Identification of isobutanol tolerance-associated pathways in the *S.cerevisiae* yeast strain

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1. Introduction

Limited supply of fossil fuels, together with increasing concern on their involvement in global warming and climate change, motivated the recent search for more sustainable alternatives. The engineering of microbial cells for production of industrially important molecules, such as biofuels, holds great promise for a switch towards a more circular bioeconomy [1].

Saccharomyces cerevisiae is a well-known model organism and one of the best studied eukaryotes. This allowed the creation of a wide range of genetic tools that can be used to manipulate this yeast and, therefore, exploit it for various biotechnological applications [2].

Importantly, it is known that *S. cerevisiae* strains are inherently capable of producing isobutanol (IB), a branched-chain alcohol considered one of the best suitable substitutes for gasoline owning to its similar physical properties [3]. In fact, isobutanol is produced in small amounts as a by-product of fermentation during valine synthesis [3, 4].

However, a major factor hindering the industrial scale production of isobutanol by this yeast strain is the low tolerance of the organism to accumulation of the biofuel inside the cell. In fact, it has been shown that *S. cerevisiae* is able to tolerate up to 2% of n-butanol in its medium [3]. Therefore, understanding the causes of isobutanol toxicity as well as the pathways involved in its tolerance can help engineer strains able to overcome stress induced by this metabolite and hence produce higher titers of this alcohol.

Moreover, the existence of pathways sharing some intermediate metabolites together with spatial separation into different cell compartments are also thought to reduce the metabolic flux towards isobutanol production [4]. Therefore, relocation of involved enzymes and blocking of competing pathways can also boost isobutanol production in yeast.

Research question

The aim of this project is to identify pathways that allow and boost production of isobutanol, a biofuel alternative to gasoline, in the yeast strain *S. cerevisiae* and that can be engineered to

grant higher end product yield.

2. Methods

A transcriptomic dataset by Kuroda et al. (2019) [5] containing data of differentially expressed (DE) genes in the *S.cerevisiae* yeast $GLN3\Delta$ mutant strain was retrieved from the GEO DataSets database of NCBI. Four conditions of interest were then selected for further analysis:

- WT (1.3% Isobutanol) / WT (0%)
- $GLN3\Delta$ (0%) / WT (0%)
- $GLN3\Delta$ (1.3% Isobutanol) / $GLN3\Delta$ (0%)
- $GLN3\Delta$ (1.3% Isobutanol) / WT (1.3% Isobutanol)

The obtained data was then run on ShinyGATOM [6, 7], a web tool that uses an active module approach to identify the most regulated metabolic subnetworks between the different conditions. For the inital network construction step, *"S. cerevisiae"* was selected as organism and the DE data was uploaded and parsed. "KEGG" was selected as network type and "metabolites" was indicated as network topology. In metabolite-level topology, nodes correspond to metabolites, and two metabolites are connected if there is a reaction where at least on carbon atom is transitioned between the two metabolites [7]. For the scoring step, default options were applied: the number of positive genes was set as scoring parameter (n = 50) and the Virgo solver was used. Finally, the highest scoring connected module that contains the most significantly changed genes was identified and reactions without highly changing genes but with high average expression were also added to the graph. Functional annotation and pathway enrichment of obtained modules by KEGG and Reactome was also calculated.
The obtained subnetworks were downloaded for additional visualization on Cytoscape (version 3.9.1) in order to identify the most significant nodes and edges and their interconnection within the whole yeast metabolism.

3. Results and discussion

Out of the 6532 total genes contained in the dataset by Kuroda et. al (2019) [5], only 4 were not mapped to an Open Reading Frame by the ShinyGATOM, all corresponding to pseudogenes.
The starting network used for the identification of most active submodules contained 906 nodes,

corresponding to metabolites, and 1243 edges, corresponding to gene regulators.

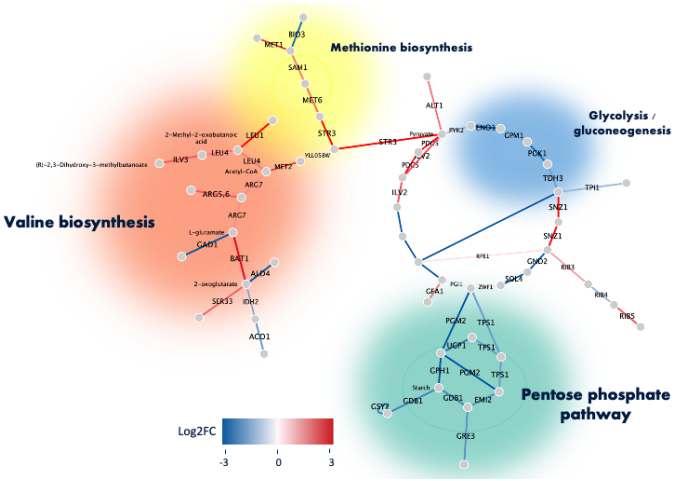

Figure 1: *Top scoring active subnetwork, WT (1.3% Isobutanol) / WT (0%)*

The first condition of interest analyzed was the control wild-type yeast strain with and without treatment with isobutanol. The final highest scoring module identified contained 52 nodes and 59 edges. Four main pathways were found to be functionally enriched: valine and methionine biosynthesis, that contained genes that were mostly upregulated, and glycolysis/gluconeogenesis and pentose phosphate pathway (PPP), which instead were found to be downregulated (Figure 1).
Inherent isobutanol production by *S. cerevisiae* occurs during the three-step catalytic breakdown of valine, during the Ehrlich pathway: valine undergoes transamination to 2-ketoisovalerate, which is catalyzed by the aminotransferase Bat2 [8]. Thus, it is not suprising that this pathway is functionally enriched after treatment with isobutanol. Another interesting result is the downregulation of PPP, which is in agreement with a paper by Liu et al. (2021) [9]: in fact, this pathway was found to be involved in isobutanol tolerance.

The most active subnetwork identified in the DE analysis of mutant versus control strain without treatment with isobutanol was composed of only 3 nodes and 2 edges, thus it was not analyzed further (Figure 2A). This result can be explained looking at the p-value distribution of the DE analysis (Figure 2B), in which it appears clear that most genes are not significantly changed in expression after deletion of $GLN3\Delta$, which encodes a transcriptional activator in the nitrogen catabolite repression system [10].

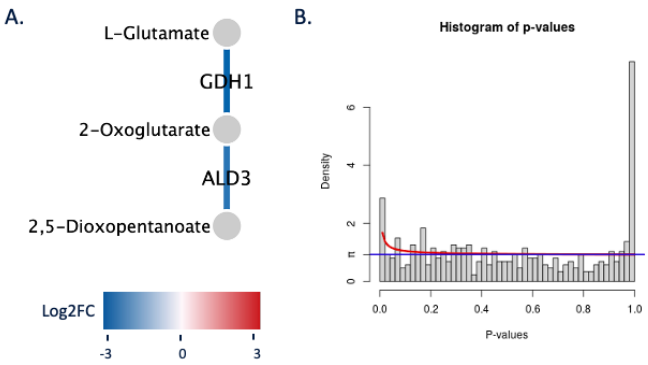

Figure 2: A. *Top scoring active subnetwork, GLN3Δ (0%) / WT (0%).* B. *p-value distribution of differentially expressed genes.*

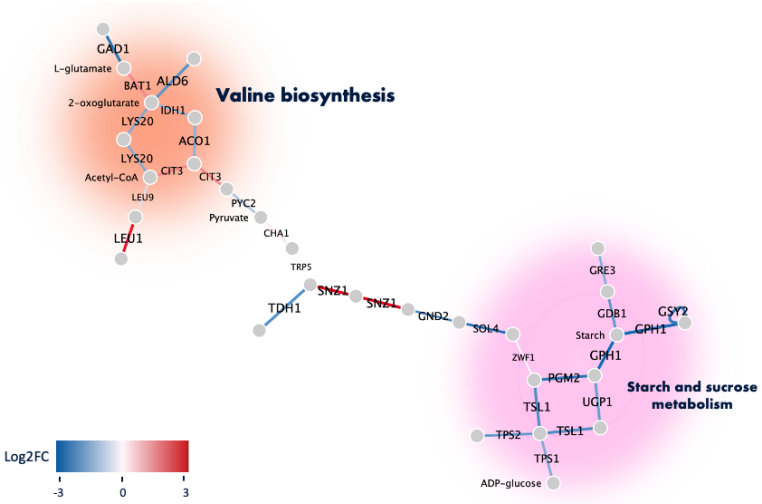

Figure 3: *Top scoring active subnetwork, GLN3Δ (1.3% Isobutanol) / GLN3Δ (0%).*

The module identified by ShinyGATOM for the data comparing treated versus untreated $GLN3\Delta$ strain contained 29 nodes and 31 edges. Two submodules were identified as functionally enriched, namely valine biosynthesis and starch and sucrose metabolism. While the first contained a mixture of genes up- and down-regulated, the second is only down-regulated (Figure 3).
This result is very similar to the one obtained when comparing the wild type strain with and without IB. Moreover, it is interesting to notice how these two pathways tend to form two distinct clusters: in fact, they appear to be very well interconnected within the submodule while two edges representing genes not differentially expressed in the dataset (CHA1 and TRP5) connect the two "communities". This is a result of the scoring algorithm, which always tries to identify the largest differentially regulated metabolic subnetwork between the two conditions.

Finally, the last condition of interest analyzed was obtained comparing the mutant $GLN3\Delta$ with the WT control, after treatment with isobutanol. The most regulated subnetwork identified was the largest between the four conditions taken into consideration, with 57 nodes and 63 edges. Furthermore, many pathways were found to be functionally enriched within the module, most of them related to the category of amino acid and secondary metabolites biosynthesis, including valine, lysine, methionine and cysteine, aspartate and glutamate, histidine and arginine. In addition, glycolysis and gluconeogenesis were also identified (Figure 4).

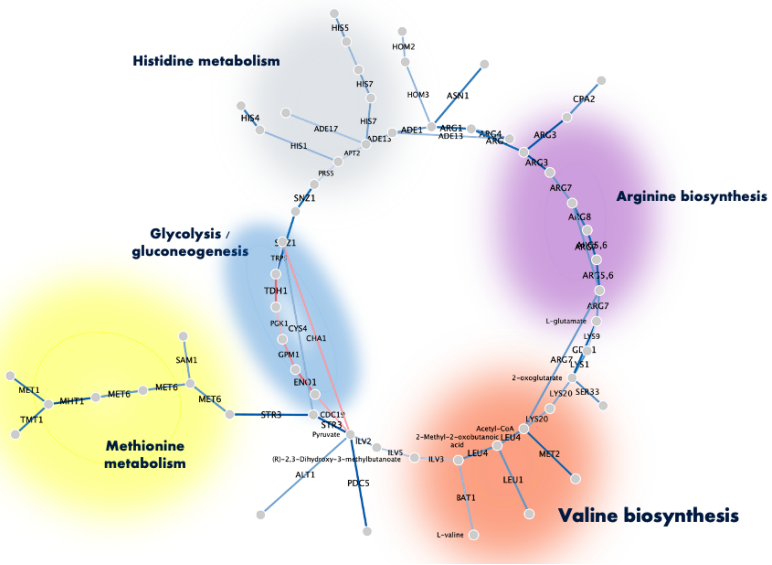

Figure 4: *Top scoring active subnetwork, $GLN3\Delta$ (1.3% Isobutanol) / WT (1.3%).*

These results are consistent with the findings by Kuroda et al. (2019) [5], in which similar

pathways were functionally annotated after differential expression analysis. However, in their results, two other important pathways were found to be induced by the presence of isobutanol in the medium: ion transport and cell wall biogenesis and organization. However, the genes associated to these pathways were not contained in the initial graph used in this analysis, since only 438 unique genes out of 6532 were included in the network representing yeast metabolism. In fact, the algorithm does not take into account a number of ubiquitous metabolites due to their extreme connectivity as well as multi-step reactions to avoid redundancy [6]. This could thus explain why the tool did not identify these pathways as functionally enriched in the submodules for the four conditions.
Another interesting result is the absence of pathways related to tryptophan metabolism, an amino acid previously identified to be involved in isobutanol tolerance [9]. In fact, TRP5, a gene encoding for a protein responsible for tryptophan synthesis, is not differentially expressed in the dataset analyzed in this project in any of the conditions.

To conclude, the results obtained in this project highlight the usefulness of using an active submodule approach to identify a range of enriched pathways in different conditions of interest using omics data. These kind of analyses could then help track down genes and metabolites that are central in the organism metabolism. In this case, discovery of the underlying mechanisms governing the observed changes in gene expression could help towards the engineering of knock-out strains with improved isobutanol production.
While this study is an important step towards a more biosustainable production of IB, there is still very few literature regarding this topic; moreover, large interaction networks often include many false positives. For this reason, future developments of these type of studies should always include verification of reproducibility in laboratory.

References

[1] Liu Z, Moradi H, Shi S, Darvishi F. Yeasts as microbial cell factories for sustainable production of biofuels. Renewable and Sustainable Energy Reviews. 2021 Jun;143:110907. Available from: https://doi.org/10.1016/j.rser.2021.110907.

[2] Parapouli M, , Vasileiadi A, Afendra AS, Hatziloukas E, and. &ltem&gtSaccharomyces cerevisiae&lt/em&gt and its industrial applications. AIMS Microbiology. 2020;6(1):1-32. Available from: https://doi.org/10.3934/microbiol.2020001.

[3] Azambuja SPH, Goldbeck R. Butanol production by Saccharomyces cerevisiae: perspectives, strategies and challenges. World Journal of Microbiology and Biotechnology. 2020 Mar;36(3). Available from: https://doi.org/10.1007/s11274-020-02828-z.

[4] Wess J, Brinek M, Boles E. Improving isobutanol production with the yeast Saccharomyces cerevisiae by successively blocking competing metabolic pathways as well as ethanol and glycerol formation. Biotechnology for Biofuels. 2019 Jul;12(1). Available from: https://doi.org/10.1186/s13068-019-1486-8.

[5] Kuroda K, Hammer SK, Watanabe Y, López JM, Fink GR, Stephanopoulos G, et al. Critical Roles of the Pentose Phosphate Pathway and GLN3 in Isobutanol-Specific Tolerance in Yeast. Cell Systems. 2019 Dec;9(6):534-47.e5. Available from: https://doi.org/10.1016/j.cels.2019.10.006.

[6] Sergushichev AA, Loboda AA, Jha AK, Vincent EE, Driggers EM, Jones RG, et al. GAM: a web-service for integrated transcriptional and metabolic network analysis. Nucleic Acids Research. 2016 Apr;44(W1):W194-200. Available from: https://doi.org/10.1093/nar/gkw266.

[7] Emelianova M, Gainullina A, Poperechnyi N, Loboda A, Artyomov M, Sergushichev A. Shiny GATOM: omics-based identification of regulated metabolic modules in atom transition networks. Nucleic Acids Research. 2022 May;50(W1):W690-6. Available from: https://doi.org/10.1093/nar/gkac427.

[8] Brat D, Weber C, Lorenzen W, Bode HB, Boles E. Cytosolic re-localization and optimization of valine synthesis and catabolism enables increased isobutanol production with the yeast Saccharomyces cerevisiae. Biotechnology for Biofuels. 2012 Sep;5(1). Available from: https://doi.org/10.1186/1754-6834-5-65.

[9] Liu HL, Wang CHT, Chiang EPI, Huang CC, Li WH. Tryptophan plays an important role in yeast's tolerance to isobutanol. Biotechnology for Biofuels. 2021 Oct;14(1). Available from: https://doi.org/10.1186/s13068-021-02048-z.

[10] Minehart PL, Magasanik B. Sequence and expression of GLN3, a positive nitrogen regulatory gene of Saccharomyces cerevisiae encoding a protein with a putative zinc finger DNA-binding domain. Molecular and Cellular Biology. 1991 Dec;11(12):6216-28. Available from: https://doi.org/10.1128/mcb.11.12.6216-6228.1991.