Carbon dioxide fixation by Calvin-Cycle enzymes improves ethanol yield in yeast

Víctor Guadalupe-Medina1,2, H Wouter Wisselink1,2, Marijke AH Luttik1,2, Erik de Hulster1,2, Jean-Marc Daran1,2, Jack T Pronk1,2 and Antonius JA van Maris1,2*

Abstract

Background: Redox-cofactor balancing constrains product yields in anaerobic fermentation processes. This challenge is exemplified by the formation of glycerol as major by-product in yeast-based bioethanol production, which is a direct consequence of the need to reoxidize excess NADH and causes a loss of conversion efficiency. Enabling the use of CO2 as electron acceptor for NADH oxidation in heterotrophic microorganisms would increase product yields in industrial biotechnology.

Results: A hitherto unexplored strategy to address this redox challenge is the functional expression in yeast of enzymes from autotrophs, thereby enabling the use of CO2 as electron acceptor for NADH reoxidation. Functional expression of the Calvin cycle enzymes phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase (Rubisco) in Saccharomyces cerevisiae led to a 90% reduction of the by-product glycerol and a 10% increase in ethanol production in sugar-limited chemostat cultures on a mixture of glucose and galactose. Co-expression of the Escherichia coli chaperones GroEL and GroES was key to successful expression of CbbM, a form-II Rubisco from the chemolithoautotrophic bacterium Thiobacillus denitrificans in yeast.

Conclusions: Our results demonstrate functional expression of Rubisco in a heterotrophic eukaryote and demonstrate how incorporation of CO2 as a co-substrate in metabolic engineering of heterotrophic industrial microorganisms can be used to improve product yields. Rapid advances in molecular biology should allow for rapid insertion of this 4-gene expression cassette in industrial yeast strains to improve production, not only of 1st and 2nd generation ethanol production, but also of other renewable fuels or chemicals.

Keywords: Metabolic engineering, Synthetic biology, Rubisco, Ribulose-1,5-bisphosphate carboxylase, Phosphoribulokinase, NADH re-oxidation, Carbon dioxide fixation, Saccharomyces cerevisiae, Glycerol, Bioethanol

Background

The yeast Saccharomyces cerevisiae is not only used for the large-scale production of fuel ethanol [1], but also for industrial production of a broad and rapidly expanding range of other chemical compounds from renewable carbohydrate feedstocks [2,3]. In anaerobic, ethanol-producing cultures of S. cerevisiae, excess NADH generated from biosynthetic reactions, such as NAD+-dependent oxidative decarboxylations involved in synthesis of the precursors acetyl-CoA and 2-oxoglutarate, is reoxidized by reducing part of the sugar substrate to glycerol [4]. In growing anaerobic yeast cultures, glycerol production typically accounts for 4-10% of the total sugar consumption and therefore has a significant impact on ethanol yields and process economy in both 1st and 2nd generation largescale bioethanol production [5,6].

Using CO2 as electron acceptor for the reoxidation of NADH would be a highly attractive metabolic engineering strategy, in particular when CO2 reduction can be coupled to the formation of the product of interest. Functional expression of the Calvin cycle enzymes phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase (Rubisco) in S. cerevisiae should enable the coupling of CO2, a major product of alcoholic fermentation, to ribulose-5-phosphate, a normal intermediate of the S. cerevisiae pentose-phosphate pathway (Figure 1). The resulting two molecules of 3-phosphoglycerate can subsequently be converted to 2...
molecules each of ethanol and CO₂, with the concomitant net oxidation of 2 molecules of NADH to NAD⁺ (Figure 1). When ribulose-5-phosphate is formed via the oxidative pentose-phosphate pathway (Figure 1), this route results in a transhydrogenase-type conversion of redox cofactors (NADP⁺ + NADH → NADPH + NAD⁺). Since the total amount of NADPH required in biosynthesis is smaller than the amount of NADH generated [7], such a transhydrogenase-like activity cannot fully replace glycerol formation as a mechanism for reoxidizing biosynthetic NADH. However, no such constraint exists when ribulose-5-phosphate is formed from intermediates of glycolysis via the rearrangement reactions of the non-oxidative pentose-phosphate pathway (Figure 1). A theoretical analysis shows that complete replacement of glycerol production with CO₂ incorporation through PRK and Rubisco can increase the ethanol yield of sugar by as much as 14% (Figure 1).

The PRK gene from Spinacia oleracea [8] has previously been expressed in the yeast Pichia pastoris [9] and is therefore an interesting candidate for heterologous
expression in *S. cerevisiae*. For Rubisco, a key enzyme in the Calvin cycle for autotrophic carbon fixation, three catalytically active forms have been described [10,11]. Prokaryotic form-II Rubisco's are encoded by single structural genes and several have been heterologously expressed in *E. coli* [12,13]. Functional expression of form-II Rubisco's in *E. coli* was shown to be strongly stimulated by the *E. coli* protein-folding chaperones GroEL and GroES [14] and expression of *Hydrogenovibrio marinus* Rubisco in *E. coli* was further stimulated by co-expression of the CbbO and CbbQ chaperones of the donor organism [15]. Very recently the structure of GroEL/GroES encapsulating Rubisco was visualized by cryo-electron microscopy [16]. Eukaryotes such as *S. cerevisiae* harbour a chaperone couple (Hsp60/Hsp10) that structurally and functionally resemble GroEL/GroES. However, these proteins are located in the mitochondria, whereas a role in Rubisco expression would require their activity in the cytosol.

In this study we investigated how to achieve functional expression of PRK and Rubisco in yeast. In view of the envisioned benefit of being encoded by single structural genes, a prokaryotic form-II Rubisco gene was expressed in *S. cerevisiae* in combination with the PRK gene from *Spinacia oleracea*. Both the promoters and coding regions for genes required for glycerol formation were left unchanged compared to the reference strain. Subsequently, the impact of the resulting CO2 incorporation on product formation was studied, with special emphasis on the yields of ethanol and the undesired by-product glycerol.

### Results and discussion

**Chaperone-mediated functional expression of Rubisco in *Saccharomyces cerevisiae***

To study a possible requirement of heterologous chaperones for expression of Rubisco in *S. cerevisiae*, the form-II Rubisco-encoding *cbbM* gene from *T. denitrificans* [17] was codon-optimised for expression in *S. cerevisiae* and expressed from a centromeric vector, both alone and in combination with expression cassettes for the *E. coli* GroEL/GroES [18] or in combination with the *T. denitrificans* chaperones CbbO2/CbbQ2 [20] or all four chaperones (IMC014). Heterologously expressed genes were codon optimised for expression in yeast and expressed from a single centromeric vector. Biomass samples were taken from anaerobic batch cultures on synthetic media (pH 5.0, 30°C), sparged with nitrogen and containing 20 g l⁻¹ glucose as carbon source. Rubisco activities, measured as ¹⁴CO₂-fixation in cell extracts, in a wild-type reference strain and in *S. cerevisiae* strains expressing *cbbM* and *cbbM*-cbbQ2-cbbO2 were below the detection limit of the enzyme assay (0.2 nmol CO₂ min⁻¹ mg protein⁻¹).

**Functional expression of phosphoribulokinase in *Saccharomyces cerevisiae***

The *Spinacia oleracea* phosphoribulokinase (PRK) gene [8], which has previously been expressed in the yeast *Pichia pastoris* [9], was integrated together with *E. coli* GroEL/GroES and *T. denitrificans* CbbO2/CbbQ2 into the *S. cerevisiae* genome at the CAN1 locus, under control that requires expression of prokaryotic enzymes in the cytosol of eukaryotes.
of the galactose-inducible GAL1 promoter. This resulted in high PRK activities (approximately 15 μmol mg protein\(^{-1}\) min\(^{-1}\)) in cell extracts of S. cerevisiae strain IMU033 taken from carbon-limited chemostat cultures on a mixture of glucose and galactose (Table 1). Although relatively high background activities were measured in the reference strain without PRK (IMU032), this activity does not contribute to pathway activity (see below). We therefore assumed that the background activity observed in the reference strain was caused by an impurity in one of the chemicals used in the assay and did not reflect formation of ribulose-1,5-bisphosphate. The engineered strain IMU033, which additionally carried the centromeric expression cassette for T. denitrificans Rubisco, was used to quantitatively analyse the physiological impacts of the expression of Rubisco and PRK.

**Carbon dioxide as electron acceptor in anaerobic chemostat cultures of Saccharomyces cerevisiae**

Quantitative physiological analysis is facilitated by the constant and highly reproducible process conditions in steady-state chemostat cultures [23,24]. Therefore, ethanol and glycerol yields of PRK- and Rubisco-expressing S. cerevisiae were compared to those of an isogenic reference strain in anaerobic, sugar-limited chemostats on a mixture of 12.5 g l\(^{-1}\) glucose and 12.5 g l\(^{-1}\) galactose. In nitrogen-sparged cultures, the glycerol yield on sugar in the strain expressing both Calvin-cycle enzymes was 68% lower than in the reference strain, while ethanol and biomass yields on sugar were 11% and 12% higher, respectively (Table 1). To investigate whether the low affinity of T. denitrificans form-II Rubisco for CO\(_2\) (\(K_{\text{CO}_2} = 0.26\) mM [17]) limited its in vivo activity in the nitrogen-sparged cultures, additional chemostats were sparged with a 10%/90% blend of CO\(_2\) and N\(_2\). Indeed, this CO\(_2\) supplementation resulted in a further decrease of the glycerol yield to a value below 10% of that of the reference strain (Table 1). Co-expression of Rubisco and chaperones without co-expression of PRK (strain IMC014) did not result in decreased glycerol yield (0.13 mol mol\(^{-1}\)) compared to the reference strain IMU032 (0.12 mol mol\(^{-1}\)) in carbon-limited chemostat cultures supplemented with CO\(_2\). This observation confirmed that expression of a heterologous phosphoribulokinase (PRK) gene is required for in vivo carbon fixation via Rubisco in yeast.

**Carbon dioxide as electron acceptor in anaerobic batch fermentations**

Since industrial-scale ethanol production is routinely performed in batch fermentations [25], the impact of the expression of PRK and Rubisco was also investigated in anaerobic, CO\(_2\)-supplemented batch cultures (Figure 3). Galactose was used as the carbon source for these experiments to enable efficient expression of PRK from the GAL1 promoter. Despite an almost 10 h difference in the lag phase, the specific growth rates of the engineered and reference strains on galactose in these anaerobic cultures were not significantly different (Figure 3) and in good agreement with values reported elsewhere for this yeast strain family [26]. Consistent with the observations in chemostat cultures, expression of the two Calvin cycle enzymes reduced glycerol formation in the batch cultures by 60% and increased the ethanol yield on galactose by 8% (Figure 3e-f). However, the biomass yield

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**Table 1 Physiological analysis of S. cerevisiae IMU033 expressing PRK and Rubisco and the isogenic reference strain IMU032 in anaerobic chemostat cultures, grown at a dilution rate of 0.05 h\(^{-1}\) on a synthetic medium (pH 5) supplemented with 12.5 g l\(^{-1}\) glucose and 12.5 g l\(^{-1}\) galactose as carbon sources**

|                      | IMU032                  | IMU033                  |
|----------------------|-------------------------|-------------------------|
|                      | (Reference strain)      | (Expressing PRK and Rubisco) |
| CO\(_2\) in inlet gas (%) | 0                       | 0                       |
| CO\(_2\) in outlet gas (%) | 0.89 ± 0.03             | 10.8 ± 0.0              |
| Phosphoribulokinase (\(\mu\text{mol mg protein}\(^{-1}\) min\(^{-1}\)) | 0.58 ± 0.09             | 0.51 ± 0.12             |
| Rubisco (nmol mg protein\(^{-1}\) min\(^{-1}\)) | < 0.2*                  | < 0.2                   |
| Biomass yield on sugar (g l\(^{-1}\)) | 0.083 ± 0.00\(^a\)     | 0.084 ± 0.000\(^{b, c}\) |
| Ethanol yield on sugar (mol mol\(^{-1}\)) | 1.56 ± 0.03\(^c\)      | 1.56 ± 0.02\(^d\)       |
| Glycerol yield on sugar (mol mol\(^{-1}\)) | 0.14 ± 0.00\(^p\)     | 0.12 ± 0.00\(^q\)       |

*Results are represented as average ± mean deviations of data from independent duplicate chemostat experiments. Data pairs labelled with the same subscripts (\(^{a, b, c}\), etc.) are considered statistically different in a standard t-test (p <0.02).

*Detection limit of enzyme activity assay.
on sugar, which in the chemostat cultures increased according to the predictions, did not increase in the anaerobic batch cultures on galactose. These observations together might indicate that under the galactose excess conditions used for these batch cultivations increased expression levels of PRK result in a small metabolic burden, but still result in an overall positive effect on the ethanol yield.

**Conclusions**

This study provides a compelling proof of principle for the replacement of glycerol formation as the predominant redox sink in anaerobic yeast metabolism by PRK- and Rubisco-mediated incorporation of CO₂ into yeast central carbon metabolism. The loss of sugar feedstock due to glycerol production in industrial bioethanol processes has been estimated at 4% of the consumed sugar.
If expression of PRK and Rubisco in industrial yeast strains were to completely eliminate this loss, this could enable an additional production of 5 billion liters of ethanol from the amount of sugar used for the 2011 global ethanol production of 110 billion liters [1]. Use of CO₂ as an external electron acceptor offers important advantages over previously proposed strategies for reducing glycerol production in yeast-based bioethanol production. Optimizing the redox cofactor specificity of nitrogen assimilation in S. cerevisiae [5] only enables a partial reduction of glycerol production and its impact further depends on the nitrogen sources present in industrial feedstocks. Similarly, a metabolic engineering strategy that enables NADH-dependent reduction of acetic acid [6] to ethanol is dependent on the presence of acetic acid in industrial feedstocks. Further optimization of PRK and Rubisco gene expression and regulation in S. cerevisiae should enable the design and construction of DNA cassettes that can be easily introduced in the genomes of industrial yeast strains. This should include the replacement of the GALI-promotor, that was used for the expression of PRK in this study, by a promotor that is compatible with fast growth at high glucose concentrations and that further balances the expression of PRK and Rubisco. Since ribulose-5-phosphate is also an intermediate in pentose metabolism by engineered S. cerevisiae strains [27], this approach should also be readily applicable to the yeast-based conversion of lignocellulosic hydrolysatess. The observed stimulatory effect of CO₂ on the engineered strains will not hinder application of this concept in industrial bioethanol production, since large-scale processes for bioethanol production are characteristically CO₂ saturated.

Our results illustrate how metabolic engineering strategies based on the functional integration of extensively studied reactions in the central carbon metabolism of distantly related organisms enables the optimization of product yields in industrial biotechnology. Although the present study focuses on ethanol production by yeast, functional integration of autotrophic carbon-fixing enzymes in the metabolic networks of industrial microorganisms should also enable optimization of yields of other existing and novel products whose synthesis results in a net positive ATP yield.

**Methods**

**Construction of expression modules**

Phosphoribulokinase (PRK) cDNA from Spinacia oleracea (spinach) [9] (accession number: X07654.1) was PCR-amplified using Phusion Hot-Start Polymerase (Finnzymes, Landsmeer, the Netherlands) and the oligonucleotides XbaI_prk_FW2 and RV1_Xhol_prk (Table 2), and was ligated in pCR®-Blunt II-TOPO® (Life Technologies Europe BV, Bleiswijk, the Netherlands). After restriction by XbaI and XhoI, the PRK-containing fragment was ligated into pTEF424 [28]. The TEF1p was later replaced by GAL1p from plasmid pSH47 [29] by XbaI and SacI restriction/ligation, creating plasmid pUDE046 (Table 3).

Rubisco form II gene cbbM from T. denitrificans [17] flanked by KpnI and SacI sites was codon optimized [30] (accession number: KC699555), synthesized at GeneArt (Life Technologies Europe BV), and ligated into pPCR-Script. The cbbM-containing fragment was ligated into the BamHI and SacI restricted vector pGPD_426 [28] creating plasmid pBTWW002. The cbbM expression cassette was transferred into pRS416 using KpnI and SacI, yielding pUDC098.

Expression cassette of the specific Rubisco form II chaperones from T. denitrificans cbbQ2 and cbbO2 [20], and chaperones groEL and groES [18] from E. coli were codon optimized [30] (accession numbers: KC699555 and KC699556, respectively). The expression cassettes contained a yeast constitutive promotors and terminator, flanking the codon optimized gene. The cassette was flanked by unique 60-bp regions obtained by randomly combining bar-code sequences used in the Saccharomyces Genome Deletion Project [31] and an EcoRV site (GeneArt). The expression cassettes were inserted in plasmid pMK-RQ (GeneArt) using the SfiI cloning sites yielding pUD230 (PGI1p-cbbQ2-TEF2), pUD231 (PGK1p-cbbO2-ADH1), pUD232 (TEF1p-groEL-ACT1), and pUD233 (TPH1p-groES-PGI1) (Table 3). The expression cassette TDH3p-cbbM-CYC1, was PCR-amplified from plasmid pBTWW002 using Phusion Hot-Start Polymerase (Finnzymes) and primers HR-cbbM-FW-65 and HR-cbbM-RV-65 in order to incorporate the 60-bp region for recombination cloning.

**Strain construction, isolation and maintenance**

All Saccharomyces cerevisiae strains used (Table 4) belong to the CEN.PK family [32,33]. All strains were grown in 2% w/v glucose synthetic media [7] supplemented with 150 mg F⁻¹ uracil when required [34] until they reached end exponential phase, then sterile glycerol was added up to ca. 30% v/v and aliquot of 1 ml were stocked –80°C. The strain IMC014 that co-expressed the Rubisco form II cbbM and the four chaperones cbbQ2, cbbO2, groEL, and groES was constructed using a previously published in vivo transformation associated recombination [35]. 200 fmol of each expression cassette were pooled with 100 fmol of the KpnI/SacI linearized pRS416 backbone in a final volume of 50 μl and transformed in CEN.PK 113-5D using the lithium acetate protocol [36] (Figure 4a). Cells were selected on synthetic medium. Correct assembly of the fragment of pUDC075 was performed by multiplex PCR on transformant colonies using primers enabling amplification over the regions used for homologous recombination (Table 2) and by restriction analysis after
| Number | Name                  | Sequence (5' to 3')                                                                 | Purpose                                                                 |
|--------|-----------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------|
| 1      | XbaI_prk_FW2          | TGACATCTAGTGTCACAACAAACAACAAATTG                                                    | Cloning of PRK into pUDE046.                                           |
| 2      | RV1 XhoI_prk          | TGACATCTAGTGTCACAACAAACAACAAATTG                                                    | Cloning of PRK into pUDE046.                                           |
|        | **Primers used for in vivo plasmid assembly** |                                                                      |                                                                        |
| 3      | HR-cbbM-FW-65         | TGTAAACACGAGCAGCTGACGCTCTATAGCTGACTCTATAGGCGGAAATTGGCTAGCGTATGCTGCTATATATAGTAC    | Rubisco cbbM cassette for plasmids pUDC075, pUDC099, and pUDC100.       |
| 4      | HR-cbbM-RV-65         | GGAATCTGTGATAGGAGGTCTGCTGCGGCGCTATGCTGACTATGCTGCTATATATAGTAC                        | Rubisco cbbM cassette for plasmids pUDC075, pUDC099, and pUDC100.       |
| 5      | linker-cbbO2-prR514   | ATACCTCTTACGAGCTGACGCTCTATAGTGGAGGAGGAGGATGCTGCTATATATAGTAC                        | Linker fragment for assembly of plasmid pUDC099.                        |
| 6      | linker-cbbM-GroEL     | GACATACGAGCATCGAGCTGAGCGAGCAATCGCATTACGATCGCTGAGAATTGGCTAGCGTATGCTGCTATATATAGTAC  | Linker fragment for assembly of plasmid pUDC100.                        |
|        | **Primers used for in vivo integration assembly** |                                                                      |                                                                        |
| 7      | FW pTDH3-HR-CAN1up    | GTGGGATCCAGTTTTTTAACTTCTGCTGCAACCTGAAAGCTTTATTTTGAGGTCTCTGACCTCTTAC               | 1st cloning expression cassette linker fragment between CAN1 upstream and PRK expression cassette (IMI229), and CAN1 up-linker and KLEU2 expression cassette (IMI232). |
| 8      | RV linker-iHR2B       | AGATAATCGAGGAGCAGCCGAACTGATGCTAGATGCTGACTCTACGATGCTGCTATATATAGTAC                | 1st cloning fragment: linker fragment between CAN1 up-linker and PRK expression cassette (IMI229). |
| 9      | RV linker-iHR6        | GCTATGACGATTTACGCTGAGCTGACTCTACGATGCTGCTATATATAGTAC                                | 1st cloning fragment: linker fragment between CAN1 up-linker and KLEU2 expression cassette (IMI229). |
| 10     | FW pGAL1-prk_HR2B    | CAACAAAGTGAGGAGCAGCTGAGCTGACTCTACGATGCTGCTATATATAGTAC                              | 2nd cloning fragment: GAL1 prk expression cassette from pUDE046.         |
| 11     | RV CYC1t-prk_HR2     | GGAATCTGAGACTGCTGCTGAGCTGACTCTACGATGCTGCTATATATAGTAC                              | 2nd cloning fragment: GAL1 prk expression cassette from pUDE046.         |
| 12     | FW HR2-cbbQ2-HR3     | GACATACGAGCATCGAGCTGAGCTGAGCTGACTCTACGATGCTGCTATATATAGTAC                        | 3rd cloning fragment: PRK cbbQ2-TEF2 cassette from pUDE046.             |
| 13     | RV HR2-cbbQ2-HR3     | GGACAGCCTGAGCAGAATGTCAAGAG                                               | 3rd cloning fragment: PRK cbbQ2-TEF2 cassette from pUDE046.             |
| 14     | FW HR3-cbbO2-HR4     | CGTCGATGACGATTGCTGACTCTACGATGCTGCTATATATAGTAC                                | 4th cloning fragment: PGK1 cbbO2-ADH1 cassette (IMI229).               |
| 15     | RV HR3-cbbO2-HR4     | CCTAGAATCTCGCAGCTGACTCTACGATGCTGCTATATATAGTAC                                | 4th cloning fragment: PGK1 cbbO2-ADH1 cassette (IMI229).               |
| 16     | FW HR4-GroEL-HR5     | ATCACTTACGAGCTGACTCTACGATGCTGCTATATATAGTAC                                | 5th cloning fragment: TEF1 groEL-ACT1 cassette (IMI229).               |
| 17     | RV HR4-GroEL-HR5     | CGGACATCAGTATCGTGAGCTGACTCTACGATGCTGCTATATATAGTAC                              | 5th cloning fragment: TEF1-groEL-ACT1 cassette (IMI229).               |
| 18     | FW HRS-GroES-HR6     | CCGTAGGATGACGATTGCTGACTCTACGATGCTGCTATATATAGTAC                              | 6th cloning fragment: TP1 groES-PG1 cassette (IMI229).                 |
| Oligonucleotides used in this study (Continued) |
|-----------------------------------------------|
| **19** RV HR5-GroES- HR6                      |
| GCTATGACCATGAATACGCAAAGC                      |
| 6th cloning fragment: TPI1-groES-PGI1 cassette (IMI229). |
| **20** FW HR6-LEU2- CAN1                    |
| CCACGTTTTTGGCATCTGAAAAATTGCGGCGCTTGGTAATGCTGACATGCTGTAAGATCCCGAGCAAG |
| 7th (IMI229) or 2nd (IMI232) cloning fragment: KILEU2 cassette from pUG73. |
| **21** RV LEU2 HR- CAN1                     |
| AGCTCATGACGGGCTTTTCTCGGTATGCTTATGCGAGTGAGAATTGGCGAAATGGCGTTGAAATGGTAGAAAGTGAATAAAACGGTCATATATCCCGGAGGTAAC |
| 7th (IMI229) or 2nd (IMI232) cloning fragment: KILEU2 cassette from pUG73. |
| **Primers used for verification of the in vivo assembled constructs** |
| **22** m-PCR-HR1- FW  |
| GGCGATTAAGTTGGGTAAACG |
| Diagnostic for assembly of plasmids pUDC075, pUDC099, and pUDC100, and integration in strain IMI229. |
| **23** m-PCR-HR1- RV  |
| AACTGAGCTCCAGCTGTACC  |
| Diagnostic for assembly of plasmids pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **24** m-PCR-HR2- FW  |
| ACGCGTGCTACGATGTAAC |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **25** m-PCR-HR2- RV  |
| GCCGCGTTCCCTCTATAATC  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **26** m-PCR-HR3- FW  |
| GTGAAATGCTGGTGATGTCGATATAC |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **27** m-PCR-HR3- RV  |
| GTAAGCAGCAAACACCTTCAG  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **28** m-PCR-HR4- FW  |
| ACCTGACCTACAGGAAAGAG  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **29** m-PCR-HR4- RV  |
| TGAAGTGGTGACGGCGATGC  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **30** m-PCR-HR5- FW  |
| ATAGCCACCCAGGCGATTTC  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **31** m-PCR-HR5- RV  |
| CCGCAGCTTCTCCATGAGG  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **32** m-PCR-HR6- FW  |
| CGACGGTTACGGTTGTAAG  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
| **33** m-PCR-HR6- RV  |
| CTCCCCGCTCTATGGTG  |
| Diagnostic for assembly of pUDC075, pUDC099, pUDC100, and integration in strain IMI229. |
Table 3 Plasmids used in this study

| Name       | Relevant genotype | Source/reference |
|------------|-------------------|------------------|
| pFL451     | AOX1Δ::prk (Spinach)-AOX1, (pHIL2-D2 HIS4 Amp centromeric) | Brandes et al. [9]. |
| pCR* Blunt II-TOPO | Bla | Life Technologies Europe BV |
| pTEF424_TEF | TRP1 2μi bla | Mumberg et al. [28]. |
| pSH47      | URA3 CEN6 ARS4 GAL1::cre-CYC1, bla | Guldener et al. [29]. |
| pUDOE4     | TRP1 2μi GAL1::prk-CYC1, bla | This study. |
| pPCR-Script | Bla | Life Technologies Europe BV |
| pGPD_426   | URA3 2μi bla | Mumberg et al. [28]. |
| pRS416     | URA3 CEN6 ARS4 bla | Mumberg et al. [28]. |
| pBTWW002   | URA3 2μi TDIH2-tdbhM-CYC1, bla | This study. |
| pUDC098    | URA3 CEN6 ARS4 TDIH3-tdbhM-CYC1, bla | This study. |
| pMK-RQ     | nptII | Life Technologies Europe BV |
| pUD230     | PGK1::cbbQ2-TEF2, nptII | Life Technologies Europe BV |
| pUD231     | PGK1::cbbQ2-ADH1, nptII | Life Technologies Europe BV |
| pUD232     | TEF1::groEL-ACT1, nptII | Life Technologies Europe BV |
| pUD233     | TPI1::groES-PGI1, nptII | Life Technologies Europe BV |
| pUDC075    | URA3 CEN6 ARS4 TDIH3-tdbhM-CYC1,PGK1::cbbQ2-TEF2,PGK1::cbbQ2-ADH1, TEF1::groEL-ACT1, TPI1::groES-PGI1, bla | This study. |
| pUDC099    | URA3 CEN6 ARS4 TDIH3-tdbhM-CYC1,PGK1::cbbQ2-TEF2,PGK1::cbbQ2-ADH1, bla | This study. |
| pUDC100    | URA3 CEN6 ARS4 TDIH3-tdbhM-CYC1, TEF1::groEL-ACT1, TPI1::groES-PGI1, bla | This study. |

retransformation of the isolated plasmid in *E. coli* DH5α. pUDC075 was sequenced by Next Gen Seq Illumina (100-bp reads paired-end, 50 Mb) and assembled with Velvet [37]. The assembled sequence did not contain mutations in any of the assembled expression cassettes. The strains IMC034 and IMC035 that expressed *cbbM/cbbQ2/cbbO2* and *cbbM/groEL/groES* respectively were constructed using the same *in vivo* assembly method with the following modification. To construct plasmids pUDC099 and pUDC100, 120 bp cbbO2-pRS416 linker and cbbM-GroEL linker were used to close the assembly respectively (Table 2), 100 fmol of each of complementary 120 bp oligonucleotides were added to the transformation. The strain IMC033 that only expressed the *cbbM* gene was constructed by transforming CEN.PK113-5D with pUDC098.

Table 4 *Saccharomyces cerevisiae* strains used in this study

| Relevant genotype | Source/reference |
|-------------------|------------------|
| CEN. PK113-5D     | MATa ura3-52     |
| CEN. PK102-3A     | MATa ura3-52 leu2-3, 112 |
| IMC014            | MATa ura3-52 pUDC075 (CEN6 ARS4 URA3 TDH3-tdbhM-CYC1, PGK1::cbbQ2-TEF2, PGK1::cbbQ2-ADH1, TEF1::groEL-ACT1, TPI1::groES-PGI1) |
| IMC033            | MATa ura3-52 pUDC098 (CEN6 ARS4 URA3 TDH3-tdbhM-CYC1) |
| IMC034            | MATa ura3-52 pUDC099 (CEN6 ARS4 URA3 TDH3-tdbhM-CYC1, PGK1::cbbQ2-TEF2, PGK1::cbbQ2-ADH1, cbbO2-pRS416 linker) |
| IMC035            | MATa ura3-52 pUDC100 (CEN6 ARS4 URA3 TEF1::groEL-ACT1, TPI1::groES-PGI1, cbbM-GroEL linker) |
| IMI229            | MATa ura3-52 leu2-3, 112 can1::GAL1::prk-CYC1, PGK1::cbbQ2-TEF2, PGK1::cbbQ2-ADH1, TEF1::groEL-ACT1, TPI1::groES-PGI1, KILEU2 |
| IMI232            | MATa ura3-52 leu2-3, 112 can1::KILEU2 |
| IMI232            | IMI232 p426_GPD (2μ URA3) |
| IMU032            | IMU033 p426_GPD (2μ URA3) |
| IMU033            | IMU033 pUDC100 (CEN6 ARS4 URA3 TEF1::groEL-ACT1, TPI1::groES-PGI1, cbbM-GroEL linker) |

To construct the strain IMU033 that co-expressed PRK, *cbbM*, cbbQ2, cbbO2, the intermediate strain IMI229 was constructed by integrating PRK, the four chaperones and *KILEU2* [38] at the CAN1 locus by *in vivo* homologous integration in CEN.PK102-3A (Figure 4b). The expression cassettes were PCR amplified using Phusion Hot-Start Polymerase (Finnzymes), the corresponding oligonucleotides and DNA templates (Table 2). Finally, the strain IMI229 was transformed with pUDC100 that carries the Rubisco form II *cbbM* and the two *E. coli* chaperones groEL and groES.

Strain IMI232 was constructed by transforming CEN. PK102-3A with the KILEU2 cassette. IMI232 was finally transformed with the plasmid p426GPD to restore prototrophy resulting in the reference strain IMU0302.

Experimental set-up of chemostat and batch experiments

Anaerobic chemostat cultivation was performed essentially as described [39] but with 12.5 g l⁻¹ glucose and 12.5 g l⁻¹ galactose as the carbon source and where indicated, a mixture of 10% CO₂/90% N₂ replaced pure nitrogen as the sparging gas. Residual glucose and galactose concentrations were determined after rapid quenching [40] using commercial enzymatic assays for glucose (Boehringer, Mannheim, Germany) and D-galactose (Megazyme, Bray, Ireland). Anaerobic bioreactor batch cultures were grown essentially as described [6], but with 20 g l⁻¹ galactose and a sparging gas...
Figure 4 Strategy for the heterologous expression of Rubisco and PRK in *S. cerevisiae*. (a) In vivo assembly of Rubisco expression plasmid pUDC075, and (b) in vivo assembly and integration of PRK and chaperone proteins in CAN1 locus of Saccharomyces cerevisiae strain IMI229. Each fragment represents a different expression cassette or plasmid backbone. All fragments used in assembly experiments were flanked by 60-bp sequences used for in vivo recombination, either enabling the assembly of plasmids or the integration assembled constructs into the *S. cerevisiae* genome. Arrows and numbers indicate primers used in the construction of the cassette.
(PerkinElmer, Groningen, The Netherlands). Protein concentrations were determined by the Lowry method [43] using standard solutions of bovine serum albumin dissolved in 50 mM Tris–HCl (pH 8.2).

Abbreviations
Rubisco: Ribulose 1,5-bisphosphate carboxylase/oxygenase; PRK: Phosphoribulokinase; NAD$: Nicotinamide adenine dinucleotide; NADP$: Nicotinamide adenine dinucleotide phosphate.

Competing interests
VGM, HWW, AJAV, and JTP are inventors on a patent application related to the content of this article.

Authors’ contributions
VGM, JMD, JTP, AJAV, and MAM designed the experiments and wrote the manuscript. HWW selected the Rubisco and PRK genes, VGM selected the chaperone genes and constructed all yeast strains. VGM and EdH carried out and analyzed the fermentation experiments. VGM and MAHL performed the PRK and Rubisco enzymatic activity determinations. All the authors have read and approved the final manuscript.

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