2 Product formation and protein expression of *Saccharomyces cerevisiae* during growth on glutamate as nitrogen source

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Introduction

Ammonium and glutamate are key components in the nitrogen metabolism of *Saccharomyces cerevisiae*. Ammonium is assimilated via the formation of glutamate [1], which is the main donor of nitrogen in the biosynthesis of amino acids [2]. The amino group of glutamate is transferred to amino acid precursors, leaving 2-oxoglutarate, which may be either excreted or further converted in the cellular metabolism. Previous studies have shown that the kind of nitrogen source affects the product formation and carbon flow in *S. cerevisiae* [3,4]. However, one would expect not only the metabolite formation to be affected by the change of nitrogen source, but also the protein expression pattern. In the current work, metabolite production and protein expression for growth on the two different nitrogen sources glutamate and ammonium were compared. Furthermore, product formation of a haploid laboratory strain of *S. cerevisiae* was compared to that of an industrial strain of baker's yeast.

Methods

A laboratory strain VW K43, a prototrophic wild type in the CEN.PK2 background, (provided by Prof. Stefan Hohmann, Dept Cell and Molecular Biology, Göteborg University, Sweden) and an industrial strain of baker's yeast [3,4] were used. Minimal mineral medium was used [3] with glucose (20 g/l) as the carbon and energy source and ammonium (5 g/l) or glutamate (3.5 g/l) as the nitrogen source. Ergosterol and Tween 80 were included in the medium in anaerobic cultivations. The aerobic cultures (medium adjusted to pH 6.0) were grown in shake flasks at 30°C (volume of flask twice as large as the volume of medium). The anaerobic cultures were grown in a bioreactor system as described in [4].

To follow the carbon flows at anaerobic conditions, [U-^{14}C]-labelled glucose or glutamate was added. The experimental setup and measurements are described elsewhere [4].

The product yields of the exponential growth phase were determined from samples taken after inoculation and after the glucose exhaustion. The time of glucose exhaustion was determined from measurements of the glucose concentration using the teststrip Diabur-Test® 5000 (Boehringer Mannheim) (aerobically grown cultures), or from on-line measurements of the carbon dioxide concentration in the exhaust gas (anaerobically grown cultures). Extracellular metabolite concentrations were measured with HPLC (Waters) [4] and glutamic acid was determined using an enzymatic kit assay (Boehringer Mannheim). Biomass concentration was determined from dry weight measurements and measurements of the optical density at 610 nm (Nova Spec II, Pharmacia).

For determination of the protein expression pattern, aerobic cultures of the laboratory strain (VW K43) were inoculated to an OD_{610} of 0.07 and grown to an OD of 0.35–0.50. A volume of 1 ml was transferred to a separate flask and 8 μl ^{35}S-labelled (3.2 MBq) L-methionine (specific activity: >37 TBq/mmol, Amersham) was added. The cultures were grown for further 30 min and then put on ice. The cells were centrifuged (15 000×g, 4°C, 5 min) and washed once with ice-cold milliQ water. After a final short centrifugation (10 s) the supernatant was discarded and the cell pellet was stored at −80°C. The proteins were extracted and the amount of incorporated ^{35}S was measured. The protein composition was determined by two-dimensional (2D) poly-acrylamide gel electrophoresis, with about 2 000 000 dpm of the protein extract loaded. The proteins are separated by the isoelectric point and the molecular mass. Resolved proteins were previously identified by microsequencing [5] or MS-MALDI [6]. The protein pattern was detected by phosphoimaging and the image analysis was performed with the PDQUEST software (v. 5.1), which is a commercial variant of the QUEST system [7]. Image data can be found on the www server (http://yeast-2DPAGE.gmm.gu.se).

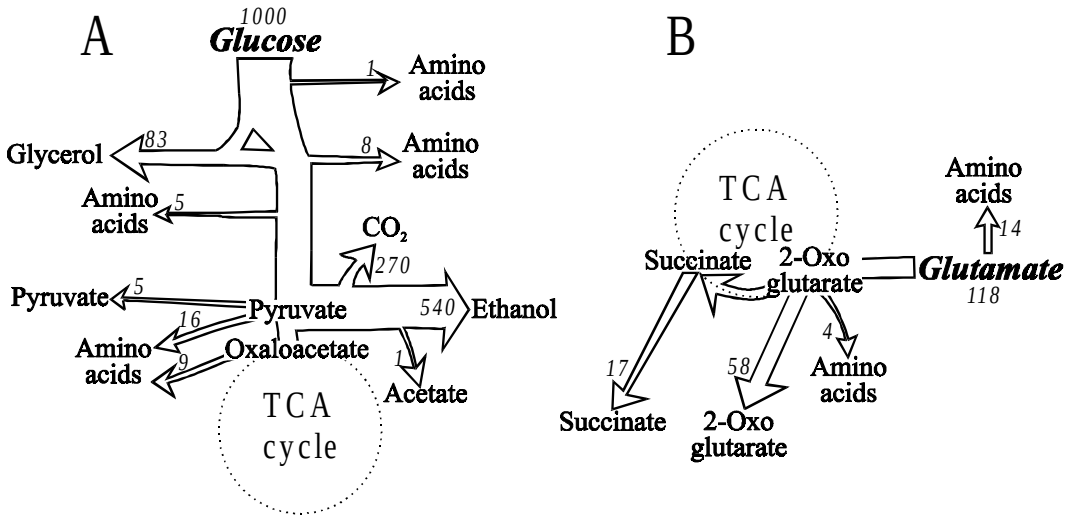

Fig. 2.1 Carbon flows during anaerobic growth of the industrial strain of *S. cerevisiae* with glutamate as the nitrogen source. The magnitudes of the fluxes, in C-mol (all scaled to glucose consumption, 1000 C-mol) are indicated and calculated from the data presented in [3,4]. The formation of 2-hydroxyglutarate is omitted. A. Carbon flow from glucose B. Carbon flow from glutamate.

Results and discussion

The carbon flows from glucose and glutamate in anaerobically grown *S. cerevisiae* are shown in Fig. 2.1. The balances of radioactive compounds closed up to 100% [4]. The carbon from glucose is primarily found in the two main anaerobic products; ethanol and glycerol, but also some organic acids and amino acids are formed from glucose. Glutamate, on the other hand, is almost exclusively converted to the TCA cycle intermediates 2-oxoglutarate and succinate. Not surprisingly, also the amino acids belonging to the "glutamate family" [2,8] are formed from glutamate. Furthermore, other cell material is only formed from glucose. There is no detectable overlap between these two flows and thus the TCA cycle does not seem to operate in a cyclic manner [4].

The product formation of glutamate grown cells differs largely compared to ammonium grown cells, Table. 2.1. The products originating from glutamate, i.e., 2-oxoglutarate, succinate and 2-hydroxyglutarate, are formed in substantially higher amounts during growth on glutamate compared to growth on ammonium as nitrogen source. This may be expected considering the large amount of 2-oxoglutarate formed from glutamate in the transamination of amino acid precursors [4]. Furthermore, the glycerol yield is decreased in glutamate cultures because of the lower demand for NADH reoxidation [3]. The glycolytic flux was found to be higher in ammonium grown cells as reflected by the higher maximal specific growth rate, and at the same time similar biomass yields during both

Table 2.1 Yields and maximal specific growth rate during anaerobic growth of the baker's yeast from [4] and during anaerobic and aerobic growth of the laboratory strain VW K43, with glucose as the carbon and energy source and glutamate as the nitrogen source. Also data for anaerobic cultures of baker's yeast with ammonium as the nitrogen source are presented, adapted from [3].

Products formed (mg/g glucose used)	Ammonium	Glutamate		
	Baker's yeast, anaerobic	Baker's yeast, anaerobic[a]	Lab strain anaerobic[b]	Lab strain aerobic[c]
ethanol	366	414 ± 15	395	400 ± 2
glycerol	107	87 ± 3	96	36 ± 1
acetic acid	3	1 ± 0	8	7 ± 0
pyruvic acid	2	4 ± 1	1	<0.1
2-oxoglutaric acid	0.4	55 ± 0	53	64 ± 0
succinic acid	2	17 ± 2	17	18 ± 0
2-hydroxyglutaric acid	ND	15 ± 2	15	12 ± 0
biomass, dry weight	90	92 ± 4	96	107 ± 1
Substrate used (mg/g glucose used)				
glutamic acid	–	106 ± 8	109	120 ± 3
Maximal specific growth rate (h^{-1})	0.45	0.33	NM	0.40[b]

ND—not detected, NM—not measured

[a]Means and standard deviations from six experiments

[b]Values from a single experiment

[c]Means and standard deviations from two experiments

conditions.

The laboratory strain was grown anaerobically in a bioreactor in order to compare it with the industrial strain previously used [4]. The yields were determined and are shown in Table. 2.1. The recovery for this culture of carbon and degree of reduction was 97.7 % and 97.3 %, respectively. The yield of carbon dioxide (1.66 mol/mol glucose), used in the recovery calculations, was experimentally determined. The product yields were very similar, with the exception of the yields of acetic and pyruvic acids.

Pyruvate is connected to the formation of both acetate and ethanol via acetaldehyde, formed by pyruvate decarboxylase (*PDC* genes), see Fig. 2.2. Aldehyde dehydrogenases (*ALD* genes) are responsible for acetate formation and alcohol dehydrogenases, mainly adh1p, for ethanol production. There exist, for both aldehyde and alcohol dehydrogenases, also other putative ORFs in the yeast genome that may be catalytically active. One may think of two scenarios resulting in the

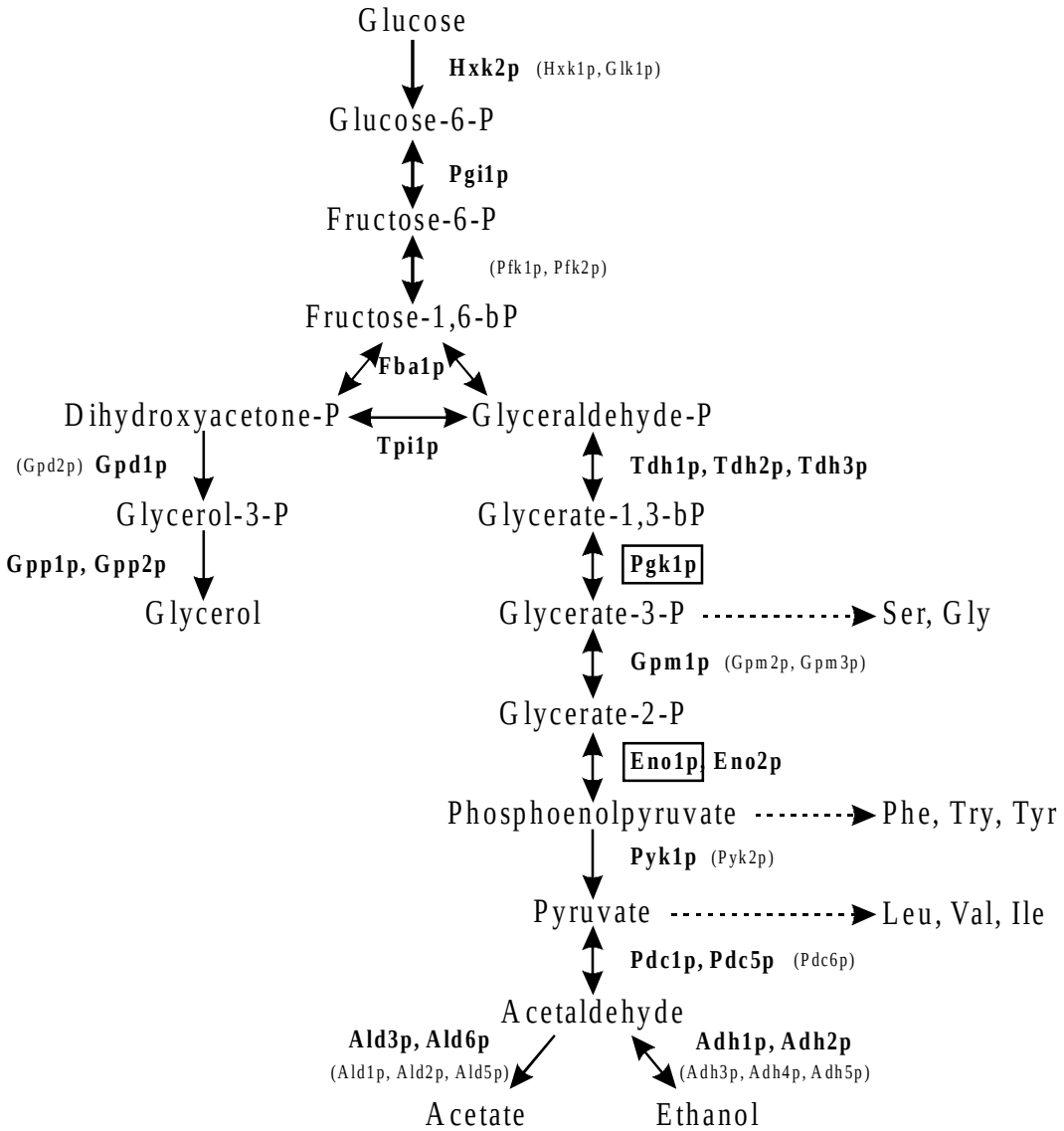

Fig. 2.2 Glycolysis in *Saccharomyces cerevisiae* marked with the enzymes which catalyse each step. The enzymes in bold are identified in the 2D gel pattern and the ones in brackets are not so far identified. The enclosed enzymes are downregulated during aerobic growth of the laboratory strain VW K43 on glutamate as the nitrogen source, compared to ammonium as the nitrogen source. Some amino acids formed from glycolytic precursors are also indicated.

present pattern of product formation. First, if the proportion of the total activity of pyruvate decarboxylase contra alcohol dehydrogenase is high, relatively more acetaldehyde may be exposed for the aldehyde dehydrogenase and converted to acetate. Secondly, if the activity of alcohol dehydrogenase contra aldehyde dehydrogenase is low or if the isoenzymes of aldehyde dehydrogenase in the actual strain are more effective, more acetate is formed. In enological fermentations it is

well known that the level of acetate formation is strain dependent [9,10]. Furthermore, it has been shown that a single gene could explain this strain-dependent difference [10].

The difference between anaerobic growth and respiro-fermentative growth was in agreement with what could be expected. The biomass yield on glucose is higher during respiro-fermentative growth than during anaerobic growth. Hence, more glutamate is used and more 2-oxyglutaric acid is formed. Glycerol formation is anaerobically important as a sink for surplus formation of NADH [8]. In the presence of oxygen, however, NADH can be reoxidised with oxygen as terminal electron acceptor, and a lower yield of glycerol is to be expected during aerobic conditions.

The protein pattern of aerobically grown *S. cerevisiae* (VW K43) with either ammonium or glutamate as the nitrogen source was compared. The protein spots on 2D gels from experiments with ammonium and glutamate grown cells were matched, giving about 500 spots, the intensity of which could be compared. Spots with intensity more than twofold different for the different nitrogen sources were then identified. With this criterion, 8 proteins were found in significantly higher concentrations, and 10 were found in significantly lower concentrations, during growth on glutamate compared to growth on ammonium. Only three of these interesting proteins have so far been identified. One identified protein, up-regulated during growth on glutamate, is a breakdown product of Adh1p. However, both the native Adh1p and Adh2p, had the same expression levels for growth on both nitrogen sources. Most of the enzymes in the glycolysis have been identified in the gels (Fig. 2.2), and two proteins, down-regulated during growth on glutamate, were found in glycolysis; Eno1p and, most probably, a modified Pgk1p. There were indications that also the native Pgk1p is decreased during growth on glutamate. All other identified glycolytic enzymes remained at practically the same expression level for growth on glutamate and ammonium. Perhaps surprisingly, neither the glutamate dehydrogenase (Gdh1p) nor glutamine synthetase (Gln1p), both essential enzymes in the glutamate/ammonium metabolism, were significantly changed.

Why are then Eno1p and probably a modified form of Pgk1p down-regulated during growth on glutamate compared to growth on ammonium salt as the nitrogen source? One may speculate that this may be due to altered branch-flows needed for synthesis of the amino acids formed from precursors of glycolysis (Fig. 2.2) relative to the flow of glycolysis for the very different requests of TCA cycle intermediates during growth on glutamate compared to ammonium. Alternatively, the down-regulation may serve the purpose of keeping the intracellular level of the metabolite pools of glycolysis constant or to alter the pool sizes. It may be that the down-regulation is merely a result of a changed flux of glycolysis, or it may also be that the proportion of these enzymes in the relation to their isoenzymes governs control on the glycolytic flux during these conditions.

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References

1. Cooper, T.G. (1982) Nitrogen metabolism in *Saccharomyces cerevisiae*. In *The molecular biology of the yeast* Saccharomyces cerevisiae, (Strathern, J.N., Jones, E.W. and Broach, J.R., Eds.), pp. 39–99. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA.

2. Jones, E.W. and Fink, G.R. (1982) Regulation of amino acid and nucleotide biosynthesis in yeast. In *The molecular biology of the yeast* Saccharomyces cerevisiae, (Strathern, J.N., Jones, E.W. and Broach, J.R., Eds.), pp. 181–299. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA.

3. Albers, E., Larsson, C., Lidén, G., Niklasson, C. and Gustafsson, L. (1996) Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. *Appl. Environ. Microbiol.* **62**, 3187–3195.

4. Albers, E., Gustafsson, L., Niklasson, C. and Lidén, G. (1998) Distribution of ^{14}C-labelled carbon from glucose and glutamate during anaerobic growth of *Saccharomyces cerevisiae*. *Microbiology* **144**, 1683–1690.

5. Norbeck, J. and Blomberg, A. (1997) Two-dimensional electrophoretic separation of yeast proteins using a non-linear wide range (pH 3–10) immobilized pH gradient in the first dimension; reproducibility and evidence for isoelectric focusing of alkaline (pI > 7) proteins.*1*

9. Garcia Moruno, E., Delfini, C., Pessione, E. and Giunta, C. (1993) Factors affecting acetic acid production by yeasts in strongly clarified grape musts. *Microbios* **74**, 249–256.

10. Giudici, P. and Zambonelli, C. (1992) Biometric and genetic study on acetic acid production for breeding of wine yeast. *Am. J. Enol. Vitic.* **43**, 370–374.