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Engineering biotin synthesis; towards vitamin independency of *Saccharomyces cerevisiae*

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Summary

Every century brings its own challenges, but the 21st century is the first in which a global transition towards circularity is required to ensure human existence on this planet. Exhaustion of planetary resources, such as oil and rare elements, must be prevented and sustainable circular value chains introduced into our industry and economy. In addition to new challenges, every century also brings new and unique solutions. Today, biotechnology may provide some of the most relevant solutions by providing scientists with the ability to decipher the code of life represented by an organism's DNA as well as with the tools to edit this code. Especially fast-reproducing microorganisms have a great potential to serve as cell factories, which can convert renewable raw materials into chemicals, materials and food ingredients and thereby support a circular bio-based economy. Recently developed biotechnological tools enable us to rewrite ('edit') the blueprint for these microbial cell factories with unprecedented precisions and at unprecedented rates. A myriad of life forms evolved over billions of years to adapt to an incredibly diverse number of habitats on our planet, which led to an immense diversity in survival strategies and metabolic capabilities. Recombining these naturally occurring DNA codes and 'novel-to-nature' DNA sequences generated in laboratories offers unique possibilities for development of novel cell factories to address challenges in our century and beyond.

Baker's yeast, *Saccharomyces cerevisiae* is one of the most intensively studied microorganisms and, as a cell factory, has a long history of successful application in industrial applications. Its story of success began thousands of years ago when processes for production of wine, beer and bread-making were first invented and, over many centuries, improved. Application of yeasts probably started as serendipitous discovery rather than as an invention, when yeast cells from the environment 'contaminated' sugar-containing food products and, by accident, turned sugars into ethanol and carbon dioxide, thus yielding the first alcoholic beverages and rising dough. All essential nutrients that yeast require for growth and fermentation were either present in the food product or generated by other microorganisms that inadvertently entered these early fermentation processes. Such a co-existence of multiple microbial species is a natural phenomenon that helps organisms thrive, but in man-made industrial settings such undefined mixed populations are often difficult to control and optimize. When researchers discovered that pure cultures of individual yeast strains were very efficient in producing transport fuels and other interesting chemicals, they therefore developed growth media that contained all essential and non-essential nutrients required for optimal yeast growth, to make these yeast cell factories as productive as possible. For over a century now, yeast cell factories have been under continual development. Classical strain improvement strategies to obtain high-producing strains, later combined with recombinant-DNA technology (genetic engineering) brought microbial production systems to a next level and helped pave the way towards a sustainable bio-based

industry. However, while studying and developing product pathways for yeast strains employed in these processes, the specific requirements of these hosts regarding essential nutrients (vitamins) did not always receive attention. Use of generic media, containing excess amounts of vitamins to ensure high productivity, increase overall production costs, complicate down-stream processing and increase contamination risks. The research described in this thesis explores genetic engineering strategies in which heterologous DNA sequences are introduced to improve vitamin synthesis under industrially relevant conditions, with the goal to enable development of fully vitamin-independent (prototrophic) *S. cerevisiae* strains. The research focusses on a number of compounds that are routinely added to synthetic media for cultivation of *S. cerevisiae* that, based on their role in human nutrition, are referred to as B-type vitamins. A special focus was laid upon one of the more expensive B vitamins, biotin. The pathway by which some *S. cerevisiae* strains synthesize biotin is still not completely resolved. By a combination of laboratory evolution, genome analysis and genetic engineering, different strategies were designed and tested to obtain biotin prototrophic and fully vitamin-independent *S. cerevisiae* strains.

Chapter 1 provides an introduction to B-type vitamins that are commonly added to synthetic media used for cultivation of *S. cerevisiae*, their roles in yeast metabolism and the pathways for their *de novo* synthesis. Furthermore, the occurrence of genes involved in synthesis of B-type vitamins in genomes of different *Saccharomyces* species was analyzed to explore their potential vitamin requirements and options to improve established medium compositions. In addition, such comparisons can help metabolic engineers identify potential bottlenecks in synthesis of these vitamins, which are often cofactors or cofactor precursors for key enzymes in metabolic pathways. In **Chapter 2**, the biodiversity of Saccharomycotina was explored for biotin prototrophic species to identify highly active orthologs of Bio1, an enzyme involved in biotin synthesis that was previously shown to rate limiting in *de novo* biotin synthesis by *S. cerevisiae*. Six orthologous *BIO1* genes, which based on literature information on *S. cerevisiae* Bio1 were assumed to encode pimelate-CoA ligase, were inferred from BLAST analysis with the genomes of six biotin prototrophic yeasts and heterologously expressed in biotin-auxotrophic *S. cerevisiae* strains. One of the six *BIO1* orthologs, isolated from the yeast *Cyberlindnera fabianii* rendered conferred different laboratory and industrial *S. cerevisiae* strains with the ability to grow fast on media that were devoid of biotin. Many yeast-based industrial processes may benefit from introducing *C. fabianii BIO1*, provided that all the other biotin synthesis genes are functional. This study illustrates how harvesting information from the rapidly growing databases of strains and genome sequences can help address biotechnological challenges. Furthermore, the newly discovered *Cf*Bio1 enzyme provided a chance to study biotin metabolism further. Based on *in silico* analysis, a hypothesis was formulated that, instead of being CoA-ligases, Bio1 enzymes are dioxygenases that cleave fatty acyl-CoAs with molecular oxygen and thereby synthesize pimeloyl-CoA. Further studies on the enzymatic mechanism of *Cf*Bio1 may finally resolve the full pathway for biotin synthesis in yeast. The discovery in Chapter 2 that yeast biotin synthesis required oxygen

contributed to the understanding of overall nutritional needs of yeast microbial cell factories. Since some of the largest industrial processes involving yeasts are performed under anaerobic conditions, it was of interest to explore strategies to also make anaerobic cultures of *S. cerevisiae* biotin independent. Since the *Cf*Bio1-expressing strains described in Chapter 2 would, when used in a cultivation, require aeration in order to grow without biotin supplementation, another strategy was explored in **Chapter 3** to obtain an oxygen-independent, biotin-prototrophic *S. cerevisiae* strain. To this end, the well-studied *Escherichia coli* pathway for *de novo* synthesis of biotin in was transplanted into biotin-auxotrophic *S. cerevisiae* backgrounds. By using CRISPR/Cas9 technology, expression cassettes for the required ten *E. coli* genes were integrated into the yeast genome in a single transformation. The resulting strains showed instantaneous anaerobic growth on medium without biotin. This result showed that strategy is a straightforward way to implement anaerobic biotin prototrophy and illustrates the enormous potential of modern genome-editing techniques to accelerate improvement of microbial strains towards industrially relevant phenotypes. After oxygen- and biotin-independent growth of the resulting strain was further optimized by evolutionary engineering, causal mutations for faster growth were successfully transferred to an industrial ethanol-producing strain. The results presented in Chapter 3 thereby provide a basis for development of yeast strains whose performance in large-scale bioethanol production processes can no longer be affected by biotin availability in industrial feedstocks. This Chapter shows how integration of laboratory evolution and rational genetic engineering can overcome metabolic engineering challenges. In particular, observation of genome duplication and hypothetically micro-homology-mediated end-joining, resulting in the same segmental aneuploidy in independently evolved mutants, demonstrated the power of accurate, high-coverage whole-genome sequencing and bioinformatics for identifying causal mutations in laboratory-evolved strains. In **Chapter 4**, metabolic engineering efforts towards single-vitamin prototrophies were merged to engineer a fully vitamin-independent *S. cerevisiae* strain. Overexpression of native and heterologous vitamin biosynthesis genes yielded a strain that was able to grow on a simple mineral salt medium and glucose without substantial changes growth rate relative to vitamin-supplemented cultures of the parental, non-engineered strain. This endeavour is an example of how modular genetic engineering strategy can quickly establish a complex and industrially relevant phenotype. In addition, the strain platform described in Chapter 4 provides a basis to explore context-dependency of its vitamin independence and any trade-offs associated with its engineered phenotype.

Samenvatting

Iedere eeuw stelt de mensheid voor uitdagingen, maar de 21e eeuw is de eerste waarin een wereldwijde transitie naar circulariteit nodig is om het menselijk voortbestaan op deze planeet zeker te stellen. Uitputting van natuurlijke hulpbronnen, zoals olie en zeldzame elementen, moet worden voorkomen en duurzame circulaire waardeketens moeten in onze industrie en economie worden verankerd. Naast nieuwe uitdagingen brengt elke eeuw ook nieuwe en unieke oplossingen. Dankzij de huidige ontwikkelingen in de biotechnologie zijn wetenschappers in staat om de code van het leven, vastgelegd in het DNA, te ontcijferen en hebben ze de middelen om deze te bewerken. Vooral snel reproducerende micro-organismen hebben een groot potentieel om als "celfabrieken" te functioneren, door hernieuwbare grondstoffen om te zetten in chemicaliën, materialen en voedselingrediënten en daarmee een circulaire "bio-based" economie te ondersteunen. Recent ontwikkelde biotechnologische gereedschappen, zoals bijvoorbeeld CRISPR-Cas-techniek, stellen ons in staat om de blauwdruk voor deze microbiële celfabrieken met ongekende precisie en snelheid te herschrijven. Een enorme verscheidenheid van levensvormen is in de loop van miljarden jaren geëvolueerd door zich aan te passen aan de vele, zeer uiteenlopende habitats op onze planeet. Deze evolutie heeft geleid tot een enorme diversiteit aan overlevingsstrategieën en metabolische eigenschappen. Het recombineren van deze natuurlijk geëvolueerde DNA-codes en 'nieuw-voor-de-natuur'-DNA-volgorden die in laboratoria zijn gegenereerd, biedt unieke mogelijkheden voor ontwikkeling van nieuwe celfabrieken om uitdagingen in deze en volgende eeuwen aan te pakken.

Bakkersgist, *Saccharomyces cerevisiae*, is een van de meest intensief bestudeerde micro-organismen en heeft een lange geschiedenis van succesvol gebruik als celfabriek in industriële toepassingen. Dit succesverhaal begon duizenden jaren geleden toen de processen voor de productie van wijn, bier en brood voor het eerst werden uitgevonden en gedurende vele eeuwen geoptimaliseerd. Gebruik van gist begon waarschijnlijk als een toevallige ontdekking in plaats van als uitvinding, toen gistcellen uit de omgeving suikerhoudende voedingsproducten 'verontreinigden' en vervolgens per ongeluk suikers omzetten in ethanol en koolstofdioxide, waardoor de eerste alcoholische dranken en brood ontstonden. Alle essentiële voedingsstoffen die gist nodig heeft voor groei en fermentatie waren ofwel aanwezig in het voedingsproduct of aangeleverd door andere micro-organismen die onbedoeld in deze vroege fermentatieprocessen terechtkwamen. Het naast elkaar bestaan van verschillende samenwerkende microbiële soorten is een natuurlijk fenomeen dat organismen helpt gedijen. Echter, in industriële omgevingen zijn dergelijke ongedefinieerde gemengde populaties vaak moeilijk te beheersen en te optimaliseren. Daarom worden in de industrie voor grote biotechnologische processen die gebruik maken van gisten zogenaamde reinculturen gebruikt die uit nakomelingen van een enkele cel bestaan. Voor productieprocessen met gisten

worden voedingsoplossingen (media) gebruikt die alle essentiële en niet-essentiële voedingsstoffen bevatten die nodig zijn om een zo hoog mogelijke productiviteit te behalen. Naast mediumoptimalisatie werden voor het optimaliseren van productieprocessen al vroeg klassieke stamverbeteringstechnieken gebruikt, later aangevuld met recombinant-DNA-technologie (genetische modificatie). Deze methoden brachten microbiële productiesystemen naar een hoger niveau en hielpen de weg vrij te maken voor een duurzame "bio-based" industrie. Door een sterke nadruk op het verbeteren van productiviteit en de optimalisatie van productvormingsroutes werden de specifieke benodigdheden van het gastheerorganisme met betrekking tot essentiële voedingsstoffen (vitamines) vaak veronachtzaamd hetgeen uiteindelijk tot een suboptimaal proces kan leiden. Veel toegepaste generieke media in de biotechnologie bevatten vaak een overmaat aan vitaminen om een hoge productiviteit zeker te stellen Deze toevoegingen verhogen echter de productiekosten, kunnen productzuivering bemoeilijken en geven daarnaast een verhoogd risico op microbiële contaminatie van processen. Het onderzoek in dit proefschrift beschrijft strategieën voor genetische modificatie om volledig vitamine-onafhankelijke (prototrofe) *S. cerevisiae*-stammen te genereren. Hiertoe werden heterologe DNA-volgorden geïntroduceerd om de vitamine-synthese onder industrieel relevante condities te verbeteren. Het onderzoek richt zich op een aantal verbindingen die routinematig worden toegevoegd aan synthetische media voor het kweken van *S. cerevisiae* en die, op basis van hun rol in de menselijke voeding, worden aangeduid als B-type vitamines. In het onderzoek werd in het bijzonder aandacht besteed aan een van de duurdere B-vitamines, biotine. De route waarlangs sommige *S. cerevisiae*-stammen biotine synthetiseren, is nog steeds niet volledig opgelost. Door gebruik van een combinatie van laboratorium-evolutie, genoom-analyse en genetische modificatie werden verschillende strategieën ontworpen en getest om biotine-prototrofe en volledig vitamine-onafhankelijke *S. cerevisiae*-stammen te verkrijgen.

Hoofdstuk 1 geeft een inleiding over de B-type vitamines die vaak worden toegevoegd aan synthetische media die worden gebruikt voor het kweken van *S. cerevisiae,* hun rol in de giststofwisseling en de routes voor hun *de novo* bio-synthese. Bovendien zijn de bij synthese van B-type vitamines betrokken genen in het genoom van verschillende *Saccharomyces*-soorten geanalyseerd om hun potentiële vitaminebehoeften te onderzoeken en daarmee rationele optimalisatie van gevestigde mediumsamenstellingen mogelijk te maken. Eerder verricht onderzoek naar de rol van B-vitaminen geeft aanknopingspunten voor identificatie van mogelijke knelpunten in de synthese van deze vitamines, die vaak co-factoren of cofactor-bouwstoffen zijn voor belangrijke enzymen in stofwisselingsroutes. In **Hoofdstuk 2** werd de biodiversiteit van *Saccharomycotina* gisten onderzocht voor ondersoorten die biotine zelf kunnen maken (prototrofen) om zeer actieve orthologen van Bio1, een enzym dat betrokken is bij biotine-synthese, te identificeren. Eerder was aangetoond dat Bio1 snelheidsbeperkend was voor de reeds aanwezige biotine-synthesecapaciteit in *S. cerevisiae*. Zes kandidaat *BIO1*-orthologen, waarvan op basis

van literatuurinformatie over *S. cerevisiae* Bio1 werd aangenomen dat ze coderen voor pimelaat-CoA-ligase, werden geïdentificeerd door BLAST-analyse met de genomen van zes biotine-prototrofe gisten. Deze genen werden vervolgens heteroloog tot expressie gebracht in biotine-auxotrofe *S. cerevisiae*-stammen. Een van de zes *BIO1*-orthologen, geïsoleerd uit de gist *Cyberlindnera fabianii*, gaf verschillende laboratorium- en industriële *S. cerevisiae*-stammen het vermogen om snel te groeien op medium zonder biotine. Veel op gist gebaseerde industriële processen kunnen baat hebben bij de introductie van *C. fabianii BIO1,* op voorwaarde dat alle andere biotinesynthese-genen functioneel zijn. Deze studie illustreert hoe het verzamelen van informatie uit de snelgroeiende databases van stammen en genoom-sequenties kan helpen bij het oplossen van biotechnologische uitdagingen. Bovendien bood het nieuw ontdekte CfBio1-enzym de kans om het biotine metabolisme in gisten verder te bestuderen. Op basis van *in silico*-analyse werd de hypothese geformuleerd dat Bio1-enzymen, in plaats van CoA-ligases, dioxygenases zijn die vetacyl-CoA's splitsen met moleculaire zuurstof en daardoor pimeloyl-CoA synthetiseren. Verdere studies naar het enzymatische mechanisme van CfBio1 kunnen helpen om eindelijk de volledige route voor biotine-synthese in gist op te helderen. De ontdekking in Hoofdstuk 2 dat voor de synthese van biotine in gist zuurstof nodig was, droeg bij aan de kennis over de algemene voedingsbehoeften van gistcelfabrieken. Aangezien enkele van de grootste industriële processen waarbij gisten betrokken zijn worden uitgevoerd onder anaërobe omstandigheden, was het van belang om strategieën te onderzoeken die het mogelijk maken om ook anaërobe culturen van *S. cerevisiae* biotine-onafhankelijk te maken. De in hoofdstuk 2 beschreven giststammen die CfBio1 tot expressie brengen, hebben beluchting nodig om te kunnen groeien zonder biotinetoevoeging. In **Hoofdstuk 3** werd een strategie onderzocht om een zuurstofonafhankelijke, biotine-prototrofe *S. cerevisiae* stam te verkrijgen. Hiervoor werd de goed bestudeerde *Escherichia coli*-route voor *de novo*-synthese van biotine geïntroduceerd in biotine-auxotrofe *S. cerevisiae*-achtergronden. Door gebruik te maken van CRISPR-Cas technologie werden expressie-cassettes voor de tien benodigde *E. coli*-genen in één transformatie geïntegreerd in het gistgenoom. De resulterende stammen vertoonden onmiddellijke anaërobe groei op medium zonder biotine. Dit resultaat toonde aan dat deze strategie een eenvoudige manier is om anaërobe biotine-prototrofie te implementeren en illustreert het enorme potentieel van moderne genoom-bewerkingstechnieken om de verbetering van microbiële stammen naar industrieel relevante fenotypes te versnellen. Nadat de zuurstof- en biotine-onafhankelijke groei van de resulterende stam verder was geoptimaliseerd door laboratoriumevolutie, werden causale mutaties voor snellere groei met succes overgebracht naar een industriële ethanolproducerende stam. De in Hoofdstuk 3 gepresenteerde resultaten vormen daarmee een basis voor de ontwikkeling van giststammen waarvan de prestaties in grootschalige bio-ethanol productieprocessen niet langer worden beïnvloed door de beschikbaarheid van biotine in industriële grondstoffen. Dit hoofdstuk laat tevens zien hoe de integratie van laboratorium-evolutie en rationele genetische modificatie uitdagingen in het verbeteren van stofwisselingsnetwerken

kan overwinnen. Tijdens dit onderzoek werd in geëvolueerde giststammen zowel genoom-duplicatie als door microhomologie gemedieerde end-joining waargenomen, waarbij een specifieke segmentele aneuploïdie werd waargenomen in onafhankelijk geëvolueerde mutanten. Deze resultaten tonen de kracht aan van nauwkeurige, 'high coverage' sequencing van het hele genoom en bio-informatica voor het identificeren van causale mutaties in laboratorium-geëvolueerde stammen. In **Hoofdstuk 4** werden strategieën voor het elimineren van auxotrofieën voor individuele vitaminen gecombineerd om een volledig vitamine-onafhankelijke *S. cerevisiae*-stam te ontwikkelen. Overexpressie van natieve en heterologe vitamine-biosynthese genen leverde een stam op die in staat was om te groeien op een eenvoudig medium bestaande uit minerale zouten en glucose, zonder dat dit leidde tot substantiële veranderingen in de groeisnelheid ten opzichte van vitamine-aangevulde culturen van de niet-gemodificeerde ouderstam. Dit onderzoek is een voorbeeld van hoe een modulaire genetische modificatiestrategie kan bijdragen aan snelle introductie van een complex en industrieel relevant fenotype. Bovendien biedt het stamplatform dat in Hoofdstuk 4 wordt beschreven een basis om de context-afhankelijkheid van de vitamine-onafhankelijkheid en eventuele compromissen die verband houden met het vitamine-onafhankelijke fenotype te onderzoeken.

Chapter 1 |

Introduction

Adapted from the publication entitled

"Vitamin requirements and biosynthesis in *Saccharomyces cerevisiae*"

by Thomas Perli[#], **Anna K. Wronska[#]**, Raúl A. Ortiz-Merino,
Jack T. Pronk and Jean-Marc Daran
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"No animal can live on only pure protein, fat, and carbohydrates, but other dietary factors are required for life" [1]. This observation eventually led to the vitamine (later changed to vitamin) theory established by Casimir Funk [2]. An organic compound is defined as a vitamin if it is essential, cannot be synthesized by the organism itself, and therefore needs to be taken up from the environment [3]. Whether a compound is a vitamin therefore depends on the organism studied and, potentially, on growth conditions.

Chemically defined media for cultivation of yeasts (CDMY) are essential for fundamental as well as applied research. In contrast to complex media, which contain non-defined components such as yeast extract and/or peptone, defined media enable the generation of highly reproducible data, independent variation of the concentrations of individual nutrients and, in applied settings, design of balanced media for high-biomass-density cultivation and application of defined nutrient limitation regimes. The use of CMDY prevents thus unwanted variations. Lot to lot variation of the complex raw materials as yeast extract may lead to up to 50% difference in growth rate and biomass levels that [4, 5]. The control of process variability is not only crucial to be in line with FDA regulations but also for maintaining high productivity and maximize process economics [6]. The CDMY that are now used in yeast research laboratories around the world are based on an early investigation of the requirements for riboflavin (B_2), biotin (B_7), thiamine (B_1), pyridoxine (B_6), inositol (B_8), nicotinic acid (B_3) and pantothenate (B_5) of over a hundred yeast species [7]. With the exception of riboflavin, which could be universally omitted, yeast species exhibited diverse auxotrophy patterns for the remaining six compounds, which were therefore included in the first CDMY. *para*-Aminobenzoic acid (*p*ABA, formerly referred to as vitamin B_{10}) was later added as it was found to stimulate growth of brewing yeasts [8]. These seven compounds with riboflavin (vitamin B_2) and folate (vitamin B_9) are still included in the widely used CDMY known as Yeast Nitrogen Base [9, 10] (YNB; Table 1.1). The concentration of the vitamins contained in YNB have been empirically defined but without quantitative assessing the exact yeast requirement [11]. In another popular CDMY, often referred to as Verduyn medium (Table 1.1), concentrations of media components were adjusted to support yeast biomass concentrations up to 10 g L^{-1} in aerobic, glucose-limited cultures that exhibit a fully respiratory metabolism [12, 13].

While meant to suit all *S. cerevisiae* strains, it may happen that in specific growth conditions or for specific strains these recipes have to be adjusted. Strains of the popular *S. cerevisiae* BY lineage [14] require additional inositol to support fast growth until glucose exhaustion in YNB medium [15]. Inositol concentration represents one of main difference between the YNB and the Verduyn media, the latest containing an inositol (B_8) concentration 12.5-fold higher to prevent occurrence of undesired growth limitation (Table 1.1). Information of yeast biomass vitamin content (per $gram_{DW}$) would allow to prepare tailor-made media based on exact nutritional requirements. However, data of intracellular vitamin concentrations remain scarce and difficult to

compare. As an example the range of measured intracellular biotin concentration in *S. cerevisiae* varies by order of magnitude likely influenced by the used detection method that oscillates between bioassay based on growth of an auxotroph organism (1.4-1.5 µg/g) [16], immunodetection (0.053-0.004 ng/g) [17] or liquid chromatography. It is obvious that more complete and accurate quantitative information regarding intracellular vitamin concentration is needed. This knowledge will be key to further understand the physiological role of class B vitamin in yeast metabolism.

Based on their essentiality in the human diet, the molecules precedently mentioned can all be classified as B vitamins, which are water-soluble compounds involved in cell metabolism. However, as will be discussed below, they have widely different chemical structures and roles in cellular metabolism [3]. Early studies already demonstrated that growth of some yeasts, including *Saccharomyces* species, was not strictly dependent on addition of all of these compounds, although omission of individual compounds might result in sub-optimal growth [18-20]. These observations suggested that these yeast strains could *de novo* synthesize some of these compounds, in which cases they should formally not be referred to as vitamins but, if their addition leads to improved growth, as growth factors.

Table 1.1 | Composition of Yeast Nitrogen Base (YNB) [26] and Synthetic Media (SM) [13] for aerobic growth. Values are for 1 L of media.

		Yeast Nitrogen Base w/o amino acids	Verduyn Synthetic Media
Nitrogen source	Ammonium sulfate ($(NH_4)_2SO_4$)	5 g	5 g
Salts	Potassium phosphate monobasic (KH_2PO_4)	850 mg	3 g
	Potassium phosphate dibasic (K_2HPO_4)	150 mg	
	Sodium chloride (NaCl)	100 mg	
	Calcium chloride ($CaCl_2$)	100 mg	3.39 mg
	Boric acid (H_3BO_3)	0.5 mg	1 mg
	Copper sulfate ($CuSO_4$)	0.04 mg	0.19 mg
	Cobalt chloride ($CoCl_2$)		0.16 mg
	Potassium iodide (KI)	0.1 mg	0.1 mg
	Ferric chloride ($FeCl_3$)	0.2 mg	
	Iron sulfate heptahydrate ($FeSO_4\,7{\cdot}H_2O$)		3 mg
	Magnesium sulfate ($MgSO_4$)	0.5 g	0.244 g
	Manganese chloride ($MnCl_2$)		0.64 mg
	Manganese sulfate ($MnSO_4$)	0.4 mg	
	Sodium molybdate (Na_2MoO_4)	0.2 mg	0.34 mg
	Zinc sulfate ($ZnSO_4$)	0.4 mg	2.53 mg
	EDTA		15 mg
Growth factors	Biotin	0.002 mg	0.05 mg
	Calcium pantothenate	0.4 mg	1 mg
	Folic acid	0.002 mg	
	Inositol	2 mg	25 mg
	Nicotinic acid	0.4 mg	1 mg
	para-Aminobenzoic acid	0.2 mg	0.2 mg
	Pyridoxine	0.4 mg	0.82 mg
	Riboflavin	0.2 mg	
	Thiamine	0.32 mg	0.79 mg

It is well established that vitamin and/or growth-factor requirements of yeasts are not only species dependent, but can also strongly vary with growth conditions. In particular, ergosterol and unsaturated fatty acids, whose synthesis by *S. cerevisiae* requires molecular oxygen, are routinely included in CDMY for anaerobic yeast cultivation [21, 22]. These anaerobic nutritional requirements of yeasts are addressed in several dedicated reviews [23, 24] and will not be discussed here. For information on the applications and physiological impacts of artificially introduced auxotrophic requirements in *S. cerevisiae*, readers are referred to a previous mini-review [25].

This Chapter aims to review current knowledge on the capability of *S. cerevisiae* for *de novo* synthesis of the seven 'vitamins' that are commonly added to CDMY and on the pathways and genes involved in their biosynthesis. Riboflavin (B_2) and folic acid (B_9) that are only present in YNB will not be discussed further. *S. cerevisiae* and more generally Saccharomycotina yeasts are B_2 prototroph under both aerobic and anaerobic conditions [7]. Folic acid (B_9) synthesis depends on *p*ABA as a rate limiting precursor, whose *de novo* synthesis and metabolic implication are reviewed below. In addition, based on the existing knowledge on *S. cerevisiae* and a comparative analysis of the genomes of *Saccharomyces* species, we present a brief assessment of the distribution of these metabolic pathways across *Saccharomyces* species.

Vitamins that act as enzyme cofactors

Pyridoxine (B_6)

Pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives pyridoxine 5'-phosphate (PNP) and pyridoxamine 5'-phosphate (PMP) can be interconverted intracellularly and together form the B_6 vitamers. A vitamer is defined as a molecule having a similar structure and the same nutritional impact as the biologically active form of the vitamin. Pyridoxine was isolated and synthesized after its identification as a substance preventing dermatitis in rats [27-29]. Its chemical structure is characterized by a tetra-substituted pyrimidine ring with one methyl, one hydroxyl and two methyl-hydroxyl groups (Figure 1.1). Pyridoxine was first reported to stimulate yeast growth in 1939 [30]. Although mainly supplied to CDMY as the vitamer pyridoxine, pyridoxal 5'-phosphate (PLP) is the active form. PLP serves as co-enzyme and/or substrate for at least 50 *S. cerevisiae* enzymes involved in amino-acid, glucose and lipid metabolism, as well as in thiamine biosynthesis and regulation (Table 1.2). PLP formation from PM, PN or PL involves a salvage pathway that is widespread in nature [31]. These three vitamers can be imported in *S. cerevisiae* by the high-affinity proton symporter Tpn1 [32]. In the yeast cytosol, PN, PM and PL are phosphorylated to form PNP, PMP and PLP, respectively, most probably by the putative pyridoxine kinase Bud16. PNP and PMP are subsequently oxidized to PLP by the pyridoxine oxidase Pdx3 [33].

De novo synthesis of PLP by *S. cerevisiae* [34] involves a single reaction catalysed by PLP synthase, which is a heterodimeric enzyme [35] (Figure 1.1). Its glutamine-

hydrolase subunit (Sno) catalyses the hydrolysis of L-glutamine, producing L-glutamate and ammonia [36]. Ammonia generated in this reaction is not released from the enzyme, but channelled to the active site of the synthase subunit (Snz), which condenses it with D-ribulose 5-phosphate and D-glyceraldehyde 3-phosphate to yield PLP [37]. The Snz protein not only catalyses PLP formation but also isomerizes dihydroxyacetone-phosphate and ribose-5-phosphate to glyceraldehyde-phosphate and ribulose-5-phosphate, respectively, with the latter being the favoured substrate for PLP formation [38].

The *S. cerevisiae* genome carries three members of the *SNO* and *SNZ* genes families (*SNO1,2* and *3, SNZ1,2* and *3*). These *SNO* and *SNZ* genes form co-localized gene pairs, each expressed from a single bi-directional promoter. The *SNZ1/SNO1* pair has been shown to be involved in *de novo* PLP biosynthesis and its transcription is activated in late stationary phase [39]. Transcriptional activation of *SNZ1/SNO1* under amino acid starvation, mediated by the Gcn4 master regulator, is consistent with the PLP requirement of aminotransferases [40]. The *SNZ1/SNO1* gene pair is co-regulated by the adenine and histidine biosynthesis transcription factor Bas1 [41-43] in the presence of glycine [44]. In contrast to the *SNZ1/SNO1* gene pair, which is located in the middle of the right arm of CHRXIII, *SNZ2/SNO2* and *SNZ3/SNO3* are found in sub-telomeric regions of CHRXIV and VI, respectively and are flanked by the thiamine biosynthetic genes *THI12* and *THI5*, respectively. Their increased expression upon thiamine depletion is consistent with the role of PLP in thiamine biosynthesis [45]. The demonstration that Snz proteins can directly interact with Thi5 proteins [46] further shows the interaction of pyridoxine and thiamine biosynthesis (Figure 1.1).

Table 1.2 | *S. cerevisiae* S288C proteins requiring pyridoxal-5-phosphate, thiamine diphosphate and biotin as cofactor or as substrate. Protein lists were obtained through advanced search in UNIPROT and manually curated (https://www.uniprot.org).

Cofactor	Protein	Protein name
Pyridoxal-5-phosphate	Uga1	4-aminobutyrate aminotransferase [47]
	Hem1	5-aminolevulinate synthase [48]
	Arg8	Acetylornithine aminotransferase* [49]
	Bio3	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase [50]
	Agx1	Alanine-glyoxylate aminotransferase 1 [51]
	Abz2	Aminodeoxychorismate lyase [52]
	Aro9	Aromatic amino acid aminotransferase 2 [53]
	Aro8	Aromatic/aminoadipate aminotransferase [54]
	Aat2	Aspartate aminotransferase 2 [55]
	Aat1	Aspartate aminotransferase 1 [56]
	Bat2	Branched-chain-amino-acid aminotransferase 2 [57]
	Bat1	Branched-chain-amino-acid aminotransferase 1 [57]
	Cha1	Catabolic L-serine/threonine dehydratase [58]
	Str3	Cystathionine β-lyase [59]
	Cys4	Cystathionine β-synthase [60]
	Cys3	Cystathionine gamma-lyase [61]
	Str2	Cystathionine gamma-synthase [62]
	Nfs1	Cysteine desulfurase [63]
	Dsd1	D-serine dehydratase [64]
	Gad1	Glutamate decarboxylase [65]
	Gcv2	Glycine dehydrogenase [66]
	Gph1	Glycogen phosphorylase [67]

	His5	Histidinol-phosphate aminotransferase [68]
	Met17	Homocysteine/cysteine synthase [69]
	Bna5	Kynureninase [70]
	Sry1	L-threo-3-hydroxyaspartate ammonia-lyase [71]
	Gly1	Low specificity L-threonine aldolase [72]
	Car2	Ornithine aminotransferase [73]
	Spe1	Ornithine decarboxylase [74]
	Ser1	Phosphoserine aminotransferase [75]
	Alt2	Probable alanine aminotransferase 2 [76]
	Alt1	Probable alanine aminotransferase 1 [77]
	Bna3	Probable kynurenine-oxoglutarate transaminase [78]
	Irc7	Putative cystathionine beta-lyase [78]
	Yll058w	Putative cystathionine gamma-synthase [79]
	Yml082w	Putative cystathionine gamma-synthase [62]
	MCY1	Putative cysteine synthase [80]
	Shm2	Serine hydroxymethyltransferase 2 [81]
	Shm1	Serine hydroxymethyltransferase 1 [81]
	Lcb1	Serine palmitoyltransferase 1 [82]
	Lcb2	Serine palmitoyltransferase 2 [82]
	Dpl1	Sphingosine-1-phosphate lyase [83]
	Ilv1	Threonine dehydratase [84]
	Thr4	Threonine synthase [85]
	Trp5	Tryptophan synthase [86]
	Yhr112c	Uncharacterized trans-sulfuration enzyme [87]
	Thi5	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase [88]
	Thi11	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase [88]
	Thi12	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase [88]
	Thi13	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase [88]
Thiamine diphosphate	Kgd1	2-oxoglutarate dehydrogenase [89]
	Ilv2	Acetolactate synthase catalytic subunit [90]
	Pxp1	Putative 2-hydroxyacyl-CoA lyase [91]
	Pdc1	Pyruvate decarboxylase isozyme 1 [92]
	Pdc5	Pyruvate decarboxylase isozyme 2 [93]
	Pdc6	Pyruvate decarboxylase isozyme 3 [94]
	Pda1	Pyruvate dehydrogenase E1 component subunit α [95]
	Pdb1	Pyruvate dehydrogenase E1 component subunit β [96]
	Thi3	Thiamine metabolism regulatory protein THI3 [97]
	Aro10	Transaminated amino acid decarboxylase [98]
	Tkl1	Transketolase 1 [99]
	Tkl2	Transketolase 2 [100]
Biotin	Acc1	Acetyl-CoA carboxylase [53]
	Hfa1	Acetyl-CoA carboxylase, mitochondrial [101]
	Bpl1	Biotin protein ligase [102]
	Pyc1	Pyruvate carboxylase 1 [103]
	Pyc2	Pyruvate carboxylase 2 [103]
	Dur1,2	Urea amidolyase [104]

Thiamine (B_1)

Thiamine, also known as vitamin B1, was first isolated by Jansen and Donath [105] and later obtained in sufficient amounts for extended chemical analysis [106]. In animals, which cannot synthesize thiamine, a lack of dietary supply causes beriberi, a disease affecting the nervous system [107]. Thiamine is essential for cellular energy metabolism and its major biologically active derivative thiamine diphosphate (TDP) serves as

cofactor for a variety of enzymes, including pyruvate and oxoglutarate dehydrogenases, transketolases, 2-hydroxy-3-oxoadipate synthase, acetolactate synthase and 2-oxo acid decarboxylases (Table 1.2). As an electrophilic cofactor, TDP forms covalent intermediates with enzyme substrates. Thiamine can also perform intramolecular proton transfers, which is a rare function among cofactors [108]. It has been proposed that a general stress-protective role of thiamine in *S. cerevisiae* is partially unrelated to its role as a cofactor [109]. Thiamine is synthesized *de novo* by plants and many microorganisms including yeast species.

Thiamine consists of two substituted aromatic moieties, 4-amino-2-methyl-5-pyrimidyl (HMP) and 5-(2-hydroxylethyl)-4-methylthiazolium (HET), which are connected by a methylene bridge (Figure 1.1). In addition to free thiamine and the biologically active form thiamine diphosphate (TDP), thiamine monophosphate (TMP), and thiamine triphosphate (TTP) are also found intracellularly. All thiamine-prototrophic organisms synthesize TDP via condensation of the precursors 5-(2-hydroxylethyl)-4-methyl thiazole phosphate (HET-P) and 4-amino-2-methyl-5-pyrimidine diphosphate (HMP-PP) to TMP by TMP-diphosphorylase (Thi6 in *S. cerevisiae*) (Figure 1.1). While bacteria can synthesize TDP from TMP in a single reaction, catalysed by a TMP kinase, eukaryotes utilize a pathway in which TMP is first dephosphorylated to thiamine, which is then pyrophosphorylated to TDP by a thiamine pyrophosphokinase (Thi80 in *S. cerevisiae*) [110]. Two transporters involved in the acquisition of exogenous thiamine have been identified in *S. cerevisiae*: a high-affinity transporter encoded by *THI10* [111] and a periplasmic acid phosphatase encoded by *PHO3* that releases thiamine from thiamine phosphates [112].

In *S. cerevisiae,* the thiamine precursor HMP-PP is synthesized in two steps. First, 4-amino-2-methyl-5-pyrimidine phosphate (HMP-P) is formed from pyridoxal-5-phosphate and histidine. The histidine used for HMP-P synthesis is provided from the active site of HMP-P synthase in a suicide reaction [88, 113]. HMP-P synthase is encoded by four highly similar *S. cerevisiae* genes (*THI5*, *THI11*, *THI12,* and *THI13*). These genes are located in sub-telomeric regions of different chromosomes, suggesting that an increased copy number conferred a selective advantage in thiamine-poor environments [114]. In a second step, HMP-P is phosphorylated to HMP-PP by HMP-P kinase in an ATP-dependent reaction [115]. The *S. cerevisiae* genome harbours two paralogous genes encoding HMP-P kinase, *THI20,* and *THI21*, of which the former encodes the major isoform [114]. Thi20 is a trifunctional protein that displays thiamine biosynthesis and thiamine degradation activities in a single protein. Its N-terminal domain is active as HMP and HMP-P kinase, while its C-terminal domain has thiaminase II activity [116]. Although molecular oxygen is not directly required for HMP biosynthesis, activity of this branch of the thiamine biosynthetic pathway was shown to be oxygen dependent [114]. However, based on gene deletion studies it has been proposed that *S. cerevisiae* can still synthesize the pyrimidyl moiety under anaerobic conditions via an alternative, as yet unidentified, pathway [117].

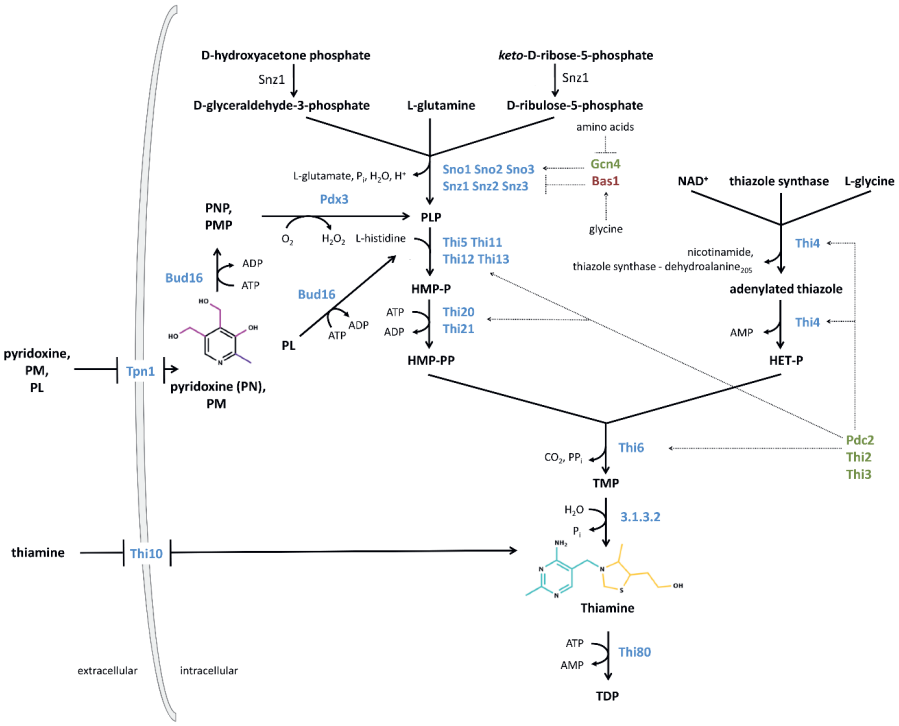

Figure 1.1 | **Pyridoxal-5'-phosphate (PLP) and thiamine diphosphate (TDP) *de novo* synthesis pathway in *S. cerevisiae*.** D-glyceraldehyde 3-phosphate, L-glutamine and *keto*-D-ribose 5-phosphate are converted to PLP by the catalytic activity of the gene products of *SNO1,2,3* and *SNZ1,2,3*. Gcn4 acts as positive regulator of *de novo* PLP biosynthesis while Bas1 acts as an inhibitor. Gcn4 is inhibited by amino acids and activated under amino-acid starvation. Bas1 instead is upregulated in the presence of glycine. Pyridoxine (PN), pyridoxamine (PM) and pyridoxal (PL) are imported by Tpn1. PN is converted at the expense of ATP to pyridoxine-5'-phosphate (PNP) by Bud16 whereupon Pdx3, produces PLP and hydrogen peroxide in an oxygen-dependent reaction. Similarly, PLP can be formed starting from PM in two steps by action of Bud16 and Pdx3, with pyridoxamine-5'-phosphate (PMP) as intermediate. Moreover, PL can also be converted to PLP by action of Bud16. PLP is used as cofactor or converted to 4-amino-2-methyl-5-pyrimidine phosphate (HMP-P) by one of the four homologous enzymes Thi5, Thi11, Thi12 and Thi13, under consumption of L-histidine. HMP-P is the intermediate for the formation of the pyrimidyl moiety of thiamine (shown in cyan). Thi20 and Thi21 further phosphorylate HMP-P to 4-amino-2-methyl-5-pyrimidine diphosphate (HMP-PP). The thiazole moiety (shown in yellow) is synthesized by activity of Thi4 in a suicide mechanism, leading to 5-(2-hyroxylethyl)-4-methylthiazole phosphate (HET-P). HMP-PP and HET-P are merged by the gene product of *THI6* to thiamine phosphate (TMP). The following reaction catalysed by an acid phosphatase (EC number 3.1.3.2) yields thiamine. Thiamine can be taken up with the aid of the transporter Thi10. Finally, thiamine is converted to its biologically active form thiamine diphosphate (TDP) under consumption of ATP by Thi80. Pdc2, Thi2 and Thi3 are responsible for the upregulation of transcription of Thi5/11/12/13, Thi20/21, Thi6 and Thi4. Alcohol and methyl substitutions on the pyridoxine pyrimide ring are shown in magenta and purple respectively. Metabolites, proteins and positive regulators are shown in bold, blue and green respectively.

For the synthesis of the thiazole moiety, eukaryotic cells use a single enzyme to form HET-P from glycine and NAD^+, encoded by *THI4* in *S. cerevisiae* [118]. Thi4 acts as a substrate in the reaction by providing the sulfur atom needed for thiazole formation in an iron-dependent sulfide transfer from a conserved cysteine. Therefore, similar to Thi5, Thi4 acts as a suicide enzyme undergoing only a single catalytic turnover [119-121]. Under thiamine-depleted conditions, Thi5 and Thi4 are among the most abundant proteins in *S. cerevisiae* [122]. Strains harbouring a *THI4* deletion have an increased sensitivity to DNA damaging agents such as UV light and methyl methanesulfonate. The mechanism of this protection is not fully understood [114, 123].

Involvement of two suicide enzymes makes *de novo* thiamine biosynthesis in yeast an energetically very expensive process: for each molecule of thiamine produced, two complete proteins (Thi4 and Thi5/11/12/13) have to be synthesized and degraded. Tight regulation of thiamine synthesis occurs mainly at the transcriptional level [124, 125]. As a result, the *THI* regulon is repressed in the presence of high intracellular levels of TDP. A strain carrying a partially inactivated form of Thi80 was shown to constitutively express the *THI* genes, suggesting that TDP is the molecule acting in this negative feedback regulation loop [126]. Three positive regulators for thiamine biosynthesis have been identified to date: Thi2, Thi3, and Pdc2 [97, 127, 128] . Elimination of any of these three proteins abolishes *THI* genes expression. The expression of *THI2* and *THI3*, but not *PDC2*, strongly increased under thiamine-depleted conditions[129]. Deletion of *THI2* results in repression of all *THI* genes except for *THI10*, whereas deletion of *THI3* causes repression of all *THI* genes. Thi3, which binds TDP, was originally proposed to also act as a 2-oxo acid decarboxylase involved in the Ehrlich for fusel alcohol biosynthesis [130] but this conclusion was later refuted [131-133]. A strain that only carried a *thi3* allele encoding a protein unable to bind TDP showed constitutive expression of *THI* genes in thiamine-containing medium, suggesting that Thi3 acts as a TDP sensor. However, Thi3 lacks a clear DNA-binding motif and is likely to act through interaction with other proteins, such as Thi2 and Pdc2. Pdc2 is a transcriptional regulator that activates both *THI* genes and *PDC* genes encoding pyruvate decarboxylases [128, 134]. These regulatory proteins therefore provide an interesting link between the biosynthesis of pyruvate decarboxylase, the most highly expressed TDP-dependent enzyme in *S. cerevisiae*, and its cofactor. A regulatory link between the biosynthesis of thiamine and that of nicotinic acid, another member of the B-complex vitamins, was demonstrated when the NAD^+-dependent histone deacetylase Hst1 was found to act as a repressor of basal *THI*-gene expression [135].

Biotin (B_7)

During the first half of the 20th century, biotin was discovered as an essential growth factor for various organisms [136, 137]. Biotin plays an important role as co-enzyme in carboxylases involved in fatty acid synthesis, sugar and amino acid metabolism [138]. The cytosolic (Acc1) [139] and mitochondrial (Hfa1) acetyl-CoA carboxylases [140], pyruvate carboxylase (Pyc1,2) [141], urea carboxylase (Dur1,2) [142] and a tRNA-

aminoacylation cofactor (Arc1) [143] are the only biotin-dependent enzymatic activities in *S. cerevisiae* (Table 1.2). Covalent linkage of the carboxyl group of biotin to an ε-lysine residue of apo-Acc1 and apo-Pyc1 and 2 is catalysed by the biotin protein ligase, Bpl1 [102, 144]. While not characterised, a similar mechanism is likely to occur for the mitochondrial acetyl-CoA carboxylase [101, 145]. Biotin can be taken up via the proton symporter Vht1 [146]. Alternatively, the biotin intermediates 8-amino-7-oxononanote (KAPA) and 7,8-diaminopelargonate (DAPA) can be transported into yeast via the Bio5 membrane protein [50].

The molecular structure of biotin is characterized by an imidazole, or ureido ring, fused with a sulfur-containing tetrahydrothiophene ring, substituted with a valeric acid chain (Figure 1.2). The reactions involved in the formation of the ring structures of biotin from KAPA are highly conserved among yeast and bacteria and require three steps starting with the conversion of KAPA to DAPA. This reaction is catalysed by Bio3, a DAPA aminotransferase that requires S-adenosyl-methionine (SAM) and pyridoxal 5'-phosphate (PLP) as cofactors. The following step, catalysed by the dethiobiotin synthetase Bio4, converts DAPA to dethiobiotin at the expense of ATP [50]. In the final step, the biotin synthase Bio2, a mitochondrial iron-sulfur-cluster protein, converts dethiobiotin to biotin by incorporating a sulfur atom [147], presumably acting as a suicide enzyme [148].

The pathway for synthesis of the valeric side chain of biotin remains elusive and probably involves Bio1 and Bio6, both of which are required for biotin-independent growth of *S. cerevisiae* [149]. Presence of Bio1 and Bio6 is strain dependent. For example, the reference strain S288C [150] lacks these two genes and is unable to grow on CDMY lacking biotin [151]. In contrast, sake strains of *S. cerevisiae* [152], *S. cerevisiae* strains isolated from cachaça fermentations [153], and the laboratory strains A364a [149] and CEN.PK113-7D [151, 154] do carry these two genes and exhibit growth, albeit very slowly, on CDMY without biotin. *BIO6* has been proposed to have evolved from a duplication and neo-functionalization of *BIO3*, after *BIO3* and *BIO4* had been simultaneously acquired by horizontal gene transfer, with *BIO1* similarly having evolved from duplication and neo-functionalization of the uncharacterized ORF *YJR154W* [149].

In view of its 55% amino-acid sequence similarity with *Escherichia coli* BioA, *BIO6* probably encodes an adenosylmethionine-8-amino-7-oxononanoate transaminase [152]. The initial formation of the pimeloyl thioester in *S. cerevisiae* remains unclear. Hall and Dietrich [149] proposed that *BIO1* encodes a coenzyme A (CoA) ligase that activates pimelic acid, a C7 dicarboxylic acid, to pimeloyl-CoA. Although such a CoA ligase (BioW) was identified in the gram-positive bacterium *Bacillus subtilis* [155], *S. cerevisiae* Bio1 protein does not show similarity to that enzyme. Additionally, biosynthesis of pimelic acid by *S. cerevisiae* has not been reported and pimelic acid feeding to a strain carrying the full biotin biosynthesis pathway was not able to stimulate growth on medium lacking biotin [156].

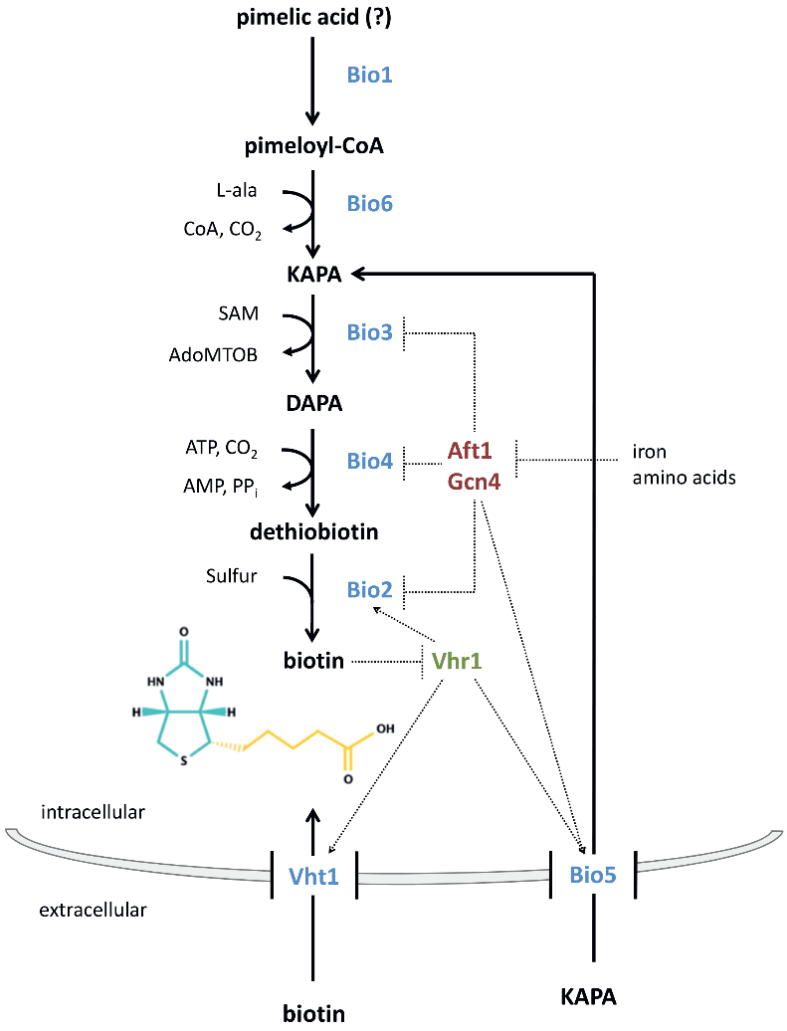

Figure 1.2 | **Biotin *de novo* biosynthesis pathway in *S. cerevisiae*.** Biotin is composed of an ureido and a tetrahydrothiophene ring (shown in cyan) fused to a valeric acid chain (shown in yellow). The five final steps of *de novo* biotin synthesis are carried out by Bio1, Bio6, Bio3, Bio4 and Bio2. Origin of pimelic acid remains elusive in *S. cerevisiae* (indicated by question mark(?)). Pimeloyl-CoA formed by Bio1 is converted via 8-amino-7-oxonanoate (KAPA) to 7,8-diaminopelargonate (DAPA) by Bio6 and Bio3. DAPA is subsequently converted by Bio4 to dethiobiotin and finally to biotin by Bio2. The intermediate KAPA and biotin can be imported via the membrane transporters Bio5 and Vht1, respectively. In the absence of biotin, the regulator Vhr1 upregulates expression of genes encoding the transporters Vht1 and Bio5 as well as Bio2. In iron and amino acid rich conditions the transcriptional regulator genes *AFT1* and *GCN4* are transcriptionally repressed, which under iron and amino acid scarce conditions would not activate transcription of *BIO3*, *BIO4* and *BIO2* and relieve *BIO5* expression. Metabolites, proteins, positive and negative regulators are shown in bold, blue, green and red respectively.

Laboratory evolution studies highlighted the role of the enigmatic Bio1 protein in biotin prototrophy of *S. cerevisiae*. Prolonged cultivation of the laboratory strain CEN.PK113-7D in biotin-free accelerostats yielded an evolved strain that showed the same high specific growth rate (0.36 h^{-1}) in the absence and presence of biotin. Whole-genome re-sequencing of evolved isolates revealed a massive 20 to 40-fold amplification of the physically linked *BIO1* and *BIO6* gene copies [154]. Overexpression of *BIO1*, but not *BIO6*, from a multi-copy plasmid sufficed to increase specific growth rates of the non-evolved strain on biotin-free CMDY without biotin from 0.01 h^{-1} to 0.15 h^{-1}. Despite its unknown function, these results show that *BIO1* is a key bottleneck of in *de novo* biotin synthesis in *S. cerevisiae* [154]. Strategies to generate biotin-prototrophic *S. cerevisiae* strains are likely to benefit from elucidation of the reaction catalysed by Bio1.

The biotin biosynthetic genes *BIO5, BIO2, BIO4, BIO3, BIO6, VHT1* and *BPL1* showed a concerted upregulation during biotin starvation [17, 152]. The promoter regions of *BIO5, VHT1, BIO2* and *BPL1* contains an upstream activating element that, in the absence of biotin, is bound by the transcription factor Vhr1 which upregulates transcription. This activation ensures expression of biotin and DAPA transporters, *de novo* biotin synthesis and enzyme biotinylation [157]. The transcriptional regulation of the biotin permease gene *VHT1* is additionally controlled by the transcription factors Aft1 and Gcn4, which are involved in iron homeostasis and global control of nitrogen metabolism, respectively. Down-regulation of biotin biosynthesis genes and a parallel upregulation of biotin transport upon low availability of iron and/or nitrogen [158] has been interpreted as a strategy to decrease the metabolic burden of *de novo* biotin synthesis under these conditions [17].

Vitamins that act as metabolic precursors for cofactor biosynthesis

Pantothenic acid (B_5)

Vitamin B_5 was discovered by 1933 [159] and, based on its presence in all animal tissues, named pantothenate (παντοθεν, from everywhere). Pantothenate is not a co-factor, but a key precursor for synthesis of coenzyme A (CoA) and acyl carrier protein (ACP), which play key roles in metabolism. When supplied to media, pantothenate is imported into *S. cerevisiae* by the plasma-membrane pantothenate-proton symporter Fen2 [32]. Only plants and microorganisms, including fungi, can perform *de novo* pantothenate biosynthesis. However, most sake strains of *S. cerevisiae* strains are entirely auxotrophic for pantothenate when grown in media that exclusively contain organic nitrogen sources and, in some cases, also when an inorganic nitrogen source is provided [160]. Many *S. cerevisiae* strains can synthesize pantothenic acid. In such strains, removal of the molecule from the medium typically results in impaired growth on glucose, but not on glycerol or acetate [161].

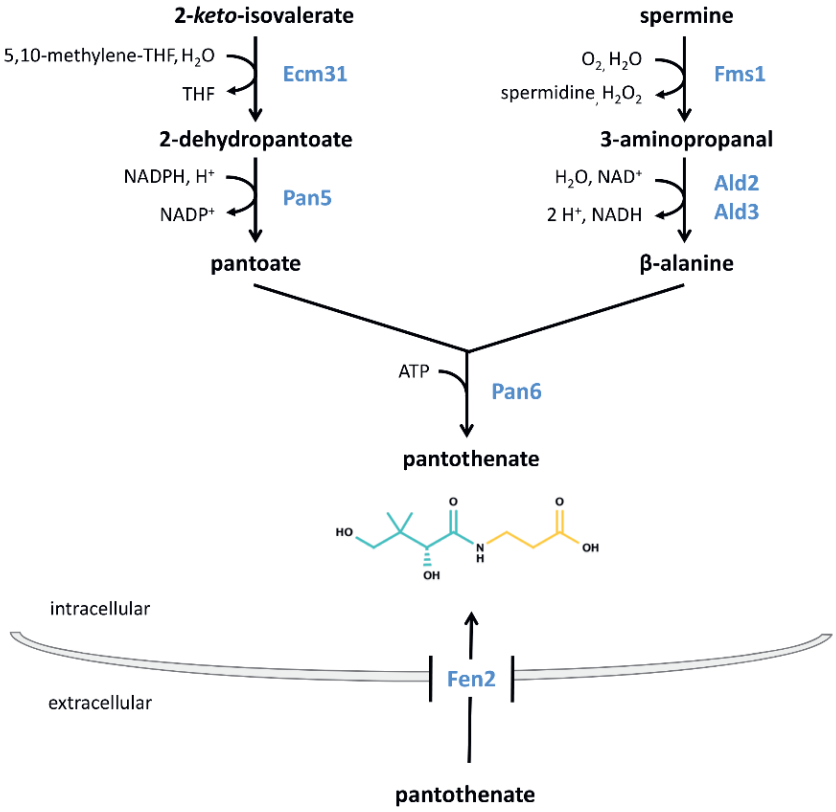

Figure 1.3 | **Pantothenate *de novo* synthesis pathway in *S. cerevisiae* and transcription profiles of pantothenate biosynthetic genes under different growth conditions.** Pantothenate can be imported by the proton symporter Fen2 or synthesized *de novo* by condensation of pantoate (shown in cyan) and β-alanine (shown in yellow) in an ATP-dependent reaction catalysed by Pan6. Pantoate is formed in a two-step pathway from 2-*keto*-isovalerate catalysed by Ecm31 and Pan5 with 2-dehydropantoate as intermediate. β-alanine is formed starting from spermine by the enzymes Fms1 and Ald2-3 via 3-aminopropanal.

Pantothenate is formed by fusion of pantoate and β-alanine, in a reaction catalysed by pantoate-beta-alanine ligase (Pan6 in *S. cerevisiae*, Figure 1.3). In *S. cerevisiae*, β-alanine is produced from spermine in two steps [161]. The first step is catalysed by the polyamine oxidase Fms1, which produces 3-aminopropanal from spermine. 3-Aminopropanal is then oxidized to β-alanine by the cytosolic aldehyde dehydrogenases Ald2 and Ald3. The reaction catalysed by Fms1 has been reported to be rate limiting for pantothenate biosynthesis and Fms1 overexpression results in the secretion of pantothenic acid [162]. Pantoate is synthesized in *S. cerevisiae* from 2-*keto*-isovalerate, an intermediate of the valine biosynthesis. After conversion of 2-*keto*-isovalerate into 2-dehydropantoate [163] by *keto*-pantoate hydroxymethyltransferase (Ecm31), 2-dehydropantoate is transformed into pantoate by 2-dehydropantoate 2-reductase (Pan5) in an NADPH-dependent reduction [164].

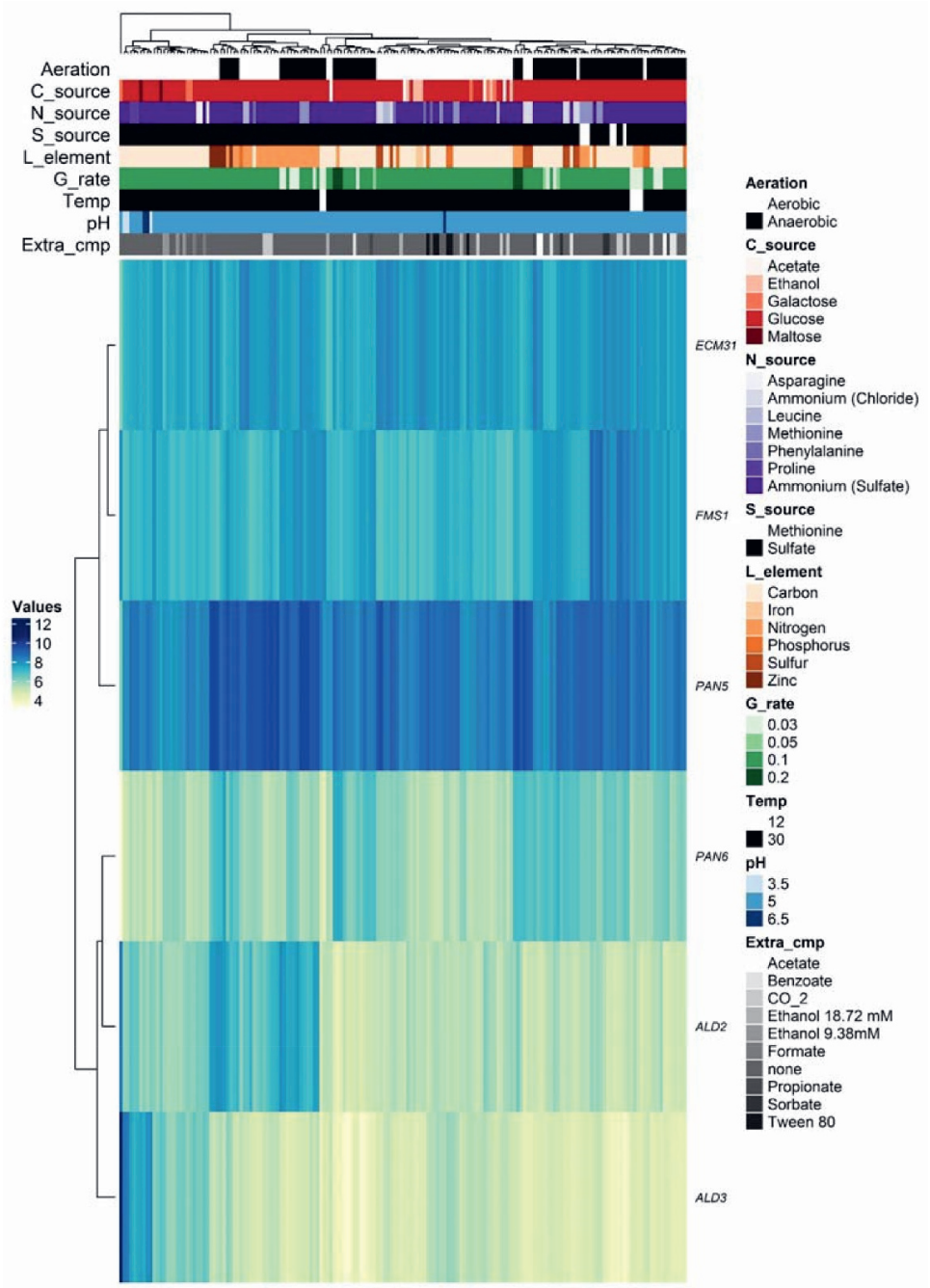

Figure 1.4 | Heatmap showing mRNA levels for pantothenate biosynthetic genes measured under 70 different conditions in chemostat cultures. Each row shows a gene involved in *de novo* pantothenate biosynthesis while each column represents one condition. Data are derived from [166, 167] and code for generating this plot is available at https://gitlab.tudelft.nl/rortizmerino/sacch_vitamins.

In comparison with the regulation of the other biosynthetic pathways discussed in this review, regulation of pantothenate acid biosynthesis in *S. cerevisiae* has not been intensively studied and, therefore, is still incompletely understood. Expression of *ECM31* and *PAN6* was shown to be low, constitutive and unaffected by extracellular pantothenate concentrations [165], while transcript levels of the pantothenic acid biosynthetic genes (*ECM31, PAN5, FMS1, ALD2, ALD3* and *PAN6*) across 70 different culture conditions [166, 167] (Figure 1.4) did not reveal indications for co-regulation.

para-Aminobenzoic acid (B_{10})

Para-aminobenzoic acid (*p*ABA), also known as vitamin B_{10}, is a water-soluble B complex vitamin. It was discovered in 1920's [168, 169]. A temporary sunscreen application after the WW2 was soon withdrawn as it caused dermatitis and auto-immune responses [170, 171]. *p*ABA is an important intermediate in the biosynthesis of folates, a class of co-factors involved in transfer of C1-units in nucleic acid and amino-acid metabolism, as well as in ubiquinone biosynthesis [172, 173]. Folates result from the association of three precursors *p*ABA (B_{10}), GTP and glutamate, out of which *p*ABA is the less abundant intracellularly and limit folic acid (B_9) synthesis. Additionally, growth deficiency in the presence of *p*ABA and absence of folic acid has not been reported before, making this vitamin dispensable for CDMY.

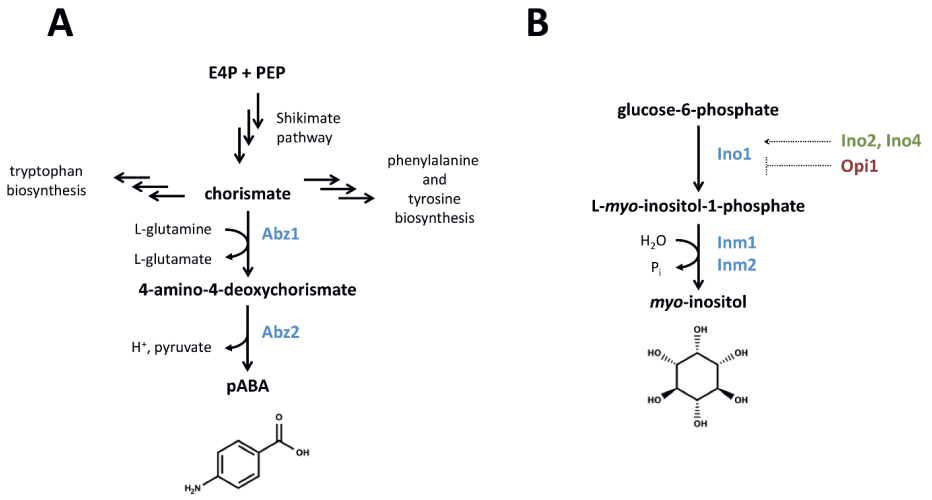

Figure 1.5 | *para*-Aminobenzoic acid (*p*ABA) and *myo*-inositol *de novo* synthesis pathways in *S. cerevisiae*. (A) The genes *ABZ1* and *ABZ2* code for a two-step pathway producing *p*ABA from chorismate via the intermediate 4-amino-4-deoxychorismate. Chorismate is synthesized from erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) via the shikimate pathway. In addition to being precursor for *p*ABA biosynthesis, chorismate also serves as precursor for tryptophan, phenylalanine and tyrosine biosynthesis. (B) *myo*-Inositol is formed from glucose-6-phosphate via Ino1 yielding L-*myo*-inositol-1-phosphate, which is in a second step converted to *myo*-inositol by Inm1 or Inm2. *INO2, INO4* genes encode *INO1* transcriptional activators while *OPI1* encodes the antagonist regulator of the gene encoding the initial step of inositol synthesis (*INO1*). Metabolites, proteins, positive regulators and positive regulators are shown in bold, blue, green and red respectively.

In *S. cerevisiae*, *p*ABA biosynthesis starts from chorismate which, as indicated by its name (χωρίζω; to separate) is located at the intersection of the biosynthesis of i) tyrosine and phenylalanine, ii) tryptophan, and iii) *p*ABA and folates. Conversion of chorismate in to *p*ABA involves two enzyme reactions (Figure 1.5A). First, amino-deoxy-chorismate synthase (Abz1) uses glutamine as amino donor to produce 4-amino-4-deoxy-chorismate. Subsequently, amino-deoxy-chorismate lyase (Abz2) removes the pyruvate moiety of chorismate, resulting in *p*ABA [174]. Chorismate is a key intermediate of the shikimate pathway for aromatic amino-acid biosynthesis. The shikimate pathway is tightly regulated, not only transcriptionally but also by allosteric feedback regulation of its first committed enzyme, 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase. *S. cerevisiae* contains two isoenzymes of DAHP, Aro3 and Aro4, which are feedback inhibited by phenylalanine and tyrosine, respectively [175, 176]. This regulation ensures that intracellular chorismate availability is strongly influenced by aromatic amino-acid concentrations. *ABZ1* and *ABZ2*, which encode the key enzymes of the *p*ABA pathway, are transcribed constitutively [177], suggesting that any regulation of *p*ABA biosynthesis occurs is post-transcriptional. Rates of fermentation and nitrogen assimilation of *S. cerevisiae* strains have been correlated with specific alleles of *ABZ1*, thereby linking *p*ABA synthesis to overall strain performance [177, 178]. This genetic heterogeneity has been exploited to engineer *S. cerevisiae* for *p*ABA production by overexpressing *ABZ1-2* alleles from wine strains that encode highly active enzymes [179].

Inositol (B_8)

Of the seven organic supplements that are added to commonly used CDMY, only inositol (Table 1.1) is not a cofactor or cofactor precursor. First isolated in 1928 [180], inositol is a polyol (cyclohexane-1,2,3,4,5,6-hexol) that serves as precursor for phosphatidylinositol (PI), a main constituent of phospholipid membranes [181]. Upon its cleavage into inositol phosphate (IP) and diacylglycerol by phospholipase C, PI also plays a central role in inositol-phosphate signalling [182]. Moreover, inositol is a precursor for the synthesis of glycosylphosphatidylinositol (GPI) anchor proteins [183].

Myo-inositol is physiologically the most common stereoisomer among the eight possible inositol enantiomers. In organisms capable of synthesizing *myo*-inositol, it is formed from glucose-6-phosphate via two enzyme-catalysed reactions. The genes involved in the *S. cerevisiae* inositol biosynthesis pathway were discovered by complementation of inositol-requiring mutants [184] (Figure 1.5B). First, L-*myo*-inositol 1-phosphate (IP) is generated from glucose-6-phosphate by L-*myo*-inositol 1-phosphate synthase (Ino1) [185]. Subsequently, *myo*-inositol is generated by dephosphorylation of L-*myo*-inositol 1-phosphate by the heterodimeric enzyme inositol 3-phosphate monophosphatase (Inm1/Inm2) [186] Lipid metabolism in eukaryotic cells, including yeasts, is rigorously regulated. Yeast cells continuously monitor lipid status and quickly respond to alterations by a dual regulatory control. Many insights into how the yeast cells regulate their phospholipid metabolism derive from research on regulatory

responses to variations in the inositol content of growth media [187]. Analysis of inositol-auxotrophic *S. cerevisiae* strains enabled discovery of *INO2* and *INO4*, which encode positive transcriptional regulators for *INO1* and a large number of other genes involved in phospholipid synthesis [188]. A key negative feedback mechanism for transcriptional regulation was discovered by the characterization of mutants able to secrete inositol, a phenotype also referred to as the Opi$^-$ phenotype [189]. The transcriptional factor Opi1 was shown to act as a negative regulator in the presence of inositol, with some mutations in *OPI1* resulting in constitutive *INO1* expression. The Opi$^-$ phenotype has also been involved in identifying other *S. cerevisiae* genes in phospholipid biosynthesis, transcription, protein processing, and trafficking [190].

Nicotinic acid (B_3)

Nicotinic acid, also known as niacin, was first isolated from liver in 1937 and was identified as "pellagra-preventing factor" and "anti-black tongue factor" [191]. Together with nicotinamide, it makes up the vitamin B_3 complex. Nicotinic acid is an important precursor for the essential redox cofactors nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). *S. cerevisiae* can either obtain NAD^+ from *de novo* biosynthesis or from salvage routes that regenerate NAD^+ from its nicotinamide degradation products [192, 193] (Figure 1.6). These pathways converge at the level of nicotinic acid mononucleotide (NaMN) and share the last two reactions towards NAD^+ formation.

In the *de novo* biosynthesis pathway, NaMN is synthesized from L-tryptophan in a series of six enzymatic reactions (catalysed by Bna1-2 and Bna4-7) and one spontaneous reaction (Figure 1.6). Three of the enzymes involved in the *de novo* biosynthesis pathway, indoleamine 2,3 dioxygenase (Bna2), kynurenine 3-monooxygenase (Bna4), and 3-hydroxyanthranilate 3,4-dioxygenase (Bna1), require molecular oxygen as a substrate, thereby explaining the strict requirement of anaerobic *S. cerevisiae* cultures for nicotinic acid supplementation [70]. In the salvage pathway, nicotinamide and nicotinic acid are converted to NaMN via the so-called Preiss-Handler pathway I [194, 195] which involves Pnc1 and Npti1 as key enzymes. Extracellular nicotinic acid can be imported into yeast cells by the plasma-membrane transporter Tna1 and then used to form NAD^+ through the salvage pathway [196, 197].

In yeast, there are other four additional pathways for NAD^+ biosynthesis: two salvage pathways from nicotinamide riboside (NR) and two salvage pathways from nicotinic acid riboside (NaR) [198-200]. Three of these salvage pathways converge first with the Preiss-Handler NAD^+ salvage pathway and then with the *de novo* NAD pathway (Figure 1.6). In the NR salvage pathway I. which is not connected to the other pathways, NR is first phosphorylated to NMN by the Nrk1 kinase and then adenylated to NAD^+ by Nma1 or Nma2 (Figure 1.6).

NAD^+ and $NADP^+$ are essential redox cofactors for many oxido-reductases [201]. In addition to its role as a redox co-factor, NAD^+ is a substrate for several enzymes in yeast including sirtuin protein deacetylases (Sir2, Hst1-4) and cyclic ADP-ribose (cADPR) synthases (Tpt1) [202, 203]. These enzymes have important roles in the maintenance and regulation of chromatin structure, calcium signalling, life-span and DNA repair [193, 204-207]. NAD^+ is also a precursor for $NADP^+$ which, like NAD^+ is involved in many cellular redox reactions [208]. Intracellular NAD^+ levels are controlled by a complex regulation network. Hst1 (Homologue of Sir2) acts as a NAD^+ sensor that represses *BNA* genes when NAD^+ is abundant. Hst1 does not bind the DNA directly but interacts with Rfm1 and Sum1 to form a repressor complex.

Mac1, which was previously characterized as a copper-sensing transcription factor, has been shown to also be involved in regulation of *BNA* genes, together with Hst1 [192, 209-211]. When NA is abundantly available, NA salvage metabolism is preferred over use of the *de novo* biosynthetic pathway, which is repressed by Hst1 [192, 211]. In *S. cerevisiae*, NAD^+ metabolism is regulated together with phosphate and purine nucleotide metabolism, although the exact mechanisms remain uncharacterised [212, 213]. NR can be produced and stored in vacuoles and then released into the cytosol by the Fun26 transporter, thereby enabling cells to feed NR stores into NAD^+ synthesis [214, 215].

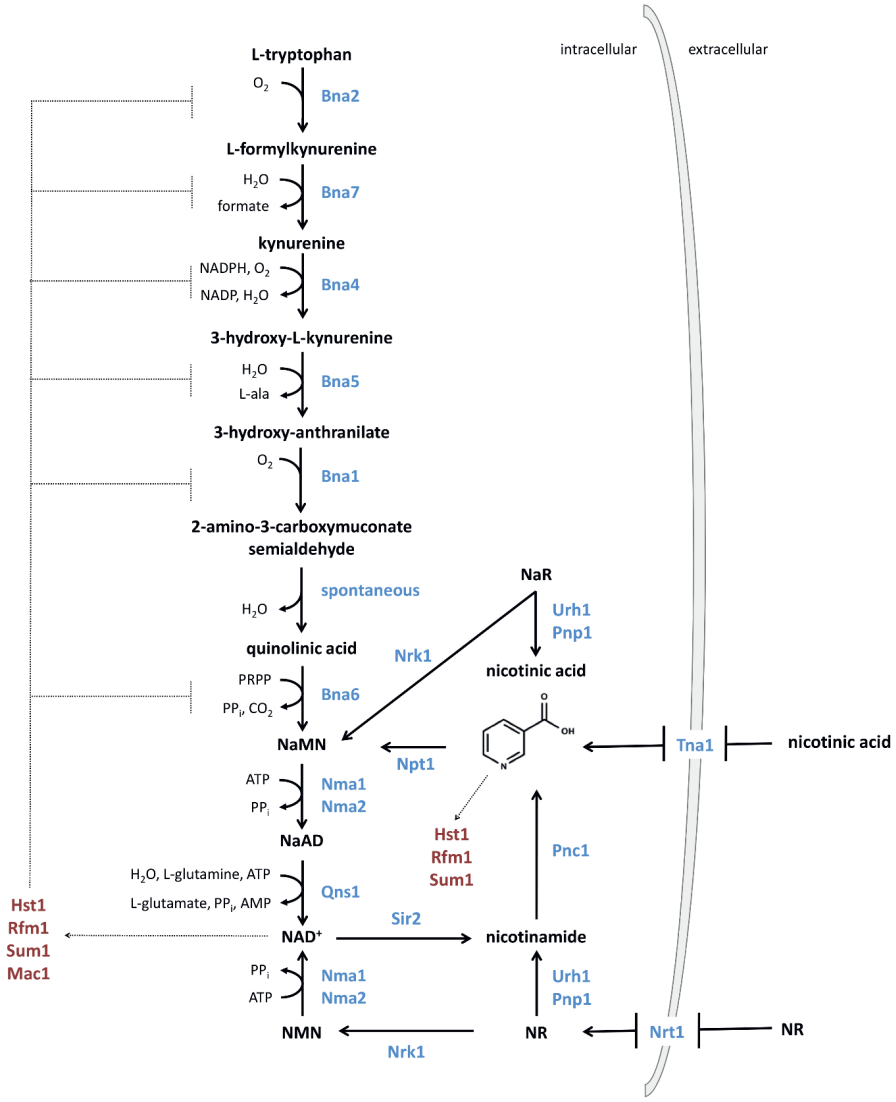

Figure 1.6 | Nicotinic acid *de novo* synthesis and salvage pathway in *S. cerevisiae*. Nicotinamide adenine dinucleotide (NAD^+) is *de novo* synthesized from L-tryptophan in nine catalytic steps involving the Bna enzyme family and enzymes Nma1, Nma2 and Qns1. Nicotinic acid can be imported into the cell via Tna1 and enters the NAD synthesis pathway as nicotinic acid mononucleotide (NaMN) by catalytic activity of Npt1. Similarly, nicotinamide riboside (NaR) can be salvaged by catalytic activity of Nrk1 to form NaMN. NaR can be also converted to nicotinic acid by Urh1 and Pnp1. Nrk1 also converts nicotinamide riboside (NR) into nicotinamide nucleotide (NMN) subsequently converted to NAD^+ by Nma1 and Nma2. NR is imported by activity of Nrt1 transporter and might be used by Pnp1 or Urh1 to form nicotinamide. Alternatively, nicotinamide can be synthesized via Sir2 from NAD^+. Pnc1 uses nicotinamide to form nicotinic acid. The regulators Hst1 (with aid of Rfm1 and Sum1) and Mac1 repress the expression of genes encoding Bna enzymes upon binding to NAD^+ and nicotinic acid. Metabolites, proteins and negative regulators are shown in bold, blue and red respectively.

Systematic search for components of the class B vitamin biosynthesis pathways in Saccharomyces species

Although strain-to-strain differences occur, the *S. cerevisiae* pan-genome harbour all necessary genetic information to synthesize inositol, biotin, thiamine, nicotinic acid, pantothenate, pyridoxine and *p*ABA. Since the work of Burkholder, McVeigh and Moyer in 1944, no systematic analysis has been performed to assess growth factor requirements of different species within the *Saccharomyces* genus. To explore this issue, we screened the genomes of the type strains of *Saccharomyces* species for annotated sequences homologous to the structural genes encoding enzymes involved in biosynthesis of class B vitamins in *S. cerevisiae* [216]. Based on this screen, the genomes of most *Saccharomyces* type species encode complete biosynthetic pathways for these compounds (Figure 1.7). Two notable exceptions are *S. arboricola*, which misses key genes required for biosynthesis of pyridoxine, thiamine, and biotin (*SNO2/3*, *SNZ2/3*, *THI5-13*, *BIO1*) and *S. kudriavzevii* which lacks genes involved in biosynthesis of pyridoxine, pantothenate, *p*ABA and inositol (*SNO1, FMS1, PAN6, ABZ1/2, INO1*). Absence of *SNO2/3* in *S. paradoxus* should not compromise its pyridoxine prototrophy as its genome does harbour the main paralog *SNO1*.

Some *Saccharomyces* species show higher copy numbers for individual vitamin biosynthesis genes than *S. cerevisiae*. In particular, *S. jurei* harbours additional copies of *SNO2/3, SNZ2/3, THI5* and *THI11-13*, while *S. paradoxus* carries two copies of *BIO1* and *BIO6*. These genes are all located in subtelomeric regions in *S. cerevisiae*. Subtelomeric regions are known hotspots for genetic plasticity that contain many gene families involved in interaction between the cell and its environment [217]. Assuming conserved synteny within the *Saccharomyces* genus, these gene amplifications may therefore reflect evolutionary adaptations to the environmental conditions these different species were exposed to.

Table 1.3 | *Saccharomyces* species involved in a comparative genome analysis of the presence of vitamin biosynthesis genes.

TaxID	Species	Strain	Other Identifiers	Reference	Biosample
1080349	*S. eubayanus*	CBS12357^T	NBRC111513^T	[219]	NA
226127	*S. uvarum*	CBS7001^T	MCYC623^T	[220]	SAMN13069661
1160507	*S. arboricola*	CBS10644^T	H-6^T	[221]	SAMN13069660
226230	*S. kudriavzevii*	CBS8840^T	IFO1802^T	[220]	NA
226126	*S. mikatae*	CBS8839^T	IFO1815^T	[220]	SAMN13069662
1987369	*S. jurei*	CBS14759^T	NCYC3947^T	[222]	SAMN13069663
226125	*S. paradoxus*	CBS432^T	NRRLY-17217^T	[223]	SAMN13069659
559292	*S. cerevisiae*	S288C	CBS8803	[150]	NA
889517	*S. cerevisiae*	CEN.PK113-7D	CBS8340	[224]	SAMN13069664

Biosamples can be accessed under bioproject accession PRJNA578688 (https://www.ncbi.nlm.nih.gov/bioproject)

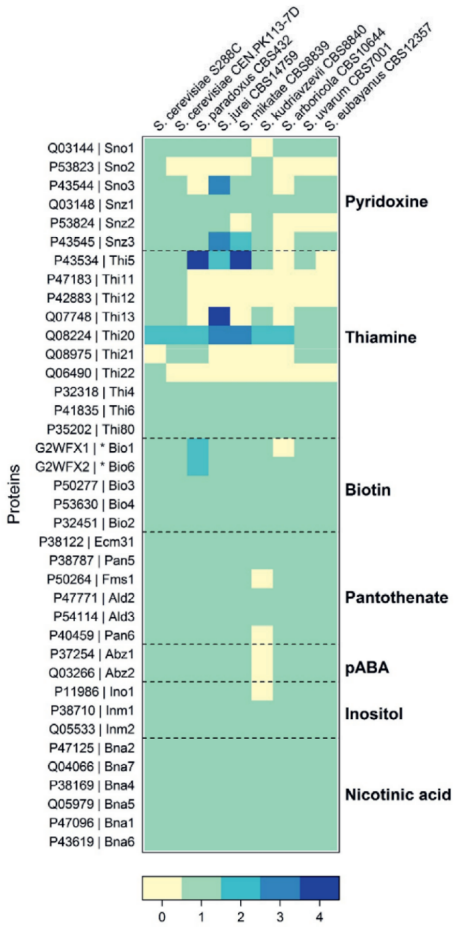

Figure 1.7 | Occurrence of vitamin biosynthesis genus in *Saccharomyces* species. A homology search was conducted using HMMER v3 [218] with *S. cerevisiae* S288C proteins as queries (left side row names) against a database of annotated proteins from the *Saccharomyces* species listed in the column headers. For Bio1 and Bio6, *S. cerevisiae* K7 proteins were used as queries (indicated with *) since S288C is known to lack such proteins. Available genome annotations from species in the monophyletic *Saccharomyces* clade (formerly known as *sensu stricto*; Table 1.3) were used to build a protein sequence database. Besides *S. cerevisiae* S288C and CEN.PK113-7D, sequences in the database belong to type strains. This database was then searched for sequence homologs using the queries listed on the left-hand side. Queries are grouped and labelled on the right-hand side and depending on the biosynthetic pathway they are involved in. Boxes are coloured depending on the number of hits (e-value > 1E-5, percentage of alignment > 75%) obtained by each query on each strain. The colour code is shown at the bottom. Hits from queries belonging to the same biosynthetic pathway were ranked according to lowest e-value, then highest percentage of alignment and best hits were uniquely assigned to each query (i.e. a sequence considered as best hit is never used more than once and best hits with a count > 1 are all identical). This last step accounts for the presence of paralogs and the high level of similarity between proteins in the same pathway, especially in the pyridoxine and thiamine pathways (see Thi5 and Thi20 for instance). Code for this search is available in https://gitlab.tudelft.nl/rortizmerino/sacch_vitamins and sequences are deposited under BioProject accession PRJNA578688 as indicated in Table 1.3.

Scope and outline of this thesis

Although strain-to-strain differences do occur, the genomes of most *S. cerevisiae* strains harbour all genetic information required for synthesis of inositol, biotin, thiamine, nicotinic acid, pantothenate, pyridoxine and *p*ABA. However, since endogenous synthesis does not always meet the demands for fast growth and high product titres, yeast cultivation in research and industry often involves supplementation of these 'vitamins' to growth media. The goals of the research described in this thesis were to gain a deeper understanding of the biochemistry and genetics of vitamin metabolism in *S. cerevisiae* and, based on this understanding, to design and test metabolic engineering strategies for construction of fully vitamin-independent yeast strains.

Biotin is one of the most expensive among B-type vitamins that are routinely added to synthetic media for cultivation of yeasts and the pathway for its *de novo* synthesis by yeasts remains incompletely understood. In **Chapter 2**, yeast biodiversity was explored in an attempt to address a bottleneck in biotin synthesis by *S. cerevisiae* that had been identified in earlier laboratory evolution experiments. Based on expression of different orthologs of *S. cerevisiae* Bio1 in a *bio1Δ* genetic background, a new and straightforward metabolic engineering strategy for conferring full biotin prototrophy was designed and tested in laboratory and industrial *S. cerevisiae* strains. In addition to this application-inspired goal, the research described in **Chapter 3** also provided a new insight into the role of oxygen in biotin synthesis by yeast.

While Chapter 2 outlines a strategy for conferring aerobic biotin prototrophy to laboratory and industrial *S. cerevisiae* strains, it did not offer a perspective for eliminating a complete biotin auxotrophy of anaerobic cultures of this yeast. Therefore, Chapter 3 explored a metabolic engineering strategy in which an entire bacterial pathway, involving ten different bacterial genes, for conversion of malonyl-CoA into the biotin-biosynthesis intermediate KAPA, was introduced into *S. cerevisiae*. In addition to CRISPR-based genome editing and *in vivo* assembly of the expression cassettes required for expression of the heterologous pathway, laboratory evolution and reverse engineering of causal mutations were applied to identify and reverse causal mutations for fast anaerobic, biotin-independent growth.

Chapter 4 addresses the ambitious goal to construct an *S. cerevisiae* platform that is fully prototrophic for all B-type vitamins that are routinely added to synthetic media for aerobic cultivation of this yeast. To this end, the strategy for eliminating the biotin requirement of aerobic *S. cerevisiae* culture was combined with similar strategies focused on other vitamins, which were inspired by a separate study [225] performed in the context of the same European Union-funded project (PAcMEN) in which the present PhD project was embedded.

Chapter 2 |

Exploiting the diversity of Saccharomycotina yeasts to engineer biotin-independent growth of *Saccharomyces cerevisiae*

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Abstract

Biotin, an important co-factor for carboxylases, is essential for all kingdoms of life. Since native biotin synthesis does not always suffice for fast growth and product formation, microbial cultivation in research and industry often requires supplementation of biotin. *De novo* biotin biosynthesis in yeasts is not fully understood, which hinders attempts to optimize the pathway in these industrially relevant microorganisms. Previous work based on laboratory evolution of *Saccharomyces cerevisiae* for biotin prototrophy identified Bio1, whose catalytic function remains unresolved, as a bottleneck in biotin synthesis. This study aimed at eliminating this bottleneck in the *S. cerevisiae* laboratory strain CEN.PK113-7D. A screening of 35 Saccharomycotina yeasts identified six species that grew fast without biotin supplementation. Overexpression of the *ScBIO1* ortholog isolated from one of these biotin prototrophs, *Cyberlindnera fabianii,* enabled fast growth of strain CEN.PK113-7D in biotin-free medium. Similar results were obtained by single overexpression of *CfBIO1* in other laboratory and industrial *S. cerevisiae* strains. However, biotin prototrophy was restricted to aerobic conditions, probably reflecting the involvement of oxygen in the reaction catalysed by the putative oxidoreductase *Cf*Bio1. In aerobic cultures on biotin-free medium, *S. cerevisiae* strains expressing *Cf*Bio1 showed a decreased susceptibility to contamination by biotin-auxotrophic *S. cerevisiae*. This study illustrates how the vast Saccharomycotina genomic resource may be used to improve physiological characteristics of industrially relevant *S. cerevisiae*.

Importance

The reported metabolic engineering strategy to enable optimal growth in the absence of biotin is of direct relevance for large-scale industrial applications of *S. cerevisiae*. Important benefits of biotin prototrophy include cost reduction during preparation of chemically-defined industrial growth media, as well as a lower susceptibility of biotin-prototrophic strains to contamination by auxotrophic microorganisms. The observed oxygen dependency of biotin synthesis by the engineered strains is relevant for further studies on the elucidation of fungal biotin biosynthesis pathways.

Introduction

Biotin is a class B vitamin (B_7) and an essential co-factor for higher eukaryotes such as mammals and birds, which have to acquire it from their diet [226]. In contrast, most prokaryotes, plants and some fungi can synthesize biotin [227]. In *S. cerevisiae*, biotin serves as an important co-factor for enzymes catalysing carbon-dioxide transfers: the cytosolic (Acc1) and mitochondrial (Hfa1) acetyl-CoA carboxylases generating malonyl-CoA for fatty acid synthesis [228] as well as the pyruvate carboxylases (Pyc1,2) responsible for anaplerotic formation of oxaloacetate [141]. In addition, biotin is involved in the reaction catalysed by urea amidolyase (Dur1,2), which releases ammonia and carbon dioxide from urea [142]. The only biotin-dependent protein in *S. cerevisiae* that is not a carboxylase is Arc1, which is involved in tRNA aminoacylation [229].

A fast, widely applicable metabolic engineering strategy for construction of fully biotin-prototrophic yeast strains has significant economic and technological relevance. Omission of biotin from media formulations would enable design of cheaper, easier to handle media with a longer shelf life. Such advantages have been reported for heterologous protein production with a *Komagatella phaffi* (formerly known as *Pichia pastoris*) strain expressing the *S. cerevisiae* biotin biosynthesis genes *BIO1* and *BIO6*. However, this biotin-prototrophic strain exhibited slower growth in the absence of biotin than in its presence [230]. Additionally, use of fully biotin-prototrophic strains of yeast in fermentation processes that do not contain biotin may reduce the impact of contamination with 'wild' yeast or even bacteria strains that grow slower or not at all in the absence of this cofactor [231-233].

The genomes of most *S. cerevisiae* strains carry all genes necessary to encode all known enzymes in the yeast biotin synthesis pathway. In some cases, *S. cerevisiae* strains lack one or more biosynthetic genes, resulting in a full biotin auxotrophy (e.g., the reference strain S288C misses both *BIO1* and *BIO6*). In the intensively studied *S. cerevisiae* strain CEN.PK113-7D [234-236], all biotin pathway genes are present [151]. Nevertheless, cultivation of this strain on synthetic medium devoid of biotin results in a much lower specific growth rate than cultivation on biotin-containing medium [151, 237]. Growth defects in absence of biotin were observed for several *S. cerevisiae* strains as well as for other budding yeasts. The extent to which growth was impaired was hypothesized to be dependent on the *BIO1-BIO6* gene cluster copy number of the specific strain [149]. Evolutionary engineering of strain CEN.PK113-7D for biotin-independent growth enabled the selection of mutants whose growth in biotin-free media was as fast as growth of the reference strain in the presence of biotin. This evolved phenotype was linked to a 20- to 40-fold amplification of the *BIO1-BIO6* gene cluster originally located on chromosome I. Karyotyping and genome sequencing revealed that extensive chromosomal rearrangements and formation of neo-chromosomes contributed to this amplification [154]. Although these experiments showed that fast biotin-independent growth of *S. cerevisiae* is possible, the responsible

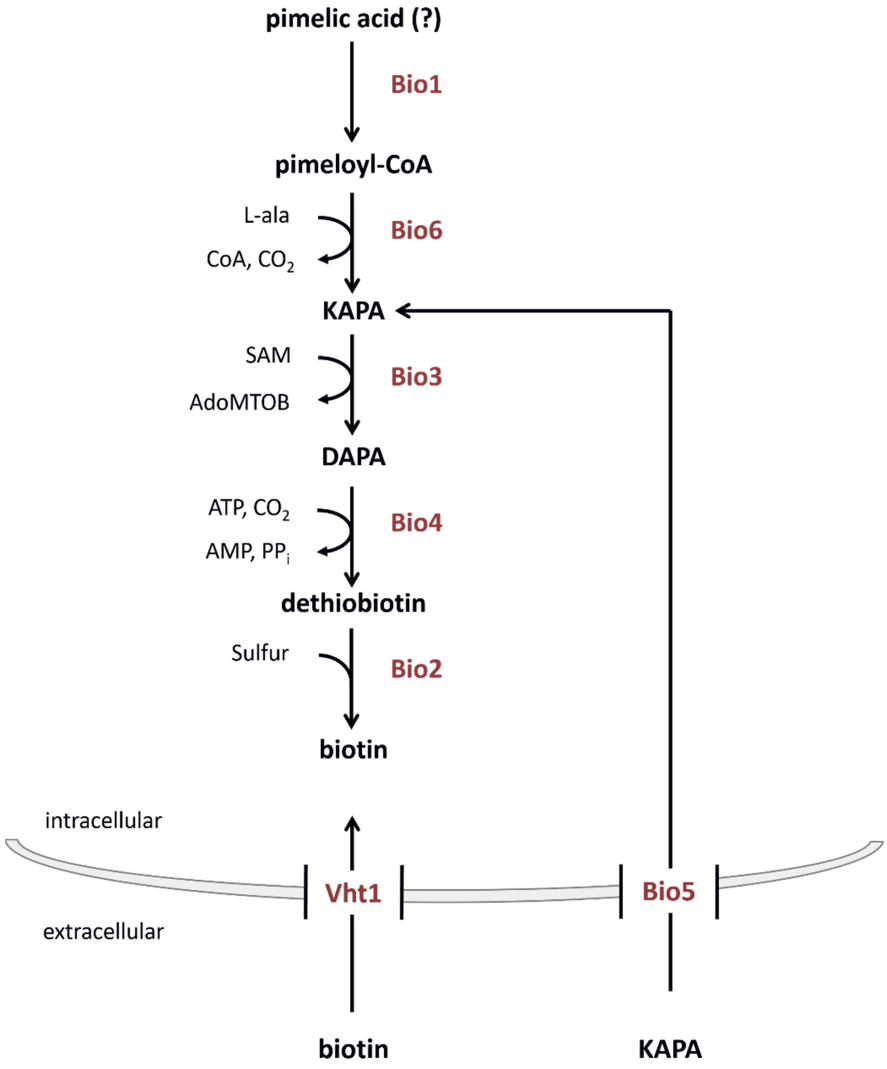

Figure 2.1 | **Yeast biotin biosynthesis pathway.** The origin of pimelic acid, the substrate of the first reaction catalysed by Bio1 is unknown and yields pimeloyl-CoA, which is further metabolized to 7-*keto*-8-aminopelargonic acid (KAPA), 7,8-diaminopelargonic acid (DAPA), dethiobiotin and biotin by the action of the enzymes Bio6, Bio3, Bio4 and Bio2, respectively. Biotin can be imported into the cell via Vht1. The intermediate KAPA can be imported by Bio5.

mutations could not be easily transferred to other strains. Attempts to overexpress either the *BIO1-BIO6* cluster or *BIO1* alone only yielded a partial growth improvement in biotin-free media. This partial success in reverse engineering of biotin prototrophy might indicate that additional mutations in genes responsible for intracellular transport or production of biotin-synthesis intermediates are necessary. Furthermore, codon-usage of *BIO* genes might be suboptimal, since these genes have been proposed to be acquired by horizontal gene transfer from bacteria [149]. To date the only option

to obtain full biotin prototrophy in *S. cerevisiae* is to perform laboratory evolution experiments which are time consuming and, moreover, lead to massive chromosomal rearrangements that might result in mutations that could negatively affect other cellular functions [238].

The genetic information required for biosynthesis of the fused-ring structure of biotin (catalysed by Bio3, Bio4 and Bio2) is well characterized in *S. cerevisiae* ([50, 239], Figure 2.1). In contrast, the origin of the pimeloyl moiety contributing to the valeric-acid chain remains elusive (Figure 2.1) [240]. Hitherto, *ScBIO1* was hypothesised to act as a CoA-ligase that activates free pimelic acid for biotin synthesis, similar to the enzyme encoded by *bioW* in *Bacillus subtilis* [155]. The hypothetical product of such a ligase, pimeloyl-CoA, could be converted into 8-amino-7-oxononanoate by yeast KAPA synthase (Bio6) using L-alanine and pyridoxal-5-phosphate. However, no evidence is available for utilization of free pimelic acid by yeast. The lack of knowledge on the origin of the pimeloyl moiety in *S. cerevisiae* has so far hindered further targeted molecular improvement of biotin synthesis.

The aim of this study was to explore new metabolic engineering solutions to enable fast, unimpaired growth of *S. cerevisiae* without biotin supplementation. To this end, a set of Saccharomycotina yeasts were screened for specific growth rate in biotin-free synthetic medium lacking biotin. After identification of the best-performing species, the genome sequences of these yeasts were interrogated for *ScBIO1* orthologs. The identified orthologous genes were functionally characterized in *S. cerevisiae* CEN.PK113-7D and evaluated for their ability to support fast growth in the absence of biotin, under aerobic as well as under anaerobic conditions. A promising candidate gene was expressed in several industrial and laboratory *S. cerevisiae* strains and its impact was evaluated. Furthermore, it was investigated how cultivation of an engineered biotin-prototrophic *S. cerevisiae* strain on biotin-free medium affected the impact of an intentional contamination with an auxotrophic strain.

Results

Characterization of biotin requirement in Saccharomycotina yeasts

Determination of nutritional requirements is a standard procedure in classical yeast taxonomy. While biotin requirements of individual species are reported in the yeast taxonomy reference book "The Yeasts" [241], this information is only qualitative. To capture quantitative information on biotin requirements, we determined the specific growth rates of a set of 35 biotechnologically relevant chosen yeasts belonging to the Saccharomycotina sub-phylum in biotin-free synthetic medium (biotin-free SMD). Of these 35 species, eleven exhibited growth, albeit at different rates. We focused further analysis on fast-growing species, based on an arbitrary threshold specific growth rate of 0.25 h^{-1}. Only strains belonging to the species *Yarrowia lipolytica*, *Pichia kudriavzevii* (*syn. Candida krusei* and *Issatchenkia orientalis*) [242], *Wickerhamomyces ciferrii*,

Cyberlindnera fabianii (*syn. Candida fabianii*), *Lachancea kluyveri* and *Torulaspora delbrueckii* met this criterion, with specific growth rates in biotin-free SMD ranging from 0.27 to 0.64 h^{-1}. Specific growth rates of *Y. lipolytica*, *P. kudriavzevii* and *C. fabianii* were not significantly higher in biotin-supplemented SMD. The other strains all showed lower specific growth rates in biotin-free media than in biotin-supplemented media, however not below 60% of the growth rate on SMD supplemented with biotin (Figure 2.2). Consistent with earlier observations [149, 151, 154], *S. cerevisiae* strain CEN.PK113-7D exhibited a specific growth rate of 0.39 ± 0.01 h^{-1} on biotin-supplemented SMD, but grew extremely slowly ($\mu < 0.01$ h^{-1}) on biotin-free SMD.

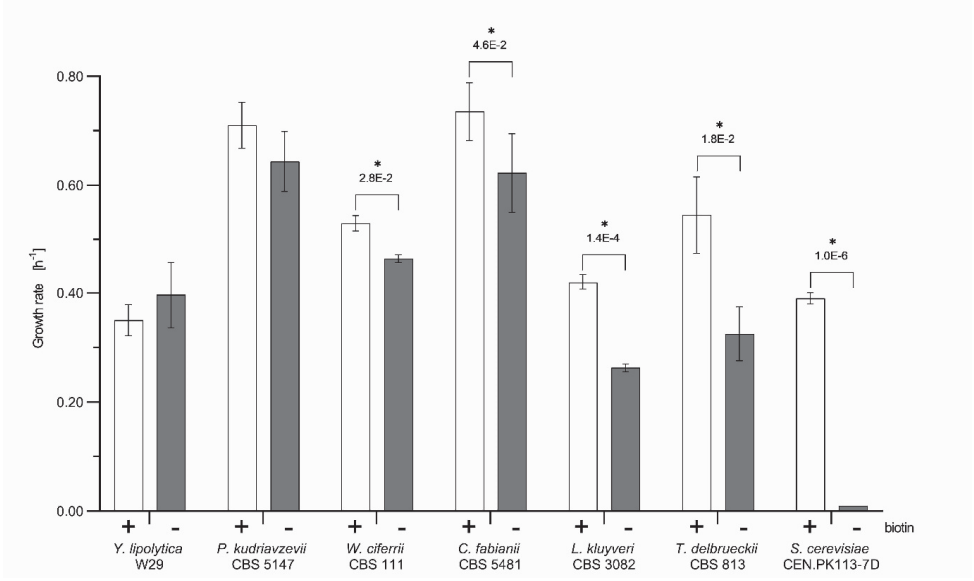

Figure 2.2 | Specific growth rates of Saccharomycotina species in SMD (+/white) and biotin-free SMD (-/grey). The bars represent average and standard deviation of three biological replicates. The data are derived from culture optical density (OD_{660}) measurements over time during the exponential growth phase of shake flask batch cultures of *Y. lypolytica* W29, *P. kudriavzevii* CBS 5147, *W. ciferrii* CBS 111, *C. fabianii* CBS 5481, *L. kluyveri* CBS 3082, *T. delbrueckii* CBS 813 and *S. cerevisiae* CEN.PK113-7D. Statistical significance between growth rates in SMD and biotin-free SMD of each strain was determined using the Holm-Sidak method. *denotes comparison with p_{value} lower than 5.0E-2.

Identification of putative *ScBIO1* orthologs in *Y. lipolytica*, *P. kudriavzevii*, *W. ciferrii*, *C. fabianii*, *L. kluyveri* and *T. delbrueckii*.

The growth performance of *Y. lipolytica, P. kudriavzevii, W. ciferrii*, *C. fabianii, L. kluyveri* and *T. delbrueckii* in biotin-free SMD indicated that the Bio1 'bottleneck' in the biotin biosynthesis pathway of *S. cerevisiae* CEN.PK113-7D was absent in these species. We therefore hypothesized that biotin prototrophy of these strains might be enabled by *ScBIO1* orthologs encoding enzymes with superior catalytic or regulatory properties.

Identification of putative *ScBIO1* orthologs was performed using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [243], more specifically tBLASTn, which uses a protein sequence as query against translation of a nucleotide database. Use of *Sc*Bio1 as a query revealed candidate proteins in five out of the six species (*P. kudriavzevii*, *W. ciferri*, *L. kluyveri*, *T. delbrueckii* and *C. fabianii*) but not in *Yarrowia lipolytica* (Table 2.1). Subsequently, the search was repeated with the *ScBIO1* orthologs found in *P. kudriavzevii*, *W. ciferri*, *L. kluyveri*, *T. delbrueckii* and *C. fabianii* as queries and using the *Y. lipolytica* W29 genome assembly as a database (PRJNA601425). Each five searches identified the same 346-amino-acids peptide sequence encoded by open reading frame YALI0A10010p (Table 2.1). Pair-wise comparisons of the identified putative Bio1 amino acid sequences using ClustalΩ [244] showed that the sequences of *Lk*Bio1 and *Td*Bio1 showed the highest amino-acid sequence similarities to the *Sc*Bio1 sequence (43-44%), while *Yl*Bio1 had the lowest similarity to the other homologs. Although its size matched perfectly with *Sc*Bio1, *Yl*Bio1 only showed 15% to 20% amino-acid sequence similarity with the *Sc*Bio1 orthologous peptides (Figure 2.3). Out of this set of putative Bio1 homologs *Cf*Bio1 and *Wc*Bio1 exhibited the highest pair-wise amino-acid similarity (62%), which is in line with the phylogenetic proximity of these yeast species [241].

Table 2.1 | Identification of heterologous *BIO1* gene by basic local alignment search tool tBLASTn, best hits for querying *Sc*Bio1 protein sequence[a], or *Td*, *Lk*, *Cf*, *Wc* and *Pk*Bio1[b].

Species	taxon ID (NCBI)	Accession N°	Sequence	Open reading frame coordinates
T. delbrueckii	txid1076872	[a]HE616748.1	Chromosome 7	18080-19111 bp
L. kluyveri	txid4934	[a]AACE03000004.1	Chromosome D SKLU-Cont10108	1095811-1094765 bp
C. fabianii	txid36022	[a]BCGI01000001.1	Scaffold 0	1136478-1137572 bp
W. ciferrii	txid1041607	[a]CAIF01000264.1	Contig 00264	39352-40449 bp
P. kudriavzevii	txid4909	[a]CP028531.1	Chromosome 1	1085523-1084447 bp
Y. lypolytica	txid284591	[b]NC_006067.1	Chromosome A	1097298-1096261 bp

To investigate whether expression of the *ScBIO1* orthologs was able to promote growth of *S. cerevisiae* CEN.PK113-7D in biotin-free medium, each *BIO1* gene was isolated from its respective genome and cloned under the transcriptional control of the *S. cerevisiae PYK1* promoter (*ScPYK1*p*)* and the *S. cerevisiae BIO1* terminator (*ScBIO1*t). Each expression module was integrated at the chromosomal *SGA1* locus of the *Spycas9*-expressing, CEN.PK-derived *S. cerevisiae* strain IMX585 by CRISPR/Cas9-mediated genome editing [245]. The resulting strains IMX1862 (*YlBIO1*↑), IMX1861 (*PkBIO1*↑), IMX1863 (*WcBIO1*↑), IMX1859 (*CfBIO1*↑), IMX1858 (*LkBIO1*↑), and IMX1857 (*TdBIO1*↑) and the control strain IMX1511 (*ScBIO1*↑*)* expressing the *S. cerevisiae BIO1* gene under the same regulatory sequences as the heterologous *BIO1* genes, were grown on biotin-containing and biotin-free SMD.

Expression of the *BIO1* ortholog of *C. fabianii* enables fast growth of *S. cerevisiae* in biotin-free medium.

All strains expressing a heterologous *BIO1* ortholog grew on biotin-containing SMD, at specific growth rates ranging from 0.35 to 0.40 h^{-1}, which were similar to the specific growth rate of the *S. cerevisiae* reference strain IMX585, which grew at 0.39 ± 0.01 h^{-1}. As anticipated, strain IMX585 showed virtually no growth on biotin-free SMD after a third transfer to biotin-free SMD. The same was observed for strain IMX1511, which contained an expression cassette for *S. cerevisiae BIO1* (IMX1511) and for the *S. cerevisiae* strains expressing the *ScBIO1* orthologs of *Y. lypolytica* (IMX1862), *P. kudriavzevii* (IMX1861), *W. ciferrii* (IMX1863), *L. kluyveri* (IMX1858) and *T. delbrueckii* (IMX1857) (Figure 2.4). Only strain IMX1859, which expressed the *C. fabianii BIO1* ortholog, showed growth in the third biotin-free SMD culture. Its specific growth rate of 0.40 ± 0.03 h^{-1} in absence of biotin was not significantly different from the specific growth rate of the reference strain IMX585 on biotin-containing SMD (Figure 2.4).

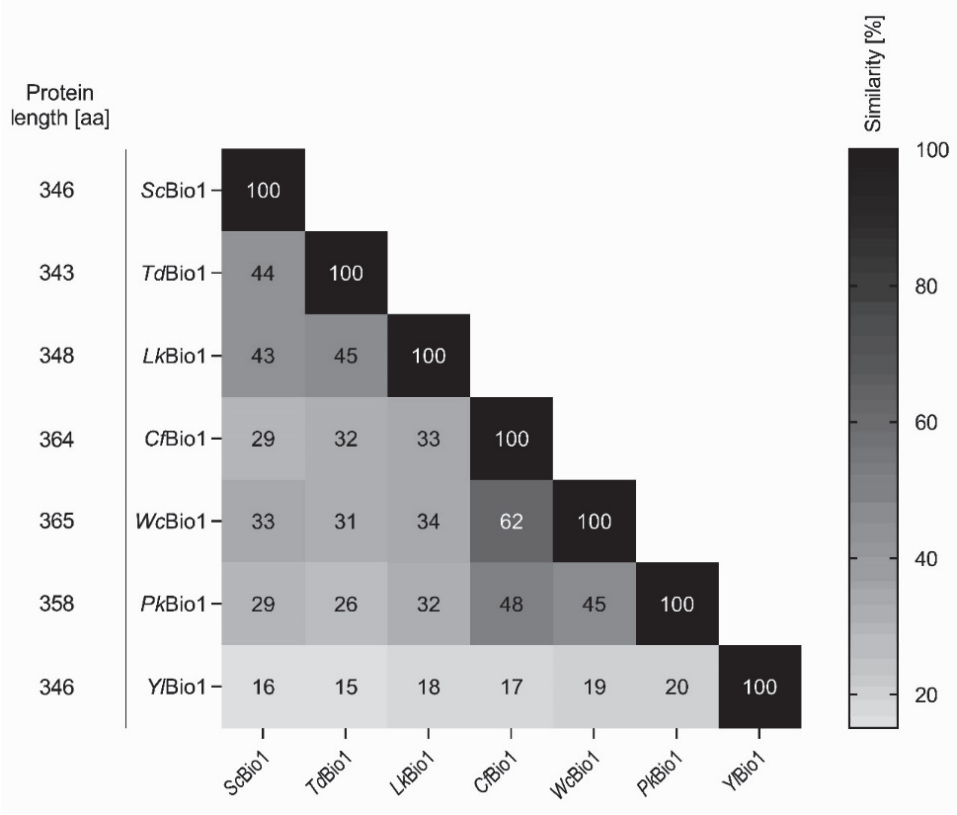

Figure 2.3 | **Amino-acid sequence similarities of putative *Sc*Bio1 orthologs.** Pair-wise alignments of amino-acid sequences were generated using ClustalΩ (similarity matrix BLOSUM 62). The amino-acid sequence similarity [%] between to orthologs is displayed in the intersecting cells. High amino-acid similarity is indicated with black and low similarity with white. Protein length for each ortholog is represented as amino acids [aa] on the left panel.

All strains expressing *ScBIO1* orthologs, including IMX1859, still harboured the native *ScBIO1* gene at its original locus. To verify that expression of the *CfBIO1* gene sufficed to achieve full biotin prototrophy, the native *ScBIO1* copy was deleted in strain IMX1859, yielding strain IMX1860 (*Scbio1*Δ *CfBIO1*↑). The specific growth rate of IMX1860 in SMD was determined as 0.39 ± 0.01 h^{-1} and in biotin-free SMD as 0.36 ± 0.00 h^{-1}, which is highly similar to the specific growth rates of strain IMX1859 on these media (Figure 2.4). Despite a slight difference in biotin-containing and biotin-free media, the specific growth rates of strain IMX1860 indicated that *CfBIO1* was sufficient to confer *S. cerevisiae* strains from the CEN.PK lineage with the ability to grow fast in the absence of biotin.

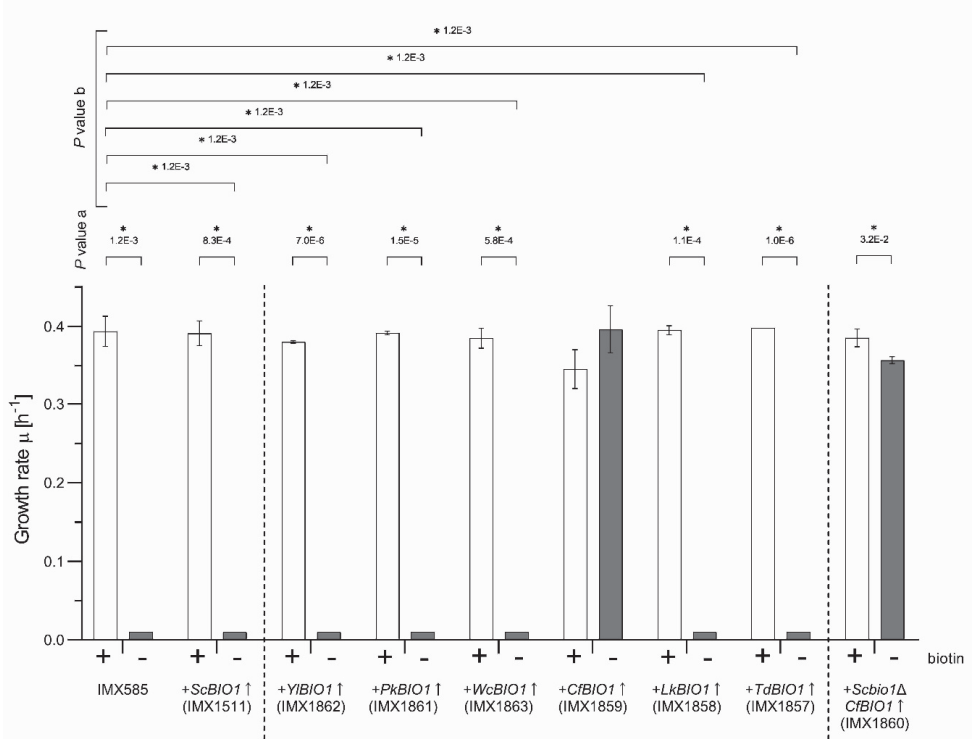

Figure 2.4 | Specific growth rates of *S. cerevisiae* CEN.PK113-7D derived strains expressing *ScBIO1* orthologs in SMD (+/white) and biotin-free SMD (-/growth). The bars data represent average and standard deviation of three biological replicates and are derived from measurement of culture optical density (OD_{660}) over time during the exponential growth phase of shake flask batch cultures of strains IMX585, IMX1511 (*ScBIO1*↑), IMX1862 (*YlBIO1*↑), IMX1861 (*PkBIO1*↑), IMX1863 (*WcBIO1*↑), IMX1859 (*CfBIO1*↑), IMX1858 (*LkBIO1*↑), IMX1857 (*TdBIO1*↑) and IMX1860 (*Scbio1*Δ *CfBIO1*↑). Statistical significance between growth rates in SMD and biotin-free SMD of each strain was determined using the Holm-Sidak method providing p_{value} a. Statistical significance between growth rates of IMX585 grown on SMD and the respective specific growth rates of engineered strains in SMD and biotin-free SMD was determined using the Holm-Sidak method providing p_{value} b. * denotes comparison with p_{value} lower than 5.0E-2.

C. fabianii BIO1 expression supports biotin prototrophy in multiple *S. cerevisiae* lineages.

To investigate whether the results obtained in the laboratory CEN.PK strain lineage could be extrapolated to other genetic backgrounds, the *CfBIO1* expression cassette was expressed in the *S. cerevisiae* laboratory strains S288C and CBS 8066, in the Brazilian bioethanol strain NCYC 3233 (syn. PE-2) and in Ethanol Red (Lesaffre, France) a commercial *S. cerevisiae* strain used for first- generation bioethanol production. Prior to expression of *CfBIO1*, none of these strains showed specific growth rates above 0.01 h^{-1} growth on biotin-free SMD. In biotin-containing SMD, strains NCYC 3233/PE-2, Ethanol Red and CBS 8066 exhibited specific growth rates ranging from 0.46 to 0.48 h^{-1}, while S288C grew at 0.34 ± 0.01 h^{-1} (Figure 2.5).

Prior to integration of the *CfBIO1* expression cassette, genome sequences of these strains were checked for the presence of biotin biosynthetic genes *BIO2, BIO3* and *BIO4* as well as *BIO1* and *BIO6* [151]. Except for S288C, all strains showed presence of these five biosynthetic genes, suggesting that only *CfBIO1* might suffice to restore biotin prototrophy. The reference *S. cerevisiae* S288C strain lacked the *ScBIO1* and *ScBIO6* genes, which usually occur as a two-gene cluster at the sub-telomeric region of chromosome I [150]. To complement the absence of *BIO6*, *CfBIO1* was co-integrated together with an expression cassette for *ScBIO6* in S288C.

Since, in contrast to the CEN.PK-derived strain IMX585, *S. cerevisiae* strains NCYC 3233/PE-2, Ethanol Red, CBS 8066 and S288C do not express Cas9, integration of the *CfBIO1* cassette at the *SGA1* locus of these strains was therefore achieved with plasmid pUDP145, which carries both the *ScSGA1* targeting gRNA and a *Spycas9* expression cassette. The *ScSGA1* Cas9-programmed double strand break was either repaired with the *CfBIO1* expression module or, in the case of S288C with two DNA fragments harbouring *CfBIO1* and *ScBIO6* cassettes.

*Cf*Bio1-expressing Ethanol Red and CBS 8066 derivatives grew on biotin-containing SMD with the same specific growth rate as their parental strains. On biotin-free SMD instead, *CfBIO1* expression in Ethanol Red and CBS 8066 lead to growth rates of 0.45 ± 0.01 h^{-1} and 0.42 ± 0.01 h^{-1}, respectively, which corresponded to 105% and 85% of their specific growth rates in biotin-containing SMD (Figure 2.5). In the NCYC 3233/PE-2 strain background, *CfBIO1* expression caused an unexpected decrease of specific growth rate on SMD with biotin, from 0.46 ± 0.02 h^{-1} to 0.17 ± 0.00 h^{-1} (Figure 2.5). The specific growth rate of strain S288C on SMD with biotin decreased slightly upon combined expression of *CfBIO1* and *ScBIO6*. Cultivation of the NCYC 3233/PE-2 and S288C-derived *CfBIO1*-expressing strains on biotin-free SMD yielded the same specific growth rates as on biotin-containing SMD (Figure 2.5).

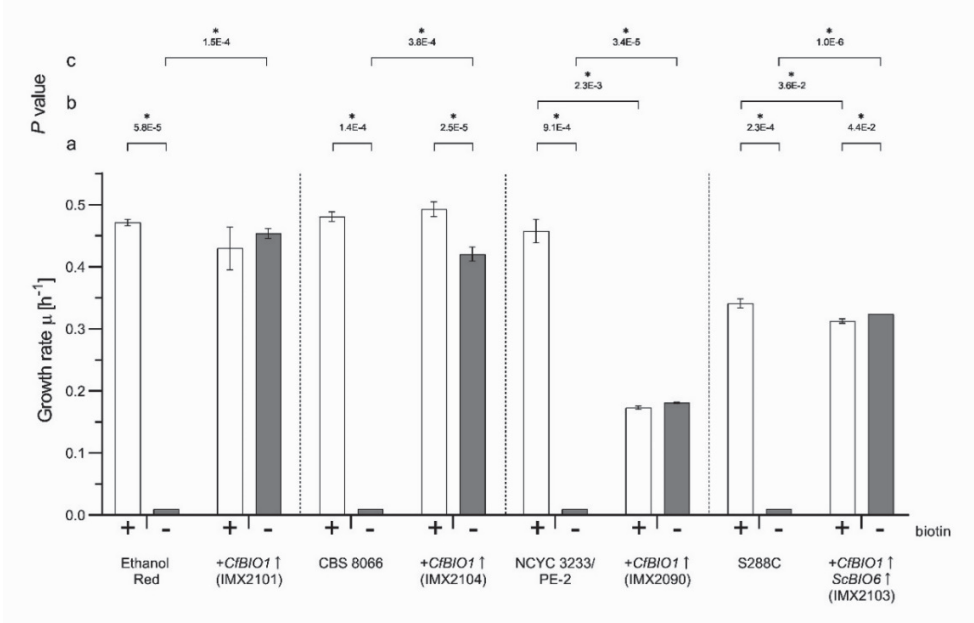

Figure 2.5 | Specific growth rates of *S. cerevisiae* strains engineered for growth without biotin in SMD (+/black) and biotin-free SMD (-/white). The bars data represent average and standard deviation of three biological replicates and specific growth rates are calculated from measurement of culture optical density (OD_{660}) over time during the exponential growth phase of shake flask batch cultures of Ethanol Red, CBS 8066, NCYC 3233/PE-2 and S288C as well as the respective engineered strains IMX2101 (Ethanol Red *CfBIO1*↑), IMX2104 (CBS 8066 *CfBIO1*↑), IMX2090 (NCYC 3233/PE-2 *CfBIO1*↑) and IMX2103 (S288C *CfBIO1*↑ *ScBIO6*↑). Statistical significance between growth rates in SMD and biotin-free SMD of each strain was determined using the Holm-Sidak method providing p_{value} a.) Statistical significance between growth rates of the initial and the engineered strain on SMD was determined using the Holm-Sidak method providing p_{value} b.) Statistical significance between growth rates of the initial and the engineered strain on biotin-free SMD was determined using the Holm-Sidak method providing p_{value} c.) * denotes comparison with p_{value} lower than 5.0E-2.

A biotin-prototrophic *S. cerevisiae* strain expressing *CfBIO1* outcompetes an auxotrophic strain.

A possible advantage of biotin-prototrophic strains upon contamination of cultures with biotin auxotrophs was investigated by co-cultivation of biotin-auxotrophic and biotin-prototrophic *S. cerevisiae* strains. *S. cerevisiae* strains IMX585 (*Spycas9*) and IMK827 (*Spycas9 Scbio1*Δ) were equipped with an expression cassette encoding Venus fluorescent protein, yielding strains IMX2212 and IMX2240, respectively. The biotin-prototrophic strain IMX1860 (*Spycas9 Scbio1*Δ *CfBIO1*↑) was engineered to overexpress the mRuby2 fluorescence protein, yielding strain IMX2238. First, the strains IMX2240 (*Spycas9 Scbio1*Δ Venus↑) and IMX2238 (*Spycas9 Scbio1*Δ mRuby2↑ *CfBIO1*↑) were mixed in a 40:60 ratio. The relative abundance of the two fluorescent populations was monitored by flow cytometry during consecutive batch cultivation cycles, both in SMD with biotin and in biotin-free SMD. In biotin-containing media, the relative

abundance of the two strains remained constant after inoculation (Figure 2.6; top panel). Instead, in biotin-free medium (Figure 2.6; bottom panel), within five hours of cultivation, 75% of the population already consisted of mRuby2-expressing, biotin-prototrophic cells, while during the third and fourth batch cultivation cycles, 99% of the culture consisted of biotin-prototrophic cells. In a similar experiment, the biotin prototroph IMX2238 (*Spycas9 Scbio1*Δ mRuby2↑ *CfBIO1*↑) was inoculated together with a fluorescent CEN.PK113-7D derived strain IMX2212 (*Spycas9* Venus↑), which carries all native biotin biosynthetic genes (Figure 2.7). The initial ratio of 60:40 was maintained three batches in biotin-containing SMD (Figure 2.7; top panel).

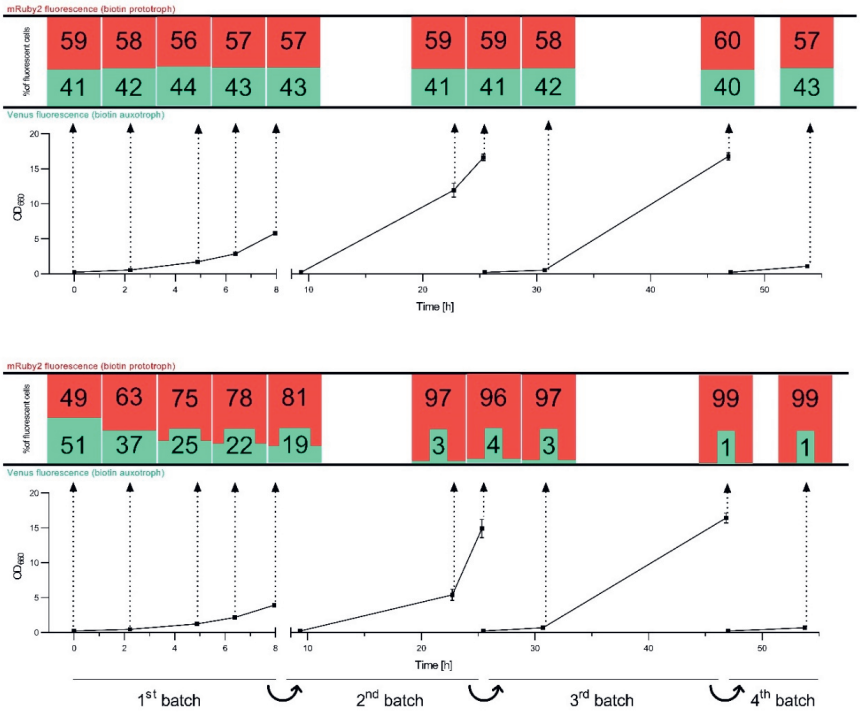

Figure 2.6 | Culture optical density (OD_{660}) and fluorescent population distribution of mRuby2 expressing strain IMX2238 (*Spycas9 Scbio1*Δ mRuby2↑ *CfBIO1*↑; biotin prototroph) and Venus expressing strain IMX2240 (*Spycas9 Scbio1*Δ Venus↑; biotin auxotroph) over time during co-cultivation in four consecutive batch culture cycles on SMD (top) and biotin-free SMD (bottom). Both conditions were analysed in two independent replicates each. Each OD_{660} time-point was sampled and analysed by flow cytometry. The distribution of mRuby2 (red bar) and Venus (green bar) fluorescent cells from the total of fluorescent cells [%] at each time-point is represented in the bar graph above (connected by arrow).

In contrast, in biotin-free SMD, abundance of the auxotroph was reduced to 2% of the total fluorescent population after three batch cultivation cycles (Figure 2.7; bottom panel). These results indicated that. while expression of *CfBIO1* (IMX2238) provided a significant growth advantage in biotin-free SMD, biotin is not released into the medium in sufficient amounts to support growth of a complete biotin auxotroph or of a strain with a very low biosynthesis capacity such as CEN.PK113-7D.

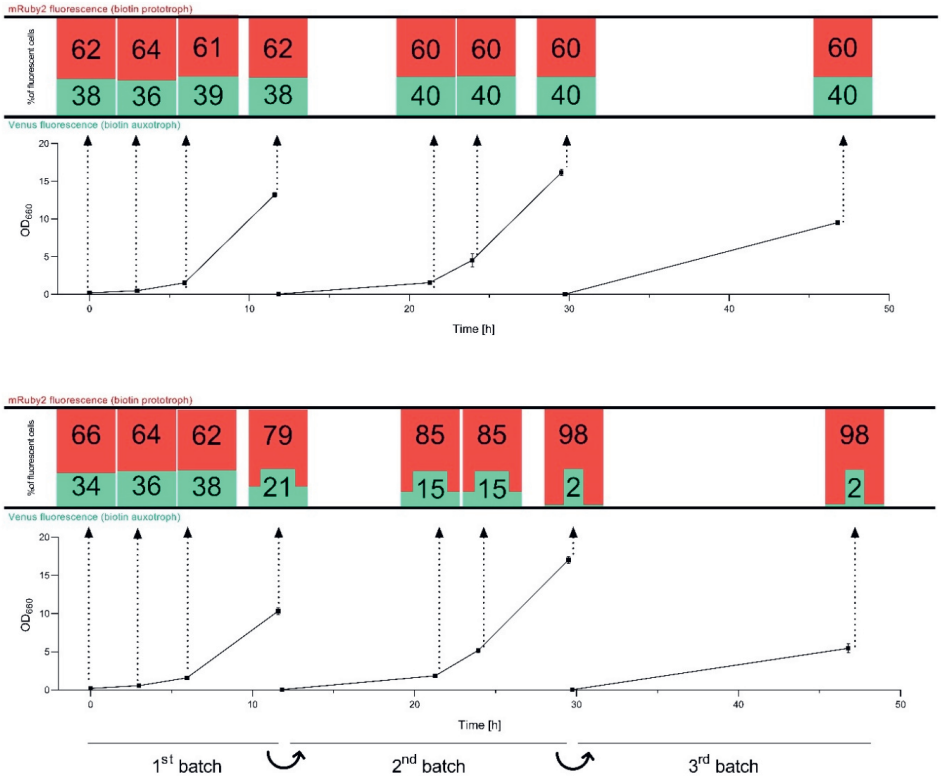

Figure 2.7 | Culture optical density (OD_{660}) and fluorescent population distribution of mRuby2 expressing strain IMX2238 (*Spycas9 Scbio1*Δ mRuby2↑ *CfBIO1*↑; biotin prototroph) and Venus expressing strain IMX2212 (*Spycas9* Venus↑; biotin auxotroph) over time during co-cultivation in three consecutive batch culture cycles on SMD (top) and biotin-free SMD (bottom). Both conditions were analysed in two independent replicates each. Each OD_{660} time-point was sampled and analysed by flow cytometry. The distribution of mRuby2 (red bar) and Venus (green bar) fluorescent cells from the total of fluorescent cells [%] at each time-point is represented in the bar graph above (connected by arrow).

Bio1 proteins show similarity to iron(II)-dependent oxidoreductases and biotin synthesis requires oxygen.

For a first exploration of the unknown reaction catalysed by *Cf*Bio1, its protein structure was predicted by homology modelling with Phyre2. The *Cf*Bio1 structure showed high structural similarity to an *Aspergillus nidulans* phytanoyl-CoA dioxygenase (5DAQ, DOI: 10.2210/pdb5daq/pdb, EC 1.14.11.18). This oxidoreductase enzyme catalyses an iron(II)-dependent reaction in which phytanoyl-CoA is oxidized to 2-hydroxyphytanoyl-CoA with 2-oxo-glutarate and oxygen as co-substrates, yielding succinate and carbon dioxide as by-products [246]. The same approach was applied to *Sc*Bio1 that showed high structural similarity to another putative oxygenase (4NAO, DOI:10.2210/pdb4NAO/pdb, EC 1.14.11.18). These two homology-'hits' were retrieved for all the orthologous Bio1 candidate polypeptides, except for *Yl*Bio1, which instead

aligned more optimally with the human phytanoyl-CoA dioxygenase (2OPW, DOI: 10.2210/pdb2OPW/pdb, EC 1.14.11.18). In addition to the structural homology to oxidoreductases (EC 1) and more specifically to enzymes using molecular oxygen (EC 1.14), all Bio1 orthologs harboured a conserved 2-oxoglutarate and iron(II)-dependent oxygenase superfamily domain (pfam03171) [247]. These structural similarities strongly suggested that the reaction catalysed by Bio1 requires oxygen. To test whether growth in biotin-free medium was indeed oxygen-dependent, the *S. cerevisiae* strains IMX1859 (*Spycas9 CfBIO1*↑) and IMS0481 (CEN.PK113-7D evolved for biotin independent growth [154]) were anaerobically incubated in biotin-free medium. In contrast to their biotin prototrophy under aerobic conditions, neither of the strains was able to grow under anaerobic conditions without biotin supplementation (Figure 2.8). These results identify biotin biosynthesis in yeast as oxygen-dependent and strongly indicate that the reaction catalysed by Bio1 requires molecular oxygen.

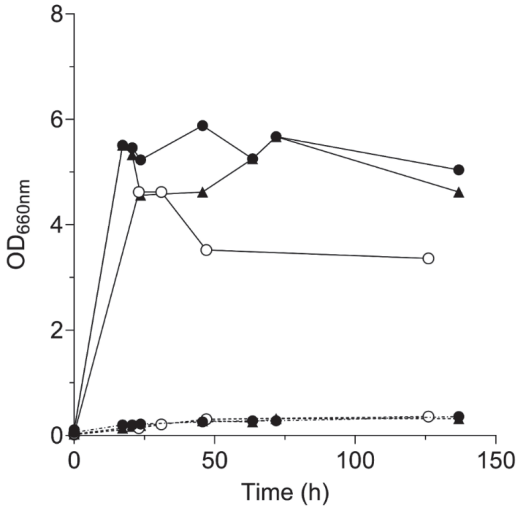

Figure 2.8 | Culture optical density (OD_{660}) over time of IMX1859 (*CfBIO1*↑; black circle), IMS0481 (isolate of biotin prototrophy evolution; white circle), IMX1511 (*ScBIO1*↑; black triangle) in SMD (solid line) and SMD without biotin (dashed line) in anaerobic conditions. Specific growth rates were measured after three consecutive transfers. The growth data (OD_{660}) presented are single batches representative for aerobic and anaerobic growth profiles of IMX1859, IMS0481, IMX1511.

Discussion

The elimination of nutritional requirements in industrially relevant microorganisms by acquisition of new metabolic functions can contribute to process robustness and economics. In this study, we report a novel and simple metabolic engineering strategy to convert biotin-auxotrophic *S. cerevisiae* strains into biotin prototrophs. The transferable *CfBIO1* expression cassette was identified by a screening of different Saccharomycotina yeasts for their ability to grow in the absence of biotin. Fast-growing, biotin-independent strains from six species belonging to diverse and distant

phylogenetic groups were identified (*Y. lipolytica* from the *Yarrowia* family, *P. krudiavzevii* from the the *Pichiaceae* family, *W. ciferrii* and *C. fabianii* from the *Phaffomycetaceae* family and *L. kluyveri* and *T. delbrueckii* from the *Saccharomycetaceae* family). Some of these yeasts have already been explored for their biotechnological properties such as *Y. lipolytica* for the production of oleochemicals [248], *P. kudriavzevii* as a multi-stress tolerant yeast for bioethanol [249] and succinic acid production [250] and *W. ciferrii* for the production of sphingolipids [251]. However, their biotin-prototrophic phenotype has not yet been exploited. *C. fabianii* (syn. *Hansenula fabianii*, *Pichia fabianii*, and *Lindnera fabianii*) is not an industrial yeast and has, in rare cases, been isolated from patients with blood infections. Its biotin prototrophy might be related to its opportunistic behaviour by providing a selective advantage in low-nutrient environments [252].

Establishing sustainable and cost-effective preparation of growth media remains an important factor in industrial process design and economics [253-255]. The benefit of microbes growing independently from the addition of organic growth factors such as biotin enables increased standardisation as well as reduced costs and time for media preparation and simplified down-stream processing [154, 256]. Economic incentives to develop simpler growth media are especially strong for production of commodity chemicals and in strictly regulated processes that require purified media components [257]. As demonstrated in this study, biotin-independent *S. cerevisiae* strains did not secrete amounts of biotin in the medium that enabled cross-feeding of fast-growing auxotrophic yeasts. This observation suggests that fermentation processes based on such prototrophs may be less susceptible to contamination by biotin-requiring 'wild' *S. cerevisiae* strains or other biotin-auxotrophic contaminants [258].

Inspection of available draft genome assemblies of the biotin-prototrophic yeast species led to the identification of candidate *BIO1* genes. Surprisingly, only the expression of the *BIO1* ortholog from *C. fabianii* supported fast growth of *S. cerevisiae* in biotin-free medium. Even the *BIO1* ortholog from *W. ciferrii*, which belongs to the same phylogenetic subdivision of the Saccharomycotina tree as *C. fabianii*, failed to support growth of *S. cerevisiae* in absence of biotin. Without a functional analysis of these *BIO1* orthologs in their original hosts, the possibility remains that these open reading frames do not encode for the enzyme performing the initial step in biotin biosynthesis, but have a different physiological role. We also cannot exclude differences in post-translation regulation between the donor organism and S. cerevisiae which could result in non-functional proteins. As shown for the elucidated biotin biosynthetic pathways in prokaryotic model organisms such as *Escherichia coli* and *B. subtilis* the precursor initiating the biosynthesis could differ. In *E. coli,* biotin is produced by the so-called BioC-BioH pathway, which diverts malonyl-CoA from fatty acid synthesis by masking the ϖ-carboxyl group of a malonyl-CoA thioester, resulting in recognition of this uncommon substrate by the *E. coli* fatty-acid synthesis machinery. In two successive rounds of fatty acid synthesis the malonyl thioester methyl ester is elongated yielding pimeloyl-ACP methyl ester which reversibly esterified to free

pimeloyl-ACP, which subsequently enters biotin synthesis [259]. In *B. subtilis* two distinct pathways have been identified. The indispensable route via the pimeloyl-CoA synthetase BioW [260] activates free pimelic acid with co-enzyme A yielding pimeloyl-CoA [155], which is used by *B. subtilis* BioF to produce KAPA [261]. Alternatively, a second, apparently redundant route [262] via a cytochrome P450-dependent carbon-bond-cleaving oxygenase encoded by *bioI* can generate pimeloyl-ACP from long chain (C_{14}, C_{16} or C_{18}) acyl-ACP molecules [263]. So it cannot be excluded that similarly the precursor to produce pimeloyl-ACP or -CoA differs in between these yeasts and *S. cerevisiae*.

While the expression of *CfBIO1* in the NCYC 3233 (PE-2) strain resulted in biotin prototrophy as in any other genetic background tested, the simple integration of this gene at the *SGA1* locus negatively impacted cell growth by reducing by half the strain specific growth rate, a phenomenon that remains enigmatic. This difference in phenotype might be the result of unwanted mutations introduce during the strain transformation procedure. As previously shown induction of aneuploidy is relatively frequent and associated with an aneuploidy associated stress response (AASR) that may lead to strain growth rate reduction [264, 265]. However, the methodology to detect this type of mutations has not used in the framework of this study such as full genome sequencing of the PE-2 derived transformants. This might also be linked to the *SGA1* genetic context, such that integration at that locus in NCYC 3233 might have disturbed expression of surrounding genes. Deregulation of *XBP1*, distal to SGA1 on CHRIX might lead to slower growth. *XBP1* is a transcriptional repressor that acts on promoter of cyclin genes and whose overexpression is known to results in decrease growth rate [266]. Integration at a different chromosomal site would be sufficient to falsify this hypothesis.

Although the amino-acid similarities of the six Bio1 orthologs ranged from 15 to 62%, all contained common structural motifs typical for phytanoyl-CoA dioxygenases and 2-oxoglutarate-iron(II)-dependent dioxygenases. These oxidoreductases catalyse reactions that incorporate oxygen from molecular oxygen (O_2) into their substrates, while oxidizing 2-oxoglutarate to succinate and carbon dioxide [267]. Consistent with the involvement of an oxygenase in biotin synthesis, our results show that *de novo* biotin biosynthesis represents a previously unidentified oxygen-requiring process in *S. cerevisiae* and other biotin-prototrophic Saccharomycotina yeasts, in addition to well-known oxygen requiring processes such as the synthesis of ergosterol, unsaturated fatty acids [21 , 22] and nicotinic acid [268].

An oxygen requirement for biotin synthesis has also been reported for *B. subtilis*, in which pimeloyl-ACP is generated by cleavage of a carbon bond of an acyl-ACP (C_{14}, C_{16} or C_{18}) molecule by the cytochrome P450-dependent, oxygen-requiring enzyme BioI [263]. This mechanism provides a direct link between biotin synthesis and fatty-acid synthesis. Five of the biotin-prototrophic yeasts identified in this study, *Y. lipolytica* [269], *W. ciferri* , *P. kudriavzevii* , *T. delbrueckii* and *C. fabianii* are classified as oleaginous yeasts [270] which might support the link between biotin and fatty acid

biosynthesis also in yeast. Biochemical studies on the reaction(s) catalysed by *Sc*Bio1 and similar proteins are essential to finally elucidate one of the very few missing reactions in fungal vitamin biosynthesis [271].

Materials and Methods

Strains, media and maintenance

Strains of 35 Saccharomycotina yeasts were tested for growth in the absence of biotin. The *S. cerevisiae* strains used and constructed in this study belong to the CEN.PK, S288C, Ethanol Red, NCYC 3233/PE-2 and CBS 8066 lineages (Table 2.2).
Yeast strains were grown on YP medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) or on synthetic medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mL L^{-1} trace element solution (4.5 mg L^{-1} $ZnSO_4 \cdot 7\ H_2O$, 0.3 mg L^{-1} $CoCl_2 \cdot 6\ H_2O$, 1 mg L^{-1} $MnCl_2 \cdot 4\ H_2O$, 0.3 mg L^{-1} $CuSO_4 \cdot 5\ H_2O$, 4.5 mg L^{-1} $CaCl_2 \cdot 2\ H_2O$, 3 mg L^{-1} $FeSO_4 \cdot 7\ H_2O$, 0.4 mg L^{-1} $NaMoO_4 \cdot 2\ H_2O$, 1 mg L^{-1} H_3BO_3, 0.1 mg L^{-1} KI, 15 mg L^{-1} EDTA), and 1 mL L^{-1} 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} pyridoxal hydrochloride, 0.2 g L^{-1} 4-aminobenzoic acid) [13]. The pH was adjusted to 6 with 2 M KOH prior to autoclaving at 120°C for 20 min. Vitamin solutions were sterilized by filtration and added to the sterile medium. Concentrated sugar solutions were autoclaved at 110°C for 20 min and added to the sterile medium to give a final concentration of 20 g L^{-1} glucose (YPD and SMD). Biotin-free SM was prepared similarly, but biotin was omitted from the vitamin solution. Similarly, after autoclaving concentrated glucose solution at 110°C for 20 min, glucose was added to biotin-free SM to a final concentration of 20 g L^{-1} (biotin-free SMD). Solid media contained 1.5% bacto agar and, when indicated, acetamide for SMD 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} $MgSO_4 \cdot 7H_2O$, 1 mL L^{-1} trace element solution and 1 mL L^{-1} vitamin solution) and 200 mg L^{-1} hygromycin for YPD hygromycin.
E. coli cells (XL1-Blue, Agilent Technologies, Santa Clara, CA) were grown in lysogeny 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 2.0% bacto agar. For maintenance, stock cultures of yeast strains were grown on YPD if not specified differently and *E. coli* cultures on LB medium with appropriate antibiotic marker until late exponential phase, complemented with sterile glycerol to a final concentration of 30% (v/v) and stored at −80°C as 1 mL aliquots until further use.

Shake flask cultivation conditions

Cultivation experiments for determination of biotin requirements of yeasts were performed as follows: 1 mL aliquot of a stock culture was inoculated in 100 mL SMD in a 500-mL-shake flask and incubated for 20 h at 30°C. A second 100 mL SMD culture

was started by inoculating 2 mL of the first shake flask culture. When the second culture reached mid-exponential phase (OD_{660} of 3-5) it was used to inoculate a third culture at an OD_{660} of 0.1-0.3. Similarly, a 1 mL aliquot of a thawed stock culture was inoculated in 100 mL biotin-free SMD in a 500-mL-shake flask and incubated for 20 h at 30°C. A second 100 mL biotin-free SMD culture was started by inoculating 2 mL of the first shake flask culture. If the second culture reached mid-exponential phase (OD_{660} of 3-5) it was used to inoculate a third culture at an OD_{660} of 0.1-0.3. Shake flasks were incubated as biological duplicates at 30°C and 200 rpm in an Innova incubator (Brunswick Scientific, Edison, NJ). Strain IMX585 and CEN.PK113-7D, which consistently failed to grow on biotin-free SMD in the third culture, were used as a negative control in all growth experiments. Growth was monitored by following cultures OD_{660} of an appropriate dilution of the third shake flask culture measured with a Jenway 7200 Spectrophotometer (Cole-Palmer, Stone, United Kingdom). Specific growth rates were calculated from a minimum of six data points during exponential growth covering 3-4 doublings of OD_{660}. Growth rate was calculated using the equation 1: $X = X_0 e^{\mu t}$ in which μ indicates the exponential growth rate. All aerobic shake flask experiments were carried out in an Innova shaker incubator (New Brunswick Scientific, Edison, NJ) set at 30°C and 200 rpm.

For growth profiling under anaerobic conditions first and second culture were grown in 40 mL SMD or biotin-free SMD in a 50 mL shake flask as described previously in aerobic conditions. 200 µL sample of mid-exponentially growing cells from the second culture were transferred to an anaerobic workstation (Bactron, Sheldon Manufacturing, Cornelius, OR) at 30°C and used to inoculate the third culture. Oxygen entry through the air lock of the anaerobic workstation was minimized with the use of a regenerated Pd catalyst for H_2-dependent oxygen removal that was introduced into the chamber. Concentrated solutions of Tween 80 and ergosterol were added to the medium aiming for final concentrations of 420 mg L^{-1} and 10 mg L^{-1} respectively. In order to deplete all the nutrients from the aerobic growth phase a fourth culture was inoculated from exponentially growing cells. OD_{660} of the fourth culture was measured with a Ultrospec® 10 cell density meter (Biochrom, Harvard Bioscience, Cambridge, United Kingdom) which was placed inside the anaerobic workstation. All anaerobic experiments were carried out in biological duplicates as described before [277]. In case of co-cultivations the strains were inoculated in 100 mL SMD and biotin-free SMD in a 500-mL-shake flask by addition of a 1 mL aliquot of a stock culture and incubated for 20 h at 30°C. A second 100 mL SMD culture was started by inoculating 2 mL of the first shake flask culture. When the second culture reached mid-exponential phase (OD_{660} of 3-5) it was used to inoculate a third culture at an OD_{660} of 0.1-0.3 and an approximate 60:40 mix of the two strains from the same medium (biotin-supplemented or biotin-free). After reaching late-exponential phase co-cultures were transferred into fresh medium to an OD_{660} of 0.2. The fifth and sixth consecutive cultures were started similarly by transferring stationary phase cultures from the previous batch. During the third, fourth, fifth and sixth culture, samples were taken to analyse OD_{660} and population distribution by flow cytometry.

Table 2.2 | List of strains used in this study.

Strain	Yeast species	Genotype	Description	Reference
CBS 5679	*Schizosaccharomyces japonicus*	Wild type		[272]
CBS 10395	*Schizosaccharomyces pombe*	Wild type		[272]
W29	*Yarrowia lipolytica*	Wild type		[272]
CBS 1993	*Kuraishia capsulata*	Wild type		[272]
CBS 4732	*Ogataea polymorpha*	Wild type		[272]
CBS 11895	*Ogataea parapolymorpha*	Wild type		[272]
CBS 2499	*Brettanomyces bruxellensis*	Wild type		[272]
CBS 5147	*Pichia kudriavzevii*	Wild type		[272]
CBS 2352	*Hyphopichia burtonii*	Wild type		[272]
CBS 767	*Debaryomyces hansenii*	Wild type		[272]
CBS 6054	*Scheffersomyces stipitis*	Wild type		[272]
CBS 7064	*Millerozyma farinosa*	Wild type		[272]
CBS 111	*Wickerhamomyces ciferrii*	Wild type		[272]
CBS 5481	*Cyberlindnera fabianii*	Wild type		[272]
CBS 3082	*Lachancea kluyveri*	Wild type		[272]
CBS 6340	*Lachancea thermotolerans*	Wild type		[272]}
CBS 270.75	*Eremothecium cymbalariae*	Wild type		[272]
CBS 6556	*Kluyveromyces marxianus*	Wild type		[272]
CBS 2359	*Kluyveromyces lactis*	Wild type		[272]
CBS 813	*Torulaspora delbrueckii*	Wild type		[272]
CBS 732	*Zygosaccharomyces rouxii*	Wild type		[272]
CBS 2163	*Vanderwaltozyma polyspora*	Wild type		[272]
CBS 4417	*Tetrapisispora phaffii*	Wild type		[272]
CBS 4309	*Naumovozyma castellii*	Wild type		[272]
CBS 2517	*Kazachstania africana*	Wild type		[272]
CBS 8638	*Kazachstania bulderi*	Wild type		[272]
CBS 2170	*Nakaseomyces delphensis*	Wild type		[272]
CBS 5792	*Saccharomyces bayanus*	Wild type		[272]
CBS 12357	*Saccharomyces eubayanus*	Wild type		[273]
CBS 10644	*Saccharomyces arboricolus*	Wild type		[272]
CBS 8840	*Saccharomyces kudriavzevii*	Wild type		[272]
CBS 8839	*Saccharomyces mikatae*	Wild type		[272]
CBS 1190	*Saccharomyces paradoxus*	Wild type		[272]
CBS 6412	*Saccharomyces cerevisiae*	Wild type	Kyokai No. 7	[272]
CEN.PK113-7D	*Saccharomyces cerevisiae*	*MATa MAL2-8c SUC2*	Laboratory strain	[274]

S288C	*Saccharomyces cerevisiae*	MATa *SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*	Laboratory strain	[275]
CBS 8066	*Saccharomyces cerevisiae*	*MATa/α HO/ho*	Laboratory strain	[272]
Ethanol Red	*Saccharomyces cerevisiae*	*MATa/α*	Industrial bioethanol production strain	Lesaffre, FR
NCYC 3233	*Saccharomyces cerevisiae*	*MATa/α*	Brazilian bioethanol production strain PE-2	[276]
IMS0481	*Saccharomyces cerevisiae*	*MATa* evolved	Evolved CEN.PK113-7D for full biotin prototrophy	[154]
IMX585	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2*	CEN.PK113-7D expressing *Spycas9*	[245]
IMX1511	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-ScBIO1-ScBIO1t ScBIO1t*	*ScBIO1*↑	This study
IMX1862	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-YlBIO1-ScBIO1t*	*YlBIO1*↑	This study
IMX1861	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-PkBIO1-ScBIO1t*	*PkBIO1*↑	This study
IMX1863	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-WcBIO1-ScBIO1t*	*WcBIO1*↑	This study
IMX1859	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-CfBIO1-ScBIO1t*	*CfBIO1*↑	This study
IMX1857	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-TdBIO1-ScBIO1t*	*TdBIO1*↑	This study
IMX1858	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-LkBIO1-ScBIO1t*	*LkBIO1*↑	This study
IMX1860	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 Scbio1Δ sga1Δ::ScPYK1p-CfBIO1-ScBIO1t*	*Scbio1Δ CfBIO1*↑	This study
IMK827	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 Scbio1Δ*	Biotin auxotroph	This study
IMX2212	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2* x-2Δ:: *ScTEF1*p-Venus-*ScTDH1*t	Venus↑ biotin auxotroph	This study
IMX2238	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 Scbio1Δ sga1Δ::ScPYK1p-CfBIO1-ScBIO1t* x-2Δ::*ScPGK1*p-mRuby2-*ScPGK1*t	mRuby2↑ biotin prototroph	This study
IMX2240	*Saccharomyces cerevisiae*	*MATa can1Δ::cas9-natNT2 Scbio1Δ* x-2Δ:: *ScTEF1*p-Venus-*ScTDH1*t	Venus↑ biotin auxotroph	This study
IMX2103	*Saccharomyces cerevisiae*	*sga1Δ::ScPYK1p-CfBIO1-ScBIO1t, ScPGK1p-ScBIO6-ScBIO6t*	S288C *CfBIO1*↑ *ScBIO6*↑	This study
IMX2104	*Saccharomyces cerevisiae*	*sga1Δ::ScPYK1p-CfBIO1-ScBIO1t*	CBS 8066 *CfBIO1*↑	This study
IMX2101	*Saccharomyces cerevisiae*	*sga1Δ::ScPYK1p-CfBIO1-ScBIO1t*	Ethanol Red *CfBIO1*↑	This study
IMX2090	*Saccharomyces cerevisiae*	*sga1Δ::ScPYK1p-CfBIO1-ScBIO1t*	NCYC 3233 *CfBIO1*↑	This study

Molecular biology techniques

DNA fragments were amplified by PCR amplification with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Landsmeer, Netherlands) and desalted or PAGE-purified oligonucleotide primers (Sigma-Aldrich, St. Louis, MO) (Table 2.3) according to manufacturers' instructions. For diagnostic PCR analysis of constructed plasmids and strains DreamTaq polymerase (Thermo Fisher Scientific) was used according to manufacturers' recommendations. PCR products were separated by electrophoresis on 1% (w/v) agarose gels in Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific) with SERVA DNA Stain G (1:100,000) (Serva, Heidelberg, Germany) and, if required, purified with a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA) or GenElute PCR Clean-Up kit (Sigma-Aldrich). Yeast strains of the CEN.PK lineage and S288C, NCYC 3233/PE-2 were transformed by the lithium acetate (LiAc) method. Yeast strains Ethanol Red and CBS 8066 were transformed using electroporation as previously described [278] with a 2 mm cuvette (Bio-Rad, Hercules, CA) using a Gene Pulser Xcell electroporation system (Bio-Rad). Yeast genomic DNA was isolated using the YeaStar Genomic DNA kit (Zymo Research) or using the SDS-LiAc protocol [279]. *E. coli* cells were chemically transformed and plated on selective LB agar medium. Plasmids from selected clones were isolated from *E. coli* with a Sigma GenElute Plasmid kit (Sigma-Aldrich).

Table 2.2 | Primers used in this study.

Primer No.	Sequence 5' → 3'
3841	CACCTTTCGAGAGGACGATG
4892	TACAGACACGACGCATGG
5328	ATTTTAGCGTAAAGGATGGG
5941	GCTGGCCTTTTGCTCACATG
6005	GATCATTTATCTTTCACTGCGGAGAAG
6006	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTC
7428	TGTGATGATGTTTTATTTGTTTTGATTGG
7469	GGAGTTGACCGTCTTAACAG
8737	ATAACGGGTTTCTCGCTGAA
10235	TTGTGCGGTTTCGTTTAGGG
10320	CATGCGCGGATGACACGAAC
10325	AGTCATCCGAGCGTGTATTG
10873	ACGTGCGGAATAGGAATCTC
11037	AGTTCTTTGTAGAACAGCTCTC
11614	GCATCGTCTCATCGGTCTCATATGATGAACACAAAATCACTCG
11615	ATGCCGTCTCAGGTCTCAGGATTTACTCTTTATCGTCATAAATAATCTTG
11618	GCATCGTCTCATCGGTCTCAATCCATATCATCATTTCTGCCACAAATATATG
11619	ATGCCGTCTCAGGTCTCACAGCCGGTAGCTTGACGTGCGGAATAG
11898	CGCGGAAACGGGTATTAGGG
11899	CTAGATCCGGTAAGCGACAG
11915	GAGTGAGTGCTTTGTTCAATGG
11945	AGCATCACCTTCACCTTCAC
12086	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAATAAGTAACAGCAGCAAAGCCGATAATTGCAGACGAAC
12108	TAAATATCTAGGAAATACACTTGTGTATACTTCTCGCTTTCCTTTTATTTTTTTTTTTGTCATGCTCAGCCGGTAGCTTG
12223	CCAGGTGGCGTGCTAAACTTTTATAATGTATAAAAACCACCACCTCATAAAGTTTACTGGATATCATCATTTCTGCCACAAATATATGTACTGAGTCTATACGTCAAAGTAAAAAAAATAA
12224	TTATTTTTTTACTTTGACGTATAGACTCAGTACATATATTTGTGGCAGAAATGATGATATCCAGTAAACTTTATGAGGTGGTGGTTTTTATACATTATAAAAGTTTAGCACGCCACCTGG

12616	CGAGTCAGTGAGCGAGGAAG
12991	GCATCGTCTCATCGGTCTCATATGGCGTCTAAGAACCAAAA
12992	ATGCCGTCTCAGGTCTCAGGATCTACTCAAAAACTGCATGGA
13038	GCATCGTCTCATCGGTCTCATATGACGTTTAGTAAGAACTTAAC
13039	ATGCCGTCTCAGGTCTCAGGATCTATTCGATAACAGGATAAA
13287	AACAAGATCCGAGTACTTAG
13290	TTCGTCTCTCTCACACTTATACGGGTCGTTAGT
13291	CACGTCTCATGAGCCCGGAATAAATCTAGTGGC
13293	ATATCGTCCCTAGTCAATTC
13596	GCTGAAGATTTATCATACTATTCCTCCGCTCGTTTCTTTTTTCAGTGAGGTGTGTCGTGATGAACTGGCCGAT AATTGCAGA
13597	ATTCTCGCCAAGGCATTACCATCCCATGTAAGAACGGAATAAAACAGCATTCGAAGGTTATGATGACC CCGTCGTCTCATT
13662	TCCTCGGGCAGAGAAACTCG
13963	CAAATAAAACATCATCACATATGACACATATAAGTAACTCGGAGTATC
13964	GCAGAAATGATGATATGGATTTATATACGAACTACTTTACAAACATCA
14139	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCAGGAAACGCTACTCGGAG TAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
14162	GGCACCTCTGGCTTGTCTTC
14167	TACTCCGAGTAGCGTTTCCT
14661	TTAGGGAGCACATCCATGCCAATAGCTCGACAAGCGGCGAGAGCCTTGCACCTATGCTATCAATAGG CACACTGTAATTG
14662	GTATATATATATATTTGATGTAAATATCTAGGAAATACACTTGTGTATACTTCTCGCTTTCTAAACTGAGCACAA GTTTC
14663	ATAGCATAGGTGCAAGGCTCTCGCCGCTTGTCGAGCTATTGGCATGGATGTGCTCCCTAATAGCTTGAC GTGCGGAATAG
14891	CATATCATCATTTCTGCCACAAATAT
14892	CAATCAAAACAAATAAAACATCATCACAATGAGTTTCAACTACGAAGACTG
14893	ATATTTGTGGCAGAAATGATGATATGTTAGTCGTATAAAACTTTACATACTT
14907	AATGCAATGGAGCTTGAGAC
14909	TGGTCGTCTCTATGCAAAGG
14925	GCATCGTCTCATCGGTCTCATATGTGCTGTACATGTACATACCA
14926	ATGCCGTCTCAGGTCTCAGGATTAGGCGCGTAAAGTTATTAC
14928	GGGAGGTCGCAATATCTCTG
15104	GCAGAAATGATGATATGGATTTAATTAACATCTGAGACTT
15105	CAAATAAAACATCATCACATATGACTGTTATAGATACCAATGA
16792	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGATTTATCATACTATTCCTCCGCTCGGCCTTGCCAA CAGGGAGTTC
16793	GTCATAACTCAATTTGCCTATTTCTTACGGCTTCTCATAAAACGTCCCACACTATTCAGGCGTTCAGGGTAA TATATTTT

Plasmid construction

Construction of part plasmids using Yeast Tool Kit principle.

Coding sequences of putative *ScBIO1* orthologs from *Y. lipolytica W29*, *P. kudriavzevii* CBS 5147, *W. ciferrii* CBS 111, *C. fabianii* CBS 5481, *L. kluyveri* CBS 3082 and *T. delbrueckii* CBS 813 were obtained by PCR with primer combinations 14925/14926, 14892/14893, 15104/15105, 13963/13964, 13291/13039 and 13290/13038 as well as 12991/12992 respectively using genomic DNA of the respective yeast as a template. In case of *S. cerevisiae* CEN.PK113-7D *BIO1*, the plasmid pUDE450 (Table 2.4) [154] was isolated from *E. coli* cultures and used as a template for PCR with primer pair 11614/11615. The *ScBIO1* terminator (*ScBIO1*t) was similarly PCR amplified using primer pair 11618/11619 and plasmid pUDE450 as template. The DNA fragments containing *BIO1* coding sequences from *Y. lypolytica, T. delbrueckii*, *L. kluyverii* and CEN.PK113-7D as well as *ScBIO1*t were *in vitro* assembled with entry vector pUD565 using BsmBI-T4

ligase directed Golden Gate cloning [280] resulting in Yeast Tool Kit type 3 plasmids pGGkp243, pGGkp169, pGGkp178 and pGGkp080 respectively and a Yeast Tool Kit type 4 plasmid with *ScBIO1*t pGGkp078 (Table 2.4). In order to remove a BsaI restriction recognition site in the coding sequence of *LkBIO1,* the open reading frame was PCR amplified from *L. kluyveri* CBS 3082 genomic DNA using two primer pairs 13291/13039 and 13290/13038 with overhangs allowing for BsmBI-T4 ligase directed Golden Gate cloning of the two DNA fragments into entry vector pUD565 [281], leaving a sequence without BsaI site behind and resulting in the Yeast Tool Kit type 3 plasmid pGGkp178. After *in vitro* assembly, plasmids were transformed into *E. coli* and plated on LB chloramphenicol for selection. The Yeast Tool Kit type plasmids pGGkp080, pGGkp169 pGGkp078 and pGGkp178 were confirmed by diagnostic PCR with primer pair 12616/4892, 12616/13287, 12616/10235and 12616/13290 respectively. Yeast Tool Kit type plasmid pGGkp243 was confirmed by restriction analysis with restriction enzymes PvuII and DraI. The promoter *ScPYK1*p was synthesized by GeneArt (Thermo Fisher Scientific) and cloned as plasmid pGGkp117. The Yeast Tool Kit type plasmids were stored in transformed *E. coli* cultures.

Construction of transcriptional modules

The control *S. cerevisiae* CEN.PK113-7D *BIO1* transcriptional module was constructed by Golden Gate cloning combining DNA fragments with compatible overhangs from plasmids pGGkd017, pGGkp117, pGGkp080, pGGkp078 yielding plasmid pUDE718. The entry plasmid pGGkd017 was also constructed by Golden Gate cloning combining DNA fragments with compatible overhangs from pYTK002, pYTK047, pYTK072, pYTK074, pYTK082 and pYTK083 [280]. The *T. delbrueckii BIO1* transcriptional module was constructed by Golden Gate cloning combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp117, pGGkp169, pGGkp078 yielding plasmid pUD788. The entry plasmid pGGkd015 was obtained by Golden Gate cloning with Yeast Tool Kit type plasmids pYTK002, pYTK047, pYTK067 and pYTK095. The *L. kluyverii BIO1* transcriptional module was constructed by Golden Gate cloning combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp117, pGGkp178, pGGkp078 yielding plasmid pUD789. The *Y. lipolytica BIO1* transcriptional module was constructed by Golden Gate cloning combining DNA fragment with compatible overhangs from plasmids pGGkd015, pGGkp117, pGGkp243, pGGkp078 yielding plasmid pUD989. The transcriptional modules of *P. kudriavzevii*, *W. ciferrii and C. fabianii BIO1* genes were constructed by Gibson assembly [282] (New England Biolabs, Ipswich, MA) using pUDE718 as plasmid backbone which includes the *ScPYK1*p and *ScBIO1*t sequences. The *BIO1* genes from *P. kudriavzevii* CBS 5147, *W. ciferrii* CBS 111, *C. fabianii* CBS 5481 were amplified by primer pairs 14892/14893, 15104/15105, 13963/13964 respectively using genomic DNA of the respective yeast as a template. DNA fragments were assembled with linearized pUDE718 backbone using primer pair 7428/14891 yielding plasmids pUD988, pUD990 and pUD790 respectively. The assembly mixes were transformed into *E. coli* and plated

on LB ampicillin for selection. The transcriptional module plasmids were confirmed by diagnostic PCR with the forward primer 10320 and a gene-specific reverse primer as follows: 13287 for *TdBIO1,* 13293 for *LkBIO1,* 14928 for *YlBIO1,* 4892 for *ScBIO1,* 14909 for *PkBIO1,* 14907 for *WcBIO1* and 14162 for *CfBIO1*. The transcriptional module for expression of fluorophore mRuby2 was constructed by Golden Gate cloning combining DNA fragment with compatible overhangs from plasmids pGGKd005, pYTK011, pYTK046, pYTK054. The entry plasmid pGGkd005 was constructed by Golden Gate cloning combining DNA fragments with compatible overhangs from pYTK002, pYTK047, pYTK067, pYTK079, pYTK083 and pYTK081. The assembly mixes were transformed into *E. coli* and plated on LB ampicillin for selection. The mRuby2 transcriptional module plasmid was confirmed by diagnostic PCR with primer pair 10320/10325 and stored as pUDE480 in transformed *E. coli* cultures.

Spycas9 expressing plasmids

The gRNA$_{ScSGA1}$ expressing plasmid pUDR244 was constructed by *in vitro* Gibson assembly. The linearized pROS11 plasmid, obtained by PCR with 6005/6006 was assembled together with a PCR amplified fragment using primer 14139 and pROS11 as a template as previously described [245]. The plasmid DNA was isolated from *E. coli* and the correct assembly of plasmid pUDR244 confirmed by diagnostic PCR with primers 3841/14167/5941. Similarly, plasmid pUDR376 was assembled with linearized pROS11 and the PCR amplified DNA fragment but using primer 10866. Assembly of pUDP145 was performed *in vitro* by BsaI–T4 DNA ligase directed Golden Gate cloning with the gRNA entry plasmid pUDP002 [283] with a *de novo*-synthesized DNA fragment (GeneArt, Thermo Fisher Scientific) encoding a gRNA targeting the Sc*SGA1*.

Strain construction

The *BIO1* transcriptional modules were PCR amplified by using primer pair 12086/12108 adding specific sequences for homologous recombination into the *SGA1* locus in *S. cerevisiae* directed by CRISPR/Cas9 [245]. The transcriptional module was amplified from plasmid pUD788 for *TdBIO1*, from plasmid pUD789 for *LkBIO1*, from plasmid pUD989 for *YlBIO1,* from plasmid pUDE718 for *ScBIO1,* from plasmid pUD988 for *PkBIO1,* from plasmid pUD990 for *WcBIO1* and from plasmid pUD790 for *CfBIO1*. Targeting at the Sc*SGA1* locus in IMX585 was directed by CRISPR/Cas9 and a target-specific gRNA expressing plasmid [245]. Each transcriptional module was co-transformed with plasmid pUDR119 expressing the gRNA to target Cas9 to *ScSGA1* in strain IMX585 using the LiAc transformation protocol. The transformed cells were plated on selective SMD acetamide and incubated for 3 days at 30°C. Genomic DNA of transformants was isolated using the SDS/LiAc protocol [279]. The desired genotype was confirmed using primer pair 11898/11899 and a gene-specific primer pair with 11898 as forward primer and the following reverse primers: 13287 for *TdBIO1,* 13293 for *LkBIO1,* 14928 for *YlBIO1,* 4892 for *ScBIO1,* 14909 for *PkBIO1,* 14907 for *WcBIO1* and 14162 for *CfBIO1*.

Table 2.4 | **Plasmids used in this study.**

Name	Characteristics	Reference
pGGkd005	*hph CEN6/ARS4 bla* ColE1 Gfp dropout	This study
pGGkd015	*Bla* ColE1 Gfp dropout	[281]
pGGkd017	*URA3* 2µ bla ColE1 Gfp dropout	This study
pGGKp078	*cat ScBIO1*t	This study
pGGKp080	*cat ScBIO1*	This study
pGGkp117	*cat ScPYK1*p	This study
pGGkp169	*cat TdBIO1*	This study
pGGkp178	*cat LkBIO1*	This study
pGGkp243	*cat YlBIO1*	This study
pROS11	*amdS* 2µ $gRNA_{CAN1}$ $gRNA_{ADE2}$	[245]
pUD565	*cat* Gfp dropout	[281]
pUD788	*bla* ColE1 *ScPYK1*p-*TdBIO1-ScBIO1*t	This study
pUD789	*bla* ColE1 *ScPYK1*p-*LkBIO1-ScBIO1*t	This study
pUD790	*URA3* 2µ *bla* ColE1 *ScPYK1*p-*CfBIO1-ScBIO1*t	This study
pUD988	*URA3* 2µ *bla* ColE1 *ScPYK1*p-*PkBIO1-ScBIO1*t	This study
pUD989	*bla* ColE1 *ScPYK1*p-*YlBIO1-ScBIO1*t	This study
pUD990	*URA3* 2µ *bla* ColE1 *ScPYK1*p-W*cBIO1-ScBIO1*t	This study
pUDC193	*URA3 CEN6/ARS4 bla* ColE1 *ScTEF1*p-Venus-*ScTDH1*t	This study
pUDE448	*URA3* 2µ *bla* ColE1 *ScPGK1*p-*ScBIO6-ScBIO6*t	[154]
pUDE450	*URA3* 2µ *bla* ColE1 *ScPYK1*p-*ScBIO1-ScBIO1*t	[154]
pUDE480	*hph CEN6/ARS4 bla* ColE1 *ScPGK1*p-mRuby2-*ScPGK1*t	This study
pUDE718	*URA3* 2µ *bla* ColE1 *ScPYK1*p-*ScBIO1-ScBIO1*t	This study
pUDP002	*hph* panARS(OPT) *bla* ColE1 *ScTDH3*p- BsaI site -*ScCYC1*t *AaTEF1*p-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*t	[283]
pUDP145	*hph* panARS(OPT) *bla* ColE1 *ScTDH3*p-HH-$gRNA_{SGA1}$-HDV-*ScCYC1*t	This study
pUDR119	*amdS* 2µ *ScSNR52*p-$gRNA_{SGA1}$-*SUP4*t	[245]
pUDR244	*amdS* 2µ *ScSNR52*p-$gRNA_{BIO1}$-*SUP4*t *ScSNR52*p-$gRNA_{BIO1}$-*SUP4*t	This study
pUDR376	*amdS* 2µ *bla* ColE1 *ScSNR52*p-$gRNA_{X-2}$-*SUP4*t	This study
pYTK002	*cat* ConLS	[280]
pYTK011	*cat ScPGK1*p	[280]
pYTK046	*cat* mRuby2	[280]
pYTK047	*cat* Gfp dropout	[280]
pYTK054	*cat ScPGK1*t	[280]
pYTK067	*cat* ConR1	[280]
pYTK072	*cat* ConRE	[280]
pYTK074	*cat URA3*	[280]
pYTK079	*cat hph*	[280]
pYTK081	*cat CEN6/ARS4*	[280]
pYTK082	*cat* 2µ	[280]
pYTK083	*bla* ColE1	[280]
pYTK095	*bla* ColE1 Gfp dropout	[280]

The correct clone was inoculated in 20 mL non-selective YPD for plasmid removal and incubated for 24 h at 30°C. The cells were plated on YPD agar in order to obtain single colony isolates. One isolate was restreaked on both SMD acetamide and YPD. When no growth was observed on SMD acetamide the respective clone was once again confirmed by PCR with gene-specific primers. Furthermore, the genetic modification at the *ScSGA1* locus was verified by Sanger sequencing (BaseClear, Leiden, Netherlands) using primers 11898/11899 to PCR amplify the modified locus and further using primers 11898, 11915 and 10235 for sequencing. The strain with the transcriptional module coding for *TdBIO1* was stocked as IMX1857, *LkBIO1* as IMX1858, *YlBIO1* as IMX1862, *ScBIO1* as IMX1511, *PkBIO1* as IMX1861, *WcBIO1* as IMX1863 and *CfBIO1* as IMX1859 in SMD.

Deletion of the native *ScBIO1* locus in strain IMX1859 and IMX585 was directed by CRISPR/Cas9 using a plasmid pUDR244 (Table 2.4) which was co-transformed with annealed repair oligo-nucleotides 12223/12224 in strain IMX1859 and IMX585 using the LiAc transformation protocol using SMD acetamide as selection marker and the deletion was. confirmed using primer pair 7469/10873.

To achieve integration of the *CfBIO1* module at the *SGA1* locus of CBS 8066, NCYC 3233/PE-2 and Ethanol Red the plasmid pUDP145 was co-transformed with a PCR amplified DNA fragment using primer pair 12086/12108 and pUD790 as template. In contrast to CBS 8066, NCYC 3233/PE-2 and Ethanol Red the *S. cerevisiae* strains S288C misses both the *ScBIO1* and *ScBIO6* gene [150], therefore the *CfBIO1* transcriptional module was amplified using primer pair 12086/14663 from pUD790 and an additional transcriptional module harbouring *ScBIO6* was amplified using primer pair 14661/14662 and plasmid pUDE448 as template. These two DNA fragments harboured homologous flanks allowing for *in vivo* assembly into the *ScSGA1* locus after co-transformation with pUDP145. Transformants selected on YPD hygromycin were tested for the desired genotype using primer pair 11898/11899 and using a *CfBIO1*-specific PCR with primer pair 11898/14162. In case of transformation into strain S288C an additional diagnostic PCR with primer pair 8737/11899 was conducted. After counter-selection the strain with the *CfBIO1* and *ScBIO6* transcriptional module in S288C was stored as IMX2103. CBS 8066 expressing *CfBIO1* was stored as IMX2104, Ethanol Red expressing *CfBIO1* as IMX2101 and NCYC 3233/PE-2 expressing *CfBIO1* as IMX2090 in SMD.

The Venus fluorophore transcriptional module was PCR amplified from pUDC193 by using primer pair 16792/16793. The mRuby2 fluorophore transcriptional module was PCR amplified from plasmid pUDE480 with primer pair 13596/13597. These two linear DNA fragments contained homologous flanks to the intergenic region X-2 [284] to enable for integration at this site when co-transformed with pUDR376 into a Cas9 expressing strain background. The Venus fluorophore gene was integrated into IMX585 and IMK827 and the mRuby2 fluorophore into IMX1860. Genomic DNA of transformants selected on SMD acetamide was isolated using the SDS-LiAc protocol. The desired genotype was confirmed by PCR using primer pair 13662/11037 and a gene-specific primer pair with 13662 as forward primer and 5328 as reverse primer to confirm mRuby2 expression cassette integration. The primer pair 13662/11945 was used to confirm the integration of the Venus expression cassette at the X-2 intergenic site. IMK827 with Venus fluorescence was stored as IMX2240, IMX585 with Venus fluorescence was stored as IMX2212 and IMX1860 with mRuby2 fluorescence as IMX2238 in SMD.

Flow cytometric analysis

Samples from aerobic 100 mL cultures in 500-mL-shake flasks were vortexed thoroughly to disrupt cell aggregates and used for flow cytometry on a BD FACSAria™ II SORP Cell Sorter (BD Biosciences, Franklin Lakes, NJ) equipped with 355, 445, 488, 561 and 640 nm lasers and a 70 µm nozzle, and operated with filtered FACSFlow™ (BD Biosciences).

Cytometer performance was evaluated prior to each experiment by running a CS&T cycle with CS&T Beads (BD Biosciences). The fluorophore mRuby2 was excited by the 561 nm laser and emission was detected through a 582 nm bandpass filter with a bandwidth of 15 nm. The fluorophore Venus was excited by the 488 nm laser and emission was detected through a 545 nm bandpass filter with a bandwidth of 30 nm. For each sample, 10,000 events were analysed and the same gating strategy was applied to all samples from the same culture. The reference sample for no fluorescent cells was a mid-exponentially growing culture of IMX585 on SMD. The Venus and mRuby2 fluorescence reference were obtained from mid-exponentially aerobic cultures on SMD of IMX2240 or IMX2212 and IMX2238 respectively. Cells without fluorescence, 'doublets' and with Venus and mRuby2 fluorescence were selected in a Venus/mRuby2 plot.

Sequence analysis and structural modelling

Genomic DNA of *Y. lypolytica* W29 was sequenced in-house on a Miseq sequencer (Illumina, San Diego, CA) to obtain a 300-cycle paired-end library with a fragment length of 550 bp using PCR-free library preparation yielding 4.28 million reads with a total sequence of 1.27 giga base. *De novo* assembly was performed using SPAdes (version 3.9.0) producing a 20.48 megabase genome comprising 409 contigs and a N50 of 181.71 kb in 36 contigs. tBLASTn (https://blast.ncbi.nlm.nih.gov) was used for identification of *BIO1* orthologs. *ScBIO1* [151, 224] amino acid sequence was queried against translation of whole genome shotgun (wgs) or nucleotide collection data of single yeast species. In a reciprocal analysis the yeast specific best hits with a minimum of 80% coverage were aligned using tBLASTn against *S. cerevisiae* CEN.PK113-7D nucleotide sequence to verify the similarity to the *ScBIO1* sequence. The identified putative *BIO1* amino acid sequences (Figure 2.3) were pair-wise aligned using ClustalΩ (Scoring matrix BLOSUM 62) [244] to determine amino-acid sequence similarities. The *BIO1* structural model was generated by homology modelling using Phyre2 [285].

Data availability

The sequencing data assembly of the *Yarrowia lipolytica* strain W29 was deposited at NCBI (https://www.ncbi.nlm.nih.gov/) under BioProject accession number PRJNA601425.

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strains, characterized constructed yeast strains, performed flow cytometric analysis of yeast cultures. E.B. performed initial physiological analysis of Saccharomycotina yeasts. M.P.H. constructed strains IMX1857, IMX1858, IMX1859, IMX1860 and IMK827 and performed physiological characterization of these strains. E.G. constructed strain IMX2238 and IMX2240. M.V.D.B. conducted *de novo* assembly of the *Y. lipolytica* W29 genome sequence. A.K.W. and J.-M.G.D. wrote the manuscript. All authors read and commented upon the manuscript and approved the final version.

Conflicts of interest

A.K.W., J.-M.G.D. and J.T.P. are inventors on a patent application related to this work. The remaining authors declare no competing interests.

Chapter 3 |

Engineering oxygen-independent biotin biosynthesis in *Saccharomyces cerevisiae*

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Abstract

An oxygen requirement for *de novo* biotin synthesis in *Saccharomyces cerevisiae* precludes the application of biotin-prototrophic strains in anoxic processes that use biotin-free media. To overcome this issue, this study explores introduction of the oxygen-independent *Escherichia coli* biotin-biosynthesis pathway in *S. cerevisiae*. Implementation of this pathway required expression of seven *E. coli* genes involved in fatty-acid synthesis and three *E. coli* genes essential for the formation of a pimelate thioester, key precursor of biotin synthesis. A yeast strain expressing these genes readily grew in biotin-free medium, irrespective of the presence of oxygen. However, the engineered strain exhibited specific growth rates 25% lower in biotin-free media than in biotin-supplemented media. Following adaptive laboratory evolution in anoxic cultures, evolved cell lines that no longer showed this growth difference in controlled bioreactors, were characterized by genome sequencing and proteome analyses. The evolved isolates exhibited a whole-genome duplication accompanied with an alteration in the relative gene dosages of biosynthetic pathway genes. These alterations resulted in a reduced abundance of the enzymes catalyzing the first three steps of the *E. coli* biotin pathway. The evolved pathway configuration was reverse engineered in the diploid industrial *S. cerevisiae* strain Ethanol Red. The resulting strain grew at nearly the same rate in biotin-supplemented and biotin-free media non-controlled batches performed in an anaerobic chamber. This study established a unique genetic engineering strategy to enable biotin-independent anoxic growth of *S. cerevisiae* and demonstrated its portability in industrial strain backgrounds.

Introduction

Typical industrial substrates derived from plant biomass such as sugarcane juice, starch, and ligno-cellulosic hydrolysates are subjected to harsh physical-chemical treatments that result in lowering nutritional properties [276] by affecting stability of vitamins [286-289]. In these substrates, biotin concentration is ranging from 10 to 80 ppb [290, 291]. Preloading of cells with vitamins during biomass propagation [292] or supplementing vitamins during fermentation showed positive impact on yeast fermentation performance [293-295] and significantly reduced occurrences of stuck wine fermentations [296, 297]. Thus, the estimation and the provision of the proper nutritional requirements of a microbial strain for industrial application are key points to improve robustness of a fermentation process [298]. In this context, vitamin prototrophic yeast strains could be highly beneficial.

Although most *S. cerevisiae* strains harbor all genes necessary to encode all known enzymes of the biotin biosynthesis pathway, these strains are bradytroph for biotin, exhibiting very low growth on media devoid of biotin. Evolutionary engineering and rational metabolic engineering strategies led to the selection of yeast strains whose growth in biotin-free medium was as fast as the growth of the reference strain in the presence of biotin [299, 300]. But in both cases, acquisition of the biotin prototroph phenotype was restricted to the presence of oxygen [300].

Several essential carboxylation reactions in eukaryotes and prokaryotes require biotin as a cofactor [240]. Despite its essentiality for prototrophic growth, *de novo* synthesis of biotin is restricted to bacteria and a limited number of plant and fungal species. The well-studied biochemical reactions involved in assembly of the fused heterocyclic rings of biotin are conserved among yeasts, bacteria and plants [301]. This assembly pathway starts with a thioester of either coenzyme A (CoA) or acyl carrier protein (Acp) with the 7-carbon dicarboxylic acid pimelate. This thioester is then further converted in four successive enzymatic steps catalyzed by 8-amino-7-oxononaote (7-*keto*-8-aminopelargonic acid, KAPA) synthase (EC 2.3.1.47), 7,8-diamino-nonanoate (DAPA) synthase (EC 2.6.1.62), dethiobiotin synthetase (EC 6.3.3.3) and biotin synthase (EC 2.8.1.6) to finally yield biotin [302]. Recently, a novel reaction involved in biotin synthesis was reported for cyanobacteria. In this reaction, the single-turnover suicide enzyme BioU converts KAPA to DAPA, using its Lys124 residue as an amino donor [303] (Figure 3.1).

The pathway for synthesis of the pimeloyl thioester that contributes to the valerate side chain of biotin is much less conserved and the origin of the pimeloyl moiety in eukaryotes remains elusive. The recent characterization of Bio1 from *Cyberlindnera fabianii* and *Saccharomyces cerevisiae*, an enzyme whose activity remains unresolved but which is essential for pimeloyl-thioester formation in yeast [149], revealed that it catalyzes an oxygen-dependent reaction [300]. A similar oxygen dependency has been reported for the *Bacillus subtilis* P450-enzyme BioI (Figure 3.1), which performs oxidative cleavage of ACP-bound long-chain fatty and thereby

generates pimeloyl-thioester for biotin synthesis [263]. Expression of *C. fabianii* Bio1 conferred full biotin prototrophy to oxic cultures of multiple laboratory and industrial strains of *S. cerevisiae* [300]. However, due to the oxygen dependence of this enzyme, this strategy is not applicable in large-scale anoxic processes such as the yeast-based production of ethanol and isobutanol.

Prokaryotic metabolism offers options for pimeloyl-thioester biosynthesis that are independent of molecular oxygen and might be suitable for implementation in *S. cerevisiae* to meet biotin demands in processes performed in absence of oxygen. In *B. subtilis*, pimeloyl-CoA can be formed by BioW, a pimeloyl-CoA synthetase that converts free pimelic acid to pimeloyl-CoA in presence of ATP and free CoA [262]. The substrate of BioW, pimelic acid (heptanedioic acid), has been proposed to be derived from fatty acid synthesis [304]. In *Escherichia coli*, a divergent pathway for pimelate thioester synthesis has been elucidated [259]. This pathway is intertwined with fatty acid synthesis and is initiated by SAM-dependent methylation of malonyl-CoA by the malonyl-[Acp] O-methyltransferase encoded by *bioC*, yielding malonyl-CoA or malonyl-[Acp]) [305]. The methyl group of malonyl-CoA methyl ester mimics the methyl ends of fatty acyl chains and removes the charge of the carboxyl group. Malonyl-CoA methyl ester then undergoes two cycles of chain elongation by a modified type-II fatty acid synthesis pathway involving FabB, a 3-oxoacyl-[Acp]-synthase (EC 2.3.1.41), as well as FabI (EC 1.3.1.9), FabZ (EC 4.2.1.59) and FabG (EC 1.1.1.100), which produce methyl pimeloyl-[Acp]. In a final step, BioH, a pimeloyl-[Acp] methyl esterase, removes the methyl group from pimeloyl-[Acp] methyl ester, thus preventing further elongation [306]. The released pimeloyl-[Acp] is then used by BioF, the first enzyme of the canonical pathway for formation of the hetero-bi-cyclic ring of biotin, which is a homolog of *S. cerevisiae* Bio6. BioF produces KAPA, which is the link between all hitherto described pathways for *de novo* syntheses of biotin. KAPA can be converted to biotin by DAPA synthase (Bio3, BioA) (or, in cyanobacteria, by (S)-8-amino-7-oxononanoate synthase BioU [303], dethiobiotin synthetase (Bio4, BioU) and biotin synthase (Bio2, BioB) [307] (Figure 3.1).

Since the multi-step prokaryotic pathway for biotin synthesis via malonyl-CoA methyl ester is not known to involve oxygen-requiring enzymes, its introduction into *S. cerevisiae* provides a possible strategy for *de novo* synthesis of biotin in anoxic cultures. To investigate this strategy, the *E. coli* genes encoding enzymes involved in KAPA synthesis, comprising *fabD*, *bioC*, *fabB*, *fabG*, *fabZ*, *fabI*, *bioH*, *bioF*, *acpP* and *acpS*, were expressed in *S. cerevisiae*. Individual transformants were evolved for fast growth in biotin-free medium conditions in absence of oxygen. Evolved biotin-prototrophic lineages were characterized by whole-genome re-sequencing and observed genetic changes were reverse engineered into *S. cerevisiae* Ethanol Red, a commercial yeast strain applied in industrial bioethanol production processes.

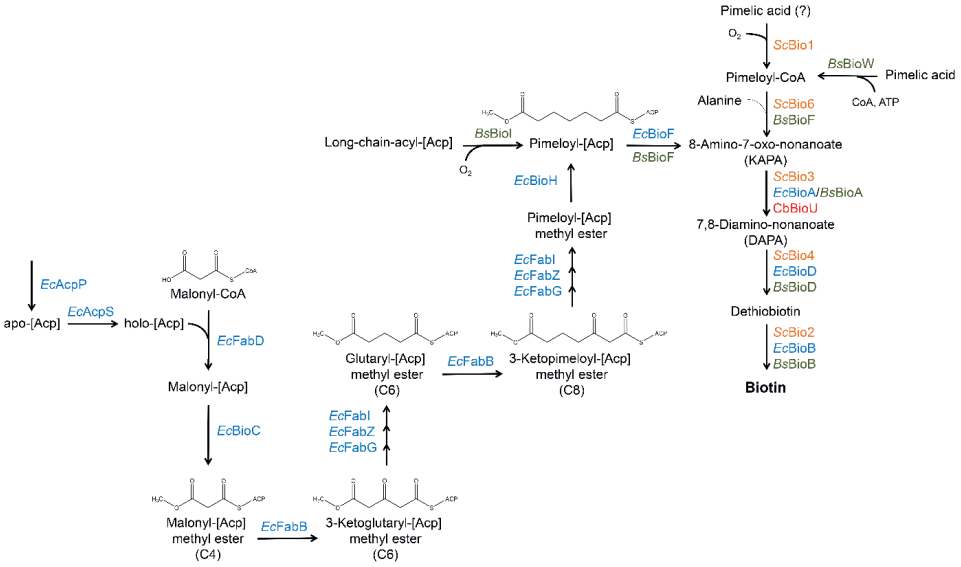

Figure 3.1 | Biotin biosynthesis pathways in *Escherichia coli* (blue), *Bacillus subtilis* (green), cyanobacteria (red) and yeast (orange). The *E. coli*-derived steps for biotin synthesis (blue) start from the acyl-carrier protein (AcpP), which is converted from its inactive apo-form into holo-[Acp] by the holo-[Acp] synthase AcpS. The malonyl-CoA-[Acp] protein transacylase FabD (EC 2.3.1.39) uses holo-[Acp] to attach the acyl-carrier protein to malonyl-CoA. The resulting malonyl-[Acp] receives a methyl group by SAM-dependent activity of the malonyl-[Acp] O methyltransferase BioC (EC 2.1.1.197). The four-carbon (C4) molecule is elongated by the 3-oxoacyl-[Acp] synthase FabB (EC 2.3.1.41). The enoyl-[Acp] reductase FabI (EC 1.3.1.9), 3-hydroxyl-[Acp] dehydratase FabZ (EC 4.2.1.59) and the 3-oxoacyl-[Acp] reductase FabG (EC 1.1.1.100) convert the product of this reaction to glutaryl-[Acp] methyl ester, which is in a subsequent step further elongated by FabB. The eight-carbon (C8) molecule is once more processed by FabI, FabZ and FabG. After two cycles of elongation the pimeloyl moiety is complete and the pimeloyl-[Acp] methyl ester esterase BioH (EC 3.1.1.85) enzyme activity removes the methyl group from pimeloyl-[Acp] methyl ester. The resulting pimeloyl-[Acp] enters after conversion by an 8-amino-7-oxononanoate synthase BioF (EC 2.3.1.47) to KAPA the yeast biotin synthesis (orange). The pathway is prolonged by three more enzymatic steps catalysed by the yeast enzymes adenosylmethionine-8-amino-7-oxononanoate aminotransferase Bio3 (EC 2.6.1.62), dethiobiotin synthetase Bio4 (EC 6.3.3.3) and biotin synthase Bio2 (EC 2.8.1.6) or in *E. coli* (blue) or *B. subtilis* (green) via the adenosylmethionine-8-amino-7-oxononanoate aminotransferase BioA (EC 2.6.1.62) or the (S)-8-amino-7-oxononanoate synthase BioU (EC 2.6.1.-) in cyanobacteria (red), the ATP-dependent dethiobiotin synthetase BioD (EC 6.3.3.3) and biotin synthase BioB (EC 2.8.1.6) to synthesise biotin. KAPA synthesis in yeast is proposed to start with pimelic acid, derived from an unknown source indicated with (?). Pimelic-acid conversion towards KAPA involves two enzymes in yeast, the putative pimeloyl-CoA synthetase Bio1 (EC 6.2.1.14) and the 7,8-diamino-pelargonic acid aminotransferase Bio6 (EC 2.3.1.47), with one of them involving putatively oxygen in the reaction. KAPA synthesis in *B. subtilis* (green) starts with the synthesis of a pimeloyl-thioester by either CoA-dependent conversion of pimelic acid by the 6-carboxyhexanoate CoA ligase BioW (EC 6.2.1.14) or oxygen-dependent cleavage of a long chain acyl-[Acp] by the biotin biosynthesis cytochrome P450 BioI (EC 1.14.14.46).

Materials and methods

Strains, media and maintenance

The *S. cerevisiae* strains used in this study are derived from the CEN.PK [308, 309] and Ethanol Red lineages (Leaf, Lesaffre, Marcq-en-Baroeul, France) (Table 3.1). Yeast strains were grown on YP medium (10 g L^{-1} yeast extract [BD Biosciences, Vianen, NL], 20 g L^{-1} peptone [BD Biosciences]) or on chemically defined medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4$, 7 H_2O, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} 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} pyridoxal hydrochloride, 0.2 g L^{-1} 4-aminobenzoic acid) [13]. The pH was adjusted to 6 with 2 M KOH prior to autoclaving at 121◦C for 20 min. Vitamin solutions were sterilized by filtration and added to the sterile medium. Concentrated sugar solutions were autoclaved at 110°C for 20 min and added to the sterile medium to give a final concentration of 20 g L^{-1} glucose (YPD and SMD). Biotin-free SM was prepared similarly, but biotin was omitted from the vitamin solution (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, 0.2 g L^{-1} 4-aminobenzoic acid) [299]. Similarly, after autoclaving concentrated glucose solution at 110°C for 20 min, glucose was added to biotin-free SM to a final concentration of 20 g L^{-1} (biotin-free SMD). Solid media contained 2% (w/v) Bacto agar (BD Biosciences) and, when indicated, acetamide for SMD 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 H_2O, 1 mL L^{-1} trace element solution and 1 mL L^{-1} vitamin solution) [310], 200 mg L^{-1} hygromycin for YPD hygromycin and 200 mg L^{-1} G418 (geneticin) for YPD geneticin. Where indicated, unsaturated fatty acids and/or sterols were added to autoclaved media as Tween 80 (polyethylene glycol sorbate monooleate, Merck, Darmstadt, Germany) and ergosterol (≥95% pure, Sigma-Aldrich, St. Louis, MO). 800-fold concentrated stock solutions of these "anaerobic" growth factors were prepared as described previously and incubated at 80°C for 20 min before diluting them in growth medium, yielding final concentrations of 420 mg L^{-1} Tween 80 and 10 mg L^{-1} ergosterol [311].

E. coli cells (XL1-Blue, Agilent Technologies, Santa Clara, CA) were grown in Lysogeny broth (LB) medium (5.0 g L^{-1} yeast extract, 10 g L^{-1} Bacto trypton [BD Biosciences], 5.0 g L^{-1} NaCl) supplemented with 25 mg L^{-1} chloramphenicol, 100 mg L^{-1} ampicillin or 50 mg L^{-1} kanamycin for selection. Solid LB medium contained 2% bacto agar.

Unless indicated otherwise, stock cultures for strain maintenance were prepared by growing yeast strains on YPD and *E. coli* cultures on LB with appropriate antibiotic markers. After reaching late exponential phase, cultures were complemented with sterile glycerol to a final concentration of 30% (v/v) and stored at −80°C as 1 mL aliquots until use.

Shake flask cultivations

For cultivation experiments for determination of specific growth rates, 1 mL aliquot of a stock culture was inoculated in 100 mL SMD in a 500-mL-shake flask and incubated for 20 h at 30°C. A second 100 mL SMD culture was started by inoculating 2 mL of the first shake flask culture. When the second culture reached mid-exponential phase, which corresponded to an optical density at 660 nm (OD_{660}) of 3-5, an aliquot was used to inoculate a third culture at an OD_{660} of 0.1-0.3. For biotin-free growth studies, the pre-cultivation steps were performed in biotin-free SMD. Strains *S. cerevisiae* IMX585 and CEN.PK113-7D, which consistently failed to grow on biotin-free SMD in the third culture, were included as negative controls in all growth experiments. Growth was monitored by measuring OD_{660} of accurately diluted culture samples of the third shake-flask culture with a Jenway 7200 Spectrophotometer (Cole-Palmer, Stone, United Kingdom). Specific growth rates were calculated from a minimum number of six data points collected during exponential growth and covering 3-4 doublings of OD_{660}. Specific growth rate was calculated using the equation $X = X_0 e^{\mu t}$ in which μ indicates the exponential growth rate. All oxic shake-flask experiments were carried out as biological duplicates in an Innova shaker incubator (New Brunswick Scientific, Edison, NJ) set at 30°C and 200 rpm. To test if growth rate averages observed for different combinations of strains and medium composition are significantly different, one-way analyses of variance (ANOVA) and Tukey's multiple comparison test with a = 0.05 were performed using GraphPad Prism 8.2.1 software (GraphPad Software, Inc., San Diego, CA).

For growth profiling under anoxic conditions, the first and second pre-culture were grown in 100 mL SMD or biotin-free SMD in a 500-mL-shake flask as described previously. A 200 µL sample of mid-exponential-phase (OD_{660} of 3-5) cells from the second culture was then transferred to a Shel Lab Bactron 300 anaerobic workstation (Sheldon Manufacturing Inc., Cornelius, OR) operated at 30°C. The gas mixture used for flushing the workspace and air lock consisted of 85% N_2, 10% CO_2 and 5% H_2. An IKA KS 260 Basic orbital shaker platform (Dijkstra Verenigde BV, Lelystad, The Netherlands) placed in the anaerobic chamber was set at 200 rpm. A palladium catalyst for hydrogen-dependent oxygen removal was introduced into the chamber to reduce oxygen contamination. Cultures were grown in 50-mL shake flasks containing 40 mL SMD or biotin-free SMD. Concentrated solutions of ergosterol and/or Tween 80 were added as indicated. Sterile growth media were pre-incubated in the anaerobic chamber for at least 48 h prior to inoculation to allow for removal of oxygen. Growth experiments in the anaerobic chamber were started by inoculating shake flasks, containing SMD or biotin-free SMD, with 200 µL of an exponentially growing oxic pre-culture. Growth was measured by periodic measurements of the OD_{600} with an Ultrospec 10 cell-density meter (Biochrom, Cambridge, UK) placed inside the anaerobic chamber. Strains IMX585 and CEN.PK113-7D grown in SMD without "anaerobic" growth factors were used as controls for absence of oxygen in all anoxic

experiments [311]. All shake flask experiments were carried out as biological duplicates.

Molecular biology techniques

DNA fragments were amplified by PCR amplification with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Landsmeer, The Netherlands) and desalted or PAGE-purified oligonucleotide primers (Sigma-Aldrich) (Table 3.3). For diagnostic PCR analysis DreamTaq polymerase (Thermo Fisher Scientific) was used according to manufacturers' recommendations. PCR products were separated by gel electrophoresis and, if required, purified with a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA) or GenElute PCR Clean-Up kit (Sigma-Aldrich). Assembly of DNA fragments was, if not mentioned differently, by Golden Gate cloning based on the Yeast Tool Kit methodology [313]. Yeast strains of the CEN.PK lineage were transformed by the lithium acetate (LiAc) method [314]. *S. cerevisiae* Ethanol Red was transformed using electroporation as previously described [278]. Electroporated cells were plated on selective YPD hygromycin or YPD geneticin (G418) agar medium. Genomic DNA of transformants was isolated using the YeaStar Genomic DNA kit (Zymo Research) or with the SDS/LiAc protocol [315]. *E. coli* cells were chemically transformed [316] and plated on selective LB agar. Plasmids from selected clones were isolated from *E. coli* with a Sigma GenElute Plasmid kit (Sigma-Aldrich) and verified by restriction analysis (Thermo Fisher Scientific) according to the manufacturer's recommendations or by diagnostic PCR.

Plasmid construction

Construction of part plasmids using Yeast Tool Kit

Coding sequences of *EcfabD, EcbioC, EcfabB, EcfabG, EcfabZ, EcfabI, EcbioH, EcbioF, EcacpP* and *EcacpS* were codon optimized for expression in *S. cerevisiae* using JCat [317] and synthesized by GeneArt (Thermo Fisher Scientific). *E. coli* cells were chemically transformed with the plasmids harbouring the coding sequences together with 5' and 3' flanks compatible with the YTK type 3 BsaI sites [313] and after selection for the antibiotic marker stored as Yeast Tool Kit type plasmids pUD671, pUD663, pUD664, pUD665, pUD666, pUD667, pUD668, pUD669, pUD661, pUD662 (Table 3.3).

The promoter sequence *ScPFK2*p was obtained by PCR application from genomic DNA of CEN.PK113-7D using primer pair 9630/9631. The promoter sequence was introduced in the entry vector pUD565 [281] using BsmBI-T4 ligase directed Golden Gate cloning resulting in Yeast Tool Kit type 2 plasmids pGGkp031. Correct assembly was confirmed by restriction analysis with enzyme PvuII (Thermo Fisher Scientific) according to manufacturer's recommendations. The Yeast Tool Kit type plasmid was propagated in *E. coli* grown in liquid LB chloramphenicol at 37°C and stored at -80°C.

Table 3.1 | List of strains used in this study

Strain	Genotype	Reference or source
CEN.PK113-7D	*MATa MAL2-8c SUC2*	[308]
CEN.PK-122	*MATa/MATa*	[308]
IMS0481	Single colony isolate of CEN.PK113-7D evolved in synthetic medium without biotin	[299]
IMX1859	*MATa can1Δ::cas9-natNT2 Scsga1Δ::ScPYK1p-CfBIO1-ScBIO1t*	[300]
IMX585	*MATa can1Δ::cas9-natNT2*	[312]
IMX2600	*MATa can1Δ::cas9-natNT2*	This study
IMX2035	*MATa can1Δ::cas9-natNT2 Scsga1Δ::SkADH1p-EcfabD-ScADH1t_SkTDH2p-EcbioC-ScTEF2t_SkPDC1p-EcfabB-ScPYK1t_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioF-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t*	This study
IMX2122	*MATa can1Δ::cas9-natNT2 Scsga1Δ::SkADH1p-EcfabD-ScADH1t_SkTDH2p-EcbioC-ScTEF2t_SkPDC1p-EcfabB-ScPYK1t_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioF-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t Scbio1Δ*	This study
IMS0994	Single colony isolate of IMX2122 evolved under anoxic conditions without biotin in bioreactor A	This study
IMS0995	Single colony isolate of IMX2122 evolved under anoxic conditions without biotin in bioreactor B	This study
Ethanol Red	*MATa/a* (diploid prototrophic industrial bioethanol production strain)	F.R. Lesaffre
IMX2555	Ethanol Red *Scsga1Δ::SkADH1p-EcfabD-ScADH1t_SkTDH2p-EcbioC-ScTEF2t_SkPDC1p-EcfabB-ScPYK1t_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioF-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t/Scsga1Δ::SkADH1p-EcfabD-ScADH1t_SkTDH2p-EcbioC-ScTEF2t_SkPDC1p-EcfabB-ScPYK1t_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioF-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t*	This study
IMX2632	Ethanol Red *Scsga1Δ::SkADH1p-EcfabD-ScADH1t_SkTDH2p-EcbioC-ScTEF2t_SkPDC1p-EcfabB-ScPYK1t_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioF-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t/Scsga1Δ::AgTEFp-kanMX-AgTEFt_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioF-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t*	This study
IMX2706	*MATa can1Δ::cas9-natNT2 Scsga1Δ::SkTDH2p-EcbioC-ScTEF2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioFΔ-ScTPIt*	This study
IMX2707	*MATa can1Δ::cas9-natNT2 Scsga1Δ::SkADH1p-EcfabD-ScADH1t_SkTDH2p-EcbioC-ScTEF2t_SkPDC1p-EcfabB-ScPYK1t_SkFBA1p-EcfabG-ScFBA1t_SePDC1p-EcfabZ-ScPDC1t_ScENO2p-EcfabI-ScPFK2t_ScPYK1p-EcbioH-ScPGI1t_ScPFK2p-EcbioFΔ-ScTPIt_ScPGI1p-EcacpP-ScGPM1t_ScHXK2p-EcacpS-ScTDH3t*	This study

The terminator sequences *ScFBA1t*, *ScTPI1t* and *ScPGI1t* were obtained by PCR with primer combinations 10757/10758, 10765/10766 and 10771/10772, respectively using genomic DNA of *S. cerevisiae* CEN.PK113-7D as template. The terminator sequences were cloned in pUD565 using BsmBI-T4 DNA ligase directed Golden Gate cloning yielding the Yeast Tool Kit type 4 plasmids pGGkp046, pGGkp042 and pGGkp044

respectively. After assembly and transformation into *E. coli*, plasmids harbouring the terminator sequences were confirmed by restriction analysis with enzyme SspI (Thermo Fisher Scientific) according to manufacturer's recommendations. The Yeast Tool Kit type plasmids were stored in transformed *E. coli* cultures.

The promoter sequence *ScHXK2*p was synthesized by GeneArt (Thermo Fisher Scientific) and is harboured by Yeast Tool Kit type 2 plasmid pGGkp096. The Yeast Tool Kit type plasmid was propagated in a chemically transformed *E. coli* culture in liquid LB chloramphenicol medium grown at 37°C on a rotary shaker and subsequently stored at -80°C.

Construction of gRNA-expressing plasmid pUDR791

The $gRNA_{EcBIOF}$ expressing plasmid pUDR791 was constructed *in vitro* by Gibson assembly. The linearized pROS11 plasmid, obtained by PCR with 6005/6006 was assembled with a PCR amplified fragment using primer 18409 and pROS11 as a template [312]. Plasmid DNA was isolated from *E. coli* and correct assembly of plasmid pUDR791 was confirmed by diagnostic PCR with primers 18457/3841/5941.

Construction of expression cassettes

The *E. coli fabD* expression cassette was constructed by BsaI–T4 DNA ligase directed Golden Gate cloning combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp062, pUD671, pGGkp037 yielding plasmid pUD978. The next expression plasmids were constructed following a similar cloning principle. The *E. coli bioC* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp063, pUD663, pGGkp038 yielding plasmid pUD979. The *E. coli fabB* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp064, pUD664, pGGkp040 yielding plasmid pUD980. The *E. coli fabG* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp065, pUD665, pGGkp046 yielding plasmid pUD981. The *E. coli fabZ* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp074, pUD666, pGGkp045 yielding plasmid pUD982. The *E. coli fabI* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp028, pUD667, pGGkp103 yielding plasmid pUD983. The *E. coli bioH* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp117, pUD668, pGGkp044 yielding plasmid pUD984. The *E. coli bioF* expression cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp031, pUD669, pGGkp042 yielding plasmid pUD985. The *E. coli acpP* expression cassettee was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp033, pUD661, pGGkp048 yielding plasmid pUD986. The *E. coli acpS* expression

cassette was constructed by combining DNA fragments with compatible overhangs from plasmids pGGkd015, pGGkp096, pUD662, pGGkp041 yielding plasmid pUD987. After assembly reaction and transformation of *E. coli* with the plasmids carrying the expression cassettes, four to eight colonies were selected for each plasmid, followed by isolation of plasmid DNA.

Table 3.2 | List of primers used in this study.

Primer No.	Sequence 5' → 3'
1719	TCCATCCGGTCTTTATCGAC
7469	GGAGTTGACCGTCTTAACAG
9630	AAGCATCGTCTCATCGGTCTCAAACGTATTCTTAGTGGATAACATGCG
9631	TTATGCCGTCTCAGGTCTCACATATTTTAGGCTGGTATCTTGATTC
10320	CATGCGCGGATGACACGAAC
10325	AGTCATCCGAGCGTGTATTG
10757	AAGCATCGTCTCATCGGTCTCAATCCGTTAATTCAAATTAATTGATATAGTTTTTTAATG
10758	TTATGCCGTCTCAGGTCTCACAGCCGCGAACTCCAAAATGAGC
10765	AAGCATCGTCTCATCGGTCTCAATCCGATTAATATAATTATATAAAAATATTATCTTCTTTTC
10766	TTATGCCGTCTCAGGTCTCACAGCCGGTACACTTCTGAGTAAC
10771	AAGCATCGTCTCATCGGTCTCAATCCACAAATCGCTCTTAAATATATACC
10772	TTATGCCGTCTCAGGTCTCACAGCGAAATAGGACCTGATATCCTCC
10873	ACGTGCGGAATAGGAATCTC
11898	CGCGGAAACGGGTATTAGGG
11899	CTAGATCCGGTAAGCGACAG
12223	CCAGGTGGCGTGCTAAACTTTTATAATGTATAAAAACCACCACCTCATAAAGTTTACTGGATATCATCATT TCTGCCACAAATATATGTACTGAGTCTATACGTCAAAGTAAAAAAATAA
12224	TTATTTTTTTACTTTGACGTATAGACTCAGTACATATATTTGTGGCAGAAATGATGATATCCAGTAAACTTTATG AGGTGGTGGTTTTTATACATTATAAAAGTTTAGCACGCCACCTGG
12450	TCTGTCAGTTGGTTAAGCGCCGCTACGATTACTACACATGCCACAGACTGATCTACAATGTATCCTCCTT TTAAACAGTTGATG
12455	ACATTGCATGGAATCAGGGCCTCAATATGTGGGAGAATGCATGAGTACGCGAGCGATCCTCCTGGT CAAACTTCAGAACTAAG
12655	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAATAAGTAACAGCAGCAAATCCGATTTCC GTGGTTGATG
12656	CCAGGGCTCAAATGGCATAAACACTGATGGAACAGGTAGCATCGAACGTGTGTCAAACGCATGTTA GCGTCAACAACAAG
12657	GGCACAGACGAATCACTGACTGATCTGTACCACTGCGTCGACATAACTTTCCAGAAGCGCGTGGGT GCGTCAACTACATC
12658	TGAGCCAGTGCATTCCATCGATGCAGATTCGCGTCCACGTAACGTATCGGAAGCATAGGCAACAAT GCCAACCCCTCTAC
12659	TGTGAGCAGTCATCCACTCGGCATAAGCCTGAATTGCACCATATCCTTGGAAGCCTGGGCGAAGCT ATCTTCCGGTTATG
12660	TCCTCGACGCGATGGCATATCCAGTGTGATAACGTATGAGAAGGTACTGGAAGCTACTGCAACACTA AACGAAGGCTATC
12663	CACTGCGTGTTAAGGATATGCCTAAGGATACATGACACGCATAGCTCATTAACCGGCACGTGGATAA CATGCGGCATTTC
12664	GCCGCGTAGACAATAGATCACCATCTAGTTGAATCCTGAGAGACTATCTCTAATGACCCGGGTAAAG TACAGCTACATTC
12665	GCGTTTGACACACGTTCGATGCTACCTGTTCCATCAGTGTTTATGCCATTTGAGCCCTGGACACACCGA GATTCATCAAC
12666	GCGCTTCTGGAAAGTTATGTCGACGCAGTGGTACAGATCAGTCAGTGATTCGTCTGTGCCATATACATA CGCTGACATGG
12667	GCCTATGCTTCCGATACGTTACGTGGACGCGAATCTGCATCGATGGAATGCACTGGCTCATCGCCAT CCTGATAATCATG
12668	GCCCAGGCTTCCAAGGATATGGTGCAATTCAGGCTTATGCCGAGTGGATGACTGCTCACATTGAAAT GACTCCGCAGTGG
12669	GCAGTAGCTTCCAGTACCTTCTCATACGTTATCACACTGGATATGCCATCGCGTCGAGGATTCCTTGGTT CCACTAATTC
12674	GAAAAAACTAGATCCGGTAAGCGACAGATCTTTGAATTTGTTTATAGCCGACTCTAAGTCCAGAATCGTT ATCCTGGCGG
12745	AGCGTAGATAGAAGCGTCAG
12746	TCCAGTTGGTGACGTTAAGG
12747	TCAGCACCCAAGTCTTCAAC
12749	TCCAGATAGCCCATTCGTTG

12750	ACACTACGCTTGTGCTACTG
12751	GAAGCACCAGTAACCAAAGC
12752	CGAAGCTGCTTACATCACTG
12759	ATTGGCTTACCTGGGAAGTG
12760	TGCTTTGGTTGACGGTAAGG
12761	GTCAGCCAACATACCAACAG
12762	ACGAAGTTGGTCCAGGTAAG
12763	CGATACCGTAAGCGATAGAC
12764	CGCTGCTATGAACGAATTGG
13280	GGTTGCTTTGAAGCAAAGAG
13281	TTTGCCACCAGATGTTGTTC
13283	CAGATACTGGCGATCATCCG
13284	CTTGGGTGTTATCGCTAGAG
13483	TCTCCAGGACCATCTGAATC
13545	TTTGTGGCAACATAGCCAAC
13718	CCAATGAGTCTTCACATGGCGCGTGTCATGTATCCTTAGGCATATCCTTAACACGCAGTGCGGTACAC TTCTGAGTAACC
13748	CGGGTCATTAGAGATAGTCTCTCAGGATTCAACTAGATGGTGATCTATTGTCTACGCGGCTTGGCAGC CATTAAACTACG
14000	AGGATCGCTCGCGTACTCATGCATTCTCCCACATATTGAGGCCCTGATTCCATGCAATGTCAGCAAAT CGTCTATATCAC
14448	CATTGTAGATCAGTCTGTGGCATGTGTAGTAATCGTAGCGGCGCTTAACCAACTGACAGACATTCTCTG CTGCTTTGTTG
17154	GCGCTGGCAGTGTTCCTGCG
17991	TCTTTCTTGTTAAAAATTTCAAGCTATACCAAGCATACAATCAACTATCTCATATACAATACGCTGCAGGTC GACAACC
17992	ATAAAATTTAAAAAATATAATTCAAAAAAATAATATCTTCATTCAATCATGATTCTTTTTCTAGTGGATCTGATAT CACC
18404	GAAAAAACTAGATCCGGTAAGCGACAGATCTTTGAATTTGTTTATAGCCGACTCTAAGTCCGGTACACT TCTGAGTAACC
18405	AGGATCGCTCGCGTACTCATGCATTCTCCCACATATTGAGGCCCTGATTCCATGCAATGTATATACATA CGCTGACATGG
18406	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAATAAGTAACAGCAGCAAAATGTTAGCGT CAACAACAAG
18407	GAACAATAGAACTAGATTTAGAGACTAGTTTAGCATTGGCCAAGAACTAACCATACGCATATCCGATTA ATATAATTATATAAAAATATTATCTTCTTTTCTTTATATCTAGTGTTATGT
18408	ACATAACACTAGATATAAAGAAAAGAAGATAATATTTTTATATAATTATATTAATCGGATATGCGTATGGTTA GTTCTTGGCCAATGCTAAACTAGTCTCTAAATCTAGTTCTATTGTTC
18409	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCCTGGCAGGAGAAAATCA ACGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
18418	CGTTGATTTTCTCCTGCCAG

Correct assembly was checked by diagnostic PCR primer combinations, with one primer binding outside the expression cassette and one within the gene sequence: 13483/12761 for *EcfabD,* 10320/10325 for *EcbioC,* 13483/12745 for *EcfabB,* 13483/12751 for *EcfabG,* 13483/12759 for *EcfabZ,* 13483/12763 for *Ecfabl,* 10320/10325 for *EcbioH,* 13483/13283 for *EcbioF,* 10320/10325 for *EcacpP* and 13483/12749 for *EcacpS*. The obtained plasmids were stored as pUD979, pUD980, pUD981, pUD982, pUD983, pUD984, pUD985, pUD986, pUD987.

Strain construction

Integration of *E. coli* bio gene expression cassettes into *S. cerevisiae*

S. cerevisiae IMX2600 was constructed by homology-directed repair by assembly and integration of two cassettes containing *Spycas9* and the natNT2 marker into the *CAN1* locus as described in [312]. The *EcbioC, EcbioH* and *EcbioF* expression cassettes were PCR-amplified with the following primer pairs adding 60-bp homologous sequences

[319]: 18406/18405 for *EcbioC* (pUD979), 12455/12450 for *EcbioH* (pUD984) and 14448/18404 for *EcbioF* (pUD985). Targeting at the *ScSGA1* locus in IMX2600 was directed by Cas9 activity and a target-specific gRNA expressing plasmid. The strain was co-transformed with the *EcbioC, EcbioH* and *EcbioF* expression cassette fragments and the plasmid pUDR119 expressing the gRNA to target Cas9 activity to the *ScSGA1* locus [318] using the LiAc transformation protocol. Transformed cells were plated on selective SMD with acetamide and incubated for 3 days at 30°C. Genomic DNA of colonies was isolated and the desired genotype confirmed by diagnostic PCR using primer combinations 11898/13545, 13284/13281, 13280/13283 and 1719/11899. A verified clone was inoculated in 20 mL non-selective YPD for plasmid removal and incubated for 24 h at 30°C. Cells were plated on YPD agar to obtain single colony isolates. One isolate was re-streaked on both selective medium and YPD. When no growth was observed on selective medium the respective clone was again checked by diagnostic PCR with above-mentioned primer combinations. The strain with *in vivo* assembled expression cassettes of the *E. coli bio* genes into *ScSGA1* was stored as IMX2706.

Integration of *E. coli* KAPA synthesis in IMX585 and Ethanol Red

Expression cassettes were PCR-amplified with the following primer pairs, thereby adding 60-bp homologous sequences [320] to enable *in vivo* assembly at the *ScSGA1* locus: 12655/12665 for *EcfabD* (pUD978), 12656/12666 for *EcbioC* (pUD979), 12657/12667 for *EcfabB* (pUD980), 12658/12668 for *EcfabG* (pUD981), 12659/12669 for *EcfabZ* (pUD982), 12660/14000 for *EcfabI* (pUD983), 12455/12450 for *EcbioH* (pUD984), 14448/13718 for *EcbioF* (pUD985), 12663/13748 for *EcacpP* (pUD986) and 12664/12674 for *EcacpS* (pUD987). The resulting expression cassettes were integrated at the *ScSGA1* locus in IMX585 and Ethanol Red, by transformation of specific gRNA encoded on plasmid pUDR119 in case of IMX585 and in case of Ethanol Red by plasmid pUDP145. Targeting at the *ScSGA1* locus in IMX585 was directed by strain-intrinsic Cas9 activity and in Ethanol Red by expression of *Spycas9* from plasmid pUDP145 [283]. Yeast strains were co-transformed with the respective plasmids and the *EcfabD, EcbioC, EcfabB, EcfabG, EcfabZ, EcfabI, EcbioH, EcbioF, EcacpP and EcacpS* expression cassettes using the LiAc transformation protocol.

Table 3.3 | List of plasmids constructed and used in this study.

Name	Characteristics	Reference or source
pGGkd015	*bla* ColE1 Gfp dropout	[281]
pGGkp028	*cat* ColE1 *ScENO2*p	[281]
pGGkp031	*cat* ColE1 *ScPFK2*p	This study
pGGkp033	*cat* ColE1 *ScPGI1*p	[281]
pGGkp037	*cat* ColE1 *ScADH1*t	[281]
pGGkp038	*cat* ColE1*ScTEF2*t	[281]
pGGkp040	*cat* ColE1 *ScPYK1*t	[281]
pGGkp041	*cat* ColE1 *ScTDH3*t	[281]
pGGkp042	*cat* ColE1 *ScTPI*t	This study
pGGkp044	*cat* ColE1 *ScPGI1*t	This study
pGGkp045	*cat* ColE1 *ScPDC1*t	[281]
pGGkp046	*cat* ColE1 *ScFBA1*t	This study

pGGkp048	*cat* ColE1 *ScGPM1*t	[281]
pGGkp062	*aphA* ColE1 *SkADH1*p	[281]
pGGkp063	*aphA* ColE1 *SkTDH3*p	[281]
pGGkp064	*aphA* ColE1 *SkPDC1*p	[281]
pGGkp065	*aphA* ColE1 *SkFBA1*p	[281]
pGGkp074	*cat* ColE1 *SePDC1*p	[281]
pGGkp096	*cat* ColE1 *ScHXK2*p	GeneArt
pGGkp103	*cat* ColE1 *ScPFK2*t	[281]
pGGkp117	*cat* ColE1 *ScPYK1*p	[300]
pUD565	*cat* ColE1 *GFP*	[281]
pUD661	*bla* ColE1 *EcacpP*	GeneArt
pUD662	*bla* ColE1 *EcacpS*	GeneArt
pUD663	*bla* ColE1 *EcbioC*	GeneArt
pUD664	*bla* ColE1 *EcfabB*	GeneArt
pUD665	*bla* ColE1 *EcfabG*	GeneArt
pUD666	*bla* ColE1 *EcfabZ*	GeneArt
pUD667	*bla* ColE1 *EcfabI*	GeneArt
pUD668	*bla* ColE1 *EcbioH*	GeneArt
pUD669	*bla* ColE1 *EcbioF*	GeneArt
pUD671	*bla* ColE1 *EcfabD*	GeneArt
pUD978	*bla* ColE1 *SkADH1p-EcfabD-ScADH1*t	This study
pUD979	*bla* ColE1 *SkTDH3p-EcbioC-ScTEF2*t	This study
pUD980	*bla* ColE1 *SkPDC1*p-*EcfabB-ScPYK1*t	This study
pUD981	*bla* ColE1 *SkFBA1*p-*EcfabG-SCfBA1*t	This study
pUD982	*bla* ColE1 *SePDC1*p-*EcfabZ-ScPDC1*t	This study
pUD983	*bla* ColE1 *ScENO2*p-*EcfabI-ScPFK2*t	This study
pUD984	*bla* ColE1 *ScPYK1*p-*EcbioH-ScPGI1*t	This study
pUD985	*bla* ColE1 *ScPFK2*p-*EcbioF-ScTPI*t	This study
pUD986	*bla* ColE1 *ScPGI1*p-*EcacpP-ScGPM1*t	This study
pUD987	*bla* ColE1 *ScHXK2*p-*EcacpS-ScTDH3*t	This study
pUDP145	*bla* ColE1 panARS(OPT) *hph ScTDH3*p-HH-gRNA$_{ScSGA1}$-HDV-*ScCYC1*t *AaTEF1*p-Spc*as9*$^{D147Y\ P411T}$-*ScPHO5*t	[300]
pUDR119	*bla* ColE1 2µ *amdS ScSNR52*p-gRNA$_{ScSGA1}$-*ScSUP4*t	[318]
pUDR244	*bla* ColE1 2µ *amdS ScSNR52*p-gRNA$_{ScBIO1}$- *ScSUP4*t	[300]
pUDR791	*bla* ColE1 2µ *amdS ScSNR52*p-gRNA$_{EcBioF}$-*ScSUP4*t	This study
pROS11	*bla* ColE1 2µ *amdS ScSNR52*p-gRNA$_{CAN1}$-*ScSUP4*t-*ScSNR52*p-gRNA$_{ADE2}$-*ScSUP4*t	[312]
pROS13	*bla* ColE1 2µ kanMX *ScSNR52*p-gRNA$_{CAN1}$-*ScSUP4*t-*ScSNR52*p-gRNA$_{ADE2}$-*ScSUP4*t	[312]

Transformed cells were plated on selective SMD with acetamide in case of IMX585 and on YPD with hygromycin in case of Ethanol Red and incubated for 3 days at 30°C Genomic DNA of colonies was isolated and the desired genotype confirmed by diagnostic PCR using following primer combinations 11898/12761, 12762/13545, 13284/12745, 12746/12751, 12752/12759, 12760/12763, 12764/13281, 13280/13283, 1719/12747 and 12750/11899. Single colony isolation and plasmid removal was performed as described for strain IMX2706. Strain IMX585 with *in vivo* assembled expression cassettes for *E. coli* KAPA synthesis into *ScSGA1* was stocked as IMX2035 and strain Ethanol Red with this modification as IMX2555 at -80°C. The genome of strain IMX2035 was sequenced by Illumina technology (Illumina, San Diego, CA) to confirm mutation-free integration of the pathway genes.

Gene deletion

To delete the native *ScBIO1* locus in *S. cerevisiae* IMX2035, it was co-transformed with plasmid pUDR244 [300] and a repair DNA fragment resulting from the annealing of oligo-nucleotides 12223/12224. Transformed cells were plated on selective SMD acetamide and incubated for 3 days at 30°C. Genomic DNA of colonies was isolated and the desired genotype confirmed by diagnostic PCR using primer pair 7469/10873. A verified clone was inoculated in 20 mL non-selective YPD for plasmid removal and incubated for 24 h at 30°C. Cells were plated on YPD agar in order to obtain single colony isolates. One isolate was re-streaked on both SMD acetamide and YPD. When no growth was observed on SMD acetamide the respective clone was once again confirmed by diagnostic PCR and stored as IMX2122. Similarly, to delete the heterologously expressed *EcbioF* gene, strain IMX2035 was co-transformed with plasmid pUDR791 and a repair DNA fragment resulting from the annealing of the oligo-nucleotides 18407/18408. After growth on selective SMD acetamide, genotyping of the resulting colonies was carried out by diagnostic PCR with primer pair 1719/12747. After plasmid removal a single colony was isolated and stored as IMX2707.

Deletion of *EcfabD*, *EcbioC* and *EcfabB* in *S. cerevisiae* IMX2555, which was derived from the diploid *S. cerevisiae* strain Ethanol Red containing the KAPA synthesis pathway, was performed by transformation with and integration of a deletion cassette. The transformed linear DNA fragment contained 60-bp flanks homologous to the *SkADH1* promoter and the intergenic region between the *EcfabB* and *EcfabG* expressional units and the KanMX expression cassette conferring resistance to geneticin [321]. The linear DNA fragment with the deletion cassette was obtained by PCR with the primer pair 17991/17992 using plasmid pROS13 as a template. Upon homologous recombination, the deletion cassette replaced one of the two copies of the three expression cassettes for *EcfabD*, *EcbioC* and *EcfabB*. Electroporated cells were plated on selective YPD G418 agar plates and incubated for 5 days at 30°C. Genomic DNA of transformants was isolated and the desired genotype was confirmed by diagnostic PCR using following primer combinations 11898/12761, 12762/13545, 13284/12745, 11898/12562, 12751/17154. The correct clone was re-streaked on YPD agar to obtain single colony isolates. A single colony was once again confirmed by diagnostic PCR with above-mentioned primer combinations and inoculated for stocking in 20 mL non-selective YPD. The Ethanol Red strain with the integration of the KanMX cassette in the ScSGA1 locus was stored as IMX2632.

Batch cultivation in bioreactors

Physiological characterization of *S. cerevisiae* IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway) was performed in anoxic bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1.0 L. All cultures were grown on biotin-free SMD; anoxic cultures were supplemented with sterile solutions of the "anaerobic" growth factors ergosterol (10 mg L^{-1}) and Tween 80 (420 mg L^{-1}), as well as with 0.2 g L^{-1} sterile antifoam C

(Sigma-Aldrich). These conditions were maintained by sparging cultures with a gas mixture of N_2/CO_2 (90/10%, < 10 ppm oxygen) at a rate of 0.5 L min^{-1}. Culture pH was maintained at 5.0 by automatic addition of 2 M KOH. All cultures were grown at a stirrer speed of 800 rpm and at a temperature of 30°C. Oxygen diffusion in the bioreactors was minimized by using Neoprene tubing and Viton O-rings, and evaporation was minimized by cooling of outlet gas to 4°C. Oxic conditions were maintained by sparging with pressurised air at a rate of 0.5 L min^{-1}. For bioreactor inocula, a 1 mL aliquot of a thawed stock culture of strain IMX2122 was inoculated in 100 mL biotin-free SMD in a 500 mL-shake flask and incubated for 20 h at 30°C. A second 100 mL biotin-free SMD culture was started by inoculating 2 mL of the first shake flask culture. Shake flasks were incubated at 30°C and 200 rpm in an Innova incubator (Brunswick Scientific). When the second culture reached mid-exponential phase (OD_{660} of 3-5) it was used to inoculate the bioreactors at an OD_{660} of 0.1-0.3. Growth in the bioreactor was monitored based on the CO_2 concentration in the off-gas. Specific growth rates were calculated from CO_2 concentration values collected during exponential growth and covering 3-4 doublings. Specific growth rate was calculated using the equation $X = X_0 e^{\mu t}$ in which μ indicates the exponential growth rate. After anaerobic cultures had reached a first CO_2 production peak and the CO_2 percentage in the off-gas subsequently decreased below more than 20% of the previously measured value, a computer-controlled peristaltic pump automatically removed *ca.* 90% of the culture volume, leaving *ca.* 10% as an inoculum for the next batch cultivation cycle that occurred after refilling the reactor with fresh medium. Specific growth rates in absence of oxygen were determined from the CO_2 profile after two empty-refill cycles in order to deplete "anaerobic" growth factors from the pre-cultures that were run in presence of oxygen [311].

Laboratory evolution

Laboratory evolution of *S. cerevisiae* IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway) for fast anoxic growth without biotin supplementation was performed in sequential-batch bioreactor cultures. Empty-refill cycles in two independent anaerobic bioreactors, operated as described above, were continued until no further increase of the specific growth rate was observed for at least five consecutive batch cultivation cycles. Single-colony isolates from reactor A were obtained after 109 cycles and from reactor B after 100 cycles by plating on biotin-free SMD.

Whole-genome sequence analysis

DNA of *S. cerevisiae* strains IMX2035, IMX2122, IMS0994 and IMS0995 grown in shake-flask cultures with SMD was isolated with a Qiagen Blood & Cell Culture DNA kit (Qiagen, Germantown, MD), following manufacturer's specifications. Paired-end sequencing was performed on a 350-bp PCR-free insert library using an Illumina HiSeq PE150 sequencer (Novogene Company Limited, Hong Kong). Sequence data was mapped to the CEN.PK113-7D genome [309], to which the sequences of the integrated expression cassettes for the heterologous genes *EcfabD, EcbioC, EcfabB,*

EcfabG, EcfabZ, EcfabI, EcbioH, EcbioF, EcacpP and *EcacpS* were manually added. Data processing and chromosome copy number analysis were carried out as described previously [299, 322].

Ploidy analysis by flow cytometry

For determination of ploidy, frozen aliquots of *S. cerevisiae* strains IMX2035, IMX2122 and the evolved strains IMS0994 and IMS0995 were thawed and used to inoculate 20-mL cultures on SMD (IMX2035 and IMX2122) or on biotin-free SMD (IMS0994 and IMS0995). After incubation at 30°C until mid-exponential phase, cells were harvested, washed twice with demineralized water and stored in 70% ethanol at 4 °C. Sample preparation and staining were performed as described previously [323, 324]. Samples were processed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and analysed using the FlowJo software package (Flowjo LLC, Ashland, OR). *S. cerevisiae* strains CEN.PK113-7D and CEN.PK122 were used a haploid and diploid references, respectively.

Proteome analysis

Frozen aliquots of *S. cerevisiae* strains IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway), IMS0994 (evolution A IMX2122), and IMS0995 (evolution B IMX2122) were thawed and used to inoculate wake-up cultures in 20 mL biotin-free SMD. After overnight incubation at 30°C, these cultures were used to inoculate two independent 100 mL cultures at a starting OD_{660} of 0.2. Once these cultures reached an OD_{660} of 4, 1 mL was collected and centrifuged at 3000 x g for 5 min, yielding a cell pellet with a volume of approximately 60 µL. After protein extraction and trypsin digestion [325], extracted peptides were re-suspended in 30 µL of 3% acetonitrile/0.01% trifluoroacetic acid. The peptide concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific) at a wavelength of 280 nm. A total of 1 µg of sample was injected in a CapLC system (Thermo Scientific) coupled to an Orbitrap Q-exactive HF-X mass spectrometer (Thermo Scientific). First, samples were captured at a flow rate of 10 µL/min on a pre-column (µ-pre-column C18 PepMap 100, 5 µm, 100 Å) and subsequently peptides were separated on a 15 cm C18 easy spray column (PepMap RSLC C18 2 µm, 10^4 pm, 150 µm x 15 cm) using a flow rate of 1.2 µL min^{-1}. A linear gradient from 4% to 76% acetonitrile in water was applied over 60 min. While spraying the samples into the mass spectrometer the instrument was operated in data dependent mode using settings as previously described in [326]. Data analysis was performed using Proteome discover 2.4 (Thermo Scientific) with fixed modifications set to carbamidomethyl (C), variable modifications set to oxidation of methionine residues, search mass tolerance set to 20 ppm, MS/MS tolerance set to 20 ppm, trypsin selected as restriction enzyme and allowing one missed cleavage. False Discovery Rate (FDR) was set at 0.1% and the match between runs window was set to 0.7 min. Quantification was only based on unique peptides and normalization between samples was based on total peptide amount. For protein search, a protein database consisting of the *S. cerevisiae* S288C proteome amino-acid sequences together with

the sequences of the heterologously expressed proteins was used. Each strain was analyzed in independent biological duplicate samples. Data processing and analysis of differentially expressed proteins was conducted as previously described in [325]. Enrichment analysis of up- and downregulated proteins in the isolates was performed using the GO Enrichment Analysis [327].

Results

Expression of the *E. coli* KAPA-biosynthesis pathway supports biotin-independent growth of *S. cerevisiae* in absence of oxygen.

Of the currently known prokaryotic biotin-biosynthesis pathways (Figure 3.1), only the variant that occurs in *E. coli* starts with malonyl-CoA, a key precursor for lipid synthesis in *S. cerevisiae*. To complete the malonyl-CoA conversion into pimeloyl-CoA, only *EcbioC* and *EcbioH* would be required in *S. cerevisiae* assuming that the other reactions could be performed by the native fatty acid elongation machinery. We also included *EcbioF* since it is unclear whether *Sc*Bio6, the protein ortholog of *Ec*BioF, can use pimeloyl-[Acp] as substrate. Integration of these three *E. coli* genes at the *SGA1* locus yielded *S. cerevisiae* IMX2706. Even after prolonged oxic incubation in biotin-free synthetic medium, this engineered strain did not show growth on biotin-free synthetic medium. To investigate whether this inability was related to the different organization of the prokaryotic and yeast fatty-acid-synthesis machineries, we introduced an additional set of expression cassettes for *E. coli* proteins involved in conversion of malonyl-[Acp]-methyl ester into pimeloyl-[Acp]-methyl ester. In addition to *EcbioC, H* and *F*, five genes involved in fatty-acid biosynthesis (*EcfabD, EcfabB, EcfabG, EcfabZ, EcfabI)* and two genes involved in acyl carrier protein formation *(EcacpP* and *EcacpS*) were introduced. In *E. coli,* the concerted action of the enzymes encoded by these genes converts malonyl-CoA into 8-amino-7-oxo-nonanoate (KAPA), a metabolic intermediate of the native *S. cerevisiae* biotin pathway. Using the *Spy*Cas9-expressing strain IMX585, the ten expression cassettes were integrated at the *SGA1* locus, yielding *S. cerevisiae* IMX2035 (↑*Ec*KAPA pathway; Figure 2.2A)

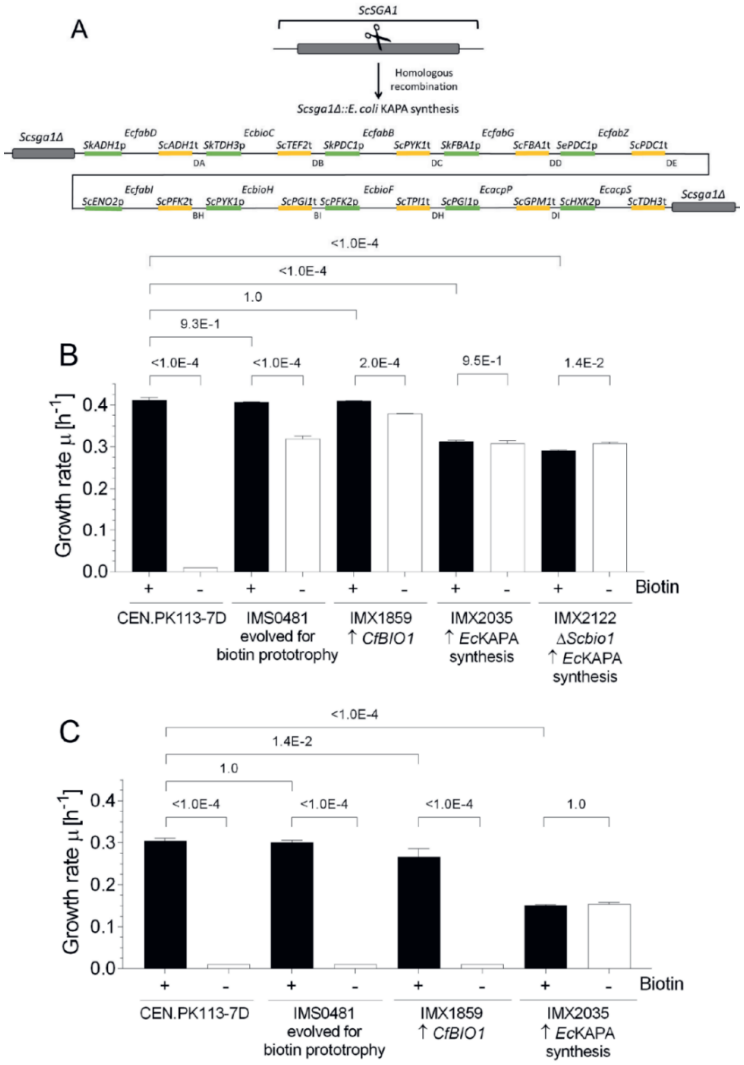

Figure 3.2 | **Expression of *E. coli* KAPA biosynthesis pathway in *S. cerevisiae***. (A) Schematic overview of genetic modifications introduced at the *ScSGA1* locus. A Cas9-induced cut in the *ScSGA1* coding sequence and *in vivo* homologous recombination enabled integration of expression cassettes for ten *E. coli* genes with different promoters (green) and terminators (yellow). Intergenic regions consisted of synthetic 60-bp-homologous recombination sequences [320]. (B) Bar graphs representing average specific growth rates of *S. cerevisiae* strains CEN.PK113-7D, IMS0481 (evolved for biotin prototrophy [299]), IMX1859 (↑*CfBIO1*, [300]), IMX2035 (↑*Ec*KAPA pathway) and IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway) under oxic conditions on glucose synthetic medium with (+, black) and without (-, white) biotin. (C) Bar graphs representing average specific growth rates of *S. cerevisiae* strains CEN.PK113-7D, IMS0481, IMX1859 and IMX2035 under anoxic conditions on glucose synthetic medium with (+, black) and without (-, white) biotin. Averages and deviations of the bar graphs were calculated from independent duplicate cultures. Brackets between two bar graphs show the p-value, which was derived from significance testing of the difference between observed growth rates by one-way analyses of variance (ANOVA) and Tukey's multiple comparison test using GraphPad prism 8.2.1 software (significance threshold $p_{\text{-value}} < 5.0\text{E-}02$).

This engineered strain showed immediate oxic growth on biotin-free synthetic medium, at a specific growth rate of 0.31 ± 0.01 h^{-1}. Under the same conditions, the reference strain CEN.PK113-7D was unable to grow [225, 299, 300] (Figure 3.2B). Compared to previous *S. cerevisiae* strains engineered (IMX1859, [300] or evolved (IMS0481, [299] for biotin prototrophy, IMX2035 grew approximately 25% slower in biotin-supplemented as well as biotin-free media (Figure 2.2B). However, in contrast to these other biotin-prototrophic strains, strain IMX2035 (↑*Ec*KAPA pathway) showed anoxic growth in biotin-free medium, at specific growth rate of 0.15 ± 0.003 h^{-1}. Also, in absence of oxygen, the specific growth rate of strain IMX2035 on biotin-supplement medium was lower than observed in cultures of reference strains (Figure 3.2C). These results demonstrated that expression of the *E. coli* KAPA pathway in *S. cerevisiae* supports conversion of malonyl-CoA into KAPA and promotes biotin-independent anoxic growth of *S. cerevisiae*.

The functionality of the *Ec*KAPA pathway in *S. cerevisiae* IMX2035 enabled us to evaluate whether the orthologs *ScBIO6* and *EcbioF* are functionally redundant. To this end, *EcbioF* was deleted in strain IMX2035, yielding strain IMX2707. This deletion strain did not grow on biotin-free medium, indicating that the yeast 7,8-diamino-pelargonic acid aminotransferase *Sc*Bio6 cannot functionally replace the *E. coli* 8-amino-7-oxononanoate synthase *Ec*BioF.

Laboratory evolution for fast biotin-independent anoxic growth

To exclude the possibility that activity of the native *S. cerevisiae* biotin pathway interfered with the interpretation of results, *ScBIO1* was deleted in strain IMX2035 (↑*Ec*KAPA pathway), yielding strain IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway). *Sc*Bio1 is proposed to catalyse an as yet unidentified reaction for synthesis of pimeloyl-CoA. In oxic cultures, strain IMX2122 showed similar specific growth rates on biotin-supplemented and biotin-free media (specific growth rates of 0.29 ± 0.00 h^{-1} and 0.31 ± 0.00 h^{-1}, respectively, Figure 3.2B). As anticipated, strain IMX2122 grew without oxygen on biotin-free medium, at a specific growth rate of 0.20 ± 0.00 h^{-1} (Figure 3.3C). As observed for strain IMX2035 (↑*Ec*KAPA pathway) biotin supplementation did not restore the specific growth rate of strain IMX2122 to that of reference strain CEN.PK113-7D, which in both cultivation regimes on biotin-supplemented media exhibits specific growth rates of 0.32 - 0.33 h^{-1} [328, 329] and 0.38 - 0.40 h^{-1} [330] ,respectively.

To explore the evolvability of full biotin prototrophy, strain IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway) was grown in two independent, anoxic sequential batch reactors (SBRs) on biotin-free synthetic medium. Throughout the course of SBR cultivation, the specific growth rate of the yeast populations in the two reactions increased to close to 0.32 h^{-1}, which corresponded closely to the reported specific growth rate on the congenic CEN.PK113-7D reference strain in absence of oxygen on chemically defined medium with biotin [328, 329] (Figure 3.3A, B). After 436 (109 batch cycles) and 400 generations (100 batch cycles) for reactor A and B, respectively, single colony isolates (SCI) were

obtained from each reactor (IMS0994 from reactor A and IMS0995 from reactor B). Both these SCI's showed specific growth rates on biotin-free medium of 0.39 ± 0.01 h^{-1}. Under anoxic conditions, specific growth rates of the SCI's were 0.33 ± 0.01 h^{-1} and 0.33 ± 0.02 h^{-1}, respectively. These specific growth rates are virtually identical to those measured in this study for the reference strain CEN.PK113-7D during growth on biotin-containing synthetic medium under both cultivation regimes (0.41 ± 0.01 h^{-1} and 0.31± 0.01 h^{-1}, respectively). Compared to the specific growth rates of their parental strain IMX2122 on biotin-free medium in presence and absence of oxygen, those of the two SCI's had increased by 34% (p = 2E-04) and 57% (p = 1E-04), respectively (Figure 3.3C).

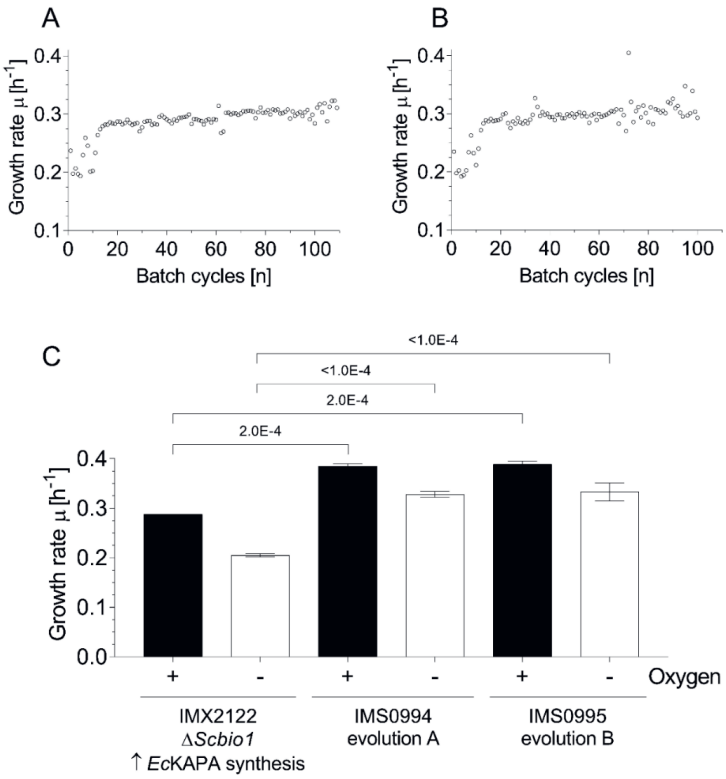

Figure 3.3 | Laboratory evolution of the engineered biotin-prototrophic *S. cerevisiae* strain IMX2122. (A) Specific growth rates of anoxic sequential batch cycles [n] of strain IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway) on biotin-free medium, reactor A. (B) Specific growth rates of anoxic sequential batch cycles [n] of strain IMX2122 on biotin-free medium, reactor B. (C) Bar graphs represent average specific growth rates of the parental strain *S. cerevisiae* IMX2122 and evolved isolates IMS0994 (evolution A IMX2122) and IMS0995 (evolution B IMX2122) on synthetic medium without biotin under oxic (+, black) and anoxic (-, white) conditions. The growth rate means and deviations of the bar graphs were calculated from biological duplicates. Brackets between two bar graphs show the $p_{\text{-value}}$, which was derived from significance testing of the difference between observed growth rates by one-way analyses of variance (ANOVA) and Tukey's multiple comparison test using GraphPad prism 8.2.1 software (significance threshold $p_{\text{-value}}$ < 5.0E-02).

Diploidization and subsequent copy-number reduction of *Ec*KAPA biosynthesis genes contribute to evolved full biotin prototrophy

To identify the genetic basis of the evolved full prototrophy of the evolved isolates IMS0994 and IMS0995, their genomes and that of their share parental strain IMX2122 were sequenced with Illumina short-read sequencing technology and analysed. Sequence reads from the three strains were aligned with a high-quality CEN.PK113-7D genome sequence [309] supplemented with the sequence of the contig comprising the expression cassettes of the engineered *E. coli* KAPA-pathway.

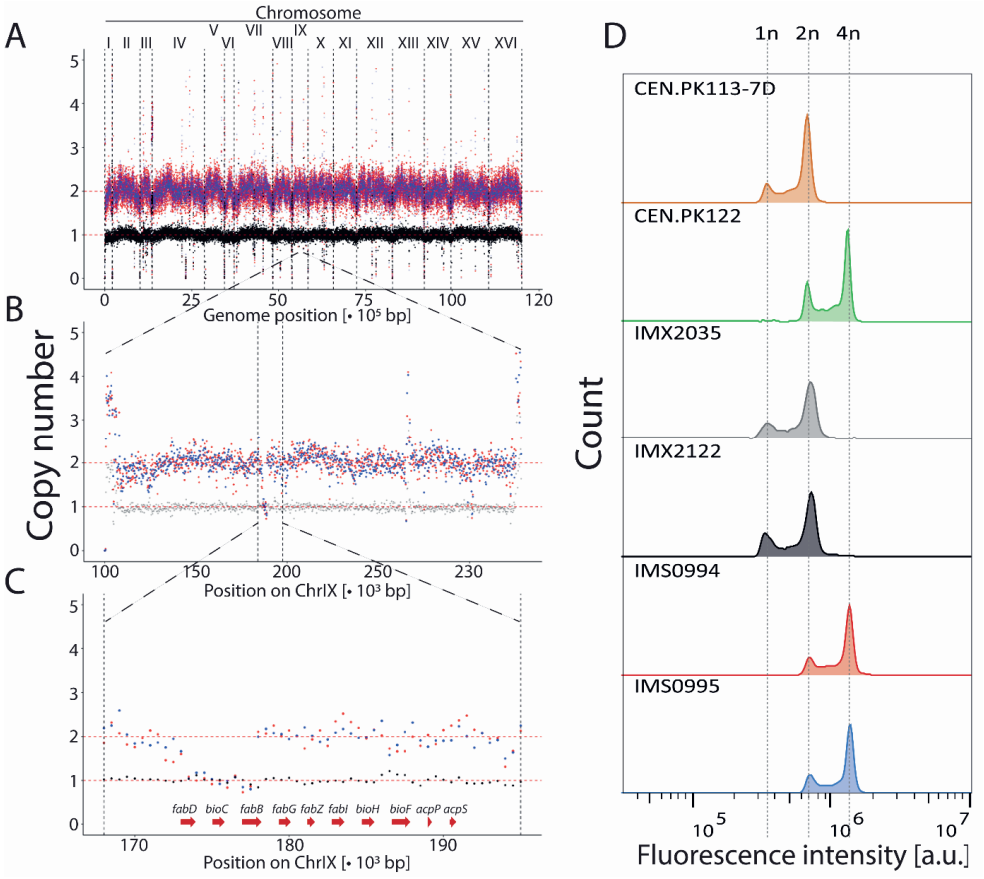

Figure 3.4 | Genetic alterations of the evolved isolates IMS0994 and IMS0995 compared to the initial engineered strain IMX2122. Copy number coverage plots of IMX2122 (*Scbio1*Δ ↑*Ec*KAPA pathway, black), IMS0994 (evolution A IMX2122, red) and IMS0995 (evolution B IMX2122, blue) over the whole genome (A), from position 100 to 250 kbp on CHRIX (B), from position 168 to 195 kbp on CHRIX, regions including the *E. coli* KAPA pathway *SGA1* integration site. The position of coding sequences of *E. coli* genes *fabD, bioC, fabB, fabG, fabZ, fabI, bioH, bioF, acpP* and *acpS* is indicated by red arrows (C). Histograms of fluorescence intensity of nucleic-acid-stained cells of haploid CEN.PK113-7D (orange), diploid CEN.PK122 (green), IMX2035 ((↑*Ec*KAPA pathway), grey), IMX2122 (dark grey), IMS0994 (red) and IMS0995 (blue). Vertical dashed lines indicate the fluorescence intensity of reference haploid (1n), diploid (2n) and tetraploid (4n) cells (D).

Mapped data were analysed for copy number variations (CNVs), structural modifications and single nucleotide variations (SNVs) in annotated coding sequences. Prior to sequence data analysis, the nominal strain ploidy of IMX2035, IMX2122, IMS0994 and IMS0995 was analysed by nucleic acid staining and subsequent flow-cytometry analysis. The genetically engineered strains IMX2035 and IMX2122 exhibited the same ploidy as the haploid reference strain CEN.PK113-7D. In contrast, a higher fluorescence intensity of both evolved SCI's (IMS0994 and IMS0995) corresponded with that of the diploid reference strain CEN.PK122 (Figure 3.4) and indicated that a whole-genome duplication had occurred in two independent evolution experiments. CNV analysis of strains IMX2122 and IMS0994-5 did reveal a segmental aneuploidy of the engineered *SGA1* locus in which the *E. coli* KAPA pathway was integrated. As anticipated, the read coverage over the contig harboring the *E. coli* KAPA-pathway cassettes in the parental strain IMX2122 was the same as that of the rest of the genome. In contrast, the evolved SCI's IMS0994 and IMS0995 showed a 50% lower coverage for a region comprising the three contiguous expression cassettes for *EcfabD*, *EcbioC* and *EcfabB* (Figure 3.4 A-B-C). This coverage reduction relative to the rest of the genome was consistent with the overall 2n ploidy of the evolved isolates (Figure 3.4 C-D). While no homozygous SNVs were found in coding regions of the two evolved SCI's, a single homozygous SNV in IMS0994 was identified in the intergenic region between *PTR2* and *MLP1* on CHRXI. In addition, the two SCI's harbored a small number of heterozygous SNVs that caused amino-acid changes in the peptide sequence encoded by the mutated allele. In IMS0994 nine heterozygous SNVs occurring in coding sequences were found to be distributed over five genes (*FLO11*, *AGA1*, *MFa1*, *TIF3* and *ADE3*). Similarly, IMS0995 harbored ten heterozygous SNVs scattered over the coding sequences of four genes (*GLT1*, *MFa1*, *ECM38* and *TIF3*). Out of these heterozygous SNVs, five observed in *TIF3* and one detected in *MFa1* were shared by the two evolved isolates suggesting that these SNVs might originate from stock cultures used to inoculate the evolution cultures. None of the affected genes showed an obvious functional relationship with biotin-related cellular processes and the individual impact of these SNVs was not further studied.

To investigate the impact of the altered gene dosage of three *E. coli* KAPA biosynthesis genes in the evolved strains, levels of the *E. coli* KAPA pathway proteins were quantified in strains IMX2122, IMS0994 and IMS0995. Consistent with their lower copy number relative to the remainder of the genome in the evolved SCI's, abundances of the 3-oxoacyl-[Acp] synthase *Ec*FabB, the malonyl CoA-acyl carrier protein transacylase *Ec*FabD and the malonyl-[Acp] O-methyltransferase *Ec*BioC in IMS0994 and IMS0995 were at least 1.8-fold lower than those of the non-evolved parental strain IMX2122 (Figure 3.5). Despite the change in ploidy, no differences in average protein abundance were observed between the three strains (Figure 3.5A). While all expressed heterologous proteins were detected, *Sc*Bio2 was the only native biotin-synthesis pathway detected in the samples.

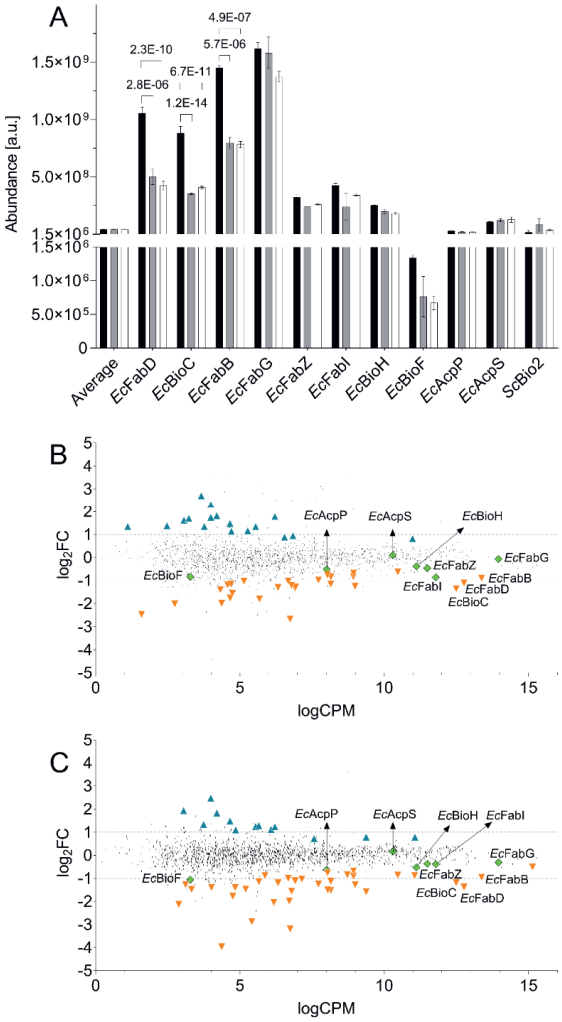

Figure 3.5| Abundance of the proteins of the *Ec*KAPA pathway in IMX2122 and its derived isolates. (A) Bar graphs representing average protein abundance [a.u.] in *S. cerevisiae* strains IMX2122 ((Δ*Scbio1* ↑*Ec*KAPA pathway), black), IMS0994 (evolution A IMX2122, grey) and IMS0995 (evolution B IMX2122, white) grown in synthetic medium without biotin. The protein abundance means and deviations of *Ec*FabD, *Ec*BioC, *Ec*FabB, *Ec*FabG, *Ec*FabZ, *Ec*FabI, *Ec*BioH, *Ec*BioF, *Ec*AcpP, *Ec*AcpS and *Sc*Bio2 calculated from biological duplicates are displayed. Significance of differential expression is shown with the upper brackets and the False Discovery Rate (FDR) adjusted $p_{\text{-value}}$ ($^{FDR}p_{\text{-value}}$ < 5.0E-02). (B) and (C) show dot plots representing the fold-change in protein abundance ($\log_2$FC) over the average protein concentration (logCPM) of annotated *S. cerevisiae* proteins in evolved strain IMS0994 (B) and IMS0995 (C) compared to strain IMX2122. Protein abundances with an insignificant change in expression ($^{FDR}p_{\text{-value}}$ > 5.0E-02) are indicated as black dashes, protein abundances with a significant increase in expression ($^{FDR}p_{\text{-value}}$ < 5.0E-02) are indicated as blue triangles and protein abundances with a significant decrease in expression ($^{FDR}p_{\text{-value}}$ < 5.0E-02) are indicated as orange down-triangles (those include *Ec*FabD, *Ec*FabB and *Ec*BioC). Green diamonds represent the heterologously expressed proteins *Ec*FabG, *Ec*FabZ, *Ec*FabI, *Ec*BioH, *Ec*BioF, *Ec*AcpP and *Ec*AcpS, which were not significantly up- or downregulated.

Only 44 native yeast proteins in strain IMS0994 and 48 in strain IMS0995 showed a significantly different abundance relative to the parental strain IMX2122, of which 22 showed a unidirectional difference in the two isolates (Figures 3.5B and 3.5C). Not fewer than 20 and 26 proteins exhibited a 2-fold reduction at least of their abundance in IMS0994 and IMS0995 relative to IMX2122, respectively. Concomitantly, 14 and 10 proteins exhibited a 2-fold increase at least of their abundance in IMS0994 and IMS0995 relative to IMX2122, respectively (Figures 3.5B and 3.5C). Proteins that showed a lower level in the two SCI's did not show GO-categories related to metabolic processes, whereas proteins that showed a higher level in IMS0994 ($^{Bonferroni}p$-value = 9.98E-07) or IMS0995 ($^{Bonferroni}p$-value = 3.41E-02) indicated an overrepresentation of proteins belonging to the GO-category 'ATP metabolic process' (GO:0046034). As members of this GO category, the ATP synthase subunit Atp20 as well as the cytochrome *c* oxidase subunits Cox5A and Cox13 showed higher levels in both isolates. In IMS0995, the cytochrome *c* oxidase subunit Cox4 and, in IMS0994, the cytochrome *b-c1* complex subunit Qcr8, ATP synthase subunits Atp7 and Atp4 as well as cytochrome *c* oxidase subunit Cox9 also showed increased levels.

Reverse engineering gene dosage of the *E. coli* KAPA biosynthesis pathway contributes to improve both an- and oxic growth rate of the industrial diploid strain Ethanol Red.

To test whether altered gene dosage of the first three genes of the oxygen-independent KAPA biosynthesis pathway relative to the downstream genes, and the corresponding lower level of the encoded proteins, was critical to enhance growth of engineered strains in biotin-free conditions, we engineered the diploid industrial strain Ethanol Red. Using CRISPR/Cas9, which enables the simultaneous modification of all gene copies in polyploid strains [278], the ten heterologous genes were introduced at the *SGA1* locus. In contrast to the parental strain Ethanol Red, the resulting strain IMX2555 readily grew in biotin-free medium under oxic as well as under anoxic conditions. However, in both cultivation conditions, strain IMX2555 grew slower than Ethanol Red in biotin-supplemented medium (0.34 ± 0.01 h^{-1} versus 0.45 ± 0.01 h^{-1} and 0.20 ± 0.01 h^{-1} versus 0.42 ± 0.00 h^{-1}, respectively; Figure 3.6). Specific growth rates of strain IMX2555 were not affected by the presence or absence of biotin (Figure 3.6)

To reproduce the genotype observed in the evolved isolates IMS0994-5, a copy of *EcfabD, EcbioC* and *EcfabB* was deleted in IMX2555 by 'pre-CRISPR' marker-assisted homologous recombination [321] as it enables deletion of only one of the two copies of a targeted region in diploid strains. The deletion yielded the heterozygous diploid strain IMX2632 (↑*EcfabD EcbioC EcfabB,G,Z,I EcbioH,F EcacpP,S* / ↑*EcfabG,Z,I EcbioH,F EcacpP,S*). The specific growth rate of strain IMX2632 in anaerobic cultures on biotin-free medium was significantly higher than that of its parental strain IMS2555 (p_{value} < 1.0E-04; 0.30 ± 0.01 h^{-1} versus 0.19±0.00 h^{-1}). A smaller but significantly higher specific growth rate (p_{-value} < 1.0E-04; 0.38 ± 0.01 h^{-1} versus 0.34 ± 0.00 h^{-1}) was observed in oxic cultures. Despite these improvements, the engineered strain IMX2632 still grew

slower than observed for the Ethanol Red strain in both biotin supplemented cultures (Figure 3.6), suggesting additional tuning of gene dosages of KAPA-pathway cassettes and/or other mutations are required for full anoxic biotin prototrophy in engineered strains.

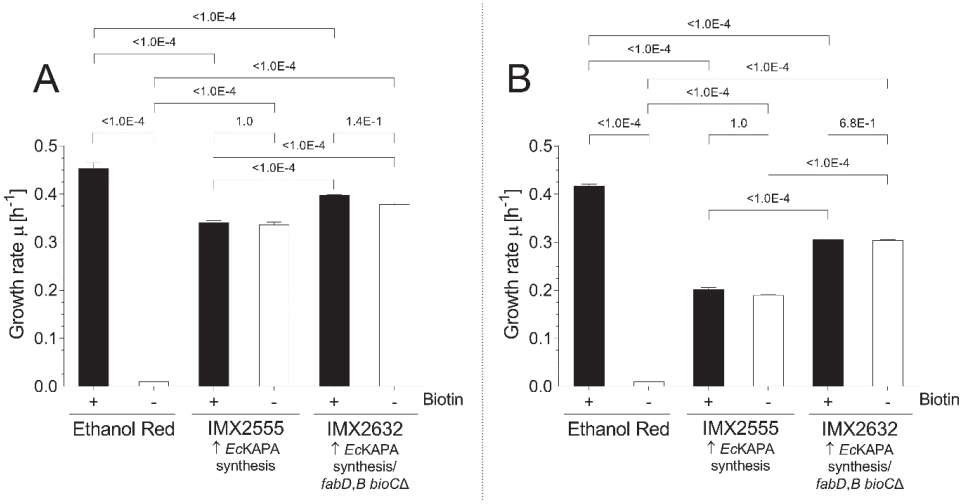

Figure 3.6 | Growth of *S. cerevisiae* Ethanol Red and engineered strains expressing *E. coli* KAPA synthesis genes. (A) Bar graphs representing average specific growth rates of *S. cerevisiae* strains Ethanol Red (diploid, industrial ethanol producer), IMX2555 (Ethanol Red ↑*Ec*KAPA pathway) and IMX2632 (Ethanol Red ↑ *Ec*KAPA pathway / *fabD,B bioC*Δ) under oxic conditions on synthetic medium with (+, black) and without (-, white) biotin. (B) specific growth rates of *S. cerevisiae* strains Ethanol Red, IMX2555 and IMX2632 under anoxic conditions on synthetic medium with (+, black) and without (-, white) biotin. The bars represent averages and standard deviations from two biological replicates. Statistical significance between growth rates in SMD with and without biotin, and between strains grown in the same conditions using one-way analyses of variance (ANOVA) and Tukey's multiple comparison test using GraphPad prism 8.2.1 software ($p_{\text{-value}}$ < 5.0E-02) is indicated.

Discussion

The native yeast pathway for biotin biosynthesis, for which the first committed reaction remains to be resolved, is oxygen dependent [300]. This study shows that functional expression of the *E. coli* KAPA pathway yields *S. cerevisiae* strains that are biotin prototrophic irrespective of the applied oxygen regime and whose specific growth rates can be further improved by tuning of the expression levels of specific KAPA-pathway enzymes. Prokaryotic biosynthesis pathways have previously been transferred between bacteria to increase biotin production by bacterial hosts such as *Pseudomonas mutabilis* [331], *Agrobacterium sp.* [332] and *E. coli* [333]. For functional expression of the *E. coli* KAPA pathway in *S. cerevisiae*, the different organization of prokaryotic and eukaryotic fatty-acid biosynthesis needed to be considered. In the type-II FAS system of *E. coli*, individual reactions in fatty-acid synthesis are catalysed by separate proteins [334]. In contrast, the type-I FAS system of *S. cerevisiae* and other fungi harbours all catalytic sites required for fatty-acid biosynthesis in domains of a

large, multi-functional single polypeptide or, as in *S. cerevisiae*, two polypeptides [335, 336]. Despite this structural difference, functional replacement of the *S. cerevisiae* type I-FAS complex by the *E. coli* type-II FAS system has been demonstrated [337]. In this study, expression of only *EcbioC, H* and *F* in *S. cerevisiae* did not support biotin prototrophy. This observation suggested that the yeast type-I FAS complex cannot convert malonyl-CoA methyl ester into pimeloyl-[Acp] or, alternatively, that the location of the acyl-carrier function on a distinct domain within a large multifunctional protein prevented *Ec*BioC from accessing its substrate. While the *S. cerevisiae* genome additionally encodes a soluble acyl carrier protein (Acp1) and its activating enzyme phosphopantetheine:protein transferase (Ppt2), these proteins participate in mitochondrial fatty-acid synthesis and are located in the mitochondrial matrix [338]. This localization issue was circumvented by additionally expressing the *E. coli* fatty-acid synthesis genes *EcfabD,B, G, Z, I* as well as *EcacpS* and *P* and, thereby, enabling cytosolic synthesis of pimeloyl-[Acp]. In the engineered biotin-prototrophic strain IMX2035, conversion of pimeloyl-[Acp] to 7-*keto*-8-aminopelargonic acid (KAPA) was enabled by expression of *EcbioF*. Deletion of *EcbioF* from this strain led to loss of its biotin prototrophy. Apparently, like its *B. subtilis* ortholog BioF, *S. cerevisiae* Bio6 cannot convert pimeloyl-[Acp] to KAPA but specifically requires pimeloyl-CoA as a substrate [261]. The biotin auxotrophy of the *EcbioF* deletion strain was unlikely to be caused by an insufficient expression level of Bio6 since expression of *Sc*Bio1 or *Cf*Bio1 suffices to confer oxic biotin prototrophy in CEN.PK strains [300].

In metabolic engineering, optimization of productivity and yield often requires balancing of the relative levels of enzymes in product pathways [339]. Such balancing may be especially challenging when, as in the present study, the product pathway is strongly intertwined with core metabolic processes of the microbial host. Optimal enzyme levels can be explored by *in vitro* [340] or *in vivo* [341, 342] approaches for combinatorial variation of the amounts of relevant enzymes. Our results illustrate how adaptive laboratory evolution [343, 344], combined with access to a high-quality reference genome [309], modern sequencing technologies, proteomics and a streamlined bioinformatics pipeline [345, 346] can provide a powerful alternative approach to gain relevant information on pathway balancing.

Evolution of strain IMX2122 for faster biotin-independent growth involved a whole-genome duplication and subsequent reduction of the copy number of three genes of the heterologous biotin-biosynthesis pathway. Ploidy changes from haploid to diploid and from tetraploid to diploid have been reported in previous studies on evolving yeast populations subjected to strong selection pressures such as repetitive carbon-source switching [347] and ethanol stress [348]. A whole-genome duplication was also observed after prolonged cultivation (over 1000 generations) of haploid *S. cerevisiae* strains on complex medium [349]. However, based on several shared homozygous and heterozygous SNVs in independently evolved isolates, we cannot exclude the possibility that a small subpopulation of diploid cells was already present in the predominantly haploid stock cultures with which the evolution experiments were inoculated.

Diploidy enabled tuning of the levels of *Ec*FabD, *Ec*BioC and *Ec*FabB relative to other KAPA pathway enzymes by gene deletion (Figures 4 and 5). Micro-homology-mediated end joining (MMEJ), an error-prone repair mechanism that involves alignment of micro-homologous sequences before joining, is typically associated with deletions and insertions that mark the original break site. In yeast, MMEJ is enhanced by homologous flanking sequences of at least 12 nucleotides [350]. Analysis of the break-point sequence in the evolved strains revealed a 18-bp (5'-CTGGTCACTCTTTGGGTG-3') direct repeat in *EcfabD* (positions 265-283) and in *EcfabB* (positions 991 and 1009) that perfectly flanked the heterozygous deletion. This observation strongly suggests that MMEJ was responsible for the deletion [351]. Deliberate introduction of short direct repeats in between clustered expression cassettes introduced into diploid or tetraploid strains by Cas9-medidated integration, followed by adaptive laboratory evolution, may be an attractive approach for exploring optimal gene dosages in heterologously expressed pathways whose *in vivo* activity can be coupled to growth or survival.

Deletion of a copy of *EcfabB, D* and *EcbioC* in the evolved diploid strains is likely to have mitigated a too strong competition for malonyl-CoA between the heterologously expressed KAPA pathway and native fatty-acid synthesis. This interpretation is consistent with the observed sub-optimal growth of the non-evolved parental strain on biotin-supplemented medium. The relevance of the segmental aneuploidy in the evolved strains was demonstrated by its reconstruction in the diploid industrial *S. cerevisiae* strain Ethanol Red. The anoxic specific growth rate of the thus engineered biotin-prototrophic strain was *ca.* 25% lower than that of biotin-supplemented cultures of non-engineered Ethanol Red. Although further targeted engineering and/or laboratory evolution is required for industrial implementation, our results demonstrate the feasibility of introducing anoxic biotin prototrophy into industrial *S. cerevisiae* strains.

Growth of wild-type *S. cerevisiae* strains on chemically defined media in absence of oxygen depends on supplementation of several nutrients, including ergosterol [352], nicotinic acid [353], pantothenate [354] and biotin [300]. Although essential for fast growth, the unsaturated fatty acid requirement of *S. cerevisiae* for anoxic growth is not absolute [311, 355]. Several metabolic strategies have recently been studied to eliminate these biosynthetic oxygen requirements. Expression of a squalene-tetrahymanol cyclase gene from *Tetrahymena thermophila* was shown to enable synthesis of the sterol surrogate tetrahymanol and anoxic growth of *S. cerevisiae* in sterol-free media [356]. Similarly, expression of fungal genes encoding an L-aspartate oxidase (NadB) and a quinolinate synthase (NadA) enabled nicotinic acid prototrophy without oxygen, while expression of heterologous L-aspartate-decarboxylases (AdcA) supported anoxic growth in the absence of pantothenate [354]. In terms of anoxic synthesis of cofactors, this leaves the puzzling case of thiamine, whose synthesis by yeast has been reported to be oxygen-dependent although the enzymes involved do not appear to require molecular oxygen [357]. Further research on engineering anoxic cofactor synthesis in yeast is therefore not only relevant for the

development of robust, prototrophic and feedstock-agnostic yeast strains for application in anoxic processes, but also for fundamental understanding of native biosynthetic pathways.

Conclusions

Functional expression of ten *E. coli* enzymes involved in KAPA synthesis enabled biotin-prototrophic growth of *S. cerevisiae* irrespective of oxygen supply. Adaptive laboratory evolution, genome resequencing, proteomics and reverse engineering of observed copy-number differences in a naive strain identified balancing of the relative levels of KAPA pathway enzymes as a key requirement for fast biotin-prototrophic growth. This metabolic engineering strategy can be used to construct *S. cerevisiae* cell factories for anaerobic bioprocesses based on feedstocks with low or variable biotin contents.

Data availability

The genome sequencing data of the *S. cerevisiae* strains IMX2035, IMX2122, IMS0994 and IMS0995 can be found in the NCBI archive BioProject under the accession number PRJNA717156. The codon optimised sequences of the heterologous genes used in this study and the raw data used to draw graphs on Figures 2, 3, 5 and 6 are available at the 4TU.Centre for research data repository (https://researchdata.4tu.nl/) under the https://doi.org/10.4121/14308007.

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Chapter 4 |

Engineering class-B vitamin biosynthesis in *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae is bradytroph for class B vitamins, it means that yeast cells exhibit slower growth in the absence of an external source of these metabolites. Alleviating these nutritional requirements for optimal growth performance would represent a valuable phenotypic characteristic for industrial strains since this would result in cheaper processes that would also be less susceptible to contaminations. In the present study, suboptimal growth of *S. cerevisiae* in absence of either pantothenic acid, *para*-aminobenzoic acid (*p*ABA), pyridoxine, inositol and biotin were corrected by single or double gene overexpression of native *FMS1*, *ABZ1/ABZ2*, *SNZ1/SNO1*, *INO1* and the *Cyberlindnera fabianii BIO1*, respectively. Several strategies were attempted to improve growth of *S. cerevisiae* CEN.PK113-7D in absence of thiamine, revealing that overexpression of *THI4* and *THI4/THI5* was able to improve growth up to 83% of the maximum specific growth rate of the reference CEN.PK113-7D in medium including all vitamins. Although the initial aim of this study was to combine all identified mutations in a single strain, the engineered strain IMX2210 only harboured genes to correct biotin, *p*ABA, pantothenate and inositol bradotrophies. Firstly, this strain was fast-growing at a maximum specific growth rate of 0.28 ± 0.01 h^{-1} in medium devoid of all vitamins. Secondly, this strain exhibited physiological variables in aerobic glucose limited chemostat cultures at a dilution rate of 0.1 h^{-1} in absence of vitamins similar to that of the reference strain CEN.PK113-7D grown in the same conditions but in a fully supplemented complete medium. These physiological similarities were further emphasized by the limited differences observed in comparative transcriptome analysis from the chemostat culture grown cells that were essentially affecting genes of the class B vitamins biosynthetic pathways. This work paves the way towards construction of the first fast growing vitamin-independent *S. cerevisiae* strain.

Introduction

A vitamin is an organic compound that an organism needs in small quantities to properly run metabolic functions. It also implies that this nutrient is essential and cannot be synthesized by the organism, either at all or not in sufficient quantities, and consequently has to be obtained through diet. Traditional chemically defined media used to cultivate the budding yeast *Saccharomyces cerevisiae* includes seven different class B vitamins (thiamine [B_1], pyridoxine [B_6], pantothenate [B_5], nicotinic acid [B_3], biotin [B_7], *myo*-inositol [B_8] and *para*-aminobenzoate (B_{10}) that act as enzyme cofactors, with the exception of inositol which acts mainly as phospholipid precursor [240].

Multiple studies have shown the dependency of vitamin dosages on productivity of yeast processes which employ pathways and enzymes requiring vitamins. Increased productions of triterpenoid and n-butanol could be achieved by increasing pantothenate dosages by either supplementation of higher quantity or deregulating the expression of the genes involved in its synthesis [162]. Pantothenate is a precursor of coenzyme A, whose availability was identified as bottleneck in the biosynthesis of these two compounds in *S. cerevisiae* [358]. In other examples, optimization of fumarate and succinate biosynthesis in *S. cerevisiae* required supplementation of higher concentration of biotin, a cofactor used by the pyruvate carboxylase that converts pyruvate to oxaloacetate a key enzyme in these metabolic pathways [359, 360]. The production of high yield yeast biomass or yeast extract as food-additive for human or animal diet requires the addition of vitamins to the yeast propagation medium [361]. From these examples it is obvious that performance optimizations through nutritional supplementation have a direct impact on the overall process cost.

Contrasting with the need to supply vitamins for optimal growth of *S. cerevisiae*, the genome sequence of this yeast carries complete pathways for class B vitamins biosynthesis (thiamine, pyridoxine, pantothenate, nicotinic acid, biotin, inositol and *para*-aminobenzoate). This genetic resource was recently exploited and resulted in almost complete alleviation of single vitamin requirement. Through adaptive laboratory evolution and subsequent reverse engineering of causative mutations, a small set of genetic alterations was identified to solve individual thiamine, pyridoxine, pantothenate and *para*-aminobenzoic acid requirement for fast growth of *S. cerevisiae* on synthetic media devoid of the respective vitamin [225]. In other case the genetic solution proposed by evolutionary engineering might be too complex to be reversed and transferred to multiple backgrounds. Evolution for biotin independent growth involved a massive 20- to 40-fold amplification of the *BIO1-BIO6* genes cluster that encode respectively the first two enzymatic steps of the pathway [154]. However, a simpler solution to solve biotin dependency was more recently found by screening yeast genetic biodiversity. The expression of the *S. cerevisiae BIO1* ortholog from the biotin prototrophic yeast *Cyberlindnera fabianii* immediately restored wild-type growth in media lacking biotin [362].

The resolution of vitamin B nutritional requirements would enable the first application of a mineral medium base that would just need to be supplemented with a carbon source for *S. cerevisiae* cultivation. Such medium would present several advantages such as an economical benefit, a simpler recipe, a higher stability, and lower susceptibility to contamination which should overall result in an increased process robustness.

The goals of this study were to investigate whether strategies can be found to eliminate single vitamin requirements and if the found solutions could be combined yielding a vitamin-independent *S. cerevisiae* strain. Next, the engineered strain was characterized for growth performance in batch and chemostat cultures and compared to a parental strain requiring vitamin supplementation for optimal growth. Finally, transcriptome data of the engineered strain grown in absence of vitamins was analyzed and compared to its isogenic reference grown in presence of vitamins.

Material and Methods

Strains, media, and maintenance

The *S. cerevisiae* strains used and constructed in this study (Table 4.1) all derived from the CEN.PK lineage [224, 308]. Yeast cultures were grown in either YP (10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone) or synthetic medium (SM) [363]. 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} pyridoxal 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 (SMDΔbio), thiamine-free SMD (SMDΔthi), pyridoxine-free SMD (SMDΔpyr), *para*-aminobenzoic acid-free SMD (SMDΔ*p*ABA), inositol-free SMD (SMDΔino), and pantothenic acid-free SMD (SMDΔpan), respectively. For medium without vitamins the vitamin solution was not added to obtain SMDΔvitamins. Drop-in media where only one vitamin was provided were prepared by adding 1 mL L^{-1} of concentrated solution of either 0.05 g L^{-1} D-(+)-biotin or 1.0 g L^{-1} thiamine hydrochloride to SMD to form SMDΔvitamins+bio or SMDΔvitamins+thi, respectively. Yeast strains were inoculated in 100 mL medium in 500-mL-shake flasks or in 20 mL medium in 100-mL-shake flasks and incubated at 30°C, 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} $MgSO_4$ $7·H_2O$, 1 mL L^{-1} trace element solution and 1 mL L^{-1} vitamin solution) [364] or 200 mg L^{-1} G418 (geneticin) for YPD G418 were added for selection of transformants.

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. After reaching late exponential phase, glycerol to a final concentration of 30% (v/v) was added to each culture prior storing at −80°C.

Table 4.1 | *S. cerevisiae* strains used in this study. * = sequence most likely missing in the strain; not verified by sequencing.

Name	Relevant genotype	Parental strain	Reference
CEN.PK113-7D	*MATaMAL2-8c*		[308]
CEN.PK113-5D	*MATa ura3-52 MAL2-8c*		[308]
IMX585	*MATa can1Δ::cas9-natNT2*	CEN.PK113-7D	[245]
IMX1651	*ura3-52Δ::ScTDH3p-THI4-ScTDH1t URA3*	CEN.PK113-5D	This study
IMX1652	*ura3-52Δ::ScCCW12p-THI5-ScENO2t URA3*	CEN.PK113-5D	This study
IMX1653	*ura3-52Δ::ScTDH3p-EcThiC-ScTDH1t URA3*	CEN.PK113-5D	This study
IMX1654	*ura3-52Δ::ScTDH3p-THI4-ScTDH1t ScCCW12p-THI5-ScENO2t URA3*	CEN.PK113-5D	This study
IMX1655	*ura3-52Δ::ScTDH3p-SNO1-ScTDH1t ScCCW12p-SNZ1-ScENO2t URA3*	CEN.PK113-5D	This study
IMX1859	*can1Δ::cas9-natNT2 sga1Δ::ScPYK1p-CfBlO1-ScBlO1t*	IMX585	[362]
IMX2044	*ura3-52Δ::ScTDH3p-INO1-ScTDH1t URA3*	CEN.PK113-5D	This study
IMX2045	*ura3-52Δ::ScTEF1p-FMS1-ScADH1t URA3*	CEN.PK113-5D	This study
IMX2088	*ura3-52Δ::ScTDH3p-ABZ1-ScTDH1t ScCCW12p-ABZ2-ScENO2t URA3*	CEN.PK113-5D	This study
IMX2089	*sga1Δ::ScTEF1p-FMS1-ScADH1t ScTDH3p-INO1-ScTDH1t ScTDH3p-SNO1-ScTDH1t* ScCCW12p-SNZ1-ScENO2t ScPGK1p-THI4-ScSSA1t* ScTDH3p-ABZ1-ScTDH1t ScCCW12p-ABZ2-ScENO2t**	IMX585	This study
IMX2210	*sga1Δ::ScTEF1p-FMS1-ScADH1t ScTDH3p-INO1-ScTDH1t ScCCW12p-SNZ1-ScENO2t ScTDH3p-ABZ1-ScTDH1t* YPRcTau3Δ::*ScPYK1p-CfBlO1-ScBlO1t*	IMX2089	This study
IMX2325	*Δx-2::ScPGI1p-THI2-ScGPM1t ScHXK2p-THI3-ScTDH3t sga1Δ::ScTEF1p-FMS1-ScADH1t ScTDH3p-INO1-ScTDH1t ScCCW12p-SNZ1-ScENO2t ScTDH3p-ABZ1-ScTDH1t* YPRcTau3Δ::*ScPYK1p-CfBlO1-ScBlO1t*	IMX2210	This study

Molecular biology techniques

DNA was PCR amplified with Phusion Hot Start II High Fidelity Polymerase (Thermo Scientific) and desalted or PAGE-purified oligonucleotide primers (Sigma Aldrich) by following manufacturers' instructions. DreamTaq polymerase (Thermo Scientific) was used for diagnostic PCR. Primers used in this study are shown in Table 4.2. 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). *E. coli* XL1-blue cells (Agilent Technologies) were used for chemical transformation and plasmid propagation [316]. Plasmids were purified from *E. coli* using a Sigma GenElute Plasmid Kit (Sigma Aldrich) and verified by either restriction analysis or by diagnostic PCR.

Yeast genomic DNA was isolated with the SDS-LiAc protocol [365]. Yeast strains were transformed with the lithium acetate method [366]. After genotyping by diagnostic

PCR, each engineered strain was restreaked twice on selective medium to ensure the selection of a single clone. The gRNA-carrying plasmid was cured as previously described [367]. One colony for each strain that lost the plasmid was inoculated in the respective SMD medium and stocked.

Plasmid cloning

The gRNA-expressing plasmid pUDR538 was Gibson assembled using the linearized pROS12, which was obtained by PCR with primer 6005 [245], and the X-2 gRNA containing linear DNA fragment amplified by PCR with pROS12 as template and 10866 as primer. Integration plasmids carrying a single gene expression module were cloned by BsaI-mediated (New England Biolabs, Ipswich, MA) Golden Gate Assembly using pYTK096 as backbone. The Yeast Tool Kit DNA parts for promoters and terminators sequences and a purified linear DNA fragment including the gene of interest were added as previously described [313]. The coding sequences for *ABZ2*, *SNO1*, *SNZ1*, *THI4*, *THI5*, *INO1*, *FMS1*, *THI2* and *THI3* 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.

Table 4.2 | **Primers used in this study.**

Primer No.	Sequence 5' → 3'
3904	GTAATTAAAACTTAGATTAGATTGCTATGCTTTC
4727	GGGCGGATTACTACCGTTGC
4728	CCAGCCCATATCCAACTTCC
5120	GGTGCACACGCGTGGCTTTTTCTTGAATTTGC
6005	GATCATTTATCTTTCACTGCGGAGAAG
7320	CTAGTTCGCCATCTAGTGTG
7331	GAGACTCGCATGAGAACATC
10710	TATATTTGATGTAAATATCTAGGAAATACACTTGTGTATACTTCTCGCTTTTCTTTTATTATTTTTCAAACTGCAAA TTCAAGAAAAAGCCAC
10866	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCGGCGACTAGGAAGAGA GTAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
12166	CATAAACAAACAAAAGATCTATGCTGTCCGATACAATTGA
12167	GCTTTATCTCGAGTTAGGATCTACATGAAAATTTGTAAGTTGC
12168	GCATCGTCTCATCGGTCTCATATGTCACTAATGGACAATTGGAAGA
12169	ATGCCGTCTCAGGTCTCAGGATTCAATATTTTGTCTTCACTGTTCCC
12170	GCATCGTCTCATCGGTCTCATATGCACAAAACCCACAGTACAATG
12172	GCATCGTCTCATCGGTCTCATATGACTGGAGAAGACTTTAAGATCAAGAG
12173	ATGCCGTCTCAGGTCTCAGGATTCACCACCCAATTTCGGAAAGT
12174	GCATCGTCTCATCGGTCTCATATGTCTGCTACCTCTACTGCTACTTCC
12175	ATGCCGTCTCAGGTCTCAGGATCTAAGCAGCAAAGTGTTTCAAAATTTG
12176	GCATCGTCTCATCGGTCTCATATGTCTACAGACAAGATCACATTTTTG
12177	ATGCCGTCTCAGGTCTCAGGATTTAAGCTGGAAGAGCCAATCTC
12212	GCATCGTCTCATCGGTCTCATATGACAGAAGATAATATTGCTCCAATC
12213	ATGCCGTCTCAGGTCTCAGGATTTACAACAATCTCTCTTCGAATCTTAGTTC
12214	ATGCCGTCTCAGGTCTCAGGATTTAATTAGAAACAAACTGTCTGATAAACCAATC
12215	TCAATTGTATCGGACAGCATAGATCTTTTGTTTGTTTATGTG
12216	ACTTACAAATTTTCATGTAGATCCTAACTCGAGATAAAGC
12217	TACAGATCATACCGATGACTAACGCACCCATGAACCACAC
12218	TTAGTTGTGAGTCGCCAGGCCAGCATTTTTCAAACTGCAAAT
12219	TTGCAGTTTGAAAAATGCTGGCCTGGCGACTCACAACTAA
12220	GTGTGGTTCATGGGTGCGTTAGTCATCGGTATGATCTGTACATG

12664	GCCGCGTAGACAATAGATCACCATCTAGTTGAATCCTGAGAGACTATCTCTAATGACCCGGGTAAAG TACAGCTACATTC
13389	TACTGCTACTTTAATTTTATGCCTGGCGACTCACAACTAA
13390	CTCACTCTTTCCTTACTCACCAGCATTTTTCAAACTGCAA
13391	TTAGTTGTGAGTCGCCAGGCATAAAATTAAAGTAGCAGTACTTCA
13392	TTGCAGTTTGAAAAATGCTGGTGAGTAAGGAAAGAGTGAGG
13662	TCCTCGGGCAGAGAAACTCG
13663	GTGAGCCTCTTACCTGTTTG
13748	CGGGTCATTAGAGATAGTCTCTCAGGATTCAACTAGATGGTGATCTATTGTCTACGCGGCTTGGCAGC CATTAAACTACG
14162	GGCACCTCTGGCTTGTCTTC
14486	GGGGACGTTCTTCACCTCCTTG
14537	GCATCGTCTCATCGGTCTCATATGAATACAGTTTCACCAGC
14538	ATGCCGTCTCAGGTCTCAGGATCTATTTCAGTAAGTCAGAGATTCG
14743	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAATAAGTAACAGCAGCAAACCTTGCCAA CAGGGAGTTC
14745	TCAGCGTGTTGTAATGATGCGCCATGAATTAGAATGCGTGATGATGTGCAAAGTGCCGTCGCATGCC GGTAGAGGTG
14746	GACGGCACTTTGCACATCATCACGCATTCTAATTCATGGCGCATCATTACAACACGCTGACAGTTCGA GTTTATCATTATCAATACTGC
14747	GCTACATCTTCCGTACTATGCTGTAGTCTCATGGTCGAGTTCTATTGCTGTTCGGCGGCACCGTTCAGG GTAATATATTTTAACCG
14748	TGCCGCCGAACAGCAATAGAACTCGACCATGAGACTACAGCATAGTACGGAAGATGTAGCCAGTT CGAGTTTATCATTATCAATACTGC
14749	CTCCACTGTACTGCATGTAGCATTCGCCGATCTGCATGATGTGTGACATTCTGCTATCGGATAAAATTAA AGTAGCAGTACTTCAACCATTAG
14750	CCGATAGCAGAATGTCACACATCATGCAGATCGGCGAATGCTACATGCAGTACAGTGGAGCAGTTC GAGTTTATCATTATCAATACTGC
16107	AGAATGATTTACAATCTAGTCGCAAAAACAAGTACAGTGCTGACGTCCCATCTTTTAATGGCCGATAATT GCAGACGAAC
16108	CATTACCAATGAATGCTGTTTTTGCAGAAATAACGAGATATCTGCAATAAAAGCAAAAGTCATGCTCAG CCGGTAGCTTG
16774	GCATCGTCTCATCGGTCTCATATGATCAATAGTAAGAGGCA
16775	ATGCCGTCTCAGGTCTCAGGATCTAGTCCTGCATGGCATATA
16776	GCATCGTCTCATCGGTCTCATATGAATTCTAGCTATACACA
16777	ATGCCGTCTCAGGTCTCAGGATTCAGTATCCAACTTGATTTT
16778	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGATTTATCATACTATTCCTCCGCTCGTATTCTTAGTG GATAACATG
16779	GTCATAACTCAATTTGCCTATTTCTTACGGCTTCTCATAAAACGTCCCACACTATTCAGGGTAACTTCAGA ATCGTTATC

Then, the *ABZ2*, *SNO1*, *SNZ1*, *THI4*, *THI5*, *INO1*, *FMS1*, *THI2* and *THI3* 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, pYTK009/pYTK056, pYTK013/pYTK053, pGGkp033/pGGkp048, and pGGkp096/pGGkp041, respectively, to yield plasmid pUDI177, pUDI178, pUDI179, pUDI201, pUDI181, pUDI182, pUDI211, pUD1103, and pUD1104, respectively (Table 4.3).

Plasmid pUDI176 for the expression of *ABZ1* was cloned by Gibson Assembly by combining equimolar amounts of a backbone fragment amplified with primer pair 12215/12216 and pUDI178 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, pUDI178, and pUDI180 were linearized by PCR using primer pair 12219/12220. Then, the expression modules for *ABZ2*, *SNZ1*, and *THI5* were amplified by PCR using primers 12217/12218. Finally, the linearized backbone containing the expression module for *ABZ1*, *SNO1*, and *THI4* was combined with the expression module for *ABZ2*, *SNZ1*, and *THI5*, respectively, yielding plasmid pUDI183, pUDI184, and pUDI185, respectively. Plasmid pUDI203 for the expression of *SNO1*, *SNZ1*, and *THI4* 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. The correctly assembled plasmids were transformed In *E. coli* and stock.

Strain construction

Strains expressing single-vitamin modules 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 *URA3* locus. Linearized plasmids pUDI180, pUDI181, pUDI188, pUDI185, pUDI184, pUDI201, pUDI182, pUDI211, pUDI183 and pUD261 were transformed in CEN.PK113-5D yielding strains IMX1651, IMX1652, IMX1653, IMX1654, IMX1655, IMX2044, IMX2045, IMX2088 and IMX2559, respectively. Strain IMX2089 carrying expression modules for *FMS1*, *INO1*, *SNO1*, *SNZ1*, *THI4*, *ABZ1*, and *ABZ2* was constructed by co-transforming the *Spycas*9-expressing strain IMX585 with the gRNA-expressing plasmid pUDR119 targeting the *SGA1* locus together with equimolar amounts of linear DNA repair fragments flanked by 60 bp unique homology sequences [320] to allow for *in vivo* homologous recombination. The expression modules for *FMS1*, *INO1*, *SNO1-SNZ1-THI4*, and *ABZ1-2* were amplified using primer pairs 14743/14745, 14746/14747, 14748/14749, 14750/10710, respectively, and plasmid pUDI211, pUDI182, pUDI203, and pUDI183 as a template, respectively. The expression module for *CfBIO1* [362] was amplified using primer pair 16107/16108 and plasmid pUD790 as template, gel-purified and transformed in IMX2089 together with the *YPRcTau3*-targeting plasmid pUDR514 to yield IMX2210. Transformants were selected on SMD plates with the exception of IMX2089 and IMX2210 that were plated on SMD acetamide and YPD G418, respectively. Linear DNA fragments with overexpression modules for *ScTHI2* and *ScTHI3* were amplified by PCR using primer pairs 13748/16778, 16779/12664 and plasmids pUD1103 and pUD1104 as a template and co-transformed with the X-2 targeting plasmid pUDR538 [369] into IMX2210, to yield strain IMX2325. Genotyping of strains IMX1651, IMX1652, IMX1653, IMX1654, IMX1655, IMX2044, IMX2045, IMX2088, and IMX2559 was performed by diagnostic PCR using gDNA as template and the primer pair 4727/4728 while genotyping of strain IMX2089 was carried out with gDNA and primer pairs 7320/3904 and 5120/7331, for IMX2210 with primer pairs 14162/14486, and

for IMX2325 with primer pairs 13662/16781 and 13663/16782 respectively. After genotyping, a correct clone was selected and stocked.

Shake flask cultures

For determination of growth rates under oxic condition, 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 grown 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, which was equivalent to an optical density at 660 nm (OD_{660}) of 3-5, an aliquot was used to inoculate a third 100 mL-culture with an OD_{660} of 0.2. Growth was monitored by measuring OD_{660} over time in the third culture using a Jenway 7200 Spectrophotometer (Cole-Palmer, Stone, United Kingdom). Specific growth rates were calculated by fitting the exponential growth function ($X = X_0 e^{\mu t}$ where μ indicates the growth rate) to a minimum of five data points and three-to-four-time doublings during exponential growth. All oxic shake flask experiments were carried out as at least biological duplicates and incubated in an Innova incubator (New Brunswick Scientific) set at 30°C and 200 rpm.

Batch and chemostat cultures in lab-scale bioreactors

Physiological characterization of engineered *S. cerevisiae* strain IMX2210 was performed in biologically independent quadruplicates in 2-L bioreactors (Applikon, Delft, The Netherlands) with 1-L working volume. Thawed stocks of strain IMX2210 were grown in 100 mL either SMD or SMDΔvitamins in 500-mL-shake flasks. 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_{660} of 4-5) from the second flask were washed twice in demi-water by centrifuging for 5 min, 3000 x g, re-suspended in 50 mL of sterile water and used to inoculate each reactor to a starting OD_{660} of 0.15-0.25. An oxic batch culture with 20 g L^{-1} glucose was followed by an oxic, carbon-limited chemostat culture at a dilution rate of 0.10 h^{-1} with 7.5 g L^{-1} glucose in the medium as previously described [370, 371]. 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 oxic conditions, air at a rate of 0.5 L min^{-1} was sparged through the culture which was stirred at 800 rpm. All cultures were grown on either SMD or vitamin-free SMD (SMDΔvitamins) 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_{660} 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_{660}/DW (g L^{-1}) calibration line to convert each OD_{660} 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). Steady state sampling of the chemostat cultures for extracellular metabolites and sugar was performed by rapid sampling technique using cold steel beads pre-cooled to -20°C [372].

Whole genome sequencing

DNA of *S. cerevisiae* strain IMX2210 was grown in shake-flask cultures with SMD and genomic DNA was isolated with a Qiagen Blood & Cell Culture DNA kit (Qiagen, Hilden, Germany), following manufacturer's specifications. 150bp paired-end sequencing was performed on a 350-bp TruSeq PCR-free insert library using an Illumina NovaSeq 6000 sequencer (Macrogen Europe, Amsterdam, the Netherlands). Sequence data was mapped to the CEN.PK113-7D genome [224] to which the sequences of the integrated expression cassettes for the heterologous genes *FMS1*, *INO1*, *SNO1, SNZ1*, *THI4*, *ABZ1*, *ABZ2*, *CfBIO1* were manually added. Data processing and single nucleotide variation (SNV) analysis were carried out as described previously [151, 373].

Total RNA isolation and transcriptomics analysis

Steady state chemostat cultures were sampled directly from the reactor in liquid nitrogen as previously described [374]. 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 [375]. 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) [376] to the CEN.PK113-7D genome [224] to which the sequence of *CfBIO1* were manually added. Expression was quantified by applying featureCounts (version 1.6.0) in paired-end mode and reversely stranded [377].

Differential gene expression (DGE) analysis was performed by using edgeR (version 3.30.3) with TMM normalization [378]. 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 between CEN.PK113-7D in SMD, IMX2210 in SMD and IMX2210 in SMDΔvitamins.

Table 4.3 | **Plasmids used in this study.** HF = homology flank. * = codon-optimized by GeneArt for *S. cerevisiae*.

Plasmid	Characteristics	Reference or source
pGGkp033	*cat* ColE1 *ScPGI1*p	[281]
pGGkp041	*cat* ColE1 *ScTDH3*t	[281]
pGGkp048	*cat* ColE1 *ScGPM1*t	[281]
pGGkp096	*cat* ColE1 *ScHXK2*p	[368]
pROS12	*bla* ColE1 2µ *hph ScSNR52*p-gRNA$_{ScCAN1}$-*ScSUP4*t *ScSNR52*p-gRNA$_{ScADE2}$-*ScSUP4*t	[245]
pUD1103	*bla* ColE1 *ScPGI1*p-*THI2*-*ScGPM1*t	This study
pUD1104	*bla* ColE1 *ScHXK2*p-*THI3*-*ScTDH3*t	This study
pUD727	*bla* ColE1 *EcthiC**	GeneArt
pUD790	*bla* ColE1 *ScPYK1*p-*CfBIO1*-*ScBIO1*t	[362]
pUDI176	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*ABZ1*-*ScTDH1*t *URA3* 3'HF^{URA3}	This study
pUDI177	*aph* ColE1 5'HF^{URA3} *ScCCW12*p-ABZ2-*ScENO2*t *URA3* 3'HF^{URA3}	This study
pUDI178	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*SNO1*-*ScTDH1*t *URA3* 3'HF^{URA3}	This study
pUDI179	*aph* ColE1 5'HF^{URA3} *ScCCW12*p-*SNZ1*-*ScENO2*t *URA3* 3'HF^{URA3}	This study
pUDI180	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*ScTHI4*-*ScTDH1*t *URA3* 3'HF^{URA3}	[225]
pUDI181	*aph* ColE1 5'HF^{URA3} *ScCCW12*p-*THI5*-*ScENO2*t *URA3* 3'HF^{URA3}	This study
pUDI182	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*INO1*-*ScTDH1*t *URA3* 3'HF^{URA3}	This study
pUDI183	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*ABZ1*-*ScTDH1*t *ScCCW12*p-*ABZ2*-*ScENO2*t *URA3* 3'HF^{URA3}	This study
pUDI184	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*SNO1*-*ScTDH1*t *ScCCW12*p-*SNZ1*-*ScENO2*t *URA3* 3'HF^{URA3}	This study
pUDI185	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*THI4*-*ScTDH1*t *ScCCW12*p-*THI5*-*ScENO2*t *URA3* 3'HF^{URA3}	This study
pUDI188	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*EcthiC**-*ScTDH1*t *URA3* 3'HF^{URA3}	This study
pUDI201	*aph* ColE1 5'HF^{URA3} *ScPGK1*p-*THI4*-*ScSSA1*t *URA3* 3'HF^{URA3}	This study
pUDI203	*aph* ColE1 5'HF^{URA3} *ScTDH3*p-*SNO1*-*ScTDH1*t *ScCCW12*p-*SNZ1*-*ScENO2*t *ScPGK1*p-*THI4*-*ScSSA1*t *URA3* 3'HF^{URA3}	This study
pUDI211	*aph* ColE1 5'HF^{URA3} *ScTEF1*p-*FMS1*-*ScADH1*t *URA3* 3'HF^{URA3}	This study
pUDR119	*bla* ColE1 2µ *amdS ScSNR52*p-*gRNA$_{ScSGA1}$*-*ScSUP4*t	[318]
pUDR514	*bla* ColE1 2µ kanMX *ScSNR52*p-*gRNA$_{YPRcTau3}$*-*ScSUP4*t	[225]
pUDR538	*bla* ColE1 2µ *hph ScSNR52*p-*gRNA$_{X-2}$*-*ScSUP4*t	This study
pYTK009	*cat* ColE1 *ScTDH3*p	[313]
pYTK010	*cat* ColE1 *ScCCW12*p	[313]
pYTK011	*cat* ColE1 *ScPGK1*p	[313]
pYTK013	*cat* ColE1 *ScTEF1*p	[313]
pYTK051	*cat* ColE1 *ScENO1*t	[313]
pYTK052	*cat* ColE1 *ScSSA1*t	[313]
pYTK053	*cat* ColE1 *ScADH1*t	[313]
pYTK055	*cat* ColE1 *ScENO2*t	[313]
pYTK056	*cat* ColE1 *ScTDH1*t	[313]
pYTK096	*aph* ColE1 5'HF^{URA3} *sfGFP URA3* 3'HF^{URA3}	[313]

Analytical methods

Biomass dry weight measurements were performed using pre-weighed nitrocellulose filters (0.45 µm, Gelman Laboratory, Ann Arbor, MI). 10 mL culture samples were filtrated and then the filters were washed with demineralized water prior to drying in a microwave oven (20 min at 360 W) and weight measurement. 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 [379]. 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 [380].

Statistical analysis

Statistical significance of differences between measurements from replicate cultures were 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 the *p*-values were corrected for multiple comparisons using Tukey's correction for multiple comparison by GraphPad Prism version 8.2.1 for Windows, (GraphPad Software, San Diego, California USA, www.graphpad.com). Significant differences were assumed when *p*-value > 5.0E-2. The batch and chemostat culture determined data were compared by using two-tailed student's t-test.

Data availability

All measurement and calculations used to prepare figure 4.2, 4.3, 4.5, 4.6, 4.7 and 4.8 as well as table 4.4, 4.5 and 4.7 of the manuscript as well as DNA sequencing data of *S. cerevisiae* strain IMX2210 are available at the 4TU.Centre for research data repository (https://researchdata.4tu.nl/) under 10.4121/17185607 (data under public embargo and accessible through the following reviewer link: https://figshare.com/s/d8b0128171d8c85ce091. The RNA sequencing data of *Saccharomyces cerevisiae* strain IMX2210 and CEN.PK113-7D are available at the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE191212 (data under public embargo and accessible through the following reviewer link: that can be opened using the token "uvahuimsdpanrkv".

Results

Selection of engineering strategies to eliminate single vitamin requirements

To start engineering *S. cerevisiae* for vitamin independent growth, strategies suitable for removing individual vitamin requirements were evaluated (Figure 4.1).

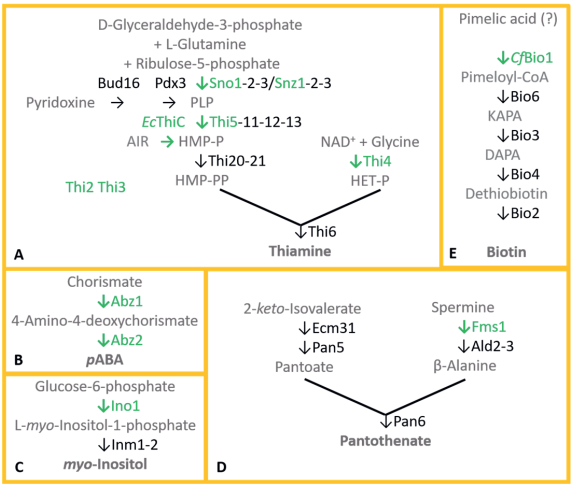

Figure 4.1 | Overview of vitamin biosynthetic pathways and genetic modifiations studied in this work. Schematic representation of the yeast native biosynthetic pathway for thiamine (A) starting with the reaction of D-glyceraldehyde-3-phosphate, L-glutamine and ribulose-5-phospahte to pyridoxal 5'-phosphate (PLP) by the PLP synthase Sno1-2-3/Snz1-2-3. PLP can also be synthesized by two reactions for pyridoxine catalyzed by the pyridoxal kinase Bud16 and the Pyridoxamine 5'-phosphate oxidase Pdx3. The Thi5 protein family resembling the function of a 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase makes hydroxymethylpyrimidine phosphate (HMP-P). The Phosphomethylpyrimidine synthase ThiC from *Escherichia coli* converts 5-phosphoribosyl-5-aminoimidazole (AIR) to HMP-P. Hydroxymethylpyrimidine diphosphate (HMP-PP), which is produced by Thi20-21, and hydroxyethylthiazole phosphate (HET-P), which is made from NAD^+ and glycine by action of Thi4, a thiamine thiazole synthase, are combined by the action of Thi6 into thiamine. Thi2 and Thi3 are thimaine biosynthesis regulators *p*ABA (B) is made from chorismate and 4-amino-4-deoxychorismate by catalytic activity of *ABZ1* and *ABZ2*, an aminodeoxychorismate synthase and aminodeoxychorismate lyase, respectively. *myo*-Inositol (C) is synthesized from glucose-6-phosphate via L-*myo*-inositol-1-phosphate by action of Ino1 an inositol-3-phosphate synthase and Inm1-2 (inositol-1-monophosphatase). Pantothenate (D) is made by reaction of pantoate and β-alanine catalyzed by Pan6 (pantoate-β-alanine ligase). Pantoate is synthesized from 2-*keto*-isovalerate and the reactions of 3-methyl-2-oxobutanoate hydroxymethyltransferase (Ecm31) and 2-dehydropantoate 2-reductase (Pan5) and β-alanine from spermine by polyamine oxidase (Fms1) and aldehyde dehydrogenase (Ald2-3). Biotin synthesis (E) starts with a putative precursor pimelic acid, which is indicated by (?). The first enzyme from *Cyberlindnera fabianii* (*Cf*Bio1) catalyses the synthesis of pimeloyl-CoA, which is converted by 7,8-diamino-pelargonic acid aminotransferase Bio6, adenosylmethionine-8-amino-7-oxononanoate aminotransferase Bio3, 8-amino-7-oxononanoate synthase Bio4 and biotin synthase Bio2 to biotin. The intermediates are 7-*keto*-8-aminopelargonic acid (KAPA), 7,8-diaminopelargonic acid (DAPA) and dethiobiotin. The names of overexpressed enzymes in this study are green. Unmodified enzyme names are shown in black and intermediates and products in grey.

Pyridoxine

In absence of pyridoxine (SMDΔpyr), *S. cerevisiae* CEN.PK113-7D grew with a growth rate of 0.29 ± 0.01 h^{-1} which represented a 28% reduction compared to the growth rate of the same strain in the presence of pyridoxine (Figure 4.2A). It was previously established that mutations in the gene encoding the transcriptional regulator Bas1 restored wild type growth in absence of pyridoxine through the relief of the transcriptional repression of gene *SNZ1* involved in pyridoxal-phosphate synthesis [225, 381]. To mitigate unwanted effects due the dual activating and repressing regulatory functions of Bas1, deregulation of *SNZ1* and *SNO1* was evaluated (Figure 4.1). In absence of vitamin B_6 the strain IMX1655 that overexpressed the pyridoxal-5'-phosphate biosynthetic genes *ScSNO1* and *ScSNZ1* grew at a growth rate of 0.37 ± 0.01 h^{-1}. Although significantly slower than the parental strain grown in presence of pyridoxine (p = 1.6E-2), IMX1655 exhibited a growth rate identical to that of *BAS1* mutants previously reported [225].

para-Aminobenzoic acid

In absence of *para*-aminobenzoic acid (*p*ABA), the strain CEN.PK113-7D exhibited a severe growth reduction (81%) relative to growth in presence of *p*ABA. However, *S. cerevisiae* previously showed fast evolvability of the genes encoding enzymes of the chorismate node, *ABZ1* and *ARO7* [225]. Mutations in these two genes could restore growth rates that were undiscernible from the CEN.PK113-7D growth rate in presence of *p*ABA. Inspired by these earlier results and following a strategy similar to that applied for pyridoxine, the two genes, *ABZ1* and *ABZ2* encoding enzymes involved in the conversion of chorismate to *p*ABA (Figure 4.1) were overexpressed. The resulting strain IMX2088 recovered a growth rate of 0.38 ± 0.00 h^{-1}, that did not significantly differ from the growth rate of CEN.PK113-7D on SMD (Figure 4.2B).

Inositol

Although growth improvement in absence of inositol can be rapidly rescued by adaptive laboratory evolution, no mutations have been linked to this phenotype. However, deregulation or constitutive expression of *INO1* has been linked to an inositol excretion phenotype [189]. The strain IMX2044 that overexpresses *ScINO1* (Figure 4.1) could grow in absence of inositol as fast as CEN.PK113-7D cultivated on regular SMD medium. IMX2044 (*ScINO1)* grew with a growth rate of 0.37 ± 0.01 h^{-1} in SMDΔino that was significantly improved relative to CEN.PK113-7D grown on the same medium ($\mu_{SMD\Delta ino\text{-}CEN.PK113\text{-}7D}$ = 0.31 ± 0.00 h^{-1}) (Figure 4.2C).

Pantothenate

Although previous research showed that mutations in global regulators as *GAL11* and *TUP1* had positive effect on growth rate in absence of pantothenate [225], the proteins encoded by these two genes have a central regulatory role and affect the expression of multiple genes [382]. To avoid the introduction of a massive transcriptional

rearrangement, other options were investigated. Pantothenic acid independent growth was achieved by overexpression of gene *ScFMS1* in CEN.PK113-7D coding for a polyamine oxidase synthesizing β-alanine (Figure 4.1), the precursor of pantothenic acid synthesis [383]. The resulting strain IMX2045 was able to grow at a rate of 0.38 ± 0.01 h^{-1}, which was an improvement of 124% when compared to growth of CEN.PK113-7D on the same medium ($\mu_{SMD\Delta pan,CEN.PK113\text{-}7D}$ = 0.17 ± 0.00 h^{-1}) and a restoration of growth as observed for CEN.PK113-7D on SMD (Figure 4.2D).

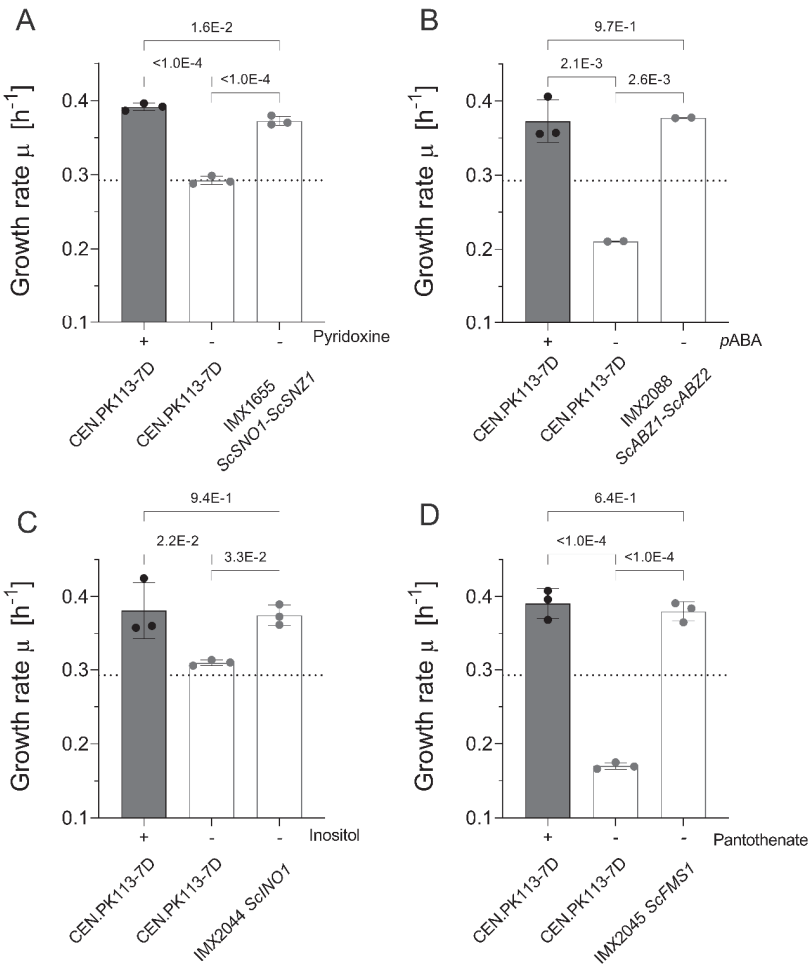

Figure 4.2 | Engineering fast-growth in the absence of single vitamins. Growth rate of the non-engineered CEN.PK117-7D strain (dark grey) and engineered derivatives (white) in synthetic medium with pyridoxine (A), *p*ABA (B), inositol (C) and pantothenate (D) added (+) or ommitted (-). Gene(s) that were overexpressed in the engineered strains are reported below the relative bar. Statistical analysis was based on a one-way analysis of variance analysis (ANOVA) followed by Tukey's multiple comparisons test (GraphPad Software, San Diego, California USA, www.graphpad.com) with means of independent cultures (n=3; except n=2 for CEN.PK117-7D in SMDΔ*p*ABA and IMX2088). Significant differences were assumed when *p*-value > 5.0E-2.

Nicotinic acid and biotin

Previous studies indicated that the presence of nicotinic acid (niacin, Vitamin B_3) was dispensable for oxic growth of *S. cerevisiae* [70]. Based on these data no genetic modifications aiming at improving nicotinic acid provision were investigated. For biotin, an already confirmed strategy consisting in the overexpression of *BIO1* from the biotin prototroph Phaffomycetaceae yeast *Cyberlindnera fabianii* was selected [362] (Figure 4.1).

Evaluation of genotypes to reduce thiamine dependency of *S. cerevisiae* growth

Of all class B vitamins, removing thiamine dependency was the most problematic after biotin. Thiamine remained the only vitamin for which ALE did not yield isolates whose growth was as fast as the parental strain in presence of thiamine [225]. Several point mutations in *CNB1* that encodes the regulatory subunit of calcineurin, in *PMR1* that encodes a high affinity Ca^{2+}/Mn^{2+} P-type ATPase, and in *FRE2* that encodes a cell-surface iron reductase as well as the overexpression of *ScTHI4* have been shown to act positively on growth rate in absence of thiamine [225]. The thiazole synthase *Sc*Thi4 that is involved in the synthesis of the thiazole moiety of thiamine operates as a co-substrate in the reaction by providing a sulfur group for thiazole formation; this implies that Thi4 undergoes a single turnover suicide reaction [384]. This unusual catalytic role identifies Thi4 as a potential bottleneck in thiamine biosynthesis and was therefore chosen for this overexpression study.

The omission of thiamin in liquid culture reduced the CEN.PK113-7D cell growth by 25% ($\mu_{SMD\Delta thi}$ = 0.29 ± 0.00 h^{-1} vs. μ_{SMD} = 0.40 ± 0.01 h^{-1}, Figure 4.3). The *ScTHI4* overexpression (IMX1651) resulted in a significant but moderate increase in growth rate ($\mu_{SMD\Delta thi\text{-}IMX1651}$ = 0.32 ± 0.01 h^{-1} vs. μ_{SMD} = 0.39 ± 0.01 h^{-1}). This increase can also be observed in strain IMX1653, which overexpresses both *ScTHI4* and *ScTHI5* ($\mu_{SMD\Delta thi\text{-}IMX1653}$ = 0.33 ± 0.01 h^{-1}). Thi4 is not the only suicide enzyme in the thiamine biosynthetic pathway.

Indeed, *ScThi5* that is responsible for the formation of the pyrimidine heterocycle in the thiamine biosynthesis pathway, catalyzes the synthesis of hydroxymethylpyrimidine phosphate from pyridoxal phosphate (Figure 4.1) and a histidine residue located in the Thi5 active site, resulting in a single turnover reaction [385]. Conversely to *ScThi4* overexpression, that of *ScTHI5* did not yield a growth improvement; this was confirmed by the phenotype of IMX1654 (*ScTHI4-5*) that was not different to that of IMX1651 (*ScTHI4*) (Figure 4.3).

Two other strategies were attempted, firstly the genes encoding the *THI* regulon transcriptional regulators, *ScTHI2* and *ScTHI3* were overexpressed (IMX2325) as described earlier to have a positive effect [386]. Secondly the *E. coli* gene *thiC* which, as opposed to *Sc*Thi5, encodes a phosphomethylpyrimidine synthase that provides 4-amino-2-methyl-5-(phosphor-oxymethyl) pyrimidine in a non-suicide mechanism [387], was overexpressed in strain IMX1653 (Figure 4.1). Of all the attempted strategies,

only the *THI4* overexpression yielded a significant positive impact on the strains growth rate in medium without thiamine (Figure 4.3).

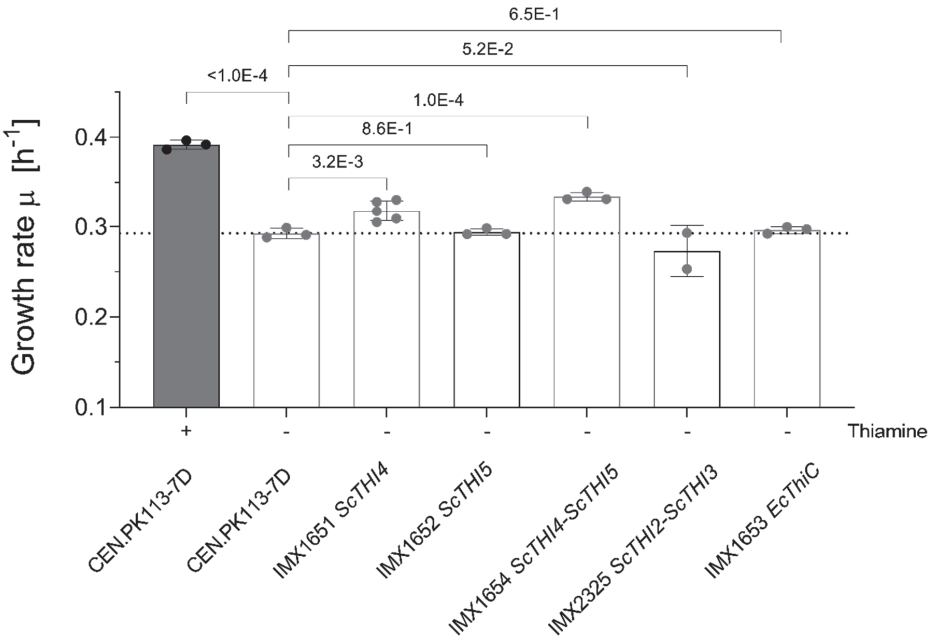

Figure 4.3 | **Engineering fast-growth in the absence of thiamine.** Growth rate of the non-engineered CEN.PK117-7D strain (dark grey) and engineered derivatives (white) in synthetic medium with thiamine (+) and without thiamine (-). Gene(s) that were overexpressed in the engineered strains are reported below the relative bar. Statistical analysis of means of independent cultures (n=3; except n=2 for IMX2325) 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 (GraphPad Software, www.graphpad.com) with significant difference when *p*-value > 5.0E-2.

Construction of a *S. cerevisiae* strain able to grow on mineral medium supplemented with glucose as carbon source.

To eliminate the dependencies for thiamine, pyridoxine, inositol, *p*ABA and pantothenic acid, the overexpression of the *S. cerevisiae* genes *THI4*, *SNO1* and *SNZ1*, *INO1*, *ABZ1* and *ABZ2*, and *FMS1* were attempted by integration at the *SGA1* locus of the *Spycas9*-expressing strain IMX585, which would yield strain IMX2089. The remaining biotin requirement was eliminated by integrating and constitutively expressing the *BIO1* ortholog from *C. fabianii* [362] in IMX2089.

The whole genome of strain IMX2210 was sequenced to verify the correct strain construction. Mapping of the sequencing data on the CEN.PK113-7D reference genome [224] added of the expected engineered gene cluster revealed that several genes (*THI4*, *SNO1* and *ABZ2*) were missing (Figure 4.4). This indicated that some of the ORFs were not correctly integrated in the *SGA1* locus as expected for strain IMX2210,

probably caused by the repeated use of several promoter and terminator sequences that caused unintended recombination events. Therefore, IMX2210 instead of harboring genetic solutions to fix biotin, *p*ABA, pantothenate, inositol, pyridoxine and thiamine, only included the modifications sufficient to repair *p*ABA (*ABZ1*↑), pantothenate (*FMS1*↑), inositol (*INO1*↑) and biotin (*CfBIO1*↑) bradytrophies.

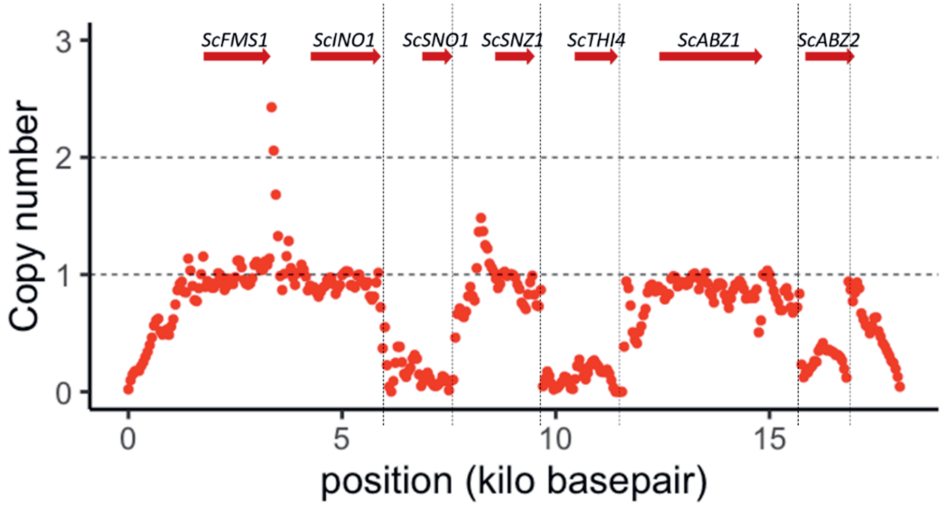

Figure 4.4 | Copy number per base pair position of *SGA1* locus in IMX2210. The copy number per every 50 bp (red dot) as indicated by the reads from whole genome sequencing of IMX2210. The expected position of the open reading frames of *FMS1*, *INO1*, *SNO1*, *SNZ1*, *THI4*, *ABZ1*, *ABZ2* (red arrows) is indicated in the relation to the sequence and a decrease in copy number from one to zero is framed by dashed lines.

The resulting strains IMX2089 (*SNZ1*↑ *INO1*↑ *ABZ1*↑ *FMS1*↑) and IMX2210 (*SNZ1*↑ *INO1*↑ *ABZ1*↑ *FMS1*↑ *CfBIO1*↑) grew as fast as CEN.PK113-7D on complete SMD (Figures 4.5A and 4.5B) indicating that even though the strain engineering did not occur as expected, it did not negatively affect overall physiology.

On a mineral medium supplemented with glucose (SMDΔvitamins) strain IMX2210 exhibited a growth rate of 0.30 ± 0.00 h^{-1} (Figure 4.5B) contrasting with the extreme slow growth of CEN.PK113-7D in the same condition (Figure 4.5B). Expectedly, the addition of only biotin to SMDΔvitamins did not significantly improve growth ($\mu_{SMD\text{-}bio\text{-}IMX2210}$ = 0.30 ± 0.00 h^{-1}) demonstrating that the expression of *CfBIO1* was sufficient to fulfil biotin requirement as previously shown, whereas addition of thiamine did result in a significant improvement (p_{value} = 2.0E-4) ($\mu_{SMD\text{-}thi\text{-}IMX2210}$ = 0.36 ± 0.01 h^{-1}), an observation in agreement with the absence of *THI4* overexpression in IMX2210 (Figure 4.5B). Although the growth rate of IMX2210 in medium devoid of all vitamins was reduced by 21% relative to that of CEN.PK113-7D in complete SMD, it is hitherto the first reported *S. cerevisiae* strain able to grow in such condition.

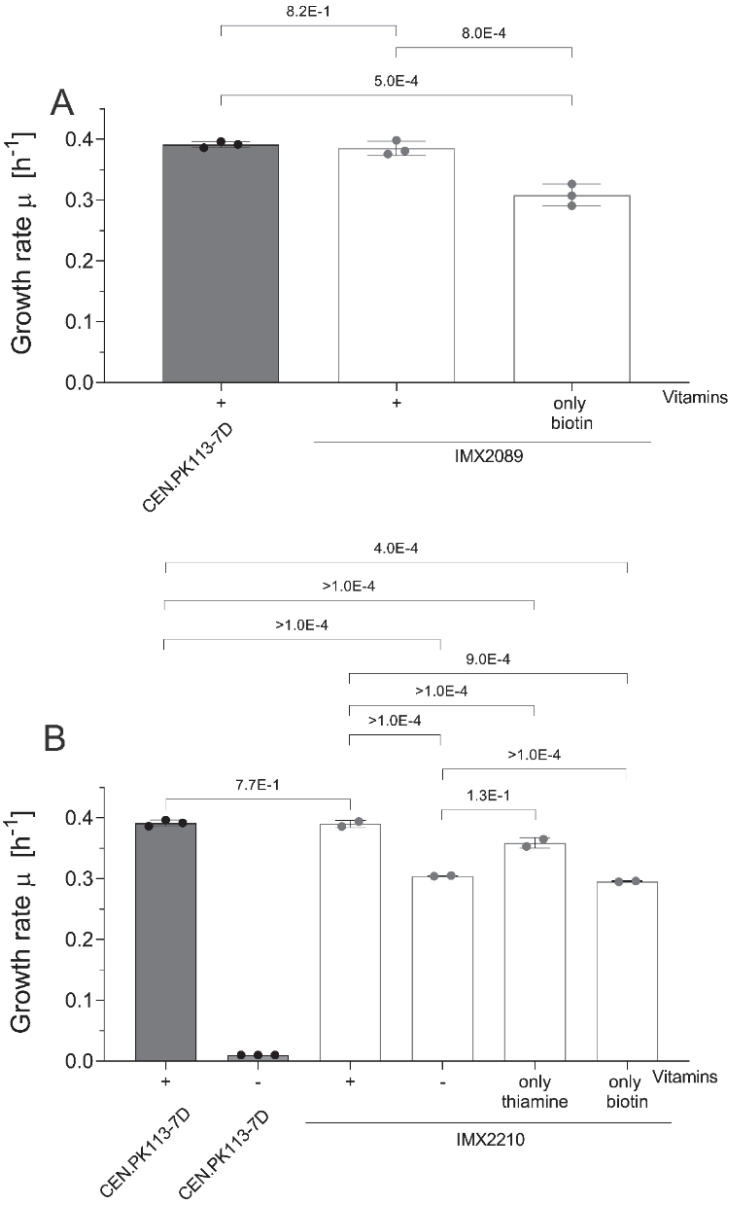

Figure 4.5 | **Engineering fast-growth in the absence of vitamins.** Growth rate of the non-engineered CEN.PK117-7D strain (dark grey) and engineered derivatives (white) IMX2089 (A) and IMX2210 (B) in synthetic medium with vitamins (+), without vitamins (-) or a single vitamin added. Statistical analysis was based on a one-way analysis of variance (ANOVA) followed in case of panel A by Tukey's multiple comparisons correction and in case of panel B by Fisher's Least Significant Difference (LSD) test (GraphPad Software, www.graphpad.com) with significant differences between the means of independent cultures (n=3; except for IMX2210 n=2) when *p*-value > 5.0E-2.

Physiological characterization of vitamin independent strain IMX2210 in aerobic glucose limited chemostat culture.

Although not entirely repaired, IMX2210 is capable of growth in medium devoid of all class B vitamins. The physiology of strain IMX2210 was characterized in controlled oxic bioreactors in batch and glucose-limited chemostat cultures in both SMD and SMDΔvitamins.

Table 4.4 | Growth rate μ, biomass yield $Y_{X/S}$, biomass specific glucose consumption rate (qGlucose), biomass specific ethanol production rate (qEthanol), of oxic batch cultures of strain IMX2210 grown on either SMD or SMDΔvitamins. Data are compared to previously published data for the reference strain CEN.PK113-7D grown in SMD [389]. Errors represent the standard deviation of the mean (n=2). Statistical significance determined using the student's t-test, with alpha = 0.05. * = significant different results. n.a. = not measured/applicable

Strain	IMX2210		CEN.PK113-7D	*p*-values		
Medium	**SMD (1)**	**SMDΔvitamins (2)**	**SMD (3)**	**1 vs. 2**	**2 vs. 3**	**1 vs. 3**
μ [h^{-1}]	n.a.	0.28±0.01	0.37±0.01	n.a.	1.7E-2*	n.a.
Y_{XS} [g_X/g_S]	0.14±0.01	0.15±0.01	0.13±0.01	8.2E-1	3.9E-1	5.6E-1
qGlucose [mmol g_X^{-1} h^{-1}]	6.03±0.29	5.92±1.02	14.8±1.1	9.3E-1	2.8E-2*	6.4E-2*
qEthanol [mmol g_X^{-1} h^{-1}]	8.04±0.41	9.71±3.38	22.0±0.5	7.1E-1	7.4E-2	2.7E-3*

During the batch phase of the chemostat cultures in the absence of all vitamins (SMDΔvitamins), strain IMX2210 showed a growth rate of 0.28 ± 0.01 h^{-1}, which is reduced compared to growth rates of similar batch cultures with CEN.PK113-7D in SMD (Table 4.4, *p*-value = 1.7E-2). The strain 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 oxic batches of *S. cerevisiae* (Figure 4.6) [388]. The biomass yield for strain IMX2210, grown either on SMD or on SMDΔvitamins, was comparable to the CEN.PK113-7D biomass yield in an oxic batch cultures (Table 4.4). The quantitative data for the biomass specific glucose uptake rate and ethanol production rate for strain IMX2210 on SMD were slightly lower compared to CEN.PK113-7D data and did not change significantly when IMX2210 was grown on SMDΔvitamins (Table 4.4).

The batches were followed by an oxic glucose-limited chemostat culture at a dilution rate of 0.1 h^{-1}. Irrespective of the culture medium, IMX2210 exhibited identical steady state biomass yield, biomass specific oxygen consumption rate, biomass specific carbon dioxide production rate, and respiratory quotient. These quantitative data were almost identical to those measured for the reference CEN.PK113-7D strain in oxic glucose limited chemostat at the same dilution rate [389] (Table 4.5). Only biomass specific glucose uptake rate of IMX2210 compared to CEN.PK113-7D was slightly reduced according to the student's t-test, as well as oxygen consumption rate and carbon recovery when grown on SMD.

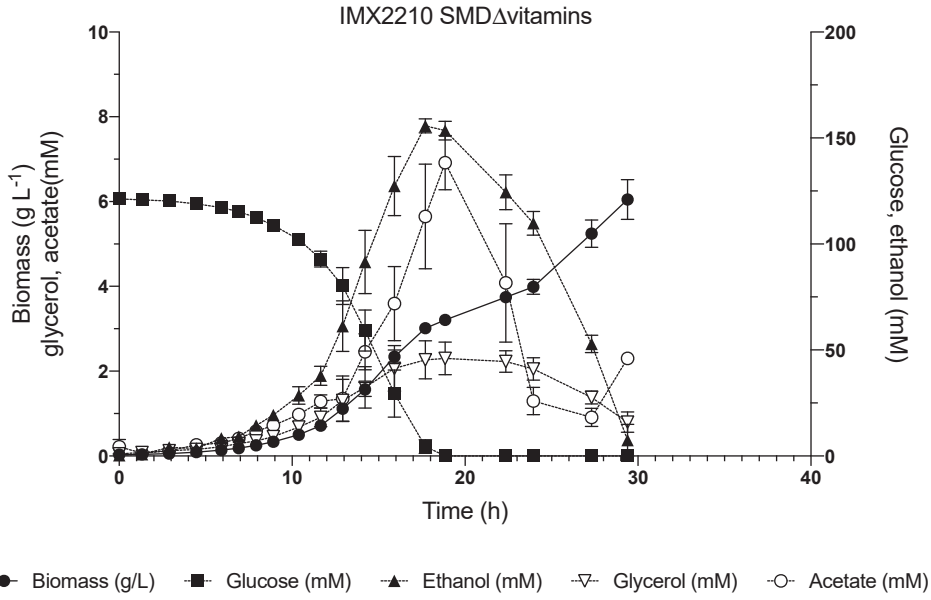

Figure 4.6 | Biomass and metabolite concentration over time of IMX2210 cultures in SMDΔvitamins in oxic batch bioreactors. Values for biomass (●), glucose (■), ethanol (▲), glycerol (▽), and acetate (○) are shown over time. Error bars represent the standard error of the mean (n=4).

IMX2210 transcriptome analysis

Chemostat cultivation offers a number of advantages for genome-wide transcriptome studies because it enables cell cultivation under tightly defined environmental conditions. This cultivation mode in particular is very well suited to compare conditions or strains that exhibit difference in growth rate in batch cultures. Aerobic glucose-limited chemostat-cultures of IMX2210 in SMD and SMDΔvitamins were sampled for analysis of the transcriptome. RNA-seq data of the cultivation with vitamins, were also compared with RNA seq-based transcriptome data of the reference strain CEN.PK113-7D grown in identical vitamin containing cultivation conditions. The pairwise IMX2210 versus CEN.PK113-7D grown in SMD comparison revealed that 123 genes were differently expressed between the two strains, 31 were upregulated in IMX2210 and 92 genes exhibited a reverse profile (|fold change| > 2 with a $q_{FDR\text{-}value} < 0.05$) (Supplemental data).

The upregulated genes set was enriched for genes involved in siderophore transmembrane transport (GO:0044718; GO: *ENB1*, *SIT1*, *ARN1*, *FET3* and *FRE3*) and in water-soluble vitamin biosynthetic process (GO:0042364: *SNZ1* (+36-fold), *INO1* (+7.8-fold), *ABZ1* (31.7-fold), *FMS1* (+26.7-fold)) (Table 4.6). The latter category corresponded to four overexpressed genes to eliminate pantothenic acid, *para*-aminobenzoic acid (*p*ABA), pyridoxine, inositol bradytrophies, to which we could add the heterologous gene *CfBIO*1. Although it was not possible to calculate a fold

change since *CfBIO1* was absent in the reference strain CEN.PK113-7D, this gene was highly expressed but about thrice lower than *ABZ1*, *FMS1* and *INO1*, and eight times lower than *SNZ1*. These data confirmed the effectiveness of the engineering strategy.

Table 4.5 | Dilution rate D, biomass yield $Y_{X/S}$, biomass specific oxygen consumption rate (qO_2), biomass specific carbon dioxide production rate (qCO_2), biomass specific glucose consumption rate (qGlucose), biomass specific ethanol production rate (qEthanol), respiratory quotient RQ, and carbon recovery of oxic, carbon-limited chemostat cultures of strain IMX2210 grown on either SMD or SMDΔvitamins. Data are compared to previously published data for the reference strain CEN.PK113-7D grown in SMD [389]. 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 α = 0.05. * = significant different results.

Strain	**IMX2210**		**CEN.PK113-7D**	***p*-values**		
Medium	**SMD (1)**	**SMDΔvitamins (2)**	**SMD (3)**	**1 vs. 2**	**2 vs. 3**	**1 vs. 3**
D [h^{-1}]	0.10±0.00	0.10±0.00	0.10	2.2E-1		
Y_{XS} [g_X/g_S]	0.48±0.01	0.48±0.01	0.49±0.01	6.4E-1	1.5E-1	7.4E-2
qO_2 [mmol g_X^{-1} h^{-1}]	2.6±0.2	2.8±0.3	2.8±0.3	4.7E-2*	9.0E-1	1.3E-1
qCO_2 [mmol g_X^{-1} h^{-1}]	2.8±0.1	2.9±0.1	2.8±0.3	7.7E-2	3.4E-1	1.0E-1
qGlucose [mmol g_X^{-1} h^{-1}]	1.1±0.05	1.16±0.04	1.1±0.0	1.9E-1	<1.0E-4*	3.2E-3*
qEthanol [mmol g_X^{-1} h^{-1}]	0.0±0.0	0.0±0.0	0.0±0.0	n/a	n/a	n/a
RQ	1.1±0.1	1.1±0.1	1.0±0.0	4.4E-1	3.8E-1	5.1E-1
Carbon recovery [%]	100±2	101±2	98±3	1.2E-2*	2.1E-1	6.6E-1

No functional categories were found enriched in the down regulated gene set of the pairwise comparison between IMX2210 versus CEN.PK113-7D grown in SMD. However, these data confirmed that the chromosomal site used for the IMX2210 strain construction *SGA1* and *CAN1* in which *Spycas9* is integrated were logically found downregulated in IMX2210. A closer scrutiny of these transcriptomic data showed that the four genes carried by a so-called 'native selfish episome', the 2-micron (2-µm) plasmid (*REP1*, *REP2*, *FLP1* and *RAF1*) were all down regulated, but more precisely were not expressed anymore in IMX2210 [390]. This would suggest that the IMX2210 strain has been converted into a cir^0 strain, a mutation characterized by the loss of the 2-µm plasmid.

The impact of the overexpression of *SNZ1*, *ABZ1*, *FMS1*, *INO1* and *CfBIO1* was evaluated by comparing the transcriptome response of IMX2210 in absence and presence of vitamins. In total, only 77 genes out of the 5475 detected were significantly differentially expressed (Figure 4.7). A set of 32 genes exhibited a lower expression in cultivation without vitamins while 45 were up-regulated (Table 4.7) in absence of vitamins.

The set of genes transcriptionally up-regulated in IMX2210 (Table 4.7) in absence of vitamins were subjected to functional category enrichment using Fischer's exact test. It revealed a significant enrichment in upregulated genes for GO biological process categories related to thiamine, biotin and pyridoxal-phosphate biosynthesis (Table 4.6). The 13 genes comprising the category GO:0006772 thiamine metabolic

process, were all upregulated in IMX2210 when vitamins were omitted to the culture medium (*THI12, THI6, THI2,1 SNZ3, THI2, THI20, SNZ2, THI11, THI4, THI5, THI22, THI13* and *PET18*). The upregulation of *PET18* [391], *THI73, THI74* [392] and *THI7* [393] was already reported in starving or low thiamine conditions.

Similarly, all six genes (*BIO2*, *BIO5*, *BIO4*, *BIO3* and *ScBIO1 and BIO6)* comprising the GO category "biotin biosynthetic process (GO:0006768)" related genes were up-regulated in vitamin less conditions. This was accompanied with the upregulation of the biotin transporter gene *VTH1* as well. The last set of enriched categories was pyridoxal phosphate biosynthesis related genes (GO:0042822) *BUD16*, *SNZ3*, *SNO3* and *SNZ2* (Table 4.7, Figure 4.7). Conversely, the gene *SNO4* that encodes a cysteine protease hypothesized to be involved in pyridoxine metabolism [394] was down-regulated. In total a minimum of 26 genes, which represented 83% of the upregulated genes were related to class B vitamins (thiamine, biotin and pyridoxine) biosynthesis.

Although, this was not significantly overrepresented, several genes encoding vitamin dependent enzymes were also upregulated in the transcriptome of IMX2210 grown in absence of vitamins. Expression of proteins requiring vitamins as cofactors was up-regulated. The pyridoxal-phosphate dependent alanine-transaminase gene (*ALT1*) [77] expression was increased by 2.1-fold, similarly the expression of the thiamine dependent broad range 2-oxo acid decarboxylase encoding gene *ARO10* [131, 395] and of the pyruvate decarboxylase isoform gene *PDC5 [394]* were increased by 3.1 and 3.2-fold, respectively. The highest alterations in mRNA abundance were observed for *THI4*, *THI5*, *THI12*, *THI13*, *THI11* and *BIO5* as well as for *FCY22,* a nucleobase cation symporter gene [396] and YCR102C, a stress tolerance gene [397] (Figure 4.7).

Table 4.6 | GeneOntology (GO) biological process complete category enrichment analysis using Fischer's exact test in up-regulated genes in the pairwise comparison of samples of IMX2210 chemostat cultures with CEN.PK113-7D in SMD and IMX2210 chemostat cultures in SMDΔvitamins and SMD media. Presented is the GO biological process the related identifier (GO_ID), the total number of genes in this category N, the number of matches belonging to this category n, the fold-enrichment in the tested set of genes, the false discovery rate (FDR) corrected for the *p*-value (FDR^{corr} *p*-value) and the genes belonging to this category.

GO biological process	GO_ID	N	n	Fold enrichment	FDR^{corr} p_{value}	Genes
$IMX2210^{SMD}$ vs $CEN.PK113\text{-}7D^{SMD}$ up regulated genes						
siderophore transport	0015891	11	5	> 100	1.65E-03	*ENB1 ARN1 SIT1 FET3 FRE3*
water-soluble vitamin biosynthetic process	0042364	50	4	18.61	1.55E-02	*ABZ1 FMS1 SNZ1 RKI1*
$IMX2210^{SMD\Delta vit}$ vs $IMX2210^{SMD}$ up regulated genes						
thiamine biosynthetic process	0009228	12	12	> 100	1.08E-18	*THI12 THI6 THI21 SNZ3 THI2 THI20 SNZ2 THI11 THI4 THI5 THI22 THI13*
thiamine diphosphate biosynthetic process	0009229	8	7	> 100	5.77E-10	*THI12 THI6 THI21 THI20 THI11 THI5 THI13*
thiamine metabolic process	0006772	13	13	> 100	3.32E-20	*THI12 THI6 THI21 SNZ3 THI2 THI20 SNZ2 THI11 THI4 THI5 THI22 THI13 PET18*
organophosphate biosynthetic process	0090407	255	11	6.69	1.14E-04	*THI12 THI6 THI21 BUD16 SNZ3 THI20 SNZ2 THI11 THI5 THI13*
thiamine diphosphate metabolic process	0042357	8	7	> 100	5.38E-10	*THI12 THI6 THI21 THI20 THI11 THI5 THI13*
biotin biosynthetic process	0009102	6	6	> 100	6.93E-05	*BIO5 BIO4 BIO3 BIO2 BIO6 BIO1*
biotin metabolic process	0006768	6	6	> 100	7.26E-05	*BIO5 BIO4 BIO3 BIO2 BIO6 BIO1*
monocarboxylic acid biosynthetic process	0072330	54	6	11.49	4.41E-02	*BIO5 BIO4 BIO3 BIO2 BIO6 BIO1*
pyridoxal phosphate biosynthetic process	0042823	9	4	68.93	2.05E-04	*BUD16 SNZ3 SNZ2 SNO3*
pyridoxal phosphate metabolic process	0042822	9	4	68.93	1.97E-04	*BUD16 SNZ3 SNZ2 SNO3*
vitamin B6 metabolic process	0042816	11	4	56.40	3.34E-04	*BUD16 SNZ3 SNZ2 SNO3*
vitamin B6 biosynthetic process	0042819	10	4	62.04	2.64E-04	*BUD16 SNZ3 SNZ2 SNO3*
aldehyde biosynthetic process	0046184	10	4	62.04	2.55E-04	*BUD16 SNZ3 SNZ2 SNO3*
organic hydroxy compound biosynthetic process	1901617	82	6	11.35	2.53E-03	*BUD16 SNZ3 SNZ2 SNO3 PDC5 ARO10*

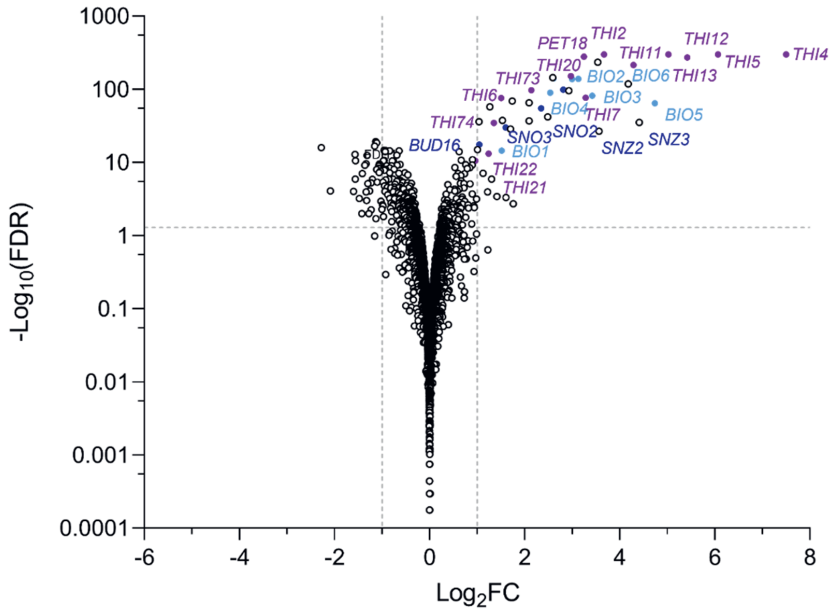

Figure 4.7 | Differentially expressed genes between IMX2210 chemostat cultures with SMD and SMD without vitamins. Genes presented were plotted by their -$\log_{10}$ of the False Discovery Rate (-$\log_{10}$(FDR)) against the $\log_2$ of the fold change ($\log_2$FC). Genes with colored labels have a $\log_2$FC > 1 with a -$\log_{10}$(FDR) > 2 and are grouped in thiamine metabolism related genes (purple), biotin metabolism related genes (light blue) and pyridoxine metabolism related genes (dark blue).

Discussion

Availability of vitamin prototrophic yeast strains would result in costs reduction for preparation of industrial growth media and would allow exploration of characteristic of strains that have hitherto never been investigated under harsh industrial conditions [240, 298]. Although, the initial ambition of this work was to construct such a strain by combining overexpression of only eight genes (*THI4*, *SNO1* and *SNZ1*, *INO1*, *ABZ1* and *ABZ2*, *FMS1* and *CfBIO1*) that in pair or individually were able to improve growth of *S. cerevisiae* in absence of a single vitamin (Figure 4.2), the constructed strain was not exactly matching the initial expectation. It combined two major genetic alterations, it missed recombination of the seven cassettes for expression of the *S. cerevisiae* genes, and during this process, it also lost the native 2-µm plasmid. While the latter mutation did not have direct impact on the ability of the strain to grow in absence of vitamins, it did not permit to easily correct the absence of *THI4*, *SNO1* and *ABZ2*, since cir^0 strains cannot maintain recombinant 2-µm plasmids. This absence prevented the subsequent transformation of CRISPR plasmid enabling the expression of the gRNA necessary to target the integration of the missing genes into a chromosomal site. For this reason, the strain IMX2210 evaluated in this work was only engineered for biosynthesis of *p*-aminobenzoate, pantothenate, inositol [240] and biotin [362].

Table 4.7 | Over-expressed genes between IMX2210 chemostat cultures with SMD and SMD without vitamins. Genes are presented with their systematic name and their gene name and log_2 of the fold change (log_2FC) and the corresponding False Discovery Rate (FDR).

Systematic name	Gene name	Log_2FC	FDR
YGR144W	*THI4*	7.497	0.00E+00
YFL058W	*THI5*	6.064	0.00E+00
YNL332W	*THI12*	5.421	3.22E-275
YDL244W	*THI13*	5.023	0.00E+00
YNR056C	*BIO5*	4.740	1.49E-65
YER060W-A	*FCY22*	4.408	3.80E-36
YJR156C	*THI11*	4.287	1.36E-215
YCR102C	YCR102C	4.177	3.94E-120
YBR240C	*THI2*	3.670	0.00E+00
YPL265W	*DIP5*	3.562	1.24E-27
YCR020C	*PET18*	3.536	1.38E-236
YNR058W	*BIO3*	3.423	1.05E-82
YLR237W	*THI7*	3.285	1.89E-77
YOL055C	*THI20*	3.247	3.19E-280
YGR286C	*BIO2*	3.128	2.14E-140
BIO6	*BIO6*	2.996	1.62E-139
YLR004C	*THI73*	2.973	7.62E-153
YEL073C	YEL073C	2.929	6.31E-97
YFL059W	*SNZ3*	2.815	5.40E-100
YNR057C	*BIO4*	2.604	3.02E-142
YNL331C	*AAD14*	2.589	2.21E-145
YGR065C	*VHT1*	2.538	4.22E-91
YJR157W	YJR157W	2.482	8.40E-43
YNL333W	*SNZ2*	2.348	4.52E-56
YPL214C	*THI6*	2.140	1.65E-98
YJL213W	YJL213W	2.095	8.67E-38
YJR154W	YJR154W	2.095	3.23E-66
YMR317W	YMR317W	1.759	1.83E-03
YJR155W	*AAD10*	1.740	5.32E-70
YLR134W	*PDC5*	1.700	1.41E-29
YDR380W	*ARO10*	1.608	4.75E-04
YFL060C	*SNO3*	1.603	5.03E-31
YDR541C	YDR541C	1.530	1.95E-38
BIO1	*BIO1*	1.514	2.98E-15
YDR438W	*THI74*	1.506	3.86E-77
YKR053C	*YSR3*	1.413	3.53E-04
YPR121W	*THI22*	1.350	1.81E-35
YIL169C	YAR068W	1.307	1.09E-06
YDR508C	*GNP1*	1.267	1.43E-58
YPL258C	*THI21*	1.239	5.95E-14
YNL334C	*SNO2*	1.221	1.04E-04
YGL263W	*COS12*	1.122	7.88E-08
YEL029C	*BUD16*	1.047	2.73E-18
YLR089C	*ALT1*	1.037	7.78E-37
YGR287C	*IMA1*	1.009	9.27E-16

Despite an incomplete gene complement, IMX2210 was able to grow in absence of vitamins in a mineral medium supplemented with glucose at 78% of maximum theoretical growth rate of the reference strain CEN.PK113-7D in presence of vitamins. Of the three vitamins derived from the improperly constructed biosynthetic pathways, thiamine had the strongest impact. The sole addition of vitamin B_1 was sufficient to increase IMX2210 growth rate to near μ_{max}. Out of the three strategies envisaged to

eliminate thiamine dependency in *S. cerevisiae*, the most promising ones involved the overexpression of *THI4* whose translational product acts as sulfur source for thiazole formation in a single turnover reaction. The transcriptome analysis of IMX2210 revealed that *THI4* was massively upregulated in absence of thiamine already, being the top expressed gene in IMX2210 (Table 4.7, Figure 4.7). This would suggest that further alleviation of thiamine dependency for growth would require an even higher increase of the *THI4* transcription. This could be achieved by increasing the native *THI4* expression cassettes copy number or combining *THI4* expression with the *CCW12* promoter that was used to drive the overexpression of *SNZ1* in IMX2210, being the most expressed gene in IMX2210 (Supplemental data). In both situations, we cannot exclude that the reuse of such a strong promoter might in the end affect the expression of the respective native gene resulting in titration of specific transcription factors and consequently affect the strain fitness. Alteration of expression of *CCW12*, which is involved in the structure and maintenance of the cell wall, has already been reported to cause reduction of growth rate [398, 399]. Alternatively, synthetic regulatory sequences generated by a machine learning approach derived from natural genomic data that demonstrated high transcriptional activity without disturbing cell growth [399], might represent a workable solution.

The ability to grow the *S. cerevisiae* strain IMX2210 in absence of vitamins offered a unique opportunity to evaluate the transcriptional regulation of the genes involved in class-B vitamin biosynthesis. Distinct from the role of the transcriptional regulator Vhr1 in the transcriptional activation of the *VHT1* and *BIO5* genes that encodes a biotin and a 7-*keto*-8-aminopelargonic acid (KAPA) transporters respectively, little is known about the regulation of the biotin genes in *S. cerevisiae* [157]. The regulation of *VHT1* and *BIO5* by Vhr1 occurs through a cis-regulatory motif ($AATCAn_8TGAYT$) and it is dependent on the concentration of biotin in the medium. The transcriptional activity of the *VHT1* promoter was maximal at biotin concentrations ranging from 0.05 to 0.5 µg L^{-1}, concentration at least hundred-fold lower than the biotin concentration used in SMD medium (50 µg L^{-1}). Our results were in line with this previous report, although necessary to grow on SMD, *VHT1* and *BIO5* showed low expression in CEN.PK113-7D and IMX2210 in this condition. The expression of the two transporter genes along the other biotin biosynthetic genes (*BIO1*, *2*, *3*, *4* and *6*) (Figure 4.1) were highly upregulated in vitamin-free conditions (Figure 4.7, Supplemental data). Search in regulatory sequences of these genes confirmed occurrence of the Vhr1 cis regulatory motif in *VHT1* and also revealed such occurrence in *BIO2*, *BIO1* and *BIO6* promoters. No such motif could be found in the 52-bp *BIO5* promoter conversely to what has been previously suggested [157], as well as in the 220-bp bidirectional *BIO4-BIO3* promoters. Analysis using of extended sequences including the coding of the physically clustered *BIO5*, *BIO4* and *BIO3* genes identified a motif within *BIO4* open reading frame that might serve for the regulation of the three contiguous genes. This peculiar organization and the role of this regulatory motif in the regulation of these genes will have to be further explored. The absence of obvious Vhr1 cis-regulatory sequence might also indicate that Vhr1 might not be the only transcriptional activator

implicated in regulation of the *BIO* genes and this unknown mechanism has yet to be uncovered (Figure 4.8).

Unsurprisingly, the largest group of genes upregulated in absence of vitamins were related to the interdependent metabolisms of pyridoxal phosphate and thiamine (Figure 4.1). This concurrent transcriptional increase is linked to overrepresentation of targets of the regulatory complex formed by Thi2-Thi3 and Pdc2. However, systematic search conducted to define a binding site consensus have hitherto failed. Physical mapping of the upregulated genes revealed two transcriptional gene clusters on CHRX and XIV. On CHRXIV, the cluster included three genes (*TH12*, *SNO2* and *SNZ2*) involved in pyridoxal-phosphate and thiamine were also accompanied by *AAD14* that encodes a putative aryl-alcohol dehydrogenase. The cluster on CHRX also linked the gene *THI11* encoding a 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase with another putative aryl-alcohol dehydrogenase encoding gene *AAD10*. The function of aryl-alcohol dehydrogenase in *S. cerevisiae* is still under debate [401]. The gene family comprises seven members whose single deletions and the combined deletion of the seven genes do not result in any obvious phenotype in so far conditions that always included thiamine and pyridoxine in the cultivation media [402, 403]. At the light of our results, the function of the aryl alcohol dehydrogenases could be revised in the context of vitamins B_1 and B_6 biosynthesis.

While the correction of the vitamin B_1, B_6, B_{10} and B_7 bradytrophies had limited impact on the transcriptional response, it had no impact on physiological characteristics in oxic, carbon-limited cultures (Table 4.5), which should facilitate implementation of the strategy in industrially relevant strains. In addition to impact cost reduction related to media recipe and downstream processes, to lowering the risk of contaminations, vitamin prototrophy could improve strain robustness in harsh industrial environments. Supplementation of preculture media with a vitamin mixture comprising pyridoxine, thiamine and biotin have a significant impact on growth and strain performance (e.g. ethanol yield) in wheat straw and corn stover hydrolysates [292]. This was further substantiated by the transcriptomic response of *S. cerevisiae* to wheat straw hydrolysate that revealed an up-regulation of the biotin and thiamine biosynthetic genes confirming the poor nutritional characteristic of industrial media whose preparation requires heating [404]. Despite, a considerable attractiveness, the genetic solution described in this study cannot be directly applied to second generation lignocellulosic processes and more generally to anoxic processes. The native biosynthesis pathways for biotin, pantothenate, nicotinic acid and thiamine are known to require oxygen [70, 240, 362, 405]. Metabolic engineering solutions to circumvent oxygen requirements of biotin, pantothenate and nicotinic acid have been reported [368][405]. The remaining challenge is the synthesis of thiamine that also requires biosynthetic oxygen [406, 407], but the exact mechanisms behind the oxygen requirement is not yet fully understood. Combining the aforementioned strategies would contribute to the construction of the first anaerobic vitamin-independent

S. cerevisiae strain, that in a near future might prove to be a key phenotypic characteristic of industrial strains.

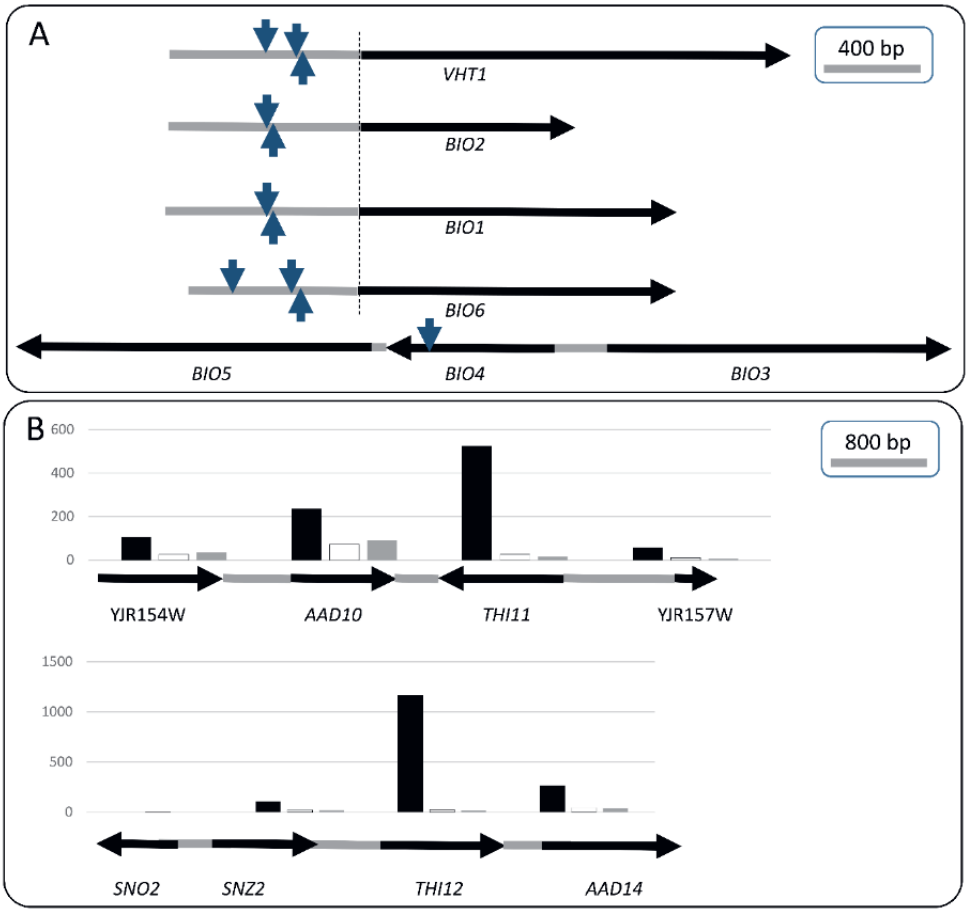

Figure 4.8 | **New insight in regulation of the biotin and thiamine biosynthetic genes.** (A) Position of Vhr1 putative cis regulatory sequence (AATCAn$_8$TGAYT) in *BIO* locus. The promoter sequences are represented in grey and the coding sequences in black. The AATCAn$_8$TGAYT motif is indicated with a dark blue arrow. Downwards arrows indicate motif located on the (+) strand conversely upwards arrows indicate motif on the (-) strand. Search for motif in the BIO loci was performed using the regulatory sequence analysis tools (RSAT; http://rsat.sb-roscoff.fr/; [400]) using the pattern matching function. (B) Co-expression of genes surrounding *THI11* and *TH12* on CHRX and XIV, respectively. The bar graphs represent the normalized transcript expression of the strains IMX2210 grown in absence of class B vitamins (black), in presence of class B vitamins (white) and CEN.PK113-7D grown in presence of vitamins (grey).

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Author contributions

AKW, TP and JMD designed the experiments and wrote a first version of the manuscript. All authors critically read this version, provided input and approved the final version. AKW, TP, constructed the yeast strains and performed shake flask culture characterization. EFAdH, AKW and TP performed the bioreactor experiments. MvdB, TP, and AKW prepared RNA libraries and analysed the transcriptome data.

Outlook

Biotechnology and genetic engineering have helped to overcome many challenges of our time. During this study, answers to challenges faced in the field of energy and health could be identified. The insights might serve as an example for solving problems in the future.

To the general public, the most visible demonstrations of the potential of biotechnological processes and products based on genetic modification are probably related to medical applications, which range from the successful replacement of slaughterhouse-derived porcine insulin by safe, body-identical recombinant human albumin to the fast development and roll-out of Covid-19 vaccines. In the coming decades, a different and even more important contribution of biotechnology is needed to enable a swift transition of our society and economy to low-carbon-emission, circular processes for production of chemicals, transport fuels and food. Current projections for replacing fossil-based fuels for road transport, shipping and aviation by sustainable, low-carbon-emission alternatives all include a significant contribution of microbial biofuels production, such as yeast-based bioethanol production. At the same time, biotechnology is expected to play a major role in the replacement of fossil-based bulk and fine chemicals by microbially production of the same compounds or of functional analogs. Especially until the environmental and economic costs of using fossil feedstocks are included in consumer prices, cell factories for production for most of these products need to become more cost-effective to be able to compete with their established, cheap fossil-based counterparts. A lower price of growth media, as well as improved stability, simplicity and scalability of processes by elimination of growth-factor requirements, which was the subject of the research described in this study, can contribute to driving down costs of production.

Current yeast-based biotechnological processes predominantly use sugars, derived from agriculture, as feedstocks. Processes for production of high-added-value compounds generally use high-purity glucose or sucrose as carbon source, chemically defined media and are performed in aerobic fed-batch cultures. In such cultures, eliminating of individual vitamins from media, e.g., by using biotin-prototrophic yeast strains (Chapter 2) or strains that are completely prototrophic (Chapter 4) simplifies medium composition and reduces costs. Moreover, it can reduce risk of contamination with auxotrophic 'wild' yeast of bacteria. In the case of biotin, it is particularly interesting to investigate whether this approach can also confer advantages for processes that require high activities of biotin dependent enzymes, such as yeast-based production of C_4-dicarboxylic acids with pyruvate carboxylase or processes that use urea as nitrogen source with the aid of urease.

To meet future demands for bulk products of engineered yeast strains, such as ethanol and isobutanol, feedstock use should move from glucose and sucrose to hydrolysates

of lignocellulosic biomass derived from 'energy crops' or non-food agricultural residues. Composition of these feedstocks can vary based on plant species, season, location and storage, resulting in variable contents of growth factors. Moreover, deconstruction of plant biomass required to release fermentable sugars from such feedstocks can involve high temperatures and extreme pH values which may inactivate vitamins [408-410]. In addition, some vitamins can also be chemically inactivated, as exemplified by biotin, which can be inactivated by exposure to aldehydes. Vitamin starvation has been proven to negatively affect ethanol yields and cell viability and be the cause of 'stuck' fermentations in a nitrogen-source dependent manner [296]. Usage of corn steep liquor as a source of vitamins, as an inexpensive alternative to yeast extract [411], in some cases still results in a requirement for addition of yeast extract and peptone as sources of vitamins to obtain highest ethanol production [412]. In addition to increasing costs, such complex medium components can also lead to increased risk of contamination with, for example, lactic acid bacteria [258]. In view of the inherent variability of lignocellulosic ('second generation') feedstocks, experimental analysis across a range of feedstocks and hydrolysis procedures is required to assess the impact of the metabolic engineering strategies presented in Chapters 3 and 4. Another process that could benefit from medium simplification is the production of yeast extracts for animal feed applications, which requires addition of vitamins in the cell propagation phase.

Further optimization of biotin prototrophic *S. cerevisiae* strains, as developed in Chapter 2 and 3, might serve as an alternative for chemical synthesis of biotin as a vitamin and feed additive. Its current synthesis uses crude oil for production of the raw materials following the method developed by Goldberg and Steinbach, and is therefore not considered to be sustainable. Biotin-producing microbes can provide a more sustainable way of biotin production, since carbon dioxide, chemical waste emissions as well as air pollution and water usage are expected to be considerably lower than for chemical biotin synthesis. Sourcing from a biological process might, moreover, be preferred by end-consumers if the biotin is added to food and cosmetic products. New microbial biotin production processes conducted locally for the European food and health market ('onshoring'), might increase the traceability and end-consumer acceptance of biotin and related-products. Research efforts to achieve microbial biotin production processes using *Pseudomonas*, *Escherichia coli* or *Corynebacterium glutamicum* as well as Bifidobacteria prove the interest in a sustainable solution for biotin production. However, these processes did not yet reach cost-competitiveness with chemical synthesis. Naturally occurring, fast-growing biotin-prototrophic yeasts such as *Cyberlindnera fabianii* (Chapter 2) may provide an interesting starting point for classical strain-improvement and/or genome-editing programmes to increase biotin content.

Awareness about the importance of the quality of food products is increasing: "The nutritional content of what we eat determines the composition of our cell membranes, bone marrow, blood, hormones, tissue, organs, skin, hair. Our bodies are replacing

billions of cells every day — and using the foods we consume as the source", as stated by the Brain World Magazine in January 2018. Although application of genetically modified yeasts for food products might encounter challenges related to consumer acceptance, vitamin-producing yeasts would offer interesting nutritional characteristics. Similarly to Golden rice, a genetically engineered variety of *Oryza sativa* that produces β-carotene intended to be grown and consumed as fortified food in areas with a shortage of dietary vitamin A [413], access to yeasts or yeast-fermented products enriched in vitamins as such as vitamin B_9 (folate) could contribute to diet fortification. Vitamin B_9 deficiency causes anaemia in adults and when occurring during pregnancy it causes neural tube defects of the new-born [414, 415]. Addition of vitamins D and B_{12} to milk is another example for fortified foods to prevent vitamin deficiencies. Nutritionally enhanced yeast and yeast extracts generated from vitamin-overproducing strains could play an important role as fortified food platforms [416, 417]. With the current rapid rise of consumer interest in alternative protein sources to replace animal protein, yeast biomass ('single cell protein') is becoming an interesting option as protein-rich food ingredient. In addition to the use of dedicated yeast strains as, enhance vitamin contents of yeast biomass could also increase the nutritional value of excess yeast produced by the brewing and bioethanol industries [361] and thereby improve its monetary value as an animal food ingredient.

In addition to providing options for industrial application, the research described in this thesis raises a number of fundamental scientific questions that can be pursued in further research. The most urgent question, arising from the research described in Chapter 2, is the identification of the reaction catalysed by Bio1 orthologs in yeast. Purification of Bio1, followed by *in vitro* incubation with acyl-CoA esters of different lengths and identification of products by mass spectrometry, NMR and/or other methods may resolve whether this enzyme indeed, as postulated in Chapter 2, catalysis an oxidative cleavage of an acyl-CoA ester that directly leads to formation of pimeloyl-CoA. In addition, it would be highly interesting to investigate why, out of a sample of five different Bio1 orthologs from biotin-prototrophic yeast species, only the *C. fabianii* enzyme conferred biotin prototrophy to *S. cerevisiae*. Availability of prototrophic *S. cerevisiae* strains also raises interesting questions about the context dependency of their prototrophies. This question not only refers to the availability of oxygen (Chapter 2) but also to possible increase vitamin conditions under conditions that require a high activity of enzymes whose activity involves a specific cofactor. In addition, quantitative physiological research on such strains may resolve trade-offs between prototrophy and fitness, for example related to resource allocation in strains in which specific enzymes involved in vitamin biosynthesis need to be overexpressed, with the single-turnover Thi4 and Thi5 enzymes of *S. cerevisiae* as interesting model systems.

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Curriculum vitae

Anna Wronska was born on the 1st of August 1993 in Braunschweig, Germany. Anna grew up in Wolfsburg, Germany and completed her high-school diploma at the Ratsgymnasium Wolfsburg in 2011. In the same year, Anna enrolled in the biotechnology bachelor programme at the Technical University of Braunschweig and performed her bachelor thesis at the microbiology section in Prof. Martina Jahns group about iron homeostasis of the human pathogen *Clostridium difficile*. In the same year she participated in the TU Braunschweig-2013 iGEM team, engineering a synthetic consortium of three inter-dependent *Escherichia coli* strains based on quorum-sensing, awarded with the price for 'Best New Application'. A year after she participated with the TU Braunschweig-2014 iGEM team with a project to engineer an immobilized methane-degrading *Escherichia coli* strain to reduce methane emissions of cattle. In 2013, Anna started a master in Cellular Biotechnology at the Technical University of Braunschweig. During her master studies she worked at the Braunschweig Helmholtz Centre for Infection Research as a research assistant at the Cell Biology group of Prof. Dr. Theresia Stradal and absolved an internship at the Microbial drugs group of Prof. Mark Stadler for identification of new antimicrobial drugs from filamentous fungi. She performed her master thesis at Prof. Jens Nielsen's Systems and Synthetic Biology group at the Chalmers University in Gothenburg, Sweden and engineered *Saccharomyces cerevisiae* for 2-butanol production under supervision of Prof. Christer Larsson. After completing her studies, Anna moved to the Netherlands and started a PhD as part of the Marie-Curie-Skłodowska-action PAcMEN ITN programme, at the Industrial Microbiology group at the Delft University of Technology under the co-supervision of Prof. Jean-Marc Daran and Prof. Jack Pronk. The results generated during Anna's PhD project are basis of this thesis. During her PhD, Anna supervised six student research projects, was inventor on a patent application and was member and chair-woman of the department- and faculty-wide PhD councils. In March 2021, Anna accepted a Scientist position at Animal Health Vision International in Zwolle, the Netherlands.

List of Publications

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List of Patents

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