RESEARCH ARTICLE

Improving heterologous protein secretion at aerobic conditions by activating hypoxia-induced genes in Saccharomyces cerevisiae

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One sentence summary: Doubling recombinant protein production by activating hypoxia-induced genes, including UPC2 which encodes an essential transcriptional regulator Upc2p for sterol biosynthesis.

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ABSTRACT

Oxygen is important for normal aerobic metabolism, as well as for protein production where it is needed for oxidative protein folding. However, several studies have reported that anaerobic conditions seem to be more favorable in terms of recombinant protein production. We were interested in increasing recombinant protein production under aerobic conditions so we focused on Rox1p regulation. Rox1p is a transcriptional regulator, which in oxidative conditions represses genes induced in hypoxia. We deleted ROX1 and studied the effects on the production of recombinant proteins in Saccharomyces cerevisiae. Intriguingly, we found a 100% increase in the recombinant fungal α-amylase yield, as well as productivity. Varied levels of improvements were also observed for the productions of the human insulin precursor and the yeast endogenous enzyme invertase. Based on the genome-wide transcriptional response, we specifically focused on the effect of UPC2 upregulation on protein production and suggested a possible mechanistic explanation.

Keywords: ROX1; α–amylase; UPC2; lipid classes

INTRODUCTION

Baker’s yeast Saccharomyces cerevisiae is a well-established host platform for the production of fuels, chemicals, food ingredients and recombinant proteins (Chemler, Yan and Koffas 2006; Huang et al. 2008; Kaufman, Pipe and Griffith 2012; Martinez et al. 2012), due to many advantages such as a large knowledge base organized in many useful databases, easy culturing in cheap defined or rich media, well-established toolbox for genetic and environmental modifications, and the GRAS status (Generally Regarded As Safe).
Oxygen is important in normal aerobic cellular metabolism, as well as in oxidative protein folding. High oxygen consumption is usually seen when there is an increased demand for protein folding and disulfide bond formation (Tyo et al. 2012) as oxygen is needed as the electron acceptor during the oxidative protein-folding process. Around 25% of the total reactive oxygen species (Tu and Weissman 2004) are generated during protein folding, which may cause oxidative stress, contribute to accumulation of misfolded proteins and induce ER stress (Gasser et al. 2008).

Most of the oxygen-regulated genes can be classified into two groups: (i) aerobic genes that are regulated under normoxic conditions and (ii) anaerobic genes that are optionally expressed under hypoxia or anoxia, and are repressed under fully aerobic conditions (Zitomer and Lowry 1992; Balasubramanian, Lowry and Zitomer 1993; Kwast et al. 1997; Poyton 1999). In response to oxygen limitation, a wide range of transcriptional changes occur resulting in extensive changes in cellular protein levels and activities related to cell respiration, heme biosynthesis, lipid metabolism, cell membrane and cell wall structures etc. (Kwast et al. 1997; Poyton 1999; Siso et al. 2012). Two regulatory mechanisms have been identified in response to hypoxia (Zitomer, Carrico and Deckert 1997): (i) the heme-dependent response which is mediated by several trans-acting factors (including Hap1p, Hap2p/3/4/5 and Rox1p (GRIVELL 1993; Poyton 1999), and (ii) the heme-independent response which is part of a global stress response (e.g. regulated through Rox3p) (Zitomer, Carrico and Deckert 1997).

Rox1p is a heme-dependent transcriptional repressor that represses the transcription of many hypoxia-induced genes, under aerobic conditions (Kwast et al. 1997; Ter Linde et al. 1999; Kwast et al. 2002). When oxygen is present, heme is synthesized which binds to the transcriptional activator Hap1 to activate its target genes (Cerdan and Zitomer 1988; Creusot et al. 1988; Zhang and Guarente 1994b; Zitomer, Carrico and Deckert 1997), including ROX1. Induction of ROX1 transcription by the heme-Hap1 complex (Keng 1992) produces the transcription factor (TF) Rox1p that represses its target genes by binding to their cognate upstream regions (Lowry and Zitomer 1988; Balasubramanian, Lowry and Zitomer 1993; Deckert et al. 1995b). About one third of the anaerobically induced genes contain a putative Rox1p-binding site in their promoter regions (Kwast et al. 2002).

The general gene repressor Tup1p–Snf6p complex is required for Rox1p-dependent repression (Deckert et al. 1995a; Smith and Johnson 2000). Mot3p/Rox7p is also involved in the suppression of anaerobically induced genes functioning either independently of Rox1p, or synergistically (Sertil et al. 2003). Many Rox1p-regulated genes have been identified in S. cerevisiae, including ANB1, COX5b, CYC7, AAC3, HEM13, HMG1, HMG2, ERG11, CPR1, OLE1 and DAN/TIR (summarized in Kwast, Burke and Poyton 1998). At oxygen-limited conditions, heme level drops, and subsequently the heme binding to Hap1 is reduced, causing decreased Hap1 activation, which results in deactivation of the transcription of its targets, including ROX1 (Zhang et al. 1993; Zhang and Guarente 1994a; Zitomer, Carrico and Deckert 1997). Non-activation of ROX1 in oxygen-limiting conditions results thus in activation of genes (due to derepression) that are otherwise under Rox1p repression (Deckert et al. 1995b).

Upc2p is a transcriptional activator regulating two groups of anaerobically induced genes, the anaerobic cell wall manno-proteins genes DAN/TIR (DAN1, DAN2, DAN3, DAN4, TIR1, TIR2, TIR3, TIR4 and TIP1) and the sterol and sphingolipid biosynthetic genes (ERG1, ERG2, ERG3, ERG6, ERG8, ERG11, ERG12, ERG13, ERG25, LCBl, LCBl2) (Abramova et al. 2001; Vik and Rine 2001). Regulation of the DAN/TIR genes by Upc2p is achieved by the consensus anaerobic response element AR1 (within the promoters of the DAN/TIR genes), in concert with the Moxlp and MoxZp repressors (Abramova et al. 2001). Expression of the sterol and sphingolipid biosynthetic genes is regulated through the putative sterol regulatory element (Vik and Rine 2001). UPC2 contains two consensus Rox1p-binding sites (Kwast et al. 2002). Under normal aerobic conditions, repression of the above genes is attained through Rox1p repression on UPC2, together with other reported repressors, including Mox1p, MoxZp, Mox3p, Tup1p/Snf6p and Rox7p (Smith and Johnson 2000; Abramova et al. 2001; Sertil et al. 2003). The repression mechanisms have not been completely defined for all the mentioned genes; however, several mechanisms have been suggested, e.g. besides direct regulation by Upc2p, there are four putative Rox1p-binding sites on DAN1, TIR2 and ERG26 (Ter Linde and Steensma 2002) indicating the possibility for both direct and indirect regulation (via Upc2p) by Rox1p.

Previously, we showed that production of a heterologous a-amylase in yeast S. cerevisiae can be significantly improved in anaerobic batch fermentations (Liu et al. 2013). Similar improvements of protein production due to low oxygen availability have also been found on recombinant glucoamylase production in S. cerevisiae, as well as on human Fab fragment production in Pichia pastoris (Ruohononen et al. 1997; Baumann et al. 2007, 2011a). In this work, we studied the effects of Rox1p regulation on the production of recombinant a-amylase. By deleting ROX1, a 100% increase in amylase yield as well as productivity was achieved. A range of subsequent experiments, including genome-wide transcriptome analysis and lipid composition measurements, indicated that the strong upregulation of the sterol biosynthesis pathway through UPC2 regulation played an essential role in improving protein production in the ROX1 deletion strain.

MATERIALS AND METHODS

Plasmids, strains and media

All the plasmids and strains used in this study are listed in Table 1. All primers are provided in Table S1 (Supporting Information).

Saccharomyces cerevisiae CEN.PK 113–11C (MATa his3Δ1 ura 3–52 MAL2–8c SUC2) (Entian and Kötter 2007) was the host strain to construct the ROX1 deletion strain, roxlΔ (MATa his3Δ1 ura 3–52 MAL2–8c SUC2 roxl1::loxP·KanMX4·loxP) which was constructed by transforming the loxP·KanMX4·loxP fragment amplified from plasmid pUG6 (Güldener et al. 1996) using primer pairs ROX_KanMX_fw/ROX_KanMX_re, and selected on YPD+G418 plate (20 g l⁻¹ peptone, 10 g l⁻¹ yeast extract, 20 g l⁻¹ agar, 200 mg l⁻¹ G418 sulfate). All yeast transformations were performed following the standard lithium acetate method (Daniel Gietz and Woods 2002).

The amylase expression vector p426GPD-AlphaAmy was constructed in several steps: (i) amplification of the alpha factor leader sequence from pUC57-NativeInsulin using primer pairs lzh014/lzh016; (ii) amplification of the a-amylase cDNA from pYapAmy (Tyo et al. 2012) using primer pairs lzh018/lzh039; (iii) fusion of the alpha factor leader with the amylase DNA using primer pairs lzh014/lzh039; (iv) cloning the fused expression cassette into p426GPD (Mumberg, Müller and Funk 1995) after digestion with SpeI and EcoRI.

The yeast alpha factor leader sequence fused with the human insulin precursor (IP) protein (codon optimized for expression in yeast, File S1, Supporting Information) was synthesized
Table 1. Plasmids and strains used in this study.

<table>
<thead>
<tr>
<th>Plasmids or Strains</th>
<th>Relevant genotypes</th>
<th>References</th>
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<tbody>
<tr>
<td>p426GPD</td>
<td>Yeast integrative plasmid, GPD1 promoter, URA3 marker</td>
<td>Mumberg, Müller and Funk (1995)</td>
</tr>
<tr>
<td>p423CYC</td>
<td>Yeast integrative plasmid, CYC1 promoter, HIS3 marker</td>
<td>Mumberg, Müller and Funk (1995)</td>
</tr>
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<td>p426GPD with KOZAX sequence, alpha factor leader, and amylase gene</td>
<td>This study</td>
</tr>
<tr>
<td>p426GPD-Alphains</td>
<td>p426GPD with KOZAX sequence, alpha factor leader, and IP gene</td>
<td>This study</td>
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<td>p423CYC-upc2-1</td>
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<th>Relevant genotypes</th>
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<td>CEN.PK 113–11C</td>
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<td>(Entian and Kötter 2007)</td>
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<td>rox1Δ</td>
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<td>This study</td>
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and ligated on plasmid pUC57-NatIvInsulin by GATC. The IP expression plasmid p426GPD-Alphans was constructed by inserting the alpha factor leader plus IP cassette into p426GPD after digestion with SpeI and SalI. The upc2-1 overexpression plasmid p423CYC-upc2-1 was constructed by inserting the upc2-1 sequence (File S2, Supporting Information) between the Sall and BamHI restriction sites of vector p423CYC (Mumberg, Müller and Funk 1995). All the vectors were constructed by following standard molecular biology techniques using Escherichia coli DH5α (Bethesda Research Laboratories).

Medium SD-2xSCAA (20 g l−1 glucose, 6.9 g l−1 yeast nitrogen base minus amino acids, 2 g l−1 KH2PO4 (pH 6 by KOH), 190 mg l−1 Arg, 108 mg l−1 Met, 52 mg l−1 Tyr, 290 mg l−1 Ile, 440 mg l−1 Lys, 200 mg l−1 Phe, 1260 mg l−1 Glu, 400 mg l−1 Asp, 380 mg l−1 Val, 220 mg l−1 Thr, 130 mg l−1 Gly, 400 mg l−1 Leu, 40 mg l−1 Trp, 140 mg l−1 His, 1 g l−1 bovine serum albumin) for batch fermentation in bioreactors was prepared as described previously (Witturp and Benig 1994). For shake flask cultivations, medium SD-2xSCAA-sf was prepared by replacing the 2 g l−1 KH2PO4 with 100 mM phosphate buffer (mixture of Na2HPO4 and NaH2PO4, pH6.0). For cultivating strains transformed with two plasmids (URA3 and HIS3 based), the 140 mg l−1 His was excluded and the glucose concentration was 17 g l−1 instead of 20 g l−1. For invertase expression, medium SD-2xSCAA-suc was used instead of medium SD-2xSCAA-sf, which replaced the 20 g l−1 sucrose instead of glucose.

Growth, cultivations and quantification of α-amylase, IP and invertase

Bioreactor fermentation: fresh colonies were inoculated into 50 ml of SD-2xSCAA medium and grew in shake flasks for 24 h at 30°C, 200 rpm. The seed cultures were inoculated into bioreactors with an initial OD600 of 0.01. Fermentations were performed in DasGip 1.0-liter stirrer-pro vessels (Drescher Arnold Schneider, Germany) with a working volume of 500 ml. The temperature was controlled at 30°C, and the stirring was at 600 rpm. Dissolved oxygen was controlled above 30% with aeration of 1 vvm throughout the experiment. The pH was maintained at 6.0 by monitoring with the pH sensor (Mettler Toledo, Switzerland) and by adding 2M KOH. Five milliliters of cell culture was filtered through a 0.45-μm filter membrane and the increased weight of the dried filter corresponded to dry cell weight. One milliliter of culture was filtered through a 0.45-μm membrane and the clear supernatant was loaded to HPX-87G column (Biorad, USA) at 65°C using 5 mM H2SO4 as mobile phase with a flow rate of 0.6 ml min−1 on a Dionex Ultimate 3000 HPLC (Dionex Softron GmbH, Germany) to measure the concentrations of exometabolites (glucose, ethanol, glycerol, etc.).

Shake flask fermentations of the α-amylase-producing strains: seed cultures were prepared as above and inoculated into 10 ml SD-2xSCAA-sf medium with an initial OD600 of 0.01 in shake flasks and shaken at 200 rpm at 30°C for 96 h. One milliliter of the culture broth was centrifuged at 10 000 g for 5 min and the clear supernatant was stored at 4°C before measurement. Activity of α-amylase was measured using the Ceralpha kit (Megazyme, Ireland) with α-amylase from Aspergillus oryzae (Sigma, USA) as standard. The actual activity of the standard amylase is 69.6 U mg−1 according to the calculation made by Liu et al. (2012). This value was used to convert the α-amylase activity (U l−1) to the amount of protein (mg l−1).

Shake flask fermentations of the IP-producing strains: fresh colonies were inoculated into 5 ml of SD-2xSCAA-sf medium in 15 ml falcon tubes and incubated at 30°C with shaking at 200 rpm for 24 h before being transferred into 10 ml of the same medium in shake flasks with an initial OD600 of 0.01. One milliliter of sample culture was taken after being cultivated at 30°C with shaking at 200 rpm for 48, 72 and 96 h, respectively. Clear supernatant from of IP culture broth was analyzed by HPLC and quantified as described in Hou et al. (2012). Humanized insulin (Sigma, St. Louis, MO, USA) was used as a standard for quantification.

Shake flask fermentations for invertase measurements: seed cultures prepared as above were inoculated with an initial OD600 of 0.05 in 10 ml SD-2xSCAA-suc medium in shake flasks and shaken at 200 rpm at 30°C for 15 h before being harvested. Invertase was measured and quantified as described in Hou et al. (2013).

Genome-wide transcriptome analysis

Samples for microarray analysis were taken from the bioreactor cultures prepared as above with an initial OD600 of 0.01 in 10 ml SD-2xSCAA-sf medium in shake flasks and shaken at 200 rpm at 30°C for 96 h before being harvested. Clear supernatant from of IP culture broth was analyzed by HPLC and quantified as described in Hou et al. (2012). Humanized insulin (Sigma, St. Louis, MO, USA) was used as a standard for quantification.

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around 1 and stored at −80°C until processing (Usaite et al. 2006). WT-Amy was performed in biological duplicates, and ΔROX1-Amy was done in triplicates. RNA was isolated using the RNeasy Minikit (Qiagen) and processed to cRNA using the Genechip 3’ IVT Express Kit (Affymetrix) and hybridized on the Yeast Genome 2.0 Array (Affymetrix) to create CEL files. The images on CEL files were analyzed using R 2.15.2 software and Bioconductor packages. Data normalization was performed using the method of Probe Logarithmic Intensity Error (PLIER) (AFFYMETRIX, INC) with perfect match probes only (PM-only). Differential expression analysis was done by moderated t-statistic and Benjamini–Hochberg’s method (FDR) (Benjamini and Hochberg 1995) was used to adjust the p-values for multiple testing. The FDR from the statistical analysis was used as input to the Reporter Features algorithm (Patil and Nielsen 2005; Oliveira, Patil and Nielsen 2008) to identify reporter gene ontologies (GO) terms and reporter TFs. The reporter analysis was performed using the Platform for Integrative Analysis of Omics (PIANO) data package (Väremo, Nielsen and Nookaew 2013).

RT-QPCR

Yeast cells were cultured in shake flasks containing 10 ml of SD-2xSCAA-sf medium at 30°C with shaking at 200 rpm for 48 h until being harvested. The total RNA was extracted and processed to cDNA as described above for microarray analysis. About 2 μl of the synthesized cDNA (corresponding to 100 ng RNA) was used as the template for the QPCR reaction to a final reaction volume of 20 μl, using Quantitect Reverse Transcription Kit (QIAGEN, Germany). Quantitative RT-PCR was performed on Stratagene Mx3000P (Agilent Technologies, USA), using DyNamo Flash SYBR Green qPCR Kit (Thermo Scientific, USA). The thermocycling program consisted of one hold at 95°C for 15 min, followed by 40 cycles of 10 s at 95°C and 20 s at 60°C, and a final cycle of 1 min at 95°C, 30 s at 55°C and 30 s at 95°C. Cycle thresholds (Ct) were normalized to the Ct value of S. cerevisiae ACT1.

Identification and quantification of whole-cell lipids and fatty acids

Cells cultured in shake flasks (initial OD<sub>600</sub> = 0.01, 30°C, 200 rpm) for 72 h were collected and dried for further processing. Lipids were extracted by CHCl<sub>3</sub>:MeOH (2:1) and quantified by HPLC-CAD as described in Khoomrung et al. (2013). Cholesterol was used as an internal standard to monitor the efficiency of the extraction. The quantification of lipids was performed using external calibration curves from known lipid standards (SE, TAG, FA, CH, ES, PA, CL, PE, PC, SM, PS and PI). Fatty acids (FAs) were extracted and esterified to FA methyl esters in hexane and Boron trifluoride (BF<sub>3</sub>) and quantified by GC-MS as described in Khoomrung et al. (2012). Heptadecanoic acid (17:0) was used as an internal standard to control the recovery rate. All experiments were done in biological triplicates.

Flow cytometry

Cells cultured in shake flasks (initial OD<sub>600</sub> = 0.01, 30°C, 200 rpm) for 48 h were collected and injected into Guava easyCite HT system (Millipore). Distribution of the cells in the population was measured based on the intensities of the forward and side scatters recorded. A total of 5000 cells from each strain were measured with the injection speed maintaining at around 500 cells μl<sup>−1</sup>. All experiments were done in biological triplicates.

RESULTS

ROX1 deletion improved α-amylase production in aerobic batch fermentations

To evaluate the effect of ROX1 deletion on α-amylase production, we transformed the α-amylase-containing plasmid p426GPD-AlphaAmy into both CEN.PK 113–11C and the roxlΔ strain. The resulting strains, designated WT-Amy and ΔROX1-Amy respectively, were evaluated in aerobic batch fermentations in bioreactors. The final titer of α-amylase in ΔROX1-Amy (70.5 mg L<sup>−1</sup>) was double compared to that of WT-Amy (34.8 mg L<sup>−1</sup>) (Fig. 1A, and Table 2). The α-amylase production was higher in ΔROX1-Amy throughout the fermentation which was also reflected by the higher amylase yield on biomass for ΔROX1-Amy during both the glucose phase (7.14 mg g<sup>−1</sup> DCW for ΔROX1-Amy vs 3.8 mg g<sup>−1</sup> DCW for WT-Amy) and the post-diauxic shift phase (10.2 mg g<sup>−1</sup> DCW for ΔROX1-Amy vs 5.89 mg g<sup>−1</sup> DCW for WT-Amy) (Table 2).

Other physiological properties of the amylase producing strains are listed in Table 2. There was no significant reduction in the specific growth rate of ΔROX1-Amy (0.31 h<sup>−1</sup>) compared to WT-Amy (0.33 h<sup>−1</sup>). The two strains showed the same biomass yields on glucose (0.122 g g<sup>−1</sup> Glc, Fig. 1B). WT-Amy had a slightly higher ethanol yield (0.37 g g<sup>−1</sup> Glc) than ΔROX1-Amy (0.35 g g<sup>−1</sup> Glc), whereas both glycerol and acetate yields in ΔROX1-Amy (0.08 g g<sup>−1</sup> Glc, 0.04 g g<sup>−1</sup> Glc) were higher than in WT-Amy (0.05 g g<sup>−1</sup> Glc, 0.03 g g<sup>−1</sup> Glc).

There was no significant difference of the amylase expression in ΔROX1-Amy and WT-Amy strains, during the exponential growth phase (microarray data in DataSet S1) and the stationary phase (at 48 h from shake flask fermentations, QPCR data in DataSet S2). Expression of amylase is initiated from the GPD1 promoter so we ensured that the GPD1 promoters driving amylase expression in both strains were not affected by ROX1 deletion, and the enhanced amylase production was due to cell response to the ROX1 deletion.

To verify if ROX1 deletion may also affect the secretion of other proteins, we tested the production of two other proteins: (i) heterologous human IP and (ii) yeast endogenous secreted enzyme invertase. The plasmid p426GPD-AlphaHins containing a gene encoding IP was transformed into both CEN.PK 113–11C and the roxlΔ strain, resulting in strains WT-Ins and ΔROX1-Ins, respectively. The production of the IP was evaluated in shake flasks, and the final titers reached 34.7 and 32.3 mg/L for ΔROX1-Ins and WT-Ins respectively at 96 h (Fig. 2A). The yeast gene SUC2 encodes two forms of endogenous invertases: an intracellular one constitutively expressed and a secreted one that is subjected to glucose repression (Carlson and Botstein 1982). The secreted invertase is highly glycosylated and contains a signal sequence that directs it to the periplasmic space. After induction by sucrose, the production of the secreted invertase was 40% higher in the ΔROX1-Amy strain (0.36 mU in ΔROX1-Amy vs. 0.25 mU in WT-Amy, Fig. 2B).

Genome-wide transcriptional changes in the ROX1 deletion strain

In order to identify the molecular mechanisms underlying the increased protein secretion, we performed genome-wide transcriptome analysis of ΔROX1-Amy and compared it to the genome-wide transcriptional response of WT-Amy, during exponential growth on glucose. Genes significantly upregulated in ΔROX1-Amy were found in the GO terms related to structural
Figure 1. Aerobic batch fermentations in bioreactors of the α-amylase-producing strains. (A) α-amylase production. (B) Glucose consumption (left axis) and biomass accumulation (right axis). The arrow indicates the sampling time (16.4 h after inoculation) for microarray study. (C) Ethanol production. (D) Glycerol production. Each data point was calculated based on the amount of α-amylase from duplicate (WT-Amy) and triplicate (∆ROX1-Amy) batch fermentations. The error bars represent standard error of the mean (SEM) at corresponding data points.

Table 2. Physiological characterization of the α-amylase producing strains in bioreactors.

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \mu_{\text{max}} )^a</th>
<th>( Y_{\text{SE}} )^b</th>
<th>( Y_{\text{SG}} )^c</th>
<th>( Y_{\text{SA}} )^d</th>
<th>( Y_{\text{Samy}} )^e</th>
<th>( Y_{\text{Xamy}} )^f</th>
<th>( Y_{\text{SX}} )^g</th>
<th>( Q_{\text{amy}} )^h</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-Amy</td>
<td>0.33 ± 0.002</td>
<td>0.37 ± 0.008</td>
<td>0.05 ± 0.003</td>
<td>0.03 ± 0.001</td>
<td>0.122 ± 0.001</td>
<td>0.461 ± 0.05</td>
<td>3.79 ± 0.45</td>
<td>5.89 ± 0.18</td>
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<tr>
<td>∆ROX1-Amy</td>
<td>0.31 ± 0.004</td>
<td>0.35 ± 0.005</td>
<td>0.08 ± 0.003</td>
<td>0.04 ± 0.000</td>
<td>0.122 ± 0.002</td>
<td>0.746 ± 0.03</td>
<td>7.14 ± 0.34</td>
<td>10.2 ± 0.9</td>
</tr>
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</table>

\( ^a \mu_{\text{max}} \), specific growth rate on glucose (h\(^{-1}\)).
\( ^b Y_{\text{SE}} \), ethanol yield on glucose (g g\(^{-1}\)).
\( ^c Y_{\text{SG}} \), glycerol yield on glucose (g g\(^{-1}\)).
\( ^d Y_{\text{SA}} \), acetate yield on glucose (g g\(^{-1}\)).
\( ^e Y_{\text{Samy}} \), α-amylase yield on glucose (mg g\(^{-1}\)).
\( ^f Y_{\text{Xamy}} \), α-amylase yield on biomass during glucose phase (mg g\(^{-1}\)).
\( ^g Y_{\text{Xamy}} \), α-amylase yield on biomass during ethanol/acetate phase (mg g\(^{-1}\)).
\( ^h Q_{\text{amy}} \), α-amylase specific production rate (mg g\(^{-1}\) h\(^{-1}\)).

Each data point represents the average and standard error from duplicate (WT-Amy) and triplicate (∆ROX1-Amy) batch fermentations.

constituent of cell wall, sterol and lipid biosynthesis, as well as heme and iron homeostasis (Fig. 3A). We also performed reporter TF analysis that enables identification of genes that have regulations controlled by common TFs (Oliveira, Patil and Nielsen 2008). With this analysis, the genes regulated by Upc2p (synthesis of anaerobic cell wall mannoproteins and sterol and lipids; Vik and Rine 2001), Hap1p (heme and iron homeostasis; Keng 1992), Mot3p as well as Tup1p (in accordance with Rox1p regulation; Smith and Johnson 2000; Sertil et al. 2003) were found significantly upregulated (Fig. 3B), which is in consistence with the reporter GO term analysis. The results of the whole genome transcriptome analysis are summarized in DataSet1.

Lipid composition changes in the ROX1 deletion strain

To check whether the transcriptional changes (Fig. 3) had any effect on the lipid and sterol biosynthesis, the lipid composition was measured in both strains. The majority of lipid classes in both strains are phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI),
followed by neutral lipids, sterol esters (SE) and triacylglycerols (TAG). Ergosterol (ES) and phosphatidylserine (PS) are less abundant than the other classes. The concentration of all these lipid classes was higher in the ΔROX1-Amy strain than that in the WT-Amy strain (Fig. 4). The difference in levels in SE was the highest (2.52-fold), followed by PI (1.42-fold), PC (1.28-fold), and TAG (1.26-fold), PE (1.18-fold), PS (1.16-fold) and lastly free fatty acids (FFA) (1.14-fold).

The distribution of the FAs is presented in Fig. 5. The majority of the FAs in both strains are C16 and C18 FA; the monounsaturated forms were four times more (C16:1 vs. C16:0) and eight times more abundant (C18:1 vs. C18:0) than the corresponding saturated forms.

The concentrations of C14:0, C14:1, C16:1 and C17:1 in ΔROX1-Amy (0.72, 0.72, 16.7 and 0.09 μg mg⁻¹) were higher than that in WT-Amy (0.24, 0.23, 14.9 and 0.06 μg mg⁻¹). In the case of other FAs (C15:0, C16:0, C18:0, C18:1, C18:2, C19:0, C19:1), the amounts were similar in the two strains.

**Morphological changes in the ROX1 deletion strain**

The basic structures of eukaryotic cell membranes are lipid bilayers, composed of diverse lipid species including phospholipids, sterols and sphingolipids (Simons and Sampaio 2011). The membranes of organelles including ER, Golgi and trafficking vesicles, etc. are also enriched with different lipids (Zinser, Paltauf and Daum 1993). With the varied levels of lipid classes (Fig. 4), we examined whether cell morphology was altered after ROX1 deletion.

The analysis was performed by flow cytometry with two non-producing strains, in the WT and rox1Δ backgrounds (WT-Emp that has the empty plasmid p426GPD in the WT CEN.PK 113-11C background; and ΔROX1-Emp [same plasmid in the rox1Δ background]), and in the two strains producing amylase (WT-Amy and ΔROX1-Amy) (Fig. 6). The forward scatter intensity (f.i) shows the size of the scattering particles (Tzur et al. 2011) and in our case indicates the size of yeast cells. After deleting ROX1, the cell size was significantly reduced (ΔROX1-Amy, f.i: 2199 < WT-Emp, f.i: 2643, p < 10⁻²); however, when amylase was overexpressed, the cells increased in size with ΔROX1-Amy (f.i: 2758) reaching even as similar size as WT-Amy (WT-Amy, f.i: 2746). It is generally agreed that any internal and surface structural irregularities, including cytoplasmic granules, vesicles, and other organelles and membrane roughness will typically contribute to side scatter signals (Tzur et al. 2011). Indicated by relative side scatter intensity (s.i), the cell density increased significantly in the ROX1 deletion strain (ΔROX1-Emp, s.i: 3360 > WT-Emp, s.i: 3105, p < 10⁻²) as well as in the amylase overproducing cells (WT-Amy, s.i: 3222 > WT-Emp, p < 10⁻²). The strain with both ROX1 deletion and amylase overexpression (ΔROX1-Amy, s.i: 3916) exhibited the highest cell density. Microscopic observation (data not shown) confirmed the cell morphology estimation by flow cytometry.

**Overexpression of upc2–1 improved amylase production**

Our transcription data shows that many genes involved in sterol biosynthesis and uptake, that are transcriptionally controlled by Upc2p, and the UPC2 gene itself (YDR213W) were differentially upregulated (> 2-fold) in the ROX1 deletion strain (DataSet S1). This pointed to the increased transcriptional activation by Upc2p. It has been recently discovered that the C-terminal domain of Upc2p is a ligand-binding domain which senses the ergosterol level in cells (Yang et al. 2015). In a sterol-rich condition (e.g. aerobic condition), Upc2p is bound to ergosterol and is present in the cytosol. On the depletion of ergosterol (e.g. anaerobic condition), unliganded Upc2p is activated and moves to the nucleus for transcriptional activation of the ERG genes (Yang et al. 2015). The UPC2–1 allele (Lewis, Taylor and Parks 1985) has a G888D mutation in the C-terminus (Crowley et al. 1998) making it constitutively active in a sterol-independent manner. To understand whether increased sterol level may result in improved protein production, we overexpressed the upc2–1 (instead of the WT UPC2) in a strain overexpressing amylase (WT-Amy), which
Figure 3. Reporter GO term and reporter TF analyses. (A) Reporter GO term. Reporter \( p \)-value \( < 10^{-6} \). (B) Reporter TF. Reporter \( p \)-value \( < 10^{-3} \). The color key shows significance of the reporter GO term and TF analyses (-log(reporter \( p \)-value)). Red color indicates that the genes regulated in the GO term, or by the specific TF, were upregulated when comparing the transcriptome of two strains; blue color indicates downregulation in the same comparison.

Figure 4. Comparisons of the lipid classes in the amylase overproducing strains. The error bars represent SEMs from triplicate experiments in shake flasks. The statistical significance tests were done with a one-tailed homoscedastic (equal variance) \( t \)-test. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). SE: sterol ester; TAG: triacylglycerol; FFA: free fatty acid; ES: ergosterol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol.

Figure 5. Comparisons of the fatty acids in the amylase overproducing strains. The error bars represent SEMs from triplicate experiments in shake flasks. The statistical significance tests were done with a one-tailed homoscedastic \( t \)-test. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).

DISCUSSION

Quite a few studies have shown that hypoxia positively affects heterologous protein production in both Crabtree-positive yeast S. cerevisiae (Ruohonen et al. 1997; Liu et al. 2013) and Crabtree-negative yeast P. pastoris (Baumann et al. 2007, 2011a,b).
Figure 6. Estimation of cell size and intracellular granularity by flow cytometry. Scales on the X and Y axis are the intensity of the dots (cells) detected by each scattering. The mean intensity from 5000 cells from each strain was plotted. The error bars represent SEMs from triplicate experiments in shake flasks. Since it is hard to show the differences among cell populations we illustrate schematically our speculations on cell sizes (FSC) and the amount of α-amylase and vesicles aside. One microscopic picture from WT-Amy and ΔROX1-Amy are demonstrated as an example, where one can get a rough idea that the granularity in ΔROX1-Amy is slightly higher than in the WT-Amy.

Figure 7. Effect of upc2–1 overexpression on amylase production. The error bars represent SEMs from triplicate experiments in shake flasks. The statistical significance tests were done with a one-tailed homoscedastic (equal variance) t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

Liu et al. (2013) focused on identifying alternative electron acceptors for protein folding in S. cerevisiae under anaerobic conditions and Baumann et al. (2011a) studied the relationship between protein secretion and ergosterol biosynthesis and membrane physics in P. pastoris. In this work, instead of culturing yeast anaerobically, we studied protein production aerobically but under the effect of anaerobically induced genes. The derepression was achieved by deleting ROX1, which allowed activation of many genes that are repressed in normoxic conditions, and normally induced only in anoxia or hypoxia. In addition to the improved production of heterologous proteins (α-amylase and IP), the production of the endogenous secreted invertase enzyme was also significantly enhanced, indicating that the efficiency of the secretory pathway was generally improved by ROX1 deletion. In comparison to the relatively larger α-amylase (478 aa) and invertase (532 aa), the production of the smaller human IP (53 aa) was only slightly improved (34.7 mg l⁻¹ vs 32.3 mg l⁻¹ at 96 h) in the rox1Δ background. This result is in accordance with our previous observation that IP production is mostly limited by synthesis and much less limited by secretion, when compared to the larger and more complex protein α-amylase (Liu et al. 2012).

In the ΔROX1-Amy strain, the transcriptional changes of the genes involved in sterol biosynthesis caused alterations in the level of the detected lipid classes, including ergosterol and sterol esters (Fig. 4). From ER where lipids are synthesized, the secretory pathway (Golgi apparatus, endosomes, vesicles, etc.) gets progressively enriched with sterols and sphingolipids, reaching the maximum at the plasma membrane (Zinser, Paltauf and Daum 1993). Secretory vesicles contain a significant amount of ergosterol, almost as much as the plasma membrane (Zinser, Paltauf and Daum 1993). The size of the ΔROX1-Amy cells did not vary much compared to the WT-Amy cells; however, the cell density increased considerably (Fig. 6). The increased lipid content may likely contribute to the increased cell density in the ΔROX1-Amy cells in the form of more lipid particles (SE and TAG) and in the enrichment of secretory vesicles (more phospholipids and ergosterol). It is possible to envisage that the efficiency of protein secretion is also increased due to the presence of more secretory vesicles. Sphingolipids and ergosterols are physically associated with the membranes, and their synthesis is coordinated (Montefusco, Matmati and Hannun 2013). The observed upregulation of the sphingolipids biosynthesis genes (TSC10, SUR2, SCS7, IPT1, LAC1, LIP1) may also result in changes in sphingolipid contents which might promote the formation of COPII vesicles, that are used for vesicular transport to the Golgi (Skrzypek, Lester and Dickson 1997).

The key driver for improved protein production in the ROX1 deletion strain seems to be altered lipid composition, which was confirmed by the overexpression of upc2–1, which led to a double amylase production in aerobic cultivation (Fig. 7). As mentioned above, under aerobic condition, wild-type UPC2 stays in the cytosol in a repressed form (Yang et al. 2015). Overexpression of UPC2 will therefore not necessarily elevate the sterol levels in the
WT-Amy strain. Overexpression of upc2-1 in the WT-Amy mimics UPC2 upregulation found in the AROX1-Amy strain, where the UPC2 might have been switched to the active form indicated by the elevated sterol levels observed (Fig. 7).

In addition to the alteration in lipid classes, there could be other factors involved in the enhancement of protein production in the rox1Δ cells, as suggested by the transcriptome analysis. For example, the upregulation of ANB1 (encoding translation elongation factor) (Saini et al. 2009) may accelerate protein synthesis. The transcriptional reprogramming from aerobic mannoproteins to the anaerobic ones may modify the fluidity and polarity of the yeast cell wall which might also contribute to enhanced protein secretion. We actually observed slight improvement in amylase production (12.7% at 72 h in shake flask) by overexpressing ANB1 in the amylase production strain (File S3, Supporting Information), which may indicate that the increased expression of ANB1 in the ΔROX1-Amy could play an important role in accelerating protein synthesis. However, we could not further increase the production of amylase by increasing the overexpression of ANB1 (data not shown) suggesting that protein synthesis is probably not the limiting step for amylase production, but rather the downstream secretion processes which could be modulated by changing lipid composition of the membranes.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSYR online.

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