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Unlocking lager's flavour palette by metabolic engineering of *Saccharomyces pastorianus* for enhanced ethyl ester production

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ABSTRACT

Despite being present in trace amounts, ethyl esters play a crucial role as flavour compounds in lager beer. In yeast, ethyl hexanoate, ethyl octanoate and ethyl decanoate, responsible for fruity and floral taste tones, are synthesized from the toxic medium chain acyl-CoA intermediates released by the fatty acid synthase complex during the fatty acid biosynthesis, as a protective mechanism. The aim of this study was to enhance the production of ethyl esters in the hybrid lager brewing yeast *Saccharomyces pastorianus* by improving the medium chain acyl-CoA precursor supply. Through CRISPR-Cas9-based genetic engineering, specific *FAS1* and *FAS2* genes harbouring mutations in domains of the fatty acid synthesis complex were overexpressed in a single and combinatorial approach. These mutations in the *ScFAS* genes led to specific overproduction of the respective ethyl esters: overexpression of $ScFAS1^{I306A}$ and $ScFAS2^{G1250S}$ significantly improved ethyl hexanoate production and $ScFAS1^{R1834K}$ boosted the ethyl octanoate production. Combinations of *ScFAS1* mutant genes with $ScFAS2^{G1250S}$ greatly enhanced predictably the final ethyl ester concentrations in cultures grown on full malt wort, but also resulted in increased levels of free medium chain fatty acids causing alterations in flavour profiles. Finally, the elevated medium chain fatty acid pool was directed towards the ethyl esters by overexpressing the esterase *ScEEB1*. The genetically modified *S. pastorianus* strains were utilized in lager beer production, and the resulting beverage exhibited significantly altered flavour profiles, thereby greatly expanding the possibilities of the flavour palette of lager beers.

1. Introduction

The spectrum of flavour molecules that defines the taste of beer is highly diverse. The interplay of these flavour molecules at varying concentrations significantly broadens the potential flavour profiles achievable in a beverage. Additionally, synergism, where flavour molecules can intensify or dampen specific aromas, even below the human sensory threshold (HST), is noteworthy (Verstrepen et al., 2003a,b; Pires et al., 2014). Traditionally, hops play a dominant role as the primary flavour-contributing ingredient in lager brewing, but the role of brewing yeast, *Saccharomyces pastorianus*, is central for flavour enhancement during fermentation. This yeast (Dunn and Sherlock, 2008, Nakao et al., 2009; Salazar et al., 2019), an interspecific hybrid of *Saccharomyces cerevisiae* and the cryotolerant *Saccharomyces eubayanus* (Libkind et al., 2011), naturally produces a wide spectrum of molecules with aromatic properties, including higher alcohols, carbonyl compounds, vicinal diketones, and esters (Nordström, 1964; Peddie, 1990; Renger et al., 1992; Verstrepen et al., 2003a,b; Pires et al., 2014).

Esters, present in only trace amounts and still being the most important aromas (Pires et al., 2014), can be categorized into two groups based on their precursors: acetate esters (e.g., isoamyl acetate, ethyl acetate and phenylethyl acetate) and ethyl esters (e.g., ethyl hexanoate, ethyl octanoate) (Fig. 1A). The formation of acetate esters involves fusel alcohols, products of the Ehrlich pathways that is involved in degradation of carbon skeleton of some class of amino acids, or ethanol and acetyl-CoA (Hazelwood et al., 2008; Romagnoli et al., 2012). This acetylation reaction is performed by alcohol acetyltransferases (AATases) encoded by *ATF1* and *ATF2* (Fujii et al., 1994, 1996; Yoshimoto et al., 1998, 1999; Mason and Dufour, 2000; Verstrepen et al., 2003a,b). On the other hand, ethyl esters, the class discussed in this manuscript, are formed by the condensation of ethanol and an acyl-CoA of which the carbon chain may vary from two to ten. Acyl-CoA and ethanol are converted to their ethyl ester derivative by acyl-coenzymeA:ethanol O-acyltransferase esterases (AEAT) encoded by *EEB1* (Saerens et al., 2006) and *EHT1* (Mason and Dufour, 2000; Saerens et al., 2006; Knight et al., 2014), with *EEB1* encoding the main enzyme

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responsible for this conversion (Saerens et al., 2006). Several hypotheses have been postulated regarding the physiological role of ester biosynthesis, but the most pertinent remains that ester formation contributes to detoxification of higher alcohols and medium chain fatty acids (MCFA) (Saerens et al., 2010). In the case of ethyl esters, their toxicity is lower than that of their respective acids, especially butanoic (C_4), hexanoic (C_6) and octanoic acid (C_8) (Hundová and Fencl, 1977; Legras et al., 2010; Besada-Lombana et al., 2017; Baumann et al., 2021). Therefore, the formation of ethyl esters mitigate accumulation of the acid and facilitate export by diffusion across the plasma membrane, which as well helps to shift the reaction equilibrium towards the ester biosynthesis (Nordström, 1964; Saerens et al., 2010; Liu et al., 2013).

In brewing context, attempts to enhance ethyl ester concentration above human sensory threshold by overexpressing acyl-coenzymeA: ethanol O-acyltransferase esterases *EEB1* and *EHT1* remained largely unsuccessful (Table 1) (Saerens et al., 2006; Yin et al., 2019). This absence of phenotype suggests that native acyl-coenzymeA:ethanol O-acyltransferase esterase capacity may not be limiting, but rather the precursor concentrations (Saerens et al., 2006, 2008). Hitherto, no studies attempted to deregulate the supply of short and medium acyl-CoA in *Saccharomyces* species alone or in combinationwith the overexpression of an acyl-coenzymeA:ethanol O-acyltransferase esterases. Thus, improving acyl-CoA precursor supply sounds a logical metabolic engineering target to enhance and tune ethyl ester formation.

In *Saccharomyces cerevisiae*, the fatty acid synthase (FAS) complex consists of two subunits, the β-chain encoded by *FAS1* that harbours the domains acetyl transferase (AT), enoyl reductase (ER), dehydratase (DH) and malonyl/palmitoyl transferase (MPT) and the α-chain encoded by *FAS2* that harbours the domains acyl carrier protein (ACP), ketoacyl reductase (KR), ketoacyl synthase (KS) and the phosphopantetheine transferase (PPT). The fatty acid synthesis starts as a two-carbon chain from acetyl-CoA, that is extended two carbons at a time by the addition of an acetyl group from malonyl-CoA. The KS domain is responsible for the condensation of an acyl substrate (either the starter molecule acetyl

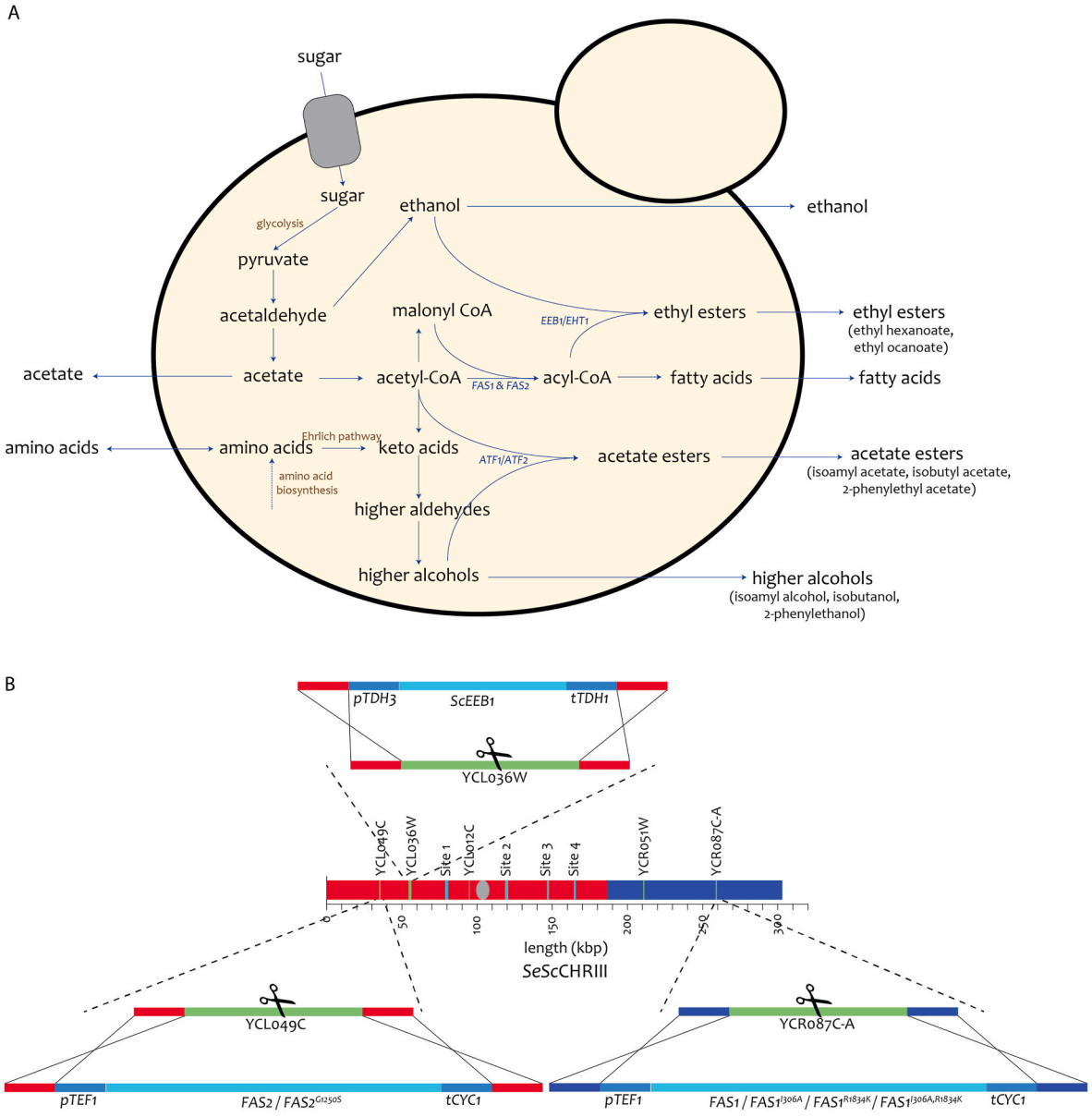

Fig. 1. A) Overview of metabolic pathways involved in ester biosynthesis in brewing yeasts. B) Strategy for integration of *ScFAS1* and *ScFAS2* genes and mutant variants in *S. pastorianus* CBS 1483 based on CRISPR-Cas9. Integration sites *Se*YCL049 (*ScFAS2* and *ScFAS2*^{G1250S}) and *Sc*YCR087C-A (*ScFAS1*, *ScFAS1*^{I306A}, *ScFAS1*^{R1834K} and *ScFAS1*^{I306A,R1834K}) on the hybrid *SeScCHRIII* (4 copies) were chosen. The *ScEEB1* gene was overexpressed in the genomic location *Se*YCL036W on the *SeScCHRIII* (4 copies).

Table 1
Flavour compounds found in beer produced by yeast, with their corresponding Human Sensory Threshold (HST) value obtained from literature, and their concentrations in lager beer brewed with CBS 1483 in tall European Brewing Convention (E.B.C.) tubes on 17 P° wort as a reference (Brickwedde et al., 2017).

Flavour Compound	Taste	HST [mg L^{-1}]	Concentrations in lager beer [mg L^{-1}]
Acetate esters			
Ethyl acetate	Sweet, fruity, solvent-like	3.0[a], 20–40[e]	49.38
Isobutyl acetate	Fruity, banana, pineapple	0.7[a]	0.16
Isoamyl acetate	Banana, pear	1.2[a]	5.26
Phenylethyl acetate	Rose, honey	0.4[a]	–
Ethyl esters			
Ethyl butanoate	Sweet, fruity, berry	0.001[g]	0.68
Ethyl hexanoate	Apple, fruity, sweet	0.15–0.25[e], 0.22[a]	0.43
Ethyl octanoate	Apple, aniseed	0.9[a]	0.76
Ethyl decanoate	Floral	0.2[f]	0.16
Higher alcohols			
Isoamyl alcohol	Alcohol	65[a]	68.31
Isobutyl alcohol	Solvent	70[a]	13.40
Propanol	Solvent	200[a]	–
2-Methyl butanol	Banana, medicinal	65[a]	–
Phenylethyl alcohol	Roses, sweet	125[a]	–
Carbonyl compounds			
Acetaldehyde	Grass, green apple	10–20[b]	–
Vicinal diketones			
Diacetyl	Buttery, butterscotch	0.07–0.15[e], 0.15[d]	82.13[h]
2,3-pentanedione	Fermented dairy	0.015[d]	25.81

[a](Stewart, 2017), [b](Olaniran et al., 2017), [c](Engan, 1972), [d](Ferreira and Guido, 2018), [e](Meilgaard, 1982), [f](Xi et al., 2011), [g](Schieberle and Hofmann, 1997), [h]Lagering period was unfinished in this fermentation.

or an ACP-bound elongated acyl-chain) with malonyl moieties. The resulting β-ketoacyl intermediate is processed sequentially by the KR, DH and ER domains, to a fully reduced acyl-chain. The resulting acyl-chain is processed in a subsequent cycle. The process repeats till the final fatty acid is cleaved off, which in *S. cerevisiae* happens typically when the fatty acid reaches 16 or 18 carbons (Wakil et al., 1983). Thus, MCFA with carbon length ranged from C_6 to C_{10}, are only intermediates of this biosynthetic process probably originating from premature release of the nascent fatty acids.

Several approaches, including overexpression of *FAS1 & 2* genes (Furukawa et al., 2003) or expression of thioesterases with specificities for shorter acyl-CoA successfully resulted in the production of MCFA (Leber et al., 2015). Moreover, specific mutations in *FAS1* or *FAS2* genes, particularly in active sites involved in chain elongation, yielding rate imbalance between the coordinated steps in fatty acid biosynthesis, have been demonstrated to enhance and tune MCFA release in *S. cerevisiae* for various purposes (e.g. beverages (Aritomi et al., 2004) and fine chemical production (Fernandez-Moya and Da Silva, 2017; Gajewski et al., 2017a, 2017b)). The I306A mutation in the AT domain of *FAS1* causes an elevated acetyl uptake. By forcing the initiation of a new fatty acid production cycle instead of elongating the existing chain, the current elongated chain is released from the FAS complex in shorter fatty acid form (Gajewski et al., 2017b). Additionally, the mutation R1834K affects the MPT domain of *FAS1* by making it less favourable for malonyl to bind. As malonyl is used for elongating the existing fatty acid chain, this mutation will result in early termination of the iterating elongation cycle (Gajewski et al., 2017b). Lastly, the G1250S mutation in the KS domain in *FAS2* prevents short chain intermediates from entering the KS domain and thus being released (Aritomi et al., 2004; Gajewski et al., 2017b).

In the present study, we aimed to modify the ethyl ester profile in *S. pastorianus* by enhancing the supply of the precursors MCFAs. This was achieved by introducing mutations in specific domains of the fatty acid synthesis complex and overexpressing single and combinatorial mutants of *ScFAS1* and *ScFAS2,* and overexpressing the esterase *ScEEB1*. This genetic engineering approach resulted in elevated levels of specific ethyl esters and alterations in flavour profiles. The genetically modified *S. pastorianus* strains were subsequently employed for lager beer production, and the resulting beverages underwent sensory evaluation to assess the modified flavour profile.

2. Materials and method

2.1. Strains and cultivation conditions

All yeast strains used in this study are listed in Table 2. Yeast were grown on complex medium that contained 10.0 g L^{-1} bacto yeast extract, 20.0 g L^{-1} bacto peptone and 20 g L^{-1} glucose (YPD). For selection of transformants or when selective pressure was required, YPD medium was supplemented with 200 mg L^{-1} of hygromycin (YPD hygR). Solid YPD media was obtained by addition of 20 g L^{-1} bacto agar. *S. cerevisiae* and *S. pastorianus* strains were cultivated at 30 °C and 20 °C, respectively, while shaking at 200 rpm in an Innova 43/43R incubation shaker (Brunswick, Nijmegen, the Netherlands). On solid media, *S. pastorianus* strains were incubated at 20 °C in a temperature controlled room. Fermentations were performed with full malt wort at either 17° P or 5.7° P (Heineken, Zoeterwoude, The Netherlands) supplemented with 1 mL L^{-1} pluronic acid 6100 PE antifoam (BASF, Ludwigshafen, Germany).

Escherichia coli XL1-Blue (New England Biolabs, Ipswich, MA) cultures were grown in 15 mL Greiner tubes containing 5 mL lysogenic broth (LB) medium supplemented with 10 mg L^{-1} ampicillin (LB Amp) for selection and cultivated at 37 °C while shaking at 200 rpm in an Innova 4000 Incubator Shaker (Eppendorf AG, Hamburg, Germany).

Table 2
Strains used in this study.

Strain	Relevant genotype	Reference
CEN. PK113-7D	MAT*a MAL2-8c SUC2*	Entian and Kötter (2007)
CBS 1483	Group II brewer's yeast, Heineken bottom yeast, isolated July 1927	Dunn and Sherlock (2008)
IMI541	CBS 1483 Δ*Sc*YCR087C::*ScFAS1*	This study
IMI542	CBS 1483 Δ*Sc*YCR087C::*ScFAS1*^{I306A}	This study
IMI543	CBS 1483 Δ*Sc*YCR087C::*ScFAS1*^{R1834K}	This study
IMI544	CBS 1483 Δ*Sc*YCR087C::*ScFAS1*^{I306A,R1834K}	This study
IMI545	CBS 1483 Δ*Se*YCL049C::*ScFAS2*	This study
IMI546	CBS 1483 Δ*Se*YCL049C::*ScFAS2*^{G1250S}	This study
IMI547	CBS 1483 Δ*Se*YCL049C::*ScFAS2* Δ*Sc*YCR087C::*ScFAS1*	This study
IMI548	CBS 1483 Δ*Se*YCL049C::*ScFAS2* Δ*Sc*YCR087C::*ScFAS1*^{I306A}	This study
IMI549	CBS 1483 Δ*Se*YCL049C::*ScFAS2* Δ*Sc*YCR087C::*ScFAS1*^{R1834K}	This study
IMI550	CBS 1483 Δ*Se*YCL049C::*ScFAS2* Δ*Sc*YCR087C::*ScFAS1*^{I306A,R1834K}	This study
IMI551	CBS 1483 Δ*Se*YCL049C::*ScFAS2*^{G1250S} Δ*Sc*YCR087C::*ScFAS1*	This study
IMI552	CBS 1483 Δ*Se*YCL049C::*ScFAS2*^{G1250S} Δ*Sc*YCR087C::*ScFAS1*^{I306A}	This study
IMI553	CBS 1483 Δ*Se*YCL049C::*ScFAS2*^{G1250S} Δ*Sc*YCR087C::*ScFAS1*^{R1834K}	This study
IMI554	CBS 1483 Δ*Se*YCL049C::*ScFAS2*^{G1250S} Δ*Sc*YCR087C::*ScFAS1*^{I306A,R1834K}	This study
IMI577	CBS 1483 Δ*Se*YCL049C::*ScFAS2*^{G1250S} Δ*Sc*YCR087C::*ScFAS1*^{I306A,R1834K} Δ*Se*YCL036W::*ScEEB1*	This study
IMI578	CBS 1483 ΔYCL036W::*ScEEB1*	This study

Solid medium was prepared by addition of 20 g L^{-1} bacto agar to the medium.

Frozen stock cultures of *S. pastorianus*, *S. cerevisiae* and *E. coli* strains were prepared by addition of 30% (v/v) glycerol and stocks were stored as 1 mL aliquots at −80 °C.

2.2. Molecular biology techniques

PCR amplifications for cloning purposes were performed using Phusion High Fidelity Polymerase (Thermo Fisher Scientific, Landsmeer, The Netherlands) according to the manufacturer's instructions. Diagnostic PCRs were performed with DreamTaq PCR Mastermix (Thermo Fisher Scientific) according to the manufacturer's instructions. All plasmids and primers (Sigma Aldrich, Zwijndrecht, The Netherlands) constructed or used in this study are listed in Tables 3 and 4, respectively. DNA was visualized by electrophoresis on 1% agarose gels with the GeneRuler™ Ladder Mix (Thermo Scientific) for size verifications. PCR products were purified from gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Alternatively, PCR mixtures were digested with FastDigest DpnI enzyme (Thermo Fisher Scientific) to remove circular templates. Digested PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) following the manufacturer's instructions using milliQ as eluent solvent. Gibson Assembly was performed with 200 fmol of each fragment using NEBuilder® HiFi DNA Assembly Master Mix (New England BioLabs) and 1 h incubation at 50 °C (Gibson et al., 2009). Golden gate assembly was done according to (Lee et al., 2015) with 20 fmol of each fragment with restriction enzyme BsaI (Thermo Fisher Scientific) and T7 DNA ligase (Thermo Fisher Scientific). Constructed plasmids were transformed to *E. coli* XL1-blue (New England Biolabs) chemically competent cells. Plasmids were isolated from the *E. coli* cells with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the supplier's manual with milliQ as eluent solvent. Diagnostic plasmid digestions were performed using Fast Digest enzymes (Thermo Fisher Scientific) and the Green Fast Digest Buffer (Thermo Fisher Scientific) on 500 ng plasmid DNA.

Table 3
Plasmids used in this study.

Plasmid	Genotype	Reference
pUDP269	*ori bla* panARS$^{(OPT)}$ *AgpTEF1-hph-AgtTEF1 ScpTDH3-HH-gRNA$_{SeYCL049C}$-HDV-SctCYC1 AapTEF1-Spcas9$^{D147Y\ P411T}$-SctPHO5*	Bennis et al. (2023)
pUDP273	*ori bla* panARS$^{(OPT)}$ *AgpTEF1-hph-AgtTEF1 ScpTDH3-HH-gRNA$_{ScYCR087C\text{-}A}$-HDV-SctCYC1 AapTEF1-Spcas9$^{D147Y\ P411T}$-SctPHO5*	Bennis et al. (2023)
p426-TEF1-amds	*ori bla* 2 μm *pTEF1-amdSYM-tTEF1 pTEF1-*empty*-tCYC1*	Solis-Escalante et al. (2013)
pUD1295	*ori bla* 2 μm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1-tCYC1*	This study
pUD1296	*ori bla* 2 μm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1^{I306A}-tCYC1*	This study
pUD1297	*ori bla* 2 μm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1^{R1834K}-tCYC1*	This study
pUD1298	*ori bla* 2 μm *pTEF1-amdS-tTEF1 pTEF1-ScFAS1^{I306A,R1834K}-tCYC1*	This study
pUD1299	*ori bla* 2 μm *pTEF1-amdS-tTEF1 pTEF1-ScFAS2-tCYC1*	This study
pUD1300	*ori bla* 2 μm *pTEF1-amdS-tTEF1 pTEF1-ScFAS2^{G1250S}-tCYC1*	This study
pGGKd015	*ampR-ColE1* GFP dropout	Hassing et al. (2019)
pYTK009	*camR pTDH3*	Lee et al. (2015)
pYTK056	*camR TDH1t*	Lee et al. (2015)
pGGKp395	*camR ScEEB1*	This study
pUD1325	*ori bla pTDH3-ScEEB1-tTDH1*	This study

2.3. Plasmid construction

The plasmids expressing *S. cerevisiae FAS* genes were constructed via Gibson assembly (Gibson et al., 2009). The plasmid backbone (p426_TEF1_amdS) containing the *ScTEF1* promotor, *ScCYC1* terminator, *E. coli* pBR322 origin of replication and ampicillin resistance marker and the yeast 2 μm origin of replication and the *Aspergillus nidulans amdS* marker (Solis-Escalante et al., 2013) was linearized by PCR amplification with primers 5921 & 7812. To introduce mutations in the *ScFAS1* and *ScFAS2* genes, the genes were PCR amplified from the *S. cerevisiae* CEN.PK113-7D genome using primers containing the mutations, and split into two or three parts depending on the number of mutations, with each fragment containing homology flanks for proper assembly. For *ScFAS1*, two fragments were obtained with primers 19699 & 19710 and 19748 & 19700 and assembled into the p426-pTEF1-amds backbone, resulting in pUD1295; *ScFAS1^{I306A}* fragments with primer pairs 19699 & 19703 and 19704 & 19700 were assembled resulting in pUD1296; *ScFAS1^{R1834K}* fragments with primer pairs 19699 & 19705 and 19706 &19700 were assembled and resulting in pUD1297; and for *ScFAS1^{I306A,R1834K}* three fragments amplified with primers 19699 & 19703, 19704 & 19705 and 19706 &19700 were assembled resulting in pUD1298. For *ScFAS2*, two fragments were obtained with primers 19701 & 19742 and 19743 & 19702, assembled into the p426-pTEF1-amds backbone and resulted in pUD1299; *ScFAS2^{G1250S}* fragments were obtained with primer pairs 19701 & 19707 and 19708 & 19702 and assembled resulting in pUD1300.

To verify the correct assembly of the *FAS* genes into the backbone, diagnostic PCRs were performed with primers 2399 & 2626. Additionally, restriction analysis of the plasmids was performed with for the *ScFAS1* encoding plasmid the restriction enzymes BamHI and KpnI and for the *ScFAS2* encoding plasmids the restriction enzymes PvuI and EcoRI.

The primers used to Sanger sequence the *ScFAS1* genes on the plasmids and PCR-amplified integration regions were 19699, 19748, 19750, 19751, 19754, 19709, 19710, 19749, 19752, 19753, 19706 (only *ScFAS1^{R1834K}*) and 19700. The primers used to Sanger sequence the *ScFAS2* genes on the plasmids and PCR-amplified integration regions were 19701, 19755, 19757, 19759, 19711, 19743 (only *FAS2*), 19707 (only *ScFAS2^{G1250S}*), 19761, 19712, 19756, 19758, 19760, 19742 (only *FAS2*), 19708 (only *ScFAS2^{G1250S}*), 19762 and 19702.

The *ScEEB1* gene was PCR amplified from *S. cerevisiae* CEN.PK113-7D genomic DNA using primers 19949 & 19950. The PCR product was cloned into the pYTK001 entry vector for golden gate cloning according to (Lee et al., 2015). Golden gate with pYTK009 (*pTDH3*), pGGKp395 (*ScEEB1*), pYTK056 (*tTDH1*) (Lee et al., 2015) and backbone pGGKd015 (Hassing et al., 2019), resulting in plasmid pUD1325, which was verified using diagnostic primers 10320 & 10325. pUD1325 was sequence verified by whole plasmid sequence (Plasmidsaurus, Eugene, OR).

2.4. Construction of yeast strains

CRISPR-Cas9 genome editing in *S. pastorianus* strains was performed by transforming *S. pastorianus* strains with 500 ng gRNA plasmid and 1000 ng repair fragment. As negative control, transformations from which the repair fragment was omitted, were performed. Yeast transformation was performed by electroporation using 50 μL of competent cells in 0.2 cm chilled Gene Pulser® Cuvettes (Bio-Rad) and pulsed with for 5 ms with 1.5 kV by the MicroPulser (Serial No. 411BR5600, Bio-Rad). The transformed cells were incubated in 0.5 mL YPD during 2 h, followed by re-suspending in 100 μL sterile demi-water and plating on selective medium (Gorter de Vries et al., 2017).

The *ScFAS* gene integrations were performed by co-transforming the gRNA plasmid and the corresponding repair fragment consisting of the *ScFAS* expression cassette flanked by 60bp homologous sequences for recombination. The *ScFAS1, ScFAS1^{I306A}, ScFAS1^{R1834K}* and *ScFAS1^{I306A,R1834K}* repair fragments were obtained by PCR amplification using

Table 4
Primers used in this study.

Number	Sequence (5' -> 3')	Purpose
***FAS* expressing plasmid construction**		
2399	AACACCCAAGCACAGCATAC	Diagnostic verification of *FAS* genes into p426-pTEF1-amdS
2626	GGTTGAAGAAGTCGCTATC	
5921	CGAGTAATCTTTCTTTCGTATCGTTAGATTAGATTCAAAA	p426-pTEF1-amdS backbone amplification
7812	TCATGTAATTAGTTATGTCACGCTTACATTC	
19699	GCTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTTTATGGACGCTTACTC CACAAG	Amplification FAS1 with p426-pTEF1-amdS backbone compatible flanks
19700	GGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGATTAGGATTGTTCA TACTTTTCCCAGTTG	
19701	GCTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGTTTTATGAAGCCGGAAG TTGAGCAAG	Amplification FAS2 with p426-pTEF1-amdS backbone compatible flanks
19702	GGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGACTATTTCTTAGTA GAAACGGCGACC	
19703	GCAATTACTGTATTATTCTTCGCTGGTGTTCGTTGTTACGAAGCATACCCAAAC ACTTCC	Introduction I306A mutation in *FAS1*
19704	CGTAACAACGAACACCAGCGAAGAATAATACAGTAATTGCTTTTCTTACGGAG ACG	
19705	TAGTTGAAGTTGTGTTCTACAAAGGTATGACTATGCAAGTTGCTGTTCC	Introduction R1834K mutation in *FAS1*
19706	ACTTGCATAGTCATACCTTTGTAGAACACAACTTCAACTAAAGATTCGATAGAC	
19707	TGAGGTTGGTAACTGTTCTGGTTCTTCTATGGGTGGTGTTTCTGCCTTACG	Introduction G1250S mutation in *FAS2*
19708	AACACCACCCATAGAAGAACCAGAACAGTTACCAACCTCAGAAACATGTACG	
19710	TCAACTTGTTTACCAGCTGGC	Amplification of *FAS1* in two fragments
19742	CCACCCATACCAGAACCAGAACAGTTACCAACCTCAGAAACATGTACG	Amplification of *FAS2* in two fragments
19743	GGTTGGTAACTGTTCTGGTTCTGGTATGGGTGGTGTTTCTGCCTTACG	Amplification of *FAS2* in two fragments
Sequencing of *FAS1* and *FAS2*		
19709	GCCGATGCTACTTTTGCTGG	Sequencing *FAS1* genes
19749	CTTCCAATGATGGGACACCAGC	
19750	CTTGACCATTGGTGCTGGTG	
19751	GCCTTTTGTTCCAGTTTTGG	
19752	CTCGAATCTACGATCCAAAACTGG	
19753	CTTCAATAAGTCACCATCGACCG	
19754	GATGGTGACTTATTGAAGTTGG	
19711	GGCTTTGAGATTTGACCGTTTGG	Sequencing *FAS2* genes
19712	GCTGGAGTTGGAGCAGGAG	
19755	CTAAAAATGGACTTGGATAACGG	
19756	GCTTGAAGTTCAGCAACAGTGTCC	
19757	CGTCTTGATCACTGGTGCTG	
19758	GCACCAATAGAACCCTTACCAGC	
19759	GGCAATAGCGCTGATGCTGC	
19760	GGGAAGTCCAGTTGAATGTTAGC	
19761	GGCTAATGACAAGAACGAATCTGCC	
19762	GGGATTACCTTCAGATCTACCC	
***ScEEB1* cloning**		
19949	AAAGCATCGTCTCATCGGTCTCATATGTTTCGCTCGGGTTACTATC	Amplification *ScEEB1* from CEN.PK113-7D genomic DNA and cloning as YTK type 3 part
19950	TTTATGCCGTCTCAGGTCTCAGGATTTATAAAACTAACTCATCAAAGCTGCC	
10320	CATGCGCGGATGACACGAAC	Diagnostic primers for pUD1325 verification
10325	AGTCATCCGAGCGTGTATTG	
Yeast genomic integration		
19744	AAAAGATGAAACCGAGTAAGCTGCTACATAATGTCTATATATCTACACATAAAATTCCGACATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCC	Adds complimentary flanks for *Sc*YCR087C site
19745	TAAGAGTATTCTGTATACAACAGCAAACGGTCTCAGTCAAGAAATATTTGTTATTACAGGGGCCGCAAATTAAAGCCTTCGAG	
18534	GAATACCTCTTCGAAACGTTGAG	Diagnostic PCR for *Sc*YCR087C integrations
18535	ATGAGTGGACTGGCAGC	
19763	TTCTATAGATGTACGTAAAGTTCTGCTCTTCTTTTATAGATAAGAGGATTGAGACTCGCCCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCC	Adds complimentary flanks for *Se*YCL049C site
19764	AGCTCAAGAAAAACCAGGCTATTCGCAGAACAGGATAACCAGCTCTGTAACCACATCAATGGCCGCAAATTAAAGCCTTCGAG	
18514	TCAGTTAGAGTGACAGTTGC	Diagnostic PCR for *Sc*YCR087C integrations
18515	GTTGTGCGTTTTACGTGC	
18517	AGATGCGGCAGCCAGTGAGGGCGTTGGGCATGATCGAAAGCCAAGACCCACCAATTCGAGACTGGCCGATAATTGCAGACG	Adds complimentary flanks for *Se*YCL036W site
18518	ATATACATATACGTACGTATGTATCTACAGAAAGAAAAAAAAAAAGATCAATGAATATATCATGATGAGCCGTGATGACCC	
18519	TCCGTCAGTATTCGAGGC	Diagnostic PCR for *Se*YCL036W integrations
18520	TCTTGGAACCTATCCTGGC	

primer pair 19744 & 19745, which incorporate compatible flanks for the *Sc*YCR087C integration site (Bennis et al., 2023), and pUD1295, pUD1296, pUD1297 and pUD1298 as template, respectively. The *ScFAS2*, and *ScFAS2*^{G1250S} repair fragments were obtained by PCR amplification using primer pair 19763 & 19764, which incorporate compatible flanks with the *Se*YCL049C site (Bennis et al., 2023), and pUD1299 and pUD1300 as template, respectively. Single *ScFAS* gene integrations were performed by co-transforming the respective *ScFAS1* repair fragment together with pUDP273 encoding the gRNA targeting *Sc*YCR087C, or the respective *FAS2* repair fragment together with pUDP269 encoding the gRNA targeting *Se*YCL049C into CBS 1483, resulting in IMI541 (*ScFAS1*), IMI542 (*ScFAS1*^{I306A}), IMI543

(*ScFAS1*^{R1834K}), IMI544 (*ScFAS1*^{I306A,R1834K}), IMI545 (*ScFAS2*) and IMI546 (*ScFAS2*^{G1250S}). To construct strains with both, *ScFAS1* and *ScFAS2* genes, the strains IMI545 (*ScFAS2*) and IMI546 (*ScFAS2*^{G1250S}) were co-transformed with pUDP273 and a respective *ScFAS1* repair fragment, resulting in the strains IMI547 (*ScFAS1 ScFAS2*), IMI548 (*ScFAS1*^{I306A} *ScFAS2*), IMI549 (*ScFAS1*^{R1834K} *ScFAS2*), IMI550 (*ScFAS1*^{I306A,R1834K} *ScFAS2*), IMI551 (*ScFAS1 ScFAS2*^{G1250S}), IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}), IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}).

The expression cassette for overexpression of *ScEEB1* was amplified from pUD1325 using primers 18517 & 18518 adding compatible flanks for *Se*YCL036W for genomic integrations. The PCR amplified fragment was co-transformed with pUDP270 (Bennis et al., 2023) into IMI554 and CBS 1483, resulting in strains IMI577 and IMI578, respectively.

Yeast genomic DNA for diagnostic purposes was isolated using the lithium acetate-SDS method (Looke et al., 2011). Gene integrations in *Se*YLC049C were confirmed with primers 18514 & 18515, *Se*YCL036W with 18519 & 18520, and *Sc*YCR087C with 18534 & 18535 (Bennis et al., 2023).

2.5. Whole genome sequencing

Yeast genomic DNA of IMI551, IMI552, IMI553, IMI554 and IMI577 was isolated using QIAGEN Genomic-tip 100/G kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Genomic DNA concentrations were measured with the BR ds DNA kit (Invitrogen, Carlsbad, CA) using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The DNA quality was verified on the TapeStation System 4150 (Agilent). In-house nanopore sequencing was performed for strain IMI551, IMI552, IMI553 and IMI554 using a MinION sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom) on a R10.4 flow cell (ONT). The sequencing library for MinION sequencing was prepared using the native barcoding sequencing kit SQK-NBD114.24 (ONT), allowing multiplexing of the four samples on one flow cell. Data acquisition from the MinION sequencer was performed using the MINKNOW software (ONT, version 23.04.6). Guppy (ONT, version 6.5.7GPU) was used for basecalling. Resulting.fastq files were filtered on length (>1 kb) and mapped to a CBS 1483 derived reference strain with *in silico* added *FAS1*, *FAS2* (mutants) and *ScEEB1* sequences using Minimap2 (Bracher et al., 2018).

Illumina sequencing of strains IMI551, IMI552, IMI553, IMI554 and IMI577 was performed on a NovaSeqX 10B system (Macrogen-Europe, The Netherlands). The libraries were prepared using a PCR-free TruSeq kit (350 bp insert size and 150 bp paired end). The sequencing reads were mapped to the CBS 1483 with *FAS1*, *FAS2* (mutants) and *ScEEB1 in silico* constructed reference genome using the Burrows–Wheeler Alignment (BWA) tool (version 0.7.15) (Li and Durbin, 2009) and further processed using SAMtools (version 1.3.1) (Li et al., 2009) (Table S1). Chromosomal copy number was estimated by the Magnolya algorithm (Nijkamp et al., 2012) (Table S2). The sequences analysed by visualizing the.bam files in the Integrative Genomics Viewer (IGV) software (version 2.8.9) (Thorvaldsdóttir et al., 2013). All sequencing data are available at NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject accession number PRJNA1087702 (To access the data use the following reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1087702?reviewer=no3vdmj443ql2kgg9g0nha3lvj).

2.6. Fermentation in septum bottles

Frozen aliquots of CBS 1483, IMI541-IMI554, IMI577 and IMI578 were inoculated in 20 mL of YPD media in 50 mL Greiner tubes with air vents at 20 °C shaking at 200 rpm. Wake-up cultures were transferred to precultures in 100 mL YPD in 500 mL shake flasks grown at 20 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 100 mL bottles sealed with rubber stopper septum containing 60 mL full malt wort at 5.7 °P for strains CBS 1483, IMI541-IMI554, IMI577 and IMI578 at a starting OD_{660} of 0.5 mL^{-1}. The cultures were incubated at 20 °C and at 200 rpm for five days with regular daily sampling to determine cell density, sugar consumption, ethanol production, esters and ketones concentrations throughout the fermentation.

2.7. Fermentation in laboratory scale mini E.B.C. tubes

Frozen aliquots of CBS 1483, IMI541-IMI554, IMI577 and IMI578 were inoculated in 20 mL of YPD media in 50 mL Greiner tubes with air vents at 12 °C shaking at 200 rpm. Wake up cultures were transferred to precultures in 100 mL YP Maltose (YPM) (6%) in 500 mL shake flasks grown at 12 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 250 mL wort (17 °P) to obtain a starting cell density of approximately 5×10^6 cells mL^{-1}. Then, 80 mL of the prepared inoculum was transferred into laboratory scale tubes with a diameter/length ratio akin to tall E.B.C. tubes (Enari, 1977) in duplicate. The laboratory scale E.B.C tubes were equipped with Ankom system gas monitors for online CO_2 measurements (Ankom, Macedon NY), which were set to release pressure at 100 mbar and recording interval to 30 s. The fermentations were performed at 12 °C in a stationary incubator. After 14 days the fermentation was stopped and the supernatant was collected for analytical measurements including sugar, ethanol, ester and ketone concentrations and fatty acid extractions.

2.8. Fermentation in tall E.B.C. tubes

Frozen aliquots of CBS 1483, IMI552-IMI554 were inoculated in 100 mL of YPD media in 500 mL shake flasks at 12 °C shaking at 200 rpm. Wake-up cultures were transferred to precultures in four times 100 mL YPM (6%) in 500 mL shake flasks grown at 12 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 5 L wort (17 °P) to obtain a starting cell density of approximately 3.5×10^6 cells mL^{-1}. Then, 2.25 L of the prepared inoculum was transferred into the tall tubes in duplicate. The tall tubes were equipped with Bronkhorst EL-FLOW® Prestige mass flow meters (Bronkhorst, The Netherlands) for online CO_2 measurements. The fermentations were performed at 12 °C using cryostats for cooling. Samples for cell counts, °Plato, pH, sugar, ethanol and flavour compounds concentration determinations were taken daily. Cell count and viability were determined using a NucleoCounter YC-100TM (Chemometec A/S, Allerod, Denmark) according to the manufacturer's recommendations. Wort density was measured using an Anton Paar DMA35 portable density meter according to the manufacturer's recommendations. After 17 days the fermentation was stopped and the beer was collected by filtering the fermentation broth using Nalgene™ Rapid-Flow™ bottle top (45 mm, 0.2 μm) (Thermo Fisher Scientific). The beer was diluted to 5% ABV using carbonated water and stored in brown-glass beer bottles at 4 °C.

2.9. Fatty acid extraction

Fatty acids in supernatant were analysed as methyl-ester derivatives by gas chromatography with flame-ionization detection (GC-FID). Supernatant was aliquoted in 10 mL portions in 15 mL Greiner tubes and to each aliquot 100 μL of internal standard solution (30 mg heptanoic acid in 15 mL hexane) was added. For acidification, 1 mL 1 M HCl was added, followed by addition of 2.5 mL of a mixture of equal amounts (v/v) of methanol and chloroform and 5 min vigorously shaking of the sample. The sample was spun down at 2000 rcf for 10 min and the chloroform layer was transferred to a glass trans-methylation tube with a glass Pasteur pipet. The chloroform was evaporated under nitrogen gas flow. For methylation, 1.5 mL methanol, 0.3 mL HCL (8% w/v solution made from 9.7 mL of concentrated HCl and 41.5 mL of methanol) and 1 mL hexane were added in the respective order. The mixture was vortexed for 1 min and heated to 80 °C for 1 h with vortexing the tubes briefly every 15 min. Thereafter, the samples were cooled on ice and 1 mL water

was added and vortexed. To separate the organic and aqueous phase, the sample was spun down for 5 min at 1900 rcf. The organic layer was transferred into a GC vial using a Pasteur pipet and loaded onto the GC.

2.10. Analytical measurements

Sugars (glucose, fructose, maltose, maltotriose) and ethanol concentrations were measured using HPLC (Agilent Technologies, 1260 HPLC system) equipped with a Bio-Rad HPX-42A column (300 × 7.8 mm, 25 μm) (Bio-Rad) and an 1260 Refractive Index Detector (RID). The column was operated at 75 °C, and the elution was performed using milliQ at 0.5 mL min^{-1} for 30 min. Compounds were measured using a RID at 35 °C. Samples were 0.2 μm filter-sterilized before analysis.

Vicinal diketone (diacetyl, 2,3-pentadione) concentrations were measured using static headspace gas chromatography in a 7890A Agilent GC (Agilent) with an electron capture detector on a CP-Sil 8 CB (50 m × 530 μm x 1 μm) capillary column. 0.5 mL of each sample was heated up to 65 °C for 30 min prior to injection. A split flow of 8 mL N_2 min^{-1} was used, with a split ratio of 1:1. The injector temperature was set at 120 °C. The oven was set at 35 °C for 3 min, followed by a 10 °C min^{-1} ramp to 95 °C. The ECD temperature was set at 150 °C with a make-up flow of 30 ml min^{-1} nitrogen.

Higher alcohols and esters were analysed using static headspace gas chromatography (GC) with a flame ionization detector FID (Agilent technologies 7890A) and a DB-WAXetr capillary column (30 m × 320 μm x 1 μm). 2.5 mL samples were heated up to 50 °C for 5 min using a CTC Combi Pal headspace auto-injector prior to the injection. A split flow of 9.88 mL N_2 min^{-1} was used, with a split ratio of 5:8:1. The temperature of the injector was 250 °C, with a flow of 10 mL N_2 min^{-1}. The oven temperature profile started at 55 °C followed by an increase of 20 °C min^{-1} to 160 °C with a hold time of 4.75 min. The FID temperature was set at 250 °C with a make-up flow of 10 ml min^{-1} nitrogen.

Fatty acid methyl esters were analysed on an Agilent Technologies 7890A GCFID system equipped with an FID-1000–220 Gas Station (Parker Balston, Haverhill, MA) and an Agilent Technologies 7693 Autosampler. A VF-5 ms column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness, Agilent part no. CP9013) was used for separation, and hydrogen was used as carrier gas at a constant flow of 25 mL min^{-1} 1 μL sample was injected at a split ratio of 10:1. The oven temperature was initially set at 50 °C and held for 4 min and then increased to 180 °C at a rate of 10 °C min^{-1}. Inlet temperature was set at 150 °C, and FID temperature at 330 °C. The Supelco FAME mix #7 C6-C10 (VWR International B.V, Amsterdam, The Netherlands) was used to calibrate the GC-FID system for quantification of individual fatty acid methyl esters. Data was corrected using the internal standard concentrations.

2.11. Sensory analysis

The institutional Human Research Ethics Committee (HREC) approved the tasting session under ID3948. Sensory analysis of the brewed beer was conducted internally by a tasting panel of 14 participants who received prior sensory training. All participants consented verbally after being informed about the study's details. An attribute list to described odour, taste, flavour and mouthfeel was composed. The attribute list served to evaluate the sensory difference of the samples, beers produced with strains IMI552, IMI553, IMS554 compared to the control beer produced by the strain CBS 1483. The participants could identify different attributes between a sample and the control and score the difference with + or – with plus denoting a higher attribute intensity and minus a lower attribute intensity relative to the control. For the evaluation, 25 ml of each sample was served in beer glasses. The samples were anonymized and served at 4 °C and examined at room temperature. The sensory data were collected manually. The tasting session was not replicated.

2.12. Data analysis and statistics

GraphPad Prism software (GraphPad Software version 10.3) was used to perform statistical analysis and data visualization. Data were analysed by one-way (bar plots) or two-way (time-course) ANOVA, followed by Tukey's multiple comparisons tests. Significance is indicated as * $p < 0.01$ and **$p < 0.0001$, unless indicated otherwise).

3. Results

3.1. Single FAS mutant overexpression resulted in specific ethyl ester production profiles

To study how mutations in the FAS complex affect ethyl ester level in lager brewing yeast fermentation, mutant alleles of Sc*FAS1* and *ScFAS2* were expressed alongside their non-mutated counterparts in *S. pastorianus* CBS 1483. For this, alleles encoding non-synonymous mutations I306A and R1384K in *Sc*Fas1 and G1250S in *Sc*Fas2 were introduced in landing sites *Sc*YCR087C and *Se*YCL049C located on CHRIII which counts four copies, respectively using CRISPR-Cas9 genome editing (Bennis et al., 2023) (Fig. 1B, Fig. S1). As such a strain set including IMI542 (*ΔSc*YCR087C::$ScFAS1^{I306A}$), IMI543 (*ΔSc*YCR087C::$ScFAS1^{R1834K}$), IMI544 (ΔScYCR087C::$ScFAS1^{I306A,R1834K}$), IMI546 (*ΔSc*YCR049C::$ScFAS2^{G1250S}$) was constructed. Control strains carrying an extra *ScFAS1* or *ScFAS2* integration IMI541 (*ΔSc*YCR087C::*ScFAS1*) and IMI545 (*ΔSc*YCR049C::*ScFAS2*) were also constructed. To prevent imbalanced in expression of the these extra *ScFAS1* and *ScFAS2* alleles, they were cloned under the control of the same regulatory sequences the Sc*TEF1* promoter and Sc*CYC1* terminator (Table 2, Supplementary information Appendix A & B).

The strains IMI541-546 were evaluated for ethyl esters production using 5.7 °P full malt wort at 20 °C. The strains were grown in bottles sealed with rubber stoppers to maintain anaerobic conditions characteristic of brewing fermentation, but it is also known that ethyl ester synthesis is notably low in this cultivation format probably caused by the building CO_2 concentration. To illustrate this, the reference *S. pastorianus* strain CBS 1483 neither produced detectable ethyl hexanoate nor ethyl octanoate under these conditions. Overexpression of wild type *ScFAS2* (IMI545) displayed a similar phenotype (Fig. 2, Fig. S2) while overexpression of *ScFAS1* (IMI541) resulted in 0.07 ± 0.00 mg L^{-1} of ethyl octanoate (Furukawa et al., 2003) (Fig. 2B and C, Fig. S2). Conversely, expression of both mutant alleles resulted in significant increase of ethyl ethers (Fig. 2B and C, Fig. S2). The expression of the $ScFAS1^{I306A}$ mutation (IMI542), located in the AT domain causing elevated uptake of acetyl moiety forcing the start of a new fatty acid synthesis cycle, specifically increased ethyl hexanoate production to reach 0.10 ± 0.00 mg L^{-1} ($p < 0.0001$), while expression of $ScFAS1^{R1834K}$ (IMI543), located in the MPT domain and lowering the affinity for malonyl moiety, increased ethyl octanoate production up to 0.15 ± 0.00 mg L^{-1} ($p < 0.0001$) (Fig. 2B and C, Fig. S2). The combination of these mutations led to production of both ethyl hexanoate and ethyl octanoate. The strain IMI544 ($FAS1^{I306A,R1834K}$) produced 0.09 ± 0.00 mg L^{-1} ($p < 0.0001$) of ethyl hexanoate and 0.095 ± 0.01 mg L^{-1} of ethyl octanoate ($p < 0.0001$) demonstrating that the expression of the double mutation in *FAS1*was additive as in contrast to single mutant FAS^{I306A} *and* $FAS1^{R1834K}$ that led to increase in ethyl hexanoate and ethyl octanoate respectively, the double mutant of *FAS1* ($FAS1^{I306A,R1834K}$) combined these improvements.The expression of the $ScFAS2^{G1250S}$ mutation (IMI546), preventing short chain intermediates from entering the KS domain, enhanced both ethyl hexanoate (0.085 mg L^{-1}, $p < 0.0001$) and ethyl octanoate (0.10 mg L^{-1}, $p < 0.0001$) production (Fig. 2B and C, Fig. S2). These mutations enhanced ethyl ester concentrations without impacting growth behaviour (Fig. 2A, Fig. S3), aligning with expected changes in fatty acid levels (Gajewski et al., 2017b) and offering potential for tailored ethyl ester profiles.

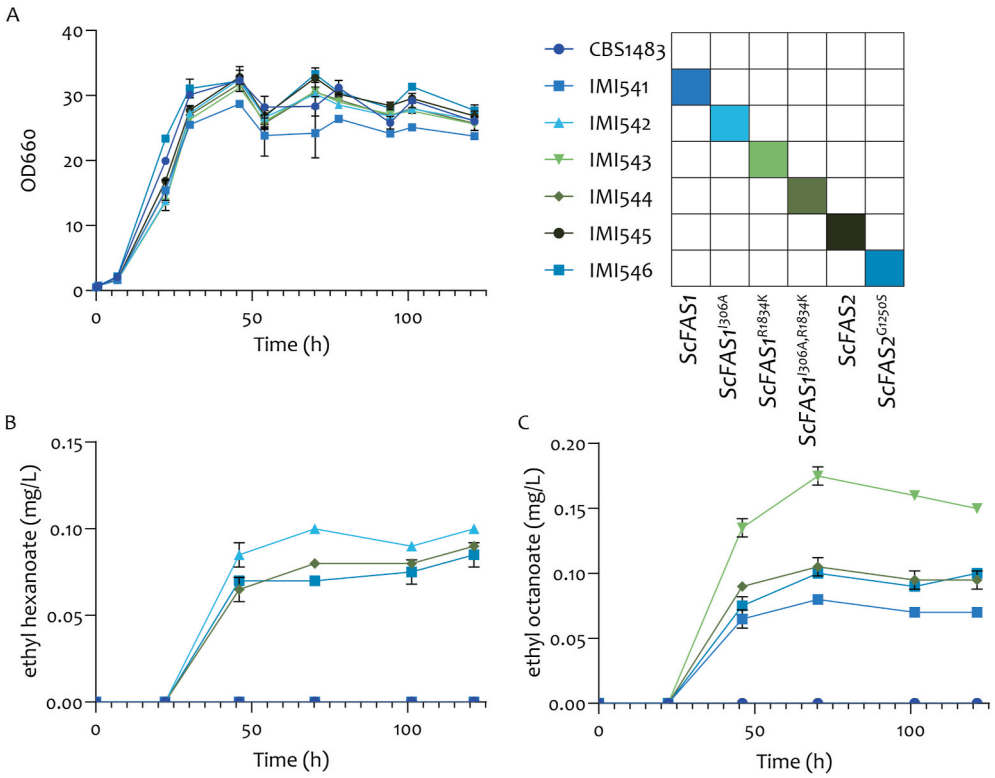

Fig. 2. Evaluation of single *ScFAS1* and *ScFAS2* mutants in CBS 1483 on ethyl ester biosynthesis. Growth and ethyl ester profiles of septum flasks fermentation (5.7 °P, 20 °C, 200 rpm) with engineered strains IMI541-IMI546 and parental strain CBS 1483 were monitored during fermentation septum flasks on 5.7 °P wort and cultivated at 20 °C shaking at 200 rpm. A) Growth, B) ethyl hexanoate and C) ethyl octanoate profiles were measured.

3.2. Combinations of ScFAS1 and ScFAS2 mutations resulted in a synergistic increase in ethyl ester production

To investigate the combined effects of mutations in the FAS complex on ethyl ester production, strains IMI545 (Δ*Se*YCL049C::*ScFAS2*) and IMI546 (Δ*Se*YCL049C::*ScFAS2*^{G1250S}) were used as host strains for integrating the Sc*FAS1* variants at the *Sc*YCR087C genomic location (Figs. 1B and 3, Fig. S1). Similar to single overexpression of native *ScFAS1*, overexpression of both native *ScFAS1* and *ScFAS2* genes (IMI547(Δ*Se*YCL049C::Sc*FAS2* ΔScYCR087C::*ScFAS1*)) resulted in the production of ethyl octanoate, confirming the contribution of Fas1 to increased production of C8 fatty acid and its ethyl ester derivative (Fig. 3C, Fig. S2). Overexpression of Sc*FAS1*^{I306A} and/or Sc*FAS1*^{R1834K} combined with overexpression of the Sc*FAS2* (IMI548, 549 and 550) resulted in similar ethyl ester production profiles compared to strains without Sc*FAS2* overexpression (IMI542, 543 and 544) (Fig. 3B and C, Fig. S2). The combination of Sc*FAS1*^{I306A} (IMI548) or Sc*FAS1*^{R1834K} (IMI549) with *ScFAS2* resulted in 20% ethyl hexanoate and in 33% ethyl octanoate increase respectively relative to the single *ScFAS1* mutants IMI542 and IMI543. Overexpression of Sc*FAS1*^{I306A,R1834K} together with *ScFAS2* (IMI550) resulted in similar production profiles (0.09 ± 0.00 mg L^{-1} ethyl hexanoate and 0.11 ± 0.00 mg L^{-1} ethyl octanoate, $p > 0.01$) as single *ScFAS1*^{I306A,R1834K} overexpression (Fig. 3B and C, Fig. S2).

Combining the *ScFAS1* variants with overexpression of *ScFAS2*^{G1250S} resulted in a systematic increase in the total ethyl ester production demonstrating the relevant role of the *ScFAS2*^{G1250S} mutation. This synergistic effect was obvious when comparing the performance of IMI542 (*ScFAS1*^{I306A}) and IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}). IMI552 exhibited a 3.4-fold significant higher ethyl hexanoate concentration. Additionally, IMI552 was also able to produce ethyl octanoate concentration up to 0.13 ± 0.00 mg L^{-1} (Fig. 3C, Fig. S2). Similarly, the combination of Sc*FAS2*^{G1250S} and Sc*FAS1*^{R1834K} (IMI553) resulted in the highest concentration of ethyl octanoate (0.33 ± 0.00 mg L^{-1}, $p < 0.0001$ compared to IMI543 and IMI546), and high concentration of ethyl hexanoate (0.18 ± 0.01 mg L^{-1}). Combining all three mutations (IMI554, Sc*FAS1*^{I306A,R1834K} Sc*FAS2*^{G1250S}) did not further increase ethyl ester synthesis but still resulted in second highest final concentrations of the ethyl esters (0.30 ± 0.01 mg L^{-1} of ethyl hexanoate and 0.25 ± 0.00 mg L^{-1} of ethyl octanoate, $p < 0.0001$ relative to IMI544 and IMI546) (Fig. 3B and C, Fig. S2). Growth and sugar metabolism remained similar to the control strain (Fig. 3A, Fig. S4). These results demonstrated that synergistically, the combination of these mutations have a direct effect on the ethyl ester production and can produce a broad range of flavour profiles.

3.3. Characterization of ethyl ester producing strains in laboratory scale mini E.B.C. tubes

In the exploration of flavour molecule production during fermentation, the design of brewing reactors holds paramount importance. To emulate brewing conditions on a laboratory scale, mini tubes with a diameter/length ratio akin to E.B.C. tubes (Enari, 1977) were employed for lab-scale fermentations. These tubes were inoculated with engineered strains IMI541 to IMI554 alongside the parental strain CBS 1483 in 17 °P wort and statically incubated at 12 °C while meticulously monitoring the cumulative pressure. Following a 14-day incubation period, the supernatant of these mini tubes was collected and subjected to analysis. Markedly, strains IMI551-IMI554, expressing the *ScFAS2*^{G1250S} allele combined with the native *ScFAS1*, exhibited remarkable performance (Fig. 4). Ethyl ester concentrations were more than quadrupled for ethyl hexanoate and doubled for ethyl octanoate and ethyl decanoate (Fig. 4A, B and 4C) relative to CBS 1483. Ethyl hexanoate concentration increased to 1.94 ± 0.07 mg L^{-1} in strain IMI552, specialized for C_6 acyl chain ester production, compared to 0.36 ± 0.03 mg L^{-1} for the parental strain CBS 1483. Similarly, strain IMI553,

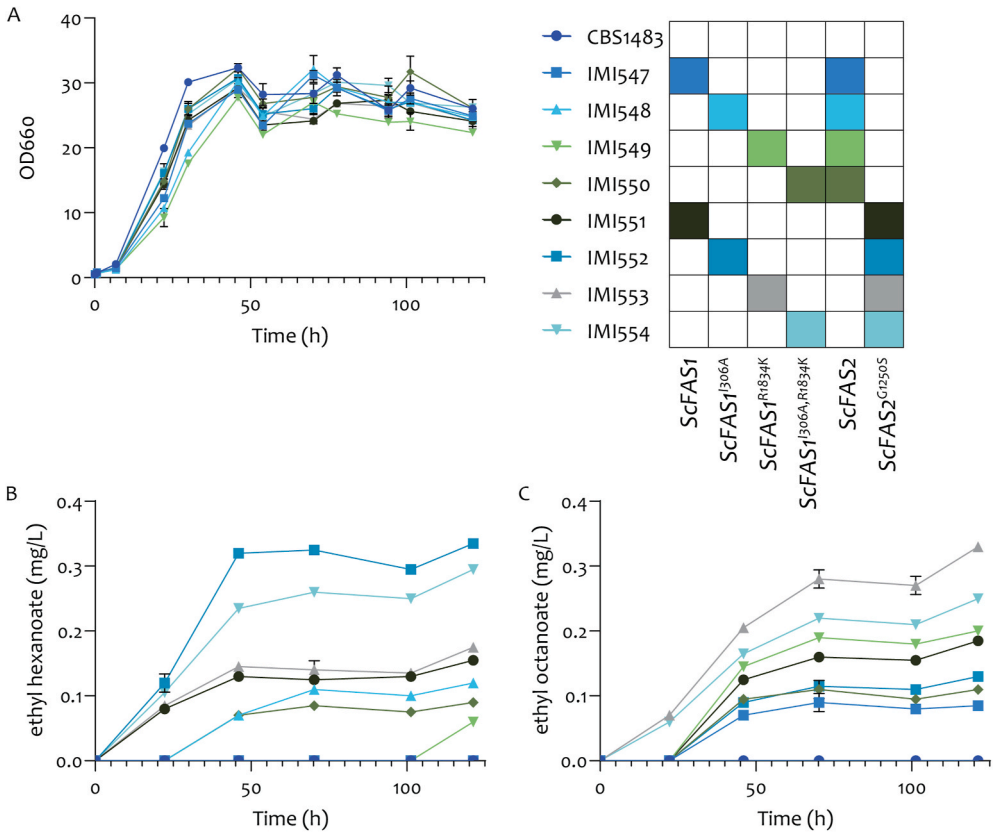

Fig. 3. Evaluation of the combinatorial effect of *ScFAS1* and *ScFAS2* mutants in CBS 1483 on ethyl ester biosynthesis.
Growth and ethyl ester profiles of septum flasks fermentation (5.7 °P, 20 °C, 200 rpm) with engineered strains IMI547-IMI554 expressing combinations of *ScFAS1* and *ScFAS2* mutant genes and the parental strain CBS 1483 were monitored during fermentation septum flasks on 5.7 °P wort and cultivated at 20 °C shaking at 200 rpm. A) Growth, B) ethyl hexanoate and C) ethyl octanoate profiles were measured.

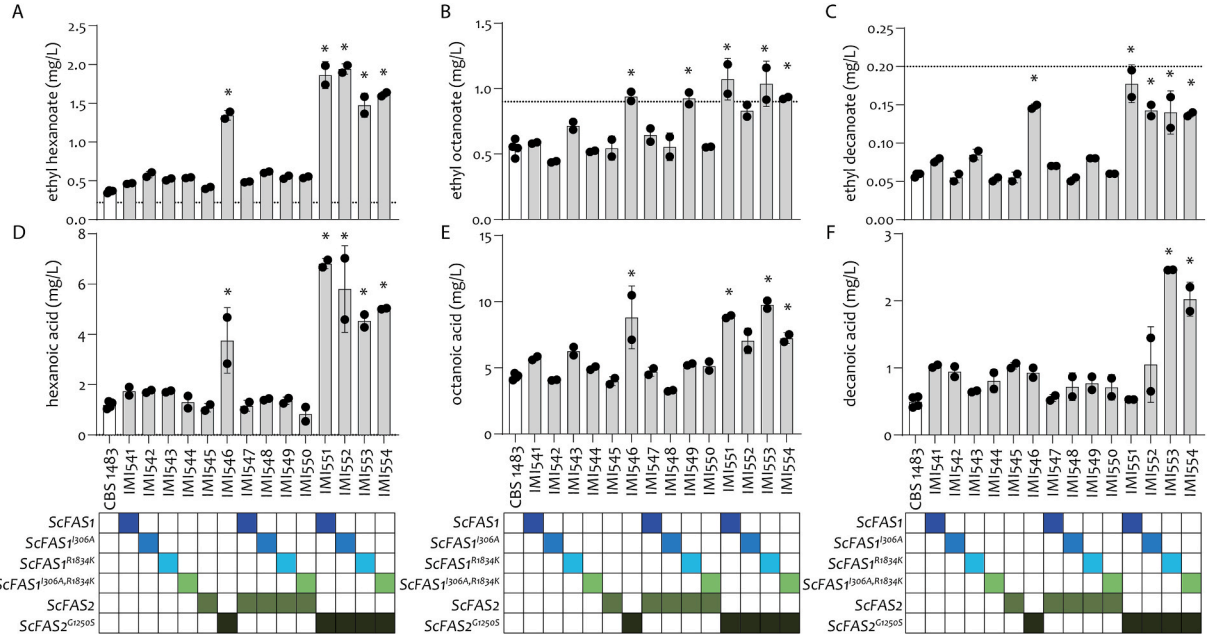

Fig. 4. Ethyl ester and fatty acid production of *ScFAS1* and *ScFAS2* engineered strains during lab scale E.B.C. tube fermentations. Ethyl esters (A,B,C) and fatty acid (D,E,F) measurements of stationary laboratory scale E.B.C. tube fermentations (17 °P, 12 °C) with engineered strains IMI541-IMI554 and parental strain CBS 1483. The dashed line indicated the human sensory threshold levels for the respective ethyl esters. (*p < 0.005 compared to CBS 1483).

tailored for C_8 acyl chain ester synthesis, yielded 1.03 ± 0.18 mg L^{-1} ethyl octanoate versus 0.50 ± 0.06 mg L^{-1} for CBS 1483. Strain IMI554, encompassing all three examined *ScFAS* mutations (*ScFAS1*^{I306A,R1834K} and *ScFAS2*^{G1250S}), produced 1.62 ± 0.04 mg L^{-1} and 0.93 ± 0.01 mg L^{-1} ethyl hexanoate and ethyl octanoate, respectively (Fig. 4A, B and 4C). While ethyl decanoate concentration fell below detection limit during septum flask cultivation, mini tube fermentations led to a significant rise in ethyl decanoate concentration for the engineered strains compared to the parental strain CBS 1483 (Fig. 4A, B and 4C).

Furthermore, an investigation into whether fatty acid intermediates accumulate in the engineered strains as a consequence of the incorporated mutations was conducted. Particularly, strains IMI551-IMI554,

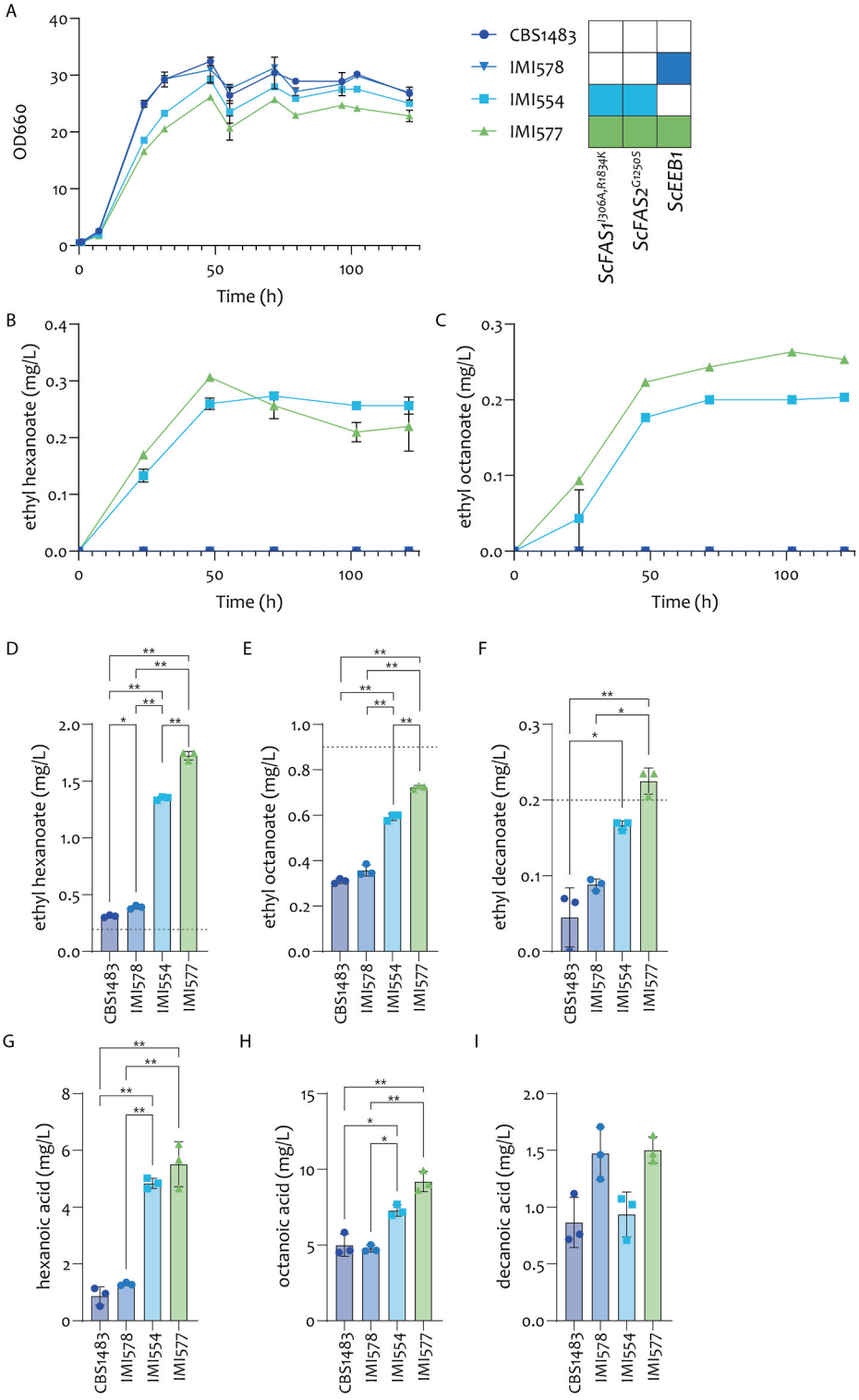

Fig. 5. Overexpression of *ScEEB1* increases ethyl ester concentrations in *FAS* mutant strains, but does not affect the fatty acid concentrations. A) Growth, B) ethyl hexanoate and C) ethyl octanoate concentrations of septum flasks fermentation (5.7 °P, 20 °C, 200 rpm) with engineered strains IMI578, IMI554 and IMI577 and parental strain CBS 1483. The strains were also cultivated in stationary lab scale E.B.C. tubes (17 °P, 12 °C). After 14 days of cultivation, the ethyl esters (D,E,F) and respective fatty acid (G,H,I) concentrations were measured (*$p < 0.01$, **$p < 0.0001$).

containing *ScFAS2*^{G1250S} and one of the *ScFAS1* variants, exhibited a significant increase in hexanoic acid excretion, paralleling the fold-change observed in the ethyl ester derivative (Fig. 4D, E and 4F). Overall, the ethyl ester concentration exhibited a correlation with the fatty acid concentration in the supernatant, indicating equilibrium in the reaction.

3.4. Enhanced ethyl ester production via combined expression of ScFAS mutants and acyl-coenzymeA:ethanol O-acyltransferase esterase ScEEB1

The extracellular concentrations of fatty acids clearly increased upon expression of combinations of *ScFAS1* and *ScFAS2* mutations (Fig. 4) suggesting that in these strains, the limitation shifted from MCFA supply by the FAS complex to the esterification reaction catalysed by the AEAT enzymes encoded by *EHT1* and *EEB1*. We hypothesized that with the increased production of acyl-CoA intermediates caused by *ScFAS1* and *ScFAS2* mutants expression, overexpressing *ScEEB1* could divert the flux from the elevated levels of acyl-CoA intermediates to ethyl esters and potentially lower extracellular fatty acid concentrations. *S. pastorianus* CBS 1483 genome harbours two *EEB1* alleles one from *S. cerevisiae* and another from *S. eubayanus* subgenomes. These two alleles shared an identity of 77% at DNA and 83% at amino acid level. Thus, to test this hypothesis, the *ScEEB1* gene was integrated into CBS 1483 and IMI554 (*ScFAS1*$^{I306A,\ R1834K}$ *ScFAS2*^{G1250S}) at the SeYCL036W genomic location (Bennis et al., 2023), resulting in strains IMI577 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S} *ScEEB1*) and IMI578 (*ScEEB1*). Solely overexpressing the main esterase encoded by *ScEEB1* (IMI578) did not notably enhance ethyl ester production in septum flask cultivation (5.7 °P full malt wort at 20 °C) (Fig. 5A, B and 5C, Fig. S5). However, in combination with *ScFAS1*^{I306A,R1834K} and *ScFAS2*^{G1250S} genes with *ScEEB1* resulted in a significant increase in ethyl octanoate production (0.20 ± 0.01 mg L^{-1} for IMI554 versus 0.25 ± 0.01 mg L^{-1} for IMI577, $p < 0.01$), while the ethyl hexanoate concentration was not significantly affected under these conditions (Fig. 5B and C). In lab-scale E.B.C. mini tubes, IMI577 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S} *ScEEB1*) exhibited a 28%, 22% and 35% increase in ethyl hexanoate, ethyl octanoate and ethyl decanoate respectively relative to IMI554 (Fig. 5D, E and 5F), reaching levels above sensory thresholds for the C_6 and C_{10} esters. In the meantime, expression of *ScEEB1* was accompanied by an increase in C_6, C_8 and C_{10} carboxylic acids (Fig. 5G, H and 5I). These results suggest that despite having a positive impact on ethyl ester profiles, the expression of *ScEEB1* was not sufficient or not well balanced with the FAS activity to reduce fatty acid concentrations.

3.5. Towards large scale brewing fermentations: S. pastorianus strain assessment in Tall E.B.C. tubes

The cultivation standard to predict and evaluate the stability of brewing characteristics of a lager yeast strain is the E.B.C. tall tube (Enari, 1977), relatively high liquid column fermenter of approximately 2L with dimension ratio close to industrial fermentation equipment. Thus, to more accurately predict the suitability of the engineered strains for producing proper ethyl ester flavour profiles at larger scale, the strains IMI552(Sc*FAS1*^{I306A} *ScFAS2*^{G1250S}), IMI553 (Sc*FAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (Sc*FAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}) were cultivated on 17 °P full malt wort in 2.25 L E.B.C. tall tubes operating at 12 °C.

The ethyl ester profile was signifcantly enhanced relative to the parental strain CBS 1483. Specifically, IMI552 (Sc*FAS1*^{I306A} *ScFAS2*^{G1250S}) exhibited a significant increase in ethyl hexanoate production, yielding 2.92 ± 0.02 mg L^{-1} compared to 0.56 ± 0.01 mg L^{-1} produced by CBS 1483 ($p < 0.0001$) (Fig. 6A). Additionally, both IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S}) also showed significantly higher ethyl hexanoate production (1.88 ± 0.02 mg L^{-1} and 2.00 ± 0.01 mg L^{-1}, respectively, $p < 0.0001$) compared to CBS 1483 (Fig. 6A). IMI552 and IMI553 produced the largest amount of ethyl octanoate (1.12 ± 0.01 mg L^{-1} and 1.06 ± 0.01 mg L^{-1}), representing a 1.42- and 1.24-fold higher concentration than that of CBS 1483 (0.85 ± 0.00 mg L^{-1}) (Fig. 6B). Moreover, ethyl decanoate levels in the engineered strains were all significantly higher than in CBS 1483 (0.09 ± 0.00 mg L^{-1}, $p < 0.0001$). IMI553 achieved the highest ethyl decanoate concentrations (0.30 ± 0.01 mg L^{-1}),

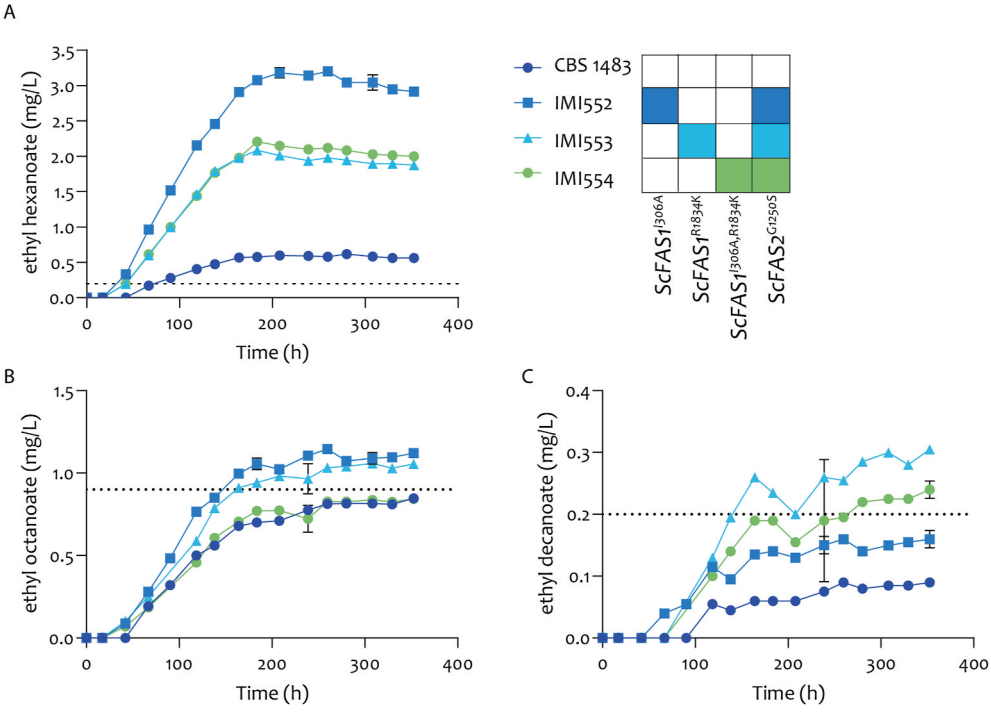

Fig. 6. Ethyl ester profiles of CBS 1483, IMI552, IMI553 and IMI554 cultivated in tall tube fermentations. A) Ethyl hexanoate, B) ethyl octanoate and C) ethyl decanoate profiles of CBS 1483, IMI552, IMI553 and IMI554 cultivated during 17 days in stationary tall tube fermentations (17 °P, 12 °C).

followed by IMI554 (0.24 ± 0.01 m L^{-1}) and IMI552 (0.16 ± 0.01 mg L^{-1}) (Fig. 6C). The engineering of the FAS complex did not alter sugar consumption and ethanol production profiles (Figs. S6 and S7). Similarly, profiles of other volatile components (e.g. diacetyl, 2,3-pentadione, isobutanol, isoamyl alcohol) remained unaffected (Figs. S7 and S8). This was contrasting with two phenotypes. i) Remarkably, throughout fermentation, the cell counts of strains expressing *ScFAS* mutant genes were lower relative to *S. pastorianus* CBS 1483 and was also accompanied by a lower cell viability near the end of fermentation. The viability of the engineered strains ranged from 89% to 93% when that of CBS 1483 never dropped below 96% (Fig. S5). ii) The level of acetate esters (ethyl acetate, ethyl butyrate, isoamyl acetate, and isobutyl acetate) were consistently lower in the engineered strains IMI552-IMI554 than in CBS 1483 (Fig. S8). In contrast, the concentration profiles of higher alcohols and vicinal diketones were not affected (Figs. S9 and S10).

3.6. Sensory analysis of beers produced by IMI552 (*ScFAS1*^{I306A} *ScFAS2*^{G1250S}), IMI553 (*ScFAS1*^{R1834K} *ScFAS2*^{G1250S}) and IMI554 (*ScFAS1*^{I306A,R1834K} *ScFAS2*^{G1250S})

At the end of E.B.C.-tall tube fermentations, the contents of the duplicate fermenters were mixed and subsequently subjected to filtration through a 0.2 μm filtration membrane. The resultant filtrate was then diluted to an alcohol by volume (ABV) concentration of 5% with carbonated water and bottled. Following a 14-day storage period at 4 °C, the four produced beers underwent sensory analysis conducted by a panel comprising 14 trained tasters. The three beers brewed with the engineered strains manifested distinguishable characteristics from the beer brewed with *S. pastorianus* CBS 1483. Notably, they showcased heightened aroma intensities attributed to the presence of ethyl esters. Specifically, notes reminiscent of pineapple and aniseed, indicative of ethyl hexanoate and ethyl octanoate, respectively, were more pronounced in beers fermented by IMI552, IMI53, and IMI554 than in the CBS 1483 beer (Fig. 7). Additionally, IMI552 exhibited a distinct red fruit note, an aroma determinant often associated with ethyl hexanoate. This observation correlated with the measured level of ethyl hexanoate in IMI552 fermentation (Fig. 6A). However, these positive sensory attributes were counterbalanced by flavours associated with MCFA. Flavours such as sweaty, carton-like, acidic, and acetic acid were detected in beers brewed with the engineered strains. Particularly the sweaty flavour identified in IMI553 and IMI554 is characteristic of hexanoic acid (Fig. 7), also known as caproate, with its etymological roots derived from "capra," Latin for goat, due to its resemblance to the scent of goat's milk, which is rich in this carboxylic acid.

Collectively, these findings underscore that elevated levels of ethyl esters resulting from yeast biosynthesis during fermentation contribute to pineapple and aniseed aromas in the finished beer. However, the presence of aroma determinants derived from MCFA also exert an influence on the overall taste profile.

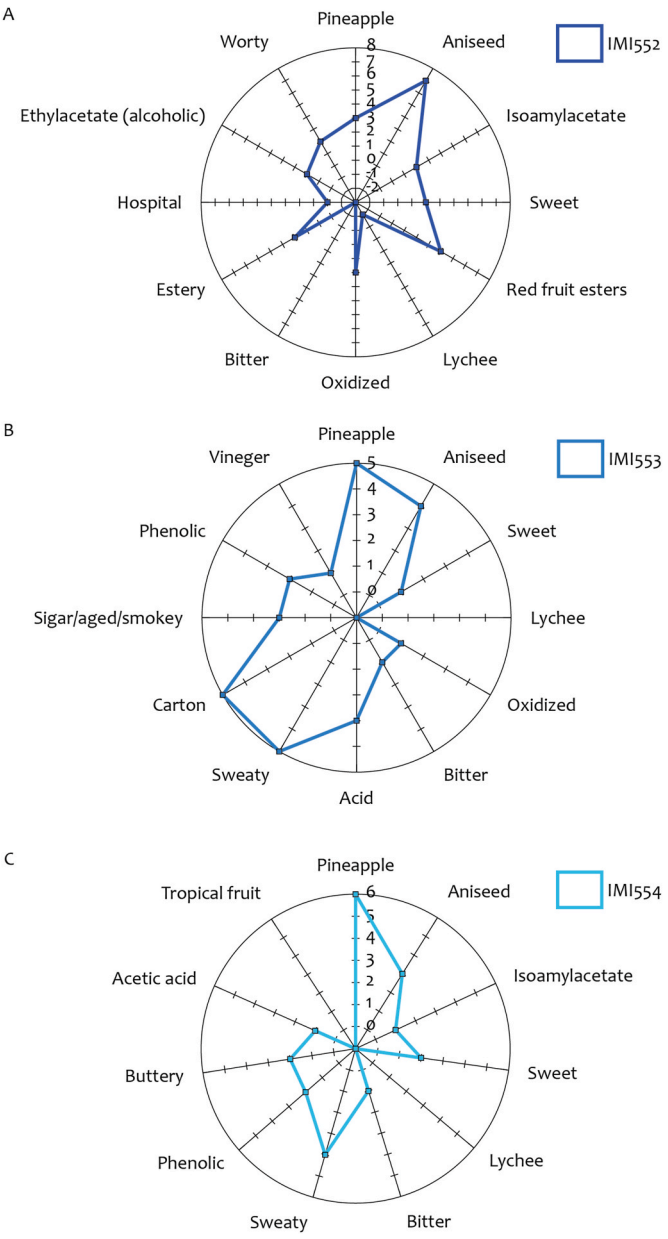

Fig. 7. Sensory evaluation of beers brewed with strains IMI552, IMI553 and IMI554 compared to the control strains CBS 1483. After 17 days of cultivation in stationary tall tube fermentations (17 °P, 12 °C), the fermentation broth was collected, the yeast was filtered out, the beer was normalized to 5% ABV (36.6 g L^{-1} ethanol) using carbonated water and stored in brown bottles at 4 °C. A panel consisting of fourteen trained accessors with a 'difference to control' analysis.

4. Discussion

The delicate balance of flavour components in beer profoundly influences its quality, as extreme concentrations of individual compounds or their synergistic interactions can detrimentally affect the overall flavour profile. Among these flavour-enhancing molecules, esters play a pivotal role despite their occurrence in relatively minute quantities (Verstrepen et al., 2003a,b). While the research focus on acetate esters has been more pronounced, ethyl esters are equally important in shaping the profile of flavoured fermentation products.

Various methods can be employed to adjust ethyl ester levels, including augmenting acetyl-CoA supply or employing 'metabolic pulling' strategies involving the overexpression of ethanol-acyltransferase alone or in combination with a thioesterase. Regardless of the medium-chain fatty acid (MCFA) species involved, these approaches consistently resulted in elevated ethyl ester levels but without exceeding HST levels. In our efforts to enhance ethyl ester production in *S. pastorianus*, we focused on increasing the supply of acyl-CoA precursors. While several strategies have been explored in *S. cerevisiae* to boost MCFA production (Fernandez-Moya and Da Silva, 2017; Wernig et al., 2020), we chose to modify the fatty acid synthase (FAS) complex by incorporating three distinct mutations, each enabling a unique mechanism to stimulate premature MCFA release. These genetic alterations not only led to an overall increase in ethyl esters, but also allowed for precise adjustment of specific ethyl ester profiles. Our findings are consistent with prior research indicating that overexpression of the native *ScFAS1* gene enhances C8-MCFA production (Furukawa et al., 2003). Specifically, mutations I306A in *ScFAS1* and G1250S in *ScFAS2*

were designed to enhance ethyl hexanoate production, with G1250S having the most significant effect. Conversely, the R1834K mutation in *ScFAS1* elevated ethyl octanoate production (Gajewski et al., 2017b). These distinct genetic modifications enable fine-tuning of ethyl hexanoate and ethyl octanoate levels, facilitating flavour customization according to user preferences.

These profiles are ultimately controlled by the ability of these esters to diffuse outside the cell. The diffusion rate over the cell membrane decreases proportionally as the chain length increases, ranging from 100% for ethyl hexanoate to 8–17% for ethyl decanoate, while even longer-chain fatty acid ethyl esters tend to remain within the cell (Nykänen et al., 1977; Suomalainen, 1981; Bardi et al., 1998, 1999; Saerens et al., 2008) which, is matching with our observations on reduced extracellular concentration. Ethyl-hexanoate levels were in most engineered strains higher than ethyl-octanoate levels which were higher than ethyl-decanoate, attributing to the reduced membrane transport.

While the endeavour to enhance the contribution of ethyl esters to the taste and aroma profile of beer was deemed successful, sensory analysis of the fermentation products, coupled with medium-chain fatty acid (MCFA) analysis, revealed a significant increase in fatty acid concentration (up to 4-fold) in the supernatant of the engineered strain fermentations (Fig. 4). This increase manifested in off-notes, including sweaty and cardboard-like flavours, characteristic of the presence of hexanoic, octanoic, and decanoic acids. Efforts to mitigate this side effect also known as "caprylic flavour" (Clapperton, 1978), by expressing an acyl-coenzymeA:ethanol O-acyltransferase esterase (*Sc*Eeb1) in strains harbouring variants of the FAS complex showed limited feat, as no significant reduction in fatty acid content of the beer was observed. Despite an increase in ethyl ester concentrations, the acyl-coenzymeA: ethanol O-acyltransferase esterase may still serve as the rate-limiting step for ethyl ester biosynthesis. In *S. cerevisiae ScEEB1* is not the only AEAT encoding gene, it harbours a paralog *ScEHT1* and another ethanol acetyltransferase encoded by *ScEAT1,* which alone or in combination could be tested to improve ethyl ester-MCFA ratio in the future.

Altering the aroma compound profile in beer necessitates maintaining a balance between the metabolites present in the final product within certain limits. This implies that strain engineering may not necessarily aim for high yield and titre of a single compound as in regular metabolic engineering studies. The ratio between ethyl esters and MCFA is critical, as MCFA can be directly converted into ethyl esters. However, the link to other metabolites may be less apparent and as critical. For instance, strains combining mutations in *ScFAS1* and *ScFAS2* (IMI552, IMI553, and IMI554) not only exhibited higher ethyl ester biosynthesis but also significantly lower levels of acetate esters (ethyl acetate, isoamyl acetate, and isobutyl acetate) (Fig. S8), which also contribute to the final aromatic bouquet. These results might connect to potential competition for acetyl CoA, which serves as the precursor for acyl-CoA biosynthesis involved in ethyl ester formation, while also serving as a substrate for acetate esters. This suggests that future strain improvement strategies should embrace metabolic pathway balancing to avoid accumulation of unwanted intermediates (e.g. carboxylic acids) by investigating gene dosage, tuning transcriptional modulation or applying post-translational variation based on for instance synthetic protein scaffolds to optimize and control metabolic fluxes.

Often regarded as non-genetically tractable, this study unequivocally demonstrates that *Saccharomyces pastorianus* can indeed undergo metabolic engineering approaches, enabling the prototyping of new lager brewing strains with customizable aroma profiles.

However, precision genetic engineering remains more complex in lager yeasts than in laboratory haploid *S. cerevisiae* strains due to the challenge of conserving the ploidy of the engineered strain. Transformation may have a mutagenic effect by altering the ploidy of *S. pastorianus*. In this study, several engineered strains showed the loss of one or more chromosome copies (Table S2) (Gorter de Vries et al., 2020). Recurrently, brewing-relevant phenotypic traits, such as the ability to consume maltotriose (e.g., strain IMI541, Fig. S3), may be lost upon transformation. This necessitates screening for transformants that have retained the phenotypic trait (Bennis et al., 2023). The reason behind this phenotype loss is still not understood. To ensure strain stability, the strain is transferred to new medium at least three times before sequencing. Further systematic deep-sequencing of engineered strains (e.g. IMI551-554) with illumia and nanopore technology did not reveal trace of recombination supporting genomic stability of engineered strains. In particular, redundancy of the *TEF1*p and *CYC1*t regulatory sequences used to control the expression of *FAS* alleles did not show chromosomal rearrangement of *ScSe*CHRIII. Despite these potential pitfalls, these brewing phenotypic determinants would be exceedingly challenging to achieve through traditional strain improvement methods. Therefore, this approach holds significant promise for the development of novel lager brewing strains with enhanced or unique characteristics, potentially changing the brewing industry.

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CRediT authorship contribution statement

Nicole X. Bennis: Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Jimme Bieseman:** Methodology, Investigation. **Jean-Marc G. Daran:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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