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Engineering *Saccharomyces cerevisiae* for fast vitamin-independent aerobic growth

Anja K. Ehrmann [a,1], Anna K. Wronska [b,1], Thomas Perli [b,1], Erik A.F. de Hulster [b], Marijke A.H. Luttik [b], Marcel van den Broek [b], Clara Carqueija Cardoso [b], Jack T. Pronk [b], Jean-Marc Daran [b,*]

[a] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, 2800 Kgs, Lyngby, Denmark
[b] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ, Delft, the Netherlands

A B S T R A C T

Chemically defined media for cultivation of *Saccharomyces cerevisiae* strains are commonly supplemented with a mixture of multiple Class-B vitamins, whose omission leads to strongly reduced growth rates. Fast growth without vitamin supplementation is interesting for industrial applications, as it reduces costs and complexity of medium preparation and may decrease susceptibility to contamination by auxotrophic microbes. In this study, suboptimal growth rates of *S. cerevisiae* CEN.PK113-7D in the absence of pantothenic acid, *para*-aminobenzoic acid (*p*ABA), pyridoxine, inositol and/or biotin were corrected by single or combined overexpression of *ScFMS1*, *ScABZ1/ScABZ2*, *ScSNZ1/ScSNO1*, *ScINO1* and *Cyberlindnera fabianii BIO1*, respectively. Several strategies were explored to improve growth of *S. cerevisiae* CEN.PK113-7D in thiamine-free medium. Overexpression of *ScTHI4* and/or *ScTHI5* enabled thiamine-independent growth at 83% of the maximum specific growth rate of the reference strain in vitamin-supplemented medium. Combined overexpression of seven native *S. cerevisiae* genes and *CfBIO1* enabled a maximum specific growth rate of 0.33 ± 0.01 h^{-1} in vitamin-free synthetic medium. This growth rate was only 17 % lower than that of a congenic reference strain in vitamin-supplemented medium. Physiological parameters of the engineered vitamin-independent strain in aerobic glucose-limited chemostat cultures (dilution rate 0.10 h^{-1}) grown on vitamin-free synthetic medium were similar to those of similar cultures of the parental strain grown on vitamin-supplemented medium. Transcriptome analysis revealed only few differences in gene expression between these cultures, which primarily involved genes with roles in Class-B vitamin metabolism. These results pave the way for development of fast-growing vitamin-independent industrial strains of *S. cerevisiae*.

1. Introduction

Vitamins are formally defined as organic compounds that cannot be synthesized by an organism but are essential for growth. Chemically defined media for aerobic growth of the budding yeast *Saccharomyces cerevisiae* typically include seven organic compounds that, based on their essential roles in human nutrition and their water solubility, are referred to as Class-B vitamins. Six of these, thiamine [B_1], pyridoxine [B_6], pantothenate [B_5], nicotinic acid [B_3], biotin [B_7] and *para*-aminobenzoate [B_{10}] are enzyme cofactors or cofactor precursors, while *myo*-inositol [B_8] mainly acts as a phospholipid precursor (Perli et al., 2020b). While supplementation of these Class-B vitamins leads to faster growth of *S. cerevisiae*, they are in most strains not strictly required for growth. In keeping with terminology commonly used in yeast research, we will nevertheless refer to them as 'vitamins'.

Multiple studies demonstrated that vitamin dosage can affect productivity of yeast-based industrial processes when they involve one or more enzymes whose activity requires a specific vitamin or cofactor derived thereof. Efficient production of *S. cerevisiae* biomass or yeast extract for application in human and animal nutrition requires addition of multiple vitamins to growth media (Bekatorou et al., 2006). Optimization of yeast-based production of individual organic compounds may require increased dosages of specific vitamins. For example, increased availability of the coenzyme A precursor pantothenate, either via its addition to media or deregulation of its synthesis, was shown to improve production of triterpenoids and *n*-butanol by engineered *S. cerevisiae* strains (Ebert et al., 2018; Schadeweg and Boles, 2016). Similarly, optimization of fumarate and succinate production by *S. cerevisiae* required increased supplementation of biotin (Xu et al., 2013; Yan et al., 2014), the cofactor of pyruvate carboxylase.

Although vitamin supplementation is required for optimal growth of *S. cerevisiae*, the genomes of most strains harbour all structural genes required to encode the enzymes required for biosynthesis of the Class-B vitamins thiamine, pyridoxine, pantothenate, nicotinic acid, biotin,

* Corresponding author.
E-mail address: J.G.Daran@TUDelft.nl (J.-M. Daran).
[1] Anja Ehrmann, Anna K. Wronska, Thomas Perli contributed equally to this publication and should be considered co-first authors.

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inositol and *para*-aminobenzoate (Salazar et al., 2017) (Fig. 1). This information was exploited in a recent study, which used adaptive laboratory evolution (ALE) to obtain a set of *S. cerevisiae* strains that showed fast aerobic growth in 'drop-out' media that each lacked a single vitamin. Reverse engineering of mutations found in these evolved strains identified small sets of genetic alterations that enabled fast aerobic growth on synthetic media that lacked either thiamine, pyridoxine, pantothenate or *para*-aminobenzoic acid (Perli et al., 2020a). ALE of *S. cerevisiae* for fast growth in biotin-free synthetic medium led to more complex genomic changes, including a massive 20- to 40-fold amplification of the adjacent *BIO1* and *BIO6* genes that encode the first two committed enzymes of its biotin-biosynthesis pathway (Bracher et al., 2017). A simple solution to eliminate biotin dependency was based on screening of yeast genetic biodiversity. Expression of the *BIO1* ortholog from the fast-growing biotin-prototrophic yeast *Cyberlindnera fabianii* supported aerobic growth of different *S. cerevisiae* strains in biotin-free media at rates equal to those observed in biotin-supplemented cultures (Wronska et al., 2020).

The abovementioned studies yielded strains that, in aerobic cultures, were fully prototrophic for individual vitamins. As yet, however, no *S. cerevisiae* strains have been reported that show fast aerobic growth in completely vitamin-free synthetic media. Such a complete elimination of Class-B vitamin requirements would enable cultivation of *S. cerevisiae* on simple mineral salts solutions supplemented with a carbon source. Potential advantages of vitamin-independent strains for industrial application include simpler and cheaper medium compositions, less complex protocols for medium preparation and sterilization as often vitamins are prepared and filter-sterilized separately, longer shelf life of media and a lower susceptibility to contamination by auxotrophic micro-organisms (Gasser et al., 2010).

The goals of this study were to design and test straightforward metabolic engineering strategies for eliminating individual vitamin requirements and, subsequently, to combine them in a single *S. cerevisiae* strain to enable fast, fully vitamin-independent aerobic growth. Performance of the resulting strains was analyzed in batch and chemostat cultures and compared to that of a parental strain that requires vitamin supplementation for optimal growth. Furthermore, transcriptome data of an engineered strain grown in the absence of vitamins were analyzed and compared to those of an isogenic reference strain grown on vitamin-supplemented medium.

2. Results

2.1. Selection of metabolic engineering strategies to eliminate individual vitamin requirements

As an essential first step towards elimination of all vitamin requirements for fast aerobic growth of *S. cerevisiae*, strategies were explored to eliminate individual vitamin requirements (Fig. 1). In these strategies, we sought to minimize risks of interference with other aspects of strain physiology that, for example, might arise from engineering global regulation mechanisms.

The maximum specific growth rate of *S. cerevisiae* CEN.PK113-7D on glucose-containing synthetic medium lacking pyridoxine ($SMD_{\Delta pyr}$), was 28% lower than on complete SMD (Fig. 2A). An earlier work showed that expression of mutant alleles of *ScBAS1*, a transcriptional regulator implicated in adenine, purine, and pyrimidine nucleotides could alleviate growth limitations in absence of pyridoxine. This effect was

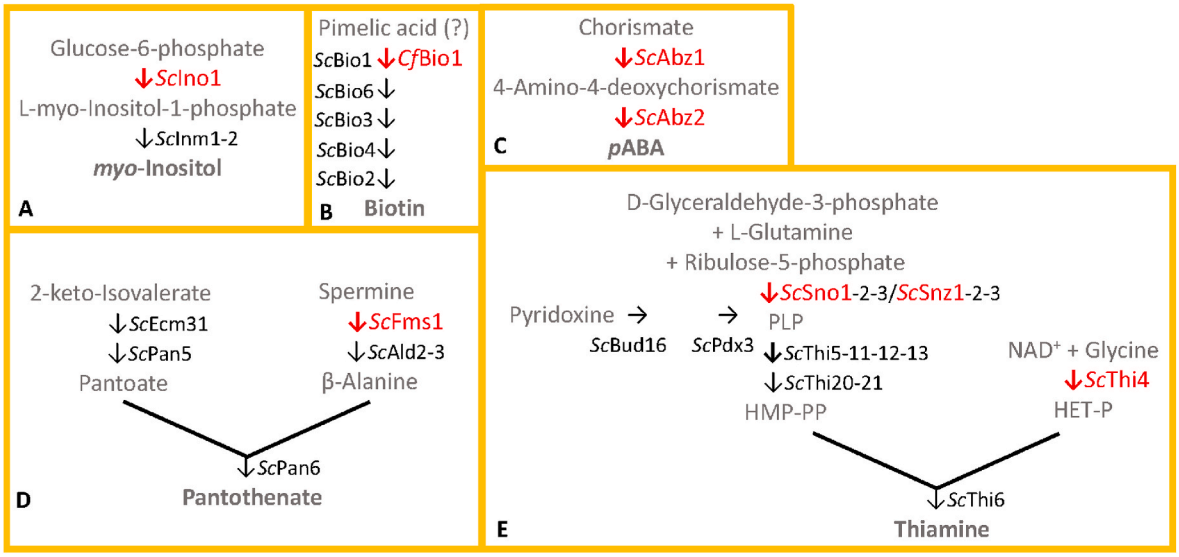

Fig. 1. Overview of vitamin biosynthetic pathways and genetic modifiations introduced in the *Saccharomyces cerevisiae* IMX2816. Schematic representation of the yeast native biosynthetic pathway for *myo*-inositol (**A**) that is synthesized from glucose-6-phosphate via L-*myo*-inositol-1-phosphate by action of *Sc*Ino1, an inositol-3-phosphate synthase and *Sc*Inm1-2 (inositol-1-monophosphatase). Biotin synthesis (**B**) starts with a precursor of pimelic acid, which is indicated by (?). The first enzyme (*Sc*Bio1 - *Cf*Bio1) catalyses the synthesis of pimeloyl-CoA, which is converted by 7,8-diamino-pelargonic acid aminotransferase *Sc*Bio6, adenosylmethionine-8-amino-7-oxononanoate aminotransferase *Sc*Bio3, 8-amino-7-oxo-nonanoate synthase *Sc*Bio4 and biotin synthase *Sc*Bio2 to biotin. *para*-aminobenzoic acid (*p*ABA) (**C**) is synthesized from chorismate by the action of the aminodeoxychorismate synthase *Sc*Abz1 and the aminodeoxychorismate lyase *Sc*Abz2. Pantothenate (**D**) derived from the conversion of pantoate and β-alanine by the pantoate-β-alanine ligase *Sc*Pan6. Pantoate is synthesized from 2-keto-isovalerate through the sequential action of a 3-methyl-2-oxobutanoate hydroxymethyltransferase (*Sc*Ecm31) and a 2-dehydropantoate 2-reductase (*Sc*Pan5). β-alanine is synthesized from spermine through the sequential action of a polyamine oxidase (*Sc*Fms1) and an aldehyde dehydrogenase (*Sc*Ald2-3). The intertwined biosynthesis of pyridoxal-phosphate and thiamine (**D**) start with the condensation of D-glyceraldehyde-3-phosphate, L-glutamine and ribulose-5-phosphate to pyridoxal 5′-phosphate (PLP) by the PLP synthase *Sc*Sno1-2-3/*Sc*Snz1-2-3. PLP can also be synthesized from pyridoxine by a two reaction conversion involving sequential action of the pyridoxal kinase *Sc*Bud16 and the pyridoxamine 5′-phosphate oxidase *Sc*Pdx3. PLP is further processed by the *Sc*Thi5 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase protein family in hydroxymethylpyrimidine phosphate (HMP-P). Next HMP-P is phosphorylated in hydroxymethylpyrimidine diphosphate (HMP-PP) by *Sc*Thi20-21. The hydroxyethylthiazole phosphate (HET-P) is derived from the condensation of NAD^+ and glycine by action of the thiamine thiazole synthase *Sc*Thi4. HMP-PP and HET-P are combined through the action of *Sc*Thi6 into thiamine. The genes encoding the enzymes indicated in red were combined and overexpressed in the *Saccharomyces cerevisiae* IMX2816.

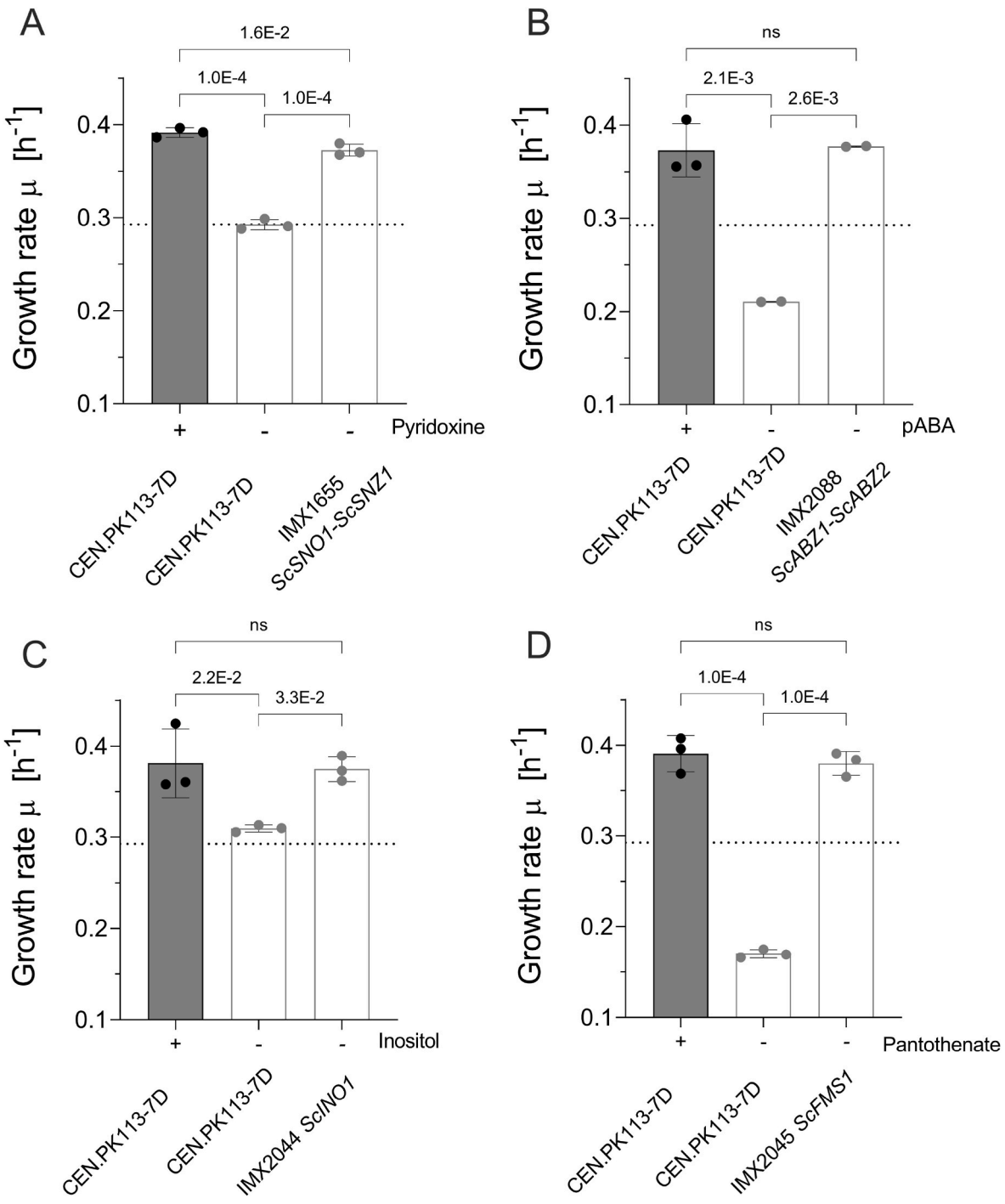

Fig. 2. Alleviating single vitamin dependency for fast-growth of *S. cerevisiae*. Maximum specific growth rates of the non-engineered *S. cerevisiae* strain CEN.PK113-7D (dark grey) and engineered derivatives (white) in synthetic medium with (+) or without (−) pyridoxine (**A**), *p*ABA (**B**), inositol (**C**) and pantothenate (**D**). Yeast strains were grown in 500 mL shake flasks containing 100 mL medium and incubated at 30 °C, 200 rpm. Gene(s) overexpressed in the engineered strains are indicated. Statistical analysis based on one-way analysis of variance analysis (ANOVA) followed by Tukey's multiple comparisons test with means of independent cultures (n = 3; except n = 2 for CEN.PK117-7D in $SMD_{\Delta pABA}$ and IMX2088 (*ScABZ1-ScABZ2*)). Differences were deemed significant with a *p*-value <5.0E-2.

attributed to the co-derepression of *ScSNZ1* and *ScSNO1*, genes that share the same bidirectional promoter, and encode a key enzyme in pyridoxal-5′-phosphate synthesis (Daignan-Fornier and Fink, 1992; Perli et al., 2020a). To avoid potential complications related to the dual transcriptional activation and repression functions of *Sc*Bas1 (Mieczkowski et al., 2006; Zhang et al., 1997), we evaluated combined overexpression of these two genes involved in pyridoxal-5′-phosphate biosynthesis (Fig. 1). The maximum specific growth rate of the resulting strain IMX1655 on $SMD_{\Delta pyr}$ (0.37 ± 0.01 h^{-1}, Fig. 2A) was identical to that of previously studied *ScBAS1* mutants (Perli et al., 2020a) and less than 10% lower than that of the parental strain CEN.PK113-7D on complete SMD (0.39 ± 0.01 h^{-1}), a growth rate for the reference strain that was on the high side, typically in this conditions the CEN.PK strain exhibits a growth rate ranged from 0.37 to 0.40 h^{-1} (Bracher et al., 2017; Hebly et al., 2015; Nijkamp et al., 2012a; Solis-Escalante et al., 2015).

The reference strain CEN.PK113-7D exhibited an 81% lower maximum specific growth rate on synthetic medium lacking para-

aminobenzoic acid ($SMD_{\Delta pABA}$) than on complete SMD (Fig. 2B). Mutations in *ScABZ1* and *ScARO7* were previously shown to eliminate this growth rate difference (Perli et al., 2020a). To avoid potential interference of mutations in *ScARO7*, which encodes chorismate mutase, in aromatic amino-acid biosynthesis, we explored overexpression of *ScABZ1* and *ScABZ2*, which encode enzymes involved in conversion of chorismate to *p*ABA (Fig. 1). The maximum specific growth rate of the resulting strain IMX2088 on $SMD_{\Delta pABA}$ (0.38 ± 0.00 h^{-1}) was not significantly different from that of strain CEN.PK113-7D on complete SMD (Fig. 2B).

The reference strain CEN.PK113-7D showed a maximum specific growth rate of 0.31 ± 0.00 h^{-1} on synthetic medium lacking inositol ($SMD_{\Delta ino}$, Fig. 2C). Growth-rate improvements on $SMD_{\Delta ino}$ were obtained in adaptive laboratory evolution experiments, but no causative mutations were identified (Perli et al., 2020a). Since constitutive expression of *ScINO1* was previously linked to inositol excretion (Greenberg et al., 1982), expression of *ScINO1* from the constitutive *ScTDH3* promoter was tested (Fig. 1). The resulting strain IMX2044, showed a maximum specific growth rate on $SMD_{\Delta ino}$ of 0.37 ± 0.01 h^{-1}. This absence of growth rate difference between IMX2044 grown on SMDΔino and CEN.PK113-7D on SMD demonstrated that inositol supply derived from the *ScINO1* overexpression was no longer limiting.

Mutations in Sc*GAL11* and Sc*TUP1*, which both encode global regulators (Cooper et al., 1994; Myers and Kornberg, 2000; van de Peppel et al., 2005), were previously shown to improve growth rate on synthetic medium lacking pantothenate ($SMD_{\Delta pan}$) (Perli et al., 2020a). To avoid major transcriptional rearrangements, an alternative strategy was investigated. Sc*FMS1*, which encodes a polyamine oxidase involved in synthesis of the pantothenate precursor β-alanine ((Landry and Sternglanz, 2003), Fig. 1), was expressed from the constitutive *ScTEF1* promoter. The resulting strain IMX2045 grew on $SMD_{\Delta pan}$ at 0.38 ± 0.01 h^{-1}, which was not significantly different from the growth rate of strain CEN.PK113-7D on complete SMD (Fig. 2D) and 124% faster than its growth rate on $SMD_{\Delta pan}$.

Previous studies showed that supplementation of nicotinic acid was not required for fast aerobic growth of *S. cerevisiae* (Panozzo et al., 2002; Perli et al., 2021c). Therefore, no genetic modifications aiming at improving nicotinic acid synthesis were investigated. To achieve fast biotin-independent aerobic growth, a previously described strategy, based on overexpression of *BIO1* from the biotin-prototrophic Phaffomycetaceae yeast *Cyberlindnera fabianii* (Wronska et al., 2020), was applied (Fig. 1).

Growth of *S. cerevisiae* CEN.PK113-7D on synthetic medium lacking thiamine ($SMD_{\Delta thi}$) was 25% slower than on complete SMD (0.29 ± 0.00 h^{-1} and 0.40 ± 0.01 h^{-1}, respectively; Fig. 3). A previous adaptive laboratory evolution study (Perli et al., 2020a) showed that point mutations in *ScCNB1*, which encodes the regulatory subunit of calcineurin, in *ScPMR1*, which encodes a high-affinity Ca^{2+}/Mn^{2+} P-type ATPase, and in *ScFRE2*, which encodes a cell-surface iron reductase, all led to faster growth on $SMD_{\Delta thi}$ (Perli et al., 2020a). However, reverse engineering did not yield isolates with a maximum specific growth rate on $SMD_{\Delta thi}$ equal to that of the parental strain CEN.PK113-7D on complete SMD. Faster growth on $SMD_{\Delta thi}$ was also observed upon overexpression of *ScTHI4*. The thiazole synthase *Sc*Thi4 operates as a co-substrate during synthesis of the thiazole moiety of thiamine by providing a sulfur group for thiazole formation and, thereby undergoes a single-turnover suicide reaction (Chatterjee et al., 2011). This reaction mechanism requires stoichiometric amounts of Thi4, which offers a plausible explanation for why fast thiamine-independent growth requires overexpression of *ScTHI4*. Expression of *ScTHI4* from the strong, constitutive *TDH3* promoter (strain IMX1651) resulted in a limited but significant increase of the maximum specific growth rate on $SMD_{\Delta thi}$ to 0.32 ± 0.01 h^{-1}). *Sc*Thi5, which generates the pyrimidine heterocycle of thiamine, has also been implicated in a single-turnover reaction (Coquille et al., 2012; Lai et al., 2012). However, the growth rate of strain IMX1654 which, in addition to carrying the *pScTDH3-ScTHI4* cassette present in strain IMX1651, expressed *ScTHI5* from the Sc*CCW12* promoter, did not lead to a significant further improvement of the maximum specific growth rate on $SMD_{\Delta thi}$ (Fig. 3).

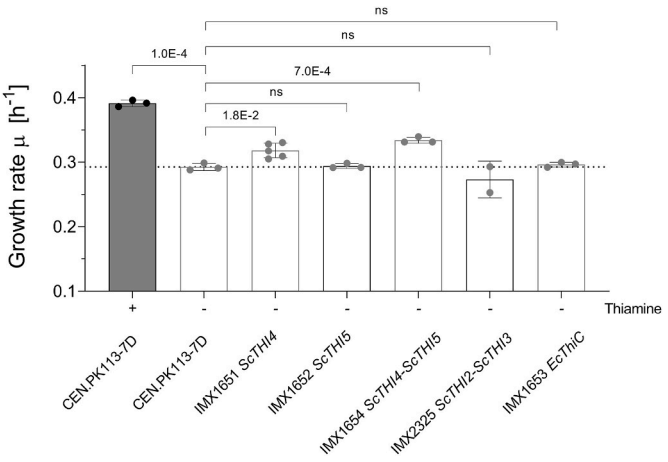

Fig. 3. Alleviating thiamin dependency for fast-growth of *S. cerevisiae*. Maximum specific growth rates of the non-engineered *S. cerevisiae* CEN.PK113-7D strain (dark grey) and engineered derivatives (white) in synthetic medium with (+) or without (−) thiamine. Yeast strains were grown in 500 mL shake flasks containing 100 mL medium and incubated at 30 °C, 200 rpm. Gene(s) overexpressed in the engineered strains are indicated. Statistical analysis of means of independent cultures (n = 3; except n = 2 for IMX2325(*ScTHI2-ScTHI3*)) was conducted using one-way analysis of variance (ANOVA) followed by significance testing of specific mean pairs by Fisher's Least Significant Difference (LSD) test with significant difference when *p*-value <5.0E-2. ns: not significant.

Two additional strategies were tested for improving growth on $SMD_{\Delta thi}$. First, based on a previous study (Nosaka et al., 2005), we attempted to overexpress *ScTHI2* and *ScTHI3*, which encode the two transcriptional regulators of the *THI* regulon. However, the resulting strain IMX2325 did not show an improved growth on $SMD_{\Delta thi}$ (Fig. 3). Subsequently, we tried expression of *E. coli thiC* which, in contrast to *Sc*Thi5, is a phosphomethylpyrimidine synthase that provides 4-amino-2-methyl-5-(phosphor-oxymethyl) pyrimidine via a non-suicide mechanism (Lawhorn et al., 2004) (Fig. 1). Since the resulting strain IMX1653 also did not show improved growth on $SMD_{\Delta thi}$ (Fig. 3), overexpression of *ScTHI4* was selected as the approach to reduce thiamine requirements for aerobic growth.

2.2. Construction of a S. cerevisiae strain capable of fast, vitamin-independent aerobic growth

To reduce or eliminate the dependencies for the six class-B vitamins thiamine, pyridoxine, inositol, *p*ABA, biotin and pantothenic acid, a strain harboring overexpression cassettes for Sc*THI4*, *ScSNO1* and *ScSNZ1*, *ScINO1*, *ScABZ1*, *ScABZ2*, *ScFMS1* and for *C. fabianii BIO1* (Wronska et al., 2020) was constructed. The cassettes were introduced in three steps into the *Spycas9*-expressing strain IMX2600, which is congenic with the reference strain CEN.PK113-7D. First, expression cassettes for *ScSNO1*, *ScTHI4* and *ScABZ2* were integrated at the X-2 locus on CHRX (Mikkelsen et al., 2012), yielding strain IMX2814. Subsequently, *ScFMS1*, *ScINO1* and *ScSNZ1* expression cassettes were integrated at the *SGA1* locus on CHRIX of strain IMX2814, yielding strain IMX2815. Integration of expression cassettes for *CfBIO1* and *ScABZ1* at the YPRcTau3 site on CHRXVI (Flagfeldt et al., 2009) of strain IMX2815 then yielded the final strain IMX2816 (Fig. S1). The genome of strain IMX2816 was sequenced to verify the correct construction. Mapping of the sequencing data on the CEN.PK113-7D reference genome (Salazar et al., 2017), to which sequences of the expression cassettes were added, revealed that all cassettes had been correctly integrated (Fig. S1). The

resulting strain IMX2816 (*CfBIO1*↑ *ScSNZ1*↑ *ScSNO1*↑ *ScINO1*↑ *ScABZ1*↑ *ScABZ2*↑ *ScTHI4*↑ *ScFMS1*↑) grew as fast as the reference strain CEN.PK113-7D on complete SMD (Fig. 4A). On a mineral salts medium supplemented with only glucose ($SMD_{\Delta vitamins}$), the strain IMX2816 exhibited a maximum specific growth rate of 0.33 ± 0.00 h^{-1}, which was only 17% lower than on complete SMD. This represented a marked contrast with the extremely slow growth of the reference strain CEN.PK113-7D on this medium (<0.001 ± 0.00 h^{-1}; Fig. 4A).

Consistent with an earlier report showing that expression of *CfBIO1* sufficed to support fast biotin-independent growth of *S. cerevisiae* (Wronska et al., 2020), supplementation of only biotin to $SMD_{\Delta vitamins}$ did not lead to faster growth (Fig. 4A). In contrast, thiamine addition resulted in a significant 9% increase to 0.36 ± 0.01 h^{-1} (p = 2.0E-4, Fig. 4A).

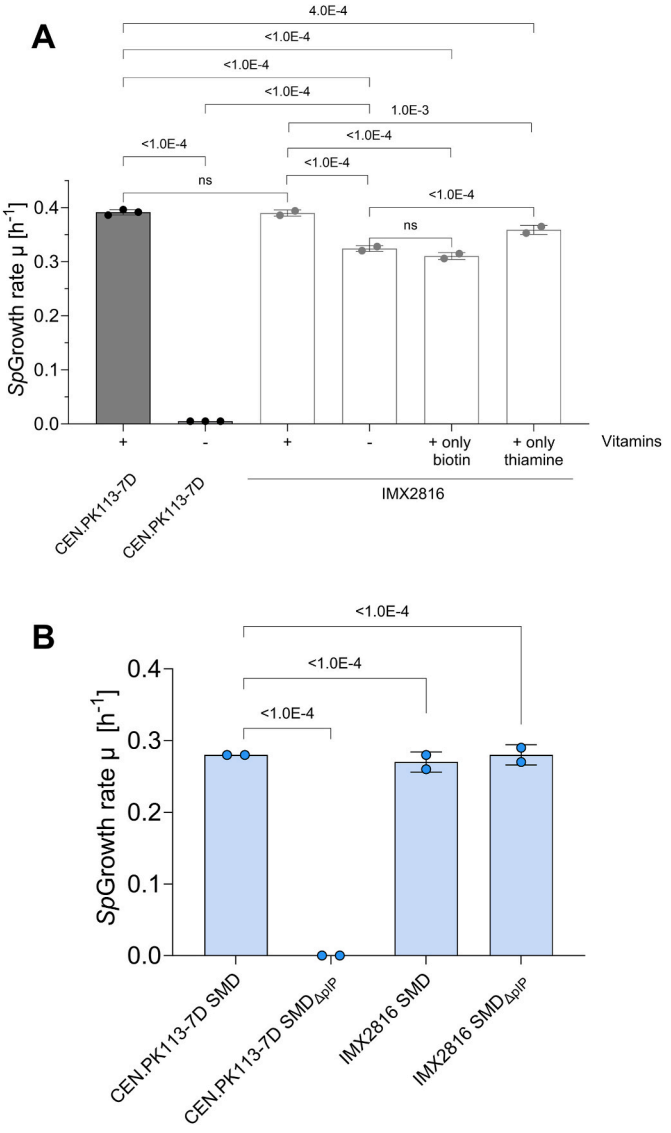

Fig. 4. Aerobic growth in medium with and without class-B vitamins. (A) Aerobic maximum specific growth rates of the non-engineered *S. cerevisiae* strain CEN.PK113-7D (dark grey) and the engineered strain (white) IMX2816 in synthetic medium with (+) or without (−) vitamins. Where indicated, a single vitamin was added to the vitamin-free medium (biotin, $SMD_{\Delta vitamins\ +\ bio}$; thiamine, $SMD_{\Delta vitamins\ +\ thi}$). Yeast strains were grown in 500-mL shake flasks containing 100 mL medium and incubated at 30 °C, 200 rpm. (B) Anaerobic maximum specific growth rates of the non-engineered *S. cerevisiae* strain CEN.PK113-7D and the engineered strain IMX2816 in SMD and $SMD_{\Delta pIP}$ (medium without of pABA, inositol and pyridoxine). Yeast strains were grown in 100-mL shake flasks containing 20 mL medium and incubated at 30 °C, 200 rpm in an anaerobic chamber. Statistical analysis was based on a one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test with significant differences between the means of at least duplicate independent cultures when p-value <5.0E-2.

While IMX2816 was unable to undergo anaerobic growth in absence of class-B vitamins due to the dependence of the pathways for biotin (Wronska et al., 2020), nicotinamide co-factor (Panozzo et al., 2002), thiamine (Wightman and Meacock, 2003), and pantothenate (Perli et al., 2021c) on oxygen, an evaluation of anaerobic growth was conducted in a medium lacking pyridoxine, *p*ABA, and inositol ($SMD_{\Delta pIP}$). For this test, IMX2816 was first grown in SMD and $SMD_{\Delta pIP}$ in aerobic shake flask. CEN.PK113-7D was grown in the same conditions and taken along as control. At the end of the first batch, the flasks were transferred in an anaerobic cabinet and used to inoculate a new flask anaerobically. After 24 h biomass was transferred to a second anaerobic flask that was monitor for growth. In presence of all class-B vitamins (SMD) CEN.PK113-7D and IMX2816 did not exhibit differential growth growing at a specific growth rate of 0.28 h^{-1} and 0.27 h^{-1} respectively (Fig. 4B). In absence of pABA, inositol and pyridoxine, CEN.PK113-7D did not exhibit growth already in the first anaerobic batch, in contrast IMX2816 was able to grow in the first batch and grew in the second batch as fast as in SMD with a specific growth rate of 0.28 h^{-1} (Fig. 4B).

2.3. Physiological characterization of the vitamin-independent strain IMX2816 in batch and carbon-limited chemostat cultures

The strain IMX2816 was capable of growth in medium devoid of all class B vitamins. The physiology of strain IMX2816 was characterized in controlled aerobic bioreactors in batch and glucose-limited chemostat cultures in both SMD and $SMD_{\Delta vitamins}$. In batch cultures in the absence of all vitamins ($SMD_{\Delta vitamins}$), IMX2816 showed a growth rate of 0.30 ± 0.01 h^{-1}, which represents a significant 23% reduction of the growth rate compared to growth rates of similar batch cultures with CEN.PK113-7D in SMD (0.375 ± 0.005 h^{-1}) (Table 1, p-value = 1.85E-04). In SMD, IMX2816 and CEN.PK113-7D did not exhibit any differences in growth rate (p-value = 6.04E-1). The strains irrespective of the growth conditions displayed a respiro-fermentative metabolism and a regular diauxic shift in which the ethanol formed during the glucose phase was subsequently completely re-consumed typical of aerobic batches of *S. cerevisiae* (Fig. 5) (van Maris et al., 2003). The biomass yield (Y_{SX}) of IMX2816 grown on either SMD or $SMD_{\Delta vitamins}$, was not significantly different than that of CEN.PK113-7D (Table 1). The quantitative mass yields and rates of product formation and substrate consumption data (Table 1) of IMX2816 and CEN.PK113-7D grown on SMD and $SMD_{\Delta vitamins}$ were highly similar with few exceptions. Both the pyruvate production rate and yield of IMX2816 grown in absence of vitamins were at least 2-fold higher in IMX2816 than in conditions with vitamins irrespectively of the strain (Table 1).

In aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1}, strain IMX2816 exhibited the same biomass yield, biomass-specific oxygen consumption rate and biomass specific carbon-dioxide production rate during growth on SMD with and without vitamins. Values of these parameters were not significantly different from those observed in similar chemostat cultures of the reference strain CEN.PK113-7D strain grown on vitamin-supplemented SMD (Table 2).

2.4. Transcriptome analysis

Chemostat cultivation allows strict control of process parameters, including the specific growth rate, which offers advantages for transcriptome comparisons between strains and/or cultivation conditions (Daran-Lapujade et al., 2009). To assess the global impact of the genetic modifications introduced into strain IMX2816, transcriptomes of aerobic glucose-limited chemostat cultures, grown at 0.10 h^{-1} on SMD and $SMD_{\Delta vitamins}$, were compared with those of chemostat cultures of the reference strain CEN.PK113-7D on SMD.

Table 1
Growth rate, yields of biomass, ethanol, glycerol and pyruvate on glucose, glucose consumption as well as ethanol, glycerol, pyruvate, acetate, citrate and succinate production rates of the *S. cerevisiae* strains IMX2816 and CEN.PK113-7D of batch cultivations performed in controlled bioreactor in chemically defined media (SMD (+) and $SMD_{\Delta vitamins}$(−). Physiological characterization of engineered *S. cerevisiae* strain IMX2816 and its isogenic reference CEN.PK113-7D was performed in 2-L bioreactors (Applikon, Delft, The Netherlands) with 1-L working volume. The data represent average and mean deviation of at least two biological replicates. Statistical significance determined by Student's t-test, with alpha = 0.01. The test with a *p*-value <0.01 are indicated in bold.

	1	2	3	Statistical significance		
	IMX2816	IMX2816	CEN.PK113-7D	Student's *t*-test p-value		
Class B vitamins	–	+	+	1 vs 2	1 vs 3	2 vs 3
Growth rate (h^{-1})	0.300 ± 0.009	0.378 ± 0.001	0.375 ± 0.005	4.89E-02	**1.85E-04**	6.04E-01
$Y_{X/S}$ ($g_{biomass}\ g_{glucose}^{-1}$)	0.337 ± 0.001	0.340 ± 0.000	0.342 ± 0.004	1.19E-01	2.64E-01	6.69E-01
$Y_{ethanol/glucose}$ ($g_{ethanol}\ g_{glucose}^{-1}$)	1.345 ± 0.053	1.421 ± 0.014	1.405 ± 0.038	3.53E-01	1.74E-01	6.24E-01
$Y_{glycerol/glucose}$ ($g_{glycerol}\ g_{glucose}^{-1}$)	0.074 ± 0.006	0.081 ± 0.013	0.091 ± 0.005	7.05E-01	2.25E-02	2.21E-01
$Y_{pyruvate/glucose}$ ($g_{pyruvate}\ g_{glucose}^{-1}$)	0.025 ± 0.000	0.008 ± 0.001	0.006 ± 0.002	**6.33E-03**	**1.19E-04**	1.86E-01
Carbon recovery (%)	94.5 ± 1.5	95.4 ± 0.9	98.1 ± 3.6	3.15E-01	2.64E-01	3.65E-01
$q_{glucose}$ (mmol $g_{biomass}^{-1}\ h^{-1}$)	−15.129 ± 0.276	−15.910 ± 0.028	−16.082 ± 0.698	1.71E-01	1.51E-01	7.59E-01
$q_{etanoloh}$ (mmol $g_{biomass}^{-1}\ h^{-1}$)	20.356 ± 1.171	22.785 ± 0.131	22.603 ± 1.147	2.39E-01	8.76E-02	9.22E-01
$q_{glycerol}$ (mmol $g_{biomass}s^{-1}\ h^{-1}$)	1.125 ± 0.110	1.439 ± 0.013	1.464 ± 0.121	1.78E-01	2.97E-02	7.20E-01
$q_{citrate}$ (mmol $g_{biomass}^{-1}\ h^{-1}$)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	NA	NA	NA
$q_{pyruvate}$ (mmol $g_{biomass}^{-1}\ h^{-1}$)	0.401 ± 0.046	0.134 ± 0.000	0.097 ± 0.023	**4.16E-03**	**4.84E-05**	1.05E-01
$q_{succinate}$ (mmol $g_{biomass}^{-1}\ h^{-1}$)	−0.004 ± 0.048	0.052 ± 0.001	0.145 ± 0.133	3.37E-01	2.18E-01	4.09E-01
$q_{acetate}$ (mmol $g_{biomass}^{-1}\ h^{-1}$)	0.394 ± 0.028	0.570 ± 0.007	0.656 ± 0.054	5.44E-02	**3.40E-03**	8.76E-02

A pair-wise comparison of the transcriptomes of strains IMX2816 and CEN.PK113-7D grown on SMD yielded only 60 differentially expressed *S. cerevisiae* genes. Of this gene set, 25 showed higher transcript levels in strain IMX2816 (|fold change| >2 and $q_{FDR\text{-}value} < 0.05$). This subset showed an overrepresentation involved in biosynthesis of co-factors (KEGG pathway: sce01240; $p_{FDR} = 8.3E\text{-}3$). As anticipated, it included all seven overexpressed *S. cerevisiae* genes (*ScTHI4* [$log_2FC = +7.3$], *ScSNZ1* [$log_2FC = +6.1$], *ScABZ2* [$log_2FC = +5.3$], *ScINO1* [$log_2FC = 2.8$], *ScABZ1* [$log_2FC4.6$], *ScFMS1* [$log_2FC = 5.1$], *ScSNO1* [$log_2FC = 4.9$]). Additionally, *CfBIO1* was also overexpressed. These eight overexpressed genes were among the 280 most highly expressed genes in strain IMX2816, with Sc*THI4* showing the highest transcript level in the entire data set (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225473). Consistent with the integration of expression cassettes in *ScSGA1* and *ScCAN1*, these genes were among 36 genes that showed a lower transcript level in strain IMX2816 (processed data available at https://doi.org/10.4121/4f77d092-450d-4354-ac61-50241f2c7e95.v1).

To further evaluate the impact of overexpressing *ScSNZ1, ScSNO1, ScABZ1*, *ScABZ2*, *ScFMS1*, *ScINO1, ScFMS1* and *CfBIO1*, the transcriptome of strain IMX2816 grown on $SMD_{\Delta vitamins}$ was compared to those of strains IMX2816 and CEN.PK113-7D grown on complete SMD. Consistently a set of 41 genes (Fig. 6) showed higher transcript levels during growth of strain IMX2816 in $SMD_{\Delta vitamins}$. Consistent with the use of constitutive promoters, the expression cassettes introduced into strain IMX2816 were not found differentially expressed.

This set of 41 upregulated genes that showed higher transcript levels during growth of strain IMX2816 on $SMD_{\Delta vitamins}$ was analyzed for enrichment of functional categories. This analysis revealed that not less than 22 genes (54%) had an annotated function related to thiamine, biotin and pyridoxal-phosphate biosynthesis (Fig. 6 and Table 3). Of eight genes comprising the category GO:0006772 "thiamine metabolic process", seven were found in this subset (Sc*PET18, ScTHI2, ScTHI6, ScTHI12, ScTHI5, ScSNZ3, ScTHI11*). The eighth gene belonging to this category, Sc*THI4*, was only recovered in the comparison with strain CEN.PK113-7D. Six additional genes related to thiamine biosynthesis that are not assigned to GO:0006772 (*ScTHI20, ScSNO3, ScSNZ2, ScTHI13, ScTHI7, ScTHI73* and *ScTHI74*) were also found in this set of 41 genes (Fig. 6 and Table 3). Upregulation of *ScPET18* (Llorente et al., 1999), *ScTHI73, ScTHI74* (Mojzita and Hohmann, 2006) and *ScTHI7* (Singleton, 1997) was previously found in *S. cerevisiae* cultures grown under thiamin limitation or starvation. Similarly, all six genes comprising the GO category "biotin biosynthetic process (GO:0006768)" (Sc*BIO2*, Sc*BIO5*, Sc*BIO4*, Sc*BIO3* and *ScBIO1 and ScBIO6)* as well as Sc*VTH1,* which encodes a biotin transporter (Stolz et al., 1999) were also found in this set of 41 genes. The final enriched category related to pyridoxal-phosphate biosynthesis (GO:0042822) comprised *ScBUD16*, *ScSNZ3*, *ScSNO3* and *ScSNZ2* (Table 3, Fig. 6). Conversely, *ScSNO4*, which encodes a cysteine protease proposed to be involved in pyridoxine metabolism (Samanta and Liang, 2003) showed lower transcript levels during growth on $SMD_{\Delta vitamins}$.

In total, 22 of these 41 genes that showed higher transcript levels during growth of strain IMX2816 on $SMD_{\Delta vitamins}$ were directly related to biosynthesis or transport of thiamine, biotin and pyridoxine (Table 3). Furthermore, several genes encoding vitamin-related enzymes also showed elevated transcript levels under these conditions. For example, transcript levels of the thiamine-pyrophosphate-dependent-decarboxylase encoding genes *ARO10* (Romagnoli et al., 2012; Vuralhan et al., 2003) and *PDC5* (Hohmann and Cederberg, 1990) were 3.1 and 3.2-fold higher, respectively.

Only one single gene consistently showed a lower transcript level during growth of strain IMX2816 on $SMD_{\Delta vitamins}$ relative to growth of either IMX2816 and CEN.PK113-7D on complete SMD. *PDR12* encodes a plasma-membrane ATP-binding cassette (ABC) transporter involved in weak organic acid (e.g. sorbate, benzoate or phenylacetate) resistance (Hazelwood et al., 2006; Piper et al., 1998). Comparison of transcriptomes of only IMX2816 cultures grown on $SMD_{\Delta vitamins}$ and complete SMD yielded a further six genes that showed lower transcript levels in cultures grown on $SMD_{\Delta vitamins}$.

In total, comparison of the transcriptomes of strains IMX2816 grown on $SMD_{\Delta vitamins}$ and CEN.PK1113-7D grown on complete SMD medium yielded only 36 genes (including *PDR12*) that showed lower transcript levels in strain IMX2816. No overrepresented GO Bioprocess terms or KEGG pathways were found to be overrepresented in this gene set.

3. Discussion

For over seven decades (Verduyn et al., 1992; Wickerham, 1951), B-class vitamins have been routinely included in chemically defined media for cultivation of *S. cerevisiae*. Omission of biotin virtually abolishes growth of many strains (Wronska et al., 2020), while leaving out several of the other B-class vitamins leads to substantially reduced growth rates in aerobic cultures (Novick and Maas, 1961; Perli et al., 2020a) (Figs. 2–4). Consistent with latter phenomenon, which is known as bradytrophy, the genomes of *Saccharomyces* yeasts typically harbour full complements of genes required for synthesis of these B-class vitamins and/or of the derived cofactors (Perli et al., 2020b). This study demonstrates that high-level expression of seven native genes and a

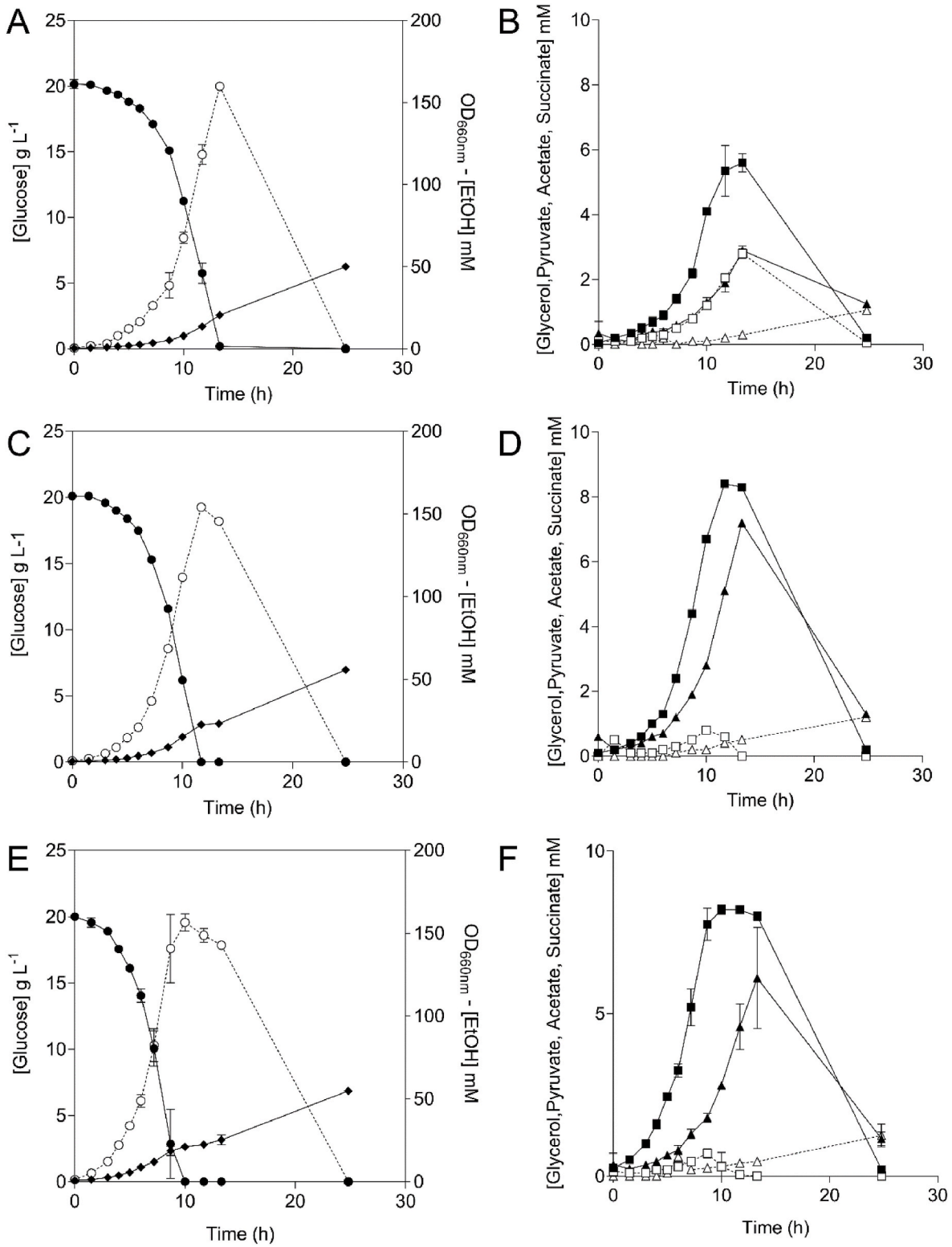

Fig. 5. Aerobic batch in controlled-bioreactor of *S. cerevisiae* strains IMX2816 and CEN.PK113-7D. Biomass expressed in OD_{660nm} unit (closed diamond and solid line), glucose (closed circle and solid line) and ethanol (open circle and dashed line) concentrations are displayed on left side of the figure while glycerol (closed square and solid line), pyruvate (open square and dashed line), acetate (close triangle and solid line) and succinate (open triangle and dashed line) concentrations are displayed on the right side of the figure. The panels **A** and **B** show data from IMX2816 grown on $SMD_{\Delta vitamins}$. The panels **C** and **D** show data of IMX2816 grown in SMD. The panel **E** and **F** show data of CEN.PK113-7D grown on SMD. The data plotted represent average and mean deviation of at least two biological duplicates.

Table 2
Dilution rate (D), biomass yield on glucose ($Y_{x/s}$), biomass-specific oxygen consumption rate (q_{O2}), biomass-specific carbon dioxide production rate (q_{CO2}), biomass-specific glucose = consumption rate ($q_{Glucose}$), biomass-specific ethanol production rate ($q_{ethanol}$), respiratory quotient (RQ), and carbon recoveries of aerobic, glucose-limited chemostat cultures of *S. cerevisiae* strain IMX2816 grown on either SMD (1) or $SMD_{\Delta vitamins}$ (2) and strain CEN.PK113-7D (3) grown on SMD. Errors represent the standard deviation of the mean (n = 4 except for CEN.PK113-7D where n = 3). Statistical significance determined by Student's t-test, with alpha = 0.05. * = statistically significant.

	IMX2816		CEN.PK113-7D	*p*-values		
Medium	SMD (1)	$SMD_{\Delta vitamins}$ (2)	SMD (3)	1 vs. 2	2 vs. 3	1 vs. 3
D (h^{-1})	0.098 ± 0.006	0.099 ± 0.005	0.097 ± 0.003	2.2E-1		
$Y_{x/s}$ ($g_{biomass}$ $g_{glucose}^{-1}$)	0.486 ± 0.007	0.484 ± 0.007	0.469 ± 0.008	6.4E-1	1.5E-1	7.4E-2
q_{O2} (mmol $g_{biomass}^{-1}$ h^{-1}]	2.68 ± 0.11	2.80 ± 0.29	2.81 ± 0.16	5.7E-2	9.0E-1	1.3E-1
q_{CO2} (mmol $g_{biomass}^{-1}$ h^{-1})	2.81 ± 0.09	2.88 ± 0.07	2.85 ± 0.22	7.7E-2	3.4E-1	1.0E-1
$q_{glucose}$ (mmol $g_{biomass}^{-1}$ h^{-1})	−1.12 ± 0.05	−1.13 ± 0.05	−1.24 ± 0.02	1.9E-1	3.2E-3*	3.2E-3*
$q_{ethanol}$ (mmol $g_{biomass}^{-1}$ h^{-1})	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	n/a	n/a	n/a
RQ	1.05 ± 0.10	1.03 ± 0.09	1.01 ± 0.07	4.4E-1	3.8E-1	5.1E-1
Carbon recovery (%)	102 ± 1.	101 ± 2	99 ± 1	1.2E-2	2.1E-1	6.6E-1

single heterologous gene enabled fast aerobic growth in a mineral salts solution supplemented with glucose. In such a simple medium, strain IMX2816 (*CfBIO1*↑ *ScSNZ1*↑ *ScSNO1*↑ *ScINO1*↑ *ScABZ1*↑ *ScABZ2*↑ *ScTHI4*↑ *ScFMS1*↑) reached 83% of the maximum growth rate of the isogenic reference strain CEN.PK113-7D on vitamin-supplemented medium.

Thiamine supplementation to vitamin-free medium led to a 9% increase of the growth rate of strain IMX2816 (Fig. 4). This remaining thiamine bradytrophy may reflect energy costs and/or protein burden associated with involvement of two single-turnover enzymes, *Sc*Thi4 and *Sc*Thi5, in synthesis of thiamine-pathway intermediates 4-methyl-5-(β-hydroxyethyl)thiazole phosphate (HET-P) and 4-amino-2-methyl-5-(diphospho-oxymethyl)pyrimidine (HMP-PP) (Fig. 1). Synthesis of HET-P depends on sulfide transfer from a conserved cysteine residue of *Sc*Thi4 to a reaction intermediate (Hazra et al., 2011), while reaction of pyridoxal phosphate with a histidine residue derived from the active site of *Sc*Thi5 has been implicated in formation of the pyrimidine ring (Coquille et al., 2012; Wightman and Meacock, 2003). *ScTHI4* was the highest transcribed gene in strain IMX2816 during growth in vitamin-free medium. Although Sc*THI5* expression was also up-regulated but remained eight-fold lower than that of *ScTHI4*. Functional expression of *Sc*Thi5 may therefore be an interesting target for further improvement. This potential limitation was also reflected in the batch physiological data of the strain IMX2816. In absence of vitamins, this strain exhibited a pyruvate yield 4.2-fold higher than that of CEN.PK113-7D grown on SMD, a trait that would point at a limitation of the pyruvate decarboxylase function, an enzyme that uses thiamine pyrophosphate as co-factor (Hohmann and Meacock, 1998; Romagnoli et al., 2012). The data provided in this study clearly identified thiamine as the most critical biosynthetic pathway to repair *S. cerevisiae* prototrophy.

In strain IMX2816, genes involved in synthesis of thiamine, biotin and pyridoxal phosphate that were not overexpressed by genetic modification still showed strongly increased transcript levels during growth on vitamin-free medium. The regulator *Sc*Vhr1 is involved in transcriptional activation of *ScVHT1* and *ScBIO5*, which encode transporters for biotin and 7-keto-8-aminopelargonic acid (KAPA), respectively, but regulation of other *S. cerevisiae* biotin-biosynthesis genes is incompletely understood (Weider et al., 2006). Transcriptional regulation of *ScVHT1* and *ScBIO5* by *Sc*Vhr1 involves a cis-regulatory motif (AATCAn$_8$TGAYT). This regulation depends on the biotin concentration, with maximum transcription at biotin concentrations being observed between 0.05 and 0.5 μg L^{-1} (Stolz et al., 1999; Weider et al., 2006). These concentrations are at least an order of magnitude below the biotin concentration found in SMD (50 μg L^{-1}). Consistent with this concentration dependency and despite the necessity for biotin uptake in the reference strain CEN.PK113-7D, *ScVHT1* and *ScBIO5* showed low transcript levels in cultures of strains CEN.PK113-7D and IMX2816 during growth on complete SMD. A common transcriptional upregulation of *ScVHT1*, *ScBIO5* and the biotin-biosynthesis-related genes Sc*BIO1*, *2*, *3*, *4* and *6* (Fig. 1) in cultures of strain IMX2816 on vitamin-free medium (Fig. 6) suggested a shared regulation mechanism. Indeed, analysis of upstream non-coding regions indicated the presence of the *Sc*Vhr1 cis-regulatory motif in the promoter region of *ScVHT1* as well as in those of *ScBIO2*, *ScBIO1* and *ScBIO6* (Fig. S2). However, in contrast to an earlier report (Weider et al., 2006), no clear *Sc*Vhr1 motif was found in the 52-bp Sc*BIO5* promoter, nor was it detected in the 220-bp bidirectional Sc*BIO4*-Sc*BIO3* promoter. Because *ScBIO5*, *ScBIO4* and *ScBIO3* are physically clustered (Hall and Dietrich, 2007), we analyzed their coding regions for potential ScVhr motifs. Indeed, the coding region of *ScBIO4* was found to harbour a ScVhr-like motif (Fig. S2), whose possible role in regulation of these three genes merits further research. These observations illustrate how engineered vitamin-independent strains such as IMX2816 provide interesting platforms to study regulation of the synthesis of class-B vitamins and derived cofactors.

Transcriptional upregulation of non-engineered genes during growth of strain IMX2816 in vitamin-free medium was not observed for genes involved in synthesis of pantothenate and nicotinic acid precursors of Co-Enzyme A and NAD^+, respectively,co-factors also known as conserved moieties (Fell, 1992), whose role does not require a large net consumption or turnover. For example, *ScFMS1* overexpression sufficed to overcome pantothenate bradytrophy (White et al., 2001) and no other genes with known roles in pantothenate synthesis showed elevated transcript levels (Fig. 6). This result suggested that the *Sc*Fms1 polyamine-oxidase activity controls the rate of pantothenate biosynthesis. Based on previous studies (Panozzo et al., 2002; Perli et al., 2020a), nicotinic acid biosynthesis was not engineered in strain IMX2816. Absence of significant differences of transcript levels of genes involved in NAD^+ synthesis or nicotinic acid transport between aerobic cultures grown with and without vitamin supplementation was consistent with full nicotinic acid prototrophy. Further research, for example based on the use of labelled nicotinic acid, is required to assess to what extent exogenous nicotinic acid is used by aerobic cultures of wild-type *S. cerevisiae* strains.

This study demonstrated that out of the seven class-B vitamins investigated *p*ABA, inositol and pyridoxine do not require oxygen and that IMX2816 could *de novo* supply these three molecules to sustain maximal growth under anaerobic condition (Fig. 4). However, vitamin requirements of yeasts are strongly influenced by oxygen availability as their native yeast biosynthesis pathways for biotin, pantothenate, nicotinic acid and thiamine all require oxygen. These oxygen requirements render *S. cerevisiae* strictly auxotrophic for these vitamins in anaerobic processes (Landry and Sternglanz, 2003; Panozzo et al., 2002; Perli et al., 2020b, 2021c; Wronska et al., 2021). Metabolic engineering solutions to eliminate oxygen requirements for the synthesis of biotin, pantothenate and nicotinic acid have recently been reported (Perli et al.,

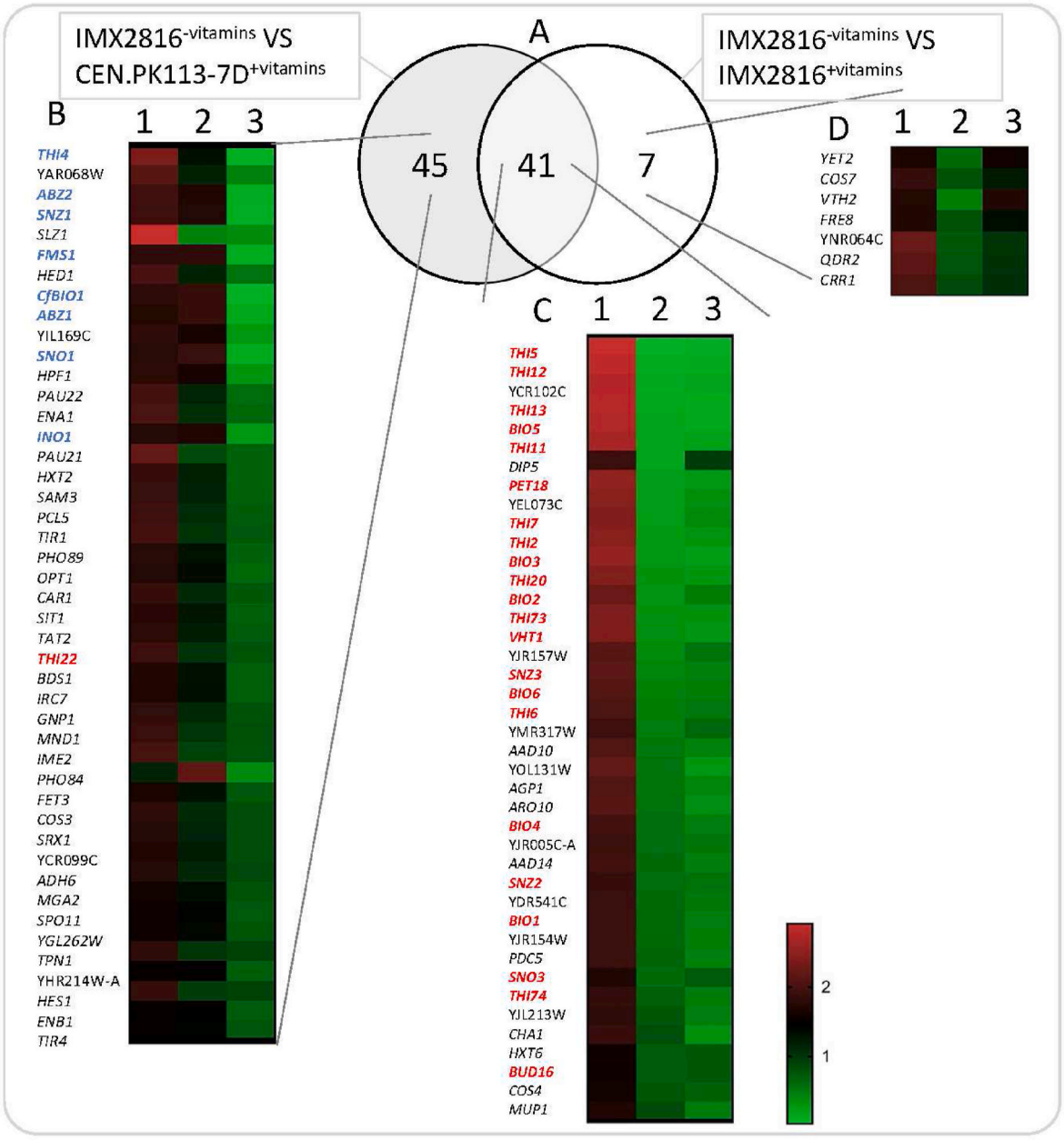

Fig. 6. Differentially up-regulated genes in absence of class-B vitamins in aerobic glucose-limited chemostat cultures. (A) Venn diagram comparing upregulated genes in IMX2816 grown in $SMD_{\Delta vitamins}$ (**1**) relative to CEN.PK113-7D grown in SMD (**3**) (left) or to IMX2816 grown in SMD (**2**) (right). (B) Heatmap of the transcript levels expressed as per gene normalized read counts of the 45 genes only significantly overexpressed in IMX2816 grown in $SMD_{\Delta vitamins}$ relative to CENPK113-7D in SMD. **C-** Heatmap of the transcript levels expressed as per gene normalized read counts of the 41 genes significantly co-overexpressed in IMX2816 grown in $SMD_{\Delta vitamins}$ relative to CENPK113-7D and IMX2612 grown in SMD. **D**- Heatmap of the transcript levels expressed as per gene normalized read counts of the 7 genes only overexpressed in IMX2816 grown in $SMD_{\Delta vitamins}$ relative to IMX2816 in SMD. Differentially over-expressed genes exhibit a fold change higher than 2 and a false discovery rate (FDR) $<$ 5.0E-2. Per gene normalized read count density boxes represent average of at least two biological duplicates.

2021c; Wronska et al., 2021). A remaining challenge concerns thiamine synthesis, which, in addition to the oxygen requirement for supply of the NAD^+ has an as yet unidentified oxygen requirement for HMP-P synthesis (Wightman and Meacock, 2003). The industrial relevance of these nutritional requirements is borne out by nutritional supplementation and transcriptome studies that link suboptimal performance of *S. cerevisiae* strains during anaerobic fermentation of plant biomass hydrolysates to thiamine and biotin availability (van Dijk et al., 2021; Zeng et al., 2017).

Several previous studies outlined the industrial relevance of eliminating vitamin requirements of microbial cell factories (Gasser et al., 2010; van Dijk et al., 2020; Wronska et al., 2021). Our results shows that complete elimination of vitamin requirements in industrial *S. cerevisiae* strains, applied in aerobic industrial processes, is a realistic target. The economic impact of a class B vitamin-independent strain can be assessed on various scales. We determined the cost based on the amount of class-B vitamins in the chemically defined medium (SMD) used in this study. Using the average catalogue price for each vitamin, we estimated their cost at 5 euro cents per litre (Supplemental Table S3), which may be perceived as remarkably cheap. It is important to note that the SMD medium was designed for the cultivation of approximately 3 g L^{-1} of biomass only (Verduyn et al., 1992). However, many industrial processes, such as fed-batch for biochemical and protein production, operate at high cell density. This necessitates an increase in vitamin

Table 3
Gene Ontology (GO) biological process complete and KEGG pathway categories enrichment analysis using Fischer's exact test in genes up-regulated in IMX2816 relative to CEN.PK113-7D in SMD and genes up-regulated in IMX2816 in $SMD_{\Delta vitamins}$ relative to IMX2816 and CEN.PK113-7D in SMD in aerobic carbon limited chemostat cultures. Unique GO_ID and KEGG pathway are indicated. The *S. cerevisiae* genes belonging to the category and found in the up-regulated set are listed. The fold-enrichment in the tested set of genes, the false discovery rate (FDR) corrected for the *p*-value (FDR^{corr} *p*-value) as determined by the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/ (Sherman et al., 2022).

Category	Term	*S. cerevisiae* genes	Fold Enrichment	P-Value	FDR
Genes upregulated in IMX2816 relative to CEN,PK113-7D in presence of vitamins					
KEGG_PATHWAY	sce01240:Biosynthesis of cofactors	*ABZ1, ABZ2, FMS1, SNO1, SNZ1, THI4*	7.88	3.98 E+12	8.31E-3
Genes up regulated in IMX2816 in $SMD_{\Delta vitamins}$ relative IMX2816 and CEN,PK113-7D in SMD (set of 41 genes Fig. 6)					
GOTERM_BP_DIRECT	GO:0009228~thiamine biosynthetic process	*SNZ2, THI5, SNZ3, THI20, THI6, THI12, THI11,THI2, THI13*	129.13	1.91E-16	1.24E-14
GOTERM_BP_DIRECT	GO:0009229~thiamine diphosphate biosynthetic process	*THI5, THI20, THI6, THI12, THI11, THI13*	12.13	2.26E-10	7.35E-09
GOTERM_BP_DIRECT	GO:0009102~biotin biosynthetic process	*BIO4, BIO5, BIO2, BIO3, BIO1, BIO6*	114.78	3.21E-06	6.95E-05
GOTERM_BP_DIRECT	GO:0042823~pyridoxal phosphate biosynthetic process	*SNZ2, SNZ3, BUD16, SNO3*	86.09	8.92E-06	1.45E-04
GOTERM_BP_DIRECT	GO:0055085~transmembrane transport	*BIO5, VHT1, THI7, AGP1, HXT6, MUP1, THI73, DIP5*	5.40	4.33E-04	0.004924
KEGG_PATHWAY	sce01240:Biosynthesis of cofactors	*THI5, SNZ3, BUD16, SNZ2, BIO4, THI20, THI6, THI12, THI11, BIO2, SNO3, THI13, BIO3, BIO1, BIO6*	14.06	6.14E-13	8.59E-12
KEGG_PATHWAY	sce00780:Biotin metabolism	*BIO4, BIO2, BIO3, BIO1, BIO6*	70.88	3.21E-06	6.95E-05

concentration (Kazemi Seresht et al., 2013; Roberts et al., 2020). Assuming a tenfold increase in vitamin concentration and a fermentation scale of 150 m^3 (Kazemi Seresht et al., 2013), the total cost of vitamins could potentially reach € 70 K (Supplemental Table S3). This illustrates that vitamin prototrophy would be economically advantageous (Gasser et al., 2010; van Dijk et al., 2020; Wronska et al., 2021).

In addition to further improving the synthesis of native cofactors, the cofactor portfolio of this yeast may be extended by introduction of pathways for synthesis of non-native cofactors, as this has been demonstrated for tetrahydrobiopterin and molybdenum cofactor (Galanie et al., 2015; Perli et al., 2021a, 2021b). Development of robust, versatile *S. cerevisiae* platforms that enable industrial use simple mineral salts media is not only relevant for microbial biotechnology, but will also inspire strategies for reducing the current complexity and costs of mammalian cell culture media for production of biopharmaceuticals and cultivated meat.

4. Methods

4.1. Strains, media, and maintenance

The *S. cerevisiae* strains used and constructed in this study (Table 4) were all derived from the CEN.PK lineage (Entian and Kotter, 2007; Salazar et al., 2017). Yeast cultures were grown in either YP (10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone) or synthetic medium (SM) (Verduyn et al., 1992). The YP and SM medium were autoclaved at 121 °C for 20 min. SM medium was then supplemented with 1 mL L^{-1} of filter-sterilized vitamin solution (0.05 g L^{-1} D-(+)-biotin, 1.0 g L^{-1} D-calcium pantothenate, 1.0 g L^{-1} nicotinic acid, 25 g L^{-1} *myo*-inositol, 1.0 g L^{-1} thiamine hydrochloride, 1.0 g L^{-1} pyridoxol hydrochloride, 0.20 g L^{-1} 4-aminobenzoic acid). A concentrated glucose solution was autoclaved separately at 110 °C and added to SM and YP at a final concentration of 20 g L^{-1} yielding SMD and YPD, respectively. Where indicated, biotin, thiamine, pyridoxine, *para*-aminobenzoic acid, inositol or pantothenic acid were omitted from the vitamin solution, yielding biotin-free SMD ($SMD_{\Delta bio}$), thiamine-free SMD ($SMD_{\Delta thi}$), pyridoxine-free SMD ($SMD_{\Delta pyr}$), *para*-aminobenzoic acid-free SMD ($SMD_{\Delta pABA}$), inositol-free SMD ($SMD_{\Delta ino}$), pantothenic acid-free SMD ($SMD_{\Delta pan}$) and pABA – inositol – pyridoxine-free SMD ($SMD_{\Delta pIP}$), respectively. Medium without vitamins, from which the vitamin solution was omitted, was indicated as $SMD_{\Delta vitamins}$. Drop-in media, which contained only a single vitamin, were prepared by adding 1 mL L^{-1} of a solution of either 0.05 g L^{-1} D-(+)-biotin or 1.0 g L^{-1} thiamine hydrochloride to $SMD_{\Delta vitamins}$ to form $SMD_{\Delta vitamins\ +\ bio}$ or $SMD_{\Delta vitamins\ +\ thi}$, respectively.

For anaerobic growth rate determination, SM medium was supplemented with 420 mg L^{-1} Tween 80 and 10 mg L^{-1} ergosterol.

Yeast strains were grown in 500-mL shake flasks containing 100 mL medium or in 100-mL shake flasks containing 20 mL medium and incubated at 30 °C and at 200 rpm in an Innova Incubator (Brunswick Scientific, Edison, NJ). Solid media were prepared by adding 1.5% Bacto agar (BD Biosciences, Franklin Lakes, NJ) and, when indicated, acetamide for $SMG_{acetamide}$ (20 g L^{-1} glucose, 1.2 g L^{-1} acetamide, 3.0 g L^{-1} KH_2PO_4, 6.6 g L^{-1} K_2SO_4, 0.5 g L^{-1} $MgSO_4$ $7{\cdot}H_2O$, 1 mL L^{-1} trace element solution and 1 mL L^{-1} vitamin solution) (Solis-Escalante et al., 2013; Solis-Escalante et al., 2013) or 200 mg L^{-1} G418 (geneticin) for YPD_{G418} or 200 mg L^{-1} hygromycin for $YPD_{hygromycin}$.

E. coli cells (XL1-Blue, Agilent Technologies, Santa Clara, CA) were grown in lysogenic broth (LB) medium (5.0 g L^{-1} yeast extract, 10 g L^{-1} bacto trypton, 5.0 g L^{-1} NaCl) supplemented with 25 mg L^{-1} chloramphenicol, 100 mg L^{-1} ampicillin or 50 mg L^{-1} kanamycin. Solid LB medium contained 2% Bacto agar. For cryostorage of strains, 30% (v/v) glycerol was added to late-exponential-phase cultures, followed by freezing and storing of aliquots at −80 °C (Solis-Escalante et al., 2013).

4.2. Molecular biology techniques

DNA was PCR amplified with Phusion Hot Start II High Fidelity Polymerase (Thermo Scientific, Waltham, MA) and desalted or PAGE-purified oligonucleotide primers (Sigma Aldrich, St-Louis, MO) by following manufacturers' instructions. DreamTaq polymerase (Thermo Scientific) was used for diagnostic PCR. Primers used in this study are shown in Table S1. PCR products were separated by agarose-gel electrophoresis and purified with either the GenElutePCR Clean-Up Kit (Sigma Aldrich) or with the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine CA). *E. coli* XL1-blue cells (Agilent Technologies) were used for chemical transformation and plasmid propagation (Inoue et al., 1990). Plasmids were purified from *E. coli* using a Sigma GenElute Plasmid Kit (Sigma Aldrich) and verified by either restriction analysis or by diagnostic PCR.

Table 4
S. cerevisiae strains used in this study.

Name	Relevant genotype	Parental strain	Reference
CEN. PK113-7D	*MATa MAL2-8c*		Entian and Kotter (2007)
CEN. PK113-5D	*MATa ura3-52 MAL2-8c*		Entian and Kotter (2007)
IMX2600	*MATa can1Δ::Spycas9*-natNT2	CEN. PK113-7D	Mans et al. (2015)
IMX1651	*MATa MAL2-8c Scura3-52::ScTDH3*p-*ScTHI4-ScTDH1*t::*ScURA3*	CEN. PK113-5D	This study
IMX1652	*MATa MAL2-8c Scura3-52::ScCCW12*p-*ScTHI5-ScENO2*t::*ScURA3*	CEN. PK113-5D	This study
IMX1653	*MATa MAL2-8c Scura3-52::ScTDH3*p-*EcThiC-ScTDH1*t::*ScURA3*	CEN. PK113-5D	This study
IMX1654	*MATa MAL2-8c Scura3-52::ScTDH3*p-*ScTHI4-ScTDH1*t::*ScCCW12*p-*ScTHI5-ScENO2*t::*ScURA3*	CEN. PK113-5D	This study
IMX1655	*MATa MAL2-8c Scura3-52::ScTDH3*p-*ScSNO1-ScTDH1*t::*ScCCW12*p-*ScSNZ1-ScENO2*t::*ScURA3*	CEN. PK113-5D	This study
IMX1859	*MATa MAL2-8c Sccan1Δ::Spycas9*-natNT2 *Scsga1Δ:ScPYK1*p-*CfBIO1-ScBIO1*t	IMX2600	Wronska et al. (2020)
IMX2044	*MATa MAL2-8c Scura3-52::ScTDH3*p-*ScINO1-ScTDH1*t::*ScURA3*	CEN. PK113-5D	This study
IMX2045	*MATa MAL2-8c Scura3-52::ScTEF1*p-*ScFMS1-ScADH1*t::*ScURA3*	CEN. PK113-5D	This study
IMX2088	*MATa MAL2-8c Scura3-52::ScTDH3*p-*ScABZ1-ScTDH1*t::*ScCCW12*p-*ABZ2-ScENO2*t::*ScURA3*	CEN. PK113-5D	This study
IMX2814	*MATa MAL2-8c Sccan1Δ::Spycas9*-natNT2 Δx-2::*ScPGK1*p-*ScSNO1-ScSSA1*t::*ScTDH3*p-*ScTHI4-ScTDH1*t::*ScCCW12*p-*ScABZ2-ScENO2*t	IMX2600	This study
IMX2815	*MATa MAL2-8c Sccan1Δ::Spycas9*-natNT2 Δx-2::*ScPGK1*p-*ScSNO1-ScSSA1*t::*ScTDH3*p-*ScTHI4-ScTDH1*t::*ScCCW12*p-*ScABZ2-ScENO2*t *Scsga1Δ::ScTEF1*p-*ScFMS1-ScADH1*t::*ScTDH3*p-*ScINO1-ScTDH1*t::*ScCCW12*p-*ScSNZ1-ScENO2*t	IMX2814	This study
IMX2816	*MATa MAL2-8c Sccan1Δ::Spycas9*-natNT2 *Δx-2::ScPGK1*p-*ScSNO1-ScSSA1*t::*ScTDH3*p-*ScTHI4-ScTDH1*t::*ScCCW12*p-*ScABZ2-ScENO2*t *Scsga1Δ::ScTEF1*p-*ScFMS1-ScADH1*t::*ScTDH3*p-*ScINO1-ScTDH1*t::*ScCCW12*p-*ScSNZ1-ScENO2*t YPRcTau3Δ::*ScPYK1*p-*CfBIO1-ScBIO1*t::*ScTDH3*p-*ScABZ1-ScTDH1*t	IMX2815	This study

Yeast genomic DNA was isolated with the SDS-LiAc protocol (Looke et al., 2011). Yeast strains were transformed with the lithium acetate method (Gietz and Woods, 2002). After genotyping by diagnostic PCR, each engineered strain was restreaked twice on selective medium (Mans et al., 2018). One colony of each strain that lost the plasmid was inoculated in the appropriate SMD medium and stocked.

4.3. Plasmid cloning

The gRNA-expressing plasmid pUDR538 was Gibson assembled using linearized pROS12, which was obtained by PCR with primer 6005 (Mans et al., 2015), and a X-2 gRNA-containing linear DNA fragment amplified by PCR with pROS12 as template and with primer 10866. Integration plasmids carrying a single gene expression module were cloned by BsaI-mediated Golden Gate Assembly (New England Biolabs, Ipswich, MA) using pYTK096 as backbone. The yeast toolkit DNA parts for promoter and terminator sequences and a purified linear DNA fragment including the gene of interest were added as previously described (Lee et al., 2015). The coding sequences for *ScABZ2*, *ScSNO1*, *ScSNZ1*, *ScTHI4*, *ScTHI5*, *ScINO1*, *ScFMS1,* Sc*THI2* and *ScTHI3* were amplified by PCR using the primer pair 12168/12169, 12170/12214, 12172/12173, 12174/12175, 12176/12177, 12212/12213, 14537/14538, 16774/16775, and 16776/16777, respectively, and CEN. PK113-7D genomic DNA (gDNA) as template. *ScABZ2*, *ScSNO1*, *ScSNZ1*, *ScTHI4*, *ScTHI5*, *ScINO1*, *ScFMS1,* Sc*THI2* and *ScTHI3* linear DNA fragments were gel purified and assembled by Golden Gate cloning with pYTK096 and the promoter/terminator part plasmids pYTK010/-pYTK055, pYTK009/pYTK056, pYTK010/pYTK055, pYTK011/pYTK052, pYTK010/pYTK055, pYTK011/pYTK052, pYTK013/pYTK053, pGGkp033/pGGkp048, and pGGkp096/pGGkp041, respectively, to yield plasmid pUDI266, pUDI265, pUDI179, pUDI201, pUDI181, pUDI182, pUDI211, pUD1103, and pUD1104, respectively (Table S2). Plasmid pUDI176 for the expression of *ScABZ1* was cloned by Gibson Assembly by combining equimolar amounts of a backbone fragment amplified with primer pair 12215/12216 and pUDI182 as a template together with an insert fragment amplified using primers 12166/12167 and CEN.PK113-7D gDNA as template. Plasmid pUDI188 for the expression of *EcthiC* was cloned by BsaI-Golden Gate Assembly. Plasmid pUD727, containing the *S. cerevisiae* codon-optimized version of *EcthiC* was synthesized by GeneArt (Thermo Fisher Scientific) and cloned with pYTK096, pYTK009 and pYTK056. Two-genes-integration plasmids pUDI183, pUDI184 and pUDI185 were cloned by Gibson Assembly by combining two single-gene expression plasmids as follows: plasmid pUDI176, pUDI265, and pUDI180 were linearized by PCR using primer pair 12219/12220. Then, the expression modules for *ScABZ2*, *ScSNZ1*, and *ScTHI5* were amplified by PCR using primers 12217/12218. Finally, the linearized backbone containing the expression module for *ScABZ1*, *ScSNO1*, and *ScTHI4* was combined with the expression module for *ScABZ2*, *ScSNZ1*, and *ScTHI5*, respectively, yielding plasmid pUDI183, pUDI184, and pUDI185, respectively. Plasmid pUDI203 for the expression of *ScSNO1*, *ScSNZ1*, and *ScTHI4* was cloned by Gibson Assembly by combining equimolar amounts of a backbone fragment amplified with primer pair 13389/13390 and pUDI184 as a template together with an insert fragment amplified using primers 13391/13392 and pUDI201 as template. *E. coli* was transformed with the correctly assembled plasmids and the resulting strains were stored at −80 °C.

4.4. Strain construction

Strains expressing single-vitamin modules (restoring single vitamin prototrophy) were constructed by transforming CEN.PK113-5D (Mata *ura3-52*) with an NotI-linearized (Thermo Scientific) integrating vector. This vector includes the single or double expression module of interest and a *URA3* selection marker flanked by 5'and 3′ homology flanks for the *ScURA3* locus. Linearized plasmids pUDI180, pUDI181, pUDI188, pUDI185, pUDI184, pUDI182, pUDI211 and pUDI183 were transformed in CEN.PK113-5D yielding strains IMX1651, IMX1652, IMX1653, IMX1654, IMX1655, IMX2044, IMX2045 and IMX2088, respectively.

Strain IMX2814 carrying expression modules for *ScSNO1*, *ScTHI4*, and *ScABZ2* was constructed by co-transforming the *Spycas9*-expressing strain IMX2600 with the gRNA-expressing plasmid pUDR538 targeting the X2 locus (Mikkelsen et al., 2012) together with equimolar amounts of linear DNA repair fragments flanked by 60 bp unique homology sequences (Kuijpers et al., 2013a, 2013b) to allow for *in vivo* homologous recombination. IMX2815 was constructed in the same way by co-transforming IMX2814 with the gRNA plasmid pUDR119 targeting the *ScSGA1* locus and repair templates carrying expression modules for *ScFMS1*, *ScINO1* and *ScSNZ1*. The final vitamin-independent strain IMX2816 was yielded by transforming IMX2815 with the YPRcTau3 (Flagfeldt et al., 2009) targeting gRNA plasmid pUDR514 and the

linearized expression cassettes for *CfBIO1* and *ScABZ1*. The expression modules for *ScSNO1, ScTHI4, ScABZ2, ScFMS1, ScINO1, ScSNZ1, CfBIO1, ScABZ1* were amplified using primer pairs 18666/14832, 14746/14747, 18667/18668, 14743/14745, 14746/14747, 18667/18671, 19162/14742, 18670/14587, respectively, and plasmid pUDI265, pUDI180, pUDI266, pUDI211, pUDI182, pUDI179, pUD790, pUDI176 as a template, respectively. All amplicons were gel-purified prior to transformation. Transformants of IMX2814, IMX2815 and IMX2816 were selected on YPD hygromycin, SMD acetamide and YPD G418 respectively. The correct recombination at each integration site was first confirmed by diagnostic PCR using gDNA as template. Integration at the *X2* locus in IMX2814 was confirmed with primer pairs 15623/13728, 13727/3927, 13733/13726, 10132/13663, and 15623/13663. For the IMX2815 strain, the integration in the *ScSGA1* locus was confirmed using the primer pairs 7326/13740, 4666/3912, 13733/10938, and 7326/10938. The integration in the YPRcTau3 locus in the final strain IMX2816 was confirmed with primer pairs 13262/5933, 10241/10123, 5905/13261, 13261/13262. Correct construction of strain IMX2816 was further checked by Illumina next generation sequencing of its full genome.

4.5. Shake-flask cultivation

For determination of specific growth rates in aerobic cultures, a frozen aliquot was thawed and used to inoculate a 20-mL culture in a 100-mL shake flask. After overnight incubation at 30 °C, the culture was used to inoculate a pre-culture in 100 mL of fresh medium in a 500-mL shake flask. When the second culture reached mid-exponential phase (optical density at 660 nm (OD_{660nm}) of 3–5), an aliquot was used to inoculate a third 100-mL culture at an initial OD_{660nm} of 0.2. Growth in the third culture was monitored by measuring OD_{660nm} over time using a Jenway 7200 Spectrophotometer (Cole-Palmer, Stone, United Kingdom). Specific growth rates were calculated by fitting the exponential growth equation ($X = X_0 e^{\mu t}$, in which μ indicates the specific growth rate) to a minimum of five data points, covering three to four doublings, during the exponential growth phase. All aerobic shake-flask experiments were carried out at least as biological duplicates and incubated in an Innova incubator (New Brunswick Scientific) set at 30 °C and 200 rpm.

For anaerobic growth shake-flask cultures were performed in Lab Bactron 300 anaerobic workstation (Sheldon Manufacturing Inc., Cornelius, OR) containing an atmosphere of 85 % N_2, 10 % CO_2, and 5 % H_2. Flat-bottom shake flasks of 100-mL containing 20 mL of media were used. After an initial aerobic overnight growth, cells were transferred to new shake-flasks at a starting OD_{600nm} of 0.5. These shake-flask were then transferred to the anaerobic workstation. Overnight growth of the cultures under anaerobic conditions ensured depletion of carry over oxygen. Subsequently, cells were transferred under anaerobic conditions to a second shake-flask to measure the anaerobic specific growth rate derived from OD_{600nm} measurements.

4.6. Controlled batch cultivation

Physiological characterization of engineered *S. cerevisiae* strain IMX2816 and its isogenic reference CEN.PK113-7D was performed in at least biologically independent duplicates in 2-L bioreactors (Applikon, Delft, The Netherlands) with 1-L working volume. Thawed stocks of strain IMX2816 were grown in 100 mL either SMD or $SMD_{\Delta vitamins}$ in 500-mL-shake flasks while CEN.PK113-7D was only propagated in SMD. After overnight incubation at 30 °C, 200 rpm, each culture was used to inoculate a second flask containing the same relative media. Exponentially growing cells (OD_{660nm} of 4–5) from the second flask were washed twice in demi-water by centrifuging for 5 min, 3000×*g*, re-suspended in 50 mL of sterile water and used to inoculate each reactor to a starting OD_{660nm} of 0.15–0.25 (Daran-Lapujade et al., 2009). The temperature was controlled at 30 °C, and the pH was maintained at 5.0 by automatic addition of 2.0 M KOH. To maintain aerobic conditions, air at a rate of 0.5 L min^{-1} was sparged through the culture which was stirred at 800 rpm. Both media SMD and vitamin-free SMD ($SMD_{\Delta vitamins}$) were supplemented with 0.2 g L^{-1} sterile pluronic PE6100 (BASF, Ludwigshafen, Germany) to avoid foam formation. For characterization of growth in batch conditions the OD_{660nm} was measured every hour using a Jenway 7200 spectrophotometer while five dry weight (DW) biomass samples were taken during the exponential growth phase and used to generate an OD_{660nm}/DW (g L^{-1}) calibration line to convert each OD_{660nm} value to DW. The carbon dioxide and oxygen concentration in the gas outflow from the bioreactors were analyzed by a Rosemount NGA 2000 analyzer (Baar, Switzerland), after cooling of the gas by a condenser (2 °C) and drying using a PermaPure Dryer (model MD 110-8P-4; Inacom Instruments, Veenendaal, The Netherlands).

4.7. Chemostat cultivation

Physiological characterization of engineered *S. cerevisiae* strains IMX2816 and CEN.PK113-7D was performed in biologically independent duplicates and triplicates, respectively, in 2-L bioreactors (Applikon, Delft, The Netherlands) with a 1-L working volume. Thawed stock cultures of strain IMX2816 were used to inoculate cultures in 500-mL shake flasks containing 100 mL of either SMD or $SMD_{\Delta vitamins}$. For CEN.PK113-7D, only SMD was used. After overnight incubation at 30 °C, 200 rpm, each culture was used to inoculate a second flask containing the same medium. Exponentially growing cells (OD_{660nm} of 4–5) from the second flask were washed twice in demi-water by centrifuging at 3000 *g* for 5 min, re-suspended in 50 mL of sterile water and used to inoculate each reactor to a starting OD_{660nm} of 0.15–0.25.

Aerobic bioreactor batch cultures on 20 g L^{-1} glucose were followed by aerobic, glucose-limited chemostat cultivation at a dilution rate of 0.10 h^{-1} with 7.5 g L^{-1} glucose in the medium as previously described (Daran-Lapujade et al., 2009; Tai et al., 2005). Temperature was controlled at 30 °C and pH was maintained at 5.0 by automatic addition of 2.0 M KOH. To maintain aerobic conditions, air was sparged through the culture, which was stirred at 800 rpm, at a rate of 0.5 L min^{-1}. Chemostat cultures were grown on either SMD or vitamin-free SMD ($SMD_{\Delta vitamins}$) supplemented with 0.2 g L^{-1} sterile Pluronic PE6100 antifoam (BASF, Ludwigshafen, Germany).

Sampling of steady-state chemostat cultures for extracellular metabolites and sugar was performed by rapid sampling, using steel balls pre-cooled to −20 °C (Mashego et al., 2003).

4.8. Whole-genome sequencing

For DNA preparation, *S. cerevisiae* IMX2816 was grown in shake-flask cultures on SMD and genomic DNA was isolated with a Qiagen Blood & Cell Culture DNA kit (Qiagen, Hilden, Germany), following the manufacturer's specifications. 300-bp paired-end sequencing was performed on a 500-bp TruSeq PCR-free insert library using an Illumina NovaSeq 6000 sequencer (Macrogen Europe, Amsterdam, the Netherlands). Sequence data were mapped to the CEN.PK113-7D genome (Salazar et al., 2017) to which the sequences of the integrated expression cassettes for the *S. cerevisiae* genes Sc*FMS1*, *ScINO1*, *ScSNO1, ScSNZ1*, *ScTHI4*, *ScABZ1*, *ScABZ2* and the heterologous *CfBIO1* were manually added. Data processing and single nucleotide variation (SNV) analysis were carried out as described previously (Bracher et al., 2017; Nijkamp et al., 2012b).

4.9. RNA isolation and transcriptome analysis

Steady-state chemostat cultures were sampled directly from the reactor into liquid nitrogen as previously described (Piper et al., 2002). Cells were stored at 80 °C for a maximum of two weeks before RNA was further purified using a phenolic acid/chloroform method as previously described (Tai et al., 2005). Total RNA was quantified by using a Qubit

RNA BR Assay Kit (Thermo Fisher Scientific) following manufacturer instructions. Total RNA quality was assessed by Nanodrop (Thermo Fisher Scientific) measurement followed by RNA ScreenTape analysis using a Tapestation System (Agilent Technologies) following manufacturer's instructions. RNA libraries were prepared with the TruSeq Stranded mRNA LT protocol (Illumina, San Diego, CA, #15031047) and subjected to 150 bp paired-end sequencing on a NovaSeq 6000 (Illumina) sequencer (Macrogen). RNAseq libraries were mapped with STAR aligner (version 2.5.3a) (Dobin et al., 2013) to the CEN.PK113-7D genome (Salazar et al., 2017) to which the sequence of *CfBIO1* was manually added. Expression was quantified by applying featureCounts (version 1.6.0) in paired-end mode and reversely stranded (Liao et al., 2014). Gene counts were TMM (Trimmed Mean of M-factors) normalized with CPM (Counts per Million) from the edgeR package (version 3.30.3) (Robinson et al., 2010). For visual display, the gene counts were per gene mean normalized and heatmap was generated using Bioconductor heatmaps (https://bioconductor.org/packages/heatmaps.).

Differential gene expression (DGE) analysis was performed by using edgeR (version 3.30.3) with TMM normalization (Robinson et al., 2010). For functional interpretation of the DGE analysis, enrichment of gene ontology terms (GO) according to the biological process was conducted with YeastMine (https://www.yeastgenome.org/goTermFinder). Analysis used a binomial test and a Bonferroni correction for multiple testing. Gene ontology terms with a p-value <0.01 were deemed as being enriched. Mean values of mRNA counts of the genes which in the DGE analysis showed $-1 < \log_2$ fold-change ($\log_2$FC) > 1 with a false discovery rate (FDR) $<$ 5.0E-2 were compared among between CEN.PK113-7D in SMD, IMX2816 in SMD and IMX2816 in $SMD_{\Delta vitamins}$ by calculating the z-score $z_i = \frac{X_i - \bar{X}}{\sigma}$.

4.10. Analytical methods

Biomass dry weight measurements were performed using preweighed nitrocellulose filters (0.45 μm, Gelman Laboratory, Ann Arbor, MI). 10 mL culture samples were filtrated, after which filters were washed with demineralized water prior to drying in a microwave oven (20 min at 360 W) and weight measurements. Metabolite concentrations in culture supernatants were analyzed by high-performance liquid chromatography (HPLC). In brief, culture supernatants were loaded on an Agilent 1260 HPLC (Agilent Technologies) fitted with a Bio-Rad HPX 87 H column (Bio-Rad, Hercules, CA). The flow rate was set at 0.6 mL min^{-1} and 0.5 g L^{-1} H_2SO_4 was used as eluent. An Agilent refractive-index detector and an Agilent 1260 VWD detector were used to detect the metabolites (Verhoeven et al., 2017). An evaporation constant of 0.008 divided by the volume in liters, was used to correct HPLC measurements of ethanol in the culture supernatants, taking into account changes in volume caused by sampling (Guadalupe Medina et al., 2010).

4.11. Statistical analysis

Statistical significance of differences between measurements from replicate cultures was calculated by using a one-way analysis of variance (ANOVA). When specific pairs of means were chosen for comparison in the experimental design, the Fisher's Least Significant Difference (LSD) test was used to calculate p-values. When all means within an experiment were compared to each other, p-values were corrected for multiple comparisons using Tukey's correction for multiple comparison as implemented in GraphPad Prism version 8.2.1 for Windows, (GraphPad Software, San Diego, CA, www.graphpad.com). Differences were assumed to be significant when p-value <0.05. Data from batch and chemostat cultures were compared by using a two-tailed Student's t-test.

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CRediT authorship contribution statement

Anja K. Ehrmann: Investigation, Writing – original draft, Validation, Visualization. **Anna K. Wronska:** Investigation, Methodology, Conceptualization, Writing – original draft. **Thomas Perli:** Investigation, Methodology, Conceptualization. **Erik A.F. de Hulster:** Investigation, Methodology, Writing – review & editing. **Marijke A.H. Luttik:** Investigation, Validation. **Marcel van den Broek:** Investigation, Formal analysis, Data curation, Visualization. **Clara Carqueija Cardoso:** Methodology, Investigation. **Jack T. Pronk:** Conceptualization, Funding acquisition, Writing – original draft. **Jean-Marc Daran:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data underlying this research are available under reviewer link: https://data.4tu.nl/datasets/4f77d092-450d-4354-ac61-50241f2c7e95/1.

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Appendix A. Supplementary data

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