

1 **Engineering the thermotolerant industrial yeast *Kluyveromyces marxianus* for anaerobic growth**

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16 Manuscript for submission in Nature Biotechnology, section: Article.

17 **Abstract**

18 Current large-scale, anaerobic industrial processes for ethanol production from renewable  
19 carbohydrates predominantly rely on the mesophilic yeast *Saccharomyces cerevisiae*. Use of  
20 thermotolerant, facultatively fermentative yeasts such as *Kluyveromyces marxianus* could confer  
21 significant economic benefits. However, in contrast to *S. cerevisiae*, these yeasts cannot grow in the  
22 absence of oxygen. Response of *K. marxianus* and *S. cerevisiae* to different oxygen-limitation regimes  
23 were analyzed in chemostats. Genome and transcriptome analysis, physiological responses to sterol  
24 supplementation and sterol-uptake measurements identified absence of a functional sterol-uptake  
25 mechanism as a key factor underlying the oxygen requirement of *K. marxianus*. Heterologous expression  
26 of a squalene-tetrahymanol cyclase enabled oxygen-independent synthesis of the sterol surrogate  
27 tetrahymanol in *K. marxianus*. After a brief adaptation under oxygen-limited conditions, tetrahymanol-  
28 expressing *K. marxianus* strains grew anaerobically on glucose at temperatures of up to 45 °C. These  
29 results open up new directions in the development of thermotolerant yeast strains for anaerobic  
30 industrial applications.

31 **Keywords:** Ergosterol, tetrahymanol, anaerobic metabolism, thermotolerance, ethanol production,  
32 yeast biotechnology, metabolic engineering

33 In terms of product volume ( $87 \text{ Mton y}^{-1}$ )<sup>1,2</sup>, anaerobic conversion of carbohydrates into ethanol by the  
34 yeast *Saccharomyces cerevisiae* is the single largest process in industrial biotechnology. For  
35 fermentation products such as ethanol, anaerobic process conditions are required to maximize product  
36 yields and to minimize both cooling costs and complexity of bioreactors<sup>3</sup>. While *S. cerevisiae* is applied in  
37 many large-scale processes and is readily accessible to modern genome-editing techniques<sup>4,5</sup>, several  
38 non-*Saccharomyces* yeasts have traits that are attractive for industrial application. In particular, the high  
39 maximum growth temperature of thermotolerant yeasts, such as *Kluyveromyces marxianus* (up to 50 °C  
40 as opposed to 39 °C for *S. cerevisiae*), could enable lower cooling costs<sup>6-8</sup>. Moreover, it could reduce the  
41 required dosage of fungal polysaccharide hydrolases during simultaneous saccharification and  
42 fermentation (SSF) processes<sup>9,10</sup>. However, as yet unidentified oxygen requirements hamper  
43 implementation of *K. marxianus* in large-scale anaerobic processes<sup>11-13</sup>.

44 In *S. cerevisiae*, fast anaerobic growth on synthetic media requires supplementation with a source of  
45 unsaturated fatty acids (UFA), sterols, as well as several vitamins<sup>14-17</sup>. These nutritional requirements  
46 reflect well-characterized, oxygen-dependent biosynthetic reactions. UFA synthesis involves the oxygen-  
47 dependent acyl-CoA desaturase Ole1, NAD<sup>+</sup> synthesis depends on the oxygenases Bna2, Bna4, and Bna1,  
48 while synthesis of ergosterol, the main yeast sterol, even requires 12 moles of oxygen per mole.

49 Oxygen-dependent reactions in NAD<sup>+</sup> synthesis can be bypassed by nutritional supplementation of  
50 nicotinic acid, which is a standard ingredient of synthetic media for cultivation of *S. cerevisiae*<sup>17,18</sup>.

51 Ergosterol and the UFA source Tween 80 (polyethoxylated sorbitan oleate) are routinely included in  
52 media for anaerobic cultivation as 'anaerobic growth factors' (AGF)<sup>15,17,19</sup>. Under anaerobic conditions, *S.*  
53 *cerevisiae* imports exogenous sterols via the ABC transporters Aus1 and Pdr11<sup>20</sup>. Mechanisms for uptake  
54 and hydrolysis of Tween 80 by *S. cerevisiae* are unknown but, after its release, oleate is activated by the  
55 acyl-CoA synthetases Faa1 and Faa4<sup>21,22</sup>.

56 Outside the whole-genome duplicated (WGD) clade of Saccharomycotina yeasts, only few yeasts  
57 (including *Candida albicans* and *Brettanomyces bruxellensis*) are capable of anaerobic growth in  
58 synthetic media supplemented with vitamins, ergosterol and Tween 80<sup>12,13,23,24</sup>. However, most currently  
59 known yeast species readily ferment glucose to ethanol and carbon dioxide when exposed to oxygen-  
60 limited growth conditions<sup>13,25,26</sup>, indicating that they do not depend on respiration for energy  
61 conservation. The inability of the large majority of facultatively fermentative yeast species to grow  
62 under strictly anaerobic conditions is therefore commonly attributed to incompletely understood  
63 oxygen requirements for biosynthetic processes<sup>11</sup>. Several oxygen-requiring processes have been  
64 proposed including involvement of a respiration-coupled dihydroorotate dehydrogenase in pyrimidine  
65 biosynthesis, limitations in uptake and/or metabolism of anaerobic growth factors, and redox-cofactor  
66 balancing constraints<sup>11,13,27</sup>.

67 Quantitation, identification and elimination of oxygen requirements in non-*Saccharomyces* yeasts is  
68 hampered by the very small amounts of oxygen required for non-dissimilatory purposes. For example,  
69 preventing entry of the small amounts of oxygen required for sterol and UFA synthesis in laboratory-  
70 scale bioreactor cultures of *S. cerevisiae* requires extreme measures, such as sparging with ultra-pure  
71 nitrogen gas and use of tubing and seals that are resistant to oxygen diffusion<sup>25,28</sup>. This technical  
72 challenge contributes to conflicting reports on the ability of non-*Saccharomyces* yeasts to grow  
73 anaerobically, as exemplified by studies on the thermotolerant yeast *K. marxianus*<sup>29-31</sup>. Paradoxically,  
74 the same small oxygen requirements can represent a real challenge in large-scale bioreactors, in which  
75 oxygen availability is limited by low surface-to-volume ratios and vigorous carbon-dioxide production.

76 Identification of the non-dissimilatory oxygen requirements of non-conventional yeast species is  
77 required to eliminate a key bottleneck for their application in industrial anaerobic processes and, on a  
78 fundamental level, can shed light on the roles of oxygen in eukaryotic metabolism. The goal of this study

79 was to identify and eliminate the non-dissimilatory oxygen requirements of the facultatively  
80 fermentative, thermotolerant yeast *K. marxianus*. To this end, we analyzed and compared physiological  
81 and transcriptional responses of *K. marxianus* and *S. cerevisiae* to different oxygen- and anaerobic-  
82 growth factor limitation regimes in chemostat cultures. Based on the outcome of this comparative  
83 analysis, subsequent experiments focused on characterization and engineering of sterol metabolism and  
84 yielded *K. marxianus* strains that grew anaerobically at 45 °C.

## 85 **Results**

### 86 ***K. marxianus* and *S. cerevisiae* show different physiological responses to extreme oxygen limitation**

87 To investigate oxygen requirements of *K. marxianus*, physiological responses of strain CBS6556 were  
88 studied in glucose-grown chemostat cultures operated at a dilution rate of 0.10 h<sup>-1</sup> and subjected to  
89 different oxygenation and AGF limitation regimes (Fig. 1a). Physiological parameters of *K. marxianus* in  
90 these cultures were compared to those of *S. cerevisiae* CEN.PK113-7D subjected to the same cultivation  
91 regimes.

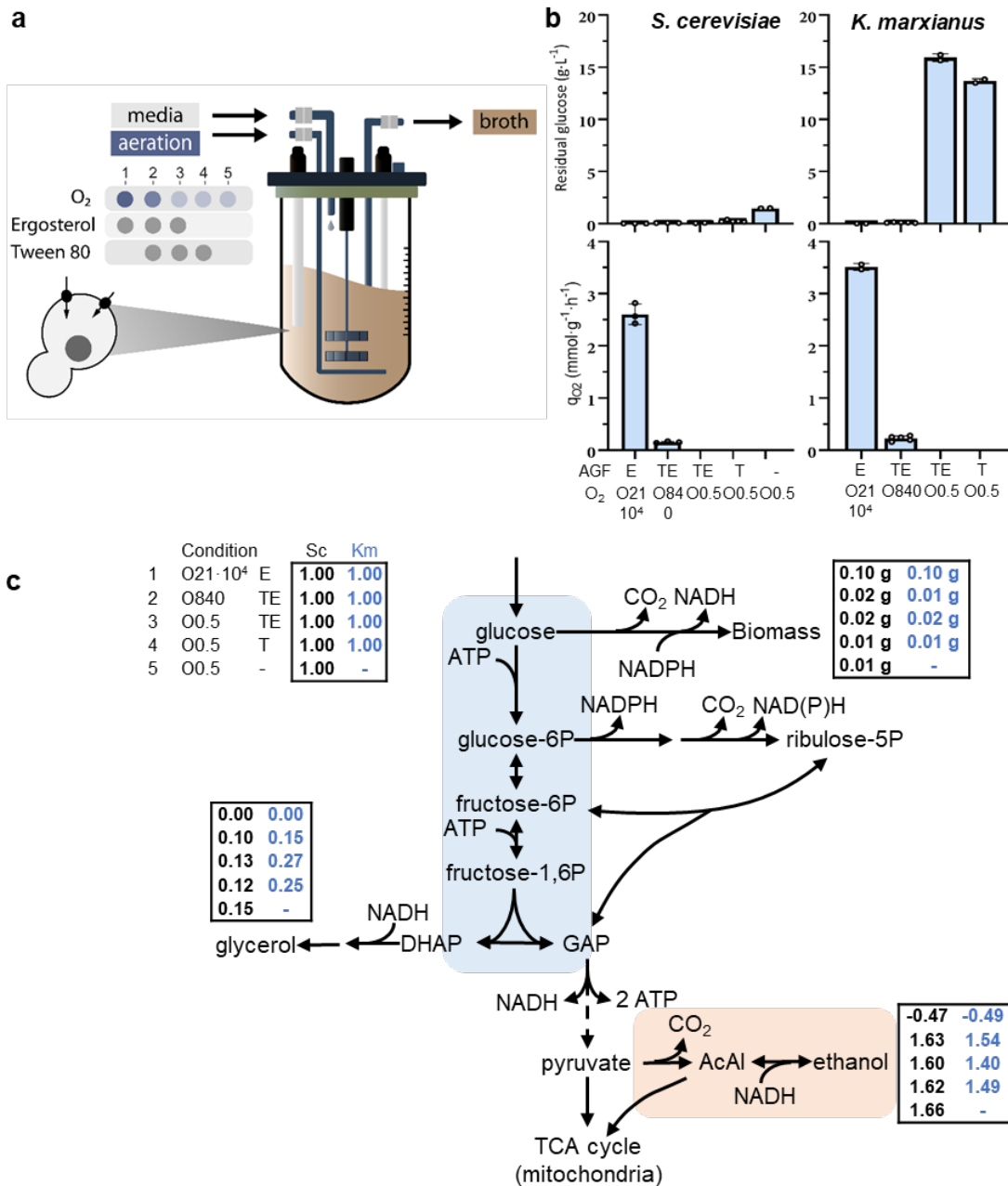
92 In glucose-limited, aerobic chemostat cultures (supplied with 0.5 L air·min<sup>-1</sup>, corresponding to 54 mmol  
93 O<sub>2</sub> h<sup>-1</sup>), the Crabtree-negative yeast *K. marxianus*<sup>32</sup> and the Crabtree-positive yeast *S. cerevisiae*<sup>33</sup> both  
94 exhibited a fully respiratory dissimilation of glucose, as evident from absence of ethanol production and  
95 a respiratory quotient (RQ) close to 1 (Table 1). Apparent biomass yields on glucose of both yeasts  
96 exceeded 0.5 g biomass (g glucose)<sup>-1</sup> and were approximately 10 % higher than previously reported due  
97 to co-consumption of ethanol, which was used as solvent for the anaerobic growth factor ergosterol<sup>32,34</sup>.

98 At a reduced oxygen-supply rate of 0.4 mmol O<sub>2</sub> h<sup>-1</sup>, both yeasts exhibited a mixed respiro-fermentative  
99 glucose metabolism. RQ values close to 50 and biomass-specific ethanol-production rates of 11.5 ± 0.6  
100 mmol·g<sup>-1</sup> for *K. marxianus* and 7.5 ± 0.1 mmol·g<sup>-1</sup> for *S. cerevisiae* (Table 1), indicated that glucose

101 dissimilation in these cultures was predominantly fermentative. Biomass-specific rates of glycerol  
102 production which, under oxygen-limited conditions, enables re-oxidation of NADH generated in  
103 biosynthetic reactions<sup>35</sup>, were approximately 2.5-fold higher ( $p = 2.3 \cdot 10^{-4}$ ) in *K. marxianus* than in *S.*  
104 *cerevisiae*. Glycerol production showed that the reduced oxygen-supply rate constrained mitochondrial  
105 respiration. However, low residual glucose concentrations (Table 1) indicated that sufficient oxygen was  
106 provided to meet most or all of the biosynthetic oxygen requirements of *K. marxianus*.

107 To explore growth of *K. marxianus* under an even more stringent oxygen-limitation, we exploited  
108 previously documented challenges in achieving complete anaerobiosis in laboratory bioreactors<sup>19,28</sup>.  
109 Even in chemostats sparged with pure nitrogen, *S. cerevisiae* grew on synthetic medium lacking Tween  
110 80 and ergosterol, albeit at an increased residual glucose concentration (Fig. 1, Table 1). In contrast, *K.*  
111 *marxianus* cultures sparged with pure N<sub>2</sub> and supplemented with both AGFs consumed only 20 % of the  
112 glucose fed to the cultures. These severely oxygen-limited cultures showed a residual glucose  
113 concentration of  $15.9 \pm 0.3 \text{ g} \cdot \text{L}^{-1}$  and a low but constant biomass concentration of  $0.4 \pm 0.0 \text{ g} \cdot \text{L}^{-1}$ . This  
114 pronounced response of *K. marxianus* to extreme oxygen-limitation provided an experimental context  
115 for further analyzing its unknown oxygen requirements.

116 *S. cerevisiae* can import exogenous sterols under severely oxygen-limited or anaerobic conditions<sup>20</sup>. If  
117 the latter were also true for *K. marxianus*, omission of ergosterol from the growth medium of severely  
118 oxygen-limited cultures would increase biomass-specific oxygen requirements and lead to an even lower  
119 biomass concentration. In practice however, omission of ergosterol led to a small increase of the  
120 biomass concentration and a corresponding decrease of the residual glucose concentration in severely  
121 oxygen-limited chemostat cultures (Fig. 1b, Table 1). This observation suggested that, in contrast to *S.*  
122 *cerevisiae*, *K. marxianus* cannot replace *de novo* oxygen-dependent sterol synthesis by uptake of  
123 exogenous sterols.



124 **Fig. 1 | Chemostat cultivation of *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 under**  
 125 **different aeration and anaerobic-growth-factor (AGF) supplementation regimes.** The ingoing gas flow  
 126 of all cultures was 500 mL·min<sup>-1</sup>, with oxygen partial pressures of 21·10<sup>4</sup> ppm (O21·10<sup>4</sup>), 840 ppm  
 127 (O840), or < 0.5 ppm (O0.5). The AGFs Ergosterol (E) and/or Tween 80 (T) were added to media as  
 128 indicated. **a**, Schematic representation of experimental set-up. Data for each cultivation regime were  
 129 obtained from independent replicate chemostat cultures. **b**, Residual glucose concentrations and

130 biomass-specific oxygen consumption rates ( $q_{O_2}$ ) under different aeration and AGF-supplementation  
 131 regimes. Data represent mean and standard deviation of independent replicate chemostat cultures. **c**,  
 132 Distribution of consumed glucose over biomass and products in chemostat cultures of *S. cerevisiae* (left  
 133 column) and *K. marxianus* (right column), normalized to a glucose uptake rate of  $1.00 \text{ mol}\cdot\text{h}^{-1}$ . Numbers  
 134 in boxes indicate averages of measured metabolite formation rates ( $\text{mol}\cdot\text{h}^{-1}$ ) and biomass production  
 135 rates ( $\text{g dry weight}\cdot\text{h}^{-1}$ ) for each aeration and AGF supplementation regime.

136 **Table 1 | Physiology of *S. cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 in glucose-grown**  
 137 **chemostat cultures with different aeration and anaerobic-growth-factor (AGF) supplementation**  
 138 **regimes.** Cultures were grown at pH 6.0 on synthetic medium with urea as nitrogen source and  $7.5 \text{ g}\cdot\text{L}^{-1}$   
 139 glucose (aerobic cultures) or  $20 \text{ g}\cdot\text{L}^{-1}$  glucose (oxygen-limited cultures) as carbon and energy source.  
 140 Data are represented as mean  $\pm$  SE of data from independent chemostat cultures for each condition.  
 141 The AGFs ergosterol (E) and Tween 80 (T) were added to the media as indicated. Cultures were aerated  
 142 at  $500 \text{ mL}\cdot\text{min}^{-1}$  with gas mixtures containing  $21\cdot 10^4 \text{ ppm O}_2$  (O21·10<sup>4</sup>),  $840 \text{ ppm O}_2$  (O840) or  $< 0.5 \text{ ppm}$   
 143  $\text{O}_2$  (O0.5). Tween 80 was omitted from media used for aerobic cultivation to prevent excessive foaming.  
 144 Ethanol measurements were corrected for evaporation (Supplementary Fig. 1). Positive and negative  
 145 biomass-specific conversion rates ( $q$ ) represent consumption and production rates, respectively.

Condition	<i>S. cerevisiae</i> CEN.PK113-7D					<i>K. marxianus</i> CBS6556			
	1	2	3	4	5	1	2	3	4
Aeration regime	O21·10 <sup>4</sup>	O840	O0.5	O0.5	O0.5	O21·10 <sup>4</sup>	O840	O0.5	O0.5
AGF	E	TE	TE	T	-	E	TE	TE	T
Replicates	3	3	2	5	2	2	5	2	2
D (h <sup>-1</sup> )	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Biomass (g·L <sup>-1</sup> )	4.22 ± 0.06	2.29 ± 0.04	1.98 ± 0.01	1.56 ± 0.03	1.12 ± 0.02	3.79 ± 0.02	1.57 ± 0.10	0.35 ± 0.02	0.50 ± 0.04
Residual glucose (g·L <sup>-1</sup> )	0.00 ± 0.00	0.07 ± 0.00	0.06 ± 0.02	0.23 ± 0.04	1.47 ± 0.01	0.00 ± 0.00	0.10 ± 0.02	15.92 ± 0.26	13.67 ± 0.16
Y biomass/glucose (g·g <sup>-1</sup> )	0.57 ± 0.01	0.12 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	0.06 ± 0.00	0.53 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.01



Y ethanol/glucose (g·g <sup>-1</sup> )	-	1.67 ± 0.06	1.63 ± 0.02	1.65 ± 0.02	1.68 ± 0.02	-	1.53 ± 0.03	1.31 ± 0.05	1.40 ± 0.02
q <sub>glucose</sub> (mmol·g <sup>-1</sup> ·h <sup>-1</sup> )	± 0.03	± 0.10	± 0.04	± 0.27	± 0.15	± 0.00	± 0.30	± 0.81	± 0.00
q <sub>ethanol</sub> (mmol·g <sup>-1</sup> ·h <sup>-1</sup> )	± 0.03	0.10	0.02	± 0.56	± 0.47	± 0.00	± 0.44	± 0.66	± 0.11
RQ	1.08 ± 0.02	52.2 ± 2.4	-	-	-	1.06 ± 0.01	49.3 ± 7.5	-	-
Glycerol/biomass (mmol·(g biomass) <sup>-1</sup> )	0.00 ± 0.00	3.67 ± 0.05	5.58 ± 0.02	6.73 ± 0.25	11.26 ± 0.40	0.00 ± 0.00	9.51 ± 0.46	16.90 ± 0.76	18.45 ± 2.09
Carbon recovery (%)	99.9 ± 0.7	101.2 ± 3.3	100.4 ± 0.1	100.1 ± 1.3	104.0 ± 0.2	100.5 ± 0.1	91.1 ± 2.0	101.6 ± 6.5	99.7 ± 3.9
Degree of reduction recovery (%)	98.4 ± 0.7	100.9 ± 0.8	100.1 ± 0.9	98.1 ± 0.6	100.1 ± 1.8	98.8 ± 0.1	94.5 ± 0.4	97.8 ± 6.2	99.1 ± 3.5

146

### 147 **Transcriptional responses of *K. marxianus* to oxygen limitation involve ergosterol metabolism**

148 To further investigate the non-dissimilatory oxygen requirements of *K. marxianus*, transcriptome

149 analyses were performed on cultures of *S. cerevisiae* and *K. marxianus* grown under the aeration and

150 anaerobic-growth-factor supplementation regimes discussed above. The genome sequence of *K.*

151 *marxianus* CBS6556 was only available as draft assembly and was not annotated<sup>36</sup>. Therefore, long-read

152 genome sequencing, assembly and *de novo* genome annotation were performed, the annotation was

153 refined by using transcriptome assemblies (**Data availability**). Comparative transcriptome analysis of *S.*

154 *cerevisiae* and *K. marxianus* focused on orthologous genes with divergent expression patterns that

155 revealed a strikingly different transcriptional response to growth limitation by oxygen and/or anaerobic-

156 growth-factor availability (Fig. 2).

157 In *S. cerevisiae*, import of exogenous sterols by Aus1 and Pdr11 can alleviate the impact of oxygen

158 limitation on sterol biosynthesis<sup>20</sup>. Consistent with this role of sterol uptake, sterol biosynthetic genes in

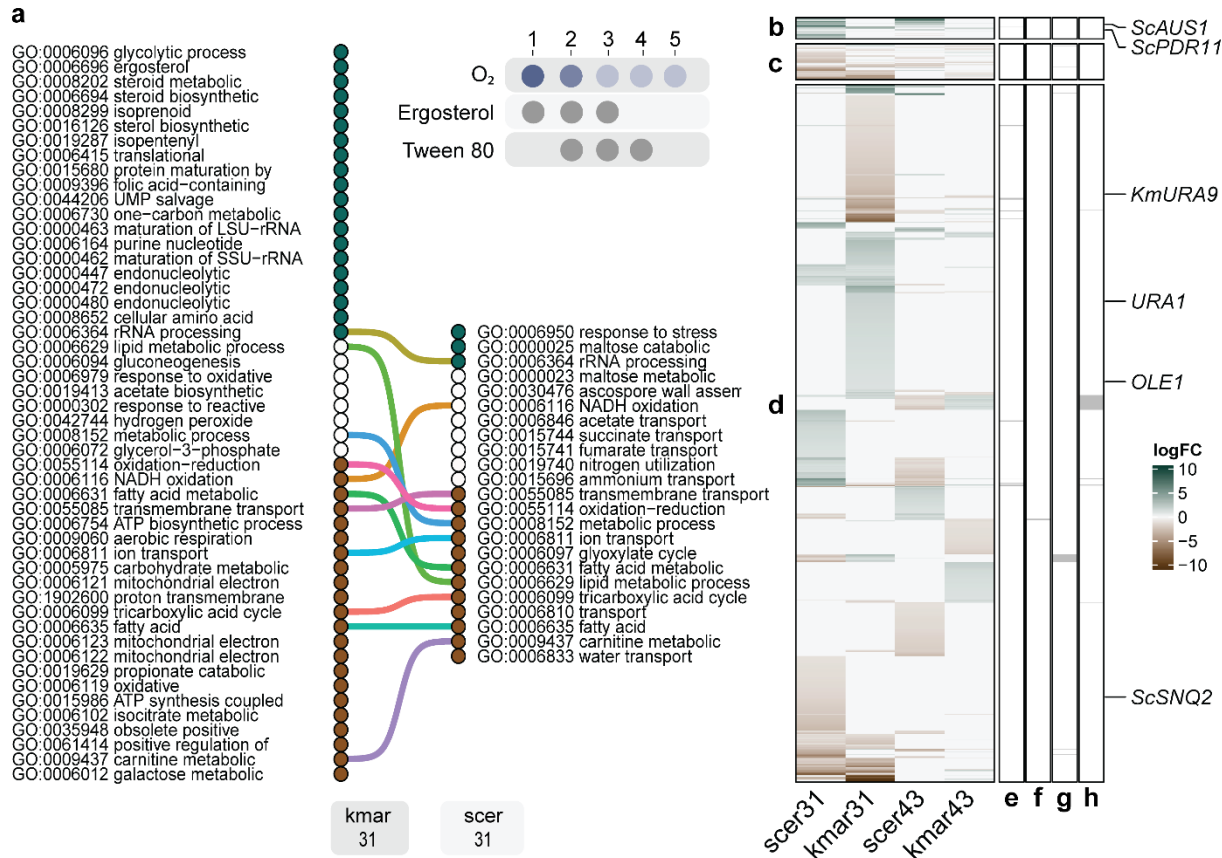
159 *S. cerevisiae* were only highly upregulated in severely oxygen-limited cultures when ergosterol was

160 omitted from the growth medium (Fig. 3b, Supplementary Fig. 6, contrast 43). Also the mevalonate

161 pathway for synthesis of the sterol precursor squalene, which does not require oxygen, was upregulated

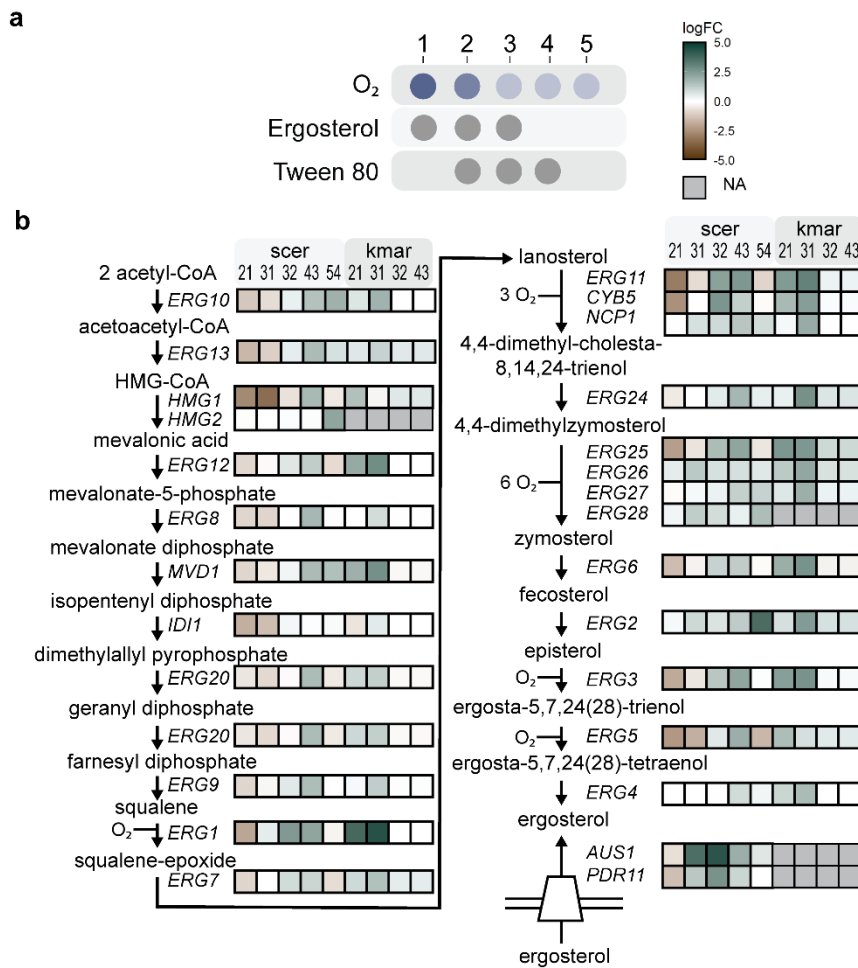
162 (contrast 43), reflecting a relief of feedback regulation by ergosterol<sup>37</sup>. In contrast, *K. marxianus* showed  
163 a pronounced upregulation of genes involved in sterol, isoprenoid and fatty-acid metabolism (Fig. 2ab,  
164 Fig. 3, contrast 31) in severely oxygen-limited cultures supplemented with ergosterol and Tween 80. No  
165 further increase of the expression levels of sterol biosynthetic genes was observed upon omission of  
166 these anaerobic growth factors from the medium of these cultures (Supplementary Fig. 6, contrast 43).  
167 These observations suggested that *K. marxianus* may be unable to import ergosterol when sterol  
168 synthesis is compromised. Consistent with this hypothesis, co-orthology prediction with Proteinortho<sup>38</sup>  
169 revealed no orthologs of the *S. cerevisiae* sterol transporters Aus1 and Pdr11 in *K. marxianus*.

170 *K. marxianus* harbors two dihydroorotate dehydrogenases, a cytosolic fumarate-dependent enzyme  
171 (KmUra1) and a mitochondrial quinone-dependent enzyme (KmUra9). *In vivo* activity of the latter  
172 requires oxygen because the reduced quinone is reoxidized by the mitochondrial respiratory chain<sup>39</sup>.  
173 Consistent with these different oxygen requirements, KmURA9 was down-regulated under severely  
174 oxygen-limited conditions, while KmURA1 was upregulated (Fig. 2b, contrast 31). Upregulation of  
175 KmURA1 coincided with increased production of succinate (Table 1).



176 **Fig. 2 | Transcriptional response of *K. marxianus* and *S. cerevisiae* to oxygen limitation and sterol,**  
 177 **Tween 80 supplementation.** Transcriptome analyses were performed for each cultivation regime (1 to  
 178 5) of *S. cerevisiae* CEN.PK113-7D (*scer*) and *K. marxianus* CBS6556 (*kmar*). Data for each regime were  
 179 obtained from independent replicate chemostat cultures (Fig. 1). **a**, Comparison of GO-term gene-set  
 180 enrichment analysis of biological processes in contrast 31 of *S. cerevisiae* and *K. marxianus* with short  
 181 description of GO-terms (Supplementary Fig. 2-5). GO-terms were vertically ordered based on their  
 182 distinct directionality calculated with Piano<sup>40</sup> with GO-terms enriched solely with up-regulated genes  
 183 (blue) at the top, GO-terms with mixed- or no-directionality in the middle (white) and GO-terms with  
 184 solely down-regulated genes at the bottom (brown). **b, c, d**, Subsets of differentially expressed  
 185 orthologous genes obtained from the gene-set analyses for both yeasts in contrasts 31 and 43, and with  
 186 genes without orthologs depicted with logFC value of 0 in the respective yeast. **b**, *S. cerevisiae* genes  
 187 previously shown as consistently upregulated under anaerobic conditions in four different nutrient-

188 limitations<sup>41</sup>. **c**, As described for panel b but for downregulated genes. **d**, Differentially expressed genes  
 189 uniquely found in this study. **e, f, g, h**, Highlighted gene-sets showing divergent expression patterns  
 190 across the two yeasts. **e**, *S. cerevisiae* genes upregulated in contrast 31 but downregulated in *K.*  
 191 *marxianus*. **f**, *S. cerevisiae* genes downregulated in contrast 31 but upregulated in *K. marxianus*. **g, h**,  
 192 Similar to e and f but for contrast 43.



193 **Fig. 3 | Different transcriptional regulation of ergosterol-biosynthesis in *K. marxianus* and *S.***  
 194 ***cerevisiae*.** **a**, RNAseq was performed on independent replicate chemostat cultures of *S. cerevisiae*  
 195 CEN.PK113-7D and *K. marxianus* CBS6556 for each aeration and anaerobic-growth-factor  
 196 supplementation regime (1 to 5; Fig. 1). **b**, Transcriptional differences in the mevalonate- and  
 197 ergosterol-pathway genes of *S. cerevisiae* and *K. marxianus* for contrasts 21 (O<sub>2</sub> 840 TE | O 21·10<sup>4</sup> E), 31

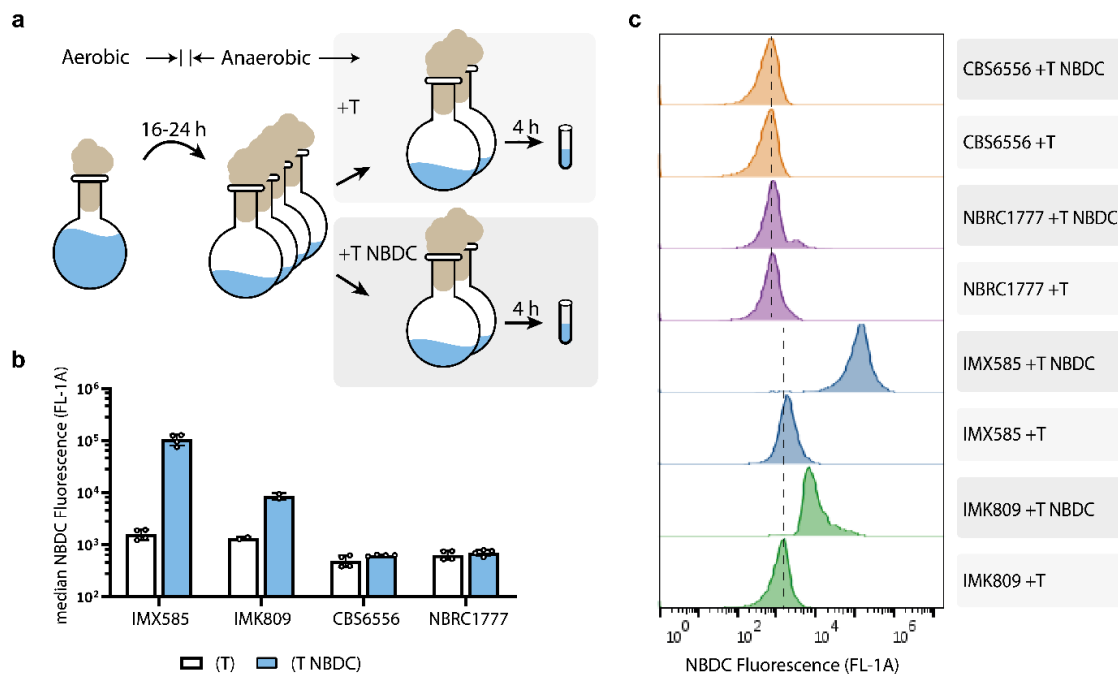
198 (O<sub>2</sub> 0.5 TE | O<sub>2</sub> 21·10<sup>4</sup> E), 32 (O<sub>2</sub> 0.5 TE | O<sub>2</sub> 840 TE), 43 (O<sub>2</sub> 0.5 T | O<sub>2</sub> 0.5 TE), 54 (O<sub>2</sub> 0.5 | O<sub>2</sub> 0.5 T).

199 Lumped biochemical reactions are represented by arrows. Colors indicate up- (blue) or down-regulation  
200 (brown) with color intensity indicating the log<sub>2</sub> fold change with color range capped to a maximum of 4.

201 Reactions are annotated with corresponding gene, *K. marxianus* genes are indicated with the name of  
202 the *S. cerevisiae* orthologs. Ergosterol uptake by *S. cerevisiae* requires additional factors beyond the  
203 membrane transporters Aus1 and Pdr11<sup>42</sup>. No orthologs of the sterol-transporters or Hmg2 were  
204 identified for *K. marxianus* and low read counts for Erg3, Erg9 and Erg20 precluded differential gene  
205 expression analysis across all conditions (dark grey). Enzyme abbreviations: Erg10 acetyl-CoA  
206 acetyltransferase, Erg13 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, Hmg1/Hmg2 HMG-CoA  
207 reductase, Erg12 mevalonate kinase, Erg8 phosphomevalonate kinase, Mvd1 mevalonate  
208 pyrophosphate decarboxylase, Idi1 isopentenyl diphosphate:dimethylallyl diphosphate (IPP) isomerase,  
209 Erg20 farnesyl pyrophosphate synthetase, Erg9 farnesyl-diphosphate transferase (squalene synthase),  
210 Erg7 lanosterol synthase, Erg11 lanosterol 14 $\alpha$ -demethylase, Cyb5 cytochrome b5 (electron donor for  
211 sterol C5-6 desaturation), Ncp1 NADP-cytochrome P450 reductase, Erg24 C-14 sterol reductase, Erg25 C-  
212 4 methyl sterol oxidase, Erg26 C-3 sterol dehydrogenase, Erg27 3-keto-sterol reductase, Erg28  
213 endoplasmic reticulum membrane protein (may facilitate protein-protein interactions between Erg26  
214 and Erg27, or tether these to the ER), Erg6  $\Delta$ 24-sterol C-methyltransferase, Erg2  $\Delta$ 24-sterol C-  
215 methyltransferase, Erg3 C-5 sterol desaturase, Erg5 C-22 sterol desaturase, Erg4 C24/28 sterol  
216 reductase, Aus1/Pdr11 plasma-membrane sterol transporter.

217 **Absence of sterol import in *K. marxianus***

218 To test the hypothesis that *K. marxianus* lacks a functional sterol-uptake mechanism, uptake of  
219 fluorescent sterol derivative 25-NBD-cholesterol (NBDC) was measured by flow cytometry<sup>43</sup>. Since *S.*  
220 *cerevisiae* sterol transporters are not expressed in aerobic conditions<sup>20</sup> and to avoid interference of  
221 sterol synthesis, NBDC uptake was analysed in anaerobic cell suspensions (Fig. 4a). Four hours after  
222 NBDC addition to cell suspensions of the reference strain *S. cerevisiae* IMX585, median single-cell  
223 fluorescence increased by 66-fold (Fig. 4bc). In contrast, the congenic sterol-transporter-deficient strain  
224 IMK809 (*aus1Δ pdr11Δ*) only showed a 6-fold increase of fluorescence, probably reflected detergent-  
225 resistant binding of NBDC to *S. cerevisiae* cell-wall proteins<sup>43,44</sup>. *K. marxianus* strains CBS6556 and  
226 NBRC1777 did not show increased fluorescence, neither after 4 h nor after 23 h of incubation with NBDC  
227 (< 2-fold, Fig. 4bc, Supplementary Fig. 7).



228 **Fig. 4 | Uptake of the fluorescent sterol derivative NBDC by *S. cerevisiae* and *K. marxianus* strains. a,**

229 Experimental approach. *S. cerevisiae* strains IMX585 (reference) and IMK809 (*aus1Δ pdr11Δ*), and *K.*

230 *marxianus* strains NBRC1777 and CBS6556 were each anaerobically incubated in four replicate shake-

231 flask cultures. NBDC and Tween 80 (NBDC T) were added to two cultures, while only Tween 80 (T) was  
232 added to the other two. After 4 h incubation, cells were stained with propidium iodide (PI) and analysed  
233 by flow cytometry. PI staining was used to eliminate cells with compromised membrane integrity from  
234 analysis of NBDC fluorescence. Cultivation conditions and flow cytometry gating are described in  
235 Methods and in Supplementary Fig. 8, Supplementary Data set 1 and 2. **b**, Median and pooled standard  
236 deviation of fluorescence intensity ( $\lambda_{\text{ex}}$  488 nm |  $\lambda_{\text{em}}$  533/30 nm, FL1-A) of PI-negative cells with variance  
237 of biological replicates after 4 h exposure to Tween 80 (white bars) or Tween 80 and NBDC (blue bars).  
238 Variance was pooled for the strains IMX585, CBS6556 and NBRC1777 by repeating the experiment. **c**,  
239 NBDC fluorescence-intensity distribution of cells in a sample from a single culture for each strain, shown  
240 as modal-scaled density function. Dashed lines represent background fluorescence of unstained cells of  
241 *S. cerevisiae* and *K. marxianus*. Fluorescence data for 23-h incubations with NBDC are shown in  
242 Supplementary Fig. 7.

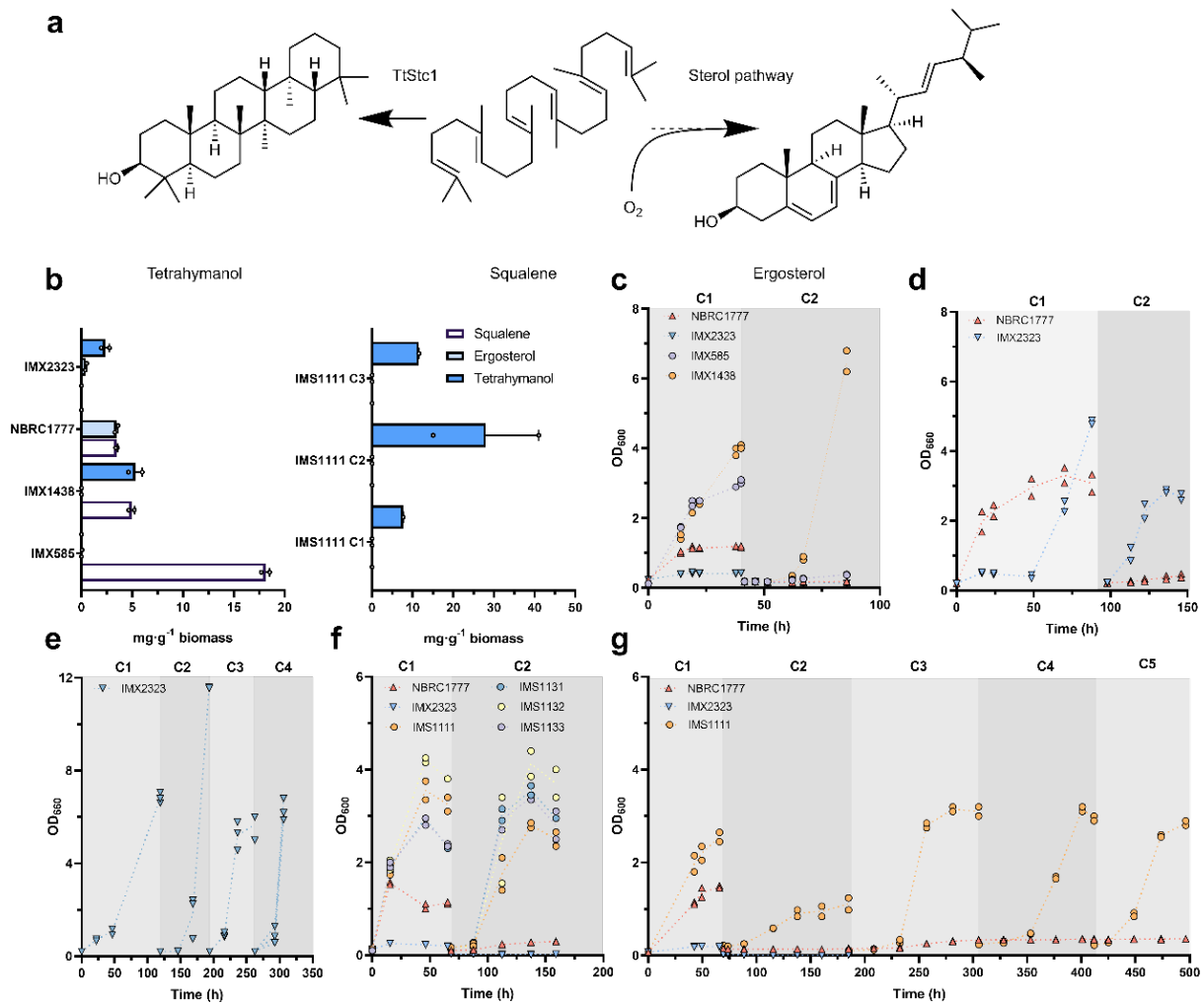
### 243 **Engineering *K. marxianus* for oxygen-independent growth**

244 Sterol uptake by *S. cerevisiae*, which requires cell wall proteins as well as a membrane transporter, has  
245 not yet been fully resolved<sup>42,43</sup>. Instead of expressing a heterologous sterol-import system in *K.*  
246 *marxianus*, we therefore explored production of tetrahymanol, which acts as a sterol surrogate in  
247 strictly anaerobic fungi<sup>45</sup>. Expression of a squalene-tetrahymanol cyclase from *Tetrahymena*  
248 *thermophila* (*TtSTC1*), which catalyzes the single-step oxygen-independent conversion of squalene into  
249 tetrahymanol (Fig. 5a), was recently shown to enable sterol-independent growth of *S. cerevisiae*<sup>46</sup>.  
250 *TtSTC1* was expressed in *K. marxianus* NBRC1777, which is more genetically amenable than strain  
251 CBS6556<sup>47</sup>. After 40 h of anaerobic incubation, the resulting strain contained  $2.4 \pm 0.4 \text{ mg} \cdot (\text{g biomass})^{-1}$   
252 tetrahymanol,  $0.4 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$  ergosterol and no detectable squalene, while strain NBRC1777 contained  
253  $3.5 \pm 0.1 \text{ mg} \cdot \text{g}^{-1}$  squalene and  $3.4 \pm 0.2 \text{ mg} \cdot \text{g}^{-1}$  ergosterol (Fig. 5b). In strictly anaerobic cultures on sterol-

254 free medium, strain NBRC1777 grew immediately after inoculation but not after transfer to a second  
255 anaerobic culture (Fig. 5c), consistent with 'carry-over' of ergosterol from the aerobic preculture<sup>19</sup>. The  
256 tetrahymanol-producing strain did not grow under these conditions (Fig. 5c) but showed sustained  
257 growth under severely oxygen-limited conditions that did not support growth of strain NBRC1777 (Fig.  
258 5de). Single-cell isolates derived from these oxygen-limited cultures (IMS1111, IMS1131, IMS1132,  
259 IMS1133) showed instantaneous as well as sustained growth under strictly anaerobic conditions (Figure  
260 5f and 5g). Tetrahymanol contents in the first, second and third cycle of anaerobic cultivation of isolate  
261 IMS1111 were  $7.6 \pm 0.0 \text{ mg}\cdot\text{g}^{-1}$ ,  $28.0 \pm 13.0 \text{ mg}\cdot\text{g}^{-1}$  and  $11.5 \pm 0.1 \text{ mg}\cdot\text{g}^{-1}$ , respectively (Fig. 5b), while no  
262 ergosterol was detected.

263 To identify whether adaptation of the tetrahymanol-producing strain IMX2323 to anaerobic growth  
264 involved genetic changes, its genome and those of the four adapted isolates were sequenced  
265 (Supplementary Table 1). No copy number variations were detected in any of the four adapted isolates.  
266 Only strain IMS1111 showed two non-conservative mutations in coding regions: a single-nucleotide  
267 insertion in a transposon-borne gene and a stop codon at position 350 (of 496 bp) in *KmCLN3*, which  
268 encodes for a G1 cyclin<sup>48</sup>. The apparent absence of mutations in the three other, independently adapted  
269 strains indicated that their ability to grow anaerobically reflected a non-genetic adaptation.



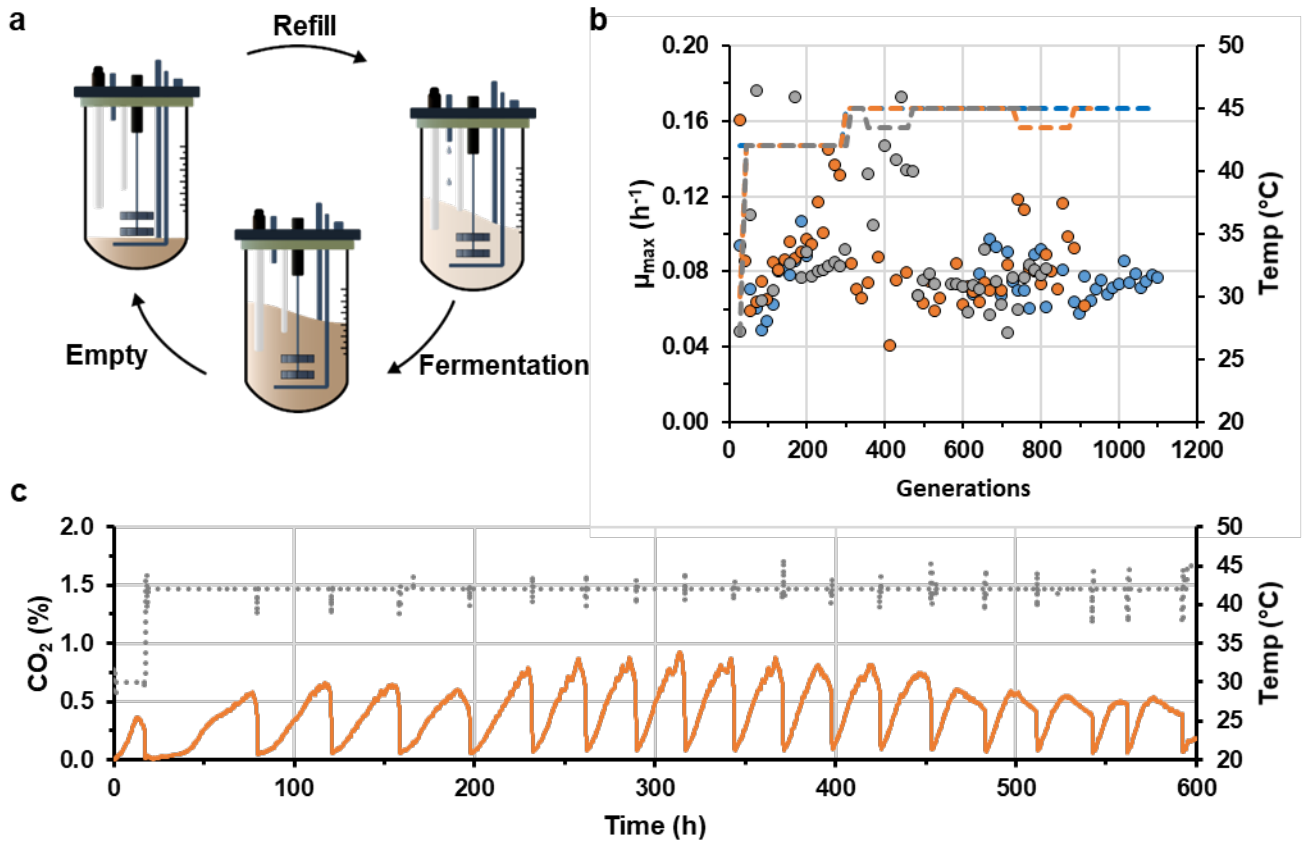


270 **Fig. 5 | Sterol-independent anaerobic growth of *K. marxianus* strains expressing *TtStc1*.** a, Oxygen-  
 271 dependent sterol synthesis and cyclisation of squalene to tetrahymanol by TtStc1. b, Squalene,  
 272 ergosterol, and tetrahymanol contents with mean and standard error of the mean of (left panel) *S.*  
 273 *cerevisiae* strains IMX585 (reference), IMX1438 (*sga1Δ::TtStc1*), and *K. marxianus* strains NBRC1777  
 274 (reference), IMX2323 (*TtStc1*). Lipid composition of single-cell isolate IMS1111 (*TtStc1*) (right panel)  
 275 over 3 serial transfers (C1-C3). Data from replicate cultures grown in strictly anaerobic (c, f, g) or  
 276 severely oxygen-limited shake-flask cultures (d, e). Aerobic grown pre-cultures were used to inoculate  
 277 the first anaerobic culture on SMG-urea and Tween 80, when the optical density started to stabilize the  
 278 cultures were transferred to new media. Data depicted are of each replicate culture (points) and the

279 mean (dotted line) from independent biological duplicate cultures, serial transfers cultures are  
280 represented with C1-C5. Strains NBRC1777 (wild-type, upward red triangles), IMX2323 (*TtSTC1*, cyan  
281 downward triangle), and the single-cell isolates IMS1111 (*TtSTC1*, orange circles), IMS1131 (*TtSTC1*, blue  
282 circles), IMS1132 (*TtSTC1*, yellow circles), IMS1133 (*TtSTC1*, purple circles). *S. cerevisiae* IMX585  
283 (reference, purple circle) and IMX1438 (*TtSTC1*, orange circles). **c**, Extended data with double inoculum  
284 size is available in Supplementary Fig. 10. **d**, Extended data is available in Supplementary Fig. 9a.

### 285 **Test of anaerobic thermotolerance and selection for fast growing anaerobes**

286 One of the attractive phenotypes of *K. marxianus* for industrial application is its high thermotolerance  
287 with reported maximum growth temperatures of 46-52 °C<sup>49,50</sup>. To test if anaerobically growing  
288 tetrahymanol-producing strains retained thermotolerance, strain IMS1111 was grown in anaerobic  
289 sequential-batch-reactor (SBR) cultures (Fig. 6) in which, after an initial growth cycle at 30 °C, the growth  
290 temperature was shifted to 42 °C. Specific growth at 42 °C progressively accelerated from 0.06 h<sup>-1</sup> to  
291 0.13 h<sup>-1</sup> over 17 SBR cycles (corresponding to ca. 290 generations; Fig. 6b). A subsequent temperature  
292 increase to 45 °C led to a strong decrease of the specific growth rate which, after approximately 1000  
293 generations of selective growth, stabilized at approximately 0.08 h<sup>-1</sup>. Whole-population genome  
294 sequencing of the evolved populations revealed no common mutations or chromosomal copy number  
295 variations (Supplementary Table 1). These data show that *TtSTC1*-expressing *K. marxianus* can grow  
296 anaerobically at temperatures up to at least 45 °C.



297 **Fig. 6 | Thermotolerance and anaerobic growth of tetrahymanol-producing *K. marxianus* strain.** The  
298 strain IMS1111 was grown in triplicate sequential batch bioreactor cultivations in synthetic media  
299 supplemented with 20 g·L<sup>-1</sup> glucose and 420 mg·L<sup>-1</sup> Tween 80 at pH 5.0. **a**, Experimental design of  
300 sequential batch fermentation with cycles at step-wise increasing temperatures to select for faster  
301 growing mutants, each cycle consisted of three phases; (i) (re)filling of the bioreactor with fresh media  
302 up to 100 mL and adjustment of temperature to a new set-point, (ii) anaerobic batch fermentation at a  
303 fixed culture temperature with continuous N<sub>2</sub> sparging for monitoring of CO<sub>2</sub> in the culture off-gas, and  
304 (iii) fast broth withdrawal leaving 7 mL (14.3 fold dilution) to inoculate the next batch. **b**, Maximum  
305 specific estimated growth rate (circles) of each batch cycle for the three independent bioreactor  
306 cultivations (M3R blue, M5R orange, M6L grey) with the estimated number of generations. The growth  
307 rate was calculated from the CO<sub>2</sub> production as measured in the off-gas and should be interpreted as an  
308 estimate and in some cases could not be calculated. The culture temperature profile (dotted line) for

309 each independent bioreactor cultivation (blue, grey, orange) consisted of a step-wise increment of the  
310 temperature at the onset of the fermentation phase in each batch cycle. **c**, Representative section of  
311 CO<sub>2</sub> off-gas profiles of the individual bioreactor (M5R) cultivation over time with CO<sub>2</sub> fraction (orange  
312 line) and culture temperature (grey dotted line), data of the entire experiment is available in  
313 Supplementary Fig. 11 (Data availability).

## 314 **Discussion**

315 Industrial production of ethanol from carbohydrates relies on *S. cerevisiae*, due to its capacity for  
316 efficient, fast alcoholic fermentation and growth under strictly anaerobic process conditions. Many  
317 facultatively fermentative yeast species outside the Saccharomycotina WGD-clade also rapidly ferment  
318 sugars to ethanol under oxygen-limited conditions<sup>26</sup>, but cannot grow and ferment in the complete  
319 absence of oxygen<sup>11,13,25</sup>. Identifying and eliminating oxygen requirements of these yeasts is essential to  
320 unlock their industrially relevant traits for application. Here, this challenge was addressed for the  
321 thermotolerant yeast *K. marxianus*, using a systematic approach based on chemostat-based quantitative  
322 physiology, genome and transcriptome analysis, sterol-uptake assays and genetic modification. *S.*  
323 *cerevisiae*, which was used as a reference in this study, shows strongly different genome-wide  
324 expression profiles under aerobic and anaerobic or oxygen-limited conditions<sup>51</sup>. Although only a small  
325 fraction of these differences were conserved in *K. marxianus* (Fig. 2), we were able to identify absence  
326 of a functional sterol import system as the critical cause for its inability to grow anaerobically. Enabling  
327 synthesis of the sterol surrogate tetrahymanol yielded strains that grew anaerobically at temperatures  
328 above the permissive temperature range of *S. cerevisiae*.

329 A short adaptation phase of tetrahymanol-producing *K. marxianus* strains under oxygen-limited  
330 conditions reproducibly enabled strictly anaerobic growth. Although this ability was retained after  
331 aerobic isolation of single-cell lines, we were unable to attribute this adaptation to mutations. In

332 contrast to wild-type *K. marxianus*, a non-adapted tetrahymanol-producing strain did not show ‘carry-  
333 over growth’ after transfer from aerobic to strictly anaerobic conditions and adapted cultures showed  
334 reduced squalene contents (Fig. 5). These observations suggest that interactions between tetrahymanol,  
335 ergosterol and/or squalene influence the onset of anaerobic growth and that oxygen-limited growth  
336 results in a stable balance between these lipids that is permissive for anaerobic growth.

337 Comparative genomic studies in Saccharomycotina yeasts have previously led to the hypothesis that  
338 sterol transporters are absent from pre-WGD yeast species<sup>11,52</sup>. While our observations on *K. marxianus*  
339 reinforce this hypothesis, which was hitherto not experimentally tested, they do not exclude  
340 involvement of additional oxygen-requiring reactions in other non-*Saccharomyces* yeasts. For example,  
341 pyrimidine biosynthesis is often cited as a key oxygen-requiring process in non-*Saccharomyces* yeasts,  
342 due to involvement of a respiratory-chain-linked dihydroorotate dehydrogenase (DHOD)<sup>53,54</sup>. *K.*  
343 *marxianus*, is among a small number of yeast species that, in addition to this respiration dependent  
344 enzyme (KmUra9), also harbors a fumarate-dependent DHOD (KmUra1)<sup>55</sup>. In *K. marxianus* the activation  
345 of this oxygen-independent KmUra1 is a crucial adaptation for anaerobic pyrimidine biosynthesis. The  
346 experimental approach followed in the present study should be applicable to resolve the role of  
347 pyrimidine biosynthesis and other oxygen-requiring reactions in additional yeast species.

348 Enabling *K. marxianus* to grow anaerobically represents an important step towards application of this  
349 thermotolerant yeast in large-scale anaerobic bioprocesses. However, specific growth rates and biomass  
350 yields of tetrahymanol-expressing *K. marxianus* in anaerobic cultures were lower than those of wild-type  
351 *S. cerevisiae* strains. A similar phenotype of tetrahymanol-producing *S. cerevisiae* was proposed to  
352 reflect an increased membrane permeability<sup>46</sup>. Additional membrane engineering or expression of a  
353 functional sterol transport system is therefore required for further development of robust, anaerobically  
354 growing industrial strains of *K. marxianus*<sup>56</sup>.

355 **online Methods**

356 **Yeast strains, maintenance and shake-flask cultivation**

357 *Saccharomyces cerevisiae* CEN.PK113-7D<sup>57,58</sup> (*MATa MAL2-8c SUC2*) was obtained from Dr. Peter Kötter,  
358 J.W. Goethe University, Frankfurt. *Kluyveromyces marxianus* strains CBS 6556 (ATCC 26548; NCYC 2597;  
359 NRRL Y-7571) and NBRC 1777 (IFO 1777) were obtained from the Westerdijk Fungal Biodiversity  
360 Institute (Utrecht, The Netherlands) and the Biological Resource Center, NITE (NBRC) (Chiba, Japan),  
361 respectively. Stock cultures of *S. cerevisiae* were grown at 30 °C in an orbital shaker set at 200 rpm, in  
362 500 mL shake flasks containing 100 mL YPD (10 g·L<sup>-1</sup> Bacto yeast extract, 20 g·L<sup>-1</sup> Bacto peptone, 20 g·L<sup>-1</sup>  
363 glucose). For cultures of *K. marxianus*, the glucose concentration was reduced to 7.5 g·L<sup>-1</sup>. After addition  
364 of glycerol to early stationary-phase cultures, to a concentration of 30 % (v/v), 2 mL aliquots were stored  
365 at -80 °C. Shake-flask precultures for bioreactor experiments were grown in 100 mL synthetic medium  
366 (SM) with glucose as carbon source and urea as nitrogen source (SMG-urea)<sup>17,59</sup>. For anaerobic  
367 cultivation, synthetic medium was supplemented with ergosterol (10 mg·L<sup>-1</sup>) and Tween 80 (420 mg·L<sup>-1</sup>)  
368 as described previously<sup>14,17,19</sup>.

369 **Expression cassette and plasmid construction**

370 Plasmids used in this study are described in (Table 4). To construct plasmids pUDE659 (gRNA<sub>AUS1</sub>) and  
371 pUDE663 (gRNA<sub>PDR11</sub>), the pROS11 plasmid-backbone was PCR amplified using Phusion HF polymerase  
372 (Thermo Scientific, Waltham, MA) with the double-binding primer 6005. PCR amplifications were  
373 performed with desalted or PAGE-purified oligonucleotide primers (Sigma-Aldrich, St Louis, MO)  
374 according to manufacturer's instructions. To introduce the gRNA-encoding nucleotide sequences into  
375 gRNA-expression plasmids, a 2µm fragment was first amplified with primers 11228 and 11232  
376 containing the specific sequence as primer overhang using pROS11 as template. PCR products were  
377 purified with genElutePCR Clean-Up Kit (Sigma-Aldrich) or Gel DNA Recovery Kit (Zymo Research, Irvine,

378 CA). The two DNA fragments were then assembled by Gibson Assembly (New England Biolabs, Ipswich,  
379 MA) according to the manufacturer's instructions. Gibson assembly reaction volumes were downscaled  
380 to 10  $\mu\text{L}$  and 0.01  $\text{pmol}\cdot\mu\text{L}^{-1}$  DNA fragments at 1:1 molar ratio for 1 h at 50 °C. Chemically competent *E.*  
381 *coli* XL1-Blue was transformed with the Gibson assembly mix via a 5 min incubation on ice followed by a  
382 40 s heat shock at 42 °C and 1 h recovery in non-selective LB medium. Transformants were selected on  
383 LB agar containing the appropriate antibiotic. Golden Gate assembly with the yeast tool kit<sup>60</sup> was  
384 performed in 20  $\mu\text{L}$  reaction mixtures containing 0.75  $\mu\text{L}$  BsaI HF V2 (NEB, #R3733), 2  $\mu\text{L}$  DNA ligase  
385 buffer with ATP (New England Biolabs), 0.5  $\mu\text{L}$  T7-ligase (NEB) with 20 fmol DNA donor fragments and  
386 MilliQ water. Before ligation at 16 °C was initiated by addition of T7 DNA ligase, an initial BsaI digestion  
387 (30 min at 37 °C) was performed. Then 30 cycles of digestion and ligation at 37 °C and 16 °C,  
388 respectively, were performed, with 5 min incubation times for each reaction. Thermocycling was  
389 terminated with a 5 min final digestion step at 60 °C.

390 To construct a *TtSTC1* expression vector, the coding sequence of *TtSTC1* (pUD696) was PCR amplified  
391 with primer pair 16096/16097 and Golden gate assembled with the donor plasmids pGGkd015 (ori  
392 ampR), pP2 (Km*PDC1*p), pYTK053 (*ScADH1t*) resulting in pUDE909 (ori ampR Km*PDC1*p-*TtSTC1*-  
393 *ScADH1t*). For integration of *TtSTC1* cassette into the *lac4* locus both upstream and downstream flanks  
394 (877/878 bps) of the *lac4* locus were PCR amplified with the primer pairs 14197/14198 and  
395 14199/14200, respectively. An empty integration vector, pGGkd068, was constructed by *BsaI* golden  
396 gate cloning of pYTK047 (GFP-dropout), pYTK079 (hygB), pYTK090 (kanR), pYTK073 (ConRE'), pYTK008  
397 (ConLS') together with the two *lac4* homologous nucleotide sequences. Plasmid assembly was verified  
398 by PCR amplification with primers 15210, 9335, 16274 and 16275 and by digestion with *BsmBI* (New  
399 England Biolabs, #R0580). The integration vector pUDI246 with the *TtSTC1* expression cassette was  
400 constructed by Gibson assembly of the PCR amplified pGGkd068 and pUDE909 with primer pairs  
401 16274/16275 and 16272/16273, thereby adding 20 bp overlaps for assembly. For this step, the

402 incubation time of the Gibson assembly was increased to 90 min. Plasmid assembly was verified by  
403 diagnostic PCR amplification using DreamTaq polymerase (Thermo Scientific) with primers 5941, 8442,  
404 15216 and subsequent Illumina short-read sequencing.

405 **Table 2 | Strains used in this study.** Abbreviations: *Saccharomyces cerevisiae* (Sc), *Kluyveromyces*  
406 *marxianus* (Km), *Tetrahymena thermophila* (Tt).

Genus	Strain	Relevant genotype	Reference
<i>S. cerevisiae</i>	CEN.PK113-7D	<i>MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2</i>	Entian and Kötter, 2007 <sup>57</sup>
<i>S. cerevisiae</i>	IMX585	CEN.PK113-7D <i>can1Δ::cas9-natNT2</i>	Mans <i>et al.</i> , 2015 <sup>61</sup>
<i>S. cerevisiae</i>	IMX1438	IMX585 <i>sga1Δ::TtSTC1</i>	Wiersma <i>et al.</i> , 2020 <sup>46</sup>
<i>S. cerevisiae</i>	IMK802	IMX585 <i>aus1Δ</i>	This study
<i>S. cerevisiae</i>	IMK806	IMX585 <i>pdr11Δ</i>	This study
<i>S. cerevisiae</i>	IMK809	IMX585 <i>aus1Δ pdr11Δ</i>	This study
<i>K. marxianus</i>	CBS6556	<i>URA3 HIS3 LEU2 TRP1</i>	CBS-KNAW*
<i>K. marxianus</i>	NBRC1777	<i>URA3 HIS3 LEU2 TRP1</i>	NBRC**
<i>K. marxianus</i>	IMX2323	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study
<i>K. marxianus</i>	IMS1111	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study
<i>K. marxianus</i>	IMS1112	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study
<i>K. marxianus</i>	IMS1113	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study
<i>K. marxianus</i>	IMS1131	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study
<i>K. marxianus</i>	IMS1132	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study
<i>K. marxianus</i>	IMS1133	Km <i>PDC1p-TtSTC1-ScADH1t-hygB</i>	This study

407



408 **Table 3 | CRISPR gRNA target sequences used in this study.** gRNA target sequences are shown with  
 409 PAM sequences underlined. Position in ORF indicates the base pair after which the Cas9-mediated  
 410 double-strand break is introduced. AT score indicates the AT content of the 20-bp target sequence and  
 411 RNA score indicates the fraction of unpaired nucleotides of the 20-bp target sequence, predicted with  
 412 the complete gRNA sequence using a minimum free energy prediction by the RNAfold algorithm<sup>62</sup>.

Locus	Target sequence (5'-3')	Position in ORF (bp)	AT score	RNA score
<i>AUS1</i>	CATTATTGTAAATGATTTGG <u>IGG</u>	320/4184	0.75	1
<i>PDR11</i>	ATCTTTCATATAAATAACAT <u>AGG</u>	1627/4235	0.85	1

413

414 **Table 4 | Plasmids used in this study.** Restriction enzyme recognition sites are indicated in superscript.  
 415 US/DS represent upstream and downstream homologous recombination sequences used for genomic  
 416 integration into the *K. marxianus lac4* locus. Abbreviations: *Saccharomyces cerevisiae* (Sc),  
 417 *Kluyveromyces marxianus* (Km), *Tetrahymena thermophila* (Tt).

Plasmid	Characteristics	Source
pGGkd015	ori amp <sup>R</sup> ConLS GFP ConR1	Hassing <i>et al.</i> , 2019 <sup>63</sup>
pGGkd068	ori kan <sup>R</sup> <sup>NotI</sup> <i>Kmlac4</i> <sub>US</sub> <sup>BsmBI</sup> ConRE' <sup>BsaI</sup> sfGFP <sup>BsaI</sup> ConLS' <sup>BsmBI</sup> hygB	This study
pP2	ori cam <sup>R</sup> <i>KmPDC1p</i>	Rajkumar <i>et al.</i> , 2019 <sup>47</sup>
pROS11	ori amp <sup>R</sup> 2μm amdSYM pSNR52-gRNA <sub>CAN1</sub> pRSNR52-gRNA <sub>ADE2</sub>	Mans <i>et al.</i> , 2015 <sup>61</sup>
pUD696	ori kan <sup>R</sup> <i>TtSTC1</i>	Wiersma <i>et al.</i> , 2020 <sup>46</sup>
pUDE659	ori amp <sup>R</sup> 2μm amdSYM pSNR52-gRNA <sub>AUS1</sub> pRSNR52-gRNA <sub>AUS1</sub>	This study
pUDE663	ori amp <sup>R</sup> 2μm amdSYM pSNR52-gRNA <sub>PDR11</sub> pRSNR52-gRNA <sub>PDR11</sub>	This study
pUDE909	ori amp <sup>R</sup> <i>KmPDC1p-TtSTC1-ScADH1t</i>	This study
pUDI246	ori kan <sup>R</sup> <sup>NotI</sup> <i>Kmlac4</i> <sub>US</sub> <i>KmPDC1p-TtSTC1-ScADH1t</i> hygB <i>Kmlac4</i> <sub>DS</sub> <sup>NotI</sup>	This study
pYTK008	ori cam <sup>R</sup> ConLS'	Lee <i>et al.</i> , 2015 <sup>60</sup>
pYTK047	ori cam <sup>R</sup> GFP dropout	Lee <i>et al.</i> , 2015 <sup>60</sup>
pYTK053	ori cam <sup>R</sup> <i>ScADH1t</i>	Lee <i>et al.</i> , 2015 <sup>60</sup>
pYTK073	ori cam <sup>R</sup> ConRE'	Lee <i>et al.</i> , 2015 <sup>60</sup>
pYTK079	ori cam <sup>R</sup> hygB	Lee <i>et al.</i> , 2015 <sup>60</sup>

418

419 **Table 5 | Oligonucleotide primers used in this study.**

Primer	Sequence (5'->3')
11228	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCCATTATTGTAAATGATTTGGGTTTTA GAGCTAGAAATAGCAAGTTAAAATAAG
11232	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCATCTTTCATATAAATAACATGTTTTA GAGCTAGAAATAGCAAGTTAAAATAAG
11233	TAGTAAAGACTGCTGTAATTCATCTCTCAGTCCCTTGCAGTCTGCTTTTTCTGGAATTAATTACCATTTTTAAATAT ATTTCTACTTTCTACTTAATAGCAATTTTAATTAATCTAATTAT
11234	ATAATTAGATTAATTAATAAATTGCTATTAAGTAGAAAGTAGAAATATATTTAAAAATGGTAATTAATTCAGAAAA GCAGACTGCAAGGACTGAGAGATGAATTACAGCAGTCTTTACTA
11241	TAGCAAAAAAATTCACAACATAACACGATAGAGTAAAAATAGAGAAGCAACGCCTCGCGGTCAGTGAATAGCGTTC CGTTAGAAAACATTCAAAAATTACCTAATACTATTCAACAGTTCT
11242	AGAAGTGTGAATAGTATTAGGTAATTTTGAATGTTTTCTAACGGAACGCTATTCAGTACCAGCGAGGCGTTGCTT CTCTAATTTTACTCTATCGTGTTTAGTTTGAATTTTTTTGCTA
11243	TGTCACTACAGCCACAGCAG
11244	TTGGTAAGGCGCCACACTAG
11251	AGAGAAGCGCCACATAGACG
11252	TGCATATGCTACGGGTGACG
11897	CACCCAAGTATGGTGGGTAG
14148	AAGCATCGTCTCATCGGTCTCATATGTCAATTTCAAAGTACTTCACTCCCGTTGCTGAC
14149	TTATGCCGTCTCAGGTCTCAGGATTTAGTTCTGTACAGGCTTCTTC
14150	TTATGCCGTCTCAGGTCTCAAGAATTAGTTCTGTACAGGCTTCTTC
14151	AAGCATCGTCTCATCGGTCTCATATGTCTTTCATAAAATCGCTGCCTTATTAG
14152	TTATGCCGTCTCAGGTCTCAGGATATCATAAGAGCATAGCAGCGGCACCGGCAATAG
14197	AAGCATCGTCTCATCGGTCTCACAATGAAAGTGATTGAAGAACCCTCAAAC
14198	TTATGCCGTCTCAGGTCTCAAGGGTTAAGCAATTGGATCCTACC
14199	AAGCATCGTCTCATCGGTCTCAGAGTTGCTTAATTAGCTTGTACATGGCTTTG
14200	TTATGCCGTCTCAGGTCTCATCGGGAAGGCCATATTGAAGACG
14339	CCCAAATCATTTACAATAATGGATCATTTATC
14340	CATGTTATTTATATGAAAGATGATCATTTATC
16366	GTCCCTAGGTTTCGTCATT
16367	CAAGATCAATGGTGGCTCTC

420

421 **Strain construction**

422 The lithium-acetate/polyethylene-glycol method was used for yeast transformation<sup>64</sup>. Homologous  
 423 repair (HR) DNA fragments for markerless CRISPR-Cas9-mediated gene deletions in *S. cerevisiae* were  
 424 constructed by annealing two 120 bp primers, using primer pairs 11241/11242 and 11233/11234 for  
 425 deletion of *PDR11* and *AUS1*, respectively. After transformation of *S. cerevisiae* IMX585 with gRNA  
 426 plasmids pUDE659 and pUDE663 and double-stranded repair fragments, transformants were selected  
 427 on synthetic medium with acetamide as sole nitrogen source<sup>65</sup>. Deletion of *AUS1* and *PDR11* was

428 confirmed by PCR amplification with primer pairs 11243/11244 and 11251/11252, respectively. Loss of  
429 gRNA plasmids was induced by cultivation of single-colony isolates on YPD, after which plasmid loss was  
430 assessed by absence of growth of single-cell isolates on synthetic medium with acetamide as nitrogen  
431 source. An *aus1Δ pdr11Δ* double-deletion strain was similarly constructed by chemical transformation of  
432 *S. cerevisiae* IMK802 with pUDE663 and repair DNA. To integrate a *TtSTC1* expression cassette into the  
433 *K. marxianus lac4* locus, *K. marxianus* NBRC1777 was transformed with 2 µg DNA *NotI*-digested  
434 pUDI246. After centrifugation, cells were resuspended in YPD and incubated at 30 °C for 3 h. Cells were  
435 then again centrifuged, resuspended in demineralized water and plated on 200 µg·L<sup>-1</sup> hygromycin B  
436 (InvivoGen, Toulouse, France) containing agar with 40 µg·L<sup>-1</sup> X-gal, 5-bromo-4-chloro-3-indolyl-β-D-  
437 galactopyranoside (Fermentas, Waltham, MA). Colonies that could not convert X-gal were analyzed for  
438 correct genomic integration of the *TtSTC1* by diagnostic PCR with primers 16366, 16367 and 11897.  
439 Genomic integration of *TtSTC1* into the chromosome outside the *lac4* locus was confirmed by short-read  
440 Illumina sequencing.

#### 441 **Chemostat cultivation**

442 Chemostat cultures were grown at 30 °C in 2 L bioreactors (Applikon, Delft, the Netherlands) with a  
443 stirrer speed of 800 rpm. The dilution rate was set at 0.10 h<sup>-1</sup> and a constant working volume of 1.2 L  
444 was maintained by connecting the effluent pump to a level sensor. Cultures were grown on synthetic  
445 medium with vitamins<sup>17</sup>. Concentrated glucose solutions were autoclaved separately at 110 °C for 20  
446 min and added at the concentrations indicated, along with sterile antifoam pluronic 6100 PE (BASF,  
447 Ludwigshafen, Germany; final concentration 0.2 g·L<sup>-1</sup>). Before autoclaving, bioreactors were tested for  
448 gas leakage by submerging them in water while applying a 0.3 bar overpressure.

449 Anaerobic conditions of bioreactor cultivations were maintained by continuous reactor headspace  
450 aeration with pure nitrogen gas (≤ 0.5 ppm O<sub>2</sub>, HiQ Nitrogen 6.0, Linde AG, Schiedam, the Netherlands)

451 at a flowrate of 500 mL N<sub>2</sub> min<sup>-1</sup> (2.4 vvm). Gas pressure of 1.2 bar of the reactor headspace was set with  
452 a reduction valve (Tescom Europe, Hannover, Germany) and remained constant during cultivation. To  
453 prevent oxygen diffusion into the cultivation the bioreactor was equipped with Fluran tubing (14 Barrer  
454 O<sub>2</sub>, F-5500-A, Saint-Gobain, Courbevoie, France), Viton O-rings (Eriks, Alkmaar, the Netherlands), and no  
455 pH probes were mounted. The medium reservoir was deoxygenated by sparge aeration with nitrogen  
456 gas ( $\leq 1$  ppm O<sub>2</sub>, HiQ Nitrogen 5.0, Linde AG).

457 For aerobic cultivation the reactor was sparged continuously with dried air at a flowrate of 500 mL air  
458 min<sup>-1</sup> (2.4 vvm). Dissolved oxygen levels were analyzed by Clark electrodes (AppliSens, Applikon) and  
459 remained above 40% during the cultivation. For micro-aerobic cultivations nitrogen ( $\leq 1$  ppm O<sub>2</sub>, HiQ  
460 Nitrogen 5.0, Linde AG) and air were mixed continuously by controlling the fractions of mass flow rate of  
461 the dry gas to a total flow of 500 mL min<sup>-1</sup> per bioreactor. The mixed gas was distributed to each  
462 bioreactor and analyzed separately in real-time. Continuous cultures were assumed to be in steady state  
463 when after at least 5 volumes changes, culture dry weight and the specific carbon dioxide production  
464 rates changed by less than 10%.

465 Cell density was routinely measured at a wavelength of 660 nm with spectrophotometer Jenway 7200  
466 (Cole Palmer, Staffordshire, UK). Cell dry weight of the cultures were determined by filtering exactly 10  
467 mL of culture broth over pre-dried and weighed membrane filters (0.45  $\mu$ m, Thermo Fisher Scientific),  
468 which were subsequently washed with demineralized water, dried in a microwave oven (20 min, 350 W)  
469 and weighed again<sup>66</sup>.

#### 470 **Metabolite analysis**

471 For determination of substrate and extracellular metabolite concentrations, culture supernatants were  
472 obtained by centrifugation of culture samples (5 min at 13000 rpm) and analyzed by high-performance  
473 liquid chromatography (HPLC) on a Waters Alliance 2690 HPLC (Waters, MA, USA) equipped with a Bio-

474 Rad HPX-87H ion exchange column (BioRad, Veenendaal, the Netherlands) operated at 60 °C with a  
475 mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub> at a flowrate of 0.6 mL·min<sup>-1</sup>. Compounds were detected by means of a  
476 dual-wavelength absorbance detector (Waters 2487) and a refractive index detector (Waters 2410) and  
477 compared to reference compounds (Sigma-Aldrich). Residual glucose concentrations in continuous  
478 cultivations were determined by HPLC analysis from rapid quenched culture samples with cold steel  
479 beads<sup>67</sup>.

#### 480 **Gas analysis**

481 The off-gas from bioreactor cultures was cooled with a condenser (2 °C) and dried with PermaPure Dryer  
482 (Inacom Instruments, Veenendaal, the Netherlands) prior to analysis of the carbon dioxide and oxygen  
483 fraction with a Rosemount NGA 2000 Analyser (Baar, Switzerland). The Rosemount gas analyzer was  
484 calibrated with defined mixtures of 1.98 % O<sub>2</sub>, 3.01 % CO<sub>2</sub> and high quality nitrogen gas N6 (Linde AG).

#### 485 **Ethanol evaporation rate**

486 To correct for ethanol evaporation in the continuous bioreactor cultivations the ethanol evaporation  
487 rate was determined in the same experimental bioreactor set-up without the yeast. To SM glucose  
488 media with urea 400 mM of ethanol was added after which the decrease in the ethanol concentration  
489 was measured over time by periodic measurements and quantification by HPLC analysis over the course  
490 of at least 140 hours. To reflect the media composition used for the different oxygen regimes and  
491 anaerobic growth factor supplementation, the ethanol evaporation was measured for bioreactor sparge  
492 aeration with Tween 80, bioreactor head-space aeration both with and without Tween 80. The ethanol  
493 evaporation rate was measured for each condition in triplicate.

#### 494 **Lipid extractions & GC analysis**

495 For analysis of triterpene and triterpenoid cell contents biomass was harvested, washed once with  
496 demineralized water and stored as pellet at -80 °C before freeze-drying the pellets using an Alpha 1-4 LD  
497 Plus (Martin Christ, Osterode am Harz, Germany) at -60 °C and 0.05 mbar. Freeze-dried biomass was  
498 saponificated with 2.0 M NaOH (Bio-Ultra, Sigma-Aldrich) in methylation glass tubes (PYREX™  
499 Borosilicate glass, Thermo Fisher Scientific) at 70 °C. As internal standard 5 $\alpha$ -cholestane (Sigma-Aldrich)  
500 was added to the saponified biomass suspension. Subsequently tert-butyl-methyl-ether (tBME, Sigma-  
501 Aldrich) was added for organic phase extraction. Samples were extracted twice using tBME and dried  
502 with sodium-sulfate (Merck, Darmstadt, Germany) to remove remaining traces of water. The organic  
503 phase was either concentrated by evaporation with N<sub>2</sub> gas aeration or transferred directly to an  
504 injection vial (VWR International, Amsterdam, the Netherlands). The contents were measured by GC-FID  
505 using Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an  
506 Agilent CP9013 column (Agilent). The oven was programmed to start at 80 °C for 1 min, ramp first to 280  
507 °C with 60 °C·min<sup>-1</sup> and secondly to 320 °C with a rate of 10 °C·min<sup>-1</sup> with a final temperature hold of 15  
508 min. Spectra were compared to separate calibration lines of squalene, ergosterol,  $\alpha$ -cholestane,  
509 cholesterol and tetrahymanol as described previously<sup>46</sup>.

#### 510 **Sterol uptake assay**

511 Sterol uptake was monitored by the uptake of fluorescently labelled 25-NBD-cholesterol (Avanti Polar  
512 Lipids, Alabaster, AL). A stock solution of 25-NBD-cholesterol (NBDC) was prepared in ethanol under an  
513 argon atmosphere and stored at -20 °C. Shake flasks with 10 mL SM glucose media were inoculated with  
514 yeast strains from a cryo-stock and cultivated aerobically at 200 rpm at 30 °C overnight. The yeast  
515 cultures were subsequently diluted to an OD<sub>660</sub> of 0.2 in 400 mL SM glucose media in 500 mL shake  
516 flasks to gradually reduce the availability of oxygen and incubated overnight. Yeast cultures were  
517 transferred to fresh SM media with 40 g·L<sup>-1</sup> glucose and incubated under anaerobic conditions at 30 °C

518 at 200 rpm. After 22 hours of anaerobic incubation  $4 \mu\text{g}\cdot\text{L}^{-1}$  NBD-cholesterol with  $420 \text{ mg}\cdot\text{L}^{-1}$  Tween 80  
519 were pulsed to the cultures. Samples were taken and washed with PBS  $5 \text{ mL}\cdot\text{L}^{-1}$  Tergitol NP-40 pH 7.0  
520 (Sigma-Aldrich) twice before resuspension in PBS and subsequent analysis. Propidium Iodide (PI)  
521 (Invitrogen) was added to the sample ( $20 \mu\text{M}$ ) and stained according to the manufacturer's  
522 instructions<sup>68</sup>. PI intercalates with DNA in cells with a compromised cell membrane, which results in red  
523 fluorescence. Samples both unstained and stained with PI were analyzed with Accuri C6 flow cytometer  
524 (BD Biosciences, Franklin Lakes, NJ) with a 488 nm laser and fluorescence was measured with emission  
525 filter of 533/30 nm (FL1) for NBD-cholesterol and  $> 670 \text{ nm}$  (FL3) for PI. Cell gating and median  
526 fluorescence of cells were determined using FlowJo (v10, BD Bioscience). Cells were gated based on  
527 forward side scatter (FSC) and side-scatter (SSC) to exclude potential artifacts or clumping cells. Within  
528 this gated population PI positive and negatively stained cells were differentiated based on the cell  
529 fluorescence across a FL3 FL1 dimension. Flow cytometric gates were drafted for each yeast species and  
530 used for all samples. The gating strategy is given in Supplementary Fig. 8. Fluorescence of a strain was  
531 determined by a sample of cells from independent shake-flask cultures and compared to cells from  
532 identical unstained cultures of cells with the exact same chronological age. The staining experiment of  
533 the strains IMX585, CBS6556 and NBRC1777 samples was repeated twice for reproducibility, the mean  
534 and pooled variance was subsequently calculated from the biological duplicates of the two experiments.  
535 The NBDC intensity and cell counts obtained from the NBDC experiments are available for re-analysis in  
536 Supplementary Data set 1, and raw flow cytometry plots are depicted in Supplementary Data set 2.

### 537 **Long read sequencing, assembly, and annotation**

538 Cells were grown overnight in 500-mL shake flasks containing 100 mL liquid YPD medium at  $30 \text{ }^\circ\text{C}$  in an  
539 orbital shaker at 200 rpm. After reaching stationary phase the cells were harvested for a total  $\text{OD}_{660}$  of  
540 600 by centrifugation for 5 min at 4000 g. Genomic DNA of CBS6556 and NBRC1777 was isolated using

541 the Qiagen genomic DNA 100/G kit (Qiagen, Hilden, Germany) according to the manufacturer's  
542 instructions. MinION genomic libraries were prepared using the 1D Genomic DNA by ligation (SQK-  
543 LSK108) for CBS6556, and the 1D native barcoding Genomic DNA (EXP-NBD103 & LSK108) for NBRC1777  
544 according to the manufacturer's instructions with the exception of using 80% EtOH during the 'End  
545 Repair/dA-tailing module' step. Flow cell quality was tested by running the MinKNOW platform QC  
546 (Oxford Nanopore Technology, Oxford, UK). Flow cells were prepared by removing 20 µL buffer and  
547 subsequently primed with priming buffer. The DNA library was loaded dropwise into the flow cell for  
548 sequencing. The SQK-LSK108 library was sequenced on a R9 chemistry flow cell (FLO-MIN106) for 48 h.  
549 Base-calling was performed using Albacore (v2.3.1, Oxford Nanopore Technologies) for CBS6556, and for  
550 NBRC1777 with Guppy (v2.1.3, Oxford Nanopore Technologies) using `dna_r9.4.1_450bps_flipflop.cfg`.  
551 CBS6556 reads were assembled using Canu (v1.8)<sup>69</sup>, and NBRC1777 reads were assembled using Flye  
552 (v2.7.1-b1673)<sup>70</sup>. Assemblies were polished with Pilon (v1.18)<sup>71</sup> using Illumina data available at the  
553 Sequence Read Archive under accessions SRX3637961 and SRX3541357. Both *de novo* genome  
554 assemblies were annotated using Funannotate (v1.7.1)<sup>72</sup>, trained and refined using *de novo*  
555 transcriptome assemblies (see below), adding functional annotation with Interproscan (v5.25-64.0)<sup>73</sup>.

## 556 **Illumina sequencing**

557 Plasmids were sequenced on a MiniSeq (Illumina, San Diego, CA) platform. Library preparation was  
558 performed with Nextera XT DNA library preparation according to the manufacturer's instructions  
559 (Illumina). The library preparation included the MiniSeq Mid Output kit (300 cycles) and the input & final  
560 DNA was quantified with the Qubit HS dsDNA kit (Life Technologies, Thermo Fisher Scientific).  
561 Nucleotide sequences were assembled with SPAdes<sup>74</sup> and compared to the intended *in silico* DNA  
562 construct. For whole-genome sequencing, yeast cells were harvested from overnight cultures and DNA  
563 was isolated with the Qiagen genomic DNA 100/G kit (Qiagen) as described earlier. DNA quantity was



564 measured with the Qubit BR dsDNA kit (Thermo Fisher Scientific). 300 bp paired-end libraries were  
565 prepared with the TruSeq DNA PCR-free library prep kit (Illumina) according to the manufacturer's  
566 instructions. Short read whole-genome sequencing was performed on a MiSeq platform (Illumina).

#### 567 **RNA isolation, sequencing and transcriptome analysis**

568 Culture broth from chemostat cultures was directly sampled into liquid nitrogen to prevent mRNA  
569 turnover. The cell cultures were stored at -80 °C and processed within 10 days after sampling. After  
570 thawing on ice, cells were harvested by centrifugation. Total RNA was extracted by a 5 min heatshock at  
571 65 °C with a mix of isoamyl alcohol, phenol and chloroform at a ratio of 125:24:1, respectively  
572 (Invitrogen). RNA was extracted from the organic phase with Tris-HCl and subsequently precipitated by  
573 the addition of 3 M Nac-acetate and 40 % (v/v) ethanol at -20 °C. Precipitated RNA was washed with  
574 ethanol, collected and after drying resuspended in RNase free water. The quantity of total RNA was  
575 determined with a Qubit RNA BR assay kit (Thermo Fisher Scientific). RNA quality was determined by the  
576 RNA integrity number with RNA screen tape using a Tapestation (Agilent). RNA libraries were prepared  
577 with the TruSeq Stranded mRNA LT protocol (Illumina, #15031047) and subjected to paired-end  
578 sequencing (151 bp read length, NovaSeq Illumina) by Macrogen (Macrogen Europe, Amsterdam, the  
579 Netherlands).

580 Pooled RNAseq libraries were used to perform *de novo* transcriptome assembly using Trinity (v2.8.3)<sup>75</sup>  
581 which was subsequently used as evidence for both CBS6556 and NBRC1777 genome annotations.  
582 RNAseq libraries were mapped into the CBS6556 genome assembly described above, using bowtie  
583 (v1.2.1.1)<sup>76</sup> with parameters (-v 0 -k 10 --best -M 1) to allow no mismatches, select the best out of 10  
584 possible alignments per read, and for reads having more than one possible alignment randomly report  
585 only one. Alignments were filtered and sorted using samtools (v1.3.1)<sup>77</sup>. Read counts were obtained

586 with featureCounts (v1.6.0)<sup>78</sup> using parameters (-B -C) to only count reads for which both pairs are  
587 aligned into the same chromosome.

588 Differential gene expression (DGE) analysis was performed using edgeR (v3.28.1)<sup>79</sup>. Genes with 0 read  
589 counts in all conditions were filtered out from the analysis, same as genes with less than 10 counts per  
590 million. Counts were normalized using the trimmed mean of M values (TMM) method<sup>80</sup>, and dispersion  
591 was estimated using generalized linear models. Differentially expressed genes were then calculated  
592 using a log ratio test adjusted with the Benjamini-Hochberg method. Absolute log<sub>2</sub> fold-change values >  
593 2, false discovery rate < 0.5, and P value < 0.05 were used as significance cutoffs.

594 Gene set analysis (GSA) based on gene ontology (GO) terms was used to get a functional interpretation  
595 of the DGE analysis. For this purpose, GO terms were first obtained for the *S. cerevisiae* CEN.PK113-7D  
596 (GCA\_002571405.2) and *K. marxianus* CBS6556 genome annotations using Funannotate and  
597 Interproscan as described above. Afterwards, Funannotate compare was used to get (co)ortholog  
598 groups of genes generated with ProteinOrtho<sup>38</sup> using the following public genome annotations *S.*  
599 *cerevisiae* S288C (GCF\_000146045.2), *K. marxianus* NBRC1777 (GCA\_001417835.1), *K. marxianus*  
600 DMKU3-1042 (GCF\_001417885.1), in addition to the new genome annotations generated here for *S.*  
601 *cerevisiae* CEN.PK113-7D, and *K. marxianus* CBS6556 and NBRC1777. Predicted GO terms for *S.*  
602 *cerevisiae* CEN.PK113-7D and *K. marxianus* CBS6556 were kept, and merged with those from  
603 corresponding (co)orthologs from *S. cerevisiae* S288C. Genes with term GO:0005840 (ribosome) were  
604 not considered for further analyses. GSA was then performed with Piano (v2.4.0)<sup>40</sup>. Gene set statistics  
605 were first calculated with the Stouffer, Wilcoxon rank-sum test, and reporter methods implemented in  
606 Piano. Afterwards, consensus results were derived by p-value and rank aggregation, considered  
607 significant if absolute Fold Change values > 1. ComplexHeatmap (v2.4.3)<sup>81</sup> was used to draw GSA results

608 into Fig. 2, highlighting differentially expressed genes found in a previous study<sup>51</sup>. DGE and GSA were  
609 performed using R (v4.0.2)<sup>82</sup>.

### 610 **Anaerobic growth experiments**

611 Anaerobic shake-flask experiments were performed in a Bactron anaerobic workstation (BACTRON300-  
612 2, Sheldon Manufacturing, Cornelius, OR) at 30 °C. The gas atmosphere consisted of 85% N<sub>2</sub>, 10% CO<sub>2</sub>  
613 and 5% H<sub>2</sub> and was maintained anaerobic by a Pd catalyst. The catalyst was re-generated by heating till  
614 160 °C every week and interchanged by placing it in the airlock whenever the pass-box was used. 50-mL  
615 Shake flasks were filled with 40 mL (80 % volumetric) media and placed on an orbital shaker (KS 130  
616 basic, IKA, Staufen, Germany) set at 240 rpm inside the anaerobic chamber. Sterile growth media was  
617 placed inside the anaerobic chamber 24 h prior to inoculation to ensure complete removal of traces of  
618 oxygen.

619 The anaerobic growth ability of the yeast strains was tested on SMG-urea with 50 g·L<sup>-1</sup> glucose at pH 6.0  
620 with Tween 80 prepared as described earlier. The growth experiments were started from aerobic pre-  
621 cultures on SMG-urea media and the anaerobic shake flasks were inoculated at an OD<sub>600</sub> of 0.2  
622 (corresponding to an OD<sub>600</sub> of 0.14). In order to minimize opening the anaerobic chamber, culture  
623 growth was monitored by optical density measurements inside the chamber using an Ultrospec 10 cell  
624 density meter (Biochrom, Cambridge, UK) at a 600 nm wavelength. When the optical density of culture  
625 no longer increased or decreased new shake-flask cultures were inoculated by serial transfer at an initial  
626 OD<sub>600</sub> of 0.2.

### 627 **Laboratory evolution in low oxygen atmosphere**

628 Adaptive laboratory evolution for strict anaerobic growth was performed in a Bactron anaerobic  
629 workstation (BACTRON BAC-X-2E, Sheldon Manufacturing) at 30 °C. 50-mL Shake flasks were filled with

630 40 mL SMG-urea with 50 g·L<sup>-1</sup> glucose and including 420 mg·L<sup>-1</sup> Tween 80. Subsequently the shake-flask  
631 media were inoculated with IMX2323 from glycerol cryo-stock at OD<sub>660</sub> < 0.01 and thereafter placed  
632 inside the anaerobic chamber. Due to frequent opening of the pass-box and lack of catalyst inside the  
633 pass-box oxygen entry was more permissive. After the optical density of the cultures no longer  
634 increased, cultures were transferred to new media by 40-50x serial dilution. For IMS1111, IMS1112,  
635 IMS1113 three and for IMS1131, IMS1132, IMS1133 four serial transfers in shake-flask media were  
636 performed after which single colony isolates were made by plating on YPD agar media with hygromycin  
637 antibiotic at 30 °C aerobically. Single colony isolates were subsequently restreaked sequentially for  
638 three times on the same media before the isolates were propagated in SM glucose media and glycerol  
639 cryo stocked.

640 To determine if an oxygen-limited pre-culture was required for the strict anaerobic growth of IMX2323  
641 strain a cross-validation experiment was performed. In parallel, yeast strains were cultivated in 50-mL  
642 shake-flask cultures with SMG-urea with 50 g·L<sup>-1</sup> glucose at pH 6.0 with Tween 80 in both the Bactron  
643 anaerobic workstation (BACTRON BAC-X-2E, Sheldon Manufacturing) with low levels of oxygen-  
644 contamination, and in the Bactron anaerobic workstation (BACTRON300-2, Sheldon Manufacturing) with  
645 strict control of oxygen-contamination. After stagnation of growth was observed in the second serial  
646 transfer of the shake-flask cultures a 1.5 mL sample of each culture was taken, sealed, and used to  
647 inoculate fresh-media in the other Bactron anaerobic workstation. Simultaneously, the original culture  
648 was used to inoculate fresh media in the same Bactron anaerobic workstation, thereby resulting in 4  
649 parallel cultures of each strain of which half were derived from the other Bactron anaerobic  
650 workstation.

651 **Laboratory evolution in sequential batch reactors**

652 Laboratory evolution for selection of fast growth at high temperatures was performed in 400-mL  
653 MultiFors (Infors Benelux, Velp, the Netherlands) bioreactors with a working volume of 100 mL for the  
654 strain IMS1111 on SMG 20 g·L<sup>-1</sup> glucose media with Tween 80 in triplicate. Anaerobic conditions were  
655 created and maintained by continuous aeration of the cultures with 50 mL·min<sup>-1</sup> (0.5 vvm) N<sub>2</sub> gas and  
656 continuous aeration of the media vessels with N<sub>2</sub> gas. The pH was set at 5.0 and maintained by the  
657 continuous addition of sterile 2 M KOH. Growth was monitored by analysis of the CO<sub>2</sub> in the bioreactor  
658 off-gas and a new empty-refill cycle was initiated when the batch time had at least elapsed 15 hours and  
659 the CO<sub>2</sub> signal dropped to 70% of the maximum reached in each batch. The dilution factor of each  
660 empty-refill cycle was 14.3-fold (100 mL working volume, 7 mL residual volume). The first batch  
661 fermentation was performed at 30 °C after which in the second batch the temperature was increased to  
662 42 °C and maintained at for 18 consecutive sequential batches. After the 18 batch cycle at 42 °C the  
663 culture temperature was again increased to 45 °C and maintained subsequently. Growth rate was  
664 calculated based on the CO<sub>2</sub> production as measured by the CO<sub>2</sub> fraction in the culture off-gas in  
665 essence as described previously<sup>83</sup>. In short, the CO<sub>2</sub> fraction in the off-gas was converted to a CO<sub>2</sub>  
666 evolution rate of mmol per hour and subsequently summed over time for each cycle. The corresponding  
667 cumulative CO<sub>2</sub> profile was transformed to natural log after which the stepwise slope of the log  
668 transformed data was calculated. Subsequently an iterative exclusion of datapoints of the stepwise  
669 slope of the log transformed cumulative CO<sub>2</sub> profile was performed with exclusion criteria of more than  
670 one standard deviation below the mean.

#### 671 **Variant calling**

672 DNA sequencing reads were aligned into the NBRC1777 described above including an additional  
673 sequence with *TtSTC1* construct, and used to detect sequence variants using a method previously  
674 reported<sup>84</sup>. Briefly, reads were aligned using BWA (v0.7.15-r1142-dirty)<sup>85</sup>, alignments were processed

675 using samtools (v1.3.1)<sup>77</sup> and Picard tools (v2.20.2-SNAPSHOT) (<http://broadinstitute.github.io/picard>),  
676 and variants were then called using the Genome Analysis Toolkit (v3.8-1-0-gf15c1c3ef)<sup>86</sup> HaplotypeCaller  
677 in DISCOVERY and GVCF modes. Variants were only called at sites with minimum variant confidence  
678 normalized by unfiltered depth of variant samples (QD) of 20, read depth (DP)  $\geq$  5, and genotype quality  
679 (GQ)  $>$  20, excluding a 7.1 kb region in chromosome 5 containing rDNA. Variants were annotated using  
680 the genome annotation described above, including the *TtSTC1* construct, with SnpEff (v5.0)<sup>87</sup> and  
681 VCFannotator (<http://vcfannotator.sourceforge.net>).

## 682 **Statistics**

683 Statistical test performed are given as two sided with unequal variance t-test unless specifically stated  
684 otherwise. We denote technical replicates as measurements derived from a single cell culture. Biological  
685 replicates are measurements originating from independent cell cultures. Independent experiments are  
686 two experiments identical in set-up separated by the difference in execution days. If possible variance  
687 from independent experiments with identical setup were pooled together, but independent  
688 experiments from time-course experiments (anaerobic growth studies) are reported separately. *p*-  
689 values were corrected for multiple-hypothesis testing which is specifically reported each time. No data  
690 was excluded based on the resulting data out-come.

## 691 **Data availability**

692 Data supporting the findings of this work are available within the paper and source data for all figures in  
693 this study are available at the [www.data.4TU.nl](http://www.data.4TU.nl) repository with the doi:10.4121/13265552.

694 The raw RNA-sequencing data that supports the findings of this study are available from the Genome  
695 Expression Omnibus (GEO) website (<https://www.ncbi.nlm.nih.gov/geo/>) with number GSE164344.

696 Whole-genome sequencing data of the CBS6556, NBRC1777 and evolved strains were deposited at NCBI  
697 (<https://www.ncbi.nlm.nih.gov/>) under BioProject accession number PRJNA679749.

#### 698 **Code availability**

699 The code that were used to generate the results obtained in this study are archived in a Gitlab  
700 repository ([https://gitlab.tudelft.nl/rortizmerino/kmar\\_anaerobic](https://gitlab.tudelft.nl/rortizmerino/kmar_anaerobic)).

#### 701 **Author's contributions**

702 WD and JTP designed the study and wrote the manuscript. WD performed molecular cloning, bioreactor  
703 cultivation experiment, transcriptome analysis and sterol-uptake experiments. JB contributed to  
704 bioreactor cultivation experiments and molecular cloning. FW contributed to the molecular cloning and  
705 sterol-uptake experiments. AK and CM contributed to bioreactor experiments and transcriptome  
706 studies. PdIT performed plasmid and genome sequencing. RO contributed to transcriptome analysis and  
707 performed sequence annotation and assembly.

#### 708 **Acknowledgements**

709 We thank Mark Bisschops and Hannes Jürgens for fruitful discussions. We thank Erik de Hulster for  
710 fermentation support and Marcel van den Broek for input on the bioinformatics analyses.

#### 711 **Competing interest**

712 WD and JTP are co-inventors on a patent application that covers aspects of this work. The authors  
713 declare no conflict of interest.

#### 714 **Funding**

715 This work was supported by Advanced Grant (grant #694633) of the European Research Council to JTP.

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917

918 **Description of Additional Supplementary Files**

919 **Supplementary Data Set 1 | Overview of flow cytometry samples with meta-data.** Meta-data Table of  
920 file names, frequency of cells compared to parent, number of cells in each group, strain name, time  
921 point of fluorescence measurement after 4 hours (1) or 23 hours (2), staining of cells with propidium-  
922 iodide (PI) with value (PI) or without PI staining (-), staining of cells with Tween 80 NBD-cholesterol (TN)  
923 or with Tween 80 only (T), with species names abbreviated *K. marxianus* (Km) or *S. cerevisiae* (Sc).

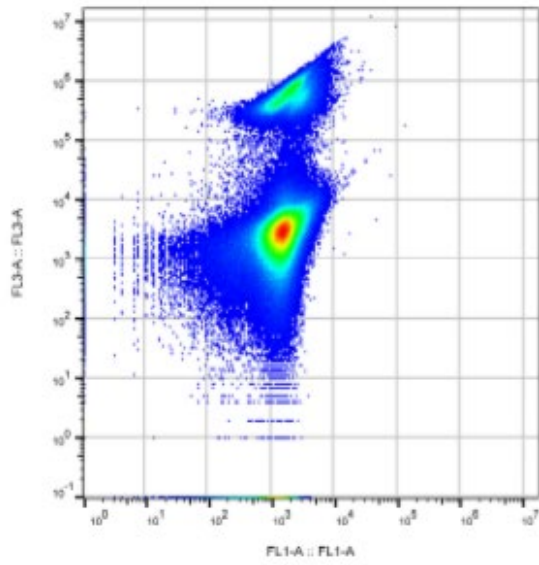
924 [Example picture of file FlowCyto\_Table.xlsx]

Filename	Strain	Time pc	PI	#	Day	Staining	Cells/P	Cells/P	Cells/P
A09 CBS6556_T_A_PI_1.fcs	CBS6556	1	PI	A		1 T	576	411000	75590
B09 CBS6556_T_B_PI_1.fcs	CBS6556	1	PI	B		1 T	625	398024	88212
A01 IMX585_T_A_1.fcs	IMX585	1	-	A		2 T	1391	3	472000

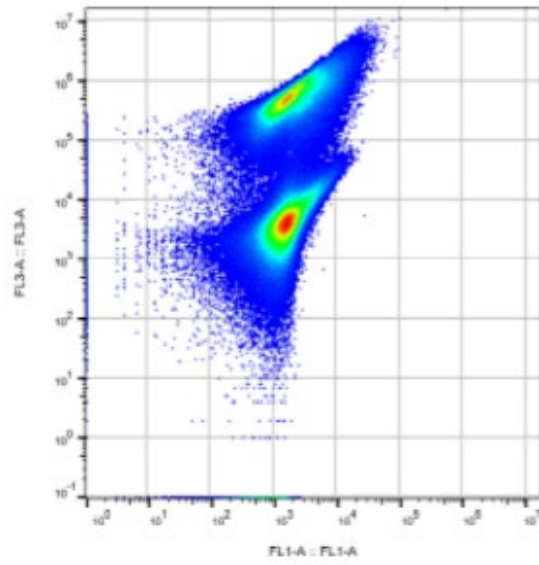
926 **Supplementary Data set 2 | Flow cytometry non-gated data of FL3-A versus FL1-A of all samples.**

927 Flow cytometry data of showing fluorescent NBDC uptake by *K. marxianus*, *S. cerevisiae* strains with for  
928 each sample the intensity of counts (pseudo-colored) for 533/30 nm (FL1) for NBDC and > 670 nm (FL3)  
929 for PI.

930 [Example of first row of FlowCyto\_FL1\_FL3.pdf]



Sample Name	Subset Name	Count
A01 IMX005_T_A_PL_1.fcs	Ungated	5.00E5



Sample Name	Subset Name	Count
A01 IMX005_T_A_PL_2.fcs	Ungated	5.00E5

931

934 **Supplemental material for:**

935 **Engineering the thermotolerant industrial yeast *Kluyveromyces marxianus* for anaerobic growth**

936 Wijbrand J. C. Dekker, Raúl A. Ortiz-Merino, Astrid Kaljouw, Julius Battjes, Frank Wiering, Christiaan

937 Mooiman, Pilar de la Torre, and Jack T. Pronk\*

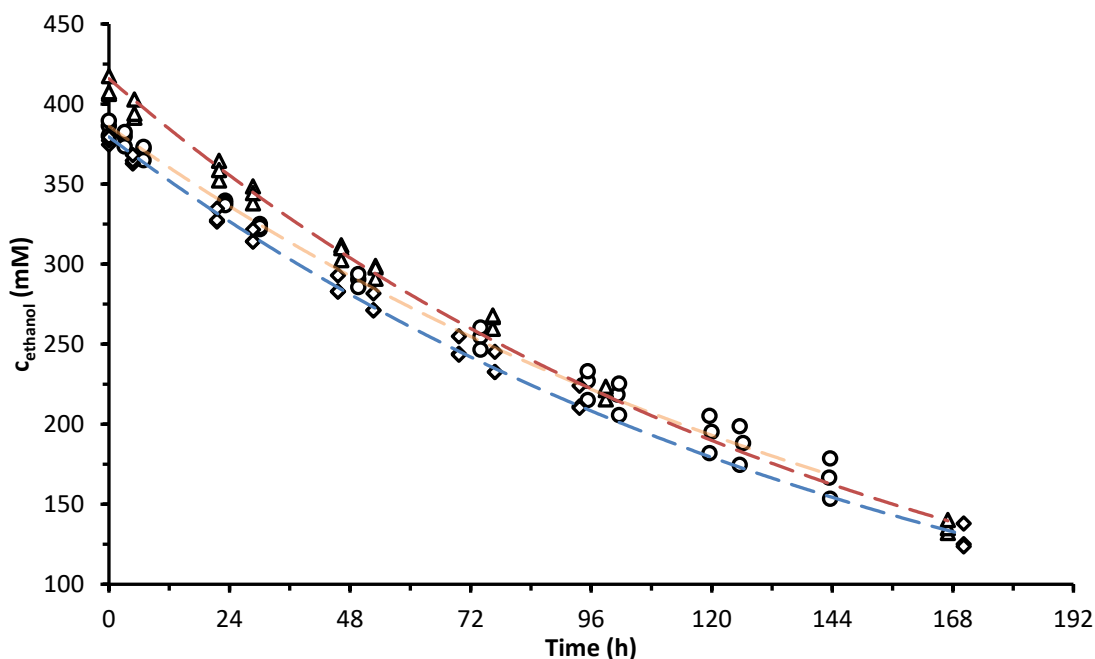
938 Department of Biotechnology, Delft University of Technology, van der Maasweg 9, 2629 HZ Delft, The

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941 9, 2629 HZ Delft, The Netherlands, E-mail: [j.t.pronk@tudelft.nl](mailto:j.t.pronk@tudelft.nl), Tel: +31 15 2783214.



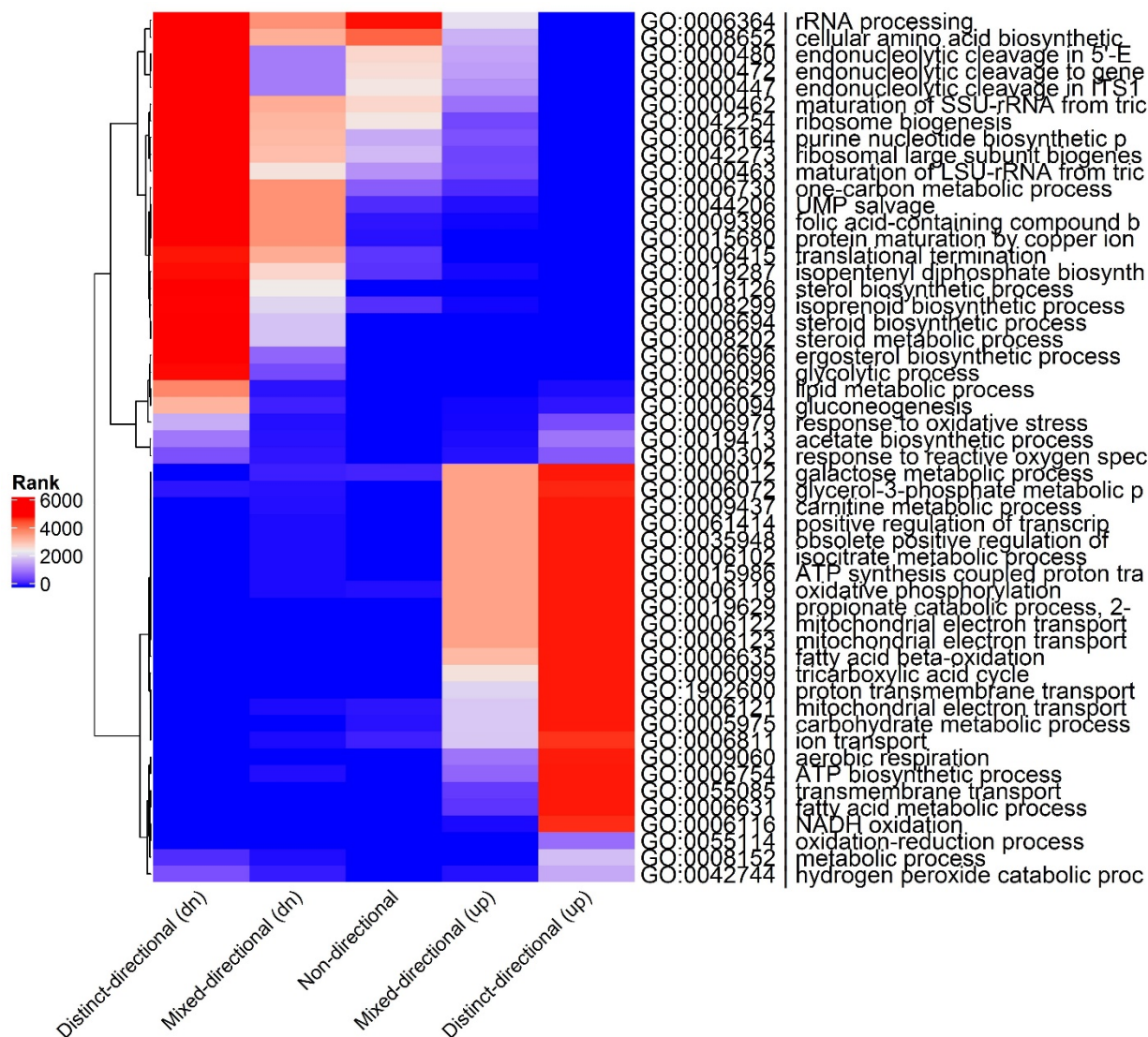


942 **Supplementary Fig. 1 | Ethanol evaporation rate.** Ethanol concentration over time with reactor volume  
 943 of 1200 mL SM glucose urea media maintained at 30 °C, stirred with 800 rpm and aerated with a  
 944 volumetric gas flow rate of 500 mL·min<sup>-1</sup>. The reactor off-gas was cooled by passing through a condenser  
 945 cooled at 2 °C. Circles and orange line represent the condition with sparge aeration and Tween 80 (T)  
 946 media supplementation, diamonds and blue line head-space aeration with Tween 80, triangle and red  
 947 line represent head space aeration and Tween 80 omission. Data represent mean with standard  
 948 deviation from three independent reactor experiments.

AGF	Aeration type	Ethanol evaporation (mmol·h <sup>-1</sup> )
T	Sparge	0.00578 ± 0.00062
T	Head-space	0.00625 ± 0.00032
	Head-space	0.00653 ± 0.00020

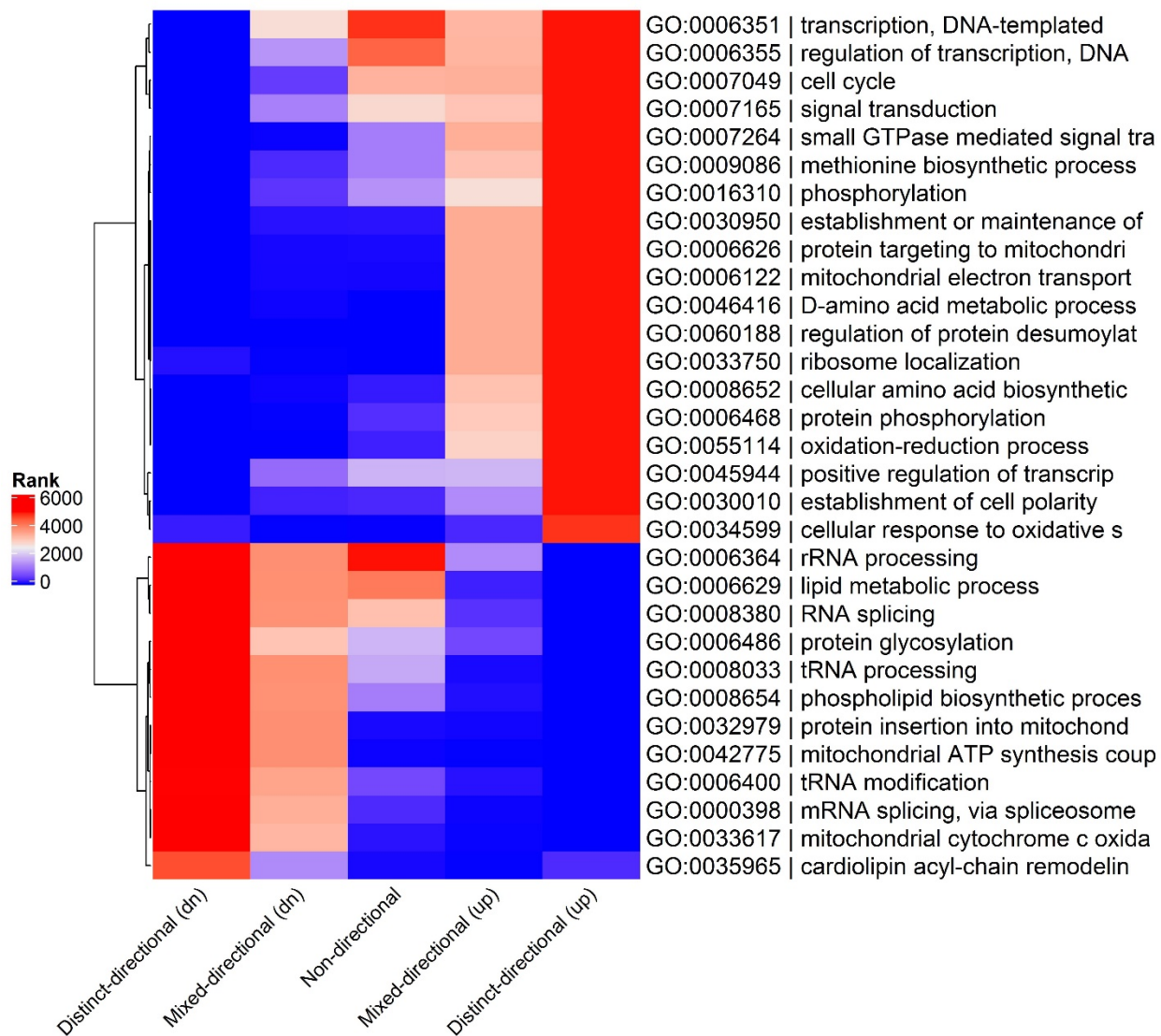
949

950



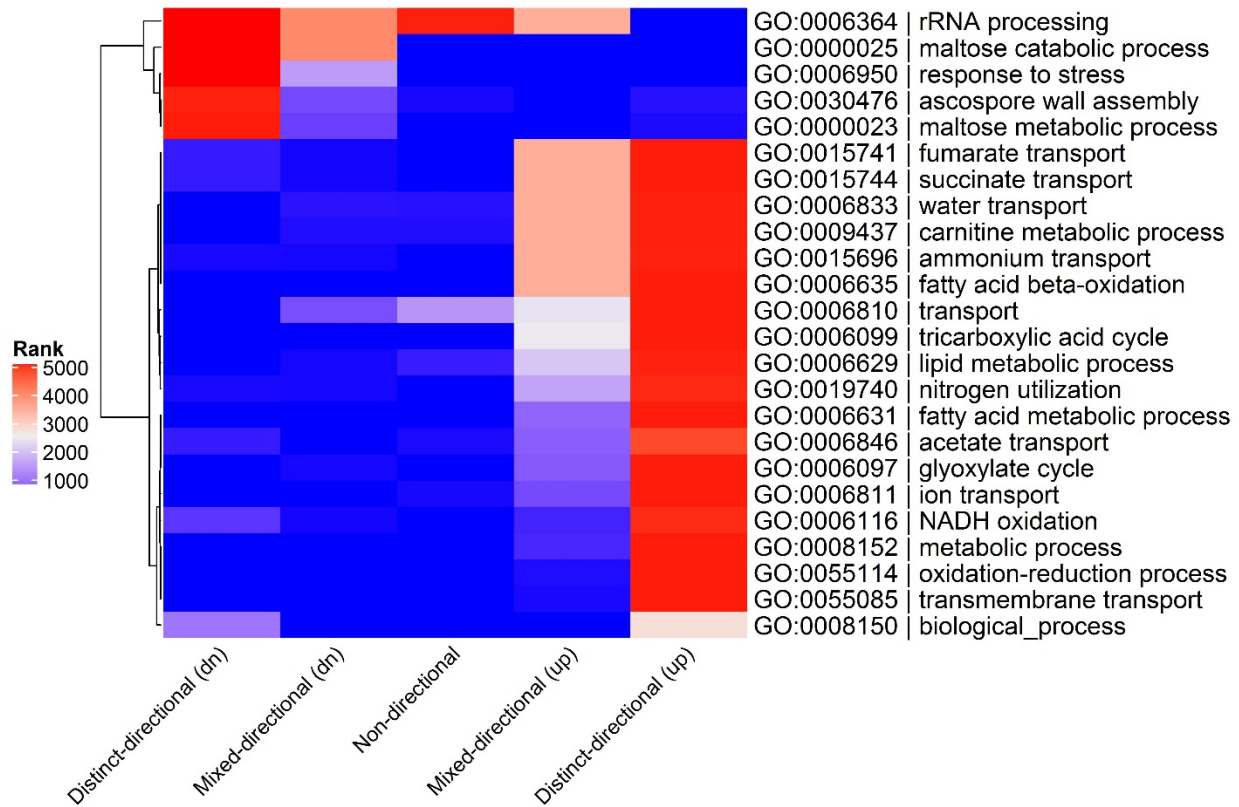
951 **Supplementary Fig. 2 | Consensus biological process GO term enrichment for *K. marxianus* contrast**

952 **31.** GO terms are clustered according to their rank. See legend of Fig. 2 for experimental details.



953 **Supplementary Fig. 3 | Consensus biological process GO term enrichment for *K. marxianus* contrast**

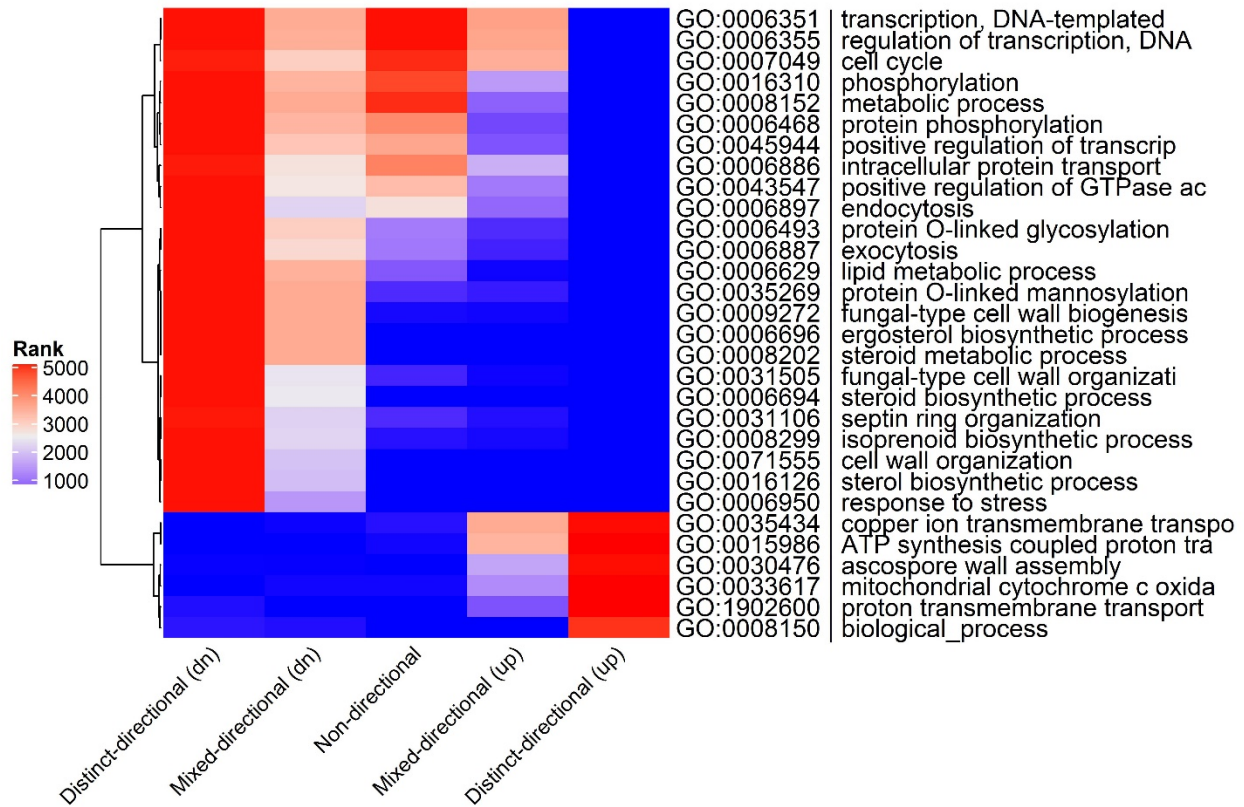
954 **43.** GO terms are clustered according to their rank. See legend of Fig. 2 for experimental details.



955

956 **Supplementary Fig. 4 | Consensus biological process GO term enrichment for *S. cerevisiae* contrast 31.**

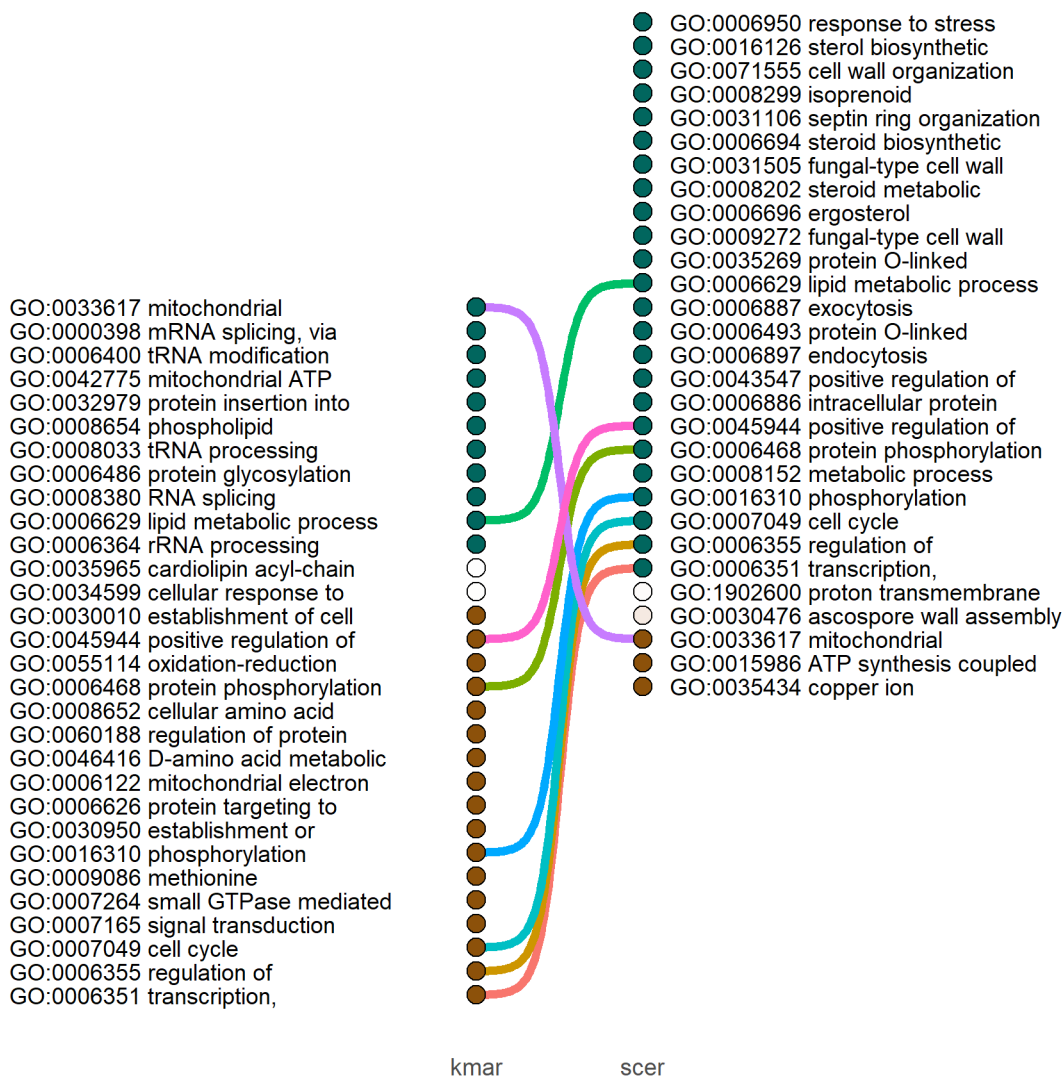
957 GO terms are clustered according to their rank. See legend of Fig. 2 for experimental details.



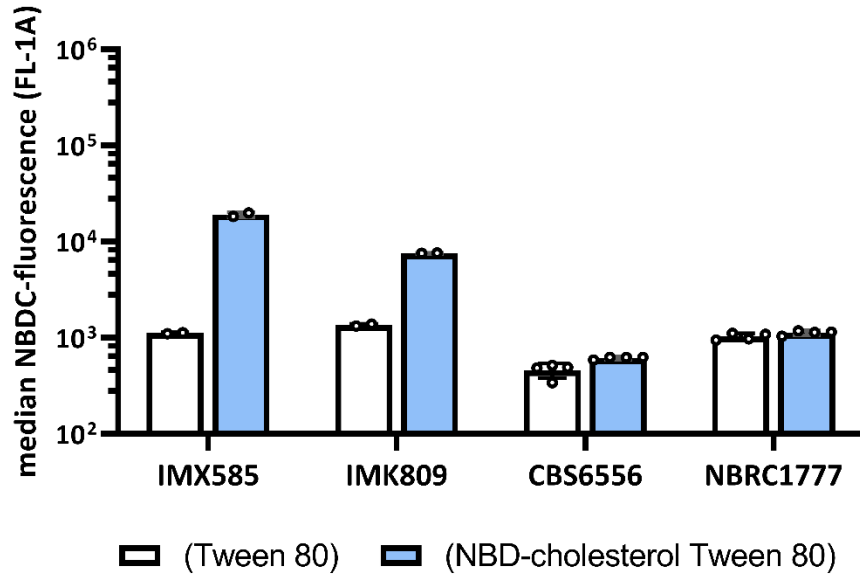
958

959 **Supplementary Fig. 5 | Consensus biological process GO term enrichment for *S. cerevisiae* contrast 43.**

960 GO terms are clustered according to their rank. See legend of Fig. 2 for experimental details.



961 **Supplementary Fig. 6 | GO term enrichment comparison of biological process of *K. marxianus* (kmar)**  
 962 **to *S. cerevisiae* (scer) of contrast 43.** GO terms were annotated with the color of distinct directionality  
 963 (up (blue) down (brown)) and the color intensity was determined by the magnitude of the inverse rank.  
 964 GO terms with significant mixed-directionality or non-directionality, as having no pronounced distinct  
 965 directionality, are colored white. Shared GO terms between *K. marxianus* and *S. cerevisiae* are  
 966 connected by a line.



967 **Supplementary Fig. 7 | Uptake of the fluorescent sterol derivative NBD-cholesterol by *S. cerevisiae* and *K.***

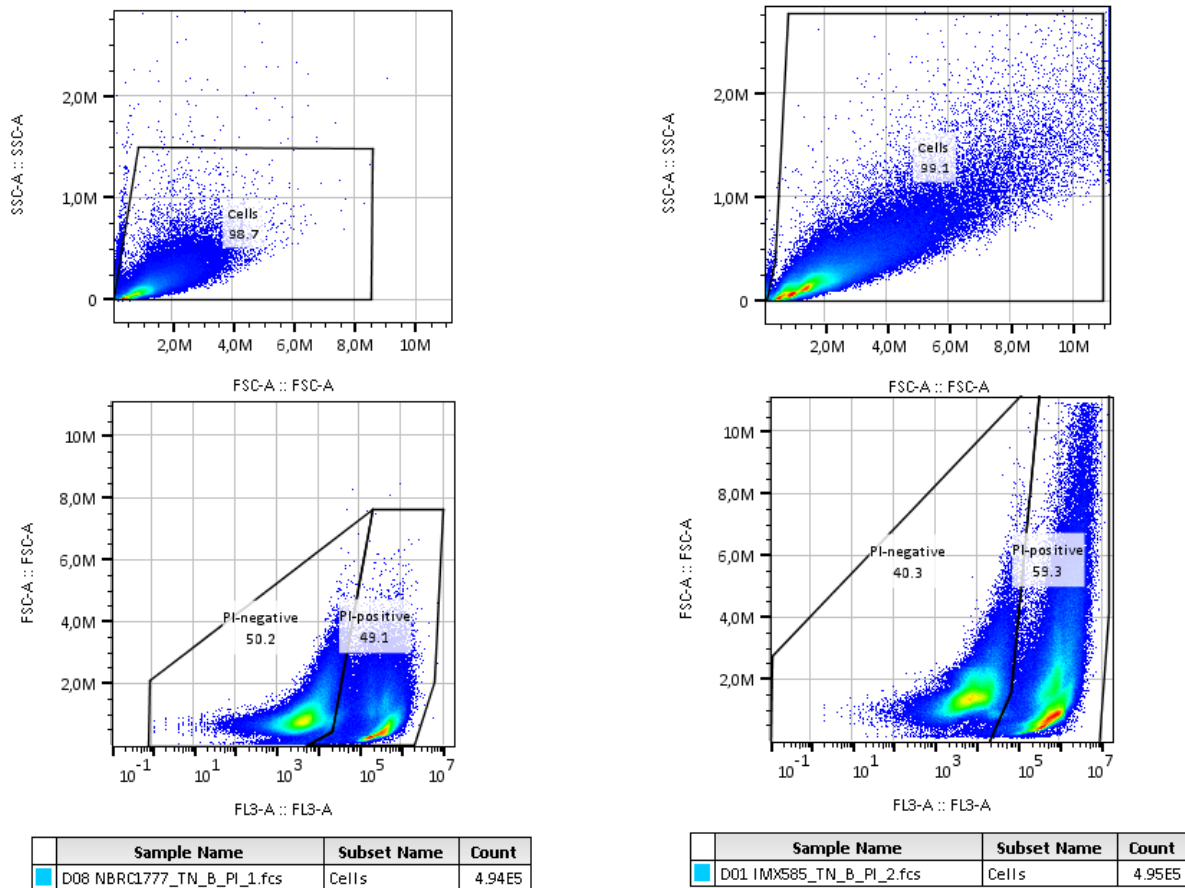
968 ***marxianus* strains after 23 h staining.**

969 Flow cytometry data of Fig. 4 with prolonged staining after pulse-addition of NBD-cholesterol to the

970 shake-flask cultures for 23 h. Bar charts of the median and pooled standard deviation of the NBD-

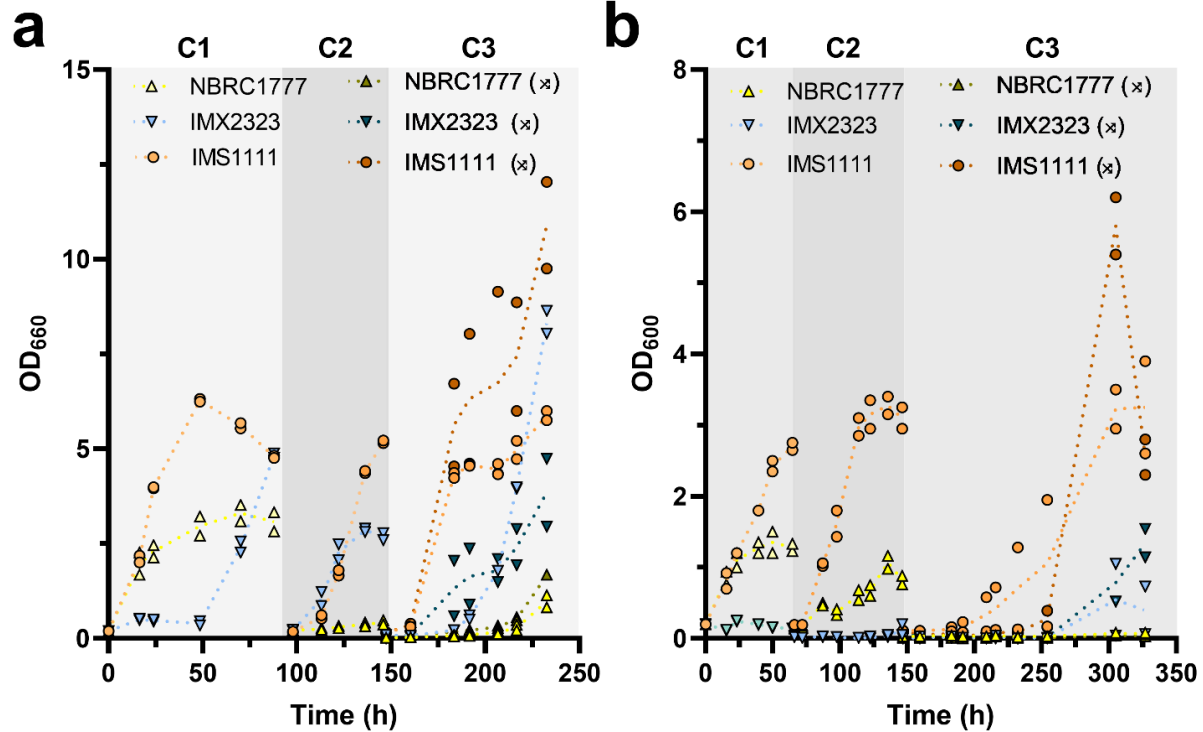
971 cholesterol fluorescence intensity of PI-negative cells with pooled variance from the biological replicate

972 cultures. See legend Fig. 4 for experimental details.

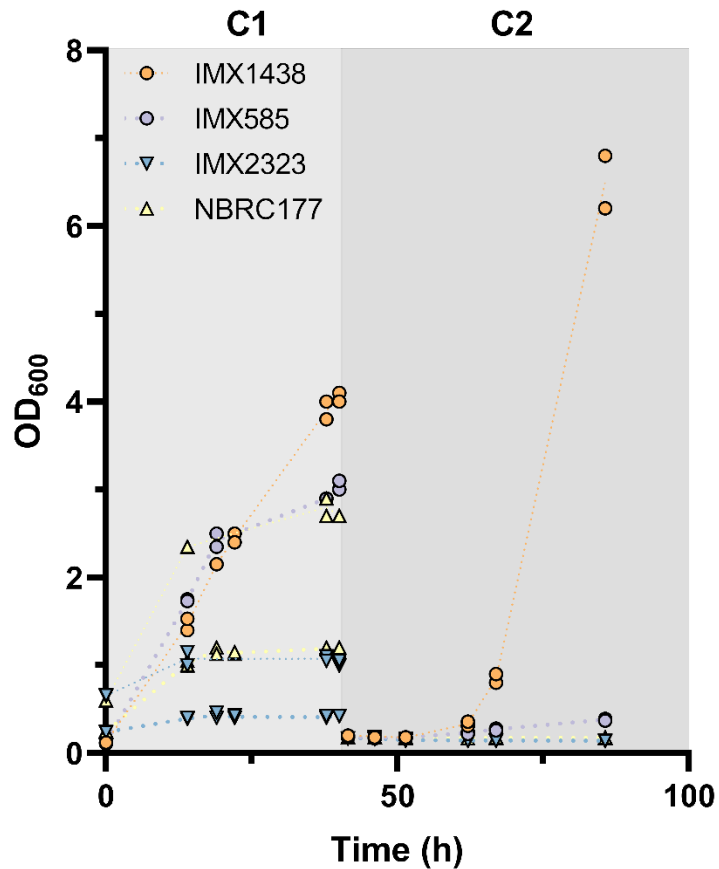


973 **Supplementary Fig. 8 | Flow cytometry gating strategy of both *K. marxianus* (left panel) and *S.***  
 974 ***cerevisiae* (right panel) samples.** Gates were set per one species for all samples independent of NBDC  
 975 staining. Density of events were calculated by FlowJo software and represented in pseudo-color (blue  
 976 low density, red high-density). The gate between PI-negative and PI-positive was inside the “Cells”  
 977 gated-population.

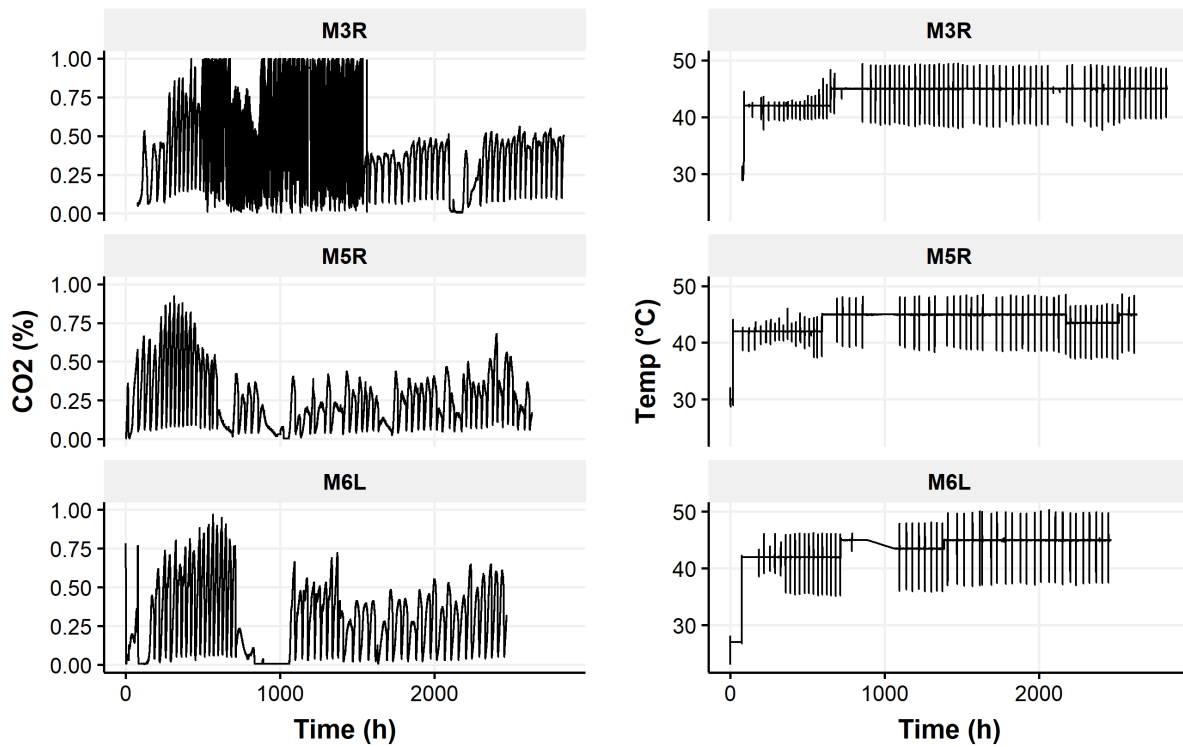




978 **Supplementary Fig. 9 | Cross-validation of oxygen-limited and anaerobic growth of *K. marxianus***  
979 **IMX2323.** Strains were grown in shake-flask cultures in an oxygen-limited (a) and strict anaerobic  
980 environment (b). To perform cross-validation between the two parallel running experiments, 1.5 mL  
981 aliquot of each culture was sealed and transferred quickly between anaerobic chambers and used to  
982 inoculate two shake-flask cultures, represented with crossed-arrows (x). The cultures from the strain  
983 NBRC1777 (x) in the third transfer (C3) in the strict anaerobic environment (b) were hence inoculated  
984 from an aliquot of the cultures of NBRC1777 (C2) grown in oxygen-limited environment (a). This resulted  
985 in a serial transfer of 26.7 times dilution from transfer C2 to C3. Aerobic grown pre-cultures were used  
986 to inoculate the first anaerobic culture on SMG-urea containing 50 g·L<sup>-1</sup> glucose and Tween 80. Data  
987 depicted are of each replicate culture (points) and the mean (dotted line) from independent biological  
988 duplicate cultures, serial transfers cultures are represented with the number of respective transfer (C1-  
989 3).



990 **Supplementary Fig. 10 | Sterol-independent anaerobic growth of *S. cerevisiae* IMX585 (reference),**  
991 **IMX1438 (*TtSTC1*), *K. marxianus* NBRC1777 (reference) and IMX2323 (*TtSTC1*).** Aerobic grown pre-  
992 cultures were used to inoculate shake-flask cultures with SMG-urea containing 50 g·L<sup>-1</sup> glucose and  
993 Tween 80 in a strict anaerobic environment at an OD<sub>600</sub> of 0.1 for all strains, and both at OD<sub>600</sub> of 0.1 and  
994 0.6 for NBRC1777 and IMX2323. Data depicted are of each replicate culture (points) and the mean  
995 (dotted line) from independent biological duplicate cultures, serial transfers cultures are represented  
996 with the number of respective transfer (C1-2).



997 **Supplementary Fig. 11 | CO<sub>2</sub> fraction in the off-gas of *K. marxianus* IMS1111.** Production of CO<sub>2</sub> as  
998 measured by the fraction of CO<sub>2</sub> in the off-gas of the individual bioreactor cultivations of the *K.*  
999 *marxianus* strain IMS1111 on SMG media pH 5.0 with 20 g·L<sup>-1</sup> glucose, 420 mg·L<sup>-1</sup> Tween 80 over time  
1000 (Left panels). The temperature profile was incrementally increased at the beginning of a new batch cycle  
1001 (right panels). After 430 h the performance of the off-gas analyzer of replicate M3R deteriorated.

1002 **Supplementary Table 1 | Mutations identified by whole-genome sequencing in comparison to the**  
 1003 **reference *K. marxianus* strain IMX2323.** Overview of mutations detected in the strains after selected for  
 1004 strict anaerobic growth IMS1111, IMS1131, IMS1132, IMS1133 compared to the *TtSTC1* engineered  
 1005 strain (IMX2323). Resequencing of IMS1111 after 4 transfers in strict anaerobic conditions is for clarity  
 1006 referred with the strain name IMS1115. Overview of mutations of the bioreactor populations after  
 1007 prolonged selection for anaerobic growth at elevated temperatures, represented by the bioreactor  
 1008 replicates (M3R, M5R, and M6L). Mutations in coding regions are annotated as synonymous (SYN), non-  
 1009 synonymous (NSY), insertion or deletions. Mutations in non-coding regions are reported with the  
 1010 identifier of the neighboring gene, directionality and strand (+/-). For *K. marxianus* genes, corresponding  
 1011 *S. cerevisiae* orthologs with the S288C identifier are listed if applicable. QD refers to quality by depth  
 1012 calculated by GATK and genotyping overviews are given per strain using the GATK fields GT: 1/1 for  
 1013 homozygous alternative, 1/0 for heterozygous, AD: allelic depth (number of reads per reference and  
 1014 alternative alleles called), DP: approximate read depth at the corresponding genomic position, and GQ:  
 1015 genotype quality. NA indicates variants were not called in that position in the corresponding strain.

Chromosome	Position	Description	Type	Kmar ID	S288cSy stID	Gene	QD	IMX2323	IMS1111	IMS1131	IMS1132	IMS1133	IMS1115	M3R	M5R	M6L
Mutation spectra of IMX2323 derived single isolates after selection for strict anaerobic growth																
3	89	Asp-747-	CDS:(SYN)	TPUv2_00	YDR283	Gcn2	3	1/1:0,	1/1:0,	NA	NA	NA	1/1:0,	1/1:0	1/1:0,	1/1:0,
	78	Asp		2092	C		2	20:99	20:99	NA	NA	NA	05:99	9:99	10:99	118:18:99
	44	Asp		2092	C		2	20:99	20:99	NA	NA	NA	05:99	9:99	10:99	118:18:99
8	59	codon: TCA	CDS:IN SERT1 ON[1]	TPUv2_00	Tran sposon		2	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,
	15	6		4766			7	7:7:21	7:7:21	NA	NA	NA	9:9:27	12:12:36	7:7:21	7:7:21
8	55	Trp-350-	CDS:(NON)	TPUv2_00	YAL040	Cln3	2	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:1,
	04	STP		4999	C		3	19:99	19:99	NA	NA	NA	43:99	9:99	17:99	98:99:99
4	45	TPUv2_0026	p3UTR :+	TPUv2_00	YGR156	Pti1	3	NA	NA	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	NA
	97	39-T1		2639	W		5	9:29	9:29	0,9:11:54	0,9:9:38	0,9:4:6:24	0,35	10:17:7:26	NA	
	50	17		TPUv2_0031	TPUv2_00	YBR283	Ssh1	2	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,	1/1:0,
5	74	61-T1	p5UTR :-	3161	C		1	NA	NA	0,9:9:27	NA	NA	0/1:1,7:8:21	NA	NA	NA
	29	90		TPUv2_00	YGR090	Utp2	3	1/1:1,11:12:34	1/1:1,11:12:34	NA	NA	1/1:1,8:9:24	1/1:1,11:11:36	NA	NA	NA
5	94	UTP22	p5UTR :+	3518	W		2	NA	NA	NA	NA	1/1:1,8:9:24	1/1:1,11:11:36	NA	NA	NA
	77						2									

Mutations in whole populations after selection for anaerobic growth at elevated temperatures

3	13	codon: AAT	CDS:D	TPUv	YLR	Lu	2	NA	NA	NA	NA	NA	NA	NA	0/1:39	
	52		ELETIO	2_00	352	g										2
	43		N[-3]	2327	W	1										2
	0															
8	63	codon: CAG	CDS:IN	TPUv	No	2	NA	NA	NA	NA	NA	NA	NA	0/1:25		
	57		SERTI	2_00	similarity										6	
	79		ON[9]	5049												

1016