

Energy-translation coupling limits anaerobic yeast growth

Corresponding Author: Professor Guan Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

This is a very solid paper describing a thorough characterization of yeast growing at C, N and P limitation at anaerobic conditions. The analysis involves multi-omics analysis and a number of interesting novel findings are reported. The paper will also stand out as an important resource. However, unfortunately there are several critical points that needs to be addressed.

Major:

- 1) It is not true that the maximum specific growth rate of yeast is up to 50% lower at anaerobic growth compared with aerobic growth. In fact the specific growth rate is in fact almost the same! I checked the references mentioned, but they do not provide specific number - just seems to further propagate a misconception. CEN.PK113-7D the strain the authors use has a reported maximum specific growth rate at anaerobic conditions of 0.36 h⁻¹, which is very close the typically reported value for aerobic conditions of 0.35-0.38 h⁻¹. This is also consistent with the fact that even in an aerobic culture the specific oxygen uptake rate at maximum growth is very low, i.e. the cells are almost fully relying on respiration.
- 2) This leads to a major problem with the paper as the authors report quite low specific growth rates in batch cultures. These are much lower than reported earlier in the literature for the same strain! This worries me as it could point to either another limitation in the medium or some other factor in the experimental setup! I could not identify this from studying the medium composition, but Tween can be quite different in composition and are the authors certain that there is provided both C16:1 and C18:1 via the Tween (yeast cannot produce the double bonds at anaerobic conditions and that is why these have to be added to the medium). The authors should check this.
- 3) My other major concern is the difference in the maximum specific growth rate in batch cultures for the different limitations! A batch culture is defined as being non-limited, and the limited substrate is the one that runs out first. Therefore the initial maximum specific growth rate should be the same at all three conditions! I do understand that differences in the maintenance coefficient (as nicely pointed out by the authors) will have impact on the measured maximum specific growth rate, but not as much as the authors report.
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Jens Nielsen

Reviewer #3

(Remarks to the Author)

This is an interesting study by Wang et al that takes a comprehensive approach using yeast, and finally identifies key metabolic constraints that are presented during continuous anaerobic growth. They suggest what constrains maximal growth rate during continuous anaerobic growth (which is the highlight of this study), and propose that it is ATP insufficiency under C & P limitation, and aminoacyl-tRNA synthase (and not necessarily amino acid production itself) during N limitation. They therefore suggest that the energy-translation coupling is the central determinant of anaerobic growth limits.

The experimental design and data produced is very good, and the question asked is important. However, in its present form, the study has made several broad assumptions through bulk measurements, ignores specific possibilities, lacks rigor in analysis, and has not contextualised these results alongside some key known studies.

Comments

1.(minor) Fig 1 is very simple (and doesn't really add much to the paper). It need not be a stand alone paper, but can highlight how anaerobic conditions were maintained and measured.

2. How was ATP production rate calculated? While total/bulk ATP can be easily measured - (i) ATP production rate is extremely difficult to calculate experimentally, and (ii) the relative contribution from glycolysis v any respiration is also hard to measure. If this being estimated (indirectly) from the amount of CO₂ and ethanol produced? With these nutrient dilutions, this calculation will result in too many incorrect assumptions. Please clarify in detail. This is particularly important, during the carbon or phosphate limitation experiments, and could show clear trade-offs that will be revealing.

3. As yeast produce ethanol, they also take up and utilise ethanol continuously, and this extent will vary under different carbon/phosphorus levels. See PMID: 35970997. How were the estimates therefore done (to separate ethanol production flux from continuous utilisation), to draw these conclusions?

4. Relatedly - to both 1 and 2 - the anaerobic growth is based on parsing N₂ into the chemostat (and is a reasonable assumption). Add some precise data on mitochondrial activity in these cells, as well as bulk oxygen levels (dissolved oxygen, and any estimates of respiration)

5. For both 2 and 3, additional inferences can be drawn through the proteome data (reflecting relative levels of the respective enzymes associated with these processes). Can also refer to existing proteome datasets eg PMID: 34903663, in addition to their own proteome datasets. Currently, this is descriptive, without bringing out key conclusions

6. Relatedly - key changes should be observed with ribosomal amounts/composition as ATP and energy charge changes. See the next section (related to fig 3/4) on the proteome - map back to ATP and energy charge, to make clearer (and significant) correlations. Currently - it is more descriptive, on growth rate v proteome (and ribosomal composition) - which is largely consistent with what we understand about growth programs in cells.

7. Again, related to carbon limitation - based on existing literature and changes in the net ATP synthesis efficiency vs rates of consumption (eg protein translation amount/rate) and tradeoffs therein. Along with this, a clearer estimate of flux through glycolysis (as the major ATP generating process).

8. The suggested translational bottleneck (due to amino acyl tRNA synthetases) is a particularly intriguing finding - and can make a lot of sense (especially since amino acids themselves do not become limiting, the pools remain large). However, any functional validation of this depletion is not shown, and it is not clear if these are really limiting, and impacting either the rate or extent of protein translation that is happening. Additional supporting validation is essential in order to make this claim. I understand that it is very hard to measure impact on tRNA charge (and proportions of charged tRNAs), but any associated impact on translation rate/amount etc can be better assessed.

9. The last section - on the changing phosphorylation landscape (especially for different metabolic enzymes, and associated impact on glycolytic flux) is particularly interesting. These data can be better contextualised and interpreted based on existing knowledge of activity based control (eg as observed in PMID: 27789812).

11. Their idea to measure the impact of 'push-pull' strategies is interesting. Assessing the push part - by over expressing transporters, is more straightforward. However, it is not clear at all if their experiments to alter pull worked at all (see points above, related to amino acid acylation, tRNA charge, overall translation).

10. Methodology details - several methodology details are missing with respect to how estimates for some rate measurements (including uptake) were calculated using only steady state metabolite data. There are no direct uptake measurements (which are usually done quantitatively over time using a labelled metabolite). Additionally, as pointed out

earlier, several conclusions regarding rate and flux are made using steady state measurements, without including other (utilisation based) measurements - for example using reductive capacity for biosynthesis (eg. amino acid biosynthesis), or ATP for protein synthesis (can be approximated based on amounts of ribosomal subunits etc).

11. In addition to some major studies omitted in this text in relation to proteome efficiency, or flux regulation, or ethanol production/utilization (pointed out earlier) - it is critical to better contextualise these results alongside some central studies related to carbon/nitrogen/phosphorus limitation, and impact (particularly) on glycolysis is critical. This includes:

- Growth limiting intracellular metabolites under nutrient limitations (PMID: 19889834)
- On impact of phosphate/inorganic phosphate in rates and flux of glycolysis based on phosphate availability) - PMID: 24436182 and PMID: 39324403
- nitrogen limitation, and large reserves in metabolic capacity PMID: 32312967

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I think the authors have addressed all my comments very well, and I rest my case that the maximum specific growth rate is almost the same at anaerobic and aerobic conditions! However, it is definitely not 50% lower! Also the issue about media limitations is addressed very well by the authors. My only remaining point is that the authors now state that the specific growth rate is lower at anaerobic conditions due to a lower ATP production! I am not sure this can be stated for certain as we do not know. As I pointed out earlier at maximum growth at aerobic conditions the oxygen uptake is close to zero, and hence ATP production is also mainly from fermentation, and there therefore must be other reasons for the lower specific growth rate at anaerobic conditions. So the authors should change this sentence, and it is OK to be vague as we do not know the exact mechanisms underlying the lower specific growth rates at anaerobic conditions. It can be mitochondrial function and hence biosynthesis of various biomass precursors, it can be uptake rates of ergosterol, and many other factors could be in play!

Excellent work and nice paper

Jens Nielsen

Reviewer #3

(Remarks to the Author)

This is a very good revision, and a much improved manuscript. The study is important, and the resource extremely useful. All my main concerns have been addressed. The manuscript requires some careful editing (there are some minor grammar errors, and a few loosely phrased statements), as well as more complete referencing (the final references cited are still selective, and some references cited in the response to reviewers should probably also be cited).

made.

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REVIEWER COMMENTS

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Response: We sincerely thank you for this help comment. We have re-examined the maximum specific growth rates under anaerobic and aerobic conditions and confirmed that the anaerobic maximum specific growth rate is substantially lower than that observed under aerobic conditions. Detailed information has been added as follows.

Clarification on the anaerobic vs. aerobic maximum specific growth rate ($\mu_{\max}$)

First, we agree with you that our original statement (a 30-50% reduction/up to 50% lower) might be interpreted as an overgeneralization. We have softened the text to accurately reflect that "anaerobic conditions considerably restrict the maximum specific growth rate compared to aerobic conditions" instead of stating a fixed percentage drop.

Regarding the $\mu_{\max}$ for the CEN.PK113-7D strain under strict anaerobiosis, we carefully re-examined the literature. To our knowledge, the value of 0.36 h^{-1} you mentioned most likely originates from an error in the publication by Bouwknecht et al¹. On page 4 of that paper, it states: "The *S. cerevisiae* reference strain CEN.PK113-7D (URA1) grew fast (0.36 h^{-1}) in anaerobic cultures on glucose-containing synthetic media with and without uracil (SMUD+ura and SMUD, respectively)." However, this

sentence contains a clear error, conflating aerobic and anaerobic results. In the same paper, Figure 2A shows that approximately 0.36 h^{-1} corresponds to the aerobic growth rate. Conversely, Figure 2B indicates that the anaerobic growth rate is roughly 0.25 h^{-1} . Moreover, the text on page 6 explicitly clarifies this, stating: "As anticipated, the reference strain... showed... specific growth rates in anaerobic cultures... ($0.24\text{-}0.25 \text{ h}^{-1}$)."

We also reached out to the corresponding author of the article, Prof. Jack T. Pronk, regarding this matter. He responded that it was indeed an oversight during their proofreading process.

[REDACTED]

Furthermore, another studies by Prof. Walter M. van Gulik's group² clearly demonstrated that under strictly anaerobic batch cultivation at $30 \text{ }^{\circ}\text{C}$, the μ_{max} of CEN.PK113-7D is $0.279 \pm 0.012 \text{ h}^{-1}$. Consistent with this, our experimental measurements in this study under C-limitation also give $0.276 \pm 0.009 \text{ h}^{-1}$.

[REDACTED]

Manuscript Revision

To prevent the propagation of errors, we have implemented the following revisions to the manuscript (**Lines 52-53**):

Compared to respiration, fermentation yields less ATP per glucose molecule and is therefore generally associated with lower maximum specific growth rates^{3, 4}.

2. This leads to a major problem with the paper as the authors report quite low specific growth rates in batch cultures. These are much lower than reported earlier in the literature for the same strain! This worries me as it could point to either another limitation in the medium or some other factor in the experimental setup! I could not identify this from studying the medium composition, but Tween can be quite different in composition and are the authors certain that there is provided both C16:1 and C18:1 via the Tween (yeast cannot produce the double bonds at anaerobic conditions and that is why these have to be added to the medium). The authors should check this.

Response: Thank you for your comment. Absolutely, *S. cerevisiae* cannot synthesize unsaturated fatty acids (UFAs) or sterols without oxygen, making the supplementation of these metabolites strictly essential for anaerobic growth.

(1). Clarification on Tween 80 composition and comparison with standard literature media

To address your concern regarding potential nutrient limitations, we systematically compared our medium composition with the medium for anaerobic

yeast cultivation established by Verduyn et al.⁵. In our study, the anaerobic factors supplemented to the medium consisted of 0.4 g/kg Tween 80 and 10 mg/L ergosterol. This corresponds to the same amount of exogenous addition in the article by Prof. Walter M. van Gulik².

The composition label of the Tween 80 we used indicates 100% polysorbate 80, with no other ingredients listed. Meanwhile, Dekker et al. (2019)⁶ study showed that anaerobic growth of *Saccharomyces cerevisiae* CEN.PK113-7D does not depend on the synthesis or supplementation of unsaturated fatty acids.

(2). The same $\mu_{\max}$ value under anaerobic batch fermentation conditions

Your concern regarding a potential medium limitation originates from the premise that the maximal anaerobic specific growth rates ($\sim 0.28 \text{ h}^{-1}$) were "too low." However, as we detailed comprehensively in our response to **Question 1**, $\sim 0.28 \text{ h}^{-1}$ is not a repressed or limited growth rate, but rather the established, theoretical, and empirical maximum growth rate for the CEN.PK strain under strictly anaerobic batch cultivation at 30°C (e.g., $0.279 \pm 0.012 \text{ h}^{-1}$ as demonstrated by Walter M. van Gulik²).

Because our formulated medium strictly adhered to the established classical composition, and yeast cells successfully achieved the physiological benchmark of anaerobic $\mu_{\max}$, it well proves that our experimental setup fully satisfied all cellular requirements.

3. My other major concern is the difference in the maximum specific growth rate in batch cultures for the different limitations! A batch culture is defined as being non-limited, and the limited substrate is the one that runs out first. Therefore, the initial maximum specific growth rate should be the same at all three conditions! I do understand that differences in the maintenance coefficient (as nicely pointed out by the authors) will have impact on the measured maximum specific growth rate, but not as much as the authors report.

Response: Thank you for highlighting this. The differences in culture media under different limitation conditions, particularly the variation in glucose concentration, may account for the differences in the maximum specific growth rate in batch cultures.

To ensure that nitrogen or phosphorus remains the sole limiting factor during the long-term continuous culture phase, we used the same medium formulation in the batch fermentation phase as in the respective chemostat phase.

Culture media (Lines 501-514):

Carbon-limited medium: 5 g/kg $(\text{NH}_4)_2\text{SO}_4$, 3 g/kg KH_2PO_4 , 0.5 g/kg

MgSO₄·7H₂O, 27.5 g/kg glucose·H₂O.

Nitrogen-limited medium: 1 g/kg (NH₄)₂SO₄, 3 g/kg KH₂PO₄, 5.3 g/kg K₂SO₄, 0.5 g/kg MgSO₄·7H₂O, 61.6 g/kg glucose·H₂O.

Phosphorus-limited medium: 5 g/kg (NH₄)₂SO₄, 0.12 g/kg KH₂PO₄, 1.9 g/kg K₂SO₄, 0.51 g/kg MgSO₄·7H₂O, 72.6 g/kg glucose·H₂O.

All media required the addition of 1 mL of trace metals solution, 1 mL of vitamins, 10 mg/L of ergosterol, 5 % defoamer, and 0.4 g/kg of Tween-80 solution. The trace metal components used included: 3 g/kg FeSO₄·7H₂O, 4.5 g/kg ZnSO₄·7H₂O, 4.5 g/kg CaCl₂·2H₂O, 1 g/kg MnCl₂·4H₂O, 0.3 g/kg CoCl₂·6H₂O, 0.3 g/kg CuSO₄·5H₂O, 0.4 g/kg Na₂MoO₄·2H₂O, 1 g/kg H₃BO₃, 0.1 g/kg KI, and 19 g/kg EDTA. The vitamin mix contained: 0.05 g/kg D-biotin, 1 g/kg D-pantothenic acid, 1 g/kg hemicalcium salt, 1 g/kg thiamin-HCl, 1 g/kg pyridoxin-HCl, 1 g/kg nicotinic acid, 0.2 g/kg 4-aminobenzoic acid, and 25 g/kg M-inositol.

The differences in media composition may be the cause of the maximum specific growth rates. Furthermore, we also attempted to perform continuous chemostat cultivations at D = 0.25 under N- or P-limited conditions, but yeast cells were washed out because they could not sustain steady-state growth at this dilution rate.

4. The good news for the authors is that these likely \"faulty\" experiments probably will not impact the results from the chemostat cultures! I therefore do believe that these experiments are OK! However, they are more difficult to evaluate and compare with past experiments as they are more novel. However, the authors should do a comparison with earlier reports from anaerobic C-limited chemostat experiments in order to make me ensured that there is not a fundamental problem with medium or experimental set-up.

Response: We appreciate your approval of our chemostat data and thank you for this suggestion.

To address this issue, we carefully compared our results with those from an anaerobic chemostat study conducted by Kuyper et al⁷, which utilized the same *S. cerevisiae* CEN.PK113-7D strain.

As shown in the comparison table below, our experimental data well match with the results by Kuyper et al.

Physiological Parameter (Anaerobic C-limitation, D=0.10 h ⁻¹)	Kuyper et al. (2003)	This Work
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Biomass yield on glucose (g/g)	0.09 ± 0.00	0.09 ± 0.00
Ethanol yield on glucose (g/g)	0.40 ± 0.00	0.39 ± 0.02
q _{Glucose} (mmol/gDW/h)	6.07 ± 0.07	6.10 ± 0.04
q _{Ethanol} (mmol/gDW/h)	9.48 ± 0.16	9.20 ± 0.09
q _{Glycerol} (mmol/gDW/h)	0.81 ± 0.00	0.94 ± 0.36
q _{CO₂} (mmol/gDW/h)	10.53 ± 0.55	11.65 ± 0.58

Minor:

1. In line 53 it is mentioned that there is produced 32 moles of ATP per mole of glucose at aerobic growth in yeast. This is definitely not the case. First of all, yeast lacks system 1 in the respiratory system decreasing the maximum P/O ratio to 2 (instead of 3) and also there is not full efficiency in the respiratory system. This number is therefore rather 20 or even lower. However, the point made is still valid, but this should be corrected so we do not continue to spread this misconception that the authors apparently also have!

Response: Thank you for clarifying this misunderstanding.

It is our oversight to apply the theoretical ATP yield (30-32 ATP) to *S. cerevisiae*. We carefully reviewed the literature and confirmed that due to the limitation of the P/O ratio, the ATP yield is indeed not that high⁸. We have corrected this in the manuscript.

Manuscript Revision:

To avoid further spreading this misconception, we have revised the text in the Introduction (**Lines 52-53**) as follows:

Compared to respiration, fermentation yields less ATP per glucose molecule and is therefore generally associated with lower maximum specific growth rates^{3, 4}.

2. Fig 3 is not necessary to have in the main text - can easily be moved as a supplementary figure. There are too many figures!

Response: Thank you for your suggestion and we have moved the original **Figure 3** to the Supplementary Information to reduce the number of main text figures. It is now designated as **Supplementary Figure S2**. The numbering of all subsequent figures in the main text has been updated accordingly.

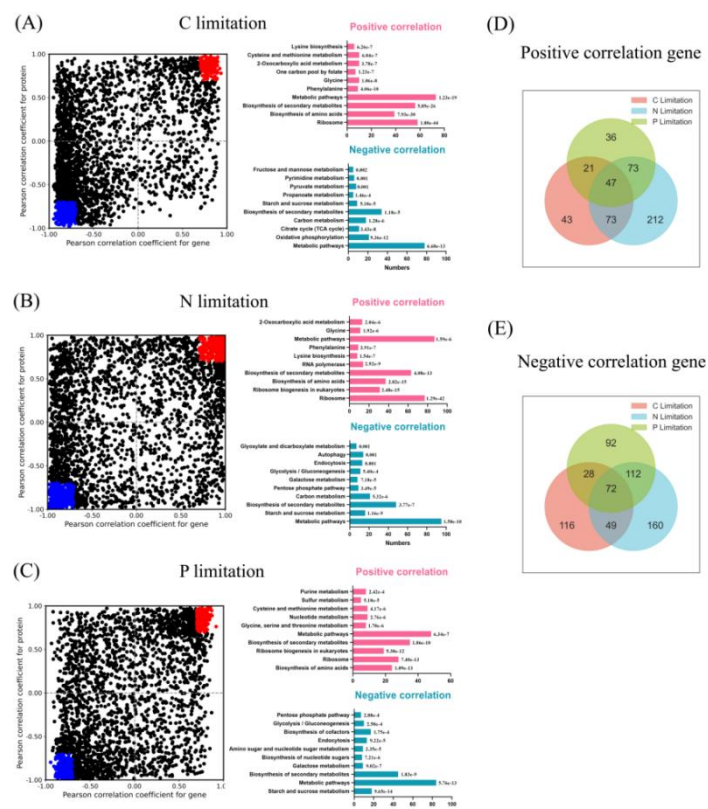


Figure S2 Hierarchical regulation of transcript and protein abundance under nutrient limitations. (A-C) Correlations of transcript levels and protein abundances with specific growth rate (μ) in chemostat cultures limited by C, N, or P. Scatter plots depict the Pearson correlation coefficients between transcript level and μ (x-axis) and between protein abundance and μ (y-axis). Genes showing strong positive ($R > 0.7$) or negative ($R < -0.7$) correlations are highlighted in red and blue, respectively. Bar charts indicate KEGG-enriched pathways associated with positively (red) and negatively (blue) correlated genes. **(D)** Venn diagram showing overlaps of positively correlated genes across all three nutrient limitations. **(E)** Venn diagram showing overlap of negatively correlated genes.

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Response: We sincerely thank you for pointing out this taxonomic inaccuracy.

Because our comparative discussion was indeed specifically referencing translational regulation in *Escherichia coli*, we have corrected the terminology for scientific precision. We have replaced the phrase "prokaryotic systems" with "bacterial systems" in the revised manuscript (**line 452**):

Comparison with **bacterial systems** highlights both conserved principles and eukaryote-specific divergence.

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The experimental design and data produced is very good, and the question asked is important. However, in its present form, the study has made several broad assumptions through bulk measurements, ignores specific possibilities, lacks rigor in analysis, and has not contextualised these results alongside some key known studies.

1. Fig 1 is very simple (and doesn't really add much to the paper). It need not be a standalone paper, but can highlight how anaerobic conditions were maintained and measured.

Response: Thank you for your suggestion, and we have now merged the original **Figure 1** and **Figure 2** into a single one (**new Figure 1**). Also, the numbering of all subsequent figures has been updated accordingly.

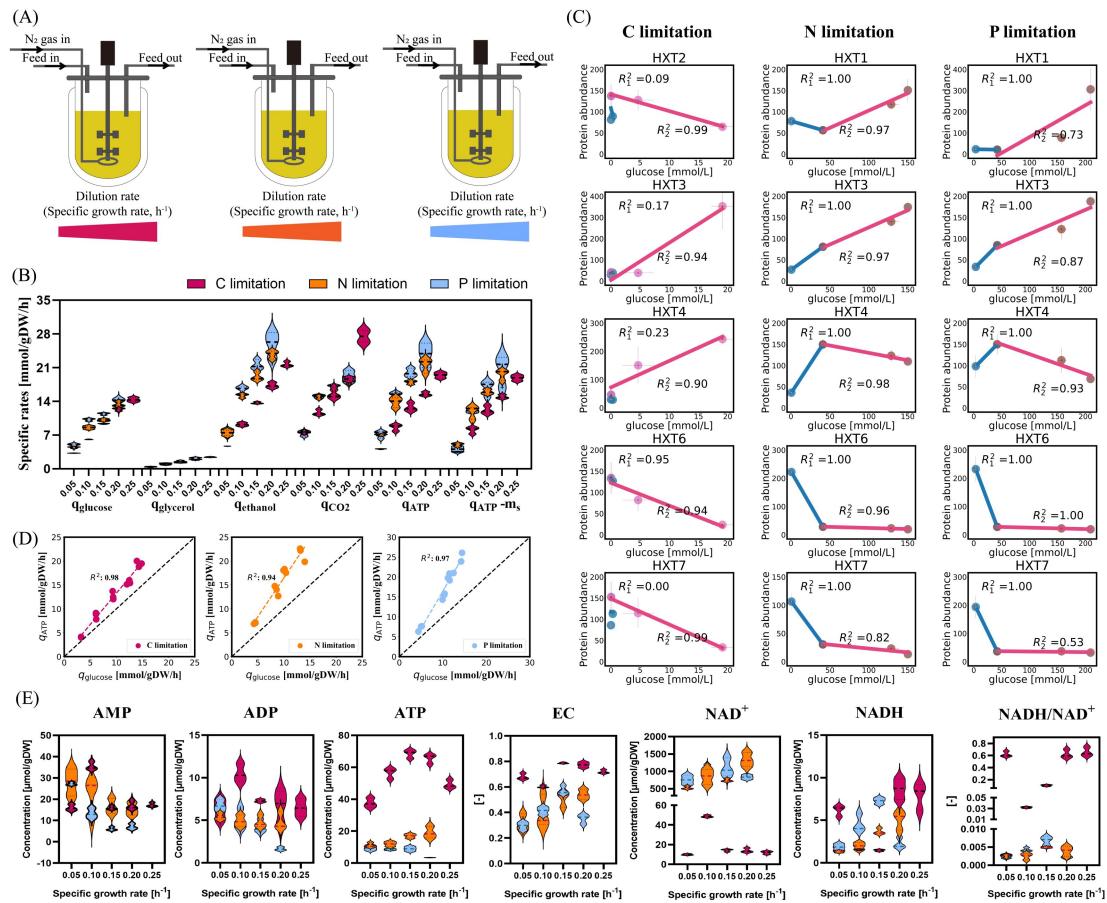


Fig. 1 Nutrient limitation reprograms energy and redox metabolism in anaerobic *S. cerevisiae* cultures. (A) Continuous cultures of *S. cerevisiae* were established under defined C, N, and P limitations. C-limited chemostats were operated at five dilution rates (0.05, 0.10, 0.15, 0.20, and 0.25 h^{-1}), whereas N- and P-limited chemostats were maintained at four dilution rates (0.05, 0.10, 0.15, and 0.20 h^{-1}). Each condition was performed in triplicate. (B) Specific glucose consumption, ethanol and glycerol production, carbon dioxide evolution, and ATP production rates at varying specific growth rates. (C) Correlation between ATP production rate and glucose consumption rate across C-, N-, and P-limited conditions. Linear fits indicate conserved stoichiometric coupling between carbon flux and energy generation. (D) Protein abundance of glucose transporters across increasing μ . Transporter switching occurred at $\mu = 0.15 h^{-1}$ under C limitation, but earlier ($\mu = 0.10 h^{-1}$) under N or P limitation, reflecting nutrient-specific control of substrate affinity. (E) Intracellular energy and redox status across growth rates, including ATP pool size and NADH/NAD⁺ ratio. Data represent means $\pm$ SEM ($n = 3$).

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produced? With these nutrient dilutions, this calculation will result in too many incorrect assumptions. Please clarify in detail. This is particularly important, during the carbon or phosphate limitation experiments, and could show clear trade-offs that will be revealing.

Response: Thank you for your question. We fully agree with you that in aerobic or respiro-fermentative cultures, calculating the exact ATP production rate (q_{ATP}) is difficult due to the complex interplay between glycolysis and the variable efficiency of oxidative phosphorylation.

(1). Under strict anaerobic conditions, *S. cerevisiae* generates energy exclusively through the glycolytic pathway

The major strength of our experimental design is the strictly anaerobic setup. Under these absolute anaerobic conditions, the mitochondrial respiratory chain is completely inactive, meaning the relative contribution from respiration to ATP generation is strictly zero.

(2). The calculation process of ATP production rate

Because ATP generation is entirely restricted to the EMP pathway, calculating q_{ATP} does not rely on physiological assumptions (such as P/O ratios), but rather on biochemical stoichiometry. Based on the established mass-balancing for anaerobic *S. cerevisiae*, originally derived by Verduyn et al⁹, the specific ATP production rate is directly tied to the fluxes of fermentation products:

- 1) The formation of 1 mole of ethanol yields exactly 1 mole of ATP.
- 2) The formation of 1 mole of glycerol consumes 1 mole of ATP (as it serves as a redox sink to reoxidize NADH generated during biomass synthesis, thereby bypassing the ATP-yielding steps of lower glycolysis).

Therefore, the specific ATP production rate is accurately defined by the stoichiometric relationship: $q_{\text{ATP}} \approx q_{\text{ethanol}} - q_{\text{glycerol}}$.

As detailed in our Methods section, our provided Equation (2) ($Y_{\text{ATP}}^{\text{biomass}} = C_{\text{biomass}} / (C_{\text{ethanol}} - C_{\text{glycerol}})$) and Equation (3) ($q_{\text{ATP}} = \mu / Y_{\text{ATP}}$) are mathematically derived directly from this exact stoichiometric balance. Because this calculation is governed strictly by the physical chemistry of the glycolytic pathway under anaerobiosis, it remains biochemically robust regardless of whether the cells are limited by carbon, nitrogen, or phosphorus.

(3). Revealing the physiological trade-offs (C, N and P-limitation)

Our results reveal obvious physiological trade-offs under P and N limitation. Despite generating ATP at a very high rate under N and P-limitation, as seen in **Figure 1B**, the respective specific growth rate (μ) is still constrained. This demonstrates that under N or P-limitation, *S. cerevisiae* enters a highly inefficient metabolic state where

a massive proportion of the generated ATP is spilled as maintenance energy ($m_s = 3.10$ mmol/gDW/h for P-limitation, 2.01 for N-limitation, 0.53 for C-limitation). This clearly illustrates the energetic trade-off required to survive extreme P or N scarcity.

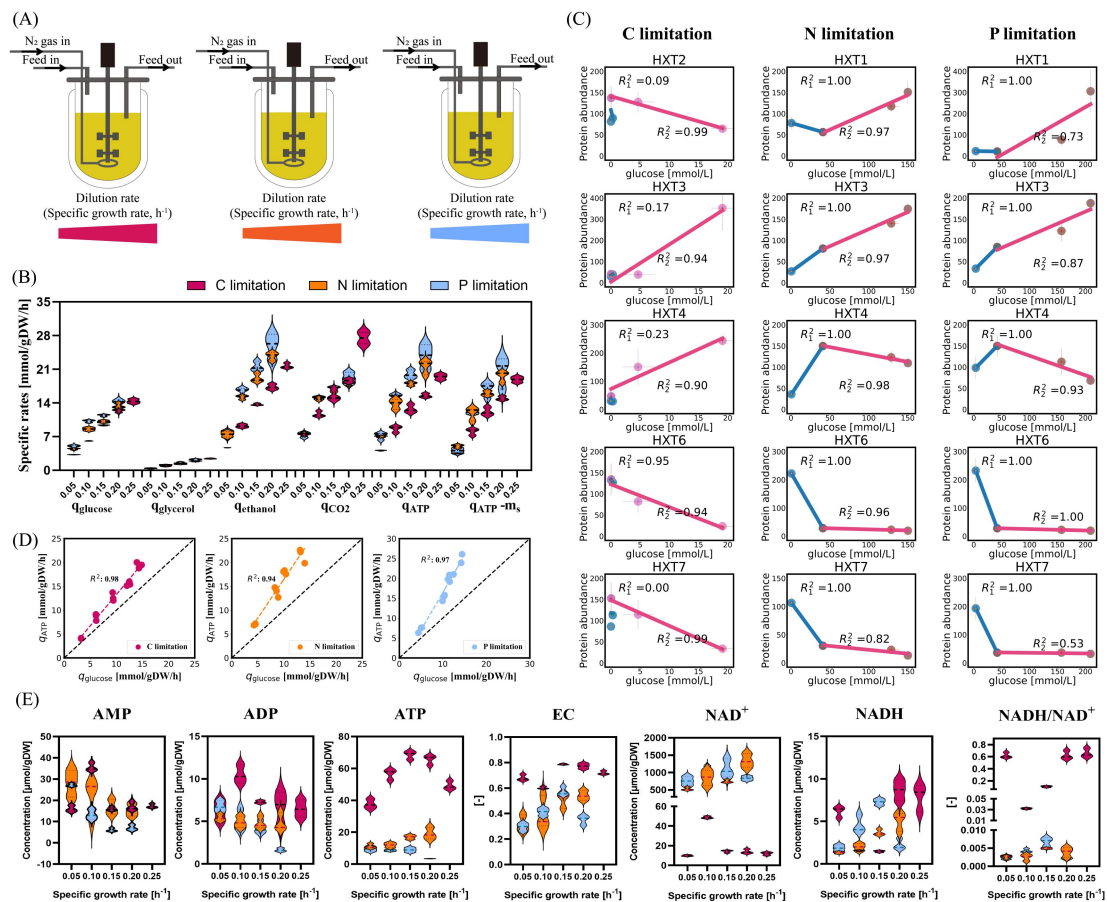


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Manuscript Revision

We have extensively revised the Methods section (Lines 672-710) to explicitly

include the stoichiometric rationale behind equations (2) and (3), citing Verduyn et al. (1991)⁹ and Boender et al. (2009)¹⁰. We also emphasized that the strictly anaerobic condition nullifies any uncertainties regarding respiratory ATP contributions:

Under steady-state chemostat conditions, the specific growth rate (μ) strictly equals the dilution rate (D). The specific macroscopic consumption rates of substrates (q_s , e.g., glucose) and production rates of extracellular metabolites (q_p , e.g., ethanol) were directly calculated using respective mass balances across the bioreactor:

$$q_s = \frac{D \times (C_{s,in} - C_{s,out})}{C_x} \quad (1)$$

$$q_p = \frac{D \times C_{p,out}}{C_x} \quad (2)$$

where $C_{s,in}$ and $C_{s,out}$ are the substrate concentrations in the feed and effluent (mmol L⁻¹), respectively, $C_{p,out}$ is the product concentration in the effluent (mmol L⁻¹), and C_x is the steady-state biomass concentration (gDW L⁻¹).

The relationship between specific substrate consumption rate and specific growth rate was described using the Herbert–Pirt equation (**Equation (3)**)^{10, 11}:

$$q_s = \frac{1}{Y_{X/S}} * \mu + m_s \quad (3)$$

where q_s represents the specific substrate uptake rate (mmol gDW⁻¹ h⁻¹), $Y_{X/S}$ is the biomass yield on substrate (gDW mmol⁻¹), μ is the specific growth rate (h⁻¹), and m_s denotes the maintenance coefficient.

Linear regression based on **Equation (3)** revealed that the maintenance energy requirement under P limitation (3.10 mmol gDW⁻¹ h⁻¹) was 5.8-fold higher than under C limitation (0.53 mmol gDW⁻¹ h⁻¹; ANOVA, $P < 0.001$). N limitation exhibited an intermediate value (2.01 mmol gDW⁻¹ h⁻¹; **Table 1**).

Under strictly anaerobic conditions, the mitochondrial respiratory chain is completely inactive, meaning that ATP is generated exclusively via substrate-level phosphorylation during glycolysis. Based on the established stoichiometry for anaerobic *S. cerevisiae*⁹, the net ATP production does not rely on variable respiratory P/O ratios, but is rigidly tied to the fermentation fluxes: the formation of 1 mole of ethanol yields 1 mole of ATP, whereas the formation of 1 mole of glycerol consumes 1 mole of ATP (as it serves as a redox sink to reoxidize biomass-derived NADH, bypassing the ATP-yielding steps of lower glycolysis).

$$Y_{\text{biomass}}^{\text{ATP}} = \frac{C_{\text{biomass}}}{C_{\text{ethanol}} - C_{\text{glycerol}}} \quad (4)$$

The ATP yield ($Y_{\text{biomass}}^{\text{ATP}}$) was determined according to **Equation (4)**⁹, where C_{biomass} (g L⁻¹), C_{ethanol} (mM), and C_{glycerol} (mM) represent the concentrations of biomass, ethanol, and glycerol, respectively.

The specific ATP production rate (q_{ATP}) was determined using **Equation (5)**¹²:

$$q_{ATP} = \frac{\mu}{Y_{ATP}} \quad (5)$$

By combining **Equations (4) and (5)**, which mathematically simplifies to the exact stoichiometric balance $q_{ATP} = q_{ethanol} - q_{glycerol}$, we determined the specific ATP production rate under each nutrient limitation condition. This absolute dependence on glycolytic stoichiometry avoids any uncertainties regarding respiratory ATP contributions, enabling a robust and quantitative assessment of energy allocation across growth regimes.

3. As yeast produce ethanol, they also take up and utilise ethanol continuously, and this extent will vary under different carbon/phosphorus levels. See PMID: 35970997. How were the estimates therefore done (to separate ethanol production flux from continuous utilisation), to draw these conclusions?

Response: Thank you so much for this enlightening question.

Under strict anaerobic conditions, *S. cerevisiae* cannot utilize ethanol as it does under aerobic conditions¹³, which means that the net ethanol excretion flux exactly equals the unidirectional ethanol production flux. We base this conclusion on two evidences:

(1). Results of transcriptomic and proteomic profiling

We mapped the multi-omics profile of the ethanol production gene (ADH1) versus the ethanol assimilation genes (ADH2, ACS1, ICL1, MLS1) across all conditions (**Fig. S1A**). As illustrated in the transcriptome data, the absolute abundance of the fermentative ADH1 is overwhelmingly dominant, whereas ADH2 and the glyoxylate shunt genes are severely repressed. Our proteomic data confirms this blockade at the translational level: while Adh1 is highly abundant, the primary assimilatory alcohol dehydrogenase (Adh2) and critical glyoxylate cycle enzymes (Icl1 and Mls1) were completely undetectable across both C-, N- and P-limitations.

(2). Results of 1-¹³C ethanol isotope tracing

To further proof that ethanol re-assimilation is completely arrested in our system, we performed a targeted high-resolution stable isotope tracing experiment using 1-¹³C ethanol (**Fig. S1B**). The experiment was carried out under a steady-state dilution rate $D = 0.2 \text{ h}^{-1}$. Samples were collected at the following time intervals: 0, 2, 5, 10, 30, 60, 70, 90, 120, 150, and 180 minutes. Pulses were performed at 0 min and 90 min, and the final concentration of the labeled ethanol added each time in the reactor was 0.5 g/L. Unlike uniformly labeled ethanol, the assimilation 1-¹³C ethanol would

exclusively yield M+1 Acetyl-CoA and subsequently M+1 downstream TCA intermediates. As shown in **Figure S1B**, we tracked the raw M+1 fraction of key metabolites alongside their theoretical natural ^{13}C abundance baselines (indicated by the red dashed lines and shaded noise zones). The M+1 fraction of Acetyl-CoA (the obligate entry point for ethanol) and downstream Malate/Fumarate remained entirely confined within or below their theoretical natural background limits, confirming zero carbon flux from ethanol.

We definitively rule out this minor shift as authentic metabolic re-assimilation based on three biochemical and analytical dimensions: (i) Mass Conservation: The obligate upstream precursor, Acetyl-CoA, was strictly unlabelled. It is stoichiometrically impossible to generate ^{13}C -enriched downstream intermediates without an enriched precursor pool. (ii) Flat Isotopic Kinetics: In a time-course isotopic pulsing experiment, authentic assimilation would display a progressive, time-dependent rise in the M+1 fraction as the label gradually incorporates into the TCA pools. Instead, the observed M+1 lines remain perfectly flat across the entire 180-minute time course. This indicates a systemic, static instrument offset rather than dynamic biological incorporation. (iii) Instrumentation Artifact: A +2~4% offset is a ubiquitous baseline shift in metabolomic mass spectrometry, commonly caused by natural isotope peak broadening of high-concentration pools or minor isobaric interferences.

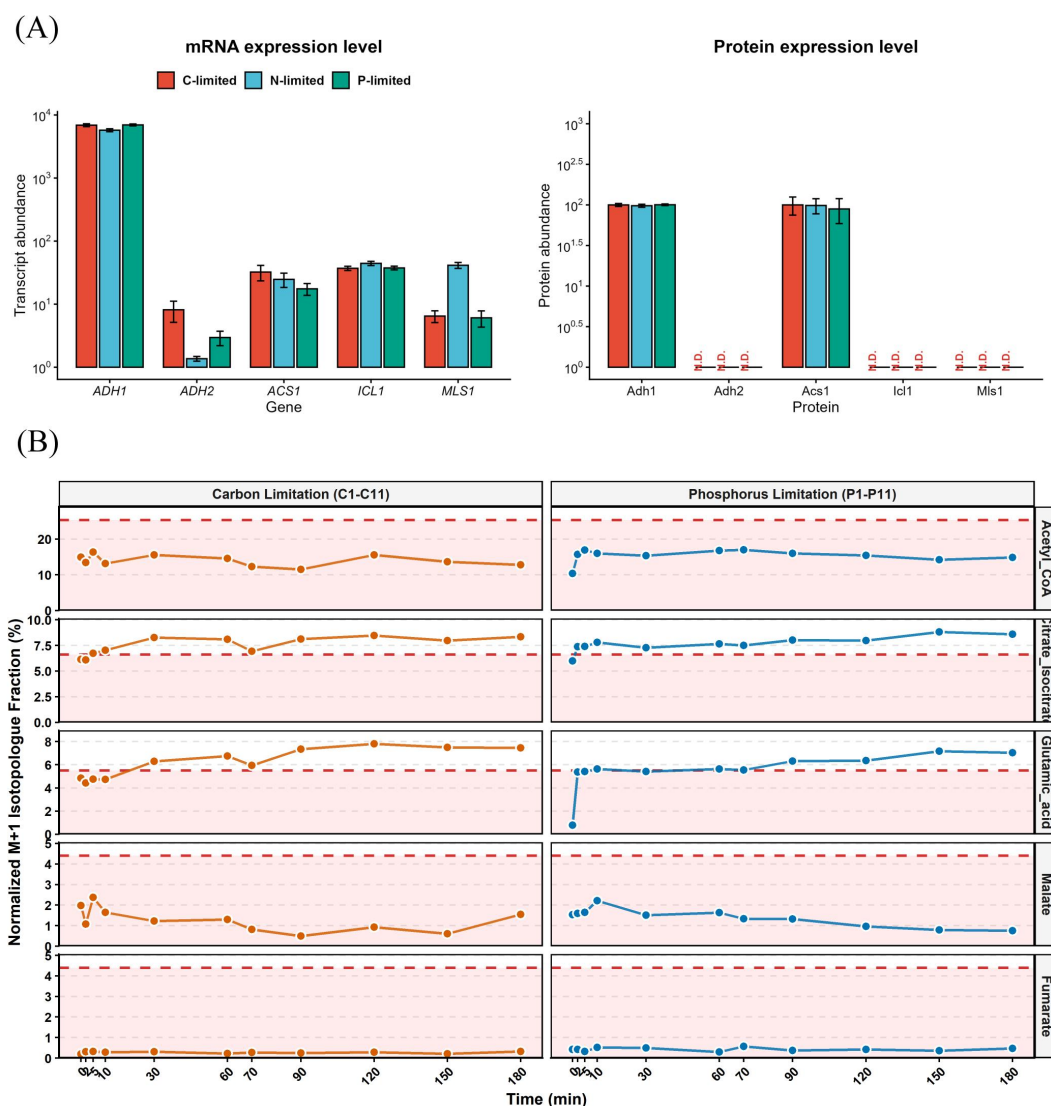


Figure S1 Validation of unidirectional ethanol production under strict anaerobiosis. (A) Transcriptomic and proteomic repression of the ethanol assimilation pathway. **(B)** Dynamic $1\text{-}^{13}\text{C}$ -ethanol isotopic tracing confirms zero metabolic re-assimilation.

Conclusion: The convergence of multi-omics profiling, and direct ^{13}C -isotopic tracing provide solid evidences that the reverse utilization of ethanol is not feasible in our anaerobic system.

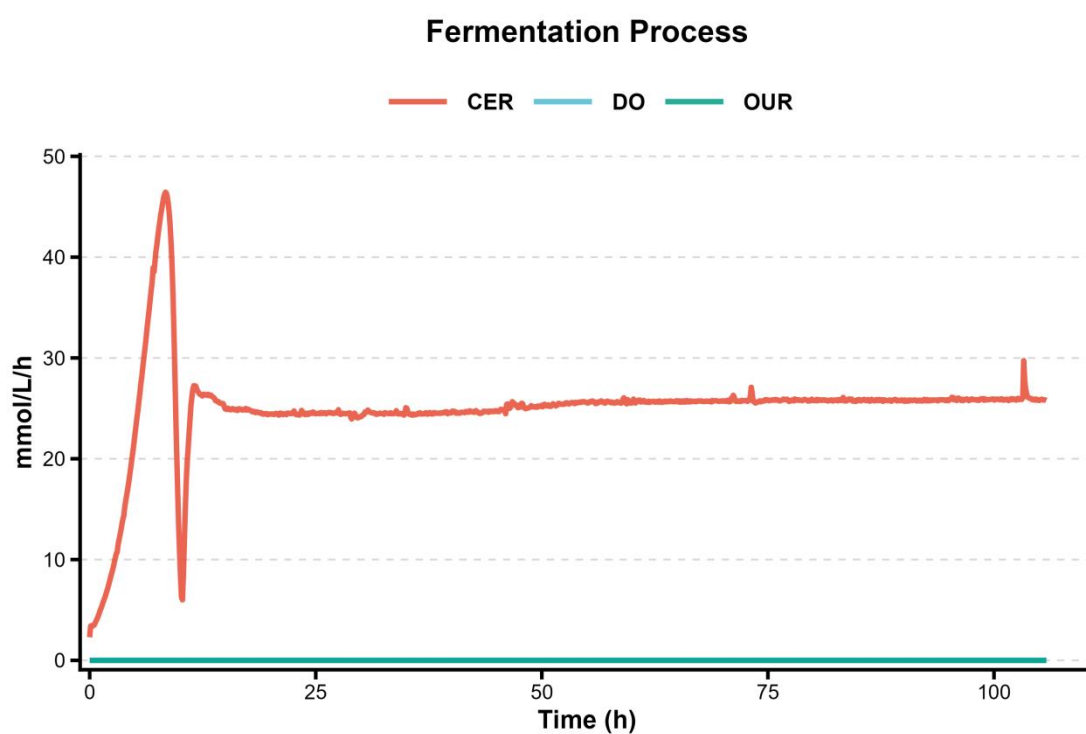
Manuscript Revision

We have rewritten the corresponding paragraphs in the Results section to explicitly incorporate these concepts (**Lines 118-122**):

Because accurate quantification of substrate-level phosphorylation requires strictly unidirectional fermentative fluxes, this assumption was experimentally validated using complementary approaches. Global proteomic profiling and dynamic $1\text{-}^{13}\text{C}$ -ethanol isotopic tracing both confirmed repression of assimilation pathways and the absence of detectable reverse carbon flux from ethanol (**Supplementary Fig. 1**).

4. Relatedly - to both 1 and 2 - the anaerobic growth is based on parsing N_2 into the chemostat (and is a reasonable assumption). Add some precise data on mitochondrial activity in these cells, as well as bulk oxygen levels (dissolved oxygen, and any estimates of respiration)

Response: Thank you for your comment. During the experiment, we continuously introduced nitrogen gas (99.999%) to maintain an anaerobic environment. At the same time, we used an optical dissolved oxygen sensor to monitor the dissolved oxygen content in the reactor, as shown in the figure below (C limitation at $D = 0.1 \text{ h}^{-1}$), where the dissolved oxygen level was 0. We also used an off-gas mass spectrometer to detect the CO_2 and O_2 fractions in the off-gas, since no oxygen is supplied, the OUR is 0.



5. For both 2 and 3, additional inferences can be drawn through the proteome data (reflecting relative levels of the respective enzymes associated with these processes). Can also refer to existing proteome datasets eg PMID: 34903663, in addition to their own proteome datasets. Currently, this is descriptive, without bringing out key conclusions

Response: We are very grateful for your nice suggestion. We have now drawn two valuable inferences regarding ATP generation (**Question 2**) and ethanol metabolism (**Question 3**):

(1). Proteomic allocation regarding ATP production (Addressing Question 2)

Carl Malina et al¹⁴. demonstrated that *S. cerevisiae* maximizes its ATP generation rate by investing its proteome mass into the highly efficient glycolytic pathway. Under anaerobic conditions, *S. cerevisiae* relies solely on the glycolytic pathway for energy generation. Our results show that as the specific growth rate increases, the expression of proteins associated with energy synthesis is upregulated (Fig. 3F).

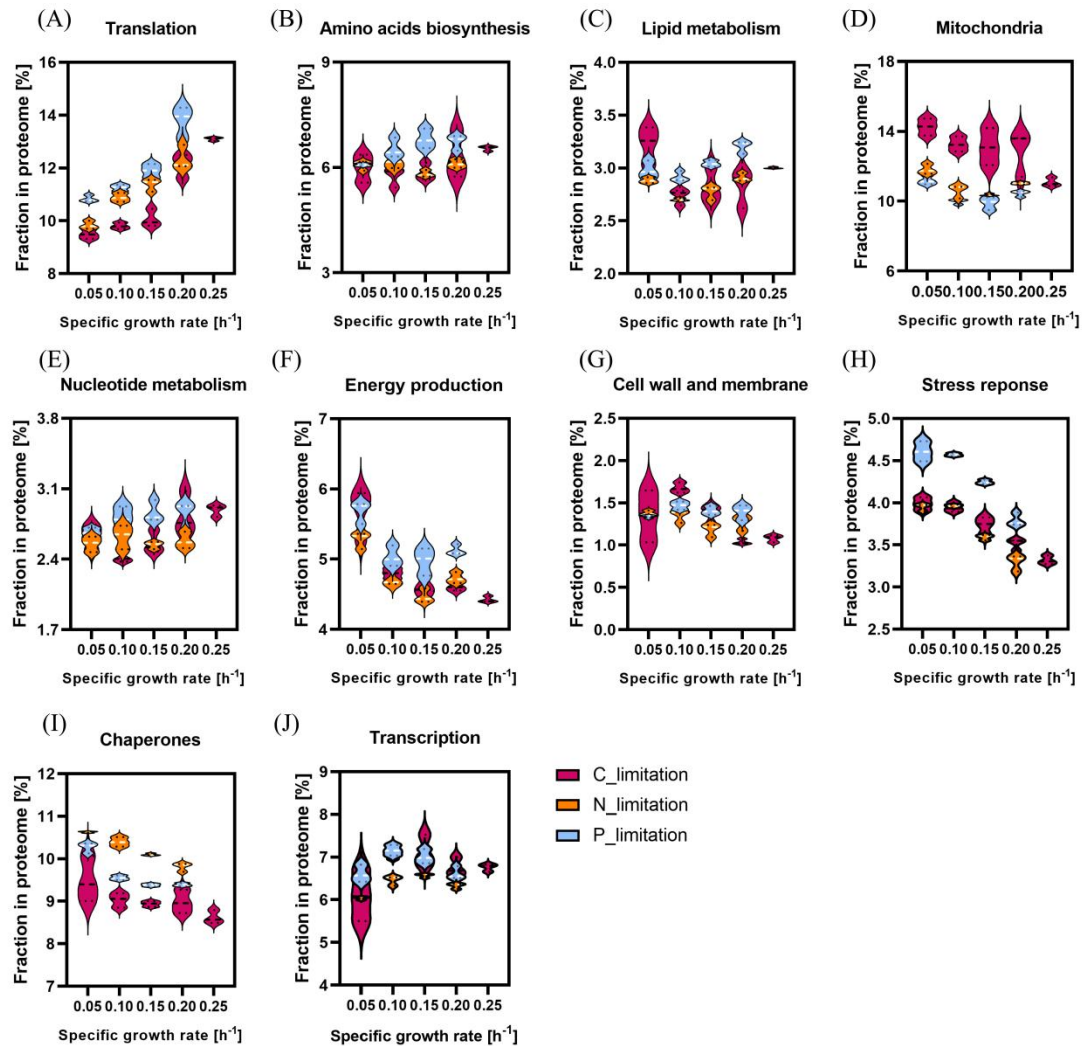


Fig. 3 Hierarchical proteomic resource allocation under different nutrient limitations. Relative abundance of proteins within major functional modules under C, N, and P limitation, expressed as a percentage of total protein content. (A) Translation machinery. (B) Amino acid metabolism. (C) Lipid metabolism. (D) Mitochondrial proteins. (E) Nucleotide metabolism. (F) Energy production. (G) Cell wall and membrane biosynthesis. (H) Stress response proteins. (I) Molecular chaperones. (J) Transcription-associated proteins. Data represent mean $\pm$ s.d. of three biological replicates (n = 3).

(2). Proteomic regarding unidirectional ethanol flux (Addressing question 3)

Relating this allocative strategy to our ethanol flux calculations, the proteomic absence of the assimilatory machinery reveals an optimized cellular economy.

Our data shows (**Fig. S1**) the complete physical absence of ethanol assimilation and glyoxylate shunt enzymes (Adh2, Icl1, Mls1). From a proteome allocation perspective, synthesizing these enzymes under anaerobiosis would not only be a waste of limited translational resources but would also risk futile carbon cycling. Therefore, the repression of these specific proteins acts as a molecular valve. This optimized proteomic architecture physically enforces the unidirectional carbon flux toward ethanol excretion, ensuring that substrate-level phosphorylation is maximized to meet the survival ATP threshold without energetic waste.

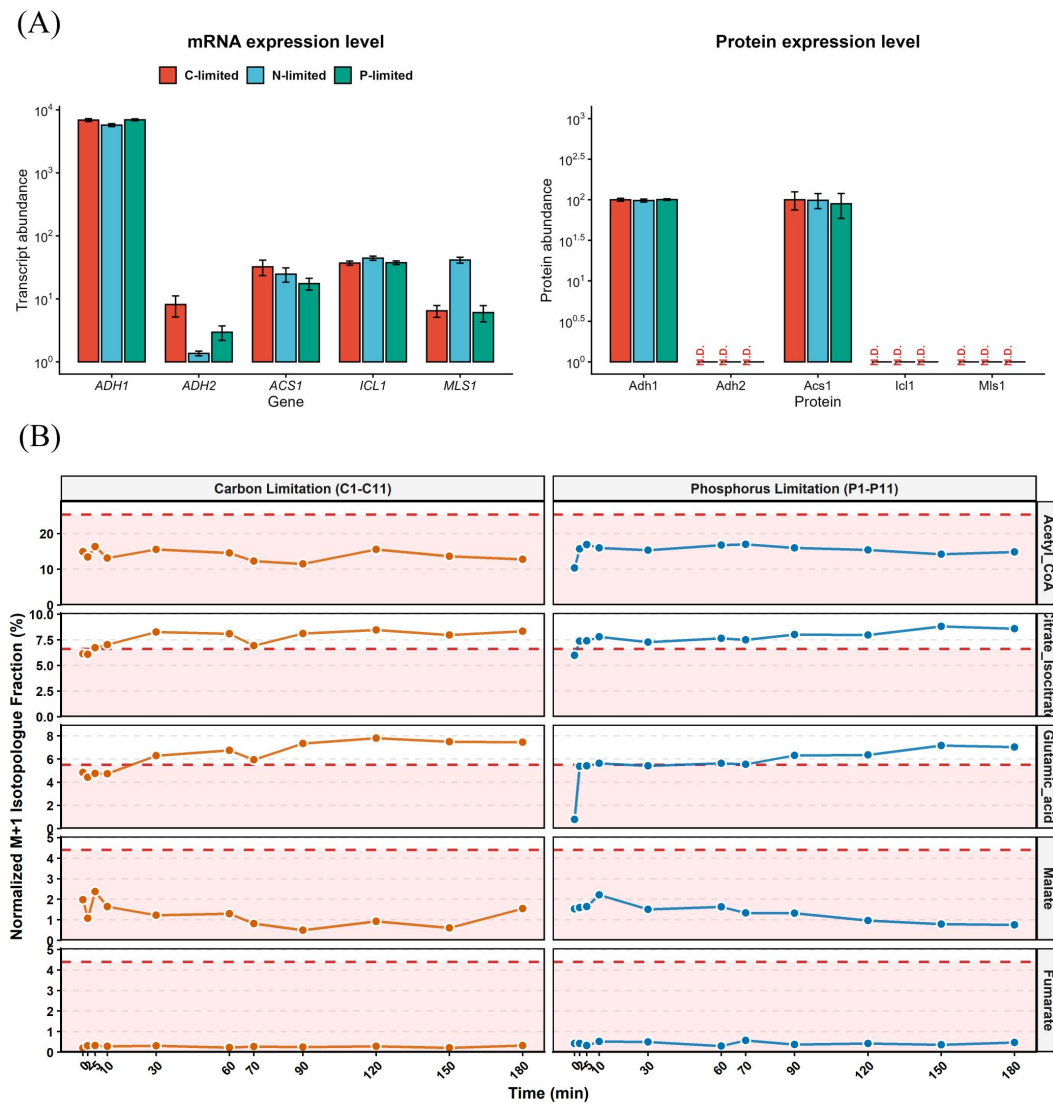


Figure S1 Validation of unidirectional ethanol production under strict anaerobiosis. (A) Transcriptomic and proteomic repression of the ethanol assimilation pathway. **(B)** Dynamic

¹-¹³C-ethanol isotopic tracing confirms zero metabolic re-assimilation.

Manuscript Revision

We have rewritten the corresponding paragraphs in the Discussion section to explicitly incorporate these concepts (**Lines 418-427**):

For example, under P limitation, intracellular ATP levels remain low despite sustained high glycolytic flux. Previous studies have shown that inorganic phosphate (Pi) functions as a critical cofactor sustaining glycolytic throughput; insufficient Pi constrains the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction, increasing the risk of decoupling ATP consumption in upper glycolysis from ATP generation in lower glycolysis¹⁵. Because strictly anaerobic cells depend exclusively on substrate-level phosphorylation for ATP production, this Pi dependency imposes a major metabolic constraint. Under P-limited conditions, cells therefore allocate increased proteomic resources to glycolysis to sustain energy production, consistent with the high glycolytic investment strategy proposed by Malina et al¹⁴.

6. Relatedly-key changes should be observed with ribosomal amounts/composition as ATP and energy charge changes. See the next section (related to fig 3/4) on the proteome - map back to ATP and energy charge, to make clearer (and significant) correlations. Currently - it is more descriptive, on growth rate v proteome (and ribosomal composition) - which is largely consistent with what we understand about growth programs in cells.

Response: We are very grateful for this nice suggestion. To explicitly address this, we calculated the total proteomic abundance (the absolute sum of all detected 40S and 60S ribosomal proteins) and mapped this massive translational investment directly against absolute ATP concentrations and Energy Charge across all conditions (see new **Fig. 2C**).

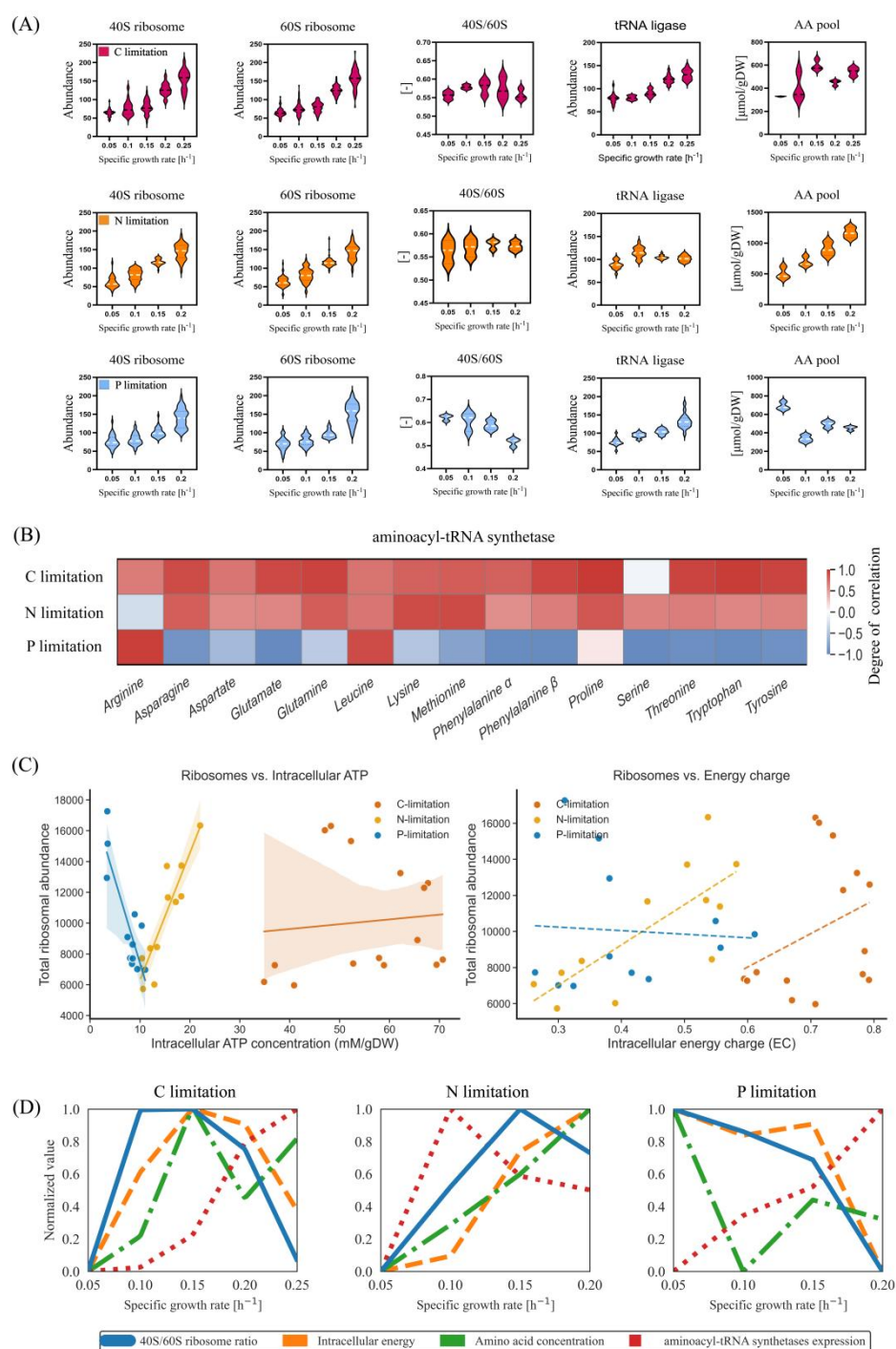


Fig. 2 Nutrient-dependent translational regulation and hierarchical control of ribosome homeostasis in *S. cerevisiae*. (A) Abundance profiles of the 40S and 60S ribosomal subunits, their stoichiometric ratio (40S/60S), aminoacyl-tRNA synthetases (aaRS), and intracellular amino acid pools across specific growth rates (μ) under C, N, and P limitations (top to bottom panels). (B) Correlations between mRNA levels and corresponding protein abundances of aaRS genes visualized as heatmaps. (C) The relationship between intracellular ATP/EC and ribosomal protein abundance. (D) Diverse translational regulation under distinct nutrient limitations. Under C

limitation, high μ leads to a decline in the 40S/60S ratio, ATP depletion, and low amino acid availability despite increased aaRS expression. Under N limitation, amino acid and ATP levels rise but translation is constrained by limited aaRS abundance. Under P limitation, rapid decreases in the 40S/60S ratio, ATP, and amino acid levels jointly restrict translation.

As shown in **Fig. 2**, when plotted against the specific growth rate, the total abundance of ribosomes increases across all limitations. However, when mapped back to actual ATP levels and Energy Charge, a completely divergent, energy-driven reallocation landscape emerges. Particularly under P-limitation, as the cell approaches its maximum allowable growth rate $D = 0.20$, the extreme maintenance energy burden causes the homeostatic system to fail, resulting in an absolute bioenergetic collapse (intracellular ATP crashes to ~ 3.4 mM and the EC plunges to < 0.35).

Strikingly, upon this EC collapse (Panel C), the cell forcefully upregulates its total ribosomal abundance to its absolute maximum. Severe ATP/GTP depletion thermodynamically limits the translation elongation rate (efficiency) of individual ribosomes. To maintain the required macroscopic translation flux under such severe energetic starvation, the cell exhibits a profound proteomic allocation trade-off. It dramatically over-invests its limited protein budget to synthesize massive quantities of translational machinery, forcefully pushing overall translation forward to compensate for the plummeting individual ribosomal efficiency.

Manuscript Revision:

We have incorporated this comprehensive 3-panel correlation analysis into the new **Fig. 2**. Furthermore, we have added a dedicated paragraph in the main text to explicitly discuss this ATP/EC-driven proteomic allocation trade-off (**Lines 192 - 209**):

Our results demonstrate that the linear proportionality between ribosomal abundance and growth rate is strongly condition-dependent, and that its relationship with intracellular energy metabolism exhibits clear element specificity (**Fig. 2C**). Plotting ribosomal abundance against intracellular ATP concentration and energy charge (EC) revealed distinct patterns across nutrient limitations. Under C limitation, ribosomal abundance increased linearly with growth rate while intracellular energy levels remained relatively stable, indicating sufficient energetic support for translational capacity; however, ATP and EC declined once μ exceeded 0.15 h^{-1} . Under N limitation, although intracellular ATP levels were lower than under carbon limitation, a positive association between cellular energy status and ribosomal abundance was still maintained. In contrast, this relationship became uncoupled under

phosphorus limitation: increasing growth rates were accompanied by declining ATP and EC levels despite continued increases in ribosomal abundance. Together, these observations indicate that as C- and P-limited cells approach their maximal growth rates, rising maintenance energy demands lead to reduced intracellular energy availability. This uncoupling highlights a physiological trade-off in which cells sustain the macroscopic translation flux required for rapid growth by allocating greater proteomic resources to ribosome synthesis, even as intracellular energy reserves progressively decline.

7. Again, related to carbon limitation - based on existing literature and changes in the net ATP synthesis efficiency vs rates of consumption (e.g., protein translation amount/rate) and tradeoffs therein. Along with this, a clearer estimate of flux through glycolysis (as the major ATP generating process).

Response: We greatly appreciate your comments.

(1). Precise estimation of glycolytic flux (ATP synthesis rate)

Because our chemostat cultivations are strictly anaerobic, the mitochondrial respiratory chain is completely inactive. Therefore, glycolysis (the EMP pathway) serves as the absolute sole source of ATP generation. As detailed in our revised Methods (and addressing **question 2**), the specific glycolytic ATP synthesis flux is precisely and accurately defined by the rigid macroscopic stoichiometric balance: $q_{ATP} = q_{ethanol} - q_{glycerol}$. This provides a calculation of the glycolytic flux driving the entire energetic system.

(2). ATP synthesis vs. translational consumption

Building on existing proteome allocation literature (e.g., MetzI-Raz et al.)¹⁶, our results reveal a different energetic landscape under C-limitation compared to the bioenergetic collapse observed under P-limitation:

High net efficiency despite Low Absolute Flux: Under C-limitation, the severe scarcity of external glucose inherently restricts the absolute glycolytic flux (yielding a lower q_{ATP}). However, the net ATP synthesis efficiency is remarkably high. Because the maintenance energy requirement under C-limitation is extremely low ($m_s = 0.53$ mmol/gDW/h, compared to 3.10 under P-limitation), the majority of the generated ATP is efficiently preserved for biomass synthesis and translation.

Because the intracellular Energy Charge (EC) remains highly stable and

preserved under C-limitation, ATP supply meets the demands of translation. Cells do not experience impending energy depletion as they do under phosphorus-limited conditions.

(3). The specific proteomic trade-off under C-Limitation

Under C-limited conditions, the trade-off between resource acquisition and translational capacity is particularly critical. Since the external carbon source is the limiting factor, cells need to increase the expression of high-affinity glucose transporters (such as Hxt proteins) to enhance substrate uptake. This necessary protein investment leaves less available protein for the translational machinery.

Manuscript Revision:

We have made the following modifications to the Discussion section (**line 428 - 436**):

This compensatory response differs fundamentally from the physiological state observed under carbon limitation. In agreement with established proteome allocation models¹⁶, C-limitation introduces a distinct systemic trade-off. Although absolute glycolytic flux quantified here using anaerobic q_{ATP} calculations is constrained by glucose availability, ATP synthesis efficiency remains high owing to reduced maintenance energy requirements ($m_s = 0.53 \text{ mmol gDW}^{-1} \text{ h}^{-1}$). As intracellular EC is largely preserved, ATP supply for translation remains stable. Consequently, cells balance ATP production and consumption by scaling translational capacity linearly with the growth rate.

8. The suggested translational bottleneck (due to amino acyl tRNA synthetases) is a particularly intriguing finding - and can make a lot of sense (especially since amino acids themselves do not become limiting, the pools remain large). However, any functional validation of this depletion is not shown, and it is not clear if these are really limiting, and impacting either the rate or extent of protein translation that is happening. Additional supporting validation is essential in order to make this claim. I understand that it is very hard to measure impact on tRNA charge (and proportions of charged tRNAs), but any associated impact on translation rate/amount etc can be better assessed.

Response: Thank you for your professional comment. To address your concern, we performed a targeted genetic intervention combined with steady-state physiological and global proteomic profiling.

(1). Relief of the translational bottleneck

We engineered a strain overexpressing WRS1 (Tryptophan--tRNA synthetase), while the translational bottleneck restricts the WT to lower dilution rates (failing to maintain steady-state above $D = 0.20 \text{ h}^{-1}$), the WRS1-OE strain successfully achieved steady-state growth at a higher dilution rate of $D = 0.27 \text{ h}^{-1}$. The 35% increase in growth rate provides conclusive evidence that the protein translation rate is indeed limited by aminoacyl-tRNA synthetase (aaRS).

(2). Increased protein expression abundance

We performed global proteome profiling on these steady-state cultures. As shown in the proteome-wide distribution profile (**Figure S22**), the engineered WRS1 exhibited a massive up-regulation $\text{Log2FC} = 1.98$. In stark contrast, the expression of the other native aaRSs remained completely unaltered at their basal N-limited levels (average $\text{Log2FC} \approx 0$). Furthermore, corroborating this targeted relief, the global abundance of the ribosomal machinery (197 detected ribosomal proteins) remained exceptionally stable between WT and WRS1-OE at $D = 0.20$ (average $\text{Log2FC} = -0.05$).

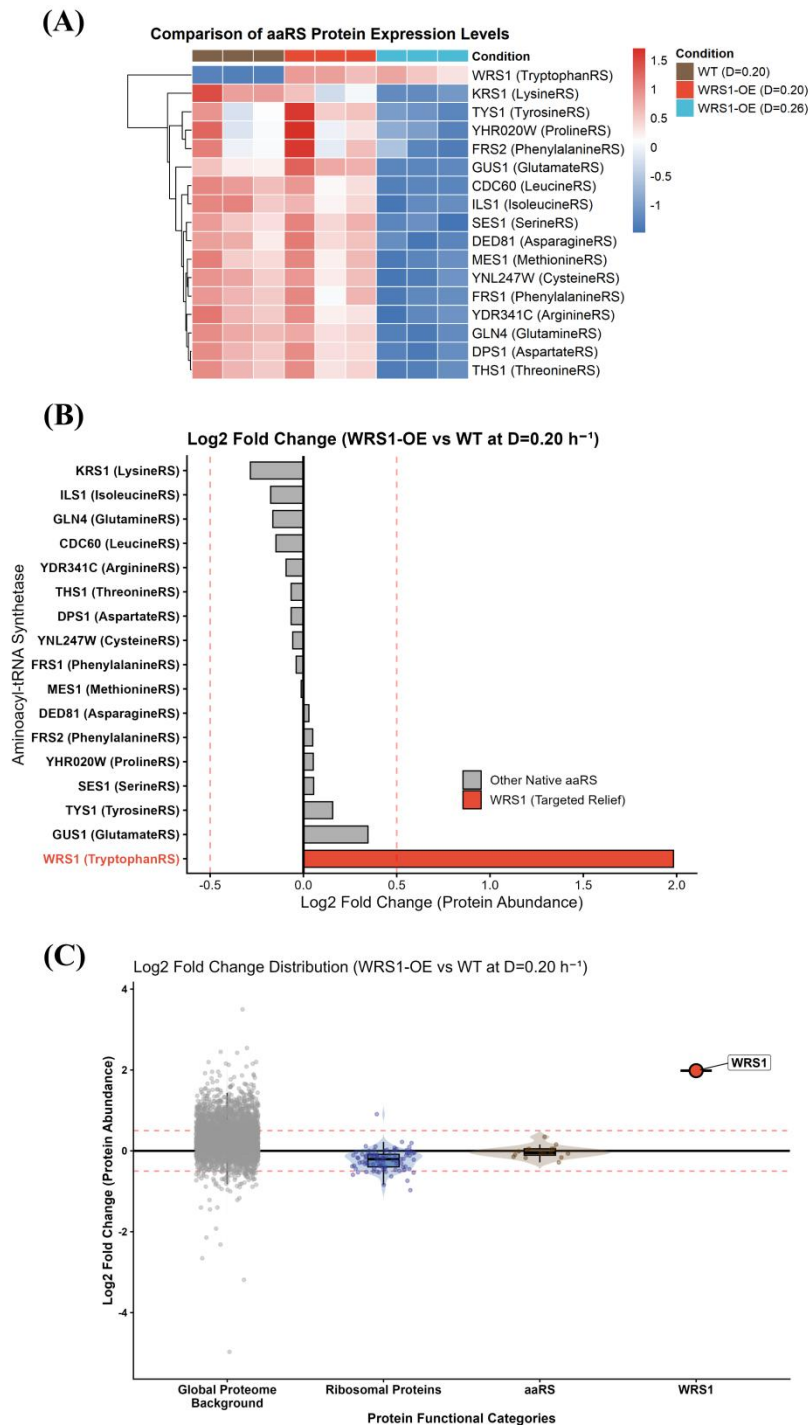


Figure S22. Validation of WRS1 overexpression (A) aaRS Heatmap. Displays the specific hyper-accumulation of WRS1 in the OE strain; other native aaRSs remain completely stable. (B) Confirms the massive, precise upregulation of WRS1 Log2FC=1.98, $P < 0.01$ without pleiotropic effects on other aaRSs (average Log2FC ≈ 0). (C) Changes in the abundance of translation-related proteins.

Because the intracellular amino acid pools were abundant, this physiological

improvement upon WRS1 overexpression provides in vivo evidence that the tRNA charging step itself, governed by aaRS availability was the bottleneck restricting the overall rate and extent of protein translation.

Conclusion: By demonstrating that the targeted restoration of aaRS availability directly accelerates the global translation-driven growth rate (from 0.20 to 0.27 h⁻¹) with proteomic specificity.

Manuscript Revision:

We have added the analysis as a new supplementary figure (**Supplementary Fig. S22**). Furthermore, we have revised the section of our Results (**line 377-384**):

To test whether the observed macroscopic growth acceleration was associated with relief of the translational bottleneck, we performed global proteomic profiling of steady-state WRS1-OE cultures. This analysis confirmed accumulation of the WRS1 target protein ($\text{Log2FC} = 1.98$, $P < 0.01$) without detectable changes in other native aaRSs (**Supplementary Fig. S22A-B**). Notably, the abundance of core components of the translational machinery remained largely unchanged (**Supplementary Fig. S22C**), indicating that the growth enhancement is more consistent with improved tRNA-charging efficiency rather than with a generalized increase in ribosome biogenesis.

9. The last section - on the changing phosphorylation landscape (especially for different metabolic enzymes, and associated impact on glycolytic flux) is particularly interesting. These data can be better contextualised and interpreted based on existing knowledge of activity-based control (eg as observed in PMID: 27789812).

Response: Thank you for this nice comment. Following your suggestion, we carefully analyzed metabolic flux and phosphoproteomic data. We plotted the macroscopic flux against site-specific phosphorylation for multiple distinct glycolytic nodes (e.g., ADH1 and GPM1) across all limitations (see newly added **Supplementary Fig. S19**).

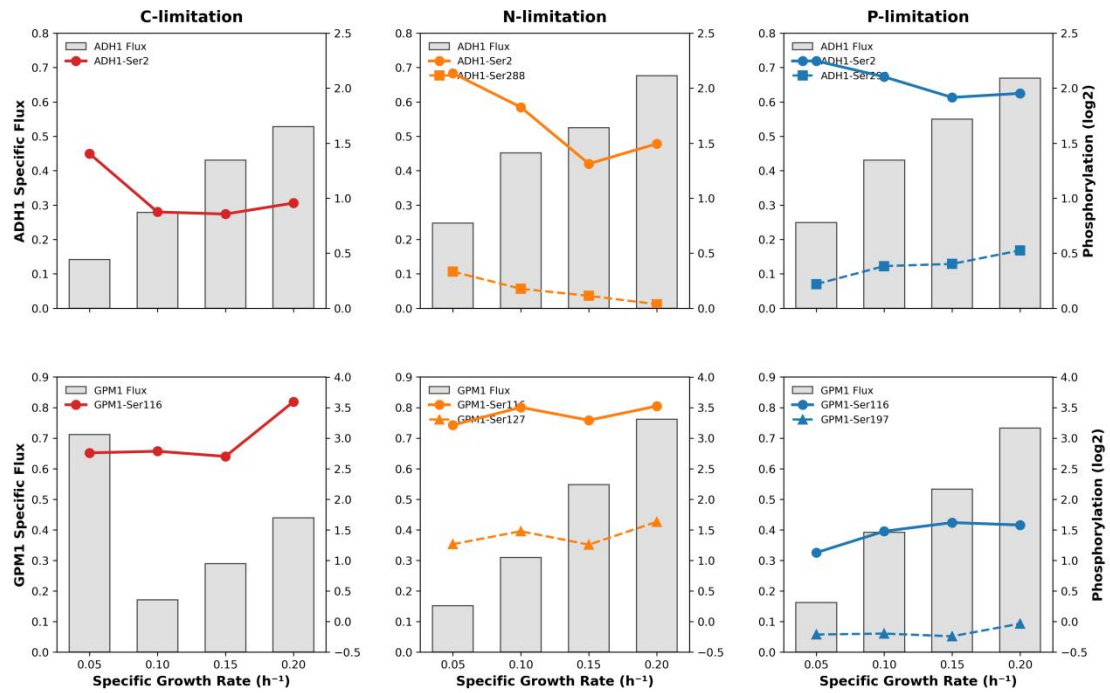


Figure S19. Information on changes in protein phosphorylation levels. The macroscopic specific metabolic flux (grey bars) is plotted against relative site-specific phosphorylation levels (colored lines) across varying specific growth rates (μ). Under C-limitation, cells rely predominantly on abundance-based control, maintaining stable or slightly decreasing baseline phosphorylation. In stark contrast, under P-limitation, where severe ATP depletion thermodynamically restricts bulk protein translation (abundance-based control fails), the cell forcefully upregulates the phosphorylation of bottleneck enzymes e.g., ADH1 at Ser2/290 to sustain the essential glycolytic flux.

As demonstrated by Hackett et al. (2016)¹⁷, adjusting metabolic flux through global changes in enzyme abundance incurs a massive proteomic and bioenergetic cost; thus, cells frequently rely on modulating enzyme activity (e.g., through allostery or PTMs).

As shown in **Supplementary Fig. S19**, macroscopic metabolic fluxes (both ADH1 and GPM1) strictly increase with the specific growth rate.

Under C-limitation, where energy is sufficient and translation scales linearly, the cell relies on abundance-based control. Consequently, the phosphorylation levels of ADH1 (Ser2) and GPM1 (Ser116) are not hyper-activated.

In stark contrast, under P-limitation, the cell faces bioenergetic collapse at high growth rates. Because the translational machinery becomes bottlenecked, the cell physically cannot execute abundance-based control (evidenced by the declining abundance of these enzymes). To maintain the required glycolytic flux, the cell is forced into an extreme, systems-wide reliance on activity-based control. It leverages a minuscule fraction of its remaining ATP to hyper-phosphorylate these bottleneck

enzymes, maintaining extraordinarily high primary phosphorylation (ADH1-Ser2, GPM1-Ser116) and sharply upregulating auxiliary functional sites (e.g., ADH1-Ser290).

This explains the transcription-translation divergence we observed, as energy-gated phosphorylation serves as a rapid, highly resource-efficient substitute for mass protein synthesis, successfully rescuing essential metabolic fluxes across multiple enzymatic nodes when translational capacity is exhausted.

Manuscript Revision:

We have added the multi-enzyme phosphorylation-flux analysis as a new supplementary figure (**Supplementary Fig. S19**). Furthermore, we have extensively revised the corresponding section of our Results (**line 315-332**):

These targeted phosphoregulatory events across diverse metabolic nodes can be interpreted within the systems-level framework of metabolic regulation proposed by Hackett et al¹⁷. According to this model, modulation of metabolic flux through global changes in enzyme abundance entails substantial bioenergetic and proteomic costs. Under conditions of severe energetic constraint, such as the pronounced decline in ATP and energy charge observed at high μ under P limitation, the capacity of the translational machinery becomes increasingly restricted, limiting the feasibility of abundance-based regulatory strategies. Under these conditions, cellular regulation appears to shift toward activity-based control. Phosphorylation provides a proteome-efficient mechanism by which a relatively small ATP investment can function as a high-affinity regulatory signal for kinase-mediated modulation. This enables selective enhancement of catalytic efficiency in key metabolic enzymes, exemplified by increased phosphorylation of Adh1 at Ser2/Ser290 and sustained activation of Gpm1 at Ser116 (**Supplementary Fig. 19**), partially compensating for declining protein abundance. Collectively, these observations suggest that the transcription–translation divergence identified here reflects an energy-dependent transition toward activity-based regulation, supporting metabolic flux maintenance when large-scale protein synthesis becomes energetically constrained.

10. Their idea to measure the impact of ‘push-pull’ strategies is interesting. Assessing the push part - by over expressing transporters, is more straightforward. However, it is not clear at all if their experiments to alter pull worked at all (see points above, related to amino acid acylation, tRNA charge, overall translation).

Response: We sincerely thank you for appreciating the rationale of our "push-pull" metabolic engineering strategy.

As detailed in our response to **Question 8**, we have now provided definitive macroscopic and proteomic proofs that our "pull" strategy successfully and specifically activated the translational machinery.

(1). Proteomic evidence

To unambiguously prove that the "pull" strategy was functionally active at the molecular level, we performed global proteomics, our data provided critical evidence. The engineered 'pull' module (WRS1) was massively and specifically upregulated (~4-fold, Log2FC=1.98). Crucially, the expression of all other native aaRSs and the ribosomal remained exceptionally stable. This precise proteomic signature provides undeniable molecular proof that the "pull" genetic intervention was successfully executed, strictly localizing the driving force to the intended tRNA charging step without triggering non-specific global amplification.

(2). Increase in specific growth rate

In systems biology, the μ is fundamentally dictated by the global rate of protein translation. As discussed in **Question 8**, simply overexpressing WRS1 (the 'pull' module) yielded a striking 30% increase in the maximum specific growth rate (from $D=0.2$ to 0.26 h^{-1}). Because intracellular amino acid pools were already abundant and non-limiting, this massive relief of the macroscopic growth limit serves as direct physiological evidence that overexpressing the specific aaRS successfully pulled the translational bottleneck forward, effectively increasing the overall translation rate.

(3). Synergistic evidence of dual targets

Furthermore, the validity of the 'pull' strategy is corroborated by the synergistic performance of the dual-overexpression strain (VMA3 + WRS1). Under nutrient-limited conditions (**Figure 5E-G**), enhancing the energy 'push' (VMA3) alone or the translation pull (WRS1) alone provided only partial relief. It was only when the translational pull capacity was successfully elevated alongside the energetic 'push' that the strain exhibited maximum synergistic improvements across all three limitations.

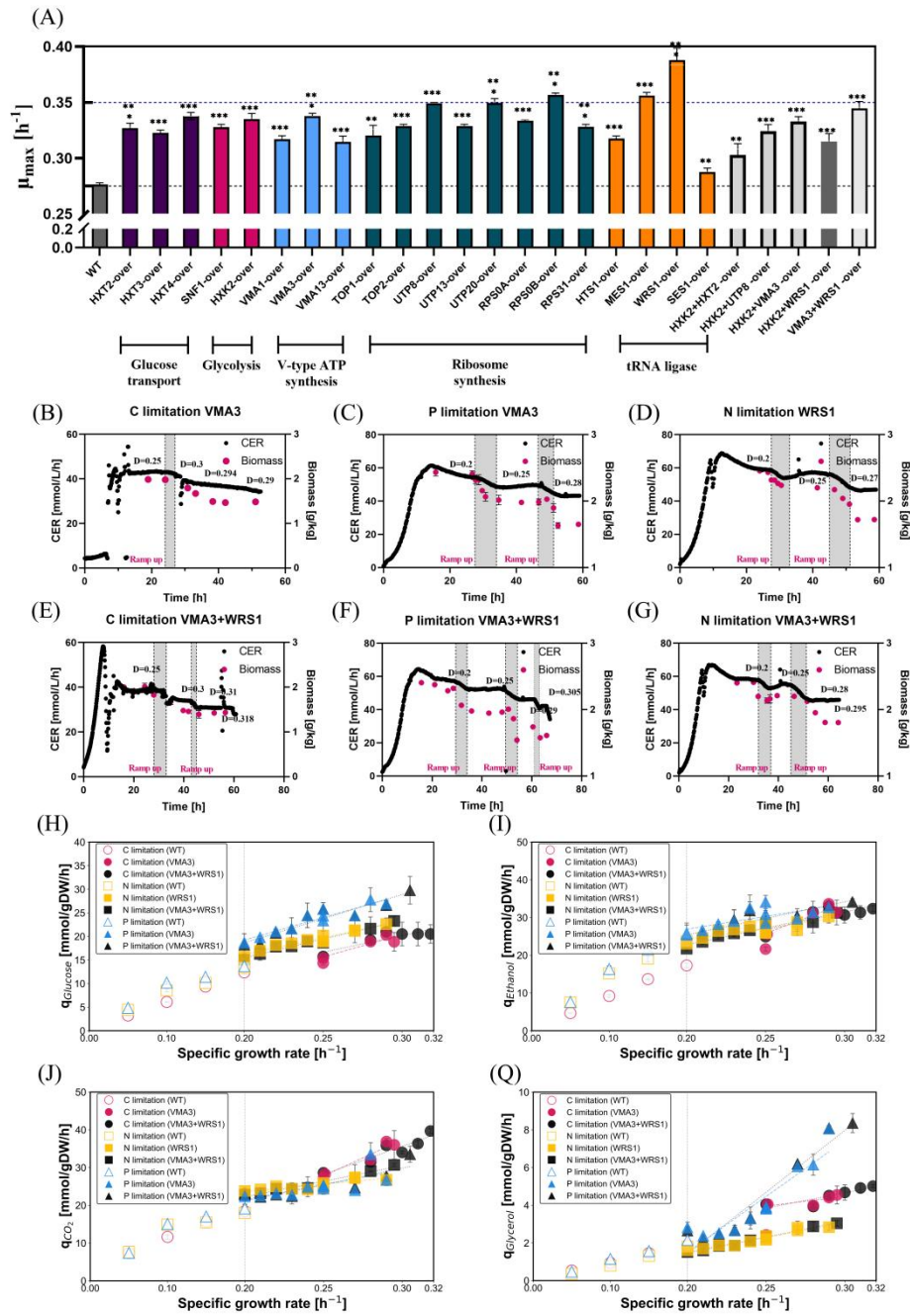


Fig. 5 Growth and metabolic verification of *S. cerevisiae* engineered strains under different nutrient limitations. (A) Maximum specific growth rates of both wild-type (WT) and engineered strains overexpressing energy-related genes (HXT, SNF1, HXK2, VMA) or translation-related genes (UTP, RPS, tRNA synthetases). (B–G) Growth of selected single- and dual-gene overexpression strains under C, N, and P limitations in chemostats. (H–Q) Comparison of glucose uptake (H), ethanol (I), CO₂ (J), and glycerol (Q) production rates between engineered and WT strains under nutrient-limited conditions.

11. Methodology details - several methodology details are missing with respect

to how estimates for some rate measurements (including uptake) were calculated using only steady state metabolite data. There are no direct uptake measurements (which are usually done quantitatively over time using a labelled metabolite). Additionally, as pointed out earlier, several conclusions regarding rate and flux are made using steady state measurements, without including other (utilisation based) measurements - for example using reductive capacity for biosynthesis (eg. amino acid biosynthesis), or ATP for protein synthesis (can be approximated based on amounts of ribosomal subunits etc).

Response: Thank you for the suggestion and we have now carefully completed methodology details.

1. Direct measurement of uptake and production rates

In a steady-state continuous chemostat culture, macroscopic fluxes are directly and accurately quantified using classical macroscopic mass balancing across the bioreactor. Because the culture is in a steady state, the specific uptake rate (q_s) and specific production rate (q_p) are directly determined by the dilution rate (D , which equals μ), the biomass concentration (C_x), and the concentration difference between the feed medium and the effluent. For example, the specific glucose uptake rate is rigidly defined as:

$$q_{glucose} = \frac{D \times (C_{glucose,feed} - C_{glucose,effluent})}{C_x}$$

2. Integrating utilization-based measurements

In fact, following your advice in **Question 6**, we have now explicitly incorporated utilization-based approximations into our bioenergetic analysis.

ATP for protein synthesis: As detailed in our response to **Question 6** (and the **Fig. 2**), we utilized the total proteomic abundance of ribosomal subunits as a direct, condition-specific proxy for the ATP utilization demand of protein translation.

Maintenance Utilization: Furthermore, we rigidly calculated the specific maintenance energy utilization (m_s) across different limitations, directly quantifying the ATP drain diverted away from biomass synthesis.

This utilization-based perspective allowed us to reveal the profound proteomic allocation trade-off where cells desperately over-invest in ribosomal mass to combat plunging ATP levels under P-limitation.

Manuscript Revision:

To ensure absolute methodological transparency, we have extensively expanded the Methods section ("Energy-specific growth rate" and analytical procedures) (**Lines**

672-710).

Under steady-state chemostat conditions, the specific growth rate (μ) strictly equals the dilution rate (D). The specific macroscopic consumption rates of substrates (q_s , e.g., glucose) and production rates of extracellular metabolites (q_p , e.g., ethanol) were directly calculated using respective mass balances across the bioreactor:

$$q_s = \frac{D \times (C_{s,in} - C_{s,out})}{C_x} \quad (1)$$

$$q_p = \frac{D \times C_{p,out}}{C_x} \quad (2)$$

where $C_{s,in}$ and $C_{s,out}$ are the substrate concentrations in the feed and effluent (mmol L^{-1}), respectively, $C_{p,out}$ is the product concentration in the effluent (mmol L^{-1}), and C_x is the steady-state biomass concentration (gDW L^{-1}).

The relationship between specific substrate consumption rate and specific growth rate was described using the Herbert–Pirt equation (**Equation (3)**)^{10, 11}:

$$q_s = \frac{1}{Y_{X/S}} * \mu + m_s \quad (3)$$

where q_s represents the specific substrate uptake rate ($\text{mmol gDW}^{-1} \text{h}^{-1}$), $Y_{X/S}$ is the biomass yield on substrate (gDW mmol^{-1}), μ is the specific growth rate (h^{-1}), and m_s denotes the maintenance coefficient.

Linear regression based on **Equation (3)** revealed that the maintenance energy requirement under P limitation ($3.10 \text{ mmol gDW}^{-1} \text{h}^{-1}$) was 5.8-fold higher than under C limitation ($0.53 \text{ mmol gDW}^{-1} \text{h}^{-1}$; ANOVA, $P < 0.001$). N limitation exhibited an intermediate value ($2.01 \text{ mmol gDW}^{-1} \text{h}^{-1}$; **Table 1**).

Under strictly anaerobic conditions, the mitochondrial respiratory chain is completely inactive, meaning that ATP is generated exclusively via substrate-level phosphorylation during glycolysis. Based on the established stoichiometry for anaerobic *S. cerevisiae*⁹, the net ATP production does not rely on variable respiratory P/O ratios, but is rigidly tied to the fermentation fluxes: the formation of 1 mole of ethanol yields 1 mole of ATP, whereas the formation of 1 mole of glycerol consumes 1 mole of ATP (as it serves as a redox sink to reoxidize biomass-derived NADH, bypassing the ATP-yielding steps of lower glycolysis).

$$Y_{\frac{\text{ATP}}{\text{biomass}}} = \frac{C_{\text{biomass}}}{C_{\text{ethanol}} - C_{\text{glycerol}}} \quad (4)$$

The ATP yield ($Y_{\frac{\text{ATP}}{\text{biomass}}}$) was determined according to **Equation (4)**⁹, where C_{biomass} (g L^{-1}), C_{ethanol} (mM), and C_{glycerol} (mM) represent the concentrations of biomass, ethanol, and glycerol, respectively.

The specific ATP production rate (q_{ATP}) was determined using **Equation (5)**¹²:

$$q_{\text{ATP}} = \frac{\mu}{Y_{\text{ATP}}} \quad (5)$$

By combining **Equations (4) and (5)**, which mathematically simplifies to the exact stoichiometric balance $q_{ATP} = q_{ethanol} - q_{glycerol}$, we determined the specific ATP production rate under each nutrient limitation condition. This absolute dependence on glycolytic stoichiometry avoids any uncertainties regarding respiratory ATP contributions, enabling a robust and quantitative assessment of energy allocation across growth regimes.

12. In addition to some major studies omitted in this text in relation to proteome efficiency, or flux regulation, or ethanol production/utilization (pointed out earlier) - it is critical to better contextualise these results alongside some central studies related to carbon/nitrogen/phosphorus limitation, and impact (particularly) on glycolysis is critical. This includes:

- Growth limiting intracellular metabolites under nutrient limitations (PMID: 19889834)
- On impact of phosphate/inorganic phosphate in rates and flux of glycolysis based on phosphate availability) - PMID: 24436182 and PMID: 39324403
- nitrogen limitation, and large reserves in metabolic capacity PMID: 32312967

Response: We are grateful to you for providing this precious information.

We have carefully incorporated these studies into our Results and Discussion sections to interpret our multi-omics and flux data:

1. Contextualizing intracellular metabolite depletion (PMID: 19889834)

Building on the foundational work by Boer et al. (2010)¹⁸, which established how the identity of the limiting macronutrient dictates specific intracellular metabolite depletions, we have expanded our metabolomics discussion. Our results show a similar trend (e.g., specific depletion of energy/nucleotide pools under P-limitation and amino acid precursor shifts under N-limitation), demonstrating that these laws of growth-limiting intracellular pools hold true even when respiratory metabolism is completely abolished.

2. The critical role of phosphate in glycolytic flux (PMID: 24436182 & PMID: 39324403)

Van Heerden et al. (2014)¹⁵ and recent systems analyses¹⁹ demonstrate how Pi availability strictly buffers glycolytic flux; without sufficient Pi, upper glycolysis outpaces lower glycolysis, leading to energetic arrest. Because our strictly anaerobic cultures rely exclusively on glycolysis for ATP, this Pi-dependent vulnerability is

amplified. We interpret the paradoxical phenotype under P limitation, characterized by extremely high glycolytic flux coexisting with a severe energy collapse, as a direct consequence of this extreme proteomic compensation. The cell invests substantial translational resources to sustain the metabolic rates required for survival, yet this investment ultimately fails to rescue the systemic bioenergetic collapse.

3. Nitrogen limitation and metabolic reserves (PMID: 32312967)

We clarify that the observed aaRS bottleneck is not a systemic metabolic failure, but rather a highly regulated adaptive strategy. Consistent with the concept of translational reserves under N-limitation (Yu et al., 2020²⁰), restricting aaRS availability functions as a precise molecular brake. It throttles the global translation rate, thereby keeping a large fraction of the pre-synthesized ribosomal pool idle (in reserve) and preventing the complete depletion of intracellular amino acid pools. Our WRS1-OE data substantiates this: the targeted release of this aaRS brake immediately unconstrained the specific growth rate (>30% increase) using the exact same ribosomal pool, providing evidence of these unexploited translational reserves.

Manuscript Revision:

We have rewritten the Discussion section (**Lines 418-427 and 444-451**) to actively engage with these concepts:

For example, under P limitation, intracellular ATP levels remain low despite sustained high glycolytic flux. Previous studies have shown that inorganic phosphate (Pi) functions as a critical cofactor sustaining glycolytic throughput; insufficient Pi constrains the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction, increasing the risk of decoupling ATP consumption in upper glycolysis from ATP generation in lower glycolysis¹⁵. Because strictly anaerobic cells depend exclusively on substrate-level phosphorylation for ATP production, this Pi dependency imposes a major metabolic constraint. Under P-limited conditions, cells therefore allocate increased proteomic resources to glycolysis to sustain energy production, consistent with the high glycolytic investment strategy proposed by Malina et al¹⁴.

Consistent with the concept of translational reserves under N-limitation²⁰, restricted aaRS availability appears to function as molecular braking mechanism on protein synthesis. By constraining global translation rates, this limitation maintains a substantial fraction of the pre-synthesized ribosomal pool in an idle, reserve state while preventing complete depletion of intracellular amino acid pools. Our WRS1 overexpression results further demonstrated that targeted alleviation of aaRS limitation increased the specific growth rate by more than 30%, indicating the

presence of previously unexploited translational reserves.

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REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

I think the authors have addressed all my comments very well, and I rest my case that the maximum specific growth rate is almost the same at anaerobic and aerobic conditions! However, it is definitely not 50% lower! Also, the issue about media limitations is addressed very well by the authors. My only remaining point is that the authors now state that the specific growth rate is lower at anaerobic conditions due to a lower ATP production! I am not sure this can be stated for certain as we do not know. As I pointed out earlier at maximum growth at aerobic conditions the oxygen uptake is close to zero, and hence ATP production is also mainly from fermentation, and there therefore must be other reasons for the lower specific growth rate at anaerobic conditions. So, the authors should change this sentence, and it is OK to be vague as we do not know the exact mechanisms underlying the lower specific growth rates at anaerobic conditions. It can be mitochondrial function and hence biosynthesis of various biomass precursors, it can be uptake rates of ergosterol, and many other factors could be in play!

Excellent work and nice paper

Response: Thank you for your suggestion.

Manuscript Revision:

To avoid further spreading this misconception, we have revised the text in the Introduction (**Lines 52-53**) as follows:

Compared to respiration, fermentation yields less ATP per glucose molecule and is therefore generally associated with lower maximum specific growth rates^{3, 4}.

Reviewer #3 (Remarks to the Author):

This is a very good revision, and a much-improved manuscript. The study is important, and the resource extremely useful. All my main concerns have been addressed. The manuscript requires some careful editing (there are some minor grammar errors, and a few loosely phrased statements), as well as more complete referencing (the final references cited are still selective, and some references cited in the response to reviewers should probably also be cited).

Response: Thank you for your suggestions, and we have carefully checked and revised these issues.