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Overexpression of alpha-ketoglutarate dehydrogenase in *Yarrowia lipolytica* and its effect on production of organic acids

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Abstract The yeast *Yarrowia lipolytica* is one of the most intensively studied "non-conventional" yeast species. Its ability to secrete various organic acids, like pyruvic (PA), citric, isocitric, and alpha-ketoglutaric (KGA) acid, in large amounts is of interest for biotechnological applications. We have studied the effect of the alpha-ketoglutarate dehydrogenase (KGDH) complex on the production process of KGA. Being well studied in *Saccharomyces cerevisiae* this enzyme complex consists of three subunits: alpha-ketoglutarate dehydrogenase, dihydrolipoyl transsuccinylase, and lipoamide dehydrogenase. Here we report the effect of overexpression of these subunits encoding genes and resulting increase of specific KGDH activity on organic acid production under several conditions of growth limitation and an excess of carbon source in *Y. lipolytica*. The constructed strain containing multiple copies of all three KGDH genes showed a reduced production of KGA and an elevated production of PA under conditions of KGA production. However, an increased activity of the KGDH complex had no influence on organic acid production under citric acid production conditions.

Keywords Alpha-ketoglutarate dehydrogenase · Alpha-ketoglutaric acid · Pyruvic acid · Isocitric acid · Citric acid · *Yarrowia lipolytica*

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Introduction

Due to its ability to utilize a wide spectrum of different substrates, like hydrophobic substrates, ethanol, glycerol and glucose, and its capability to secrete high amounts of metabolites and proteins, the ascomycetous yeast *Yarrowia lipolytica* is an important microorganism with relevance for biotechnological applications. Protein expression, dimorphism, genetics, and physiology as well as hydrophobic substrate utilization and biotechnological applications were well reviewed (Barth and Gaillardin 1996, 1997; Barth et al. 2003; Fickers et al. 2005; Finogenova et al. 2005; Bankar et al. 2009). The yeast *Y. lipolytica* is unique in its ability to secrete high amounts of a broad range of organic

acids (up to 250 gL^{-1}) including intermediates of the tricarboxylic acid cycle (TCA), citric (CA), isocitric (ICA), and alpha-ketoglutaric acid (KGA), as well as pyruvic acid (PA). The production of these acids depends on the availability of the carbon source (e.g. plant oils, fats, glycerol, ethanol, molasses and starch hydrolysates, alkanes, and glucose) and nutrition factors, like N-source, thiamine, or the mineral salt components P, S or Mg. Under excess of carbon source nitrogen exhaustion triggers secretion of CA and ICA, while limitation by thiamine at low pH values results in the secretion of mainly alpha-ketoglutarate and pyruvate (Stottmeister et al. 1982; Barth and Gaillardin 1996, 1997; Aurich et al. 2003; Mauersberger et al. 2003; Finogenova et al. 2005; Kamzolova et al. 2008). Previous studies revealed that changes in enzyme activities of TCA enzymes can influence the amounts and ratios of produced organic acids in *Y. lipolytica*. Particularly with regard to the production of CA and ICA, the involvement of different TCA and glyoxylate cycle enzymes in the product formation was shown for selected mutants (Akiyama et al. 1973; Stottmeister et al. 1982; Ermakova et al. 1986; Finogenova et al. 1986, 1991, 2002, 2005; Il'chenko et al. 2002; Kamzolova et al. 2008). In previous studies, we have demonstrated that a high-level expression of aconitase (ACO) and isocitrate lyase (ICL) caused by amplification of the encoding genes and vice versa a loss of the ICL changes the CA/ICA ratio on different carbon sources (Förster et al. 2007a, b; Holz et al. 2009). Therefore we investigated the influence of further TCA and glyoxylate cycle enzymes with regard to the production of organic acids by *Y. lipolytica*. Here we present the results of our study on the effects of changes in activity of alpha-ketoglutarate dehydrogenase (KGDH) which catalyzes the oxidative decarboxylation of KGA to succinyl-coenzyme A and carbon dioxide. In *Saccharomyces cerevisiae*, KGDH is a mitochondrial macromolecular multienzyme complex consisting of three components: alpha-ketoglutarate dehydrogenase (Kgd1p), dihydrolipoyl transsuccinylase (Kgd2p), and lipoamide dehydrogenase (Lpd1p). Each of these components is present in multiple copies in this macromolecular complex (Ross et al. 1988; Repetto and Tzagoloff 1989, 1990, 1991; Huh et al. 2003). Whereas Kgd1p and Kgd2p are exclusive components of KGDH, Lpd1p forms also an essential component of the glycine decarboxylase and pyruvate dehydrogenase complexes (Pronk et al. 1996). Repetto and Tzagoloff (1991) examined the assembly of the KGDH complex and revealed that a regulated and balanced production of Kgd1p and Kgd2p is relevant for attaining the correct subunit stoichiometry during complex assembly.

In this study, we wanted to reveal how overexpression of all subunits together influences enzyme activity of the KGDH complex in *Y. lipolytica*. To understand whether the alpha-ketoglutarate dehydrogenase activity plays a role in organic acid production in *Y. lipolytica* we studied enzyme activity of KGDH and organic acid production in wild-type and transformant strains harboring multiple copies of all three genes.

Methods and materials

Strains, media and growth conditions

The *Y. lipolytica* strains H222 (*MATA*, wild type; Barth and Gaillardin 1996), the recipient strain H222-S4 (*MATA ura3-302::pXPR2-ScSUC2*; Mauersberger et al. 2001), the strain E150 (*MATB his-1 leu2-270 ura3-302 xpr2-322;* Barth and Gaillardin 1996), and the newly constructed *KGD1, KGD2, LPD1* multicopy transformant H222-MH1 (*MATA ura3-302::ScSUC2 ura3d4 KGD1*mc, *KGD2*mc, *LPD1*mc, mc for multicopy) as well as for cloning the *E. coli* strain DH5αc were used in this study. For cultivation 500-ml shaking flasks were used. The strains were grown at 28 °C and 220 rpm in 20 to 30 ml complete medium YPD (Barth and Gaillardin 1996) or in 100 to 200-ml minimal medium Mg (Mauersberger et al. 2003), MpC [medium for citric acid production] (Mauersberger et al. 2003) or MpA [medium for alpha-ketoglutaric acid production] (Weissbrodt et al. 1989) containing (a) mineral salts: 5 gL^{-1} NH_4Cl, 2 gL^{-1} KH_2PO_4, 1 gL^{-1} $MgSO_4 \times 7\ H_2O$, 10 mgL^{-1} $FeSO_4 \times 7\ H_2O$, 44 mgL^{-1} $ZnSO_4 \times 7\ H_2O$, 60 mgL^{-1} $CaCl_2$; (b) trace elements: 0.04 mgL^{-1} $CuSO_4 \times 5\ H_2O$, 0.4 mgL^{-1} $MnSO_4 \times 4\ H_2O$, 0.2 mgL^{-1} $FeCl_3 \times 6\ H_2O$, 0.2 mgL^{-1} $Na_2MoO_4 \times 2\ H_2O$, 0.4 mgL^{-1} $ZnSO_4 \times 7\ H_2O$, 0.1 mgL^{-1} KI and 0.5 mgL^{-1} H_3BO_3; (c) vitamin: 3.5 μgL^{-1} thiamine hydrochloride (0.05 mgL^{-1} in preculture) added in 0.5 $\mu g\ L^{-1}$ aliquot in intervals of 24 h. The pH of the final medium M was 3.5–4.0. The concentration of the carbon sources was 1–2% (in Mg) and 10% (in Mp). During cultivation, the pH was adjusted using 10 N NaOH.

For cultivation in a bioreactor (1.4 L Multifors, Infors HT, Bottmingen Switzerland) the strains were grown at 30 °C in 600-ml production medium (Weissbrodt et al. 1989, modified) containing: (a) mineral salts: 6,17 gL^{-1} $(NH_4)_2SO_4$, 2 gL^{-1} KH_2PO_4, 1 gL^{-1} $MgSO_4 \times 7\ H_2O$, 10 mgL^{-1} $FeSO_4 \times 7\ H_2O$, 44 mgL^{-1} $ZnSO_4 \times 7\ H_2O$, 60 mgL^{-1} $CaCl_2$; (b) trace elements as described above; (c) vitamin: 0.020 mgL^{-1} thiamine hydrochloride. After a growth phase the production phase was started by changing pH from pH 5.0 to 3.8 and oxygen saturation rate from 50% to 10%. During cultivation the pH was regulated with 32% NH_4OH. The concentration of glycerol was 12% at the beginning of fermentation.

Vector construction

Based on the multicopy integrative plasmid p64PT (Gerber 1999) the vectors p64KGD1, p64KGD2, and p64LPD1

were constructed for multiple integration of all three genes of alpha-ketoglutaric acid dehydrogenase (*KGD1*, *KGD2*, *LPD1*) in the yeast genome. Therefore p64PT was treated with *Apa*I and religated after elimination of the 2-kb *Apa*I-fragment containing the major part of *pICL1D*. The new vector, called p64T, served as basic vector for vector construction. DNA from *Y. lipolytica* strain E150 was used as template for PCR amplification (CombiZyme DNA Polymerase, Invitek GmbH, Berlin). A 5.77-kb fragment of the *KGD1* gene with promoter and terminator regions was amplified by PCR with the primer pair KGD1for (ATATAAGATCTGCATGTACGTGTCATCTGTCC, creating the new underlined *Bgl*II restriction site on one end of the fragment) and KGD1rev (ACCACTCTACCTGCA CAACC, located directly downstream of the *Bgl*II site in the terminator). The primer pair KGD2for (ATATAA GATCTATCGCTCCTGGTACTGTACG, creating the new underlined *Bgl*II restriction site on one end of the fragment) and KGD2rev (ATATAAGATCTATGTCC GACTACCACGATGC, creating the new underlined *Bgl*II restriction site on the other end of the fragment) were used for amplification of a 3.16-kb fragment of the *KGD2* gene with promoter and terminator regions. A 3.78-kb fragment of the *LPD1* gene with promoter and terminator regions was amplified with the primer pair LPD1for (ATATACATATGCTAACTCGGTCTCCTGGTTC, creating the new underlined *Nde*I restriction site on one end of the fragment) and LPD1rev (ATATAACGCGTGCT TAACACGCAGAACGTCG, creating the new underlined *Mlu*I restriction site on the other end of the fragment). The *Bgl*II-treated *KGD1* and *KGD2* fragments were each ligated with the purified vector fragment obtained from p64T after *Bgl*II digestion. The *Nde*I- and *Mlu*I-treated *LPD1* fragment was ligated with the purified vector fragment obtained from p64T after *Nde*I and *Mlu*I digestion. For each vector plasmids without any amino acid exchange in the respective ORF were selected. These multicopy vectors p64KGD1, p64KGD2, and p64LPD1 contained the promoter truncated, defective *ura3d4* allele as multicopy selection marker, a rDNA sequence as integration target sequence and the complete expression cassette for either *KGD1*, *KGD2*, or *LPD1*, as previously described for the respective *ICL1* multicopy vectors (Juretzek et al. 2001; Förster et al. 2007a, b).

Transformation

Integrative transformation of the *Y. lipolytica* strain H222-S4 was performed by the lithium acetate method (Barth and Gaillardin 1996). The plasmids p64KGD1, p64KDG2, and p64LPD1 were digested by *Sac*II before transformation.

Chromosomal DNA preparation and southern blotting

Chromosomal DNA was prepared according to Hoffmann and Winston (1987) and digested with *Xba*I. For Southern blotting, 800-ng digested and separated DNA samples were analyzed after vaccum blotting to Hybond-N membranes using the Gene Images^{TM} CDP-Star^{TM} Detection Module and Hyperfilm^{TM} ECL^{TM} (GE Healthcare Life Sciences, Germany). The *KGD1* DNA probe containing the *Van91*I fragment of the *KGD1* gene (1.7 kb) was prepared from plasmid p64KGD1, *KGD2* DNA probe from p64KGD2 (2.5-kb *KGD2*-containing *Bgl*II/*Xba*I fragment), and *LPD1* DNA probe from p64LPD1 (3.5-kb *LPD1*-containing *Mlu*I/*Xba*I fragment). All DNA probes were labeled with flourescein using the Gene Images^{TM} Random-Prime Labelling Module (GE Healthcare Life Sciences, Germany).

Enzyme assay

Activity of alpha-ketoglutaric acid dehydrogenase was determined in cell-free supernatant fractions by a spectrophotometric assay according to Brown and Perham (1976). Cell-free supernatants were prepared as described in Holz et al. (2009), using 0.5 M potassium phosphate buffer, pH 7.4, as cell disruption buffer. Protein content was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Analytical determination of organic acids

The determination of organic acid content in cell-free samples of the fermentation broth was carried out using the DX320 ion chromatography system (column IonPac AS19, Dionex, Sunnyvale, CA, USA). The ion separation was carried out under following conditions: sample injection volume, 10 µl; eluent flow rate, 0.3 mlmin^{-1}; KOH eluent gradients, 5-mM hold for 25 min, 5–38 mM in 9 min, 38-mM hold for 2 min, 38–5 mM in 6 min. The organic acids were quantified using calibration curves with the software Chromeleon 6.8 (Dionex, Sunnyvale).

Determination of glycerol was performed using a commercial enzymatic test kit (No. 10148270035, R-Biopharm, Germany).

Results

Bioinformatical analysis of alpha-ketoglutarate dehydrogenase genes in *Y. lipolytica*

In *S. cerevisiae*, as well as in *Escherichia coli*, the alpha-ketoglutarate dehydrogenase complex consists of three subunits (Repetto and Tzagoloff 1989). Alpha-

ketoglutarate dehydrogenase subunit is encoded by the gene *KGD1* (YIL125w) and dihydrolipoyl transsuccinylase by *KGD2* (YDR148c; Repetto and Tzagoloff 1989, 1990, 1991; yeastgenome.org). Lpd1p, which is also part of pyruvate dehydrogenase complex and glycine decarboxylase in *S. cerevisiae*, is encoded by the gene *LPD1* (YFL018c; Pronk et al. 1996; yeastgenome.org). These three subunits are characterized as mitochondrial-located proteins (Huh et al. 2003; Reinders et al. 2007).

In *Y. lipolytica*, three genes with highly similar sequences to *KGD1*, *KGD2* and *LPD1* were found after analysis of *Y. lipolytica* genome database (Consortium Génolevures: http://cbi.labri.fr/Genolevures/; Casaregola et al. 2000; Dujon et al. 2004). The intron-containing gene YALI0E33517g encodes a putative Kgd1p gene product of 1,004 amino acids, exhibiting 67% homology to ScKgd1. A putative Kgd2p gene product of 447 amino acids is encoded by the gene YALI0E16929g, which shows 60% homology to ScKgd2. Furthermore, there is the intron-containing gene YALI0D20768g encoding a putative gene product of 499 amino acids and exhibiting a 63% homology to ScLpd1. According to the specific analysis program MitoProt (Claros and Vincens 1996), all deduced proteins contain N-terminal mitochondrial-targeting signal sequences of 29 (Kgd1p), 61 (Kgd2p), or 32 amino acids (Lpd1p). These *Y. lipolytica* genes were chosen for the overexpression of alpha-ketoglutarate dehydrogenase in *Y. lipolytica* and referred to as *KGD1*, *KGD2* and *LPD1* hereafter.

Overexpression of alpha-ketoglutarate dehydrogenase

To examine whether an increase of KGDH expression level influences the enzyme activity and the production of organic acids in *Y. lipolytica*, a recombinant *Y. lipolytica* strain was constructed containing multiple copies of *KGD1*, *KGD2* and *LPD1*. As described in Material and methods, the integrative multicopy vectors p64KGD1, p64KGD2, and p64LPD1 were constructed by integration of the PCR-amplified ORF of the appropriate gene as well as 5′ upstream and 3′ downstream areas of approximately 1 kb each in the recipient vector p64T. For transformation in the recipient *Y. lipolytica* strain H222-S4 (*ura3-302*), these vectors were linearized by *Sac*II. Afterwards, the prototrophic transformant H222-MH1 was obtained. Southern blot analysis evidenced the multiple integration of each vector, additionally to the single genomic copies of *KGD1*, *KGD2* and *LPD1* which are present in all strains (Fig. 1a, b). Due to similar intensities of detected vector-related signals we assume that an equal copy number of each plasmid was integrated in the genome of recipient strain H222-S4.

Furthermore, alpha-ketoglutarate dehydrogenase activity was determined in the transformant H222-MH1 and wild-type strain H222 to prove whether the multiple integration of *KGD1*, *KGD2* and *LPD1* have an influence on KGDH expression level. The strains were cultivated on 2% glucose or glycerol as carbon sources in minimal medium Mg under conditions for normal growth (no thiamine-exhaustion). Measured KGDH activities after 8 h of cultivation are shown in Fig. 1c. During growth in glucose 1.8- to 2.1-fold higher KGDH activities in transformant H222-MH1 were determined in comparison to the wild-type strain (Fig. 1c). The highest KGDH activities were measured in the early growth phase between 8 and 14 h for H222-MH1 and the wild-type strain (0.2 Umg^{-1} in H222, 0.4 Umg^{-1} in H222-MH1). However, the maximal KGDH activities of glycerol-grown cells were comparable to the activities in glucose-cells of H222-MH1 and H222, respectively.

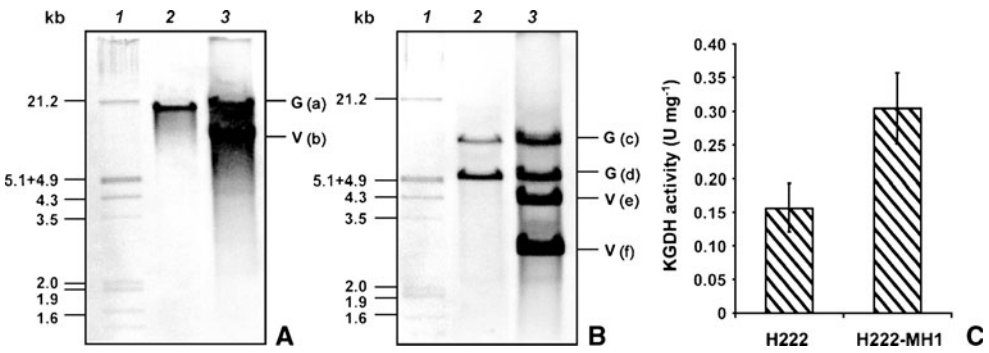

Fig. 1 Southern blot analysis (**A** and **B**) and KGDH activity (**C**) of *Y. lipolytica* wild-type strain H222 and the *KGD1, KGD2, LPD1* multicopy integrative transformant H222-MH1 evidenced high-copy integration of the *KGD1, KGD2, and LPD1* containing vector in the multicopy transformant. Genomic DNA was digested with *Xba*I and probed with specific fragments of *KGD1* (**A**), *KGD2* and *LPD1* (**B**). *1* Molecular weight standard, λ-DNA *Eco*RI/*Hin*dIII digested, *2* H222, *3* H222-MH1. 16.2-kb fragment of single copy genomic *KGD1* (*a*); 8.5-kb *KGD1* containing fragment of the integrated vector p64KGD1 (*b*); 5.1-kb fragment of single copy genomic *KGD2* (*d*); 2.7-kb *KGD2* containing fragment of the integrated vector p64KGD2 (*f*); 8.0-kb fragment of single copy genomic *LPD1* (*c*); 4.1-kb *LPD1* containing fragment of the integrated vector p64LPD1 (*e*). **C** KGDH activity during cultivation in minimal medium Mg with 2% glucose in wild-type strain H222 and *KGD1*, *KGD2*, *LPD1* multicopy transformant H222-MH1 after 8 h of cultivation in shaking flasks

Organic acid production

To examine whether the elevated KGDH expression level also influences the production of organic acids in *Y. lipolytica*, the *KGD1*, *KGD2*, *LPD1* multicopy transformant H222-MH1, and the wild-type strain H222 were cultivated in shaking flasks and in bioreactors under the conditions for CA or KGA production. The production of KGA was initiated by thiamine-exhaustion (starting concentration 20 $\mu g L^{-1}$ in bioreactors) under excess of the carbon source glycerol (Weissbrodt et al. 1989). In Fig. 2, a normal course of KGA production during cultivation in bioreactors is shown. KGA and PA production started when cells passed from logarithmic to stationary growth phase after shift of pH to 3.8 and pO_2 to 10%. The production of PA rose faster than KGA production at the beginning of organic acid production, but after 46 h of cultivation PA amounts declined until the end of fermentation. The most striking results of these KGA production experiments were the reduction of produced KGA and the increase of produced PA amounts for multicopy transformant H222-MH1 in contrast to the wild-type strain H222. KGA amounts up to 97 gL^{-1} could be determined in the cultivation broth of H222 and up to 72 gL^{-1} for the transformant H222-MH1 after 114 h of fermentation (Fig. 2). The volumetric rate of product formation (Q_{KGA}) was reduced to 76% (0.9 $gL^{-1}h^{-1}$ for H222 and 0.7 $gL^{-1}h^{-1}$ for H222-MH1) and the specific rate of product formation (q_{KGA}) for KGA to 74% in the transformant (0.035 $gg^{-1}h^{-1}$) compared to the wild-type strain (0.047 $gg^{-1}h^{-1}$). Furthermore, PA production was increased up to 128% in H222-MH1 compared to H222. We determined PA amounts of 66 gL^{-1} and 3.95 g g^{-1} for H222-MH1 and 52 gL^{-1} and 3.06 gg^{-1} for H222. The PA amounts detected in the cultivation broth of H222-MH1 rose faster at the beginning of fermentation and declined slower until the end than these of H222. At the end of fermentation, the detected PA in the cultivation broth of H222-MH1 was still 1.7 times higher than PA amounts of H222. We also determined KGDH activity during cultivation in bioreactor. In Fig. 3, the specific KGDH activity of H222 and H222-MH1 after 12 (growth phase) and 61 h (production phase) of fermentation are shown. Comparable to the detected KGDH activities under normal growth conditions (medium Mg) H222-MH1 developed 1.7 to 1.9 times higher activities than H222. After 61 h of cultivation, specific KGDH activities in both strains were approximately 5 times higher than KGDH activities after 12 h.

Apart from the influence on KGA production, we also examined whether there is any influence on CA and ICA production by the elevated KGDH expression level. Wild-type strain H222 and transformant H222-MH1 were cultivated in shaking flasks under the condition of CA and ICA production (medium MpC) from excess of the carbon source (10%) initiated by N-exhaustion (Mauersberger et al. 2003; Förster et al. 2007a, b; Holz et al. 2009). Comparable to our previous studies a CA/ICA ratio of 89–90% CA and 10–11% ICA on glucose as carbon source was detected for the wild-type strain H222. Maximal CA amounts of 42 to 45 gL^{-1} and 5.6 to 6.1 gL^{-1} ICA were produced by H222 after 216 to 240 h of cultivation. Transformant H222-MH1 showed comparable amounts of CA and ICA (42 to 44 gL^{-1} CA and 4.6 to 5.3 gL^{-1} ICA) and CA/ICA ratio (89–90%) after 216 to 264 h of cultivation.

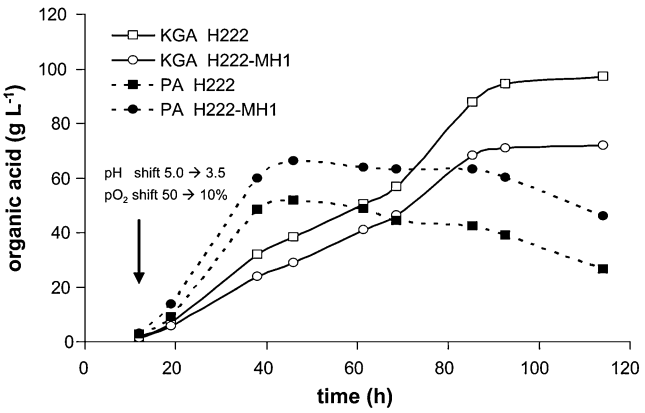

Fig. 2 Kinetics of organic acid production (KGA and PA) for the wild-type strain H222 and the *KGD1*, *KGD2*, *LPD1* multicopy transformant H222-MH1 with glycerol as substrate. Cultivation in 1.4-L bioreactors with 600 ml production medium for AKG production. *Arrow* indicates shift of pH and pO_2 values, carried out after 12 h cultivation associated with start of KGA and PA accumulation

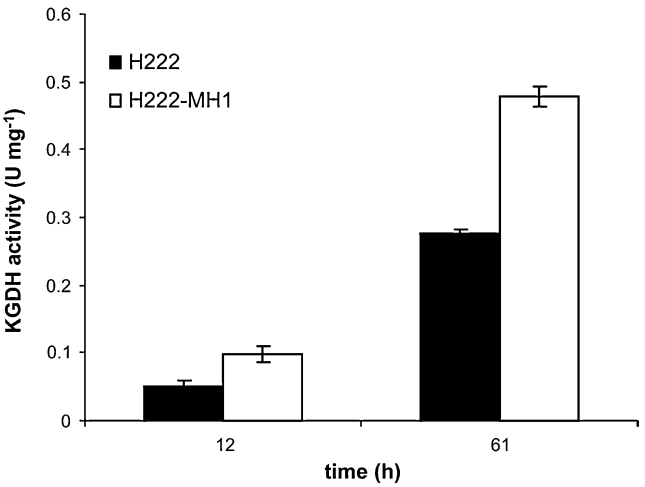

Fig. 3 KGDH activity of *H222* and *H222-MH1* during cultivation in bioreactor under KGA production conditions with glycerol as carbon source after 12 h (growth phase) and 61 h (production phase) of cultivation

Discussion

Overexpression of alpha-ketoglutarate dehydrogenase

In this study, we have demonstrated that the gene products of *KGD1* (YALI0E33517g), *KGD2* (YALI0E16929g), and *LPD1* (YALI0D20768g) showing the highest homology to the *S. cerevisiae* alpha-ketoglutarate dehydrogenase genes *ScKGD1* (YIL125w), *ScKGD2* (YDR148c), and *ScLPD1* (YFL018c) are obviously encoding a KGDH complex functioning in the mitochondrial TCA cycle in *Y. lipolytica*. The increased copy numbers of these three genes in the multicopy transformant H222-MH1 obtained in this study caused a 1.7- to 2.1-fold increase in the total KGDH activity due to a gene–dose effect, thus leading to the conclusion that these genes actually encode the KGDH complex. In previous studies, the overexpression of *Y. lipolytica* genes encoding aconitase (*ACO1*) and isocitrate lyase (*ICL1*) through integrative transformation of the gene-containing multicopy plasmid resulted in 8 to 15 times higher enzyme activities (Förster et al. 2007b; Holz et al. 2009). As described in Juretzek et al. (2001), at least 10–12 copies of the defective *ura3d4* marker gene, which is part of the integrative plasmid, are necessary for full complementation of the *ura3* gene disruption in the recipient strain. In contrast to the overexpression of *ACO1* and *ICL1*, KGDH overexpression requires a simultaneous overexpression of the genes *KGD1*, *KGD2* and *LPD1* being located on separate plasmids. For complementation of the uracil auxotrophy a lower copy number of each multicopy plasmid-containing *ura3d4* is sufficient which leads to a lower increase in enzyme activity in comparison to previous overexpression experiments. Furthermore, the complete size of *KGD1*, *KGD2* and *LPD1* promoter areas in *Y. lipolytica* is not known until now. In this study, a 5′ upstream region harboring the putative promoters with a length of approximately 1 kb was chosen for overexpression of KGDH genes. In previous studies it was shown that 5′ upstream regions of approximately 1 kb can be sufficient for gene expression in *Y. lipolytica*, e. g., for *ACO1* (Holz et al. 2009) or *XPR2* (Nicaud et al. 1989), but it was also demonstrated for *ICL1* that larger upstream regions are necessary for full expression (Juretzek et al. 2001). To achieve a possible improvement of expression of KGDH genes, an intensive study of their promoter elements would be necessary. Another possible explanation for the only slight increase of KGDH activity would be an unbalanced rate of produced KGDH subunits. As described in Repetto and Tzagoloff (1990, 1991), the assembly of the KGDH complex seems to be dependent on a regulated and balanced production of Kgd1p and Kgd2p and the correct subunit stoichiometry during complex assembly. They revealed that overproduction of Kgd2p relative to Kgd1p resulted in a predominance of incompletely assembled complexes leading to a decrease of KGDH activity whereas an excess of Kgd1p leads to increased enzyme activity. It is not clear if this assembly mechanism in *Y. lipolytica* is similar to that in *S. cerevisiae*, but it should still be considered that an overexpression of *KGD1* and *KGD2* by integration of separate plasmids in the genome of *Y. lipolytica* may cause an imbalance in Kgd1p and Kgd2p molecule amounts and thus might also lead to a disturbed complex assembly and effects on enzyme activity.

Organic acid production

Furthermore, we demonstrated in this study that the selective increase in KGDH activity in *KGD1*, *KGD2*, *LPD1* multicopy transformant *Y. lipolytica* H222-MH1 significantly changes the produced amounts of KGA and PA. In comparison to wild-type strain *Y. lipolytica* H222, the KGA production was decreased by 25.8%, but PA production was increased 1.3-fold. The changes in organic acid production observed in this study are possibly related to an influence of the thiamine availability on account of the overproduction of thiamine-dependent enzyme complex KGDH in H222-MH1. As described in several publications dealing with KGA production in *Y. lipolytica*, this yeast is unable to synthesize thiamine and therefore requires the addition of the vitamin to the medium. Under conditions of thiamine deficit, *Y. lipolytica* produces KGA and PA. This effect is traced to the thiamine-dependence of the PA- and KGA-metabolizing enzymes pyruvate dehydrogenase (PDH) and KGDH. A deficit of thiamine presumably leads to a reduction of enzyme activity and thus to an overflow of PA and KGA (Stottmeister et al. 1982; Barth and Gaillardin 1996; Kim 1999; Chernyavskaya et al. 2000; Mauersberger et al. 2003; Finogenova et al. 2005). In this study, the overexpression of KGDH genes *KGD1*, *KGD2* and *LPD1* along with an enhanced amount of functional KGDH molecules possibly caused a consumption of thiamine in favor of the KGDH complex molecules and to the disadvantage of the PDH complex molecules. Consequently, the amount of produced PA increased due to its decreased metabolization by PDH and the amount of produced KGA decreased possibly due to the minimized carbon flux from PA to KGA and due to the increased specific activity of KGDH complex. In the yeast *Torulopsis glabrata,* it was already shown that large amounts of PA can be accumulated, when the PA-converting enzymes PDH, pyruvate decarboxylase (PDC), and pyruvate carboxylase (PC) are limited due to the multi-vitamin auxotrophy of *T. glabrata* (Hua et al. 1999; Miyata and Yonehara 1999; Li et al. 2001; Huang et al. 2006; Zhang et al. 2009). However, carbon flux in *T. glabrata* can be redistributed from PA to KGA through manipulating the

specific activity of related enzymes by changing the contents of metabolic co-factors thiamine and biotin or by overexpression of the *PDC1* gene (Huang et al. 2006; Liu et al. 2007; Zhang et al. 2009). In *Y. lipolytica*, there was hitherto a lot of emphasis on improving KGA production. Several groups were able to increase KGA production by isolation of suitable mutants and by varying cultivation conditions (thiamine content, carbon source, pH, pO_2, nitrogen concentration) (Chernyavskaya et al. 2000; Il'chenko et al. 2002; Finogenova et al. 2005). But depending on the carbon sources used in cultivation-varying amounts of PA were detected. A maximal PA production of 50–61 gL^{-1} and a proportion of produced organic acid of up to 90% PA was described for *Y. lipolytica* strains cultivated with glucose or glycerol as carbon sources and whereas cultivation on n-alkane- and ethanol-containing media results in a prevailing production of KGA. (Stottmeister et al. 1982; Stottmeister and Hoppe 1991; Kim 1999; Chernyavskaya et al. 2000; Il'chenko et al. 2002; Finogenova et al. 2005). The *Y. lipolytica* strain H222-MH1 obtained in this study is due to the reduced KGA production obviously not suitable for an improvement of KGA production, but for a further focus on PA production with *Y. lipolytica*, this strain could be interesting because of the increased PA amounts. However, the main problems concerning PA production in *Y. lipolytica* are the re-uptake of produced PA during cultivation and the efficient manipulation of PA-converting enzymes. As described in this study, the overproduction of KGDH complex resulted in an increase of produced PA amounts, but KGDH overproduction might be improved by optimization of *KGD1* and *KGD2* copy numbers, the ratio of Kgd1p and Kgd2p molecules and variation of promoter lengths and thus may result in an enhanced shift of organic acid production from KGA to PA. On the other hand, research should be focussed on the enzymes PDH and PC which convert PA to acetyl coenzyme A and oxaloacetate. In *T. glabrata*, PDH and PC, but also PDC which seems not to be present in *Y. lipolytica* (no analogous gene was found in *Y. lipolytica* genome database), play an important role for PA production and can be influenced by variation of thiamine (PDH) and biotin (PC) contents (Hua et al. 1999; Miyata and Yonehara 1999; Li et al. 2001; Huang et al. 2006; Zhang et al. 2009). It must be regarded that *Y. lipolytica* shows thiamine auxotrophy, but in contrast to *T. glabrata* it is prototroph for biotin (Barth and Gaillardin 1997; Chernyavskaya et al. 2000; Il'chenko et al. 2002; Finogenova et al. 2005). Whereas PDH activity could be affected by optimization of thiamine contents, a change of PC activity might be only possible by manipulation of the PC-encoding gene *PYC1* (YALI0C24101g). Flores and Gancedo (2005) already reported the cloning and characterization of the PC-encoding gene *PYC1* in *Y. lipolytica* and the effects of *PYC1* deletion on growth. It was shown that the absence of PC activity does not inhibit growth in glucose-ammonium medium, but organic acid production was not examined until now.

We also demonstrated in this study that the selective increase in KGDH activity in *KGD1*, *KGD2*, *LPD1* multicopy transformant *Y. lipolytica* H222-MH1 does not change the produced amounts of CA, ICA and CA/ICA ratios with glucose or glycerol as carbon sources in comparison to the wild-type strain H222. In the study of Il'chenko et al. (2002), central metabolism enzymes of TCA and glyoxylate cycles under conditions of KGA and CA production were determined. Only low activities of KGDH were described on ethanol, glucose, and n-alkanes as carbon sources under nitrogen deficiency. A doubling of KGDH activity under these conditions might result in a still very low activity and therefore does not affect CA and ICA production significantly. For a better understanding of the processes in cells of *Y. lipolytica* H222-MH1 under N-limitation conditions, enzyme activities of further TCA and glyoxylate enzymes should be measured in further studies.

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