Engineering Yarrowia lipolytica to produce Fatty Acyl esters of Hydroxy Fatty Acids

Nathan Mau Qian Poh[1,2], Maybelle K. Go[1], Yuen Siong Chua[1], Wen Shan Yew[1,2], Yong-Su Jin[1]

[1]Illinois Advanced Research Center at Singapore (IARCS)

[2]National University of Singapore Synthetic Biology for Clinical and Technological Innovation (SynCTI)

Introduction

Fatty Acyl esters of Hydroxy Fatty Acids (FAHFAs) are a distinct class of lipids with notable biological activities, first discovered in murine models in 2014[1]. Among FAHFAs, palmitic acid ester of hydroxy stearic acids (PAHSAs) in particular has displayed anti-diabetic and anti-inflammatory properties and are positioned as potential therapeutic treatments for diabetes and inflammatory diseases[1]. However, given their low abundance in natural sources and complex chemical synthesis routes[2], large scale production of FAHFAs has remained economically unfeasible. We propose an alternative biosynthetic method utilizing the oleaginous yeast *Yarrowia lipolytica* as a microbial cell factory for the intracellular production of PAHSAs. This is achieved through recombinant protein expression of oleate hydratase (OH) and *Candida antarctica* lipase A (CalAp) in *Y. lipolytica* to develop an intracellular PAHSA synthesis pathway (Figure 1).

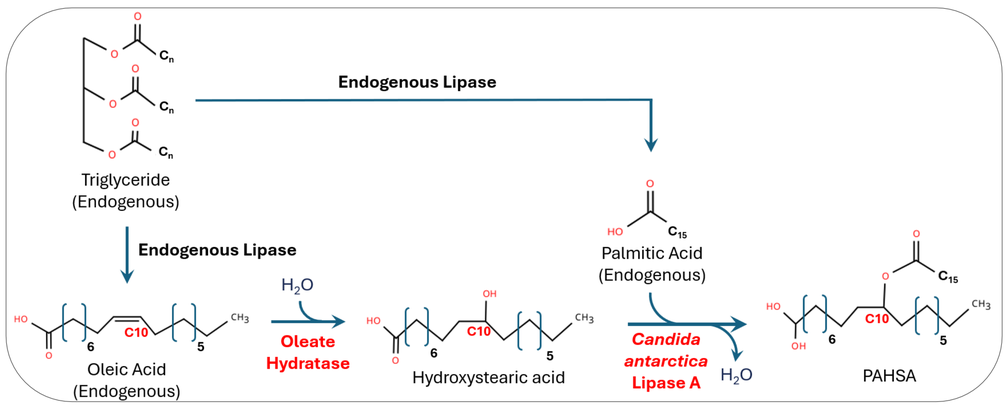
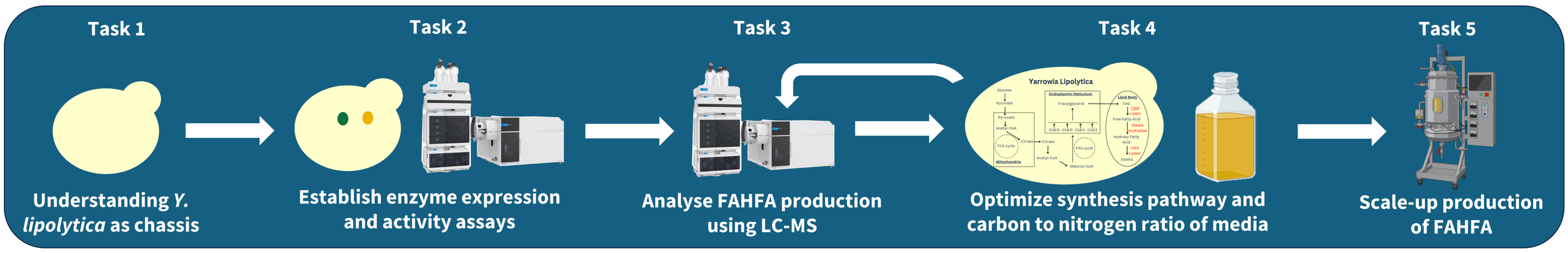


Fig 1. Proposed intracellular PAHSA synthesis pathway in *Y. lipolytica*. Enzymes to be heterologously expressed are highlighted in red.

Methodology



Results and Discussion

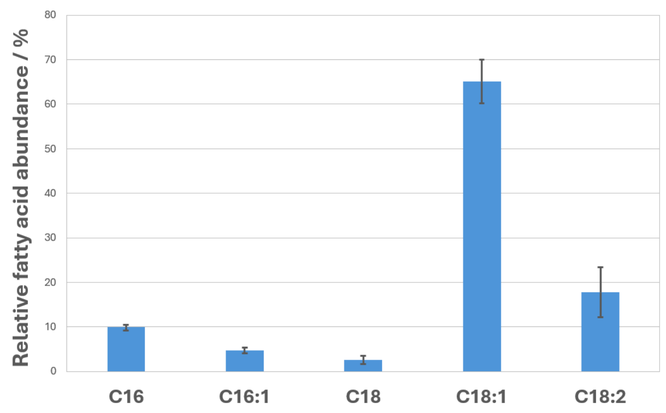


Fig 2. Fatty acid profile of *Y. lipolytica* Po1g.

Task 1

A total lipid analysis utilizing Fatty Acid Methyl Esters (FAMEs) derivatization and gas chromatography – mass spectrometry (GC-MS) was performed on lyophilized cell pellets of *Y. lipolytica* Po1g to study its natural fatty acid composition (Figure 2). Predominant fatty acids present were oleic acid C18:1 (~65%), linoleic acid C18:2 (~18%) and palmitic acid C16:0 (~10%). Given that palmitic and oleic acids are precursors of PAHSAs, their presence validates *Y. lipolytica* as a favourable chassis for the construction of an intracellular PAHSA biosynthesis pathway. The endogenous abundance of these substrates reduces the need for extensive pathway reconstruction or exogenous substrate supplementation and provides a metabolically favourable environment for PAHSA biosynthesis. Furthermore, given the extensive metabolic engineering toolkits available for *Y. lipolytica*, its fatty acid synthesis pathway can be rationally modified to further optimize the abundance of palmitic and oleic acids to increase expected PAHSA yields[3].

Task 2

Oleate hydratases catalyse the hydration of oleic acid to form hydroxystearic acid (HSA), a key step in the PAHSA biosynthetic pathway as HSA serves as the direct precursor to PAHSA. OHs reported in literature exhibit strong regioselectivity for hydroxylation at the C10 position[4], resulting in 10-HSA formation. Consequently, incorporation of these enzymes into a PAHSA biosynthetic pathway is expected to yield 10-PAHSA which has been shown to have anti-diabetic properties[5]. To identify a suitable orthologue for expression in *Y. lipolytica*, a sequence similarity network was generated from a characterised OH (Uniprot ID C7DLJ6) using the Enzyme Function Initiative–Enzyme Similarity Tool. Gene sequences of 30 orthologues spanning a range of sequence identities (designated OH1–OH30), were synthesised and genomically integrated into *Y. lipolytica* Po1g for heterologous expression. An activity assay (Figure 3) was performed on cell lysates of the OH–expressing *Y. lipolytica* strains to test enzyme functionality (Figure 4).

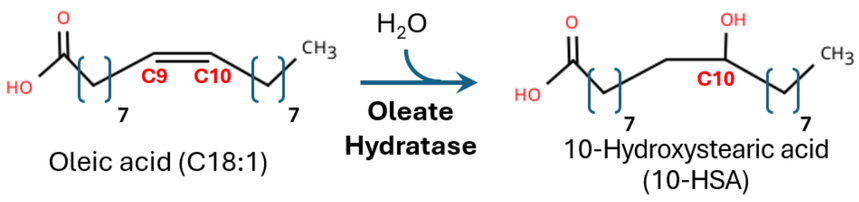


Fig 3. Reaction mechanism of OH activity assay. Cell lysate samples of OH-expressing strains were incubated in excess oleic acid. Samples were subsequently derivatized to FAMEs and analysed by GC-MS for presence of 10-HSA.

	OH 1	OH 3	OH 6	OH 7	OH 8	OH 9	OH 10	OH 11	OH 12	OH 13	OH 14	OH 15	OH 16	OH 17	OH 18	OH 19	OH 20	OH 21	OH 22	OH 23	OH 24	OH 25	OH 26	OH 27	OH 28	OH 29	OH 30
9-HSA																											
10-HSA																											

- Absent
- Present

Fig 4. Qualitative analysis of functionality of OH heterologously expressed in *Y. lipolytica.*

OH orthologues 7, 8, 9, 10, 15, 17, 20, 26 and 30 exhibited successful heterologous expression and enzymatic activity when expressed in *Y. lipolytica as* confirmed by the detection of 10-HSA following *in vitro* incubation of cell lysates with excess oleic acid. These OH orthologues were therefore selected for further characterisation. Notably, all functional OHs displayed strict regioselectivity for hydroxylating at the C10 position. Future work can look towards engineering OH to hydroxylate at the C9 position to produce 9-HSA as a precursor for producing 9-PAHSA which has shown to exert both anti-diabetic and anti – inflammatory effects in murine models [1].

Future Work

- Test *in vivo* production of 10-HSA resulting from oleate hydratase expression
- Screen CalAp orthologues to identify best performing orthologues
- Co-expression of best performing CalAp and OH orthologues for intracellular PAHSA production
- Optimization of carbon to nitrogen ratio and PAHSA synthesis pathway to maximise yield and titre of PAHSA.

Conclusion

Y. lipolytica has proven to be a promising host for the intracellular construction of a PAHSA biosynthetic pathway owing to its naturally high abundance of oleic and palmitic acids. We have also demonstrated the successful heterologous expression of functional oleate hydratases in *Y. lipolytica* PO1G, representing a key step towards validating the proposed PAHSA biosynthesis strategy. The next step will be to evaluate these functional oleate hydratase strains for in vivo production of 10-HSA, thereby establishing the first committed precursor toward intracellular PAHSA formation.

References and Acknowledgements

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