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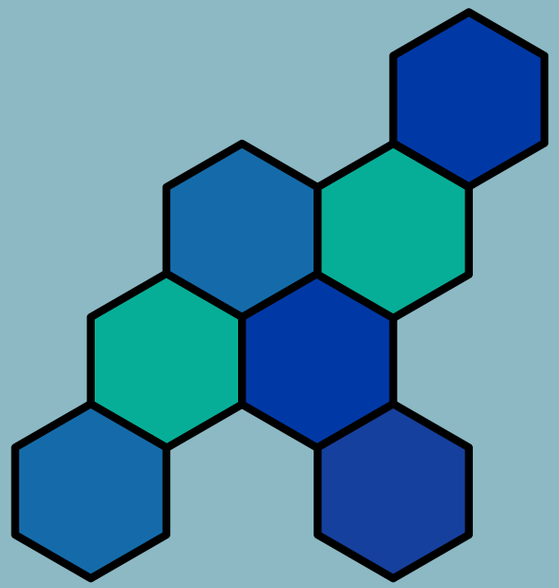

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Impact of liquid and solid-state cultures on hemoglobin production and oxidative state in *Saccharomyces* cerevisiae

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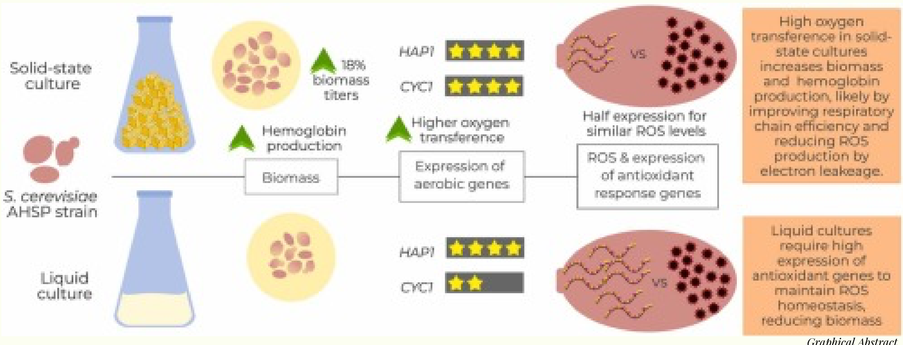
https://doi.org/10.1016/j.jbiotec.2025.01.020

Highlights

- Hemoglobin in solid culture was higher than in liquid due to higher biomass formation.
- CYC1 overexpressed in solid culture, which may indicate higher oxygen transference.
- Efficient electron transfer may relate to CYC1 overexpression in solid culture.
- Antioxidant gene overexpression in liquid culture may reduce biomass formation.
- Solid culture is a suitable platform to improve recombinant protein production.



Graphical Abstract

ABSTRACT

Recombinant human hemoglobin gained attention due to its potential use as a blood-free oxygen carrier substitute. To enhance human hemoglobin production in Saccharomyces cerevisiae, various genetic engineering strategies have been employed, including: increasing intracellular heme levels, minimizing heme and protein degradation pathways, and co-expressing the α-hemoglobin stabilizing protein (AHSP). Solid-state culture (SSC) may enhance hemoglobin production by increasing heme biosynthesis, as it relates to intracellular oxygen availability. A comparative analysis of heme and hemoglobin production was conducted between liquid culture LC) and SSC using the S. cerevisiae AHSP strain. While both systems exhibited comparable heme and hemoglobin yields per cell, a significant 18 % increase in biomass was observed in SSC. The expression of the aerobic master gene HAP1 remained consistent between both systems, however, CYC1 (regulated by HAP1) was two-fold overexpressed in SSC, indicating higher oxygen transference and possibly more efficient electron transport. Several antioxidant genes were downregulated in the SSC, suggesting that LC may be more susceptible to electron leakage during oxidative phosphorylation, potentially due to the lower expression of CYC1. It is proposed that high expression of antioxidant genes in LC inhibits biomass production due to the metabolic burden of maintaining redox homeostasis. These differences between LC and SSC may explain the suitability of SSC as a platform for recombinant protein production.

Introduction

Recombinant human hemoglobin offers a promising alternative to human blood transfusions, mitigating several critical concerns including risks of supply, cold chain deliveries, contamination with known and unknown blood pathogens (Yang et al., 2017) or adverse immune reactions (Schönborn et al., 2017, Ackfeld et al., 2022). Escherichia coliand Saccharomyces cerevisiae have been explored as production platforms of recombinant hemoglobin (Vasseur-Godbillon et al., 2006, Liu et al., 2014, Martínez et al., 2015, Ishchuk et al., 2021). S. cerevisiae is particularly interesting for the production of therapeutic proteins like human hemoglobin due to its GRAS status (Madhukar et al., 2024), long tradition in production biotechnology (Niemelä et al. 2024) and the capability to perform the post-translational modifications required for proper folding and function (Walsh, 2010).

The proper function and folding of hemoglobin critically depend on the heme cofactor. Yeast naturally synthetizes heme as an essential cofactor for several intracellular enzymes. In S. cerevisiae, improved heme biosynthesis has been genetically engineered, mainly by the overexpression of the HEM3 gene. This genetic modification aims to address the increased heme demand driven by the strong expression of globin genes (Liu et al., 2014). In addition, the deletion of: i) transcription factors involved in the activation of aerobic and anaerobic responses (HAP1 and ROX1); and ii) heme oxygenase (HMX1) involved in the degradation of heme. Heme has several biological roles in cells including the sensing of extracellular oxygen (Hon et al., 2003, Zhang et al., 2017, Hoque and Weinert, 2023). Under aerobic conditions, heme activates Hap1, a key transcription factor that regulates the expression of numerous aerobic genes (Hon et al., 1999, Lan et al., 2004). Because of these reasons, it was particularly interesting to evaluate hemoglobin production within a system characterized by a high oxygen transference, which is expected to favor heme biosynthesis. The traditional liquid culture has the disadvantage of low oxygen transference to the medium and subsequently to the cells, which can be overcome somehow by forced aeration (Miranda et al., 2024). On the contrary, solid-state cultures use solid substrates that increase the specific area in which oxygen transfers to the medium, providing higher oxygen levels to the cells (Viniegra-González et al., 2003). The oxygen transfer in solid-state culture does not require stirring or forced aeration, resulting in lower energy requirements and costs compared to the liquid culture.

Production of lignocellulose biomass-hydrolyzing enzymes by Trichoderma SG2 was significantly higher in solid-state fermentation compared to liquid fermentation, with an order of magnitude increase observed (Nanjundaswamy and Okeke, 2020). Similarly, invertase and biomass production from Aspergillus niger were higher in solid-state cultures compared to liquid culture (Romero-Gómez et al., 2000). Solid-state culture also shows additional advantages over liquid culture such as higher resistance to catabolic repression. In A. niger, laccase production using glucose as a carbon source was higher in solid-state culture than in liquid culture when recombinant laccase was expressed under an amylase promotor (Téllez-Jurado et al., 2006). Research on recombinant enzyme production in yeast using solid-state and liquid culture is scarce. López et al. (2010)investigated recombinant laccase production in Pichia pastoris using both solid-state and liquid cultures. Although biomass production was higher in the solid system, laccase production was higher in the liquid culture. This discrepancy may be attributed to suboptimal induction of laccase transcription in solid-state conditions. The use of a methanol-inducible promotor may hinder the proper induction of laccase transcription since methanol may not diffuse properly through the solid support.

This work aimed to compare the production of heme, hemoglobin, and biomass production by S. cerevisiae, in a system that favors oxygen transference such as solid-state culture, and compare it with the traditional liquid culture.

Results and Discussion

3.1. Heme determinations

Heme is the cofactor of hemoglobin and it is involved in the correct folding, stability and function of hemoglobin. Therefore, a high supply of heme is required during hemoglobin biosynthesis (Liu et al., 2014, Martínez et al., 2015, Ishchuk et al., 2021). Liquid and solid-state cultures were compared in terms of heme production (total heme, free heme and bound heme) (Fig. 1). For total heme, no significant differences were observed between both systems at 24 h (Fig. 1A). However, free heme levels were 33 % lower in the solid-state culture compared to the liquid culture (Fig. 1B). Bound heme levels were similar between both culture systems (Fig. 1C). Many enzymes use heme as a cofactor including cytochromes, and catalases A and T (Hörtner et al., 1982, Martínez et al., 2015). It is possible that the high oxygen transference in SSC allows the cell to increase biosynthesis of heme-proteins, reducing free heme pool levels and driving the need for correct heme incorporation. The deletion of the HAP1 gene reduced aerobic metabolism and increased free heme levels in S. cerevisiae (Martínez et al., 2015). This was accompanied by a reduction in transcription of several cytochrome subunits, which use heme as cofactor. It is likely that high oxygen transference from the solid-state culture reduces the free heme pools due to a higher Hap1 activity, redirecting heme to cytochrome synthesis. However, no significant differences were observed between both systems in terms of bound heme.

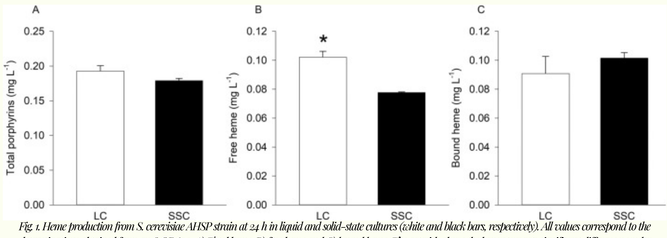


Fig. 1. Heme production from S. cerevisiae AHSP strain at 24 h in liquid and solid-state cultures (white and black bars, respectively). All values correspond to the determinations obtained from an 8 OD600. A) Total heme, B) free heme and C) bound heme. The asterisk above the bar represents significant differences under Student's t-test (P < 0.05).

Acknowledgements and References

Ackfeld et al., 2022, T. Ackfeld, T. Schmutz, Y. Guechi, C. Le Terrier
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