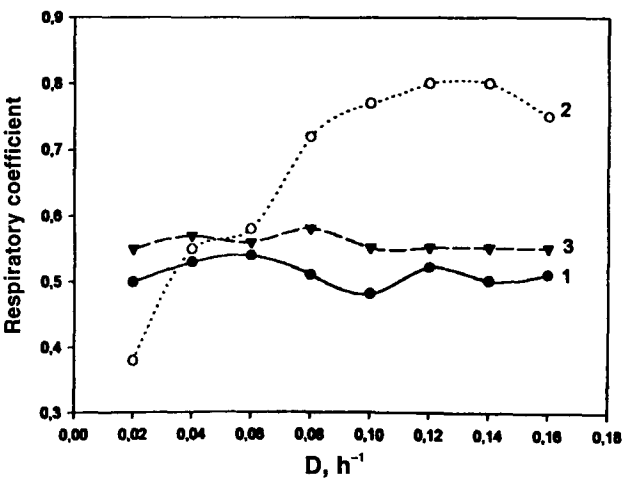

Figure 4. Dynamics of the RQ values of *H. polymorpha* strains. (1) DL-1, (2) R22-2B and (3) LAC-56 cultivated in chemostat at different growth rates.

The next changes in the activities of key enzymes of methanol utilization (decreasing the rate of formaldehyde synthesis by reducing the MO activity and enhancing the rate of its oxidation by increased FADH activity) were a response that alleviate the toxic effect of the accumulated formaldehyde. However, the changing of the enzymes activities were not enough to reduce the level of formaldehyde in LAC-56 to that of the wild strain DL-1.

At the physiological level these biochemical responses decreased the efficiency of substrate conversion into biomass (especially, in the conditions of deep substrate limitation) and reduced V_{max}.

4. Conclusions

In general, the studies of LAC-56 demonstrated that the regulatory network of the recombinant cell "interprets" the overproduction of the foreign protein as reduction of the cell energy status which is normally modulated by substrate availability. In other words, the overproduction mimics the substrate limitation in conditions of its actual availability. And because of the reduced energy status the recombinant cell (growing in the conditions of substrate availability) reduces the activities of the enzymes of energy dependent step(s) of substrate assimilation. This, in turn, leads to accumulation of toxic intermediate(s) of incomplete substrate utilization. The next steps of response of regulatory network of the cell are directed at the diminishing of its self-toxication. Of course, the enzymes activities were assayed in *in vitro* conditions. While the assays provide an estimate of the total amount of the enzymes in cells under different conditions, it may be difficult to correlate these gross estimates to actual regulatory phenomena occurring *in vivo*. This limitation could be solved by the direct measuring of the enzymes activities *in vivo*.

The final consequence of overproduction of foreign proteins could be the decrease of the yields of corresponding overproducers which in turn, during the large scale industrial cultivations could considerably reduce the productivity of fermentations. The attempt to increase the yield of the recombinant strain by genetically engineered enhancement of the efficiency of substrate utilization (strain R22-2B) show that, in fact, the enhancing activity of the key enzyme of assimilation (DHAK) increases the efficiency of substrate dissimilation (not assimilation!) and decreases the value of V_{max}. This, in turn during the large scale industrial cultivations will also decrease the productivity of fermentations.

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