DEVELOPMENT OF NOVEL BIOCATALYSTS AND BIOSYSTEMS FOR GREEN CHEMISTRY

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“To strive, to seek, to find, and not to yield”

Ulysses, Alfred Tennyson

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Abstract

The chemical synthesis currently employed in the manufacture of cosmetics presents limitations such as unwanted side reactions and the need of strong chemical conditions. In order to overcome these drawbacks, novel enzymes have been developed to catalyse the targeted bioconversions contributing to the solution of the environmental concerns of the industrial activities moving them towards sustainable biotechnologies. This work was mainly aimed at developing improved biocatalysts based on feruloyl esterases (FAEs) and glucuronoyl esterases (GEs) for the production of compounds with antioxidant activity.

Novel fungal FAEs and GEs, identified through a bioinformatics approach from the analysis of 300 fungal genomes by the "Westerdijk Fungal Biodiversity Institute", were expressed in *Pichia pastoris* and characterized. FAE from *Aspergillus wentii* was selected as the most promising enzyme to be subjected to site-directed mutagenesis to further fine-tune the enzyme towards its application in bioconversions. A homology model of this enzyme was developed and five site-directed variants were designed, expressed in *P. pastoris* and characterized assessing substrate specificity, solvent and thermo tolerance. This analysis led to the development of a rational designed variant with tenfold improved hydrolytic activity and a variant with enhanced thermo and solvent tolerance.

As a second approach to develop improved biocatalysts based on FAEs, directed evolution was applied to the already characterized FAEs from *Fusarium oxysporum* (FoFaeC) and from *Myceliophthora thermophila* (MtFae1a). Two complete methodologies for the construction and the automated screening of evolved variants collections were developed and applied to the generation of 30,000 mutants libraries and their screening. Randomly mutated variants of FoFaeC and MtFae1a were generated through error prone-polymerase chain reaction and expressed in *Yarrowia lipolytica* and *Saccharomyces cerevisiae,* respectively. Thanks to the development of *ad hoc* chromogenic substrates for high-throughput assays on solid and in liquid media, screening for higher extracellular FAE activity than the wild type enzymes led to the selection of improved enzyme variants. The best evolved variants of both MtFae1a and FoFaeC were characterized for their thermotolerance, solvent tolerance and specificity towards methylated cinnamic substrates and subjected to small molecular docking studies to assess substrate interactions. In addition, MtFae1a evolved variants were tested in transesterification reactions in detergentless microemulsions for the production of target compounds selected for their potential antioxidant activity. Thus, although the screening strategy was based on the selection of evolved variants with improved hydrolytic activity, it was possible to obtain MtFae1a variants with both hydrolytic and synthetic enhanced activities to be potentially exploited in cosmetic industry.

Finally, with the aim of identifying novel FAEs with non-conserved sequences, different fungal strains isolated from lignocellulosic biomasses during biodegradation under natural conditions and belonging to the microbial collection of Department of Agriculture (University of Naples "Federico II") were screened for the production of different enzymes having potentially synergistic actions on lignocellulose conversion. This led to the selection of a novel fungal species showing FAE activity production induced by different carbon sources. Genome and transcriptome sequencing and analysis confirmed the presence of genes related to plant cell wall degrading enzymes.

Riassunto

L'esaurimento delle riserve di combustibili fossili, i cambiamenti climatici causati da gas serra e problemi riguardanti lo smaltimento dei rifiuti, rendono necessario l'utilizzo di risorse di energia e materiali rinnovabili. L'US Environmental Protection Agency (EPA) definisce la chimica verde come "l'utilizzo della chimica per la prevenzione dell'inquinamento e la progettazione di prodotti chimici e processi più rispettosi dell'ambiente" (http://www.epa.gov/greenchemistry/whatis.htm). La chimica verde si prefigge di sostituire un sistema economico basato esclusivamente sulla petrochimica con tecnologie basate su materiali rinnovabili che possono essere convertiti in prodotti biologici per diverse applicazioni industriali. Le fonti alternative devono essere rinnovabili, sostenibili, efficienti, economicamente vantaggiose, convenienti e sicure (Prasad et al. 2007); tali nuovi processi devono essere coerenti con i dodici principi della chimica verde introdotti da Anastas e Warner nel 1998. Le biotecnologie, in particolare le biotecnologie bianche, assumono un ruolo chiave nello sviluppo di tali processi attraverso biotrasformazioni e fermentazioni usando enzimi e microrganismi, rispettivamente. In particolare, i biocatalizzatori possono fornire diversi vantaggi rispetto ai processi catalizzati chimicamente, come l'uso di condizioni di reazione più deboli, cioè un minor utilizzo di energia, un ridotto utilizzo dei solventi e produzione di rifiuti e la minima generazione di sottoprodotti dovuti all'elevata specificità degli enzimi. Enormi progressi sono stati raggiunti grazie allo sviluppo di biocatalizzatori migliorati (attraverso l'evoluzione guidata, tecnologie computazionali, progettazione di enzimi *in silico*, ingegneria proteica, ecc.) e all'ingegneria metabolica per reindirizzare i percorsi metabolici attraverso la modifica di reazioni biochimiche specifiche o l'introduzione di nuove con l'utilizzo di tecnologie ricombinanti. Un'ampia gamma di prodotti ad alto valore aggiunto, come enzimi, biocombustibili, acidi organici, biopolimeri, molecole per le industrie alimentari e farmaceutiche, possono essere ottenute utilizzando la chimica verde e processi biotecnologici (Liguori et al. 2013, Martins and Ferreira 2017, Ventorino et al. 2017).

Produzione di esteri lipidici e esteri di zuccheri con attività antiossidante

La sintesi chimica di ingredienti per l'industria cosmetica, come gli antiossidanti, non è un processo diretto ma necessita di catalizzatori chimici forti che funzionano solo a temperature elevate. Inoltre, queste reazioni sono non selettive e i prodotti risultano in una miscela di esteri, rappresentando un ostacolo per produrre sostanze pure e di alta qualità con potenziale utilizzo nell'industria cosmetica o farmaceutica (Kiran e Divakar 2001). In questo scenario, è necessario sostituire i processi chimici attualmente impiegati per la produzione di molecole con attività antiossidante con bioconversioni caratterizzate da un impatto ambientale ridotto.

Gli acidi fenolici e idrossicinamici (ferulico, *p*-coumarico, caffeico, sinapinico) hanno un diffuso potenziale industriale grazie alle loro proprietà antiossidanti e antimicrobiche (Maurya e Devasagayam 2010, Razzaghi-Asl et al 2013). Gli acidi fenolici più comuni sono solubili in acqua, limitando la loro applicazione nei processi a base di oli per prodotti alimentari, rappresentando un grande svantaggio nel caso in cui sia prevista una fase acquosa (Topakas et al. 2003a). La modifica di questi composti mediante esterificazione con molecole alifatiche (come gli alcoli) può essere utilizzata come strumento per alterare la solubilità in formule ed emulsioni a base di oli. Inoltre, gli esteri di grassi fenolici non solo mantengono l'attività antiossidante dell'acido di partenza, ma mostrano un incremento nell'effetto antiossidante (Chalas et al. 2001, Vafiadi et al. 2008a). Gli alcoli aromatici o alifatici

invece possono essere esterificati con uno zucchero (con attività antiossidante) per ottenere esteri di zuccheri che siano più idrofili rispetto al composto antiossidante di partenza, rendendo tali prodotti dei candidati ideali per l'impiego nelle industrie che utilizzano fomule a base di acqua, ampliando il loro uso nell'industria cosmetica (Stamatis et al. 2001, Katapodis et al. 2003). Negli ultimi anni, si è sviluppato un interesse crescente sulla sintesi enzimatica di esteri e glicosidi bioattivi basati su reazioni di (trans)esterificazione, (trans)glicosilazione o reazioni di ossidazione per la produzione di cosmeceutici. Tali composti sono prodotti cosmetici che contengono ingredienti biologicamente attivi che offrono un beneficio terapeutico farmaceutico. Le più importanti modifiche enzimatiche che portano alla sintesi degli ingredienti con proprietà attraenti per l'industria cosmeceutica sono l'esterificazione diretta o la transesterificazione eseguita da esterasi (come le lipasi, le feruloil esterasi o le tannasi) e le proteasi, la glicosilazione (idrolisi inversa) o la transglicosilazione eseguita da transferasi e β-glucosidasi e l'oligomerizzazione eseguite dalle laccasi.

Feruloil esterasi e glucoronoil esterasi come biocatalizzatori per la produzione di antiossidanti

Gli esempi di sintesi enzimatica o di modifiche dei composti naturali con attività antiossidante precedentemente descritti, evidenziano la possibilità di sviluppare ingredienti con proprietà antiossidanti, anti-invecchiamento, antimicrobico, antirughe, fotoprotettivo o sbiancante per la pelle in condizioni di reazione deboli, mantenendo la loro attività biologica ed evitando la formazione di sottoprodotti. L'uso di biocatalizzatori come le feruloil esterasi (FAE) e le glucoronil esterasi (GE) può essere esplorato per sviluppare vie di produzione biotecnologiche di esteri industrialmente rilevanti con ridotto impatto ambientale.

Le feruloil esterasi (FAE) catalizzano l'idrolisi del legame estereo tra acidi idrossicinnamici (acido ferulico e acido *p*-cumarico) e zuccheri (polimeri di xilano e pectina) presenti nelle pareti cellulari vegetali. Esse agiscono come enzimi accessori (o ausiliari) facilitando altri enzimi, come le xilanasi, nell'accesso al loro sito d'azione durante la conversione delle biomasse (Yu et al. 2003, Wong 2006, Faulds 2010). Una prima classificazione ha diviso le FAE in quattro tipi (A, B, C e D) in base alle loro specificità di substrato verso gli esteri metilici sintetici degli acidi idrossicinnamici e sulle loro identità di sequenza amminoacidica (Crepin et al. 2004). È stata proposta un'ulteriore classificazione (Benoit et al 2008) basata su un allineamento di sequenze multiple ottenuto utilizzando le sequenze di varie FAE da genomi fungini. L'albero filogenetico risultante ha contribuito a identificare sette sottofamiglie (SF1-7) dimostrando che tali enzimi si sono evoluti da famiglie di esterasi altamente divergenti non mostrando un antenato comune. Nel 2011 è stato costruito un nuovo e completo schema di classificazione (Udatha et al. 2011) che comprende 365 sequenze FAE di origine fungina, batterica e vegetale, risultando in una riclassificazione delle FAE in 12 subfamiglie. Tuttavia, la scoperta di nuove FAE utilizzando l'analisi di genomi e analisi filogenetiche ha condotto allo sviluppo una nuova e più completa classificazione delle FAE fungine in 13 sottogruppi (SF1-13) considerando sia la filogenesi che la specificità di substrato (Dilokpimol et al. 2016).

L'interesse verso questa classe enzimatica è in continuo aumento per un gran numero di potenziali applicazioni in varie industrie come quelle di sostanze chimiche, carburanti, tessili e lavanderia, mangimi, alimenti e agricoltura e farmaceutici, come illustrato da più di 100 brevetti su tali biocatalizzatori (Topakas et al. 2007). Inoltre, tali enzimi hanno ottenuto l'attenzione per le loro potenziali applicazioni nell'ottenimento di acido ferulico da materiali di scarto agro-industriali come quelli

prodotti da macinazione, birra e industrie dello zucchero (Fazary e Ju 2008). Oltre alla loro capacità idrolitica, in condizioni operative appropriate, le FAE sono in grado di catalizzare reazioni di (trans)esterificazione, modificando gli acidi idrossicinamici e i loro esteri con conseguente sintesi degli ingredienti con proprietà attraenti per le industrie alimentari, cosmetiche e farmaceutiche. Le esterificazioni catalizzate dalle FAE sono state principalmente condotte in microemulsioni prive di detergenti, ossia un sistema ternario costituito da un idrocarburo, *n*-propanolo e acqua formando una dispersione termodinamica stabile di microgrocce acquose nel solvente idrocarburico (Khmelnitsky et al. 1988).

Le glucoronoil esterasi (GE), una famiglia di enzimi recentemente scoperta, svolgono un ruolo significativo nella decomposizione della biomassa separando l'emicellulosa dalla lignina attraverso l'idrolisi del legame estereo tra residui di acido 4-O-metil-D-glucuronico di glucuronoxilani e alcoli aromatici di lignina (Špániková e Biely 2006, Ďuranová et al. 2009, Arnling Bååth et al. 2016). Gli esperimenti su substrati sintetici che imitano i legami esterei presenti nella parete cellulare vegetale hanno dimostrato che le GE riconoscono l'unità uronica e non l'alcool dell'estere e che mostrano preferenza verso gli esteri dell'acido 4-O-metil-D-glucuronico piuttosto che D-glucuronico (Špániková et al. 2007). La prima sequenza aminoacidica di una GE apparteneva a *Hypocrea jecorina* (Li et al. 2007), utilizzata per ricercare geni omologhi in diversi funghi filamentosi e batteri portando alla nascita di una nuova famiglia di CE15 nel database CAZy (http://www.cazy.org/). Ad oggi solo alcuni membri di questa famiglia sono stati caratterizzati utilizzando una serie di nuovi substrati sintetici che comprendono gli esteri metilici degli acidi uronici e dei loro glicosidi (Špániková and Biely 2006; Li et al. 2007; Vafiadi et al. 2009; Ďuranová et al. 2009; Topakas et al. 2010, Huttner et al. 2017).

Le GE costituiscono candidati interessanti per la produzione di biocarburanti e bioprodotti a partire da biomassa vegetale grazie al loro coinvolgimento nell'idrolisi dei complessi lignina-carboidrati (Katsimpouras et al. 2014, D'Errico et al. 2016). Rispetto alla già nota capacità di biotrasformazione delle FAE, la capacità sintetica dei GE deve essere esplorata per la produzione di derivati alchilici come tensioattivi non ionici e con buone proprietà di superficie, compresa la biodegradabilità. Inoltre, grazie alla loro assenza di sapore, irritazione cutanea e tossicità, questi composti bioattivi trovano impieghi diversi nelle industrie cosmetiche e farmaceutiche (Moreau et al. 2004).

Le FAE e GE in natura catalizzano reazioni idrolitiche, mentre per eseguire reazioni biosintetiche efficaci sono necessari sistemi di reazione privi di acqua o a basso contenuto di acqua. L'ottimizzazione di entrambi i biocatalizzatori, comprese la loro regioselettività e stereoselettività, nonché la loro specificità e le condizioni di reazione, porteranno alla sostituzione dei processi chimici attualmente utilizzati per la produzione dei suddetti composti biologicamente attivi realizzando bioconversioni economiche, efficienti ed ecocompatibili a basso costo.

Nell'ambito del progetto OPTIBIOCAT (Optimized esterase biocatalysts for cost-effective industrial production" (613868), questo progetto di dottorato ha previsto lo sviluppo di biocatalizzatori basati su FAE e GE per la produzione di composti con attività antiossidante mediante l'utilizzo di diversi approcci.

- **Sviluppo di biocatalizzatori basati su FAE e GE attraverso la loro espressione ricombinante e mutagenesi sito-diretta**

L' attuale disponibilità delle sequenze di genomi di funghi rende possibile l'esplorazione di tale regno per l'identificazione di nuove FAE e GE (Benoit et al. 2008). La ricerca di sequenze esplorando genomi al fine di identificare nuovi enzimi è una strategia robusta e consolidata per aumentare il repertorio di enzimi necessario per applicazioni biotecnologiche. Inoltre, le strutture cristalline disponibili di FAE e GE possono consentire l'ingegnerizzazione della struttura proteica per aumentare la stabilità termica dei corrispondenti biocatalizzatori.

FAE e GE già caratterizzate sono state utilizzate come *query* in una ricerca *blast* all'interno di un database contenente circa 300 genomi di funghi appartenente al Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology, Università di Utrecht. Le sequenze ottenute sono state utilizzate per un'iniziale analisi e filogenesi, portando alla selezione di 1,636 ipotetiche FAE e 166 presunte GE utilizzate per la costruzione di alberi filogenetici, separando le FAE e GE rispettivamente in 12 e 8 sottogruppi. Dopo la correzione del modello genetico, i cDNA di 20 GE e 30 FAE sono stati sintetizzati ed espressi in *P. pastoris* per validare la strategia di analisi dei genomi per l'identificazione di nuovi enzimi. La caratterizzazione di questa collezione di FAE e GE ha migliorato la comprensione della varietà delle proprietà di tali enzimi da diversi gruppi/sottofamiglie e fornisce una classificazione univoca (Dilokpimol et al. 2016).

La FAE AwFaeG da *Aspergillus wentii* è stata scelta come l'enzima più promettente da sottoporre a mutagenesi sito-diretta per migliorare ulteriormente l'enzima verso la sua applicazione nelle bioconversioni. È stato sviluppato un modello di struttura tridimensionale per omologia di tale enzima e sono state progettate 5 varianti sito-dirette, espresse in *P. pastoris* e caratterizzate in base alla loro specificità di substrato, tolleranza ad elevate temperature ed esposizione a solventi. Tali analisi hanno portato allo sviluppo di una variante progettata razionalmente con attività idrolitica contro il *p*NP-Fe dieci volte maggiore dell'enzima *wild type* e una variante con una maggiore tolleranza sia ad alte temperature che solventi.

- **Sviluppo di biocatalizzatori basati su FAE attraverso evoluzione guidata**

Un altro approccio ben consolidato e complementare alla mutagenesi razionale per sviluppare biocatalizzatori migliorati è rappresentato dall'evoluzione guidata che imita l'evoluzione naturale per alterare le proprietà degli enzimi come la specificità, l'attività, la stabilità e la solubilità utilizzando metodi per creare diversità genetica. Questa strategia consiste in un processo iterativo a due fasi: a) un primo step di generazione di una libreria di numerose varianti di una proteina di interesse; b) *high throughput screening* per identificare i mutanti con migliori proprietà. I mutanti selezionati attraverso lo *screening ad hoc* possono essere utilizzati come modello per ulteriori cicli di evoluzione guidata fino al raggiungimento del livello di miglioramento desiderato. Rispetto alla progettazione razionale delle proteine, l'evoluzione guidata mostra il vantaggio di non richiedere informazioni sulla struttura proteica o sugli effetti di sostituzioni di aminoacidi specifici, che sono molto difficili da prevedere *a priori*. Tuttavia, sono pochi gli esempi di applicazioni di successo di strategie di evoluzione in vitro di feruloil esterasi, soprattutto a causa della mancanza di substrati adatti per i saggi di attività *high throughput*.

Le FAE già caratterizzate da *Fusarium oxysporum* (FoFaeC) e da *Myceliophthora thermophila* (MtFae1a) sono state sottoposte a evoluzione guidata. Due metodologie

complete per la costruzione e lo *screening* automatizzato delle collezioni di varianti evolute sono state sviluppate e applicate alla generazione di collezioni di 30,000 mutanti e loro *screening* utilizzando postazioni automatizzate con i Robot Colony Picker e Biomek NX. Mutanti random di FoFaeC e MtFae1a sono stati generati mediante reazione di polimerizzazione a catena a bassa fedeltà (error prone PCR) ed espresse rispettivamente in *Yarrowia lipolytica* e *Saccharomyces cerevisiae*. Grazie allo sviluppo di substrati cromogenici *ad hoc* per saggi di attività *high throughput* su mezzo solido e liquido, lo *screening* per maggiore attività extracellulare FAE rispetto agli enzimi *wild type* ha portato alla selezione di varianti enzimatiche migliorate. Le migliori varianti evolute di MtFae1a e FoFaeC sono state caratterizzate per la loro termotoleranza, tolleranza al solvente e specificità nei confronti dei substrati metil esteri degli acidi cinnamici e sottoposti a studi di docking molecolare per valutare le interazioni con il substrato. Inoltre, le varianti evolute di MtFae1a sono state testate in reazioni di transesterificazione in microemulsioni prive di detergenti per la produzione di composti target selezionati per la loro potenziale attività antiossidante. Pertanto, sebbene la strategia di screening sia basata sulla selezione di varianti evolute con attività idrolitica migliorata, è stato possibile ottenere varianti MtFae1a con migliori attività sia idrolitiche che sintetiche potenzialmente applicabili nell'industria cosmetica.

- **Selezione di funghi per l'identificazione di FAE con sequenze non conservate**

Insieme alla ricerca di nuovi geni in genomi già noti, lo *screening* funzionale di specifici ceppi fungini con sequenze di genomi non sequenziati potrebbe ampliare notevolmente il repertorio delle sequenze di DNA disponibili per feruloil e glucuronoil esterasi. Il microbiota da fonti lignocellulosiche può essere considerato una risorsa importante di ceppi di funghi utili a indirizzare la conversione della lignocellulosa verso tecnologie "più ecologiche". Circa 1000 ceppi microbici isolati dalle biomasse lignocellulosiche durante la biodegradazione in condizioni naturali dal Dipartimento dell'agricoltura dell'Università di Napoli "Federico II" (Prof. Olimpia Pepe, Divisione di Microbiologia, Portici, Napoli, Italia) sono stati sottoposti a *screening* per la produzione di diverse attività enzimatiche coinvolte nella decostruzione della lignocellulosa. La loro morfologia è stata analizzata osservandone la forma e il colore, mentre la micromorfologia dei conidi è stata analizzata mediante microscopia ottica. Al fine di valutare la produzione di attività FAE, funghi selezionati sono stati scelti per lo *screening* funzionale. Tali funghi sono stati coltivati su terreno solido contenente indolil ferulato o etil ferulato, come unica fonte di carbonio, dimostrando di essere in grado di crescere su terreno solido contenente indolil ferulato, ma non di formare aloni blu di attività. L'induzione della produzione di attività FAE è stata ulteriormente studiata in terreno di coltura liquida in presenza di crusca di grano e crusca di grano senza amido come unica fonte di carbonio. Inoltre, è stata esplorata anche l'influenza dell'acido ferulico come induttore della produzione di attività di FAE, aggiungendola a crusca di grano non trattata in mezzo di crescita liquido. Ciò ha portato alla selezione di una nuova specie fungina che mostra produzione di attività FAE indotta da diverse fonti di carbonio. Il sequenziamento e l'analisi del genoma e del trascrittoma hanno confermato la presenza di geni correlati agli enzimi coinvolti nella degradazione, sintesi e modifica dei carboidrati (CAZy), tra cui putative feruloil esterasi.

Conclusioni

In sintesi questo progetto di dottorato ha previsto:

- l’espressione ricombinante in *Pichia pastoris* di nuove feruloil esterasi e glucoronoil esterasi identificate analizzando una collezione di circa 300 genomi fungini mediante approccio bioinformatico e la loro caratterizzazione in base alla specificità di substrato; tra tali enzimi, la selezione della nuova FAE AwFaeG da *Aspergillus wentii* con la migliore attività idrolitica contro i derivati metil esteri degli acidi idrossicinnamici;
- lo sviluppo di nuovi biocatalizzatori basati sulla nuova feruloil esterasi AwFaeG ottenuti mediante l’elaborazione di un modello di struttura tridimensionale, la progettazione delle mutazioni, l’espressione di 5 mutanti sito-diretti e la loro caratterizzazione; in particolare, un mutante con attività idrolitica contro il *p*NP-Fe di circa 10 volte maggiore rispetto all’enzima *wild type* e un mutante con maggiore tolleranza all’esposizione in solventi e a 55°C;
- lo sviluppo di due metodologie per la costruzione e la selezione automatizzata di collezioni di varianti evolute e la loro applicazione per la produzione di 30000 mutanti per entrambe le già caratterizzate feruloyl esterase MtFae1a e FoFaeC e la selezione per attività idrolitica delle migliori varianti;
- l’ottenimento di varianti di MtFae1a con attività di transesterificazione migliori di quelle dell’enzima *wild type* per la sintesi di composti ad attività antiossidante quali il gliceril ferulato, il prenil caffeato, il prenil ferulato e il butil ferulato;
- l’identificazione di una nuova specie fungina da *Arundo donax* lasciato naturalmente a biodegradare e isolata per la sua capacità di produrre enzimi capaci di degradare biomasse lignocellulosiche; il sequenziamento e l’analisi del genoma di tale fungo hanno rivelato la presenza di numerosi geni codificanti per enzimi coinvolti nella degradazione, sintesi e modifica dei carboidrati (CAZy), tra cui putative feruloil esterasi.

List of abbreviations

BFA: Butyl ferulate

CAZy / CAZyme: Carbohydrate-Active Enzymes

FAE: Ferulic Acid Esterase / Feruloyl Esterase

FoFaeC: FAE from *Fusarium oxysporum*

AwFaeG: FAE from *Aspergillus wentii*

GE: Glucuronoyl Esterase

GFA: Glyceryl ferulate

HTS: High Throughput Screening

MCA: Methyl caffeate

MFA: Methyl ferulate

M*p*CA: Methyl *para* coumarate

MSA: Methyl sinapate

MtFae1a: FAE from *Myceliophtora thermophila*

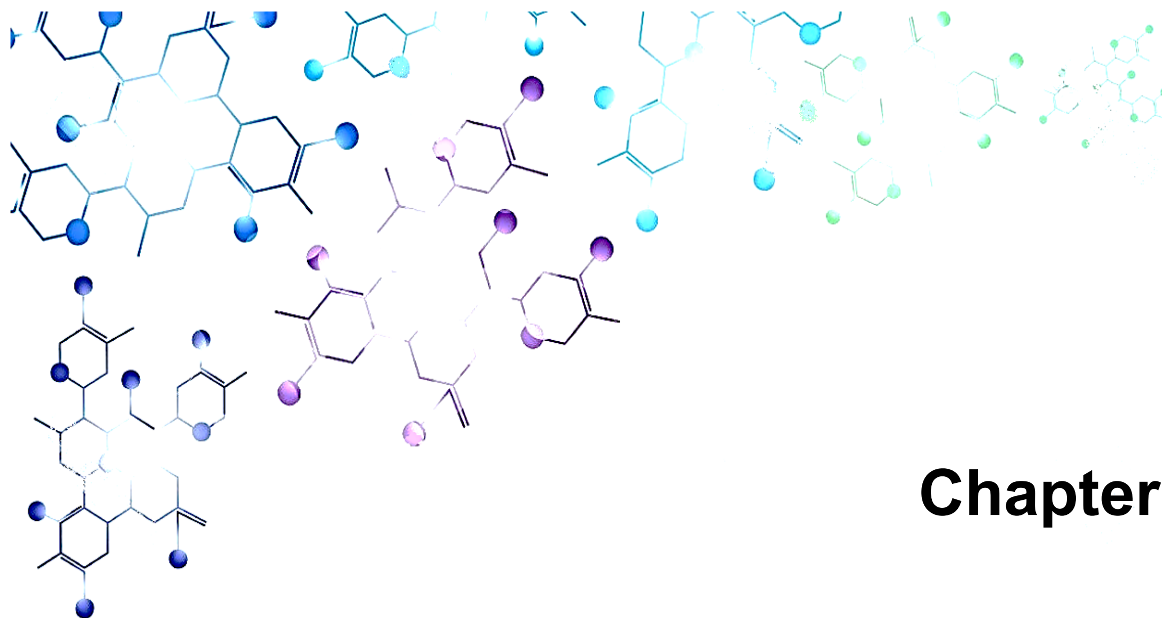
PCA: Prenyl caffeate

PCR: Polymerase Chain Reaction

PFA: Prenyl ferulate

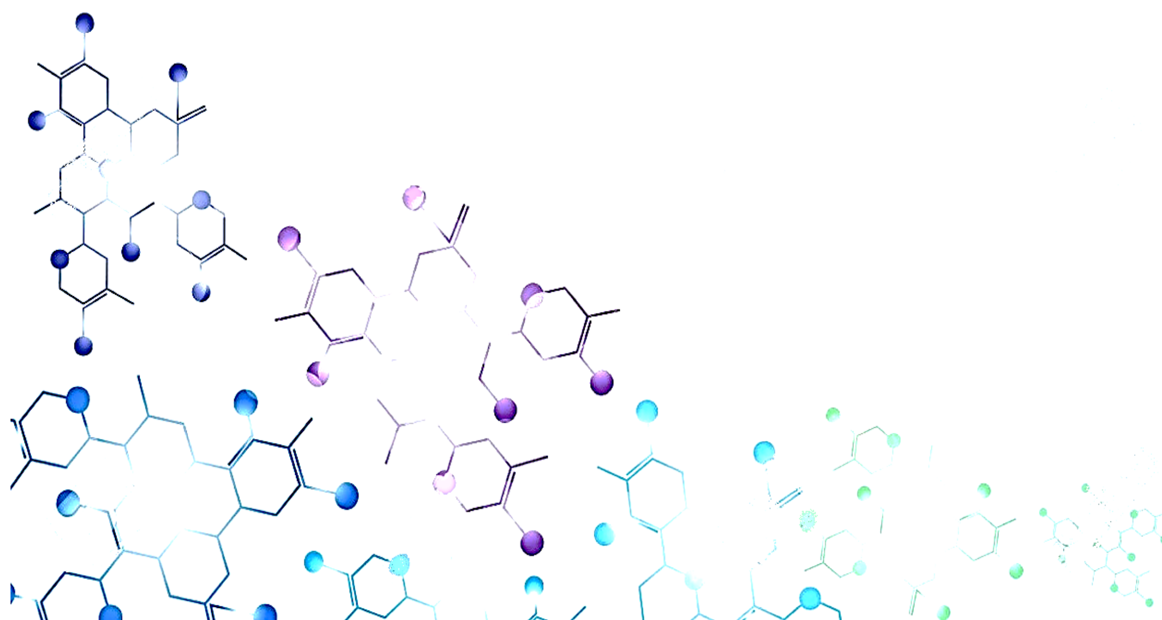
***p*NP-Fe**: *para* nitro phenyl ferulate

SDS-PAGE: Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis



Chapter I

Introduction



Chapter I: Introduction

1.1 Green chemistry

Fossil fuel reserves depletion, climate changes caused by greenhouses gases and problems in waste recycling make necessary the use of renewable resources of energy and materials. US Environmental Protection Agency (EPA) defines green chemistry as the "use of chemistry for pollution prevention, and design of chemical products and processes that are more environmentally benign" (http://www.epa.gov/greenchemistry/whatis.htm). Green chemistry is aimed at replacing an exclusively petrochemical based economic system with technologies based on renewable materials which can be converted in biochemicals for various industrial applications. Alternative sources need to be renewable, sustainable, efficient, cost effective, convenient and safe (Prasad et al. 2007); also, new bio-based processes need to be coherent with the twelve principles of green chemistry introduced by Anastas and Warner in 1998. Biotechnology, particularly white biotechnology, takes a key role into development of these processes through biotransformations and fermentations using enzymes and microorganisms, respectively. In particular, biocatalysts can give several advantages in comparison to the chemically catalyzed processes such as the use of milder reaction conditions, i.e. a lower use of energy, a reduced use of solvents and production of wastes, and minimum generation of byproducts due to the high enzyme specificity. Huge advancements in green chemistry have being achieved by both development of improved biocatalysts (through directed evolution, computational technologies, in silico enzymes design, protein or cofactor engineering, etc.), and metabolic engineering to redirect metabolic pathways through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant technology.

A wide range of high added value products, such as enzymes, biofuels, organic acids, biopolymers, bioelectricity, and molecules for food and pharmaceutical industries, can be obtained by using green chemistry and biotechnology processes (Liguori et al. 2013, Martins and Ferreira 2017, Ventorino et al. 2017). Bio-based products can be produced in biorefineries, which can be considered as an integral unit that can accept different biological feedstocks and convert them to a range of useful products including chemicals, energy and materials (Clark et al. 2006).

1.2 Production of phenolic fatty- and sugar- esters with antioxidant activity

The chemical synthesis of ingredients for the cosmetic industry, such as antioxidants, are not straight processes requiring strong acid, alkaline or metal-based (Lewis acid) chemical catalysts that function only at high temperatures. Moreover, these reactions are unselective and the products will always be present as a mixture of esters, which represent an obstacle to produce clean and high-quality substances with the potential use in the cosmetics or pharmaceutical industry (Kiran and Divakar 2001). In this scenario, it is necessary to replace chemical processes currently adopted for production of molecules with antioxidant activity with bioconversions characterized by reduced environmental impact. This will contribute to the solution of the environmental concerns of the industrial activities moving them towards sustainable biotechnologies.

Phenolic and hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic, sinapinic) have a widespread industrial potential due to their antioxidant and antimicrobial properties (Maurya and Devasagayam 2010, Razzaghi-Asl et al. 2013). Most common phenolic acids are water soluble, limiting their usefulness in oil-based food processing, which has been reported as a serious disadvantage if an aqueous phase is also present (Topakas et al. 2003a). The modification of these compounds via esterification with aliphatic molecules (such as alcohols) can be used as a tool to alter solubility in oil-based formulas and emulsions. In addition, phenolic fatty esters not only maintain the antioxidant activity of the starting acid, but possibly show increased antioxidant effects (Chalas et al. 2001, Vafiadi et al. 2008a). On the other hand, aromatic or aliphatic alcohols can be esterified with sugar (having antioxidant activity) to obtain more hydrophilic sugar esters compared with the starting antioxidant compound, which constitutes them ideal candidates for use in water-based industries, broadening their use in cosmetic industry (Stamatis et al. 2001, Katapodis et al. 2003).

In the following review, the most important enzymatic modifications that result to the synthesis of ingredients with attractive properties for the cosmeceutical industry are documented. Direct esterification or transesterification performed by esterases (such as lipases, feruloyl esterases, or tannases) and proteases, glycosylation (reverse hydrolysis) or transglycosylation performed by transferases, and β-glucosidases and oligomerization performed by laccases are the modification occurring to obtain molecules with as anti-oxidant, anti-inflammatory, anti-microbial, skin-whitening, and photoprotective effects.

1.2.1 Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application (Paper I)

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MINI-REVIEW

Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application

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Abstract Cosmeceuticals are cosmetic products containing biologically active ingredients purporting to offer a pharmaceutical therapeutic benefit. The active ingredients can be extracted and purified from natural sources (botanicals, herbal extracts, or animals) but can also be obtained biotechnologically by fermentation and cell cultures or by enzymatic synthesis and modification of natural compounds. A cosmeceutical ingredient should possess an attractive property such as anti-oxidant, anti-inflammatory, skin whitening, anti-aging, anti-wrinkling, or photoprotective activity, among others. During the past years, there has been an increased interest on the enzymatic synthesis of bioactive esters and glycosides based on (trans)esterification, (trans)glycosylation, or oxidation reactions. Natural bioactive compounds with exceptional theurapeutic properties and low toxicity may offer a new insight into the design and development of potent and beneficial cosmetics. This review gives an overview of the enzymatic modifications which are performed currently for the synthesis of products with attractive properties for the cosmeceutical industry.

Keywords Lipases · Feruloyl esterases · Tannases · Transferases · Glycosidases · Proteases · Laccases · Anti-oxidant · Anti-microbial · Anti-inflammatory · Skin whitening · Anti-wrinkling · Anti-aging · Photoprotective · Fungal · Bacterial

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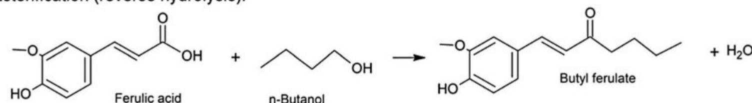
Introduction

Articles defined as cosmetics are intended for human body application aiming at increased beauty and attraction or cleaning use, without affecting the body structure or function (Nelson and Rumsfield 1988). During the last few years, the cosmetic industry is searching for bioactive compounds that also promote health benefits. This combination resulted in a new term called "cosmeceutical" where cosmetic products assert medical benefits (Choi and Berson 2006). Cosmeceuticals are different from cosmetics and drugs, as they affect the function and structure of skin, while having drug-like effects that are marketed using skin appearance-based claims. Cosmeceutical industry numbers over 400 manufacturers worldwide including Estée Lauder, L'Oréal, Procter & Gamble, and Avon, with 80 % of the US and European market dedicated to skin care (Brandt et al. 2011). In 2008, Japan was by far the biggest market in cosmeceuticals valued at $6–8 billion, followed by the USA ($5–6 billion) and EU ($3–5 billion) (Kim and Wijesekara 2012). Market growth is expected to rise in economies like China, Brazil, the Russian Federation, and India (Brandt et al. 2011). Nevertheless, the Food and Drug Administration (FDA) does not recognize cosmeceutical as a term even if it is widely used in industry, while in the EU, most are considered as cosmetics (Sharma 2011). There is no regulation of cosmeceuticals in EU, the USA, and Japan; however, as the interaction between cosmetic and skin is complex, there is an increased attention towards the need of toxicological tests of the final product and its bioactive ingredients (Nohynek et al. 2010). Target ingredients of cosmeceuticals may include phytochemicals,

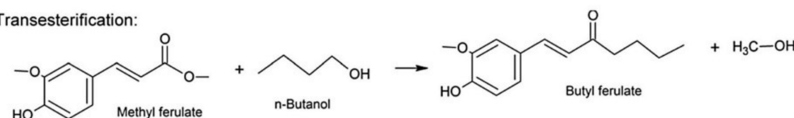
vitamins, peptides, enzymes, essential oils among others, which are incorporated into lotions, creams, and ointments dedicated to skin treatment. Desired properties, such as anti-oxidant, anti-aging, anti-microbial, anti-wrinkling, photoprotective, or skin whitening, are preferentially offered by natural compounds derived from plant or sea organisms, instead of chemically synthetic compounds. The guidelines of the Council of Europe define a natural cosmetic as a product that consists of natural substances of botanical, mineral, or animal origin, exclusively obtained through physical, microbiological, or enzymatic methods, with certain exceptions for fragrances and preservatives. This demand has increased the sales of personal care products based on natural ingredients; however, often a modification of the bioactive compounds is required prior to their application in the final product, e.g., by increasing its lipophilicity or improving its biological properties. Modification with fatty compounds generally results in more lipophilic products, whereas modification with sugars results in more hydrophilic derivatives. Chemical approaches have numerous disadvantages such as the protection and de-protection of groups resulting in many reaction steps, use of strong acid as catalyst, high temperatures (150–200 °C), formation of unwanted products, dark color, burnt taste of product, and high energy consumption (Kiran and Divakar 2001). Enzymatic modification is employed under mild conditions, is highly selective, and includes one single step.

In this review, the most important enzymatic modifications that result to the synthesis of ingredients with attractive

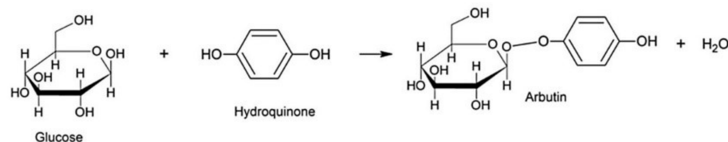
Esterification (reverse hydrolysis):



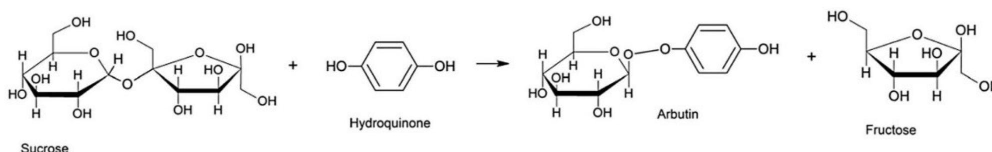
Transesterification:



Glycosylation (reverse hydrolysis):



Transglycosylation:



Oligomerization:

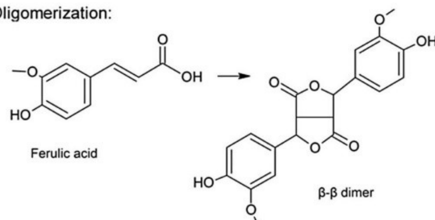


Fig. 1 Reaction examples

properties for the cosmeceutical industry are documented. Properties such as anti-oxidant, anti-inflammatory, anti-microbial, skin-whitening, and photoprotective effects were criteria for the selection of the reported modification reactions. A modification may follow different mechanisms: direct esterification or transesterification performed by esterases (such as lipases, feruloyl esterases, or tannases) and proteases, glycosylation (reverse hydrolysis) or transglycosylation performed by transferases, and β-glucosidases and oligomerization performed by laccases. Examples of such modification reactions are presented in Fig. 1.

Esterases

Except for their hydrolytic ability, esterases are able to perform (trans)esterification reactions. Triaglycerol lipases (EC 3.1.1.3) are most commonly used due to their broad specificity, as shown in Table 1. Less popular, ferulic acid esterases (FAEs; EC 3.1.1.73) generally catalyze the hydrolysis of the ester bond between the main chain polysaccharides of xylans or pectins and the monomeric or dimeric ferulic acid in plants; however, they are able to modify hydroxycinnamic acids and their esters. Tannases (tannin acyl hydrolases, EC 3.1.1.20) are known to be active on complex polyphenolics, catalyzing the hydrolysis or synthesis of the “ester bond” (galloyl ester of an alcohol moiety) or the “depside” bond (galloyl ester of gallic acid) (Battestin et al. 2008). Low water content is essential for the thermodynamic shift of equilibrium towards synthesis. Different systems have been employed including organic co-solvents, ionic liquids, solvent-free systems, supercritical fluids, and molecular sieves as water removal agents. The ideal solvent should aid solubilization of substrates, not affect enzyme activity, have low toxicity, and enable easy product recovery (Wei et al. 2002). Ionic liquids are a good alternative since they generally do not deactivate esterases and have exceptional tailorability and low volatility (Zeuner et al. 2011). However, a number of issues including the cost involved in large-scale usage are to be addressed. Aids as microwave irradiation and ultrasound treatment have been employed in lipase-catalyzed reactions (Costa et al. 2014; Cui et al. 2013). Detergentless microemulsions, so far employed in FAE-catalyzed reactions, consist of a hydrocarbon, a short-chained alcohol, and water representing thermodynamically stable dispersions of aqueous microdroplets in the hydrocarbon solvent (Khmelnitsky et al. 1988). An important advantage of these mixtures is the separation of reaction products and enzyme reuse, while the solubility of relatively polar phenolic acids is high owing to the presence of large amount of polar alcohol.

α-Hydroxy acid derivatives

α-Hydroxy acids (AHAs) are composed of carbon backbones containing a carboxyl group and a hydroxyl group on the adjacent carbon. Among them, glycolic acid, lactic acid, and malic acid have been well known in cosmetics as beauty aids and peeling agents due to their hygroscopic, emulsifying, and exfoliating properties (Tung et al. 2000). Short-chain AHAs as lactic acid are more active in regulating the rate of skin regeneration and improving dryness (Wei et al. 2002). However, limiting factors for application are their acidicity and the rapid penetration into the deep epiderm, causing irritant effects at concentrations >10 %. To control their concentration and penetration to the skin’s intercellular spaces, AHAs have been grafted onto alkylglycosides, fatty acids, or fatty alcohols so they can be gradually released by the epidermis esterases. Short-chain alkylglycosides have been reported to relieve the irritant effects on skin after UV radiation (Wei et al. 2003). A major concern regarding enzymatic modification is that lactic acid can undergo self-polymerization at high temperatures and low water content forming linear polyesters or lactones because of the presence of groups that act as acyl donor and nucleophile at the same time (Roenne et al. 2005). A key factor is the choice of enzyme that favors the desired reaction. Lactic acid does not act as nucleophile when the lipase B from *Candida antarctica* (CALB) is used as biocatalyst due to steric hindrance at the enzyme’s active site (Form et al. 1997). Another obstacle is the severe inactivation of enzymes in high concentrations of lactic acid or in solvent-free systems, as it decreases the logP of the reaction medium (Pirozzi and Greco 2004). Polar solvents aid lactic acid solubilization at higher concentrations and seem to prevent enzyme inactivation because they show an acid-suppressive effect due to their basicity (Hasegawa et al. 2008). However, esterification of glycolic acid has been favored in apolar hexane producing high yield of glycolate ester (91 % after 24 h) (Torres and Otero 1999). Limitation of lactic acid self-polymerization has been achieved in hexane although the esterification with fatty acids resulted in lower yields (35 %) (Torres and Otero 2001). Transesterification between α-butyl glycoside and butyl lactate in a solvent-free system eliminating the butanol coproduct under reduced pressure resulted in more than 95 % conversion and very high concentration of a less irritant product (170 g/L) in a single batch reaction (Bousquet et al. 1999).

Kojic acid derivatives

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) is an inexpensive water-soluble fungal secondary metabolite produced by *Aspergillus* and *Penicillium* species. It possesses valuable biological properties such as anti-oxidant, anti-microbial, and anti-inflammatory, while as an iron and copper chelator has the capacity to prevent photodamage,

Table 1 Lipase-catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
Examples of α-hydroxy acid derivatives							
C6–C18 lactates	C6–C18 fatty alcohols	Lactic acid	Novozym 435	Acetonitrile	94–96 % (48 h)	30	Torres and Otero 1999
C6–C18 glycolates	C6–C18 fatty alcohols	Glycolic acid	Novozym 435	Hexane	91 % (48 h)		
Ethyl glycoside lactate	Ethyl glycoside	Butyl lactate	Novozym 435	Solvent-free	95 % (36 h)	60	Wei et al. 2002
β-Methyl glycoside malate/glycolate/lactate	β-Methyl glycoside	Malic/glycolic/lactic acid	Novozym 435	*t*-Butanol	48–75 % (120 h)	60	Park et al. 2001
Palmitoyl or stearoyl lactic acid	C16 or C18:0 fatty acid	Lactic acid	Lipozyme IM20	Ethyl methyl ketone	37.5–40 % (72 h)	37	or 60
Kiran and Divakar 2001							
Examples of kojic acid derivatives							
Kojic acid monoricinoleate	Ricinoleic acid	Kojic acid	Lipozyme TL IM	Solvent-free	87.4 % (6 h)	80	El-Boulifi et al. 2014
Kojic acid monooleate	Oleic acid	Kojic acid	Amano G	Acetonitrile	36.7 % (48 h)	50	Liu and Shaw 1998
Kojic acid monopalmitate	Palmitic acid	Kojic acid	RM IM	Acetonitrile	29.30 % (12 h)	50	Lajis et al. 2013
Examples of lipoic acid derivatives							
Pyridoxine-O-lipoate (5′ and 4′)/tyrosol-8-O-lipoate/tyramin-8-N-lipoate	Pyridoxine (vitamin B6) Tyrosol/tyramine	Lipoic acid	CNTs-C6-NH2-CaLB or CNTs-C11-CH3-CaLB	(mtoa)NTf2 (bmim)PF6	91.1–99.5 % (72 h)	60	Papadopoulou et al. 2013
Phenolic lipoates	4-Hydroxybenzyl alcohol/vanillyl alcohol/4-hydroxyphenyl ethanol/coniferyl alcohol/dihydroxybenzyl alcohol/dihydroxyphenyl ethanol	Lipoic acid	Novozym 435	2-Butanone: hexane	64–80 % (15 h)	25	Kaki et al. 2012
Octanyl lipoate	n-Octanol	α-Lipoic acid	Whole-cell lipase from *Aspergillus oryzae* WZ007	Heptane	75.2 % (48 h)	50	Yang et al. 2009
Examples of arbutin derivatives							
Arbutin lipoate	α-Lipoic acid	β-Arbutin	Type B lipase from *C. antarctica*	*t*-Butanol	– (7 days)	55	Ishihara et al. 2010
C2–C18 alkyl arbutin esters	Vinyl esters of C2–C18 aliphatic alcohols	β-Arbutin	Immobilized lipase from *Penicillium expansum*	Anhydrous THF	82–99 % (0.5–72 h)	35	Yang et al. 2010a
Arbutin phenolic acid esters	Vinyl esters of aromatic acids				30–99 % (4–96 h)	50	Yang et al. 2010b
Arbutin fatty acid esters	Saturated fatty acids (C6–C18)	β-Arbutin	Chirazyme L-2 C2	Acetonitrile	Up to 45 % (2 days)	60	Nagai et al. 2009
Arbutin ferulate	Ferulic acid	β-Arbutin	Type B lipase from *C. antarctica*	*t*-Butanol	57 % (7 days)	55	Ishihara et al. 2010
	Vinyl ferulate	p-Arbutin	Novozym 435	Acetonitrile	50 % (–)	45	Chigorimbo-Murefu et al. 2009
Examples of vitamin derivatives							
L-Ascorbyl palmitate	Palmitic acid	L-Ascorbic acid	Lipase from *Bacillus stearothermophilus* SB1	Hexane	97 % (6 h)	50	Bradoo et al. 1999
	Methyl palmitate		Lipase from *Burkholderia multivoras*	Solvent-free (under microwave irradiation)	83 % (40 min)	80	Kidwai et al. 2009
	Ethyl palmitate		Lipozyme TL IM	*t*-Butanol	20 % (120 h)	40	Reyes-Duarte et al. 2011

Table 1 (continued)

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
	Vinyl palmitate				100 % (120 h)		
	Tripalmitin				50 % (140 h)		
L-Ascorbyl oleate	Oleic acid	L-Ascorbic acid	Novozym 435	*t*-Amyl alcohol	82 % (52 h)	65	Viklund et al. 2003
	Methyl oleate		Lipozyme TL IM	*t*-Butanol	50 % (–)	60	Reyes-Duarte et al. 2011
	Triolein		Novozym 435	*t*-Amyl alcohol	84 % (140 h)	40	Moreno-Perez et al. 2013
	Olive oil		Novozyme 435-PEI		85 % (48 h)	45	
Conjugated linoleoyl ascorbates	C9t11CLA	L-Ascorbic acid	Chirazyme L-2 C3	Acetone	~80 % (~48 h)	50	Watanabe et al. 2008
L-Ascorbyl benzoate	Benzoic acid	L-Ascorbic acid	Novozym 435	Cyclohexanone	47.7 % (48)	66.6	Lv et al. 2008
L-Ascorbyl acetate	Vinyl acetate	L-Ascorbic acid	Lipozyme TLIM	Acetone	99 % (4)	40	Zhang et al. 2012
Vitamin E succinate	Succinic anydride	Rac-all-α-tocopherol	Succinyl-Novozym 435	DMSO:*t*-butanol	94.4 % (48 h)	40	Yin et al. 2011
Vitamin E acetate	Vinyl acetate	δ-Tocopherol α-Tocopherol	Novozym 435	*t*-Amyl alcohol	65 % (16 days) >45 % (16 days)	60	Torres et al. 2008b
Vitamin E ferulate	Ethyl ferulate	Vitamin E	Novozym 435	Solvent-free	25.2 % (72 h)	60	Xin et al. 2011
Sugar or ascorbyl retinyl adipates	Sorbitol/fructose/glucose/saccharose/maltose/ascorbic acid	Retinyl adipate	Novozym 435	*t*-Amyl alcohol	22–80 % (45 h)	40	Rejasse et al. 2003
Vitamin A lactate	Lactic acid	Vitamin A acetate	Immobilized lipase from *C. antarctica*	Hexane	32 % (7 h)	30	Liu et al. 2012
	Methyl lactate	Retinol	Lipozyme		86 % (50 h)	55	Maugard and Legoy 2000
Vitamin A oleate	Methyl oleate			Hexane	90 % (50 h)		
	Oleic acid	Retinyl acetate	Immobilized lipase from *C. antarctica*		79 % (7 h)	30	Liu et al. 2012
Vitamin A saturated fatty acid esters (C6–C18)	C6–C18 saturated fatty acids			Hexane	51–82 % (7 h)		
Vitamin A methyl succinate	Dimethyl succinate	Retinol	Lipozyme	Hexane	77 % (50 h)	55	Maugard and Legoy 2000
Examples of flavonoid derivatives							
Quercetin derivatives	C18–C12 fatty acids	Isoquercetin	Novozym 435	Acetone or acetonitrile	81–98 % (18–24 h)	45–	60
	Ziaullah 2013						
	Ethyl esters of C4–C18 fatty acids		Novozym 435	*t*-Amyl alcohol	38–66 % (72 h)	65	Salem et al. 2010
	Cinnamic acids		Novozym 435	*t*-Butanol	17–89 % (7 days)	60	Stevenson et al. 2006
	Dibenzyl malonate		Lipase from *C. antarctica*	Me_2CO: pyridine	74 % (12 days)	45	Riva 1996
	Vinyl acetate	PSL-C II	Acetone		50	Ch-	ebil et al. 2007
				84 % (96 h)			
Quercetin							
				100 % (24 h)			
Silybin derivatives	Divinyl ester of decanoic acid	Silybin	Novozym 435	Acetonitrile	26–66 % (72 h)	45	Vavrikova et al. 2014
	Vinyl butanoate		Novozym 435	Acetone	100 % (24–96 h)	50	Theodosiou et al. 2009
	Vinyl acetate		Novozym 435	Acetone	92 % (48 h)	35	Gazak et al. 2010
Esculin derivatives	Fatty acids, dicarboxylic acids, other cyclic acids	Esculin	Novozym 435	*t*-Amyl alcohol	13–90 % (12 h)	60	Ardhaoui et al. 2004a
	Palmitic acid		Novozym 435	TOMA TF2N	>96 % (6 days)	60	Lue et al. 2010
	Vinyl butyrate		Novozym 435	[Bmim]BF6	90.6 % (72 h)	60	Katsoura et al. 2007
Phloridzin derivatives	C2–C18 fatty acids	Phloridzin	Novozym 435	Acetonitrile	70–90 % (7 days)	65	Milisavljecic et al. 2014

Table 1 (continued)

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
	Ethyl cinnamate		Novozym 435	Solvent-free	100 % (4 h)	80	Enaud et al. 2004
Hesperedin derivatives	Decanoic acid	Hesperidin	Novozym 435	[Bmim]BF4: acetone	53.6 % (96 h)	50	Branco de Araujo et al. 2011
	Palmitic acid		Novozym SP435	*t*-Amyl alcohol	Up to 40 % (12 h)	60	Ardhaoui et al. 2004b
	Vinyl acetate	Hesperetin	PSL-C II	Acetonitrile	30 % (96 h)	50	Chebil et al. 2007
Rutin derivatives	C4–C18 fatty acids	Rutin	CALB	*t*-Amyl alcohol	27–62 % (168 h)	60	Viskupicova et al. 2010
	Ethyl linoleate		Novozym 435	Acetone	50 % (96 h)	50	Mellou et al. 2006
	Methyl palmitate		Novozym 435	*t*-Amyl alcohol	30 % (48 h)	60	Passicos et al. 2004
	Vinyl esters of fatty acids		Novozym 435	[Bmim]BF4	15–65 % (96 h)	60	Katsoura et al. 2006
	Dicarboxylic acids, fatty acids, other cyclic acids		Novozym 435	*t*-Amyl alcohol	10–90 % (−)	60	Ardhaoui et al. 2004a
	Divinyl dicarboxylate		Novozym 435	*t*-Butanol	36 % (4 days)	50	Xiao et al. 2005
	Dibenzyl malonate		Lipase from *C. antarctica*	Me2CO: pyridine	79 % (12 h)	45	Riva 1996
	Vinyl cinnamate		Chirazyme L-2	Acetone	28 % (14 h)	37	Ishihara et al. 2002
Naringin derivatives	α-Linolenic acid, linoleic, oleic acid	Naringin	Novozym 435	*t*-Amyl alcohol	83.2–90.1 % (72 h) (assisted by ultrasound irradiation)	50	Zheng et al. 2013
	Stearic acid		Novozym 435	*t*-Amyl alcohol	46 % (24 h)	60	Duan et al. 2006
	Vinyl butyrate		Novozym 435	$[Bmim]BF_4$	86.9 % (100 h)	60	Katsoura et al. 2007
	Methyl palmitate		Novozym 435	*t*-Amyl alcohol	92 % (48 h)	60	Passicos et al. 2004
	C10–C12 vinyl esters of saturated fatty acids		Novozym 435	Acetone	22–70 % (96 h)	50	Mellou et al. 2005
	PUFA from byfish products		Novozym 435	*t*-Amyl alcohol	30 % (96 h)	50	Mbatia et al. 2011
	Vinyl laurate		Lipozyme IM TL	*t*-Amyl alcohol	90 % (30 min)	52	Luo et al. 2013
	Lauric acid		Chirazyme L-2 C2	Acetonitrile	~45 % (~30 h)	60	Watanabe et al. 2009
	Ricinoleic acid		Immobilized lipase from *C. antarctica*	Acetone	24 % (120 h)	50	Almeida et al. 2012
	Castor oil				33 % (120 h)		
	Vinyl cinnamate		Chirazyme L-2	Acetone	64 % (14 days)	37	Ishihara et al. 2002
	Dibenzyl malonate		Lipase from *C. antarctica*	Acetone: pyridine	69 % (12 days)	45	Riva 1996
	Vinyl acetate	Naringenin	PSL-C II	Acetonitrile	100 % (96 h)	50	Chebil et al. 2007
Examples of hydroxycinnamic acid derivatives							
Feruloylated lipids	Ferulic acid	Glycerol	Chirazyme L2 C-2	Solvent-free	80 % (>3 h)	80	Matsuo et al. 2008
		Trilinolenin	Novozym 435	Hexane:2-butanone	14 % (5 days)	55	Sabally et al. 2006
		Flaxseed oil	Novozym 435	$SCCO_2$ medium	57.6 % (27.5 h)	80	Ciftci and Saldana 2012
	Ethyl ferulate	Oleyl alcohol	Novozym 435	Hexane	99.17 % (4 days)	60	Chen et al. 2011b
		Triolein	Novozym 435	Toluene	77 % (144 h)	60	Compton et al. 2000
		Olive oil	Novozym 435	2M2B: toluene	59.6 % (2.34 h)	60	Radzi et al. 2014
		Tributyrin	Novozym 435	Solvent-free	94.2 % (120 h)	50	Zheng et al. 2008
		Monostearin	Novozym 435	Ethanol	97.8 % (23 h)	74	Sun et al. 2013a, b
		Soybean oil	Novozym 435	Glycerol	70 % (140 h)	60	Laszlo and Compton 2006
		Fish oil	Novozym 435	Toluene	80.4 % (5 days)	70	Yang et al. 2012
		Phosphatidylcholine	Novozym 435	Chloroform	40.51 % (4 days)	55	Yang et al. 2013
		Glycerol	Novozym 435	EMIMTF2N	100 % (12 h)	70	Sun et al. 2013a, b
		Oleic acid	Novozym 435	Solvent-free	96 % (1.33 h)	60	Sun et al. 2007

Table 1 (continued)

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
	Glyceryl ferulate	Oleic acid	Novozym 435	[Bmim]PF6	100 % (3 h)	80	Sun et al. 2009
	Vinyl ferulate	Triolein	Novozym 435	Solvent-free	91.9 % (62 h)	55	Yu et al. 2010
Methyl caffeate	Caffeic acid	Methanol	Novozym 435	[Bmim][Tf_2N]	99.79 % (9 h)	75	Wang et al. 2015
Propyl caffeate	Methyl caffeate	1-Propanol	Novozym435	[Bmim][CF_3SO_3]	99.5 % (2.5 h)	60	Wang et al. 2013
Sitosteryl hydroxycinnamates	Vinyl ferulate/caffeate/sinapate	Sitosterol	Lipase type VII from *Candida rugosa*	Hexane:2-butanone	30–90 % (−)	45	Tan and Shahidi 2011; Tan and Shahidi 2012; Tan and Shahidi 2013
Examples of galloyl derivatives							
Propyl gallate	Gallic acid	1-Propanol	Immobilized lipase from *Staphylococcus xylosus*	Hexane	90 % (6 h)	52	Bouaziz et al. 2010
Mono-, di-, and tri- acetylated EGCG	Vinyl acetate	EGCG	Lipozyme RM IM	Acetonitrile	87.37 % (1.13 h)	40	Zhu et al. 2014
Catechin 5-O and 7-O acetate	Vinyl acetate	Catechin	PCL	Acetonitrile	70 % (48 h)	45	Lambusta et al. 1993

Novozym 435: lipase B from *Candida antarctica* immobilized on a macroporous acrylic resin (CALB); lipozyme IM20/lipozyme RM IM: lipase from *Rhizomucor miehei* immobilized on duolite anion exchange resion; lipozyme TL IM: lipase from *Thermomyces laniginosus* immobilized on silica granulation; amano G: lipase from *Penicillum camemberti*; CNTs-C_6-NH_2-CaLB, CNTs-C_{11}-CH_3-CAL-B: novozym 435 functionalized with various multi-walled carbon nanotube groups; chirazyme L-2: immobilized lipase B from *C. Antarctica*; succinyl-novozym 435: novozym 435 modified with succinic anhydride; PSL-C II, PCL: lipase from *Pseudomonas cepacia*

hyperpigmentation, and skin wrinkling. Its primary use in cosmetics is as a skin whitening agent but there are concerns regarding its skin compatibility, oil solubility, and storage stability even at ordinary temperatures. Additionally, there is evidence of toxicity, irritancy, and carcinogenicity (Lajis et al. 2012). The first attempts on the enzymatic modification of kojic acid focused on the synthesis of kojic acid glycosides using a sucrose phosphorylase from *Leuconostoc mesenteroides*, an α-amylase from *Bacillus subtilis* and an immobilized β-galactosidase from *Bacillus circulans* (Nishimura et al. 1994; Kitao and Serine 1994; Hassan et al. 1995). However, many lipophilic derivatives such as saturated fatty (C6-C18) acid esters and the unsaturated kojic acid monoricinolate and monooleate have been synthesized by commercial lipases (Liu and Shaw 1998; Lajis et al. 2013; Khamaruddin et al. 2008; El-Boulifi et al. 2014; Ashari et al. 2009). A phospholipase from *Streptomyces* sp. has synthesized phosphatidylkojic acid at 60 % yield from a dipalmitoylphosphatidyl residue (Takami et al. 1994). Kojic acid has two OH– groups, the primary at C-7 and the secondary one at C-5 which is essential to the radical scavenging and tyrosinase interference activity. Many derivatives have been synthesized by modifying the 5-OH group; nevertheless, CALB showed moderate yield (53 %) synthesizing a laurate product esterified at the primary C-7 (Kobayashi et al. 2001; Chen et al. 2002).

Lipoic acid derivatives

α-Lipoic acid is a dithiol compound containing two sulfur atoms at the C-6 and C-8 carbons connected by a disulfide bond. It takes part in the anti-oxidant defense system of the cell through its ability to scavenge free radicals both in lipid and aqueous environments. This amphiphilicity constitutes it an ideal candidate for use in both oil- and water-based formulations. Moreover, it participates in the regeneration of anti-oxidants (i.e., vitamic C, vitamin E) and in the de novo synthesis of endogenous anti-oxidants (i.e., glutathione) and shows metal ion chelating activity, while it can repair oxidative damage in macromolecules (Papadopoulou et al. 2013). Other attractive properties include anti-inflammatory activity, aid in the treatment of diseases such as diabetes, atherosclerosis, cardiovascular, heavy-metal poisoning, radiation damage, cancer, Alzheimer's, and AIDS (Maczurek et al. 2008). Synthesis of lipoic acid phenolic derivatives by CALB showed that a prior aromatic hydroxylation of the donor offered higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity to the products. The hydroxytyrosol ester of lipoic acid showed similar anti-oxidant activity to α-tocopherol but higher than the commercial butylated hydroxytoluene (BHT) (Kaki et al. 2012). Lipoic acid is found in a racemic mixture, where the (R)-enantiomer is much more active than the (S)-enantiomer. Only lipases from *Candida rugosa* and *Aspergillus oryzae* (whole cell) have been reported to enable kinetic resolution of racemic α-lipoic acid (Yan et al. 2009; Fadnavis et al. 1998).

Hydroquinone derivatives

Hydroquinone, a phenolic compound with two –OH groups at the para positions of the benzene ring, has been commercially used in cosmetics at concentrations <1 % as an anti-oxidant, fragrance, reducing agent, or polymerization inhibitor (Andersen et al. 2010). Its most promising use is as a skin whitening agent; however, it is prone to cause irritations and dermatitis (Kang et al. 2009). Its glycosylated derivative, arbutin, has attracted attention as a better tyrosinase inhibitor when compared to conventional agents as it inhibits melanogenesis without causing melanocytotoxicity (Sugimoto et al. 2005). It also plays an important role in scavenging free radicals, as an anti-inflammatory, and an anti-microbial agent (Lee and Kim 2012). Arbutin has two isomers (α- and β-). The first is synthesized by chemical or enzymatic methods and shows higher efficiency and stability while the latter is extracted from natural sources such as bearberry, cranberry, blueberry, and pears (Seo et al. 2012a). α-Arbutin possesses a 10-fold stronger inhibitory effect on the activity of tyrosinase from human malignant melanoma cells compared to its anomer, whereas β-arbutin reduces tyrosinase activities from mushroom and mouse melanoma (Seo et al. 2012b). α-Arbutin shows extremely increased browning resistance to light irradiation compared to hydroquinone (Kitao and Sekine 1994). Lipases have been used for the acylation of β-arbutin with aromatic or fatty acids showing absolute regioselectivity at the 6′ position. This phenomenon can be attributed to the hypothesis that the primary OH– group of the sugar moiety is less hindered so it can enter more easily into the active site of the lipase and attach the acyl-enzyme intermediate. Studies on immobilized lipase from *Penicillium expansum* showed that the elongation of the donor chain length (C2–C8) results in higher initial yields perhaps due to stronger interactions with the hydrophobic acyl binding site of the enzyme. Branched-chain acyl donors affect negatively the initial rate due to steric strain while the presence of a conjugated C–C double bond adjacent to the carbonyl moiety decreases the rate substantially (Yang et al. 2010a). The radical scavenging activity of acyl (C6–C18) arbutin is independent of the chain length (Nagai et al. 2009). Fatty acid derivatives of arbutin show higher anti-melanogenesis and anti-oxidant activity than arbutin which could be allied to the improved membrane penetration, due to increased lipophilicity (Watanabe et al. 2009). Synthesized by CALB, arbutin ferulate was found to have 19 % higher activity against the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical than ferulic acid and be 10 % more efficient towards low-density lipoprotein (LDL), showing higher anti-oxidant than Trolox, a well-

known analog of vitamin E and commercial anti-oxidant (Chigorimbo-Murefu et al. 2009).

Vitamin derivatives

L-Ascorbic acid (vitamin C) is a potent water-soluble natural anti-oxidant that has been used in cosmetics as a preservative, pH adjuster, or/and an active compound. It has been proved that ascorbates promote collagen synthesis in human skin fibroblasts in vitro up to eightfold capacity, while they show photoprotective activity against UVA and UVB irradiation and have wound healing properties (Murad et al. 1981). Drawbacks as instability, poor liposolubility, and low skin penetrability have led to modifications. Common saturated fatty acid derivatives, as ascorbyl palmitate and ascorbyl stearate, have been synthesized showing that there is no negative effect on the radical scavenging activity by introducing a saturated group at the C-6 position of ascorbic acid (Watanabe et al. 2003). Enzymatic synthesis of ascorbyl palmitate is focused on the esterification of palmitic acid with a vast use of CALB in organic solvents or ionic liquids. Other commercial lipases have been employed offering varying yields (6–97 %) (Gulati et al. 1999; Costa et al. 2014; Park et al. 2003; Hsieh et al. 2006, Bradoo et al. 1999). However, saturated fatty acid esters still show moderate solubility in oils. Further improvement can be done by introducing a double bond in the fatty acid, resulting in superior products in terms of solubility and radical scavenging capacity. For instance, oleic acid is readily available and inexpensive (Viklund et al. 2003). There are reports on esterification of olive oil, palm oil, or lard offering a simple, direct, and natural route for synthesis (Moreno-Perez et al. 2013; Zhao et al. 2014; Burham et al. 2009). Derivatization of L-ascorbic acid requires mild conditions to prevent oxidation of both acid and its esters and high regioselectivity for the 6-*O*-position which is achieved by lipases (Zhang et al. 2012). However, the demand of polar solvents for enhancing substrate solubility tends to be deleterious for their stability. Coating is an effective way to protect immobilized lipases from denaturation reducing the interactions with the solvent (Moreno-Perez et al. 2013). The use of vinyl ester donors increases the reaction rate, but implies the release of fatty acids from oils and their further activation. For instance, CALB offered 100 % conversion of vinyl palmitate in *t*-butanol (Reyes-Duarte et al. 2011). When methyl esters are used, the by-product methanol is insoluble in oils, gets adsorbed onto the surface of the immobilized lipase, and leads to negative effects on enzyme activity and operational stability.

Vitamin E is a general term for a group of amphiphilic lipids, comprising of four tocopherols, having a saturated phytyl side chain attached to the chromanol core and four tocotrienols having an attached unsaturated isoprenoid side chain. The analogs differ with each other by the presence and placement of methyl groups around the aromatic ring. In nature, vitamin E occurs only in the *RRR*-form, while *RRR*-α-tocopherol is the most bioactive. Synthetic vitamin E (α-tocopherol) is a racemic mixture of eight stereoisomers in equal amounts (*all-rac*-α), of which not all are as bioactive as the natural form (Torres et al. 2008a). Vitamin E is non-irritant to the eyes and skin, has high anti-oxidant activity with anti-aging and anti-tumor potential, inhibits the UVB-induced lipid peroxidation, and shows skin-improving properties with anti-inflammatory and beneficial effect on the skin barrier function (Zondlo Fiume 2002). However, it is readily destabilized by light and oxygen. Non-enantioselective acetylation of vitamin E at the C-6 carbon has been performed only by CALB among other tested enzymes which can be explained by studies that show that the acceptor binding site is deeper in lipase B (Torres et al. 2008b; Pleiss et al. 1998). δ-Tocopherol gave higher rates due to its lower methylation degree, while competitive acetylation experiments indicated that there is steric hindrance caused by the aliphatic chain and not the chromanol ring. Vitamin E succinate has been synthesized by modified CALB yielding 94 % and by a lipase from *C. rugosa* with moderate yields (Yin et al. 2011; Jiang et al. 2013). Synthesized at lower yields (25.2 %) by CALB, novel vitamin E ferulate inhibits melanogenesis in human melanoma cells, being an attractive candidate as a skin-whitening agent (Xin et al. 2011).

Vitamin A includes a group of unsaturated compounds, i.e., retinol, retinoic acid, and retinaldehyde, which are widely used in cosmetic and skin care products because of their anti-oxidant, anti-aging, and skin-whitening properties. Retinol is the most active form of vitamin A; however, retinoids are readily oxidized in air and inactivated by UV light while they are water-insoluble and skin-irritating (Maugard and Legoy 2000). The most common modification of retinol is retinyl palmitate, which is stable, slightly irritating, and not sensitizing at concentrations between 0.1 and 1 % (CIR 1987). It has been synthesized by the esterification of palmitic acid using CALB, a lipase from *Candida* sp. and a modified lipase from *Pseudomonas fluorescens* (Ajima et al. 1986; Yin et al. 2006; Liu et al. 2012). Other vitamin A modifications include saturated fatty acid esters, oleate, lactate, and succinate/methylsuccinate derivatives catalyzed by CALB or *Rhizomucor miehei* lipase (Maugard and Legoy 2000; Liu et al. 2012). Rejasse et al. (2003) proposed a vitamin A inter-esterification reaction using CALB. The first step included esterification of adipic acid with retinol in *t*-amyl alcohol, while after 24 h, a polyol was added resulting in products with varying yields (22–80 %).

Flavonoid derivatives

Aglycon and glycosylated flavonoids are natural compounds of plant origin that have aroused interest for their anti-viral,

anti-allergic, anti-inflammatory, anti-oxidant activities, and the protection against cardiovascular diseases and cancer (Salas et al. 2011). Their basic structure is derived from 2-phenylbenzo-γ-pyran, where the original skeleton is substituted with numerous OH– groups that result in a considerably hydrophilic nature. The effect of acyl donors on esculin and rutin modification by CALB has been studied in microreactors offering conversion rates higher than 9.5 10^{-2} mmol L^{-1} h^{-1}(Ardhaoui et al. 2004a). Naringin esterification in a continuous flow microreactor offered more than 85 % conversion to 6-O″-monoesters. Regioselective acylation in microreactors offers mild reaction conditions, short reaction times, and high yields (Luo et al. 2013). Vinyl esters of saturated fatty acids are more reactive giving approximately a threefold increase in the conversion of naringin (Mellou et al. 2005). The enzymatic acylation of two isolated chrysoeriol glucosides by CALB resulted in products with higher anti-oxidant and anti-microbial activity against Gram-negative and Gram-positive bacteria that can be attributed to the increased interaction of the hydrophobic chain with the cell membrane due to modified lipophilicity. Irilone, chrysin, and dihydromyricetin acetate have been synthesized by *Pseudomonas* (syn *Burkholderia*) *cepacia* lipases and an immobilized lipase from *P. expansum* (Nazir et al. 2009; Chebil et al. 2007; Li et al. 2015). Orientin, vitexin, salicin fatty acid esters, and helicin butyrate have been synthesized by CALB (Liu et al. 2015; Katsoura et al. 2007). Silibyn, which occurs in nature as an equimolar mixture of two diastereoisomers (A and B) with different biological activities, has been acylated by CALB at the C-23 position producing new anti-viral and anti-tumor compounds (Gazak et al. 2010). Modification (e.g., methylation) of the C-7 OH which bears a pro-oxidant potential significantly improves the anti-radical activity of silybin.

The nature of flavonoid and the origin of lipase are crucial for product formation. Generally, flavonoids with a primary OH– group on the glycosyl moiety as naringin are more reactive than those with secondary OH– groups only, as rutin. Chebil et al. (2007) showed that isoquercetrin, the glycosylated form of quercetin, could be acylated by both CALB and *P. cepacia* lipase (PSL) although only the latter could acylate quercetin. In the absence of the 4′-OH group of quercetin (hesperetin), PSL showed preference for the 7-OH group, followed by the 3′-OH group which can be explained by steric hindrance from the C-4′ methoxy group. Chrysin was acylated only at the 7-OH group since the 5-OH group is not reactive when a 4-oxo group is present in the structure of the flavonoid. Molecular modeling regarding the regioselectivity of CALB showed that the aglycon part of both rutin and isoquercitrin was localized at the entrance of the enzyme's binding pocket stabilized by hydrogen bond and hydrophobic interactions (de Oliveira et al. 2009). The sugar part was placed close to the pocket bottom. Only the primary 6′-OH group of isoquercitrin glucose and the secondary 4″-OH group of rutin rhamnose were expected to be acetylated as they were the only ones to stabilize simultaneously near the catalytic histidine and the acetate bound to the catalytic serine. CALB synthesized monoesters on the primary OH of glucose moiety of esculin and on the secondary 4‴-OH of the rhamnose residue of rutin (Ardhaoui et al. 2004b). Acylation of quercetin was not achieved as the 4′-OH is conjugated with the C-4 carbonyl group favoring a planar formation of the molecule, which may not be suitable for the catalytic site of the enzyme (Nazir et al. 2009).

Hydroxycinnamic acid derivatives

Hydroxycinnamic acids (HCAs; ferulic, FA; *p*-coumaric, p-CA; caffeic, CA; sinapic, SA) are a class of phenylpropanoids known as more active anti-oxidants than hydroxybenzoic acids due to the presence of the C=COOH group (Widjaja et al. 2008). They are ubiquitous in nature as a component of arabinoxylans in plant cell walls offering linkage with lignin while they present broad spectrum of biological activities including anti-bacterial, anti-viral, anti-inflammatory, anti-carcinogenic, anti-HIV, and anti-tumor effects (Tan and Shahidi 2012). However, their solubility is poor in hydrophilic and lipophilic media. Among many natural photoprotective agents, feruloylated lipids have gained attention due to their strong anti-oxidant, skin-whitening, anti-wrinkling, and UV absorptive ability (Radzi et al. 2014). FA is believed to suppress melanin generation by antagonizing tyrosine because its structure is similar to tyrosine (Chandel et al. 2011). Enzymatic synthesis of green sunscreens offers stability and selectivity in contrast with chemical synthesis that is limited due to heat sensitivity and oxidation susceptibility of FA in alkaline media. A two-step synthesis of feruloylated diacylglycerols using CALB has been proposed by Sun et al. (2007) including the transesterification of ethyl ferulate with glycerol and the subsequent transesterification of glyceryl ferulate with oleic acid offering high yield of products (up to 96 %). Esterification of FA to glyceryl ferulate by CALB has been performed in a continuous reactor reaching 80 % conversion and productivity of 430 kg/m^3/reactor day (Matsuo et al. 2008). Biocatalysis under vacuum-rotary evaporation contributes to increased conversion by eliminating external mass transfer resistance, effective interaction among different phases of enzymatic reaction, minimizing the negative effects of by-product ethanol (when ethyl ferulate is used as donor) on lipase activity (Xin et al. 2009). 1,3-Diferuloyl-*sn*-glycerol has been synthesized by CALB in a pilot plant scale bed reactor as by-product of the transesterification of ethyl ferulate with soybean oil (Compton and Laszlo 2009). One hundred

twenty kilograms of diferuloyl glycerol by-product could be isolated annually. Enzymatic esterification of olive, flaxseed, and fish oil offers low cost and greener configurations to the process (Ciftci and Saldana 2012; Yang et al. 2012; Radzi et al. 2014). Transesterification of ethyl ferulate with castor oil, catalyzed by CALB, yielded 62.6 % lipophilic and 37.3 % hydrophilic products (Sun et al. 2014). Esterification of FA with fatty (C2–C8) alcohols improves the anti-oxidant capacity towards the oxidation of HDL, LDL, and total serum. Probably, the lipophilic properties of anti-oxidants affect their incorporation into the lipid part of lipoproteins reaching the site of lipoperoxidation, accounting for the increased anti-oxidant activity (Jakovetic et al. 2013).

Transesterification of methyl caffeate to propyl caffeate by CALB was performed in a continuous flow packed bed microreactor offering 99.5 % yield. The calculated kinetic constant K_m was 16 times lower than than of a batch reactor (Wang et al. 2013). Caffeic acid phenethyl ester (CAPE) is a flavonoid-like compound and one of the major components of honeybee propolis possessing anti-inflammatory, anti-carcinogenic, and neuroprotective properties (Widjaja et al. 2008). High yield CAPE synthesis has been performed by CALB in a packed bed reactor, using ultrasound treatment or in one-pot reactions using organic solvents or ionic liquids (Chen et al. 2010, 2011a; Ha et al. 2012; Wang et al. 2014). One-pot synthesis of a CAPE analog, 3-cyclohexyl caffeate, has been performed by esterification of caffeoylquinic acids derived from coffee beans with methanol using a chlorogenate hydrolase followed by the transesterification of methyl caffeate with 3-cyclohecylpropyl caffeate using CALB in [Bmim][NTf_2] (Kurata et al. 2011). Synthesized by a *C. rugosa* lipase, phytosteryl caffeate showed twofold increase in oxygen radical absorbance capacity (ORAC) comparing to the parent vinyl HCA, while phytosteryl ferulate showed a 10-fold increased anti-oxidant activity compared to Trolox and a twofold increase comparing to vinyl ferulate (Tan and Shahidi 2011, 2012). Chigorimbo-Murefu et al. (2009) synthesized arbutin and hydroxyl steroid esters of FA in *t*-methyl-ethyl ether showing higher anti-oxidant activity than Trolox and their starting hydroxycinnamate. Arbutin ferulate possessed 19 % higher anti-radical activity against ABTS free radical than FA and inhibited 10 % more efficiently LDL oxidation than its precursors.

Although FAEs are less stable in organic media and low water content than lipases, they show higher substrate specificity (Zeuner et al. 2011). Some examples of FAE-catalyzed reactions are presented in Table 2. In 2001, Giuliani et al. succeeded for the first time the synthesis of 1-pentyl-ferulate using a FAE from *Aspergillus niger* in a water-in-oil microemulsions. Since then, novel FAEs from filamentous fungi such as *Fusarium oxysporum*, *Myceliophthora thermophila* (syn *Sporotrichum thermophile*), and *Talaromyces stipitatus* have been employed in detergentless microemulsions for the transesterification of methyl donors to alkyl hydroxycinnamates, feruloylated-arabino-oligosaccharides showing regioselectivity for the primary hydroxyl group of the non-reducing arabinofuranose ring and other sugar ferulates (Topakas et al. 2003a; Vafiadi et al. 2005, 2006, 2007b, 2008a). Although esterification with fatty alcohols generally results in more lipophilic products, the glyceryl esters of HCAs have been proved more hydrophilic than their donors. Fed-batch esterification of FA with diglycerin was performed by a FAE from *A. niger* under reduced pressure yielding 69 % feruloyl and 21 % diferuloyl diglycerols (Kikugawa et al. 2012). The major product (FA-DG1) showed highest water solubility while all products maintained their radical scavenging activity against DPPH and their UV absorption properties. Diferuloyl diglycerols showed a twofold increase of anti-oxidant activity comparing to feruloyl diglycerols and FA. Esterification of SA and *p*-CA with glycerol yielded 70 % glycerol sinapate and 60 % glycerol-*p*-coumarate, respectively, with indication of the formation of minor dicinnamoyl glycerol esters (Tsuchiyama et al. 2007). The ability of glycerol sinapate to scavenge DPHH radicals was higher than BHT while it maintained its UV absorption properties.

Galloyl derivatives

Tannins, natural occurring polyphenols that can be found in pine and spruce bark, vegetables, and fruits, are categorized into hydrolysable, condensed, and complex. The simplest hydrolysable tannins, commonly named gallotannins, consist of gallic acid molecules esterified to a core polyol. The biocatalytic synthesis of gallic acid esters is performed mainly by tannases and may follow different routes: (1) hydrolysis of tannic acid into gallic acid and further esterification to galloyl esters, (2) direct esterification of tannic acid into a galloyl ester, or (3) transesterification of galloyl esters into another ester. Examples of tannase-based reactions are presented in Table 3. A well-known synthetic galloyl ester widely used in skin cleaning/care products, make up, sunscreen, and tanning products is propyl gallate. Its biological activities are not limited to the free-radical scavenging ability as it exhibits anti-microbial, anti-nociceptive activity, ultraviolet (UV) radiation protection, anti-cariogenesis, anti-mutagenesis, and anti-carcinogenesis effects. However, in cosmetic formulations, its concentration is low (up to 0.1 %) due to indications for skin irritation or sensitization (CIR 2007). Applications of propyl gallate expand into the food, pharmaceutical, adhesive, lubricant, and biodiesel industry where it has been used as an anti-oxidant additive, for more than 20 years (Zhang 2015).

The majority of tannases used for the synthesis of propyl gallate are carrier-bound or modified. A mycelium-bound tannase from *A. niger* esterified gallic acid at 65 % yield (Yu

Table 2 Ferulic acid esterase-catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
1-Pentyl ferulate	Ferulic acid	1-Pentanol	FAEA	CTAB: hexane: pentanol: buffer	60 % (n.q.)	40	Giuliani et al. 2001
1-Butyl ferulate	Methyl ferulate	1-Butanol	CLEAs immobilized Ultraflo L	Hexane: 1-butanol: buffer	97 % (144 h)	37	Vafiadi et al. 2008a
1-Butyl sinapate	Methyl sinapate	1-Butanol	AnFaeA	Hexane: 1-butanol: buffer	78 % (120 h)	35	Vafiadi et al. 2008b
2-Butyl sinapate	Methyl sinapate	2-Butanol	AnFaeA	Hexane: 2-butanol: buffer	9 % (120 h)	37	Vafiadi et al. 2008a
1-Butyl caffeate	Methyl caffeate	1-Butanol	StFae-A	Hexane: 1-butanol: buffer	Up to 25 % (144 h)	35	Topakas et al. 2004
1-Butyl-*p*-coumarate	Methyl *p*-coumarate	1-Butanol	FoFae-I	Hexane: 1-butanol: buffer	Up to 70 % (144 h)	35	Topakas et al. 2003a
1-Propyl-*p*-hydroxyphenylacetate	*p*-Hydroxyphenylacetic acid	1-Propanol	FoFae-II	Hexane: 1-propanol: buffer	75 % (224 h)	30	Topakas et al. 2003b
1-Propyl-*p*-hydroxylphenylpropionate	*p*-Hydroxylphenylpropionic acid				70 % (224 h)		
Glycerol sinapate	Sinapic acid Methyl sinapate	Glycerol	AnFaeA	[C_5OHmim][PF_6]: buffer	76.7 % (24 h) Up to 7 % (120 h)	50	Vafiadi et al. 2009
Glycerol ferulate	Ferulic acid	Glycerol	FAE-PL	Glycerol: DMSO: buffer	81 % (n.q.)	50	Tsuchiyama et al. 2006
Diglycerol ferulates (mixture of isomers)	Ferulic acid	Diglycerin S	FAE-PL	Diglycerin S: DMSO: buffer	95 % (12 h)	50	Kikugawa et al. 2012
Glycerol *p*-coumarate	*p*-Coumaric acid	Glycerol	FAE-PL	Glycerol: DMSO: buffer	~60 % (72 h)	50	Tsuchiyama et al. 2007
L-Arabinose ferulate	Methyl ferulate Ethyl ferulate	*L*-Arabinose	StFae-C	Hexane: *t*-butanol: buffer	Up to 50 % (120 h) 6.3 % (−)	35	Vafiadi et al. 2005
D-Arabinose ferulate	Methyl ferulate	*D*-Arabinose		Hexane: *t*-butanol: buffer	45 % (−)	35	Vafiadi et al. 2007a
	Ferulic acid	*D*-Arabinose	Multifect P3000	Hexane: 1-butanol:buffer	36.7 % (144 h)	35	Couto et al. 2010
D-Galactose ferulate	Ferulic acid	*D*-Galactose	Depol 670		61.5 % (144 h)		
D-Xylose ferulate	Ferulic acid	D-Xylose		Hexane: 2-butanone:buffer	37.3 % (144 h)		
Feruloyl raffinose	Ferulic acid	Raffinose	Depol 740L	Hexane: 2-butanone:buffer	11.9 % (7 days)	35	Couto et al. 2011
Feruloyl galactobiose	Ferulic acid	Galactobiose		Hexane: 1,4-dioxane:buffer	26.8 % (144 h)		
Feruloyl xylobiose	Ferulic acid	Xylobiose		Hexane: 2-butanone:buffer	9.4 % (144 h)		
Feruloyl arabinodiose	Ferulic acid	Arabinodiose			7.9 % (144 h)		
Feruloyl sucrose	Ferulic acid	Sucrose			13.2 % (n.q.)		
Feruloyl FOS	Ferulic acid	FOS			9.6 % (n.q.)		

FAEA: FAE from *Aspergillus niger*; Ultraflo L, Depol 740L: multi-enzymatic preparation from *Humicola insolens*; AnFaeA: type A FAE from *A. niger*; StFae-A, StFae-C: FAE from *Sporotrichum thermophile* ATCC 34628; FoFae-I, FoFae-II: FAE from *Fusarium oxysporum*; FAE-PL: FAE from *A. niger* purified from the commercial preparation Pectinase PL"Amano"; Multifect P3000: multi-enzymatic preparation from *Bacillus amyloliquefaciens*; Depol 670: multi-enzymatic preparation from *Trichoderma reesei*

et al. 2007), whereas its microencapsulation by a chitosan-alginate complex showed more moderate results (Yu and Li 2005). Mycelia could protect the enzyme from the harshness of organic solvents as an immobilization matrix does and offer avoidance of costly and time-consuming purifications. Tannases from *Aspergillus* species, *Lactobacillus plantarum*, and *Emericella nidulans* immobilized on different carriers, catalyzed the esterification of tannic acid in organic and aqueous media offering high yields (43–88 %) (Fernandez-Lorente et al. 2011; Prasad et al. 2011; Nie et al. 2012a; Goncalves et al. 2013). A bioimprinted commercial tannase esterified tannic acid with propanol resulting in 50 % yield increase compared to the non-imprinted enzyme. Bioimprinting locks the enzyme into a favorable conformation for catalysis during lyophilization through the addition of the targeted substrate prior to freezing. Moreover, the ligand may impede the formation of inactive "microconformations" in the active site (Aithal and Belur 2013). Bioimprinting methods can activate tannase remarkably offering a 100-fold increase of conversion (Nie et al. 2012b). Techniques such as pH tuning, interfacial activation, and cryogenic protection have been applied (Nie et al. 2012a, 2014). Free tannases from *Aspergillus* species,

Table 3 Tannase-catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
Methyl gallate	Gallic acid	Methanol	Tannase from *Aspergillus niger*	Hexane	90.7 % (8 h)	50	Sharma and Saxena 2012
Propyl gallate		1-Propanol			94.8 % (8 h)		
	Tannic acid		CNBr-agarose immobilized tannase from *Emericela ridulans*	Buffer	88 % (48 h)	60–75	Goncalves et al. 2013
	Methyl gallate		CNBr-agarose immobilized tannase from *Lactobacillus plantarum*	Buffer	55 % (–)	25	Fernandez-Lorente et al. 2011
C1–C12 acyl gallates	Gallic acid	C1–C12 fatty alcohols	Tannase from *Aspergillus niger* immobilized on alkylaminosilanized porous silica	Solvent-free	10–95 % (18–48 h)	RT	Weetall 1985
C3–C5 diol gallates (strong indication of more than one form of ester)		Diols			50–80 % (24 h)		
Catechin gallate	Gallic acid	Catechin	Tannase from *Aspergillus niger* immobilized on Eupergit C	[BMIM][MEESO$_4$]: buffer	1.3 % (20 h)	RT	Raab et al. 2007
Epicatechin gallate		Epicatechin			5.4 % (20 h)		
Epigallocatechin gallate		Epigallocatechin			3.1 % (20 h)		

Penicillium variable, and *Bacillus massiliensis* (whole-cell) have synthesized propyl gallate in organic solvents (Yu and Li 2008; Sharma and Gupta 2003; Sharma and Saxena 2012; Beena et al. 2011). Regarding other galloyl esters, Toth and Hensler (1952) reported the synthesis of methyl and ethyl esters but not the phenyl ester of gallic acid in the presence of tannase dissolved in buffer, revealing for the first time the ability of soluble tannases to esterify. Gallic acid esters were synthesized by an immobilized tannase from *A. niger* performing esterification of gallic acid with alcohols (C1–C12) and with several diols. This system proved that the enzyme had broad specificity for alcohols but absolute specificity for the acid moiety (Weetall 1985).

Representing proanthocyanidin monomers, green tea catechins mainly comprising of epicatechins (ECs), epigallocatechins (EGCs), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) have gained attention for their strong anti-oxidant and cardiovascular protective activity. Green tea is considered a useful agent for promoting skin regeneration or treatment for psoriasis, rosacea, and actinic keratosis and repairs UV-damaged skin in vivo, which leads to the improvement of wrinkles (Hong et al. 2012). EGCG is an anti-inflammatory and anti-irritant anti-oxidant, which is responsible for health benefits like the stimulation of collagen production while reducing oxidative damage within the skin. EGCG vehiculated in cosmetic formulations presents good skin penetration and retention favoring its skin effects (dal Belo et al. 2009). Among epicatechin derivatives, EGC is the most effective photoprotector, following in a descending order by EGCG, EC, and ECG (Hong et al. 2013). However, it is present in natural green tea preparations in low amounts compared to EGCG, which is the most abundant catechin in green tea (Cao and Ito 2004). Low-yield galloylation of epicatechins has been achieved by an immobilized commercial tannase from *A. niger* in ionic liquids (Raab et al. 2007). It is evident that tannases could be proved to be a powerful biocatalyst in order to modify the active constituents of green tea and synthesize tailor-made compounds with preferred biological activities for use in different cosmeceutical products. High yield acetylation of catechin and ECGG has been reported using commercial lipases from *R. miehei* and *P. cepacia* (Lambusta et al. 1993; Zhu et al. 2014).

Proteases

Besides catalyzing the cleavage of peptide bonds for the production of peptide cosmeceuticals, proteases (EC 3.4) find application in transesterification reactions in organic solvents, lowering the cost of ester production and increasing reaction specificity. Enzymes from different sources display different features; for example, contrary to serine proteases, thermolysin (a metallo-protease from *Bacillus*

thermoproteolyticus) is not generally used in transesterifications (Pedersen et al. 2002). Studies have proved that the S1 pocket of thermolysin active site can bind medium and large hydrophobic amino acids, suggesting that the vinyl group can bind as well, allowing the possibility of using thermolysin for the synthesis of sugar esters. For these reasons, the use of proteases for ester production in the cosmetic field is of great interest and potential (Fornbacke and Clarsund 2013). The main compounds synthesized by proteases are summarized in Table 4.

As a typical flavonoid glycoside with anti-oxidant activity, rutin has been enzymatically esterified with different acyl donors to enhance its solubility and stability in lipophilic media. The regioselective transesterification of rutin with divinyl carboxylates in pyridine was performed at 50 °C for 4 days by an alkaline protease from *B. subtilis* (Xiao et al. 2005). Only 3″-*O*-substituted rutin ester was obtained in these conditions showing that regioselective acylation can be controlled by regulation of solvents and enzymes. Pre-irradiated alkaline protease from *B. subtilis* increased transesterification of troxerutin with divinyl dicarboxylates by 31 % in pyridine using an ultrasound bath (150 W and 80 kHz) (Xiao et al. 2011). Ultrasonic treatment is an environmentally benign method based on the cavitation phenomenon, which causes the formation, expansion, and collapse of cavities generating high temperatures and pressures of the neighboring surroundings (Khan and Rathod 2015). Thus, cavitation can accelerate enzymatic reactions maintaining ambient conditions of the overall environment. Ultrasonic treatment represents an efficient route of performing transterification in order to modify solubility of anti-oxidant molecules.

Arbutin derivative with undecylenic acid located at its sugar moiety has been enzymatically synthesized using an alkaline protease from *B. subtilis* in a mixture of dimethylformamide and water (95:5) (Tokiwa et al. 2007a). The produced arbutin undecylenic acid ester showed to inhibit the activity of tyrosinase from mushroom; in addition, the arbutin ester seemed to have high dermal absorption and did not show the peculiar smell of undecylenic acid which commonly prevents its application in cosmetics. Further studies have proven that after 6 days of incubation of B16 melanoma cells with arbutin undecylenic ester, melanin production levels were decreased to approximately 30 % compared with that of the control cells (Tokiwa et al. 2007b). Alkaline protease from *B. subtilis* was also applied in regioselective esterification of the hydroxyl group at C-7 position of kojic acid to produce diverse lipophilic kojic acid esters in dimethylformamide (Raku and Tokiwa 2003). Kojic acid esters were effective as scavengers against DPPH radical, and they are expected to prevent oxidational stress in vivo. Moreover, their

Table 4 Protease-catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
7-*O*-Vinyl adipoyl kojic acid	Kojic acid	Divinyl adipate	Bioprase from *Bacillus subtilis*	Dimethylformamide	25 % (7 days)	30	Raku and Tokiwa 2003
7-*O*-Hexanoyl/octanoyl/decanoyl kojic acid		Vinyl hexanoate/octanoate/decanoate			13–27 % (7 days)		
6-*O*-Undecylenoyl p-hydroxyphenyl β-D-glucopyranoside	Arbutin	Undecylenic acid vinyl ester	Bioprase from *Bacillus subtilis*	Dimethylformamide	62 % (7 days)	40	Tokiwa et al. 2007b
3″-*O*-Vinylsuccinyl or vinylsebacoyl-rutin	Rutin	Divinyl succinate/sebacate	Subtilisin from *Bacillus subtilis*	Pyridine	12.8/19.8 % (4 days)	50	Xiao et al. 2005
Vinylsuccinyl/vinylglutaryl/vinyladipoyl/dinylnonanedioyl/vinylsebacoyl/vinyltridecanedioyl-troxerutin	Troxerutin	Divinyl succinate/glutarate/adipate/nonanedioate/sebacate/decanedioate	Subtilisin from *Bacillus subtilis*(-enzyme pre-irradiated)	Pyridine	10.6–33.10 % (4 h)	50	Xiao et al. 2011
2-*O*-Lauroyl-sucrose	Sucrose	Vinyl laurate	Alkaline protease from *Bacillus pseudofirmus*	Dimethylformamide:pyridine	50–60 % (24 h)	45	Pedersen et al. 2003
6-O-Vinyladipoyl-D-glucose/-D-mannose/-D-galactose/-methyl D-galactoside	D-Glucose/D-mannose/D-galactose/α-methyl D-galactoside	Divinyl adipate	Alkaline protease from *Streptomyces* sp.	Dimethylformamide	49–74 % (7 days)	35	Kitagawa et al. 1999

Table 5 Transferase catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
EGCG glycosides (EGCG-G1, EGCG-G2A, EGCG-G2B)	Sucrose	EGCG	Glucansucrase from *Leuconostoc mesenteroides*	Buffer	62.2 % (6.5 h)	28	Moon et al. 2006a
EC glycosides (EC3A, EC3B, EC3C, EC4A)	β-Cyclodextrin	(−)-Epicatechin	β-Cyclodextrin transferase from *Paenibacillus* sp.	Buffer	18.1 % (24 h)	50	Aramsangtienchai et al. 2011
Catechin 3′-O-α-D-glucopyranoside	Maltose	(+)-Catechin	Glycosyltransferase from *Streptococcus sobrinus*	Buffer	13.7 % (24 h)	45	Nakahara et al. 1995
Catechin 3′-O-α-D-glucopyranoside (main product)	Starch		Cyclodextrin glucanotransferase from *Bacillus macerans*		18.3 % (40 h)	40	Funayama et al. 1993
Catechin 3′-O-α-D-glucopyranoside	Maltose		Enzyme with transfer activity from *Xanthomonas campestris* WU-9701		57.1 % (36 h)	45	Sato et al. 2000
Hydroquinone fructoside	Sucrose	Hydroquinone	Levansucrase from *Leuconostoc mesenteroides*	Buffer	14 % (6 h)	28	Kang et al. 2009
β-Arbutin-α-G1/β-arbutin-α-G2	Sucrose	β-Arbutin	Glucansucrase from *Leuconostoc mesenteroides* B-1299B	Buffer	27.1 % (10 h)	28	Moon et al. 2007a
	Starch	β-Arbutin	Cyclomaltodextrin glucanotransferase from	Buffer	70 % (16 h)	40	Sugimoto et al. 2003
α-Arbutin-α-G1/β-arbutin-α-G2		α-Arbutin	*Bacillus macerans*	Buffer	70 % (16 h)	40	Sugimoto et al. 2005
α-Arbutin	Sucrose	Hydroquinone	Amylosucrase from *Deinococcus geothermalis*	Buffer	90 % (24 h)	35	Seo et al. 2012a
α-Arbutin (in a mixture of products, G2–G7)	α-Cyclodextrin		Cyclodextrin glycosyltranferase from *Thermoanaerobacter* sp. (Toruzyme 3.0 L; after amyloglucosidase treatment)	Buffer	30.0 % (24 h)	40	Mathew and Adlercreutz 2013
β-Arbutin-α-glycoside	Sucrose	β-Arbutin	Amylosucrase from *Deinococcus geothermalis* DSM 11300	Buffer	98 % (−)	35	Seo et al. 2009
Kojic acid glycosides (5-*O*-α-D- and 7-*O*-α-D-glucopyranoside)		Kojic acid	Sucrose phosphorylase from *Leuconostoc mesenteroides*	DMSO:buffer	19.7 % (24 h)	42	Kitao & Serine 1994
Quercetin glycosides (3′-*O*-α-D and 4-*O*-α-D glycopyranoside)		Quercetin	Glucansucrase from *Leuconostoc mesenteroides*	Acetone	23.1 % (5 h)	28	Moon et al. 2007b
Ampelopsin glycosides up to 5 units(4′-*O*-α-D-glycopyranoside as main product)		Ampelopsin	Dextransucrase from *Leuconostoc mesenteroides*	DMSO: buffer	87.3 % (1 h)	28	Woo et al. 2012
Astragalin glycosides (kaempferol-3-*O*-β-D-isomaltoside, 3-*O*-β-D-nigeroside, polymerized 3-*O*-β-D-isomaltooligosaccharides)	Sucrose	Astragalin			24.5 % (5 h)	28	Kim et al. 2012
Ascorbic acid glycosides (2-*O*-α-D-glucopyranosyl L-ascorbic acid as main product)	α-Cyclodextrin	Ascorbic acid	Cyclomaltodextrin glucanotransferase form *Bacillus stearothermophilus*	Buffer	45.6 % (1 h)	60	Aga et al. 1991
Benzoyl glycosides (1-O-benzoyl-α-D-, 2-O-benzoyl-α-D- and 2-O-benzoyl-β-D-glucopyranoside)	Sucrose	Benzoic acid	Sucrose phosphorylase from *Streptococcus mutans*	Buffer	70 % (48 h)	37	Sugimoto et al. 2007

biodegradability exceeded 60 %, allowing their application in cosmetics for the production of eco-friendly and oil-based product products.

Transferases

A broad variety of bioactive glycosides has been synthesized using glycosyltransferases (GTFs; EC 2.4); enzymes that catalyze the transfer of sugar moieties from an activated donor to specific acceptors forming glycosidic bonds. Novel EGCG mono- and di-glycosides with increased UV irradiation stability, browning resistance, and water solubility regardless of the position or linkage of the glycosylation have been synthesized by transferases from *L. mesenteroides* (Kitao et al. 1995; Moon et al. 2006a). EC mono-, di-, and tri-glycosides have been synthesized by a β-cyclodextrin glucosyltransferase from *Paenibacillus* sp. while various catechin derivatives by

amylosucrases from *Deinococcus geothermalis*, *Streptococcus sobrinus*, a cyclodextrin glucanotransferase from *Bacillus macerans* and an enzyme with glycosyl transfer activity from *Xanthomonas campestris* (Aramsangtienchai et al. 2011; Cho et al. 2011; Nakahara et al. 1995; Funayama et al. 1993; Sato et al. 2000). Transferase-based modification of hydroquinone has been focused on its glycosylation or the production of arbutin (α- and β-) glycosides. A two-step synthesis of α-arbutin including prior treatment of α-cyclodextrin with an amyloglucosidase from *A. niger* and subsequent transfer reaction using a commercial cyclodextrin glucano transferase from *Thermoanaerobacter* sp. has been reported (Mathew and Adlercreutz 2013). Before treatment, hydroquinone was glycosylated with up to 7 glucose units while after treatment, α-arbutin was an absolute product. Results on the synthesis of arbutin glycosides show that the α-glucosidic linkage plays an important role in the inhibitory effect on human tyrosinase (Sugimoto et al. 2005).

2-*O*-α-D-glycopyranosyl L-ascorbic acid has been synthesized by a cyclomaltodextrin glucanotransferase from *Bacillus stearothermophilus* and a sucrose phosphorylase from *Bifidobacterium longum* (Aga et al. 1991; Kwon et al. 2007). The first transglycosylation of CA was performed by a sucrose phosphorylase from *B. longum* in aqueous CO_2 supercritical media resulting in the formation of caffeic mono- and di-glycosides (Shin et al. 2009). Ampelopsin is a flavonoid with numerous activities such as anti-inflammatory, anti-microbial, anti-oxidant, anti-hypertension, hepatoprotective, anti-carcinogenic, anti-viral, and skin-whitening effects. A dextransucrase from *L. mesenteroides* synthesized a mixture of novel ampelopsin glycosides with up to 5 attached glycoside units. The primary product, ampelopsin-4′-*O*-α-D-glucopyranoside, reached an optimal yield of 34 g/L while it showed an 89-fold increase in water solubility, 14.5-fold increase in browning resistance comparing to ampelopsin, and 10-fold higher tyrosinase inhibition comparing to β-arbutin. Browning resistance was similar to ECGC glycosides and anti-oxidant activity superior to ampelopsin (Woo et al. 2012). Another major flavonoid found in plants, astragalin, was modified by a dextransucrase from *L. mesenteroides* giving products with increased inhibitory effects on matrix metalloproteinase-1 expression, anti-oxidant effect, and melanin inhibition (Kim et al. 2012). Quercetin glycosides were synthesized by a glucansucrase from *L. mesenteroides* showing increased water solubility, slower scavenging activity, and no improvement in tyrosinase inhibition (Moon et al. 2007b). Three main benzoyl and two main kojic acid glycosides were synthesized by a sucrose phosphorylase from *Streptococcus mutans* and *L. mesenteroides*, respectively (Sugimoto et al. 2007; Kitao and Serine 1994). Examples of transferase catalyzed reactions are presented in Table 5.

Glucosidases

Glucosidases, such as α- (EC 3.2.1.20) and β-glucosidases (EC 3.2.1.21), are a group of enzymes that hydrolyze individual glucosyl residues from various glycoconjugates including α- or β-linked polymers of glucose under physiological conditions. However, these enzymes are able to synthesize a broad variety of sugar derivatives under defined conditions in two different manners: transglycosylation or reverse hydrolysis (Park et al. 2005). Active compounds that have been obtained by enzyme-catalyzed glucosidation include vitamin and arbutin derivatives as presented in Table 6. Pharmacological properties of vitamin E can be improved by increasing its water solubility, absorbtivity and stability through glycosylation. A novel water-soluble vitamin E derivative, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG) has been synthesized from

Table 6 Glucosidase-catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
4-Hydroxyphenyl-β-isomaltoside	Sucrose	Arbutin	α-Glucosidase from *Saccharomyces cerevisiae*	Buffer	50 % (20 h)	40	Milosavić et al. 2007
Hydroquinone α-D-glucopyranoside	Maltose	Hydroquinone	α-Glucosidase from *Saccharomyces cerevisiae*	Buffer	13 % (20 h)	30	Prodanović et al. 2005
Hydroquinone α-D-isomaltoside					15 % (20 h)		
2-(α-D-Glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol	Maltose	2-Hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol (vitamin E derivative)	α-Glucosidase from *Saccharomyces* sp.	DMSO	(20 h)	40	Murase et al. 1998
β-D-Glucosyl-(1-6)-arbutin β-D-Glucosyl-(1-4)-arbutin β-D-Glucosyl-(1-3)-arbutin	Cellobiose	Arbutin	β-Glucosidase from *Thermotoga neapolitana*	Buffer	2.8 % (12 h)	80	Jun et al. 2008

2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol (TM) and maltose using α-glucosidase from *Saccharomyces* sp. in a transglycosylation reaction (Murase et al. 1998). Anti-oxidant activity of TMG was investigated on peroxidation of phosphatidylcholine-liposomal (PC)-suspension, which is usually adopted as model for cellular biomembranes. TMG showed higher efficacy on lipid peroxidation than ascorbic acid, when peroxidation was provoked by lipid-soluble radical generator such as 2,2′-azobis(2,4-dimethylvaleronitrile (AMVN). Moreover, TMG showed to inhibit cupric ion-induced peroxidation of (PC)-suspension and rat brain homogenate while it delayed the generation of cholesteryl ester hydroperoxides when exposing human plasma to lipid or water-soluble radical generators.

A β-glucosidase from *Thermotoga neapolitana* has synthesized arbutin-β-glycosides to be used as novel skin whitening agents (Jun et al. 2008). β-D-glucosyl-(1–3)-arbutin has been proved to inhibit mushroom tyrosinase and it has been tested on B16F10 murine melanoma cell line showing the strongest inhibitory effect on melanin synthesis in a dose-dependent manner without causing cytotoxicity. β-D-glucosyl-(1–3)-arbutin showed to be a more efficient inhibitor of melanin synthesis compared to arbutin. Similarly, arbutin has been glycosylated by a α-glucosidase from *Saccharomyces cerevisiae* to produce 4-hydroxyphenyl-β-isomaltoside (Milosavić et al. 2007), whose inhibitory capacity on tyrosinase is being investigated. α-Glucosidase from *S. cerevisiae* also catalyzed the synthesis of hydroquinone α-D-glucopyranoside and hydroquinone α-D-isomaltoside (Prodanović et al. 2005). Glycosylation of hydroquinone increased its water solubility and enhanced pharmaceutical properties such as skin whitening and anti-bacterial capacity.

Laccases

Laccases are dimeric or tetrameric glycosylated proteins, which usually bear four copper atoms per monomer distributed in three redox sites (Gianfreda et al. 1999). These enzymes are able to catalyze the one-electron oxidation of phenols generating phenoxy radicals and simultaneously reducing molecular dioxygen to water (Kudanga et al. 2011). Due to their features, including broad substrate specificity, catalysis in air without using H_2O_2, and production of water as only by-product, laccases are considered the ideal green catalysts. Besides catalyzing catabolic and depolymerization processes, based on reaction conditions, these enzymes can also carry out synthetic processes including the oxidization of aromatic compounds followed by heteromolecular coupling with co-substrates or simple oligomerization (Mikolasch and Schauer 2009). The main compounds that have been synthesized by laccase-catalyzed reactions include flavonoids, HCAs, and other phenolics. Conditions of their production are described in the following sections and summarized in Table 7.

The anti-oxidant activity of flavonoids derives from the B-ring, which is important for the H-transfer, and 2–3 double bond ensuring electron delocalization. Moreover, in vitro studies have demonstrated the importance of the 3-OH group for the anti-oxidant capacity. Rutin has been oxidized by a laccase from *Myceliophtora thermophyla* to produce flavonoid polymers (Kurisawa et al. 2003a). The same result was achieved by using *Pycnoporus coccineus* and *Pycnoporus sanguineus* laccases as biocatalysts. Oxidized poly-rutin showed enhanced anti-oxidant, anti-inflammatory, and anti-aging capacities compared to the rutin monomer (Uzan et al. 2011). Enzymatic oxidation of catechin was also performed by a laccase from *M. thermophyla* producing poly-catechin with greater superoxide scavenging and inhibitory capacity of xanthine oxidase (Kurisawa et al. 2003b). Laccase-catalyzed oxidation has been applied in order to enhance the anti-oxidant property of FA. Two dimeric products, β-5 and β-β, were obtained by oxidation of FA in organic media using a laccase from *Trametes pubescens* (Adelakun et al. 2012b). Reaction was performed in a biphasic system, as the concentration of ethyl acetate increased, and in monophasic system using dioxane or ethanol as co-solvents. The β-5 dimer showed higher anti-oxidant capacity than FA evaluated by DPPH and Trolox equivalent antioxidant capacity (TEAC) assays. Different oxidized products of HCAs were used to improve anti-oxidant and anti-microbial activities of polymers, such as chitosan. A laccase from *M. thermophyla* was used to functionalize chitosan with oxidated FA and ethyl-ferulate (Aljawish et al. 2012). Both derivatives showed higher anti-oxidant activity than the substrates, especially the FA chitosan. The same strategy was applied to functionalize chitosan with CA using a laccase from *Trametes versicolor*, obtaining a functionalized polymer with higher anti-oxidant and anti-microbial activity than the substrates (Božič et al. 2012b). These results indicated that the addition of an H-atom donating group, produced by laccase-mediated oxidization, could generate a good chain breaking anti-oxidant. Laccase-mediated oxidation is proved to be a good strategy to develop functionalized polymers with enhanced anti-oxidant and anti-microbial activities.

Oxidation of tannic acid by a laccase from *T. versicolor* resulted in a variety of products including gallic acid, gallic acid dimers, partially gallic acid-esterified glucose, and glucose, while oxidation of quercetin offered an oligomeric derivative (Božič et al. 2012a). Both oxidative products of gallic acid and quercetin showed higher anti-oxidant activity than the origin compounds. Furthermore, tannic acid or quercetin was used to functionalize chitosans by laccase without organic or acidic solvents. Both chitosan derivatives exhibited amplified radical scavenging ability and anti-microbial activity compared to the untreated chitosans. The

Table 7 Laccase-catalyzed reactions

Product	Donor	Acceptor	Enzyme	Solvent system	Yield (time)	T (°C)	Reference
Caffeic acid-chitosan	Caffeic acid	Chitosan	Laccase from *Trametes versicolor*	Phosphate buffer	– (24 h)	30	Božič et al. 2012a
Gallic acid-chitosan	Gallic acid				– (24 h)		
Quercetin-chitosan	Quercetin	Chitosan			– (24 h)		Božič et al. 2012b
Gallic acid-chitosan	Tannic acid						
Starch–sodium lignosulfonate graft copolymers	Sodium lignosulfonate	Starch		Sodium acetate	– (4 h)	30	Shogren and Biswas 2013
3,3,5,5-Tetramethoxy biphenyl-4,4-diol	2,6-Dimethoxyphenol	2,6-Dimethoxyphenol	Laccase from *Trametes pubescens*	Ethyl acetate Acetone	– (24 h) – (24 h)	28	Adelakun et al. 2012a
Ferulic acid dimers (5-β, β-β)	Ferulic acid	Ferulic acid		Ethyl acetate or dioxane or ethanol	– (24 h)		Adelakun et al. 2012b
Poly 8-hydroxyquinoline	8-Hydroxyquinoline	8-Hydroxyquinoline		Acetone	76 % (24 h)	30	Ncanana and Burton 2007
Resveratrol trans-dehydrodimer	Resveratrol	Resveratrol	Laccase from *Myceliophtora thermophyla* (supported on glass beads)	Ethyl acetate n-Butanol	18 % (4 days) 31 % (4 days)	45	Nicotra et al. 2004
Ethyl-ferulate-chitosan	Ethyl ferulate	Chitosan	Laccase from *Myceliophtora thermophyla*	Phosphate buffer	(4 h)	30	Aljawish et al. 2012
Ferulic acid-chitosan	Ferulic acid				(4 h)		
Poly-catechin	(+) - Catechin	(+) - Catechin		Acetone	95 % (24 h)	RT	Kurisawa et al. 2003b
Poly-rutin	Rutin	Rutin		Methanol	79 % (24 h)	RT	Kurisawa et al. 2003a
Oligorutin	Rutin	Rutin	Laccase from *Pycnoporus coccineus* Laccase from *Pycnoporus sanguineus*	Glycerol/ ethanol/ buffer	67 % (24 h)	RT	Uzan et al. 2011

laccase grafting method was also applicable to other phenolic compounds, as in the case of graft copolymers of starch with lignosulfonates (Shogren and Biswas 2013). Enzymatic polymerization of 8-hydroxyquinoline was achieved by using a laccase from *T. pubescens* (Ncanana and Burton 2007). Oxidization of 8-hydroxyquinoline was established to generate aromatic radicals which combined to form a polymeric product with a powerful anti-oxidant capacity and anti-radical efficiency. Laccase-mediated oxidization was also performed in organic solvents, due to their advantages as medium in biocatalysis. Oxidation of 2,6-dimetho xyphenol by *T. versicolor* laccase was investigated in biphasic or monophasic systems, leading the formation of a dimeric product with anti-oxidant capacity twofold higher than the substrate. The dimer production was increased in the monophasic solvent using acetone as co-solvent, while its production increased as the concentration of ethyl acetate was increased to 90 % in the biphasic system. Organic solvents were also applied in the synthesis of resveratrol dimers catalyzed by laccases from *M. thermophyla* and *T. pubescens* (Nicotra et al. 2004). *M. thermophyla* laccase-catalyzed dimers were obtained in butanol saturated with buffer; and resveratrol dimers catalyzed by *T. pubescens* laccase were synthesized using a biphasic system of ethyl acetate and buffer. The products may serve as lead for the development of new drugs and as nutraceuticals, showing anti-oxidant activity comparable to resveratrol and its analogs.

Conclusions

A large variety of compounds with potential cosmeceutical application can be obtained through biotechnological processes. The reported examples of enzymatic synthesis or modification of natural compounds so far exploited highlight the possibility of developing biologically active ingredients with anti-oxidant, anti-aging, anti-microbial, anti-wrinkling, photoprotective, or skin-whitening properties. The use of esterases (such as lipases, feruloyl esterases, tannases), transferases, glucosidases, proteases, and laccases allows the modification of target compounds under mild conditions,

maintaining their biological activity and avoiding the formation of by-products. These advantages fit the increasing demand for natural cosmetics, boosting eco-friendly design and production of active compounds in order to replace chemical processes currently used.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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1.3 Feruloyl esterases and glucuronoyl esterases as biocatalysts for antioxidants production

The examples of enzymatic synthesis or modification of natural compounds reported in the previous paragraph highlight the possibility of developing biologically active ingredients with anti-oxidant, anti-aging, anti-microbial, anti-wrinkling, photoprotective or skin-whitening properties under mild conditions, maintaining their biological activity and avoiding the formation of by-products. The use of appealing biocatalysts such as feruloyl esterases (FAEs) and glucuronoyl esterases (GEs) can be explored to develop biotechnological routes of production of industrially relevant esters with reduced environmental impact.

Feruloyl esterases (FAEs), also known as cinnamoyl esterases (E.C. 3.1.1.73), represent a subclass of carboxylic acid esterases (E.C. 3.1.1) that catalyze the hydrolysis of the ester bond between hydroxycinnamic acids (ferulic acid and *p*-coumaric acid) and sugars (xilan and pectin polymers) present in plant cell walls. They act as accessory (or auxiliary) enzymes that facilitate other enzymes, such as xylanases, in accessing to their site of action during biomass conversion (Yu et al. 2003, Wong 2006, Faulds 2010).

A first classification divided FAEs into four types (A, B, C, and D) based on their substrate specificities towards synthetic methyl esters of hydroxycinnamic acids (Figure 1) and on their amino acid sequence identity (Crepin et al. 2004). Type A FAEs show preference for the phenolic moiety of the substrate containing methoxy substitutions, as occurs in ferulic and sinapinic acids; moreover, they prefer hydrophobic substrates with bulky substituents on the benzene ring. On the contrary, type B FAEs are unable to release the dimeric forms of ferulate and show preference to substrates containing one or two hydroxyl substitutions as found in *p*-coumaric or caffeic acid. Type A FAEs show high sequence similarity with lipases, whilst type B have higher similarity acetyl xylan esterases. Type C and D FAEs possess broader substrate specificity with activity towards all four model substrates, but only type D can release diferulic acid from plant cell walls (Table 1). Type C and D FAEs have a high level of sequence identity with tannases and xylanase, respectively (Crepin et al. 2004).

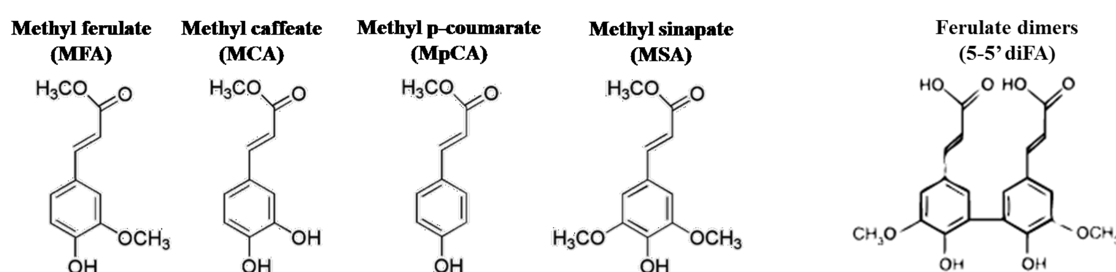
Table 1: Classification of fungal feruloyl esterases based on specificity for methyl esters (Crepin et al. 2004)

FAE Type	MFA	MCA	MSA	M*p*CA	diFA
A	yes	no	yes	yes	yes (5,5′)
B	yes	yes	no	yes	no
C	yes	yes	yes	yes	no
D	yes	yes	yes	yes	yes (5,5′)

A further modification on the classification has been proposed (Benoit et al. 2008) based on a multiple sequence alignment obtained using the sequences of various feruloyl esterases from fungal genomes. The resulted phylogenetic tree helped to identify seven subfamilies (SF1-7) of putative feruloyl esterases demonstrating that these enzymes evolved from highly divergent esterase families (tannases (SF1-4), acetyl xylan esterases (SF6), and lipases (SF7)) and do not have a common ancestor. In 2011, a new and comprehensive classification scheme was built (Udatha et al. 2011) including 365 FAE related sequences of fungal, bacterial and plant origin,

which led to a reclassification of FAEs in 12 subfamilies. However, the discovery of new FAEs using genome mining and phylogenetic analysis of the publicly accessible fungal genomes led to the development of a new subfamily classification (SF1-13) of fungal FAEs considering both phylogeny and substrate specificity (Dilokpimol et al. 2016). In comparison with the previous classification, members of SF1–SF3 and SF5–SF7 remain classified to the same subfamilies. In addition, subfamily SF8 contains FAEs for which there were no homologs found in the previous study. The new subfamily SF9 separated from SF4, and three tannases positioned in SF11, indicating that the enzymes of this subfamily may actually possess tannase activity or potentially dual-activity and may not be true FAEs. The study also resulted in new subfamilies SF10 and SF13. Some FAE sequences, which could not be classified in any group, are referred to as ungrouped sequences (U1–U10).

Figure 1: Synthetic methyl cinnamates and 5-5' ferulate dimer



So far, relatively few studies have been performed to elucidate the functional relationships between sequence-diverse FAEs. The crystal structures of only two fungal FAEs, *Aspergillus niger* (AnFaeA – Hermoso et al. 2004) and A*spergillus oryzae* (AoFaeB – Suzuki et al. 2014) have been published. These FAEs have a common α/β-hydrolase fold and a catalytic triad (Ser-His-Asp) with the Ser residue located at the centre of a universally conserved pentapeptide with the consensus sequence GXSXG that constitutes the nucleophilic elbow.

Thanks to their characteristics, there has been increasing interest in a large number of potential applications of FAEs in various industries such as chemicals, fuel, textile and laundry, animal feed, food and agriculture, and pharmaceutical, as illustrated by more than 100 patent applications on these biocatalysts (Topakas et al. 2007). Also, these enzymes have gained attention for their potential applications in obtaining ferulic acid from agro-industrial waste materials such as those produced by milling, brewing, and sugar industries (Fazary and Ju 2008). In particular, ferulic acid can perform several biological functions such as UV absorber, anti-oxidant (Kikuzaki et al. 2002) and anti-inflammatory (Murakami et al. 2002) activity. Antioxidant activity of phenolic acids is largely due to their chemical structure and the presence of hydroxy groups on the aromatic ring, explaining the higher antioxidant efficiency of caffeic acid with two hydroxy groups compared to one on ferulic acid (Chen and Ho 1997).

In addition to their hydrolytic ability, in appropriate operative conditions, FAEs are able to perform (trans)esterification reactions, modifying hydroxycinnamic acids and their esters resulting to the synthesis of ingredients with attractive properties for the food, cosmetic and pharmaceutical industries. FAE catalyzed (trans)esterifications have been mainly described in detergentless microemulsions, which represent a ternary system consisting of a hydrocarbon, *n*-propanol and water representing a thermodynamically stable dispersion of aqueous microdroplets in the hydrocarbon

solvent (Khmelnitsky et al. 1988). Transesterification of methyl ferulate with 1-butanol has been reported in detergentless microemulsions using different FAEs such as StFae-A and StFae-C from *Sporotrichum thermophile* ATCC 34628, FoFae-I from *Fusarium oxysporum*, AnFaeA from *Aspergillus niger* (Topakas et al. 2003b, 2004, 2005; Vafiadi et al. 2008a,b). Moreover, five feruloyl esterases (FaeA1, FaeA1, FaeB1, FaeB2 and MtFae1a) from *Myceliophthora thermophila* have been used as biocatalysts for the synthesis of prenyl ferulate and L-arabinose ferulate optimizing various reaction parameters such as pH and temperature, enzyme load and (Antonopoulou et al. 2017a,b).

Glucuronoyl esterases (GEs), a recently discovered family of enzymes suggested to play a significant role in biomass degradation disconnecting hemicellulose from lignin through the hydrolysis of the ester bond between 4-O-methyl-D-glucuronic acid residues of glucuronoxylans and aromatic alcohols of lignin (Špániková and Biely 2006, Ďuranová et al. 2009, Arnling Bååth et al. 2016). Kinetic experiments on synthetic substrates mimicking the ester bonds naturally occurring in plant cell wall have shown that GEs recognize the uronic rather than the alcohol moiety of the ester and that they exhibit preference towards esters of 4-O-methyl-D-glucuronic acid rather than D-glucuronic acid (Špániková et al. 2007).

The first reported amino acid sequence belonged to a GE from *Hypocrea jecorina* (Li et al. 2007), which was used to search for homologous genes in several filamentous fungi and bacteria leading to the emergence of a new family of CE15 on the CAZy database (http://www.cazy.org/). To date, only a few members of this family have been characterized using a series of new synthetic substrates comprising methyl esters of uronic acids and their glycosides (Špániková and Biely 2006; Li et al. 2007; Vafiadi et al. 2009; Ďuranová et al. 2009; Topakas et al. 2010, Huttner et al. 2017). Moreover, the only 3D structures of CE-15 family available are that of Cip2_GE from the mesophilic fungus *H. jecorina* (Pokkuluri et al. 2011) and that of StGE2 from *M. thermophila* (Charavgi et al. 2013), which confirmed the triad arrangement of the putative catalytic residues Ser-His-Glu.

GEs constitute interesting candidates for production of biofuels and bioproducts from plant biomass due to their implication in the hydrolytic breakdown of lignin-carbohydrate complexes (Katsimpouras et al. 2014, D'Errico et al. 2016). Compared to the known biotransformation ability of FAEs, synthetic ability of GEs needs to be explored towards the production of alkyl branched glucuronic acid derivatives which are non-ionic surfactants and have good surface properties, including biodegradability. In addition, due to their tastelessness, non-skin irritation and non-toxicity, these bioactive compounds find diverse uses in the cosmetic and pharmaceutical industries (Moreau et al. 2004).

FAEs and GEs naturally perform hydrolytic reaction, whereas to perform efficient biosynthetic reactions water free/low water content reaction systems are required. The optimization of both biocatalysts, including their regioselectivity and stereoselectivity as well as specificity, and conditions of reaction will lead towards the replacement of chemical processes currently used for the production of aforementioned biologically active compounds with cost-effective, energy efficient and eco-friendly bioconversions.

1.4 Aims of the thesis

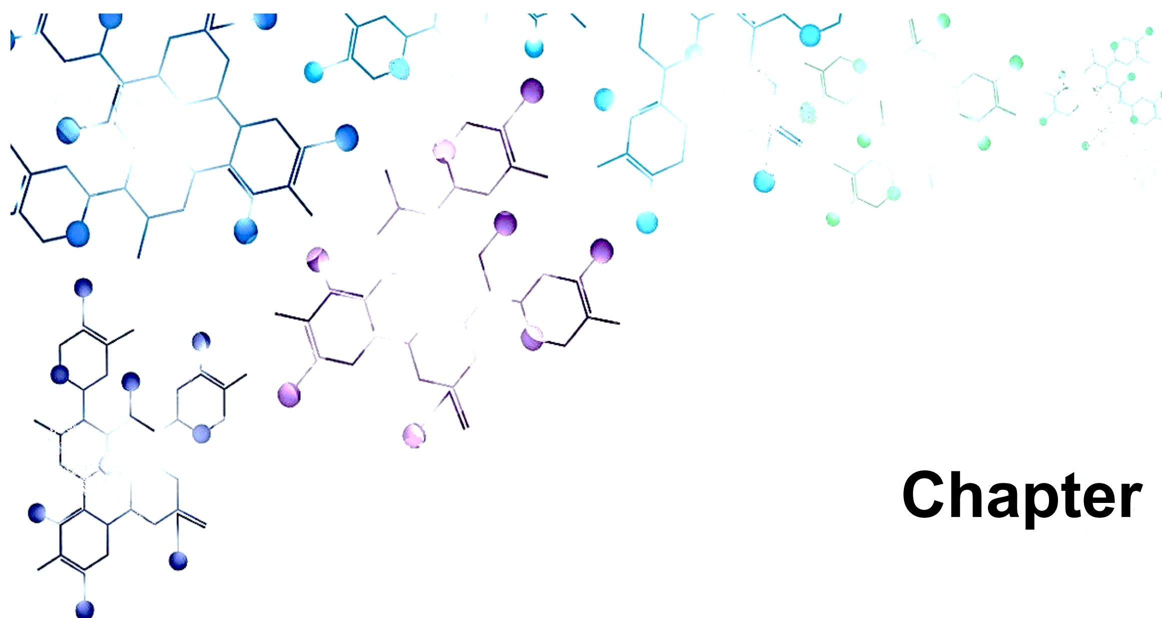
The overall aim of this PhD project is to develop improved biocatalysts based on FAEs and GEs for the production of compounds with antioxidant activity. The activities of this PhD project were carried out in the frame of OPTIBIOCAT, a large collaborative SME-targeted project aimed at the development of "Optimized esterase biocatalysts for cost-effective industrial production" (613868) funded by European Commission in the frame of FP7 Knowledge Based Bio-Economy (KBBE).
The first objective of this PhD project is aimed at the development of biocatalysts based on FAEs and GEs through through their recombinant expression and site-directed mutagenesis. Novel fungal FAEs and GEs, identified through a bioinformatics approach and expressed in *Pichia pastoris,* will be used to accomplish this objective by:

a) Selection of the most interesting enzyme to be subjected to mutagenesis
b) Identification of the amino acids that are most suitable for specific point mutations and design of desired mutations
c) Expression of the mutated cDNAs in *P. pastoris* and characterization of the mutated enzymes.

The second objective of this PhD project consists in developing biocatalysts applying directed evolution to the already characterized FAEs from *Fusarium oxysporum* (FoFaeC) (Moukouli et al. 2008) and from *Myceliophthora thermophila* (MtFae1a) (Topakas et al. 2012). This objective will be achieved by:

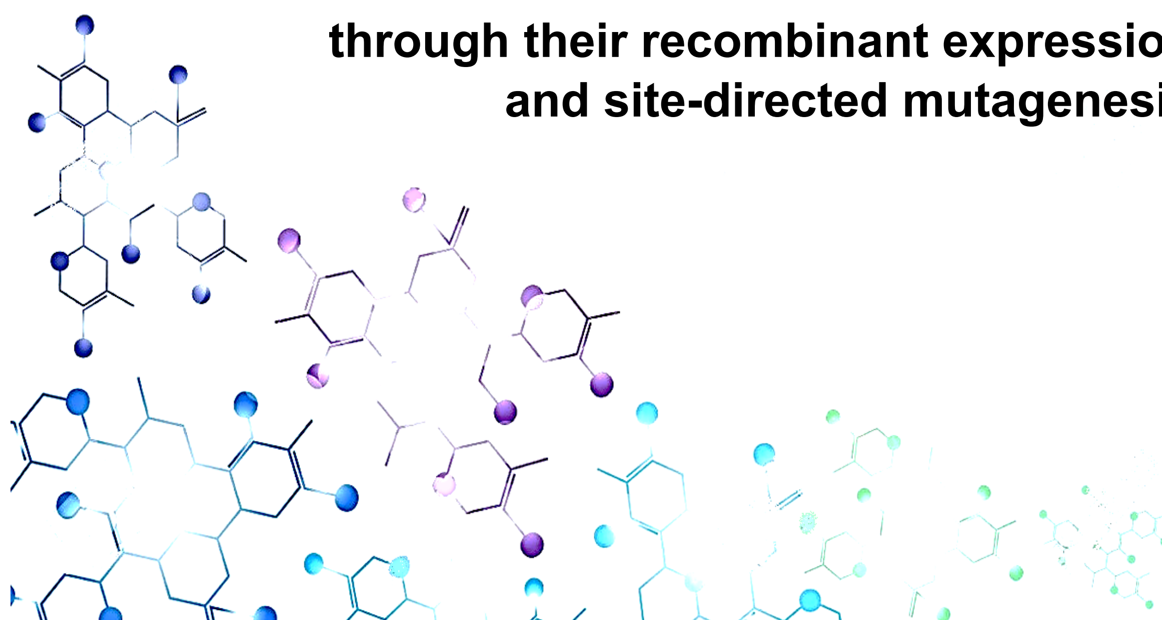
a) Construction of libraries of 30,000 randomly mutated variants of FoFaeC and MtFae1a through error prone polymerase chain reaction and expression in appropriate yeasts (*Yarrowia lipolytica* and *Saccharomyces cerevisiae*)
b) Screening of random mutant libraries performed by using a rapid method for high-throughput screening (HTS) with the automated workstation consisting of Robot Colony Picker and Biomek NX to identify mutants with higher activity than wild type enzymes. In particular, the screening will be based on:
    - set up of HTS methods of FAEs both in solid medium and in liquid medium in multi well-plates using novel substrates developed within OPTIBIOCAT
    - selection of active variants on solid medium with novel chromogenic substrate and analysis in liquid medium of selected evolved variants activity in multi well-plates with the novel substrates developed within OPTIBIOCAT
c) Characterization of the selected evolved variants by assessing substrate specificity, temperature and solvent tolerance
d) Application of the best variants in transesterification reactions for the production of antioxidants.

The third objective of this PhD project is targeted to the identification of novel FAEs with non-conserved sequences. For this purpose, different fungal strains isolated from lignocellulosic biomasses during biodegradation under natural conditions by Department of Agriculture of University of Naples "Federico II" will be screened for the production of FAE activity on solid and in liquid media.
The operative objectives previously described aim at the general objective of developing competitive and eco-friendly bioconversions based on esterification reactions catalyzed by FAEs and GEs for the production of molecules with antioxidant activity such as phenolic fatty esters and sugar esters for cosmetic industry.



Chapter II

Development of biocatalysts based on novel FAEs and GEs through their recombinant expression and site-directed mutagenesis



Chapter II: Development of biocatalysts based on novel FAEs and GEs through their recombinant expression and site-directed mutagenesis

2.1 Introduction

Since early 1990s several reports have been published on the purification and characterization of FAEs from mostly fungal kingdom. The current availability of fungal genome sequences makes possible the exploration of this kingdom to mine for novel putative FAEs and GEs (Benoit et al. 2008). Mining genomes for new enzymes is a robust and established strategy to increase the enzymatic repertoire required for biotechnological applications. Moreover, available crystal structures of FAEs and GEs can also allow the engineering of the protein structure in order to increase the thermal stability of the corresponding biocatalysts. In this way, the combination of theoretical work for selecting enzymes suited for the reactions of choice with site-directed mutagenesis will further fine-tune them towards the selected reactions, achieving significant improvement for the reaction conditions.

This chapter deals with the results concerning the development of biocatalysts based on recombinant expression of novel FAEs and GEs identified through bioinformatics analyses of fungal genomes and their improvement using rational design. In detail, in sections 2.2.1 and 2.2.2, papers **"Fungal glucuronoyl esterases: genome mining based enzyme discovery and biochemical characterization"** and **"Fungal feruloyl esterases: functional validation of genome mining based enzyme discovery including uncharacterized subfamilies"** describe the genome mining strategy used to identify novel GEs and FAEs, respectively, and their biochemical characterization.

In more details, already characterized FAEs and GEs were used as queries in a blast search against an internal fungal genome database at Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology, Utrecht University, containing approximately 300 fungal genomes. Obtained sequences were used for an initial alignment and phylogeny analysis. This resulted in a residual 1,636 putative FAE and 166 putative GE protein sequences used for the construction of phylogenetic trees, which separated the FAEs and GEs into 12 and 8 subgroups, respectively. After gene model correction, 20 putative GEs and 30 putative FAEs cDNA were synthesized and expressed in *P. pastoris* to validate the genome mining strategy. Among these putative enzymes, 10 FAEs and 7 GEs were studied in this PhD project.

Characterization of this collections of putative FAEs and GEs has improved understanding of the variation in properties of FAEs and GEs from different groups/subfamilies as well as providing biochemically supported univocal classification (Dilokpimol et al. 2016).

Section 2.2.3 describes the selection of the most active FAE among the characterized ones, its study through homology modeling, rational design and recombinant expression of mutations to optimize enzyme performance and stability for both hydrolytic and synthetic applications.

2.2 Results

2.2.1 Fungal glucuronoyl esterases: genome mining based enzyme discovery and biochemical characterization (Paper II)

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Fungal glucuronoyl esterases: Genome mining based enzyme discovery and biochemical characterization

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ABSTRACT

4-*O*-Methyl-D-glucuronic acid (MeGlcA) is a side-residue of glucuronoarabinoxylan and can form ester linkages to lignin, contributing significantly to the strength and rigidity of the plant cell wall. Glucuronoyl esterases (4-*O*-methyl-glucuronoyl methylesterases, GEs) can cleave this ester bond, and therefore may play a significant role as auxiliary enzymes in biomass saccharification for the production of biofuels and biochemicals. GEs belong to a relatively new family of carbohydrate esterases (CE15) in the CAZy database (www.cazy.org), and so far around ten fungal GEs have been characterized. To explore additional GE enzymes, we used a genome mining strategy. BLAST analysis with characterized GEs against approximately 250 publicly accessible fungal genomes identified more than 150 putative fungal GEs, which were classified into eight phylogenetic sub-groups. To validate the genome mining strategy, 21 selected GEs from both ascomycete and basidiomycete fungi were heterologously produced in *Pichia pastoris*. Of these enzymes, 18 were active against benzyl D-glucuronate demonstrating the suitability of our genome mining strategy for enzyme discovery.

Introduction

4-*O*-methyl-D-glucuronic acid (MeGlcA) is a side-residue of xylan (β-1,4-linked D-xylose) that is found in both glucuronoxylan and glucuronoarabinoxylan, which are the principle components present in the secondary cell walls of eudicotyledonous plants and both cell wall layers of commelinoid monocots, respectively (Fig. 1) [1–3]. A large proportion of MeGlcA in xylan can form ester linkages to lignin alcohol; for example 30% and 40% of MeGlcA are esterified to lignin in beechwood and birchwood, respectively [4,5]. In nature, these lignin-carbohydrate complexes (LCCs) contribute significantly to the strength and rigidity of the plant cell wall, rendering it recalcitrant to digestion. However, they impede the industrial applications of plant biomass by restricting the removal of lignin e.g. from cellulosic pulp in pulping processes and hindering efficient enzymatic hydrolysis of biomass in bioethanol production [6–9].

Glucuronoyl esterases (4-*O*-methyl-glucuronoyl methylesterases, GEs) can cleave the ester bond between MeGlcA and lignin, and therefore may play a significant role as auxiliary enzymes in biomass saccharification for the production of biofuels and biochemicals. The first GE was reported in 2006 from a white-rot like fungus *Schizophyllum commune* [10], and belongs to carbohydrate esterase family 15 (CE15) in the CAZy database [11,12]. From 182 members in CE15, only 21 are from fungi, and of these so far only around 10 GEs have been characterized (Table 1). Among these, the structures of the *Trichoderma reesei* (*Hypocrea jecorina*) Cip2 [13] and the *Myceliophthora thermophila* (*Sporotrichum thermophile*) StGE2 [14] have been resolved by X-ray crystallography. The first structure revealed the Ser-His-Glu as the putative catalytic triad of GEs, whereas in the latter case the catalytic serine mutant in complex with methyl 4-*O*-methyl-β-D-

Abbreviations: CE, carbohydrate esterase; GE, glucuronoyl esterase

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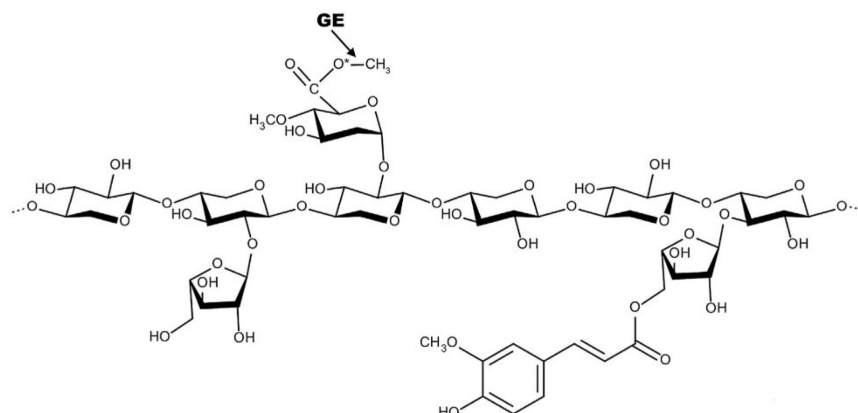

Fig. 1. Model structure of 4-*O*-methyl-D-glucurono(arabino)xylan [modified from 12, 15]. GE indicates glucuronoyl esterase. In nature *O is typically linked to lignin instead of a methyl group.

Table 1
Characterized GEs with their properties.

Origin	Enzyme	Sub-group	Production[a]	Molecular mass (kDa)[2]	pH		Temperature (°C)		pI[b]	Reference
					Optimum	Stability	Optimum	Stability		
Fungi										
Schizophyllum commune	ScGE	4	Pur	44	7.0	–	50	–	3.5	[10]
	(rScGE)		HP	53 (42)	–	–	–	–	3.7	[23]
Hypocrea jecorina (Trichoderma reesei)	Cip2	5	HT	55	5.5	4.0–8.0	40–60	< 40	7.9	[12]
Phanerochaete chrysosporium	PcGE1	4	HAv, Pc, Sc	47	5.0–6.0	–	45–55	–	6.5 (5.5)	[25,34]
Phanerochaete chrysosporium	PcGE2	4	HSc	42	5.0–6.0	–	45–55	–	4.7 (4.8)	[25]
Myceliophthora thermophila (Sporotrichum thermophile)	StGE1	8	Pur	58	6.0	7.0–8.0	60	< 55	6.7	[28]
Myceliophthora thermophila (Sporotrichum thermophile)	StGE2	1	HP	43	7.0	4.0–10.0	55	< 50	(5.8)	[30]
Phanerochaete carnosa	PcGCE	4	HP, HAt	72 (42)	6.0	–	40	–	–	[24]
Podospora anserina	PaGE1	5	HP	63	–	–	–	–	7.6 and 8.2 (6.9)	[27]
Cerrena unicolor	CuGE	4	HAo	58 (48)	–	–	–	–	–	[26]
Neurospora crassa	NcGE	8	HP	44	7.0	4.0–7.0	40–50	< 70	–	[29]
Acremonium alcalophilum	AaGE1	5	HP	72 (53)	–	7.0–11.0	–	< 50	–	[34]
Wolfiporia cocos	WcGE1	4	HP	45 (44)	–	7.0[c]	–	< 40	–	[34]
Bacteria										
Ruminococcus flavefaciens	cesA	–	HE	46	–	–	–	–	–	[35]
uncultured bacterium	MZ0003	–	HE	46	8.0	7.0–9.5	35	< 30	–	[33]

[a] Pur, purified from the original source; H, homologous expression (Ao, *Aspergillus oryzae*; At, *Arabidopsis thaliana*, Av, *Aspergillus vadensis*; E, *Escherichia coli*; P, *Pichia pastoris*; Pc, *Pycnoporus cinnabarinus*; T, *Trichoderma reesei*; Sc, *Schizophyllum commune*).
[b] Parentheses indicate calculated values.
[c] pH stability varied on the buffer [34].

glucopyranuronate was also reported revealing substrate binding within the active site and indicating possible catalytic mechanism of GEs.

The European Union (EU) collaborative project 'Optimized esterase biocatalysts for cost-effective industrial production' (OPTIBIOCAT, www.optibiocat.eu), granted in 2014 under the 7th EU Framework Programme (FP7), aims to replace chemical processes by enzymatic bioconversion via transesterification of esterases such as GEs for the production of cosmetics. To explore additional fungal GE enzymes, we used a genome mining analysis towards approximately 250 publicly accessible fungal genomes [15]. In this study, we report the genome mining strategy to identify novel fungal GEs and verify the strategy by biochemical characterization of the heterologously produced selected GEs, from both ascomycete and basidiomycete fungi, representing different phylogenetic sub-groups.

Materials and methods

Bioinformatics

Genome mining was performed by BLASTP search against 247 published fungal genomes [15] using 15 amino acid sequences from characterized and putative GEs (A.1, A.2 in Supplementary materials). All resulting amino acid sequences with an expected value lower than

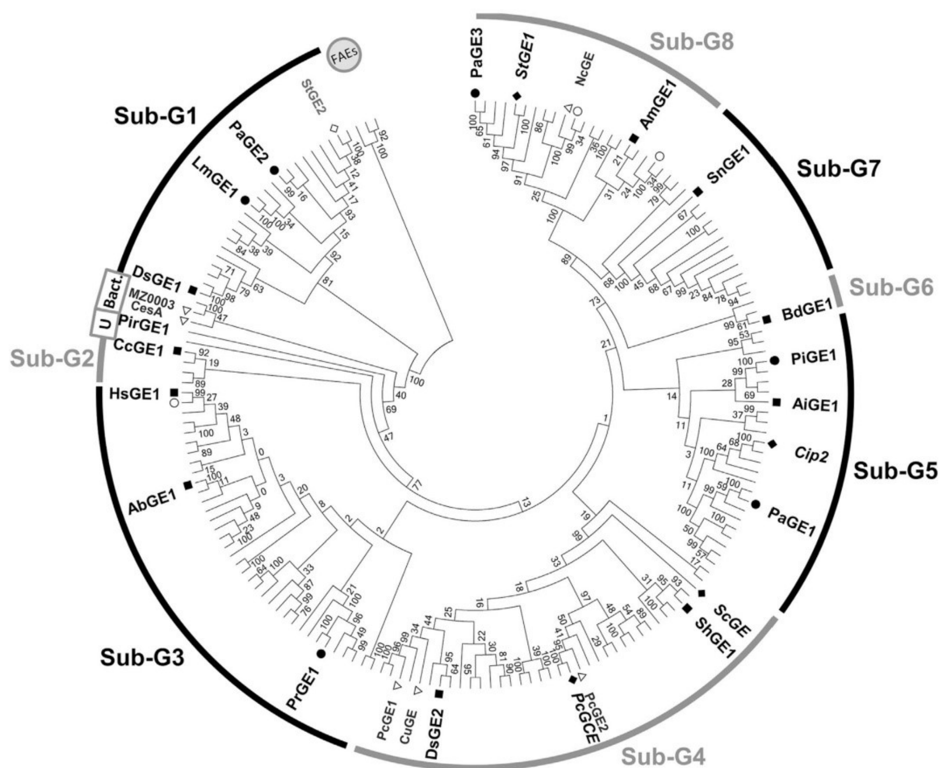

Fig. 2. Phylogenetic relationships among the (putative) fungal GEs. △, characterized GEs. The sequences used for BLASTP search in genome mining analysis are indicated as ◊ for characterized GE and ○ for putative GEs. Filled symbols indicate selected GEs for characterization in the present study. Bact. indicates group of bacterial GEs. U indicates ungrouped sequences. Feruloyl esterases (FAEs) were used as an outgroup. The full phylogenetic tree is given in A.1 in Supplementary materials. Complete enzyme names, details and sequences are given in Table 2 and A.2 in Supplementary materials.

$1E^{-40}$ were collected. Duplicate, unusually long and incomplete sequences as well as sequences with ambiguous amino acids (X) were discarded. Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/ [16]) and removed from all candidate sequences. The sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) [17]. Phylogenetic analysis was performed using the maximal likelihood method with complete deletion of gaps and the Poisson correction distance of substitution rates (statistical support for phylogenetic grouping was estimated by 1000 bootstrap re-samplings) of the Molecular Evolutionary Genetics Analysis (MEGA 7) program [18]. A few feruloyl esterase sequences were included as an outgroup. Theoretical molecular masses and p*I* values were calculated by ExPASy–ProtParam tool (http://www.expasy.ch/tools/protparam.html [19]).

Cloning of ge genes

The genes encoding the selected GEs without signal peptide were codon optimized and synthesized for expression in *P. pastoris* by NZYTech (Lisbon, Portugal). The gene products were digested by *Eco*RI *and Not*I (Thermo Fisher Scientific), and cloned in frame with α-factor secretion signal in pPNic706 (ProteoNic, Leiden, the Netherlands). The obtained plasmids were purified from *Escherichia coli* DH5α (Invitrogen) transformants selected on Luria Bertani medium supplemented with 50 μg/mL kanamycin, fully sequenced (Macrogen, Amsterdam, the Netherlands), linearised by *Sal*I (Thermo Fisher Scientific), and transformed into *P. pastoris* strain GS115 *his*4 according to the manufacturer's recommendation.

Ten transformants were selected for the enzyme production screening, which was performed in 96 deep-well plates containing 0.8 mL medium. The selected clones were grown first in buffered minimal glycerol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 1% w/v glycerol). The plates were sealed with AeraSeal™ (Sigma Aldrich) and were incubated overnight at 30 °C, 900 rpm (INFORS HT Microtron, Bottmingen, Switzerland). A volume of cells equal to an OD_{600} of 1.0 was harvested and resuspended in 0.8 mL buffered minimal methanol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 0.5% methanol) for induction. The induction was done at 30 °C, 900 rpm for 72 h before being harvested. The cultures were supplemented with 80 μL of 0.5% (v/v) methanol every 24 h.

Table 2
Molecular mass, production level and specific activity of characterized GEs in this work[a].

Fungal species	Accession number	Sub-group	Name[b]	Calculated molecular mass (kDa)	Apparent molecular mass (kDa)	Deglycosylated protein (kDa)	Calculated p*I*	Production (mg/L)	Relative activity[c] (nkat/mg)
Podospora anserina	CAP59671	1	PaGE2	44.3	nd	nd	8.3	np	na
Leptosphaeria maculans	CBX90574	1	LmGE1	41.6	nd	nd	8.2	np	Active[d]
Dichomitus squalens	jgi|Dicsq1|58498	1	DsGE1	41.8	65–70	45	5.0	2	1159
Coprinopsis cinerea	jgi|Copci1|5044	2	CcGE1	43.4	60–75	45	6.0	52	na
Penicillium rubens	CAP91804	3	PrGE1 (Pc13g07350)	40.2	50–60	42	6.3	14	162
Agaricus bisporus	jgi|Agabi_varbisH97_2|209748	3	AbGE1	46.5	nd	nd	5.9	np	na
Hypholoma sublateritium	jgi|Hypsu1|50423	3	HsGE1	47.4	48.8	nd	5.6	336	2334
Schizophyllum commune	XP_003026289	4	**ScGE**	40.2	40	36	4.3	25	4
Phanerochaete carnosa	AFM93784	4	**PcGCE**	42.5	nd	nd	4.6	330	4501
Stereum hirsutum	jgi|Stehi1|96554	4	ShGE1	47.2	nd	nd	4.6	296	333
Dichomitus squalens	jgi|Dicsq1|107426	4	DsGE2	46.7	75–100	50	4.2	38	46
Podospora anserina	XP_001903136	5	**PaGE1**	49.2	60	60	8.1	26	60
Trichoderma reesei	AAP57749	5	**Cip2**	46.7	71.4	nd	6.4	323	333
Ascobolus immersus	jgi|Ascim1|226781	5	AiGE1	47.6	nd	45.5	7.7	<1	225
Piriformospora indica	CCA74892	5	PiGE1	47.9	nd	48.9	8.3	5	67
Botryosphaeria dothidea	jgi|Botdo1|13681	6	BdGE1	39.0	nd	42.4	7.2	1	77
Stagonospora nodorum	jgi|Stano2|2908	7	SnGE1	39.5	nd	nd	7.8	np	Active[d]
Myceliophthora thermophila (Sporotrichum thermophile)	AEO60464.1	8	**StGE1**	40.3	40	40	5.6	66	31
Podospora anserina	CAP65970	8	PaGE3	40.3	50–60	40	8.5	44	36
Apiospora montagnei	jgi|Apimo1|126025	8	AmGE1	40.0	40–50	40	6.7	69	17
Piromyces sp. E2	jgi|PirE2_1|60981	U	PirGE1	40.6	42	40	6.1	<1	520

[a] nd, not detected; np, not produced; na, not active.
[b] Name in bold indicates the previously reported GEs.
[c] The assay performed at 45 °C using 2 mM benzyl-D-glucuronic acid ester in 73 mM phosphate buffer, pH 6.0.
[d] The enzyme was active but specific activity cannot be calculated because of undetectable protein level on the SDS-PAGE.

Production and biochemical properties of recombinant GEs

P. pastoris transformants were grown according to [20]. Induction was continued for 96 h at 28 °C with methanol being supplemented to 0.5% (v/v) every 24 h. Culture supernatants were harvested (4000 × *g*, 4 °C, 20 min), filtered (0.22 µm; Merck Millipore, Darmstadt, Germany) or concentrated (10 kDa cut off; Merck Millipore) and stored at −20 °C prior further analysis. Molecular mass determination and deglycosylation were performed as previously described [20]. Protein concentrations were assessed from SDS-PAGE gels by densitometry method using ImageJ program [21] with bovine serum albumin (Pierce, Thermo Scientific) as standard.

Enzyme activity assay of GEs

Activity of the recombinant GEs towards benzyl D-glucuronate (Taros Chemicals, Dortmund, Germany) was performed in 200 µL reaction mixtures adapted from [22]. The reactions were performed in the presence of 2 mM substrate, 73 mM phosphate buffer, pH 6.0, and 50 µL of culture supernatant at 45 °C for 30 min. Detection of glucuronic acid release was performed by using D-Glucuronic/D-Galacturonic Acid Assay Kit (Megazyme, Wicklow, Ireland) according to the manufacturer's recommendation. The culture supernatant of *P. pastoris* harboring pPNic706 plasmid without insert was used as negative control. All assays were performed in triplicate. One unit was defined as the amount of enzyme de-esterifying 1 µmol of benzyl-D-glucuronic acid ester per min under the assay conditions.

Results and discussion

Genome mining and phylogenetic analysis of novel fungal GEs

To identify the putative fungal GEs, a genome mining strategy was conducted by BLAST analysis with characterized and putative GEs against the published fungal genomes [15]. More than 150 putative fungal GEs were identified, which can be classified into 8 phylogenetic sub-groups (Fig. 2, A.1, A.2 in Supplementary materials). The first characterized GE (*S. commune*, ScGE, [10,23]) located to Sub-group 4 together with GEs from the white-rot fungi *Phanerochaete carnosa* (PcGCE, [24]), *Phanerochaete chrysosporium* (PcGE1, PcGE2; [25]) and *Cerrena unicolor* (CuGE, [26]) (Fig. 2). The ascomycete GEs, *Trichoderma reesei* GE (Cip2, [12]) and *Podospora anserina* GE (PaGE1 [27],) clustered in Sub-group 5. Sub-group 8 consisted of the GEs from the ascomycete fungi *Myceliophthora thermophila* (StGE1 [28],) and *Neurospora crassa* (NcGE, [29]), whereas Sub-group 1 consisted of a second GE from *M. thermophila* (StGE2, [30]). No characterized GE belongs to

Sub-group 2, 3, 6 and 7. Sub-group 3 consists of more than 30 members and Sub-group 7 consists of 15 members, whereas Sub-group 2 and 6 are small sub-groups containing 3–4 GE candidates. One GE candidate from an anaerobic fungus *Piromyces* sp. E2 (PirGE1) did not locate to any of the sub-groups. This ungrouped sequence may develop into a new sub-group if homologs for it are discovered. Two characterized bacterial GEs were included in the analysis, and clustered separately from the fungal GEs (Fig. 2).

Recently, a new classification of GEs was reported based on peptide pattern recognition (PPR) [31], which separate putative GEs into 24 PPR groups. Fungal GEs were clustered in PPR groups 1, 8, and 18. In comparison to our phylogenetic classification, the members from PPR group 8 belonged to phylogenetic Sub-group 1, whereas the members from PPR group 1 were divided in different phylogenetic groups. PPR group 18 contained only one member (GenBank XP_001832002.2 from *Coprinopsis cinerea*) representing an unusually long sequence (containing 3438 amino acids), hence it was not included in our phylogenetic analysis. In addition, a new database for Carboxylic Ester Hydrolases (CEH) was launched – CASTLE (CArboxylic eSTer hydrolase, http://castle.cbe.iastate.edu/, Iowa State University [32]). However, GEs are currently grouped together with acetyl xylan esterases in CEH8 in CASTLE database.

Sequence analysis and catalytic triad of selected fungal GEs

Twenty-one candidates (five characterized and 16 putative fungal GEs) were selected from both ascomycete and basidiomycete fungi with focus on wood rotting fungi (e.g. *Dichomitus squalens*, *P. carnosa*, *Schizophyllum commune*, *Stereum hirsutum*), saprophytic fungi living on dead plant or herbivore dung (e.g. *Podospora anserina*, *Ascobolus immersus*, *Apiospora montagnei*), plant pathogens (e.g. *Botryosphaeria dothidea*, *Stagonospora nodorum*, *Leptosphaeria maculans*) as well as industrially exploited fungi (e.g. *T. reesei*, *Penicillium rubens*), covering all eight sub-groups from the phylogenetic tree, including one ungrouped sequence (PirGE1), for heterologous production using *P. pastoris* as a host and subsequent biochemical characterization (Table 2). The selection of the number of putative GEs was solely based on the size of the phylogenetic sub-group. The amino acid sequence alignment of 16 putative fungal GEs and all characterized GEs are present in A.3 in Supplementary materials. The fungal GEs were relatively conserved and the signature motif of CE15 family (G-C-S-R-X-G, [30]) was well aligned, except for PirGE1 which has Tyr instead of Arg. In addition, two bacterial GEs (CesA and MZ0003) have Val and His, respectively, instead of Cys in the signature motif. Among the catalytic triad, Ser and His are well conserved in all sequences, whereas Glu is not highly conserved among CE15 enzymes and is substituted by Asp, Gln, Asn and Ala, as well as Ser (StGE2, PaGE2–*Podospora anserina*, DsGE1–*Dichomitus squalens*) and Cys (LmGE1–*Leptosphaeria maculans*, CesA and MZ0003) [33].

Biochemical properties of selected fungal GEs

The putative GE-encoding genes were heterologously expressed in *P. pastoris*. Only two (PaGE2 and AbGE1–*Agaricus bisporus*) out of 21 GE candidates were not successfully produced. The production level varied from 2 to 336 mg/L, and four enzymes (HsGE1–*Hypholoma sublateritium*, PcGCE, Cip2, ShGE1–*Stereum hirsutum*) were produced up to 300 mg/L. From the 19 produced GE candidates, 18 were active towards benzyl-D-glucuronic acid ester (Table 2). The highest specific activity (> 1000 nkat/mg) was detected for PcGCE, HsGE1 and DsGE1. SnGE1–*Stagonospora nodorum* and LmGE1 showed low activity (0.156 nkat/ml and 0.097 nkat/ml, respectively) and were produced at low level as they were not visible in Coomassie blue stained SDS-PAGE gel. CcGE1 protein from *Coprinopsis cinerea* was highly produced but not active towards the tested substrate at different pH values (4–8).

Conclusions

In the present study, we showed that genome mining is a powerful strategy for enzyme discovery to identify fungal GE encoding genes. Our phylogenetic analysis categorized the putative fungal GEs into eight sub-groups. We further demonstrated that from 16 putative fungal GEs, 13 possessed GE activity towards benzyl D-glucuronate. The members from Sub-groups 1, 4, 5 and 8 were previously characterized and shown to possess GE activity (Table 1). Here we demonstrated that the candidates from Sub-groups 3, 6, and 7 also possessed GE activity (Table 2). Because of the limited availability of substrates used for the assessment of GE activity, currently it is not possible to verify if the phylogenetic grouping also reflects functional differences among GEs, such as substrate specificity or possible site of action. In comparison with the previously characterized fungal GEs used in this study, most of the new GEs showed comparable activity. This indicates that they may have potential in saccharification of plant biomass or other industrial applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.nbt.2017.10.003.

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2.2.2 Fungal feruloyl esterases: functional validation of genome mining based enzyme discovery including uncharacterized subfamilies (Paper III)

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Full length article

Fungal feruloyl esterases: Functional validation of genome mining based enzyme discovery including uncharacterized subfamilies

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ABSTRACT

Feruloyl esterases (FAEs) are a diverse group of enzymes that specifically catalyze the hydrolysis of ester bonds between a hydroxycinnamic (e.g. ferulic) acid and plant poly- or oligosaccharides. FAEs as auxiliary enzymes significantly assist xylanolytic and pectinolytic enzymes in gaining access to their site of action during biomass saccharification for biofuel and biochemical production. A limited number of FAEs have been functionally characterized compared to over 1000 putative fungal FAEs that were recently predicted by similarity-based genome mining, which divided phylogenetically into different subfamilies (SFs). In this study, 27 putative and six characterized FAEs from both ascomycete and basidiomycete fungi were selected and heterologously expressed in *Pichia pastoris* and the recombinant proteins biochemically characterized to validate the previous genome mining and phylogenetical grouping and to expand the information on activity of fungal FAEs. As a result, 20 enzymes were shown to possess FAE activity, being active towards *p*NP-ferulate and/or methyl hydroxycinnamate substrates, and covering 11 subfamilies. Most of the new FAEs showed activities comparable to those of previously characterized fungal FAEs.

Introduction

Esterified or etherified to polymers within the lignocellulosic matrix, ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) and to a lesser extent *p*-coumaric acid (4-hydroxycinnamic acid) are the most abundant hydroxycinnamic acids in plant cell walls [1,2]. These hydroxycinnamic acids can be linked to arabinoxylans (*O*-5 position of α-L-arabinofuranosyl residues), which are the unique structural components in commelinid monocots (Family Poales, e.g. wheat, rice and barley). They can also be linked to neutral pectic side-chains of rhamnogalacturonan I (*O*-6 position of β-D-galactopyranosyl residues in (arabino)galactan, and *O*-2 or *O*-5 position of α-L-arabinofuranosyl residues in arabinan), which are mainly found in eudicotyledons (Order 'core' Caryophyllales, e.g. sugar beet) [3–8]. FA can form diferulic acids (mainly 5,5′-, 8-*O*-4′-, 8,5′-, 8,8′-diferulic acids) which cross-link two polysaccharide chains or a polysaccharide chain to lignin [7,9–11]. Phenolic cross-links increase the physical strength and integrity of plant cell walls and reduce their biodegradability by microbial invaders and hydrolytic enzymes [2,12].

Feruloyl esterases (or ferulic acid esterases, FAEs) [E.C. 3.1.1.73] represent a subclass of the carboxylic acid esterases (E.C. 3.1.1) and catalyze the hydrolysis of ester linkage between a phenolic acid and a poly- or oligosaccharide releasing hydroxycinnamic acids from plant cell wall polysaccharides [13,14]. FAEs are able to release FAs and other phenolic acids from natural plant sources and agro-industrial byproducts. They facilitate the degradation of complex plant cell wall

Abbreviations: FA, ferulic acid; FAE, feruloyl esterase; SF, subfamily

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polysaccharides by removing the ester bonds between plant polymers providing accessibility for glycoside hydrolases and polysaccharide lyases [15–17]. Apart from being used as accessory enzymes in the saccharification process, FAEs are also potential biocatalysts for synthesis of a broad range of novel bioactive components for use in the food, cosmetics and pharmaceutical industries [18,19]. In 2014, an EU collaborative project 'OPTIBIOCAT' was granted by the 7th Framework Programme (FP7), which aims to use microbial esterases such as FAEs as biocatalysts for synthesis of potential antioxidants for cosmetic products [19]. As these enzymes are relevant for various industries, different types of FAE are required to fit specific conditions such as pH and temperature.

Recently, we reported a genome mining strategy for FAE discovery, in which more than 1000 putative fungal FAE sequences were identified and, by using a phylogenetical analysis, classified into 13 subfamilies (SFs) [19]. In contrast to the high number of the putative FAE encoding genes, a limited number of fungal FAEs have been characterized in detail and they only cover SF1, 2, 5, 6, 7 and 13. To validate our genome mining strategy and expand the information on activity and properties of fungal FAEs, in this study we report the heterologous expression and biochemical characterization of selected recombinant FAEs discovered through genome mining, and covering the previously uncharacterized SFs.

Materials and methods

Bioinformatics

Genome mining and phylogenetic analysis were performed based on [19]. Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; [20]). The gene model correction of selected sequences was performed manually based on BlastX to identify and remove putative introns [21]. Sequence alignment was performed using Multiple Alignment using Fast Fourier Transform (MAFFT) [22]. Theoretical molecular masses and p*I* were calculated by the ExPASy–ProtParam tool (http://www.expasy.ch/tools/protparam.html [23]).

Cloning of fae genes

The genes of the selected FAEs without signal peptide and introns were codon optimized and synthesized for expression in *P. pastoris* by NZYTech (Lisbon, Portugal). The gene products were digested by *Psi*I and *Not*I (Thermo Fisher Scientific), and cloned in frame with α-factor secretion signal in pPNic706 (ProteoNic, Leiden, the Netherlands). The obtained plasmids were purified from *Escherichia coli* DH5α (Invitrogen), transformants selected on Luria Bertani medium supplemented with 50 μg/mL kanamycin, fully sequenced (Macrogen, Amsterdam, the Netherlands), linearised by *Sal*I (Thermo Fisher Scientific), and transformed into *P. pastoris* strain GS115 *his*4 according to the manufacturer's recommendation.

Ten transformants were selected for the enzyme production screening, which was performed in 96 deep-well plates containing 0.8 mL medium. The selected clones were grown first in buffered minimal glycerol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 1% w/v glycerol). The plates were sealed with AeraSeal™ (Sigma Aldrich) and incubated overnight at 30 °C, 900 rpm (INFORS HT Microtron, Bottmingen, Switzerland). A volume of cells equal to an OD_{600} of 1.0 was harvested and resuspended in 0.8 mL buffered minimal methanol medium (1% yeast nitrogen base, 0.1 M potassium phosphate buffer pH 6.5, and 0.5% methanol) for induction. The induction was performed at 30 °C, 900 rpm for 72 h before being harvested. The cultures were supplemented with 80 μL of 0.5% (v/v) methanol every 24 h.

Production and biochemical properties of recombinant FAEs

P. pastoris transformants were grown according to [24]. Induction was continued for 96 h at 28 °C with methanol being supplemented to 0.5% (v/v) every 24 h. Culture supernatants were harvested (4000 × g, 4 °C, 20 min), filtered (0.22 μm; Merck Millipore, Darmstadt, Germany) or concentrated (10 kDa cut off; Merck Millipore) and stored at −20 °C prior further analysis. Molecular mass determination and deglycosylation were performed as previously described [24]. Protein concentrations were assessed from SDS-PAGE gels by densitometric method using ImageJ program [25] with bovine serum albumin (Pierce, Thermo Scientific) as a standard.

Enzyme activity assay of FAEs

Activity of the recombinant FAEs towards *p*NP-ferulate (Taros Chemicals, Dortmund, Germany) was performed in 275 μL reaction mixtures adapted from [26]. The *p*NP-ferulate substrate solution was prepared by mixing 10.5 mM *p*NP-ferulate (in dimethyl sulfoxide) and 100 mM potassium phosphate buffer, pH 6.5 containing 2.5% Triton-X (1:9, v/v). The reactions were performed in the presence of 250 μL *p*NP-ferulate substrate solution incubated with 25 μL of culture supernatant at 37 °C. The release of *p*-nitrophenol was spectrophotometrically quantified by following the absorbance at 410 nm for 30 min with a 2 min interval and calculation according to [26]. All assays were performed in triplicate. One unit of FAE activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol from *p*NP-ferulate per min under the assay conditions.

Activity towards methyl substrates [methyl caffeate, methyl ferulate, methyl *p*-coumarate, and methyl sinapate (Apin Chemicals Limited, Oxon, United Kingdom)] was assayed in 250 μL reaction mixtures according to [24] at 37 °C for 5–30 min. Detection of substrates reduction was performed at 340 nm with a 2 min interval. The activity was determined from the standard curves of the substrates (0.001–0.5 mM). Alternatively, the activities were assayed by HPLC (Agilent 1260 Infinity) using Kinetex 2.6 u C18 100A column (Phenomenex). The quantification was performed by using calibration curves of the methyl substrates and their corresponding acids, and the detection was performed at 320 nm for methyl caffeate, methyl ferulate and methyl sinapate, and at 308 nm for methyl *p*-coumarate. The 1 mL reaction mixtures contained 850 μL MOPS buffer (pH 6.0), 100 μL enzyme and 50 μL substrate (1 mM). The reactions were stopped by adding 1 vol 0.1% trifluoroacetic acid:acetonitrile solution (80:20). Chromatographic separation was performed by isocratic method with 80% 0.1% trifluoroacetic acid and 20% acetonitrile as solvents. The culture supernatant of *P. pastoris* harboring pPicZαA plasmid without insert was used as negative control. All assays were performed in triplicate.

Enzyme activity assay of tannases

Methyl gallate (Sigma Aldrich, St. Louis, MO) was used for the assessment of tannase activity [27]. The reactions were performed in 125 μL reaction mixtures containing 6.25 μL of 100 mM methyl gallate stock solution (in dimethylformamide), 31.25 μL of 100 mM phosphate buffer, pH 6.0, and 25 μL water incubated with 62.5 μL of culture supernatant at 30 °C for 15 min. To detect the release of gallic acid, 75 μL of 0.667% rhodanine (in methanol) (Sigma Aldrich) was added to the reaction mixture followed by 5 min incubation at 30 °C, addition of 100 μL of 0.5 M KOH, further incubation at 30 °C for 5 min, and addition of 1 mL water prior to quantification by measuring the absorbance at 520 nm. The activity was determined from the standard curves of the substrates (0.006–0.6 mM). Alternatively, tannase activity was assayed by the HPLC method described above by detecting methyl gallate at 280 nm and using InfinityLab Poroshell 120 SB-AQ column (Agilent).

Enzyme activity assay of lipases

For assessment of the lipase activity, *p*NP-palmitate (Sigma Aldrich) was used as a substrate. Ten mM *p*NP-palmitate stock solution was prepared in isopropanol and diluted in 100 mM potassium phosphate buffer, pH 6.5. The reaction was performed in 1 mL reaction mixtures containing 900 μL of 0.2 mM *p*NP-palmitate in 100 mM potassium phosphate buffer, pH 6.5, and 100 μL of culture supernatant at 40 °C. The release of *p*-nitrophenol was spectrophotometrically quantified by following the absorbance at 410 nm for 30 min with a 2 min interval and calculation according to [26].

Results and discussion

Discovery of novel fungal FAEs

A genome mining strategy was previously conducted by BLAST analysis with characterized FAEs against published fungal genomes to identify the putative fungal FAEs [19]. In the current study, four additional genomes from *Aspergillus* spp., i.e. *Aspergillus sydowii*, *Aspergillus wentii* DTO 134E9, *Aspergillus carbonarius* ITEM 5010 and *Aspergillus tubingensis* [28], were added to the analysis (A.1 in Supplementary materials). Over 1000 putative fungal FAE sequences were identified, which were classified into 13 SFs. Of these, 33 FAE sequences (six previously characterized and 27 putative FAEs) from both ascomycetes and basidiomycetes, which covered five previously uncharacterized FAE subfamilies (SF3, 8, 9, 10, 12), were selected for biochemical characterization (Table 1). The enzymes from SF11 were not included in this study because they most likely possessed tannase activity.

Recombinant enzyme production of selected fungal FAEs

Heterologous expression of putative FAEs was performed using *P. pastoris* as a host organism. FAE sequences from both ascomycetes and basidiomycetes were produced as active enzymes in *P. pastoris*. Three of the selected enzymes, i.e. An09g05120 (*A. niger*), CsFae3 (*Ceriporiopsis subvermispora*) and ShFae1 (*Stereum hirsutum*), were produced at a concentration greater than 1000 mg/L and five, namely GmFae1 (*Galerina marginata*), OrpFAE (*Orpinomyces* sp.), AnFaeJ, An11g01220 (*A. niger*) and CsFae1 (*C. subvermispora*), were produced at a concentration greater than 100 mg/L. However, the production level of eight and 12 of the enzymes was less than 100 mg/L and 10 mg/L respectively, and five of them were not produced. Almost half of the recombinant enzymes showed higher molecular masses compared to the calculated ones, but the molecular mass reduced to the expected size after treatment with Endoglycosidase H indicating glycosylation by *P. pastoris*.

Activity and substrate preference among the selected fungal FAEs

To screen for FAE activity, *p*NP-ferulate was used as a substrate. Among 27 enzymes, 20 were active, with GmFae1 (*G. marginata*) showing the highest activity (Table 1). With respect to the substrate specificity, four methyl substrates (methyl *p*-coumarate, methyl caffeate, methyl ferulate, and methyl sinapate) were used. In agreement with the previous reports [29–31], AnFaeB (*A. niger*) and AsFaeF (*A. sydowii*) from SF1 hydrolyzed three methyl substrates: methyl *p*-coumarate, methyl caffeate and methyl ferulate (Table 2). Docking simulation of methyl sinapate on a structure of a member of SF1, AoFaeB (from *A. oryzae*, PDB: 3WMT), indicated that its narrow active site hindered the binding of the bulky structure of methyl sinapate [32]. AsFaeI (*A. sydowii*) from SF13 was also able to hydrolyze three methyl substrates similarly to the enzymes from SF1, whereas the other FAEs from SF13 showed either low or no activity towards methyl ferulate. The majority of the active FAEs from SF5 and SF6 hydrolyzed all four substrates, except FoFae2 (*Fusarium oxysporum* from SF6) and CcFae2 (*Coprinopsis cinerea* from SF6), which did not hydrolyze methyl sinapate, and Settu1|102085 (*Setosphaeria turcica* from SF5), which was only active towards methyl caffeate and methyl sinapate. Because Settu1|102085 as well as some other esterases (see below) were not detected to hydrolyze methyl ferulate, they were referred to as hydroxycinnamoyl esterases (HCE) instead of FAEs.

AnFaeA (*A. niger*) from SF7 hydrolyzed only methyl ferulate and methyl sinapate, which is consistent with the previous reports [29,31,33]. The crystal structure of AnFaeA showed a long and narrow cavity displaying hydrophobic residues that stabilize the aromatic moiety of the substrate [34], and replacing the bulky aromatic residues (Tyr80 or Trp260) to smaller residues broadened the substrate specificity of the enzyme [35].

Three FAEs from SF2, namely FoFaeC (*F. oxysporum*), AwFaeG (*A. wentii*) and GlFae1 (*Gymnopus luxurians*), showed no obvious substrate specificity pattern. FoFaeC hydrolyzed all tested substrates with low activity on methyl sinapate, which is consistent with the previous report [36]. AwFaeG also showed low activity towards methyl sinapate, but did not hydrolyze methyl *p*-coumarate. GlFae1 showed the highest activity towards methyl sinapate and lower activity towards methyl ferulate, but was not active towards methyl *p*-coumarate and methyl caffeate. The enzymes from SF3 (Aspca3|176503 from *A. carbonarius*), SF9 (Asptu1|30001 from *A. tubingensis*) and SF12 (Galma1|254175 from *G. marginata*) were not active towards methyl ferulate and *p*NP-ferulate, but showed activity towards only one methyl substrate with no specific pattern. Therefore, these esterases were also referred to as HCEs. The enzyme from SF10 (OrpFAE from *Orpinomyces* sp.) was not active towards methyl substrates, but showed activity towards *p*NP-ferulate.

Previously, members of SF1, 2, 5, 6, 7 and 13 were shown to possess FAE activity [19]. In the present study, we reported that enzymes from SF8, 9 and 10 showed limited activity towards synthetic FAE substrates, as well as towards methyl gallate for SF9. Recently, an esterase from *Auricularia auricularia-judae* (EstBC), belonging to SF8 was described that acted efficiently on both artificial cinnamic and benzoic acid esters, but was not active on complex natural FAE substrates [37]. Hence, the enzymes from these subfamilies should be further tested towards feruloylated saccharides or natural substrates, e.g. wheat bran or sugar beet pectin, to confirm their true FAE activity.

Enzymes with other activity

Five enzymes (CsTan1–*C. subvermispora*, AnFaeJ–*A. niger*, Aspsy1|41271–*A. sydowii*, An11g01220–*A. niger*, An09g05120–*A. niger*) were active towards methyl gallate. These enzymes belong to SF9, SF10 and SF13, indicating that the representatives of these SFs may be tannases. Recently, two FAEs from *Schizophyllum commune* were reported to hydrolyse methyl gallate [38]. It is possible that these enzymes are the bridge in the evolution from tannases to FAEs or *vice versa*. Surprisingly, one enzyme (Aspca3|176503 from *A. carbonarius*) was active towards *p*NP-palmitate, although this enzyme does not share amino acid sequence similarity to known lipases.

Conclusions

In the present study, we have confirmed the ability of the genome mining strategy to identify fungal FAE encoding genes, by demonstrating that 20 out of 27 putative fungal FAEs possessed FAE activity towards *p*NP-ferulate and/or methyl hydroxycinnamate substrates. Previously, members of SF1, 2, 5, 6, 7 and 13 were shown to possess FAE activity [19]. In the present study, we also showed that the enzymes from SF8, 9 and 10 are active towards the synthetic FAE substrates. However, it should be noted that most of the enzymes from SF9 possessed tannase activity. Additional experiments are needed to confirm whether the enzymes of these subfamilies are true FAEs. The selected esterases from SF3 and SF12 were active towards methyl caffeate

Table 1
Molecular mass, production level and specific activity (towards *p*NP-ferulate) of characterized FAEs in this study[a].

Fungal species	Phylum[b]	Accession number	SF[c]	Name[d]	Calculated molecular mass (kDa)	Apparent molecular mass (kDa)	Calculated p*I*	Production (mg/L)	Specific activity[h] (mU/mg)	Remark[i]
Aspergillus niger	Asco	Q8WZI8.1	1	**AnFaeB**	55.8	100 (55)	4.95	55	2	
Aspergillus sydowii	Asco	jgi|Aspsy1| 293049	1	AsFaeF	55.4	55[e]	4.89	< 1	4	
Fusarium oxysporum	Asco	jgi|Fusox1| 5438	2	**FoFaeC**	59.4	70 (61)[f]	6.89	6	Active	
Aspergillus wentii	Asco	jgi|Aspwe1| 156253	2	AwFaeG	56.0	57	4.99	< 1	Active	
Gymnopus luxurians	Asco	jgi|Gymlu1| 46632	2	GlFae1	56.8	58	4.53	3	na	
Aspergillus carbonarius	Asco	jgi|Aspca3| 176503	3	–	56.1	58	5.02	4	na	Lipase activity
Aspergillus sydowii	Asco	jgi|Aspsy1| 901052	3	–	55.8	nd	4.88	np	na	
Aspergillus nidulans	Asco	EAA62427.1	5	**AnidFAEC**	25.8	30 (30)[f]	4.47	30	2	
Aspergillus sydowii	Asco	jgi|Aspsy1| 154482	5	AsFaeC	25.9	30 (30)[f]	4.44	15	2	
Aspergillus sydowii	Asco	jgi|Aspsy1| 48859	5	AsFaeD1	26.6	nd	3.93	np	na	
Setosphaeria turcica	Asco	jgi|Settu1| 102085	5	–	26.9	26	8.90	< 1	na	
Myceliophthora thermophila (*Sporotrichum thermophile*)	Asco	AEO62008.1	6	**MtFae1a**	29.5	35 (30)[f]	4.44	45	3	
Fusarium oxysporum	Asco	jgi|Fusox1 |8990	6	FoFae2	29.6	30	8.44	4	Active	
Aspergillus sydowii	Asco	jgi|Aspsy1| 1158585	6	AsFaeE	29.5	32[e]	4.24	7	4	
Stagonospora nodorum	Asco	jgi|Stano2| 8578	6	–	31.3	30	5.16	12	na	
Coprinopsis cinerea	Basidio	jgi|Copci1| 3628	6	CcFae2	37.7	38	6.63	< 1	Active	
Ceriporiopsis subvermispora	Basidio	jgi|Cersu1| 68569	6	CsFae1	36.9	70	4.58	280	7	
Galerina marginata	Basidio	jgi|Galma1| 144217	6	GmFae1	35.2	42	5.83	600	120	
Aspergillus niger	Asco	CAA70510	7	**AnFaeA**	28.6	40 (35)[f]	4.19	55	4	
Aspergillus clavatus	Asco	jgi|Aspcl1| 3045	8	–	39.1	40[e]	6.34	51	na	
Ceriporiopsis subvermispora	Basidio	jgi|Cersu1| 89153	9	CsTan1	55.0	90 (60)[g]	4.59	np	na	Tannase activity
Aspergillus tubingensis	Asco	jgi|Asptu1| 30001	9	–	57.6	57	4.50	< 1	na	
Aspergillus niger	Asco	An15g05280	9	AnFaeJ	58.6	68 (60)[g]	4.93	500	na	Tannase activity
Aspergillus sydowii	Asco	jgi|Aspsy1| 41271	9	–	57.6	60[e]	4.99	32	na	Tannase activity
Orpinomyces sp.	Neo	AAF70241.1	10	**OrpFAE**	59.0	68	5.06	600	10	
Aspergillus niger	Asco	An11g01220	10	–	55.0	88	4.11	500	na	Tannase activity
Aspergillus sydowii	Asco	jgi|Aspsy1| 194109	10	–	52.7	nd	4.67	np	na	
Dichomitus squalens	Basidio	jgi|Dicsq1| 136925	12	–	56.3	nd	4.78	np	na	
Galerina marginata	Basidio	jgi|Galma1| 254175	12	–	56.0	59	6.90	3	na	
Aspergillus sydowii	Asco	jgi|Aspsy1| 160668	13	AsFaeI	59.4	55[e]	4.90	< 1	4	
Stereum hirsutum	Basidio	jgi|Stehi1| 73641	13	ShFae1	58.1	67	4.40	2160	13	
Aspergillus niger	Asco	An09g05120	13	–	53.1	66	4.79	3400	na	Tannase activity
Ceriporiopsis subvermispora	Basidio	jgi|Cersu1| 150639	13	CsFae2	55.1	nd	4.28	2400	1	

[a] na, no activity detected; nd, no protein band detected; np, no protein produced or the protein level was lower than detection limit.
[b] Asco, ascomycete; Basidio, basidiomycete; Neo, Neocallimastigomycete.
[c] According to [19].
[d] Name in bold indicates the previously reported FAEs [19]. The abbreviation of the enzyme code is based on the convention protein names for different species and not on the types of FAEs.
[e] indicates the protein band was visible only after deglycosylation by Endoglycosidase H.
[f] indicates molecular mass after deglycosylation by Endoglycosidase H.
[g] indicates molecular mass after deglycosylation by PNGase F.
[h] One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol from *p*NP-ferulate per min under assay conditions. Active indicates the enzyme was active but the specific activity could not be calculated.
[i] Tannase and lipase activities were evaluated using methyl gallate and *p*NP-palmitate as substrate, respectively.

Table 2
Relative FAE activity towards four methyl substrates[a].

			Relative activity (%)[d]			
Fungi	SF[b]	Name[c]	Methyl *p*-coumarate	Methyl caffeate	Methyl ferulate	Methyl sinapate
Aspergillus niger	1	**AnFaeB**	100	77	59	na
Aspergillus sydowii	1	AsFaeF	100	66	60	na
Fusarium oxysporum	2	**FoFaeC**	100	67	29	9
Aspergillus wentii	2	AwFaeG	na	100	88	29
Gymnopus luxurians	2	GlFae1	na	na	18	100
Aspergillus carbonarius	3	–	na	100	na	na
Aspergillus nidulans	5	**AnidFAEC**	98	42	100	46
Aspergillus sydowii	5	AsFaeC	91	45	100	40
Aspergillus sydowii	5	AsFaeD1	na	na	Low[e]	na
Setosphaeria turcica	5	–	na	100	na	91
Myceliophthora thermophila	6	**MtFae1a**	100	77	90	36
Fusarium oxysporum	6	FoFae2	100	60	16	na
Aspergillus sydowii	6	AsFaeE	95	53	100	60
Stagonospora nodorum	6	–	na	na	na	na
Coprinopsis cinerea	6	CcFae2	100	41	23	na
Ceriporiopsis subvermispora	6	CsFae1	na	na	na	na
Galerina marginata	6	GmFae1	Low[e]	Low[e]	Low[e]	Low[e]
Aspergillus niger	7	**AnFaeA**	na	na	100	81
Aspergillus clavatus	8	–	na	na	Low[e]	na
Aspergillus tubingensis	9	–	na	100	na	na
Aspergillus niger	9	AnFaeJ	na	Low[e]	Low[e]	Low[e]
Aspergillus sydowii	9	–	na	na	na	na
Orpinomyces sp.	10	OrpFAE	na	na	na	na
Aspergillus niger	10	–	Low[e]	Low[e]	Low[e]	Low[e]
Galerina marginata	12	–	100	na	na	na
Aspergillus sydowii	13	AsFaeI	100	65	60	na
Stereum hirsutum	13	ShFae1	na	na	Low[e]	na
Aspergillus niger	13	–	na	Low[e]	Low[e]	Low[e]
Ceriporiopsis subvermispora	13	CsFae2	na	na	na	na

[a] na, no activity detected.
[b] According to [19].
[c] The abbreviation of the enzyme code is based on the conventional protein names for different species and not on the types of FAEs. Name in bold indicates the previously reported FAEs.
[d] The relative activity was calculated as a percentage of the highest activity for each enzyme that was set to 100%.
[e] Low indicates activity which was lower than the reliable detectable range.

and methyl *p*-coumarate, respectively, but not towards methyl ferulate. In comparison with the previously characterized fungal FAEs, most of the new FAEs showed similar levels of specific activity. Thus, they may potentially be eligible candidates for related biotechnological applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.nbt.2017.11.004.

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2.2.3 Design, recombinant expression and characterization of site-directed FAE mutants

Results

Design of AwFaeG site-directed mutants

According to the outputs of enzyme characterization described in Paper III, the novel fungal FAE AwFaeG (SF2.3, *Aspergillus wentii*) was selected as the most promising enzyme to be subjected to site-directed mutagenesis to further fine-tune the enzyme towards its application in bioconversions.

The mutagenesis process (developed in collaboration with the group of Fungal Physiology Westerdijk Fungal Biodiversity Institute & Fungal Molecular Physiology, Utrecht University) consisted in the generation of homology models for AwFaeG and in the choice of sites suitable for mutagenesis, based on amino acid analysis/alignment and algorithms for predicting structural changes. To perform this task, four different programs were used and FAE B from *Aspergillus oryzae* (3WMT, Suzuki et al. 2014) was used as template for AwFaeG (>50% sequence identity). The four models were then evaluated using the Protein Model Check option in WHAT IF (http://swift.cmbi.ru.nl/servers/html/index.html), giving the same result for each model. The putative active site residues of AwFaeG were determined to be Ser172, Asp384 and His424, located in a groove in the middle of the protein. The MODELLER output was chosen as the main model for further analysis. To predict the amino acid residues which could possibly enhance the thermal stability of the protein, PoPMuSiC - Prediction of Protein Mutant software was used. The amino acids with high negative score/contributor from PoPMuSic were then evaluated for mutation possibility using a PyMol (https://www.pymol.org/) as molecular visualization system. Some of the risky residues were evaluated by hydrophobic cluster analysis (HCA) using DrawHCA (http://bioserv.impmc.jussieu.fr/hca-seq.html) to analyse the possibility of introducing a change in the 3D-structure. According to the analysis above, the amino acid residues that gave the best score and the minimum conflict in the 3D structure when changed, were chosen even though quite conserved. Residues to be mutated and the designed mutations are showed in figure 2. From this point further, the mutants will be named with the mutation codes reported in Table 2.

	Mutation/ Code
AwFaeG Mut1	N391V
AwFaeG Mut2	N391L
AwFaeG Mut3	G470F
AwFaeG Mut4	G195C
AwFaeG Mut5	S89V

Figure 2: Positions of the residues of AwFaeG to be mutated and the five designed mutations.

Expression and characterization of AwFaeG site-directed mutants

The *P. pastoris* clones expressing the five AwFaeG mutants were grown in 50 ml of the medium BMMY at 28°C. Samples of culture broth were harvested after three

days of growth and, after biomass removal, subjected to analysis of FAE activity towards *p*NP-Fe (Table 3). Only N391V mutant showed higher activity than AwFaeG towards *p*NP-Fe with an activity around sevenfold higher than that of the wild-type. This increase in hydrolytic activity could be due to the fact that the mutated amino acid is located near the active site, changing the Asp residue with Val residue.

All five AwFaeG site-directed mutants were analyzed for their thermotolerance, assaying their activity towards *p*NP-Fe after the incubation of 1 hour at 55°C. Moreover, AwFaeG site-directed mutants were analyzed in terms of solvent tolerance after 1 hour of incubation in 25% acetone and in 5% n-butanol at 37°C. Residual enzymatic activity after heat and solvent exposure was measured against *p*NP-Fe and the percentages of residual activity of AwFaeG and its site-directed mutants are reported in table 2. All the site-directed mutants, except for N391V, showed higher residual activity than wild type enzyme after 1 hour incubation at 55°C. This result confirmed that the designed mutations increased the thermostability of the enzyme. In particular, G470F variant showed the highest thermotolerance after 1 hour at 55°C. Moreover, G470F, G195C and S89V showed at least twofold higher residual activity than AwFaeG after exposure to 25% acetone, and higher residual activity than AwFaeG after exposure to 5% butanol. In particular, G470F variant showed the highest residual activity after exposure to 25% acetone, whilst G195C variant showed the highest activities after 5% butanol exposure.

Table 2: Analysis of FAE activity of the AwFaeG site-directed mutants, thermotolerance (1h at 55°C) and solvent tolerance after exposure to 25% acetone and 5% butanol (1h at 37°C).

		% residual activity		
sample	***Activity (mU/mL)***	***55°C***	***25% acetone***	***5% butanol***
AwFaeG	25.07	5.43	41.55	144.07
N391V	**304.26**	1.56	2.80	8.01
N391L	22.06	25.59	39.56	142.75
G470F	2.48	**197.06**	**227.52**	**881.67**
G195C	2.69	79.51	**310.70**	**767.21**
S89V	8.95	21.34	120.97	203.79

Substrate specificities of the AwFaeG site-directed mutants were analyzed by assaying their enzymatic activity towards MFA, MCA, MSA and M*p*CA (table 3). All the site-directed mutants gained activity towards different substrates compared to the wild type enzyme which was able to hydrolyze only MFA. N391V variant gained activity towards MSA and MCA, whilst S89V variant showed the highest activity towards MSA and gained affinity towards M*p*CA. However, substrate specificity of AwFaeG and its site-directed mutants did not show a specific pattern.

Table 3: Relative activity of AwFaeG site-directed mutants towards MFA, MCA, MSA and M*p*CA.

	Relative activity (%)[d]			
sample	**MFA**	**MCA**	**M*p*CA**	**MSA**
AwFaeG	100	0	0	0
N391V	100	17.4	0	36.7
N391L	0	0	15	100
G470F	98	0	100	87.7
G195C	9.6	100	65.9	0
S89V	32.2	0	71.2	100

[d]The relative activity was calculated as a percentage of the highest activity for each enzyme that was set to 100%

Materials and Methods

Design of point mutations

Four different programs were used to build homology models of AwFaeG:
1) MODELLER –Program for Comparative Protein Structure Modelling by Satisfaction of Spatial Restrains (http://salilab.org/modeller/, (Eswar et al., 2006)²²²
2) HHpred –Homology detection & structure prediction by HMM-HMM comparison (http://toolkit.tuebingen.mpg.de/hhpred, (Söding et al., 2005)
3) I-TASSER –Iterative Threading ASSEmbly Refinement protein structure and function predictions (http://zhanglab.ccmb.med.umich.edu/I-TASSER/, (Zhang, 2008)
4) Phyre –Protein Homology/analogY Recognition Engine V 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index, (Kelley and Sternberg, 2009).
FAE B from *Aspergillus oryzae*, 3WMT, (Suzuki et al., 2014)¹¹¹ was used as template for FAE801 (>50% sequence identity). The models were then evaluated using the Protein Model Check option in WHAT IF (http://swift.cmbi.ru.nl/servers/html/index.html).

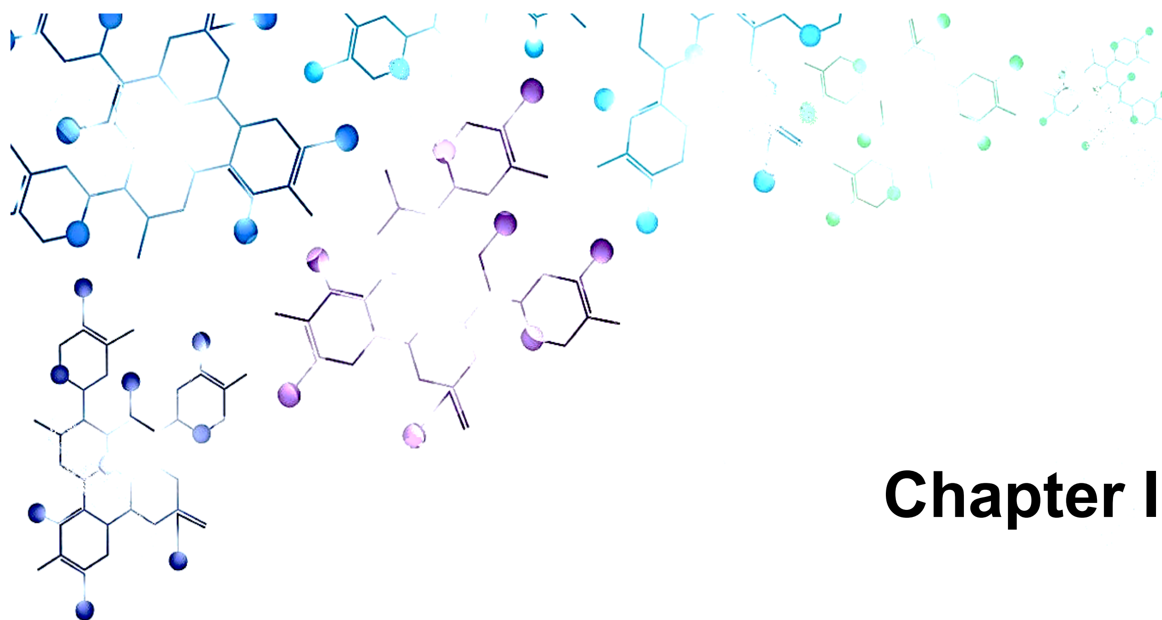
To predict the amino acid residues which could possibly enhance the thermal stability of the protein, PoPMuSiC - Prediction of Protein Mutant was used. The amino acids with high negative score/contributor from PoPMuSic were then evaluated for mutation possibility using a molecular visualization system PyMol (https://www.pymol.org/). Some of the risky residues were evaluated by hydrophobic cluster analysis (HCA) using DrawHCA (http://bioserv.impmc.jussieu.fr/hca-seq.html) to analyse the possibility of introducing a change in the 3D-structure.

Production and biochemical properties of recombinant FAEs

P. pastoris transformants were grown in in 250 mL flasks with 50 mL of induction medium BMMY. Induction was continued for 96 h at 28°C with methanol being supplemented to 0.5% (v/v) every 24 h. Culture supernatants were harvested (7500 rpm, 4°C, 20 min) and concentrated (10 kDa cut off; Sartorius). The amount of protein production was detected by the Bradford method (Sigma, Saint-Louis, USA).

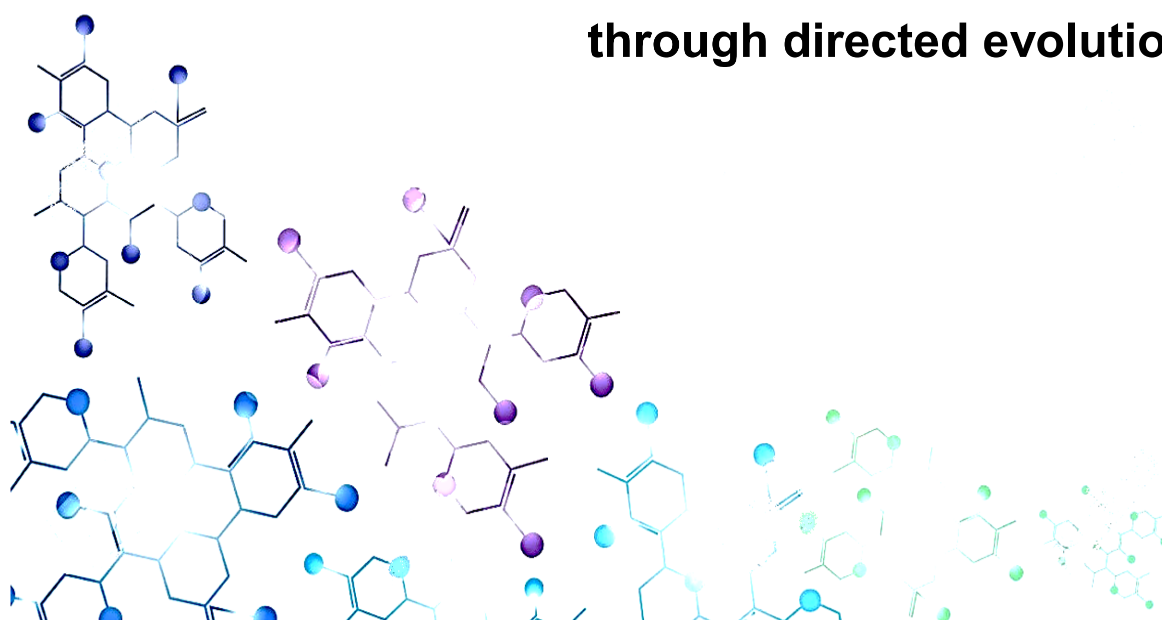
Enzyme activity assay of FAEs

Activity of the recombinant FAEs towards *p*NP-Fe (Taros chemicals, Dortmund, Germany) was performed in 1100 µL reaction mixtures adapted from. The *p*NP-Fe substrate solution was prepared by mixing 10.5 mM *p*NP-ferulate (in dimethyl sulfoxide) and 100 mM potassium phosphate buffer, pH 6.5 containing 2.5% Triton-X (1:9, v/v). The reactions were performed in the presence of 1000 µL *p*NP-Fe substrate solution incubated with 100 µL of culture supernatant at 37°C for 60 min. The release of *p*-nitrophenol was spectrophotometrically quantified by measuring the absorbance at 410 nm. One unit of FAE activity is defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol from *p*NP-Fe per min under the assay conditions. Activity towards MFA, MCA, MSA and M*p*CA (Apin Chemicals Limited, Oxon, United Kingdom) was assayed in 1 mL reaction mixtures at 37°C for 5-30 min. Detection of substrates' reduction was performed at 320 nm.



Chapter III

Development of biocatalysts based on novel FAEs through directed evolution



Chapter III: Development of biocatalysts based on FAEs through directed evolution

3.1 Introduction

Another well-established approach complementary to the rational mutagenesis to develop improved biocatalysts is represented by directed evolution, which mimics the natural evolution to alter enzymes properties such as specificity, activity, stability and solubility by methods for creating genetic diversity. This strategy consists in an iterative two-step process including: a) a first step of generation of a library of several variants of a protein of interest; b) screening in a high throughput way to identify the mutants exhibiting better properties. The best mutants selected through the tailor-made screening could be used as templates for further rounds of directed evolution until the achievement of the desired level of improvement.

In comparison to rational protein design, directed evolution brings the advantage of not requiring knowledge of the protein structure or the effects of specific amino acid substitutions, which are very difficult to predict *a priori.* However, there are only few examples of successful applications of accelerated *in* vitro evolution strategies to carbohydrate and feruloyl esterases so far, mainly due to a lack of suitable substrates for high-throughput assays.

This chapter deals with the results concerning the development of biocatalysts through directed evolution of already characterized FAEs to obtain improved variants in terms of activity, thermostability and/or solvent resistance.

In detail, in sections 3.2.1 and 3.2.2, manuscripts **"Development of a methodology and its application to generation of thousands of directed evolved mutants of the Type B feruloyl esterase from *Myceliophtora thermophila* MtFae1a and selection of improved variants for antioxidants synthesis"** and **"Development of improved variants of the feruloyl esterase from *Fusarium oxysporum* FoFaeC by directed evolution through high-throughput screening and molecular docking analysis of their substrate interactions"** describe the development and the application of complete methodologies to generate, express and screen diversity for the FAEs MtFae1a and FoFaeC in a high-throughput system.

The docking simulations and the evaluation of synthetic abilities of the selected feruloyl esterases MtFae1a and FoFaeC in detergentless microemulsions were carried out during a stage in the Biochemical Process Engineering Laboratory of Prof. Dr. Paul Christakopoulos (Division of Chemical Engineering) at Luleå University of Technology (Sweden) in the frame of OPTIBIOCAT project.

3.2 Results

3.2.1 Evolution of the type B feruloyl esterase MtFae1a from *Myceliophtora thermophila* towards improved catalysts for antioxidants synthesis

(manuscript submitted to Applied Microbiology and Biotechnology)

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Keywords: directed evolution, high throughput screening, *Myceliophtora thermophila*, library, feruloyl esterase

Abstract

The chemical syntheses currently employed for industrial purposes, including in the manufacture of cosmetics, presents limitations such as unwanted side reactions and the need for harsh chemical reaction conditions. In order to overcome these drawbacks, novel enzymes are developed to catalyse the targeted bioconversions. In the present study, a methodology for the construction and the automated screening of evolved variants library of a Type B feruloyl esterase from *Myceliophtora thermophila* (MtFae1a) was developed and applied to generation of 30,000 mutants and their screening for selecting the variants with higher activity than the wild type enzyme. The library was generated by error prone-PCR of *mtfae1a* cDNA and expressed in *Saccharomyces cerevisiae.* Screening for extracellular enzymatic activity towards 4-nitrocatechol-1-yl ferulate, a new substrate developed *ad hoc* for high-throughput assays of feruloyl esterases, led to the selection of thirty improved enzyme variants. The best four variants and the wild type MtFae1a were investigated in docking experiments with hydroxycinnamic acid esters using a hybrid model of 3D structure of MtFae1a. These variants were also used as biocatalysts in transesterification reactions leading to different target products in detergentless microemulsions and showed enhanced synthetic activities, although the screening strategy had been based on improved hydrolytic activity.

Introduction

Feruloyl esterases (FAEs, also known as ferulic or cinnamic acid esterases, EC 3.1.1.73) are enzymes whose function in Nature is to remove L-arabonofuranosyl-linked monomeric or dimeric ferulate moieties from the polysaccharide main chain of xylan in plant cell wall. They act as accessory (or auxiliary) enzymes that facilitate other enzymes, such as xylanases, xylosidases, arabinofuranosidases, etc., in accessing to their site of action during biomass conversion [1–3]. FAEs classification is based on their specificity towards the hydrolysis of methyl esters of hydroxycinnamic acids: methyl caffeate (MCA), methyl ferulate (MFA), methyl sinapate (MSA) and methyl *p*-coumarate (M*p*CA) [4–6]. However, the discovery of new FAEs using genome

mining and phylogenetic analysis of current publicly accessible fungal genomes led to the development a new subfamily classification of fungal FAEs considering both phylogeny and substrate specificity [7,8].

In addition to their hydrolytic ability, in appropriate tailor-made operative conditions, some FAEs are able to perform (trans)esterification reactions, conjugating hydroxycinnamic motif from its acid or related ester forms and leading to the synthesis of compounds with attractive properties for the cosmeceutical industry [9]. Enzymatic (trans)esterification meets the requirement for greener processes and the consumers' preference for natural products, encouraging the development of sustainable and competitive biotechnological processes which can give several advantages in alternative to the entirely chemo-catalyzed processes such as milder reaction conditions, high selectivities, and shorter synthetic pathways [10].

Type B FAE from the thermophilic fungus *Myceliophthora thermophila* ATCC 42464 (synonym *Sporotrichum thermophile)* (MtFae1a) belonging to CE1 family of the CAZy database[11] (SF 6 according to Dilokpimol et al.[7]) has been previously heterologously expressed in *Pichia pastoris* and characterized [12]. Besides its hydrolytic capabilities [13,14], MtFae1a has also been applied to the synthesis of a variety of esters and in particular of prenyl ferulate (PFA) and 5-*O*-feruloyl-l-arabinose (AFA) in detergentless microemulsions [15,16].

This study was aimed at developing a methodology for the construction and the automated screening of evolved variants library of MtFae1a, allowing the selection of variants with higher activity than the wild type enzyme. Therefore, the objectives were to generate a library of mutants by error prone polymerase chain reaction (ep-PCR) in *Saccharomyces cerevisiae,* using an expression platform previously adopted in our laboratory [17] and apply this strategy in conjunction with a high-throughput method to select the best variants. Crude supernatants of these new variants were characterized for their thermo- and solvent tolerance and the best four variants were investigated in docking experiments on hydroxycinnamic esters using a hybrid structure of MtFae1a. Moreover, the best four variants were tested in transesterification reactions in detergentless microemulsions for the production of target compounds selected for their potential antioxidant activity.

Results and discussion

Construction and screening of MtFae1a mutants library

The ability of MtFae1a to act in plant biomass biodegradation together with its (trans)esterification capability, make of considerable interest the discovery of new FAEs with novel properties and applications [19,20]. Moreover, enlarging the spectrum of biologically active compounds obtainable by FAE bioconversions and improving the (trans)esterification yields could led to the synthesis of new hits for the cosmetic sector. In this frame, directed evolution, that mimics the natural evolution, has proven to be a suitable strategy to improve or alter enzyme features such as selectivities, activity, stability and solubility by methods of genetic diversity integration [21].

To develop improved variants of *M. thermophila* feruloyl esterase MtFae1a, the enzyme was subjected to directed evolution experiments. Random mutations were introduced into *mtfae1a* cDNA by ep-PCR. S. *cerevisiae* system with pSAL4 vector was adopted for the recombinant expression of MtFae1a enzyme. Random mutated cDNAs and pSAL4 vector were used to transform S. *cereviasiae* and create a 30,000 evolved variants collection, applying the system previously adopted for directed evolution of an α-L-arabinofuranosidase from *Pleurotus ostreatus* [17]. *S. cerevisiae* was chosen as recombinant expression platform for directed evolution, thanks to its

several advantages compared to other yeasts, such as *P. pastoris*, which is able to secrete large amounts of proteins but suffers from low transformation efficiency and random gene integration in the genome limiting its use for high-throughput screening (HTS). *S. cerevisiae* exhibits the possibility to exploit its DNA recombination machinery avoiding ligation steps, together with high transformation efficiency and post-translational modifications [22].

The MtFae1a mutants library was analysed by an automated HTS strategy designed and developed to select variants with higher activity than the wild type enzyme towards 4NTC-Fe, a new substrate developed *ad hoc* due to the lack of suitable compounds for high-throughput assays of FAEs. The absence of a HTS method based on the evaluation of the synthetic abilities of FAEs implied the use of a screening strategy based on the hydrolysis of the ester bond of 4NTC-Fe that leads to 4-nitrocatechol release correlated with a colour change, which can be readily monitored. This compound enables the evaluation of FAE activity in both qualitative way in solid culture medium based and quantitative measurement in liquid medium [18]. An automated workstation including the robot colony picker QPIX 450 (Molecular Devices, LLC, CA, USA) and the robot BioMek NXP (Beckman Coulter, CA, USA) was adopted. The robot colony picker QPIX 450 was used to transfer the obtained clones of *S. cerevisiae* from selective solid medium to liquid medium in 96-well plates (Figure 1, A, Picking). Following growth by incubation at 28°C for 2 days, the mutants were transferred Q-Tray bioassay plates containing agarose and ammonium iron(III) citrate in the presence of 4NTC-Fe (Figure1, B, Gridding) and activity was detected after 1 hour at 37°C. A total of around 30,000 mutants were prepared and analysed by the developed automated HTS. The primary screening performed on agarose growth medium containing 4NTC-Fe and ammonium iron (III) citrate to identify the active mutants, allowed us to identify the 2,584 positive in the assay with the chromogenic substrate corresponding to around 10% of the library.

The clones showing activity halos were selected and transferred from 96-well plates to 96 deep-well microplates (Figure 1, C Re-arraying) for the secondary screening, focused on the detection of MtFae1a variants with higher activity than the wild type enzyme in solution. After growth at 28°C for 24 h, 100 µL of culture broth were transferred in 900 µL of liquid medium in 96 deep-well microplates by using the robot BioMek NXP (Figure 1, D) and the cultures were incubated at 28°C for 72 h. After biomass removal by centrifugation (Figure 1, E), samples of culture supernatant were subjected to analysis of FAE activity production towards 4NTC-Fe (Figure 1, F).

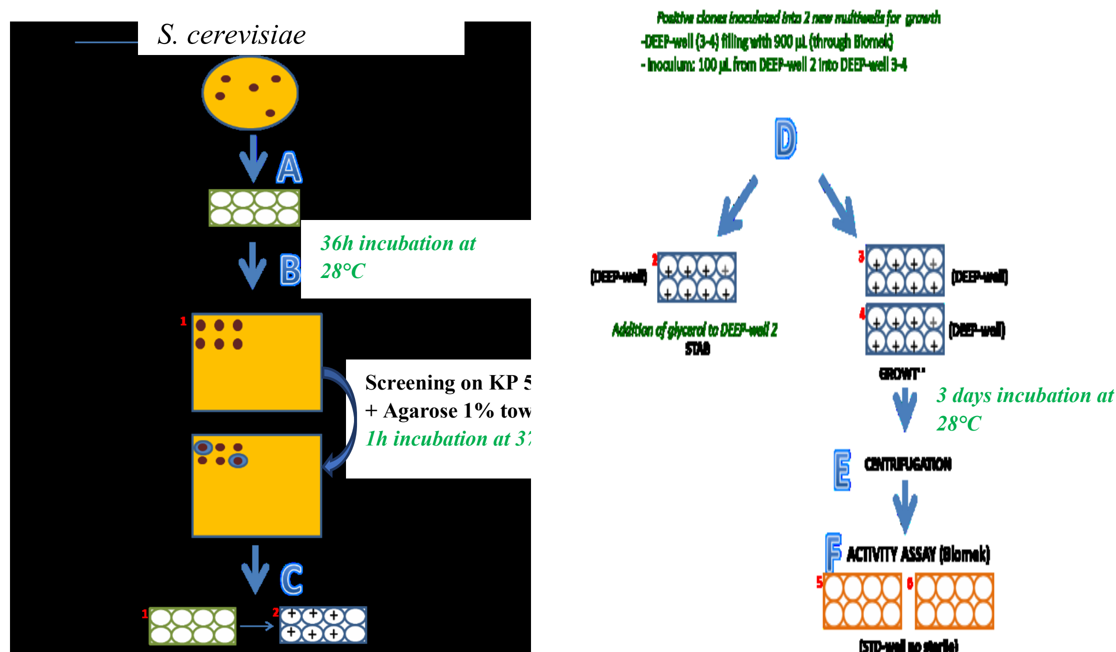

Figure 1: Schematic representation of the high-throughput screening strategy developed to analyse *S. cerevisiae* mutants expressing feruloyl esterase activity.

Characterization of mutated and wild type MtFae1a

The 30 most active evolved variants having at least twofold higher activity than the wild type enzyme towards 4NTC-Fe in microscale, were chosen to scale-up the growth in 20 mL of SG medium. Crude supernatants of improved variants were analyzed for FAE activity production towards 4NTC-Fe, their thermotolerance at 55°C for 1 hour and solvent tolerance in 25% acetone evaluating residual activity towards 4NTC-Fe. These analyses allowed the selection of the best four evolved variants, which crude supernatants showed at least twofold higher activity, around 1.3–fold higher solvent resistance and at least twofold higher thermotolerance than wild type MtFae1a (Table 1). Sequence analysis of mutated *mtfae1a* cDNAs (Table 1) showed that two out of the selected clones have different amino acid substitutions in the same position (G49D and G49A) while only one variant has a double mutation (F251L/H 105Y) one of which was shared with another variant (H105Y). In particular, G49D crude supernatant showed both the highest residual activity after heat and solvent exposure, fourfold and 1.5-fold increase respectively, compared to wild type MtFae1a.

Table 1: Activity values of MtFae1a selected clones and percentages of residual activity after solvent and heat exposure.

	Activity (mU/OD) (100mL-Flask)	***Thermo tolerance (%) (1h at 55°C)***	***Solvent resistance (%) (1h in 25% acetone)***
MtFae1a wt	25.7 (6.4)	10.8	67.2
H105Y	79.3 (21.0)	34.3	85.3
F251L/H105Y	50.0 (14.6)	21.4	94.5
G49D	54.5 (21.5)	46.2	95.9
G49A	49.6 (12.8)	37.4	95.5

Numbers in the parentheses are the estimates of standard errors

Substrate specificity of the four selected evolved variants crude supernatants was analysed in comparison to that of MtFAE1a wild type evaluating their activity towards MFA, MCA, MpCA, MSA and 4-Nitrophenyl ferulate (*p*NP-Fe) (Figure 2). MtFae1a and its mutated variants showed activity towards all the tested substrates. Wild type MtFae1a expressed in *S. cerevisiae* in this work followed a MpCA > MFA > MCA > MSA pattern, showing a slightly different behaviour from MtFae1a expressed in *P. pastoris*, as reported by Topakas et al. [23]. However, MtFae1a expressed in *S. cerevisiae* behaviour is consistent with the classification of type B FAEs, which show a preference for the phenolic moiety of the substrate containing one or two hydroxyl substitutions (as found in *p*-coumaric and caffeic acids, respectively) and no activity towards MSA [4]. In particular, H105Y crude supernatant exhibited twofold increased activity towards MFA, M*p*CA and *p*NP-Fe and a threefold increase towards MCA, together with F251L/H105Y. Moreover, G49A crude supernatant showed fourfold higher activity that wild type MtFae1a crude supernatant towards MCA.

According to the new phylogenetic classification of FAEs [7], MtFae1a belongs to subfamily SF6 together with NcFae1 and ClFaeB2, which showed activity towards MCA and MpCA but no activity towards MSA [24,25]. The same affinity pattern applies also to FoFae2 and CcFae2, whilst AsFaeD and MtFae1a showed activity towards all the methyl hydroxycinnamates [8]. ChFae and FaeB (*Talaromyces funiculosus*) are also classified in SF6, but the latter exhibits strict substrate specificity with activity only towards MFA [26,27].

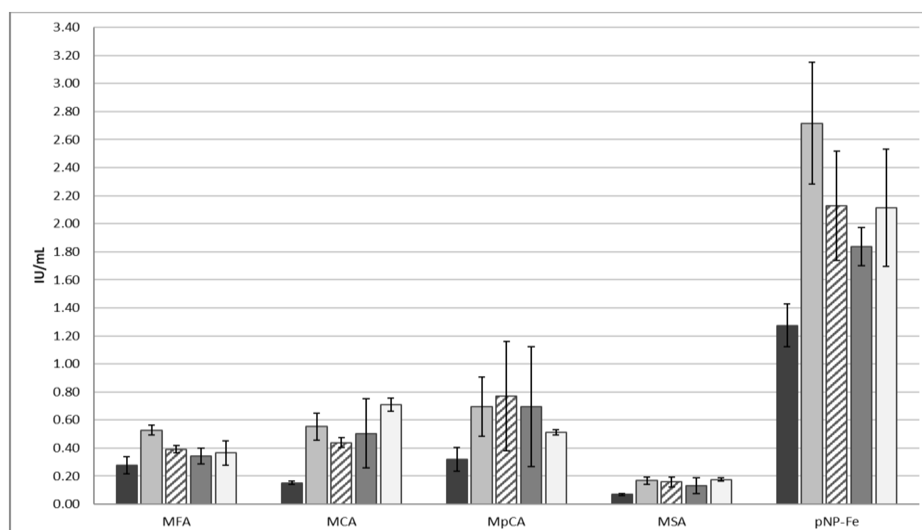

Figure 2: Substrate specificity of selected MtFae1a evolved variants towards methylated cinnamic acids and *p*NP-Fe. *Black* MtFae1a wild type, *light grey* H105Y, *striped* F251L/H105Y, *dark grey* G49D and *white* G49A.

<u>*Docking of hydroxycinnamate on the hybrid structure of MtFae1a and mutants*</u>

Using a similar approach as adopted by Antonopoulou et al. [34], docking of hydroxycinnamates on a hybrid structure of MtFae1a and on the selected evolved mutants was performed to study substrate interaction. MtFae1a belongs to the SF6 subfamily of phylogenetic classification evolved from acetyl xylan esterases [7]. Homology modeling resulted in a hybrid structure with a located putative catalytic triad (Ser118-His257-Asp201) and formation of disulfidic bond between Cys29 and Cys64. The hybrid mutants had a low RSMD (<1 Å) compared to the wild type. Mutations on Gly49 of G49A and G49D and mutation on Phe251 at F251L/H105Y were located in the second shell around the catalytic serine (within 12-13 Å) extending to different directions. Mutation on His105 on F251L/H105Y and 2D6D10

is located further from Ser108 and within a distance up to 30 Å (Fig. 3 B, C). MD in 25% acetone at 37°C resulted in low RSMD (0.1955 Å) for H105Y and higher RSMD (2.0303, 1.7128 and 1.8507 Å) for F251L/H105Y, G49D and G49A, respectively, comparing to structures before simulation. Binding of 4NTC-Fe on structures before and after solvent exposure revealed that there was a 0.8 kcal/mol reduction (from 6.7660 kcal/mol to 5.9250 kcal/mol) in the highest binding energy for the wild type and a reduction in the genetic runs resulting to a "correct orientation" of ligand (accommodating the hydroxycinnamic moiety inside the cavity). Thermal exposure seemed that had a greater impact on the highest binding energy causing a 1.3775 kcal/mol reduction (from 6.7660 kcal/mol to 5.3910 kcal/mol) for the wild type. Furthermore, the orientation of 4NTC-Fe binding onto MtFae1a wild type was severely modified after MD simulations while it was quite similar after solvent and thermal exposure (Fig. 3D) possibly contributing to the detrimental impact on residual activities. On the other hand, docking of 4NTC-Fe onto evolved mutants resulted in modification in the ligand orientation but did not affect significantly measures as the binding energy after acetone exposure aligning with the increased determined solvent resistance of evolved mutants. Regarding thermal exposure, highest binding energies were reduced while the distance of carbonyl carbon from catalytic serine was increased. Binding of methyl hydroxycinnamates and bulkier esters such as *p*NP-Fe and 4NTC-Fe on structures with ionized state at pH 6.0 revealed similar binding energies for all methyl substituted ligands (around 5 kcal/mol) and increased binding energy for *p*NP-Fe and 4NTC-Fe (around 6.5 kcal/mol), perhaps due to better stabilization of bulkier moieties around the active site. Generally, results validated the broad specificity of MtFae1a towards hydroxycinnamates. Orientation of binding onto wild type and mutants revealed that there was more deviation during binding of methoxy substituted esters than hydroxy substituted esters (Fig 4).

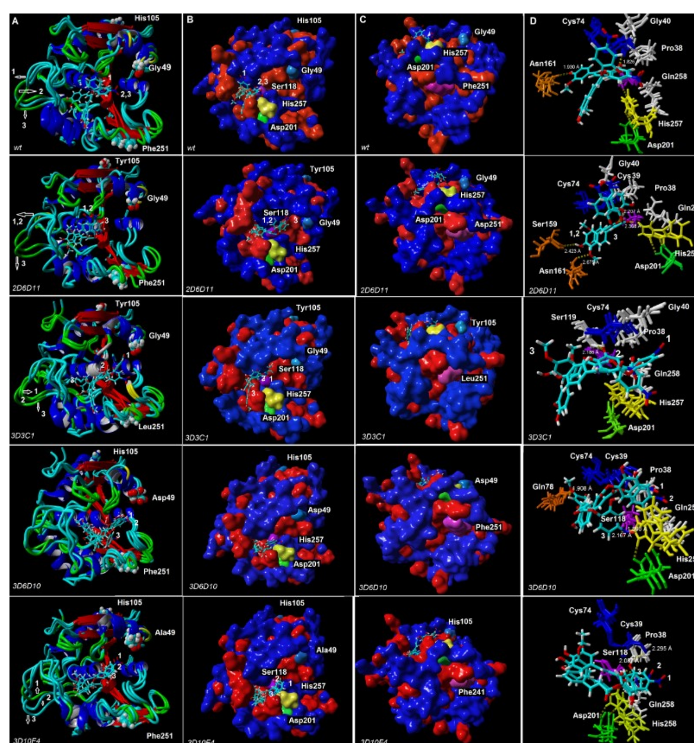

Figure 3: A) Superposition of secondary hybrid structures including docking of 4NTC-Fe, B) and C) Position of mutations on the surface of receptors D) Stabilization of esters in binding cavity 1) Initial condition 2) After acetone 3) After thermal exposure. Numbering of residues does not include the signal peptide.

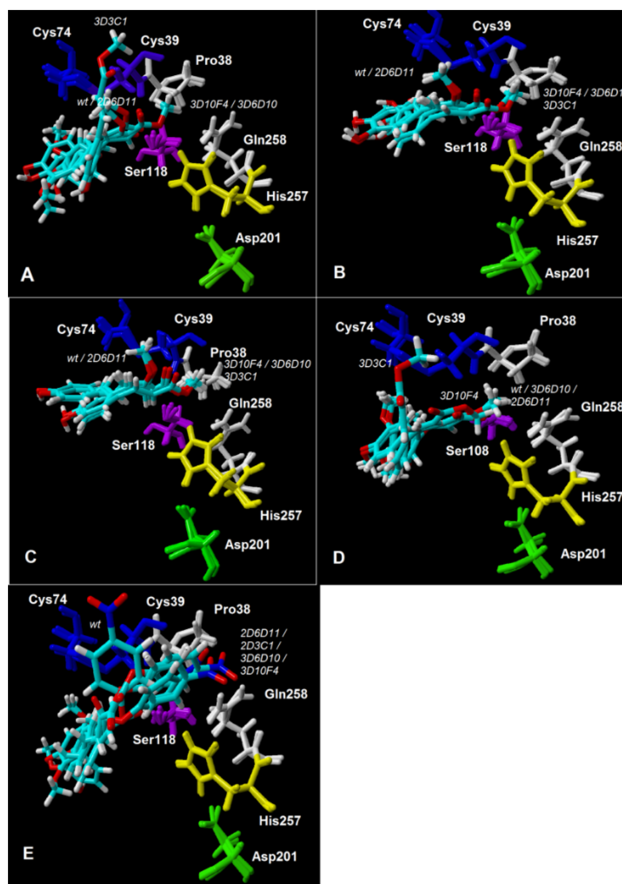

Figure 4: Docking of hydroxycinnamates onto the binding cavity of MtFae1a and evolved mutants A) MFA B) MCA C) M*p*CA E) MSA D) *p*NP-Fe. Numbering of residues does not include the signal peptide.

Evaluation of synthetic abilities of MtFae1a evolved mutants

Wild type MtFae1a and the selected four best evolved variants were evaluated for their ability to catalyse the transesterification of vinyl ferulate and caffeate with various fatty alcohols and carbohydrates in a ternary system of *n*-hexane:*t*-butanol:buffer forming detergentless microemulsions. All the tested MtFae1a evolved variants and the wild type enzyme were able to synthesize four target compounds (Table 3), namely prenyl ferulate (PFA), prenyl caffeate (PCA), glyceryl ferulate (GFA), *n*-butyl ferulate (BFA), 5-*O*-feruloyl-l-arabinose (AFA). Furthermore, both wild type MtFae1a and its evolved variants showed preference to more hydrophilic than hydrophobic alcohols, following a glycerol > 1-butanol > prenol pattern. However, hydrolytic activity as a side reaction was still detected.

Wild type MtFae1a achieved conversion yields in GFA, PFA, PCA and BFA synthesis similar to recombinantly expressed MtFae1a in *P. pastoris*, as previously reported in Antonopoulou et al. [35].

The highest transesterification yield was achieved with H105Y crude supernatant (67%) in GFA synthesis, followed by G49A variant. Moreover, H105Y crude supernatant, together with G49D, showed the highest conversion rates in GFA synthesis (Figure 5). However, wild type MtFae1a crude supernatant showed the highest selectivity in GFA synthesis, giving a GFA/FA ratio of 4.0. H105Y variant gave the highest yields and rate in PFA synthesis as well, followed by F251L/H105Y variant. Conversion yields around 60% were achieved in BFA synthesis with G49A, H105Y and F251L/H105Y crude supernatants. These variants achieved comparable

yields in PCA synthesis with similar BFA/FA and PCA/CA ratios. The best transesterification rates in BFA synthesis were obtained using G49D and H105Y crude supernatants, whilst the best in PCA synthesis were achieved with H105Y and F251L/H105Y variants.

The lowest transesterification yields were achieved in the synthesis of AFA by all the tested enzymes, indicating a less preference of MtFae1a and its evolved variants for sugar acceptors in the reaction conditions applied. Moreover, the AFA/FA ratio was less than 1, showing that the hydrolytic activity was much more prevalent that the synthetic one. Overall, MtFae1a and its evolved mutants belonging to the SF6 subfamily of phylogenetic classification are superior biocatalytic tools for synthesis of aliphatic hydroxycinnamates, aligning with previous findings regarding FAEs [35].

Table 3: Conversion yields and product selectivity of the wild type MtFae1a and its evolved variants.

Enzyme	PFA		PCA		GFA		BFA		AFA	
	yield (%)	ratio PFA/FA	yield (%)	ratio PCA/FA	yield (%)	ratio GFA/FA	yield (%)	ratio BFA/FA	yield (%)	ratio AFA/FA
MtFAE1a wt	36.8	1.35	39.3	1.55	59.2	4.02	56.9	1.62	12.3	0.16
H105Y	**50.6**	1.22	**57.7**	1.57	**67.1**	2.90	**59.6**	1.53	3.6	0.04
F251L/H105Y	**48.8**	1.14	**57.5**	1.54	63.7	2.57	**59.4**	1.52	6.5	0.07
G49D	27.5	1.25	44.0	1.65	54.6	2.92	52.4	1.66	**14.5**	0.19
G49A	35.7	0.79	**56.2**	1.59	**65.6**	2.61	**60.1**	1.56	6.0	0.07

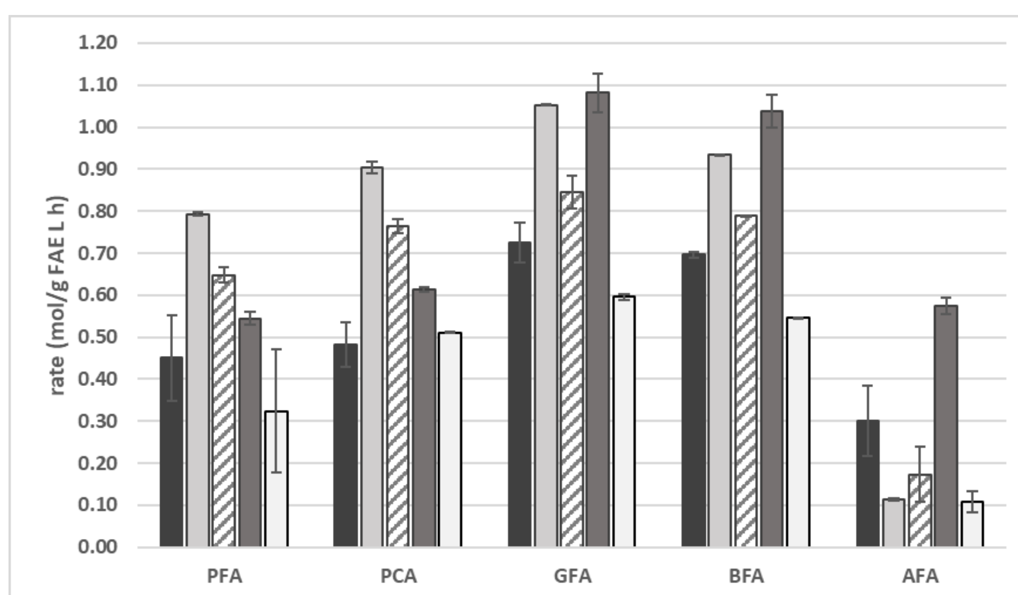

Figure 5: Transesterification rates of the MtFae1a wild type and its evolved variants. *Black* wild type MtFae1a, *light grey* H105Y, *striped* F251L/H105Y, *dark grey* G49D and *white* G49A.

Conclusions

In this study, a methodology for the construction and the automated screening of several thousand evolved variants library of MtFae1a was set up. A library of around 30,000 evolved variants of MtFae1a was generated by error prone PCR of *mtfae1a* cDNA and recombinantly expressed in *S. cerevisiae.* Screening for extracellular enzymatic activity towards 4NTC-Fe of the collection led to the selection of four variants exhibiting higher activity than wild type MtFae1a and improved thermo- and solvent tolerance. Although the screening strategy was based on the selection of

evolved variants with improved hydrolytic activity, it was possible to obtain variants with enhanced synthetic activities. In particular, H105Y crude supernatant exhibited twofold increased hydrolytic activity towards MFA, M*p*CA and *p*NP-Fe and a threefold increased activity towards MCA. Moreover, this variant showed enhanced abilities in GFA, PCA, PFA and BFA syntheses. Docking of hydroxycinnamates and bulkier esters such as *p*NP-Fe and 4NTC-Fe on a hybrid model of 3D structure of MtFae1a and mutants confirmed the preference of the enzymes towards bulkier substitutions and the broad substrate specificity of MtFae1a. In conclusion, the developed methodology allowed the selection of evolved variants with broader substrate specificity than the wild type enzyme together with the potential to expand the range of antioxidant compounds through (trans)esterification reactions.

Materials and methods

Chemicals

Casaminoacids, yeast nitrogen base (without amino acids and without ammonium sulphate) and agar were purchased from Difco (Paris, France). QIAprep kit from Qiagen (Hilden, Germany) was used for plasmid extraction and PCR fragment purifications. Restriction enzymes were purchased from Promega, (Wisconsin, USA) and methyl esters of cinnamic acids substrates were provided by Apin Chemicals ltd, Oxford, UK.pNP-Fe and 4-nitro4NTC-Fe, which was necessary for the screening of the complete library, were provided by Taros Chemicals (Dortmund, Germany). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

Vectors, strains and culture media

The Escherichia coli strain Top 10 (F-mcrA D (mrr-hsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG) was used in all DNA manipulations and its grown was performed in Luria–Bertani (LB) medium (in g l-1: 10 bactotryptone, 10 NaCl, 5 yeast extract), supplemented with 100 µg ml-1 of ampicillin for the selection of transformed clones.

The S. cerevisiae strain used for heterologous expression was W303-1A (MATa, ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100). The plasmids used for S. cerevisiae expression was pSAL4 (copper-inducible CUP1 promoter) carrying URA3 gene for auxotrophic selection28 (. Both S. cerevisiae strain and pSAL4 plasmid were kindly provided by Dr. T. Tron (Institut des Sciences Moléculaires de Marseille CNRS, Aix Marseille Université Service 342, Faculté des Sciences de Saint-Jérôme)29. S. cerevisiae was grown on a selective medium (6.7 g l−1 yeast nitrogen base without amino acids and ammonium sulfate, 5 g l-1 casaminoacids) supplemented with 30 mg l-1 adenine, 40 mg l-1 tryptophan, 50 mM succinate buffer (pH 5.3), 20 g l-1 galactose and 600 µM copper sulfate. For solid media, 15 g l-1 agar was added.

Random mutagenesis

The random mutagenesis was performed by ep-PCR with the GeneMorph II random mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacture's protocol using the recombinant vector pSAL4-mtfae1a as template. Primers, pSal4Fw (CCAACGCAATATGGATTGTCAG) and pSal4Rev (CAAGTGTAGCGGTCACGCTGCG) used in amplification experiments are complementary to the two ends of pSAL4 polylinker sequence. Cycling parameters were 95°C for 2 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min and final elongation step of 72°C for 10 min.

Construction of a mutants library

cDNAs resulting from ep-PCR were ligated in pSAL4 vector digested with EcoRI/HindIII restriction enzymes, exploiting homologous recombination expression system of S. cerevisiae. Transformation was done by using a lithium acetate protocol [30] using 1 µg EcoRI/HindIII restricted pSAL4 and 300 ng ep-PCR mtfae1a product. The cells were spread on the selective medium and the plates incubated upside down for 4 days at 28°C.

Library screening

Screening on solid medium was performed on 22 × 22 cm Q-Tray bioassay plates containing 50 mM KP pH 6.8, 1% Agarose, 0.5 mM Ammonium Iron (III) citrate supplemented with 75

μg/mL of chromogenic substrates, 4NTC-Fe as reported in Gherbovet et al.[18]. The strategy for screening was set up in high-throughput version using the automated workstation including the robot colony picker QPIX 450 (Molecular Devices, LLC, CA, USA) and the robot BioMek NXP (Beckman Coulture, CA, USA). Readings of the absorbance for screening in liquid medium were performed in a plate reader Multi Detection SystemGloMax® Discover System (Promega, Wisconsin, USA).

DNA extraction and sequencing

The recombinant plasmidic DNA was extracted following the protocol reported by Robzyk and Kassir [31] and amplified in E. coli TOP10. Plasmidic DNA bearing the mutated mtfae1a genes were purified and sequenced (Eurofins Genomics s.r.l.) using primers pSal4Fw and pSal4Rev.

Characterization toward methyl cinnamates

Activity of wild type and mutated MtFae1a was assayed against the substrates MFA, MSA, MCA, MpCA. Substrates stocks (1.18 mM) was prepared in 100 mM MOPS buffer, pH 6.0. The esterase activity measurement was performed by Beckman DU7500 spectrophotometer in 1 mL of reaction mixture (100 mM MOPS buffer, pH 6; 100 μl culture supernatant; 30 μl substrate stock) at 37°C for 15 minutes.

Thermo- and solvent tolerance conditions

The cultures were incubated at 28°C in 20 mL SG for 3 days and, after biomass removal, the supernatant was analysed for FAE activities against the substrate 4NTC-Fe. The crude broths were analyzed for thermotolerance at 55°C for 1 hour and tolerance to 25% of acetone at 37°C for 30 minutes towards 4NTC-Fe. The amount of protein production was detected by the Bradford method (Sigma, Saint-Louis, USA) and the homogeneity was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Blue. The FAE content (%, w/w) was estimated by SDS-PAGE and subsequent quantification was done by a densitometric method using JustTLC software (Sweday, Sweden).

Simulations

A MtFae1a wild type model was built by homology modeling based on the recently determined structure of an acetyl xylan esterase from *Aspergillus awamori* (5X6S_A) [32] using SWISS-MODEL [36]. The model passed both evaluation tests (GMQE 0.77, QMEAN -1.69) having high similarity with the template protein (93% query cover, 42% identities, 61% similarities, 2% gaps). Small molecule docking simulations (SMD), molecular dynamic simulations (MD) and visualization were performed in YASARA Structure in one monomer of the dimeric structure. Mutations were added to the MtFae1a hybrid model by swapping residues forming mutants, 2D3E6, 3D3C1, 3D6D10 and 3D10F4. Receptors were cleaned, their hydrogen bonding system was optimized while the pK_a values of the ionisable groups were predicted and assigned protonation states based on pH 6.0, 0.9% NaCl prior to energy minimization that was performed using AMBER14. Prior to SMD simulations, a simulation cell was created around the catalytic serine (cube of approximately 6 Å extensions, forming a cube of 16.95 Å per side), large enough to include ligands but small enough to exclude non-catalytically relevant binding. Ligands (MFA, MCA, MpCA, MSA, pNP-Fe and 4NTC-Fe) were designed, their structure was cleaned and their geometry was optimized. SMD was done on wild type and mutants by Autodock VINA [33] performing 25 docking runs per simulation. Results were evaluated based on the resulting binding energy (more positive energies indicate stronger binding and negative energies mean no binding), the dissociation constant, the number of clusters (runs are clustered into distinct complex conformations, differing by at least 5.0 Å heavy atom RMSD after superposing on the receptor), the number of genetic runs per cluster, the orientation of ligand per cluster and the distance of carbonyl carbon from the catalytic serine. MD simulations were performed at desired temperature creating a simulation cell of 10.0 Å around all atoms of wild type and mutant structures filled with 25% acetone, 75% water, 0.9% NaCl or 100%, 0.9% NaCl, water at defined temperature. The system was energy minimized prior to simulation while structures were used after the simulation for docking against 4NTC-Fe.

Transesterification reactions and quantitative analysis

Transesterification reactions in a ternary system of n-hexane:t-butanol:buffer forming detergentless microemulsions and quantitative analysis of products were performed following the protocol developed by Antonopoulou et al. [35]. Target compounds were prenyl ferulate (PFA), prenyl caffeate (PCA), glyceryl ferulate (GFA), n-butyl ferulate (BFA), l-arabinose ferulate (AFA) using 50 mM of vinyl ferulate and vinyl caffeate as donors. 800 mM of glycerol, prenol, 1-butanol and 50 mM of l-arabinose were used as acceptors at 45°C and pH 6.0. All reactions were performed in duplicate.

Transesterification yield was calculated as the molar amounts of generated PFA compared to the initial amount of donor, expressed as a percentage. Product selectivity was defined by the molar concentration of transesterification product divided by the molar concentration of hydrolysis product. Transesterification rate was calculated as the molar concentration of product formed by 1 g of expressed FAE in 1 hour (mol/g FAE L h).

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3.2.2 Directed evolution of the type C feruloyl esterase from *Fusarium oxysporum* FoFaeC and molecular docking analysis of its improved variants

(manuscript submitted to New Biotechnology)

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Keywords: directed evolution, high throughput screening, Fusarium oxysporum, library, feruloyl esterase

Abstract

The need to develop competitive and eco-friendly processes in the cosmetic industry leads to the search for new enzymes with improved properties for industrial bioconversions in this sector. In the present study, a complete methodology to generate, express and screen diversity for the type C feruloyl esterase from *Fusarium oxysporium* FoFaeC was set up in a high-throughput fashion. A library of around 30,000 random mutants of FoFaeC was generated by error prone PCR of *fofaec* cDNA and expressed in *Yarrowia lipolytica.* Screening for enzymatic activity towards the substrates 5-bromo-4-chloroindol-3-yl and 4-nitrocatechol-1-yl ferulates allowed the selection of 96 enzyme variants endowed with improved enzymatic activity that were then characterized for thermo- and solvent- tolerance. The best five mutants in terms of higher activity, thermo- and solvent- tolerance were selected for analysis of substrate specificity. L432I variant was shown able to hydrolyze all the tested substrates, except methyl sinapate, with higher activity than wild type FoFaeC towards methyl *p*-coumarate, methyl ferulate and methyl caffeate. Moreover, E455D variant showed to completely maintain its hydrolytic activity after two hour incubation at 55°C, whilst L284Q/V405I variant showed both higher thermo- and solvent-tolerance than wild type FoFaeC. Small molecule docking simulations were applied to the best five novel selected variants to examine the binding pattern of substrates used for enzyme characterization for FoFaeC wild type and its selected evolved variants.

Introduction

The interest in feruloyl esterases (ferulic acid esterases, FAEs), also known as cinnamoyl esterases (E.C. 3.1.1.73), is recently growing due to the large number of potential biotechnological applications in several industrial sectors based on their capability of hydrolyzing the ester bond between hydroxycinnamoyl motifs and sugars present in plant cell walls [3].

These enzymes release phenolic components such as ferulic, *p*-coumaric, caffeic, and sinapic acids [4], with widespread industrial potential due to their antioxidant and antimicrobial properties [5,6]. The modification of these compounds via esterification with aliphatic molecules (such as alcohols) can be used as a tool to alter solubility in oil-based formulas and emulsions, making them ideal candidates for application in

oil-based industries maintaining the antioxidant activity of the starting acid. This reaction catalyzed by FAEs in absence/low content of water represents a further route of application of these enzymes that is receiving increasing interest for the huge industrial potential [7]. As a consequence, the need to develop competitive and eco-friendlier bioconversions based on esterification reactions catalyzed by FAEs leads to search for new enzymes with improved properties for industrial applications. Directed evolution, that mimics the natural evolution, has proved to be a strategy suitable for improving or altering enzyme properties such as specificities, activity, stability and solubility by methods of genetic diversity integration [8]. However, it is of note that this approach, of time-consuming and cost-intensive nature, is based on procedures that use model substrates to detect target activities in order to provide more detailed qualitative data on enzyme properties. So far, the analysis of FAEs activity were not straightforward, mainly due to a lack of suitable compounds for practical high-throughput assays [9,10].

In a previous study, it was reported that the fungus *Fusarium oxysporum* showed multiple FAE enzymes for its ability to grow on varied materials such as wheat straw and corn cobs [11,12]. In particular, heterologous recombinant expression of type C FAE, belonging to the SF2 subfamily, was carried out in *Pichia pastoris* and the recombinant enzyme was purified and characterized using different substrates including methyl esters of hydroxycinnamates [13].

This study was aimed at developing evolved variants of FoFaeC with higher activity and improved resistance for temperature and solvent exposure than the wild type enzyme. The objectives were therefore to generate a library of mutants by error-prone polymerase chain reaction (ep-PCR) in *Yarrowia lipolytica* and apply this platform in conjunction with a high-throughput method to selecting the best variants. In addition, docking studies were employed to examine the affinity of the different substrates with the wild type and the selected evolved variants of FoFaeC.

Results and discussion

Development of recombinant expression system of FoFaeC

In order to develop improved variants of the feruloyl esterase from *F. oxysporium* FoFaeC by directed evolution, a recombinant expression system for this enzyme was developed using *Y. lipolytica*. Among the higher eukaryotic systems currently used for heterologous gene expression, *Y. lipolytica* generally allows higher yields of industrially relevant enzymes than *P. pastoris* and *Saccharomyces cerevisiae*, together with a high secretion ability, efficient tools for post-translational modification and appropriate glycosylation [14,15]. Moreover, *Y. lipolytica* represents a more appropriate platform for directed evolution than *P. pastoris* [13,16] which is characterized by low transformation efficiency and random gene integration in its genome [17].

In this study, *Y. lipolytica* was used in conjunction with the JMP62-TEF-ppLIP2-LIP2 expression vector that has been previously shown to be a correct platform for expressing and optimizing a lipase gene [14,18].

With the aim of optimizing the level of extracellular recombinant protein, the cDNA coding for the feruloyl esterase FoFaeC including the signal peptide was synthesized with the sequence optimized for the codon usage of the host and two different cloning strategies were performed to express the enzyme either with its own native secretion signal (JMP62/FoFaeC +PS) or as a fusion with LIP2 prepropeptide (ppLIP2), to direct the secretion of recombinant proteins (JMP62/FoFaeC -PS).

Transformation efficiency of *Y. lipolytica* ($4x10^3$ CFU/µg) was compatible with the construction of large mutant libraries.

Validation of the recombinant platform

In order to check if clonal variability took place among the different *Y. lipolytica* transformed clones, ten mutants for each transforming vector were cultured in liquid medium and the time-course of FAE activity production was evaluated until the 8th day of growth. Since no clonal variability among different clones was revealed, transformants for each recombinant expression system were randomly chosen and subjected to further analysis of activity production. The two recombinant systems were compared analyzing FAE activity on solid growth medium containing the chromogenic substrate X-Fe. Based on these analyses, the best results were obtained for the construct containing the native secretion signal (FoFaeC + SP), since only the yeast transformed with FoFaeC + SP construct showed blue halo activity on solid medium. Therefore, the FoFaeC + SP was selected for further experiments of mutants library generation.

Construction and screening of FoFAEc mutant library

The system *Y. lipolytica*/JMP62/FoFaeC + SP was shown to be a suitable platform for expressing the *fofaec* cDNA and therefore it was adopted for directed evolution. The variants of FoFaeC esterase were created using the strategy previously reported [18] applying a medium mutation frequency for ep-PCR (4.5 – 9 mutations/kb). At the end of the third step of PCR providing for the construction of the expression cassette by overlap PCR, the resulting mutated fragments were introduced into genome of strain JMP1212 through the homologous recombination system of the yeast.

Around 30,000 mutants were prepared and analyzed by high-throughput screening (HTS) using an automated workstation. To identify active mutants, a primary screening on YNBG agar plates containing X-Fe was performed. Around 10% of the complete library, corresponding to 3,313 clones, was proven positive on the solid culture medium with the chromogenic substrate and selected for a secondary screening focused on the detection of FoFaeC variants with higher activity than the wild type enzyme in liquid culture medium. Positive clones from the primary screening were grown in microscale for 48 h and culture supernatants were subjected to analysis of FAE activity production using of 4NTC-Fe as substrate in microscale.

The 96 most active evolved variants having at least two-fold higher activity than the wild type enzyme towards 4NTC-Fe in microscale were chosen to scale-up the growth in 20 mL of YT2DH5 medium. The improved variants were analyzed for FAE activity production towards 4NTC-Fe, their thermo-tolerance at 55°C for 2 hours and solvent tolerance in 25% acetone (v/v). The best five mutants in terms of higher activity, thermos- and solvent tolerance were sequenced and the corresponding mutations and the activity values of crude supernatants of the selected clones and percentages of residual activity after solvent and heat exposure are reported in Table 2. Scaling-up the growth of the evolved variants to 20 mL in flask, resulted in a lower FAE activity production. This result is due to changes of growth conditions in comparison with micro-scale [21]. The Table 2 reports the results in flask on the best five evolved variants showing at least twofold higher activity than wild type FoFaeC and improved thermo- and solvent tolerance. In particular, L432I, R308H and M278L/V313I variants showed twofold, threefold and fourfold increased activities compared to wild type FoFaeC. Moreover, E455D variant showed 1.3–fold higher

solvent tolerance and L284Q/V405I variant showed both 1.3-fold higher thermo-tolerance and 1.6–fold higher solvent tolerance than wild type FoFaeC.

Table 2: Activity values of crude supernatants of FoFaeC selected clones and percentages of residual activity after solvent and heat exposure.

	Activity (mIU/OD) (100mL-Flask)	***Thermo-tolerance (%) (2 h at 55°C)***	***Solvent resistance (%) (1 h in 25% acetone, v/v)***
FoFaeC wt	2.28 ± 0.54	80	70
M278L/V313I	9.02 ± 1.20	42	55
L432I	4.67 ± 0.28	nd[a]	63
R308H	6.63 ± 1.60	45	54
E455D	1.66 ± 0.24	69	94
L284Q/V405I	2.74 ± 0.10	102	114

[a]nd, not detected

(The reported data are the average of three experiments, each one carried out in duplicate)

Biochemical characterization of selected optimized mutants and wild type FoFaeC

To investigate substrate specificities of the variants selected by directed evolution on FoFaeC in *Y. lipolytica*, the hydrolytic ability of the crude supernatants of selected mutants was analyzed by assaying their activities towards a synthetic substrate, *p*NP-Fe and methyl cinnamates, MFA, MCA, M*p*Ca and MSA, in comparison to the wild type (Figure 1). FoFaeC wild type followed a M*p*CA > MCA> MFA pattern but showed no affinity for MSA. This result is consistent with findings by Moukouli et al. [13] for FoFaeC recombinantly expressed in *P. pastoris* which shows to not have broad specificity of substrate despite being a type C FAE according to Crepin et al. [21] classification. According to the new phylogenetic classification of FAEs [22], FoFaeC falls into SF2, together with AwFaeG (*A. wentii*) and GlFae1 (*Gymnopus luxurians*). AwFaeG showed low activity towards MSA as FoFaeC, but did not hydrolyse M*p*CA. On the other hand, GlFae1 showed the highest activity towards MSA and lower activity towards MFA, but no affinity towards M*p*CA and MCA. Similarly to the wild type FoFaeC, the crude supernatants [23] of the evolved variants showed activity toward MFA, MCA and M*p*CA but none of them have affinity for MSA. The crude supernatant of R308H variant hydrolyzed all the tested substrates, except MSA, with higher activity than the supernatant of wild type FoFaeC towards M*p*CA, MFA and MCA (1.4-fold, 1.8-fold and 2-fold, respectively). The supernatant of M278L/V313I variant showed an improved activity of 1.5-fold towards M*p*CA compared to FoFaeC wild type. Moreover, the supernatant of E455D variant showed to completely maintain its hydrolytic activity after two hour incubation at 55°C, whilst that of L284Q/V405I variant showed both higher thermo- and solvent tolerance than wild type FoFaeC.

Interestingly, L432I is the only variant bearing a single mutation taking place in the region of the substrate binding pocket, which reflected in an improvement of affinity towards *p*NP-Fe but affected negatively the activity towards methyl cinnamates. All the other substitutions occurred on the surface of the enzyme, particularly in the area forming a lid to the active site. It is also noteworthy that R308H variant, bearing a single amino acid substitution, lost FoFaeC wild type ability to hydrolyze MCA and M*p*CA. The same applies to E455D variant, which maintained affinity towards M*p*CA exclusively.

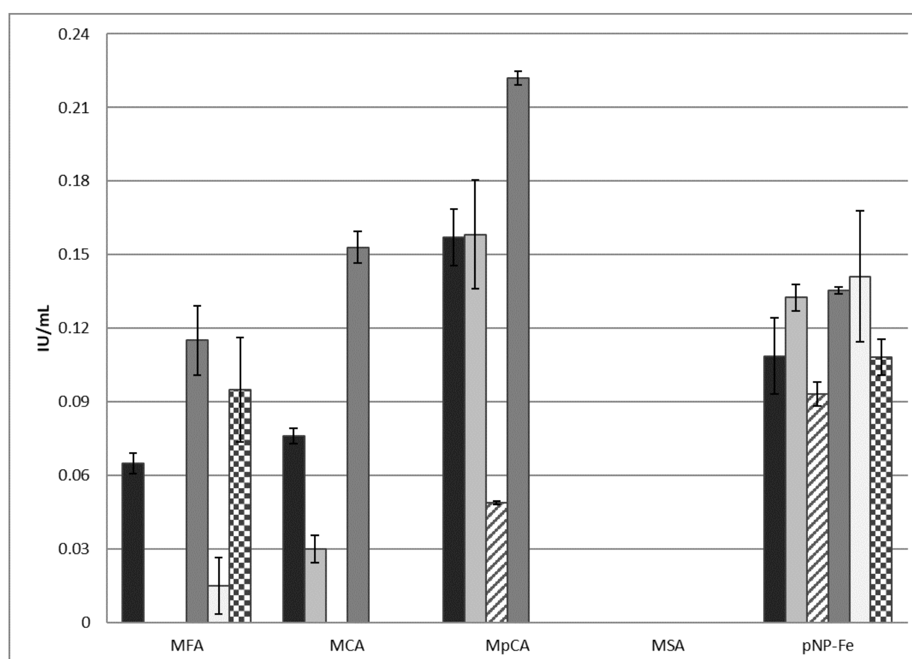

Figure 1: Activity of crude supernatants of selected FoFaeC evolved variants towards methyl cinnamates and *p*NP-Fe. *Black* wild type FoFaeC, *light grey* M278L/V313I, *striped* E455D, *dark grey* R308H, *white* L432I and *squared* L284Q/V405I.

Docking of hydroxycinnamic acid esters on the hybrid structure of FoFaeC and mutants

The feruloyl esterase from *Aspergillus oryzae* AoFaeB [1] was chosen as template (3WMT_A from PDB) to identify the position of the putative catalytic triad and disulfidic bonds of FoFaeC using YASARA software since it displays an identity of 49% with FoFaeC sequence (assessed using BLASTp). The docking process was employed to understand the binding pattern of substrates used for enzyme characterization to wild type FoFaeC and its selected evolved variants. It was shown that ligands dock onto the receptors (wild type and mutants) in the following order of preference in terms of mean/highest binding energy (determined at the correct orientation; where the hydroxycinnamoyl moiety is placed inside the binding cavity while the substitution extends outside the cavity): *p*NP-Fe, 4NTC-Fe (>7 kcal/mol) > methyl esters (5-5.8 kcal/mol) (Table S1 of supplementary material). Furthermore, it was determined that for all methyl substituted ligands three main clusters were formed in a descending order of binding energy: docking in the correct orientation (with substitution extending outside the binding cavity), in the reversed orientation (with substitution accommodated inside the binding cavity) and in a “displaced” reversed orientation (ligand was stabilized outside the cavity with the ester substitution extending towards the cavity). The latter, allowed stabilization of ligand in the highest proximity to catalytic serine (<2.8 Å) comparing to other clusters, included the majority of genetic runs per simulation (6-8 genetic runs out of 20) but resulted in lower binding energies (4-4.8 kcal/mol). Regarding the size of substitutions, MFA and MSA (containing two methoxy groups) were docked most often in the reversed orientation (with higher mean binding energy), while the two most favorable acquired orientations (reversed and correct) resulted in a non-catalytic distance between Ser166 and the ester carbonyl carbon (>5 Å) for MSA. This phenomenon, could explain the absence of activity of FoFaeC towards MSA and aligns with previous findings [26]. On the other hand, bulky substituted esters such as *p*NP-Fe and 4NTC-Fe docked preferably in the reversed orientation but all main clusters (with correct, reversed and displaced orientations) resulted in high proximity to the catalytic serine,

explaining the increased activity towards these substrates. An additional indication for increased activity towards *p*NP-Fe and 4NTC-Fe is the stabilization of both the methoxy moiety by Asn430 and nitryl moiety by Gln199. Although no systematic differences could be detected for binding of ligands onto the selected mutants and wild type, it was shown that ligands M*p*CA, *p*NP-Fe and 4NTC-Fe had smallest deviation in docking orientation (Figure 2) among wild type and mutants, proving the increased determined activity against these substrates. The contacting residues during docking of ligands on the predicted structures of wild type and mutants are presented in Table S2 of the supplementary material.

MD simulations in 25% acetone (v/v) and at 55°C resulted in distorted structures with RSMD up to 1.8 Å for acetone exposure and up to 2.3 Å for thermal exposure (Table S3 of supplementary material). This aligns with experimental findings, where thermal exposure has greater impact than acetone in residual activity. Furthermore, MD simulations imposed an increase in volume of the binding cavity that was higher after thermal exposure than solvent exposure (Table S4 of supplementary material), allowing stabilization of 4NTC-Fe in the "correct" orientation (clusters with higher mean binding energy and/or genetic runs) in the cases of M278L/V313I, L432I and R308H. Binding of 4NTC-Fe remained in the preferred initial "reversed" orientation (allowing proximity with catalytic serine) for wild type, 3455D and L284Q/V405I (Table S5 of supplementary material). This could explain the increased stability of these enzymes to both heat (80 and 102% residual activity, respectively) and acetone (102 and 114% residual activity, respectively) (Figure 3). The contacting residues during docking of 4NTC-Fe on the predicted structures of wild type and mutants after MD simulations are presented in Table S6 of the supplementary material.

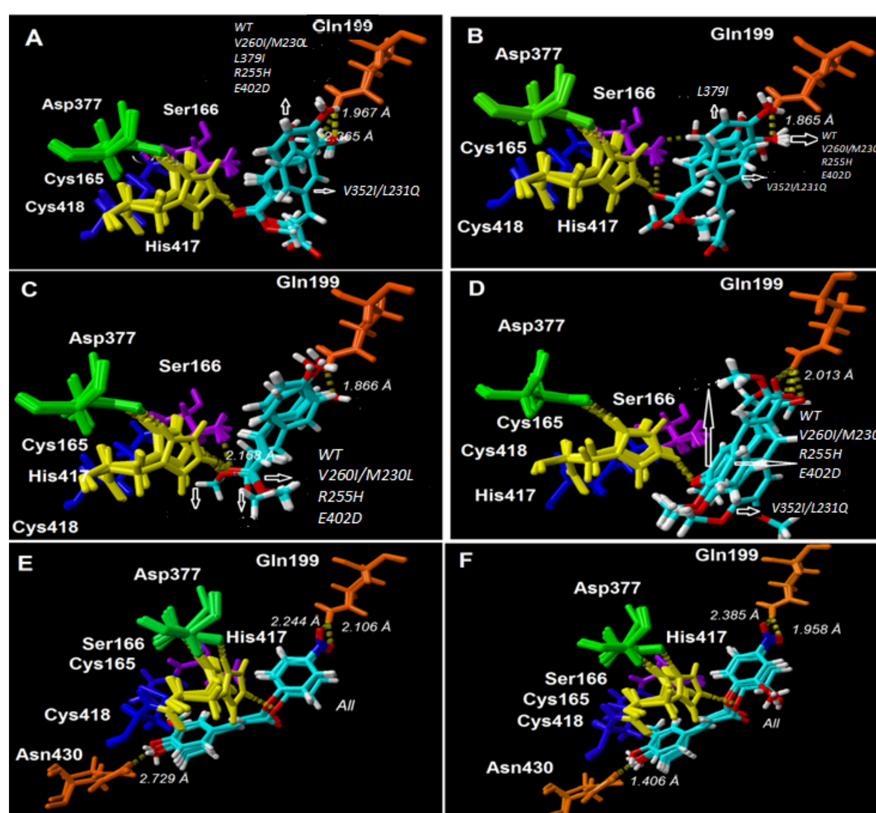

Figure 2. Docking of hydroxycinnamates onto the binding cavity of FoFaeC and evolved mutants Numbering of residues does not include the signal peptide A) MFA B) MCA C) M*p*CA D) MSA E) *p*NP-Fe F) 4NTC-Fe. The selected cluster reflects highest binding energy. Numbering of residues does not include the signal peptide

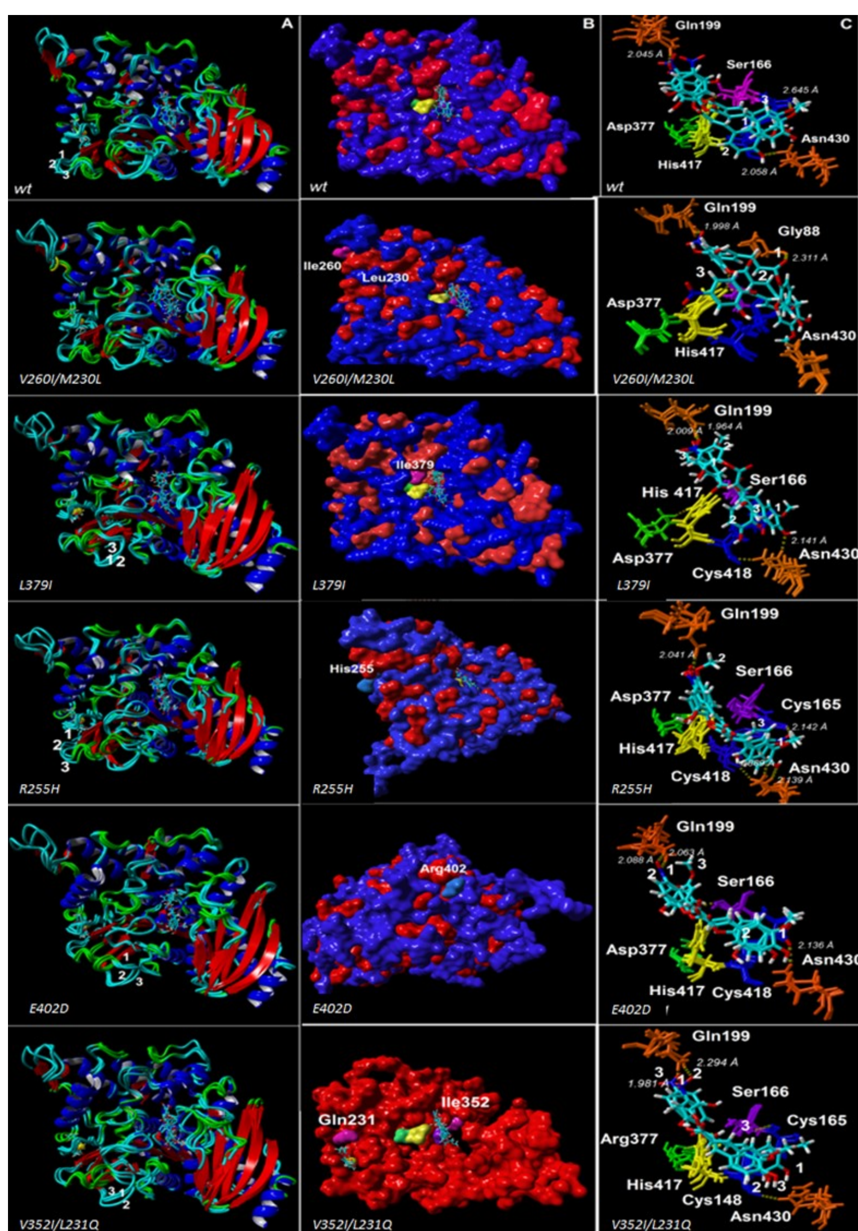

Figure 3. A) Superposition of secondary hybrid structures including docking of 4NTC-Fe, B) Position of mutations on the receptors C) Stabilization of esters in binding cavity 1) Initial condition 2) After acetone 3) After thermal exposure. Numbering of residues does not include the signal peptide.

Conclusions

In this study, a library of around 30,000 evolved variants of FoFaeC was generated by epPCR of *fofaec* cDNA and recombinantly expressed in *Y. lipolytica.* Screening for extracellular enzymatic activity towards X-Fe and 4NTC-Fe of the collection led to the selection of five variants producing higher activity than wild type enzyme, from which two were subsequently found to exhibit improved thermo- and solvent tolerance. L432I variant crude supernatant was shown able to hydrolyze all the tested substrates, except MSA, with higher activity than wild type FoFaeC towards M*p*CA, MFA and MCA. SMD simulations on the five selected evolved variants revealed that M*p*CA and *p*NP-Fe had smallest deviation in docking orientation among wild type and mutants, explaining the increased determined activity against these substrates. Moreover, E455D variant crude supernatant showed to completely maintain its hydrolytic activity after two hour incubation at 55°C, whilst L284Q/V405I variant crude supernatant showed both higher thermo- and solvent tolerance than

wild type FoFaeC. These findings were confirmed by MD simulations in 25% acetone and at 55°C, which showed how binding of 4NTC-Fe remained in the preferred initial reversed orientation allowing proximity with catalytic serine. In conclusion, generation and screening of 30,000 evolved variants library of FoFaeC allowed the selection of variants endowed with improved characteristics compared to the wild type enzyme. Properties of these enzymes could be exploited for industrial bioconversions.

Materials and methods

Chemicals

Yeast extract, bacto tryptone, bacto peptone and yeast nitrogen base (without amino acids and without ammonium sulphate) were purchased from Difco (Difco, Paris, France). Other chemicals were purchased from Sigma–Aldrich (Sigma–Aldrich, St.Louis, MO). QIAprep kit from Qiagen (Hilden, Germany) was used for plasmid extraction and PCR fragment purifications. Enzymes were purchased from Promega, Wisconsin, USA and methyl esters of cinnamic acids substrates were provided by Apin Chemicals ltd, Oxford, UK. 5-bromo-4-chloroindol-3-yl ferulate (X-Fe) was provided by LISBP, Université de Toulouse (CNRS, INRA, INSA, Toulouse, France). 4-nitrophenyl ferulate (pNP-Fe) and 4nitro-catechol ferulate (4NTC-Fe) were provided by Taros Chemicals GmbH & (Dortmund, Germany). Other chemicals were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO).

Vectors, strains and culture media

The *Escherichia coli* strain Top 10 was used for transformations and manipulations of recombinant plasmids and its growth was performed at 37°C in Luria–Bertani (LB) medium (10 g/L bacto tryptone, 10 g/L NaCl, and 5 g/L yeast extract) supplemented with 100 μg ml−1 of ampicillin or 40 μg ml−1 of kanamycin to select of transformed clones. The JMP62-TEF-ppLIP2-LIP2 expression vector (Nicaudet al. 2002) was used for the cloning of the faeC gene from *F. oxysporum* and its mutants in *Yarrowia lipolytica* strain JMP1212. The Ura3 transformants obtained by yeast transformation, were selected on YNBcasa medium (1.7 g/L Yeast Nitrogen Base, sterilized by filtration; 10 g/L Glucose; 5 g/L NH4Cl; 50 mM Phosphates Buffer Na/K pH 6.8: 10 % (v/v); 2 g/L Casamino acids, sterilized by filtration) and grown in rich medium YPD (10 g/L Bactopeptone; 10 g/L Yeast Extract; 10 g/L glucose) and YT2DH5 (10 g/L Yeast extract; 20 g/L Tryptone; 50 mM phosphate buffer Na/K pH 6.8 supplemented with 50 g/L Glucose). For solid media, 20 g/L agar was added.

Recombinant vectors construction

Two types of cloning were performed using cDNA sequence of *fofaec* gene synthesized and optimized following *Y. lipolytica* codon usage in which three different restriction sites, BamHI, BsrGI and AvrII, were inserted. The gene was cloned either with its own optimized native signal sequence (indicated as FoFaeC + SP) or by fusing the sequence of the mature protein with preproLIP2 (indicated as FoFaeC – SP), a pro-peptide that has been shown to increase in some cases the level of extracellular recombinant protein (Boonvitthya et al. 2013).

The plasmid JMP62-TEF-ppLIP2-LIP2 and *fofaec* gene were digested by restriction enzymes BamHI/AvrII and BsrgI/AvrII (Promega, Wisconsin, USA). In order to prevent circularization and re-ligation of linearized DNA, dephosphorylation of the linearized vector ends was performed using Calf intestinal alkaline phosphatase (CIAP) (Promega, Wisconsin, USA).

The ligation of DNA fragments with cohesive ends was carried out over night at 4°C in presence of T4 DNA ligase (Promega, Wisconsin, USA) and, after plasmid amplification in *E. coli*, linearization by NotI (Promega, Wisconsin, USA) was performed according to manufacturer's instruction.

Error-prone PCR strategy

The expression cassette dedicated to the library construction of mutants was obtained by overlapping PCR amplification as described by Bordes et al. (2011). PCR reactions were carried out in order to amplify DNA sequence of interest using MyCyclerTIM thermal cycler (Bio-Rad, North America). Primers used in PCR reactions are listed in Table 1.

Table 1: list of primers used into the PCR strategy.

Primer name	PCR reaction	Sequence
PCR1_d	upstream region / faithfull	GATCCCCACCGGAATTGC
PCR1_RT		GCACCTGGGGAATGAAGCCGAGCACGAGCACG
PCR2_d	gene of interest + downstream Zeta /epPCR	CGTGCTCGTGCTCGGCTTCATTCCCCAGGTGC
PCR2_RT		GGAGTTCTTCGCCACCCC
PCR3_d	Fusion of PCR fragments PCR 1and 2	CCGCCTGTCGGGAACCGCGTTCAGGTGGAACAGGACCACC
PCR3_RT		CCGCACTGAAGGGCTTTGTGAGAGAGGTAACGCCG

Y. lipolytica preparation and transformation

Competent cells preparation and transformation of *Y. lipolytica* wild type was performed as previously described in Boonvitthya et al. (2013).

Wild type FoFaeC purification

The crude cell-free extract was concentrated by ultrafiltration (Amicon chamber 8200, cut off 10kDa membrane Millipore, Billerica, MA) and purified by immobilized metal ion affinity chromatography (IMAC). The concentrated sample was loaded a column His Trap 1 mL (GE Healthcare). The column was first washed with 20 mM Sodium phosphate, 100 mM NaCl, 10 mM imidazole (pH 7) and then a linear salt gradient was applied, a flow rate of 1mL min^{-1}, from 0 to 100% of elution buffer (20 mM Sodium phosphate, 100 mM NaCl, 500 mM imidazole, pH 7). The active fractions were pooled and analyzed by means of SDS-PAGE analyses.

Library screening

The strategy for screening was set up in high-throughput version using the automated workstation including the robot colony picker QPIX 450 (Molecular Devices, LLC, CA, USA) and the robot BioMek NXP (Beckman Coulter, CA, USA). According to the screening strategy adapted from Bordes et al. (2007), clones of *Y. lipolytica* obtained by transformation with op-PCR product were grown on selective medium into petri dishes at 28 °C. The robot colony picker QPIX 450 was used to transfer the colonies obtained different sets of transformation from solid medium to 200 µL of liquid medium in 96-well plates Following growth by incubation at 28°C for 16 h, mutants were transferred on Q -Tray containing selective medium in the presence of 60 µg/mL of 5-bromo-4-chloroindol-3-yl ferulate (X-Fe) from an initial solution of 60 mg/mL X-Fe in DMSO and activity was detected after 4-12 hour at 28°C. A first layer of solid medium without substrate was poured and after complete solidification, a second layer supplemented with X-Fe was deposited. Plates were incubated, and active clones were detected via the appearance of a blue halo around colonies). In order to analyze the activity towards 4-nitrocatechol-1-yl ferulate (4NTC-Fe) as described in Gherbovet et al. (2016), the positive clones were selected and transferred from 96-well plates to 96 deep-well microplates for growth at 28°C for 16 h. Afterwards, 100 µL of culture broth were transferred in 900 µL of liquid medium in 96 deep-well microplates by using the robot BioMek NXP and the cultures were incubated at 28°C for 48h. Samples of culture supernatant after biomass removal by centrifugation were subjected to analysis of FAE activity production by 4NTC-Fe assay with in microscale.

Characterization toward methylated cinnamic acids

Activity of wild type and mutated FoFaeC was assayed against the substrates methyl ferulate (MFA), methyl sinapate (MSA), methyl caffeate (MCA), methyl *p*-cumarate (M*p*CA). 1.18 mM substrates stock were prepared in 100 mM MOPS buffer, pH 6.0. Esterase activity measurement was performed by spectrophotometer in 1mL of reaction mixture (100 mM MOPS buffer,pH 6; 100 µl culture supernatant; 30µl substrate stocks) at 37°C following the consumption of substrate for 15 minute.

Thermo- and solvent tolerance conditions

The cultures were incubated at 28°C in 20 mL YTD for 48 hours and, after biomass removal, the supernatant was analyzed for feruloyl esterase activity against 4NTC-Fe. The crude broths were analyzed for enzyme thermotolerance at 55°C for 2 hours and tolerance to 25% of acetone at 37°C for 30 minutes measuring the residual activity towards 4NTC-Fe.

DNA extraction and sequencing
The genomic DNA was extracted following the protocol reported in Jaafar (2003). Mutated *fofaec* genes were amplified by high-fidelity PCR and sequenced (Eurofins Genomics s.r.l.) using primers PCR2_d and PCR3_RT.
Molecular docking and molecular dynamic simulations
In the present analysis, docking study was aimed to examine the affinity of different substrates with the wild type and selected evolved variants of FoFaeC. A hybrid structure of FoFaeC wild type was built by homology modeling using SWISS-MODEL and based on the known structure of a feruloyl esterase from *Aspergillus oryzae* (AoFaeB) (3WMT_A; Suzuki et al. 2014; query cover 94%, identities 49%, positives 66%, gaps 2%). The acquired model passed both quality tests (GMQE 0.73, QMEAN -1.84). Small molecule docking (SMD), molecular dynamic (MD) simulations and protein visualization was done using YASARA Structure. Mutations were added to the wild type hybrid model by swapping residues resulting in five mutant models: 1D2B3, 6D4F4, 6D1H2, 7D12B1 and 7D12B8. Heteroatoms were removed, receptors were cleaned, their hydrogen bonding system was optimized while the pK_a values of the ionisable groups were predicted and were assigned protonation states based on pH 6.0, 0.9% NaCl. Following, structures were energy minimized using AMBER14 force field. Prior to SMD, a simulation cell was created around the catalytic serine (cube of approximately 6 Å extensions, forming a cube of 16.95 Å per side), large enough to include ligands but small enough to exclude non-catalytically relevant binding. Ligands (MFA, MCA, M*p*CA, MSA, *p*NP-Fe and 4NTC-Fe) were designed, their structure was cleaned and their geometry was optimized. SMD was done on wild type and mutants by Autodock VINA (Trott and Olson, 2010) performing 25 docking runs per simulation. Results were evaluated based on the resulting binding energy (more positive energies indicate stronger binding and negative energies mean no binding), the dissociation constant, the number of clusters (runs are clustered into distinct complex conformations, differing by at least 5.0 Å heavy atom RMSD after superposing on the receptor), the number of genetic runs per cluster, the orientation of ligand per cluster and the distance of carbonyl carbon from the catalytic serine. MD simulations were performed at desired temperature creating a simulation cell of 10.0 Å around all atoms of wild type and mutant structures filled with 25% acetone, 75% water, 0.9% NaCl or 100%, 0.9% NaCl water at defined temperature for specific simulation time. The system was energy minimized prior to simulation while receptor structures were used after the simulation for docking against 4NTC-Fe.

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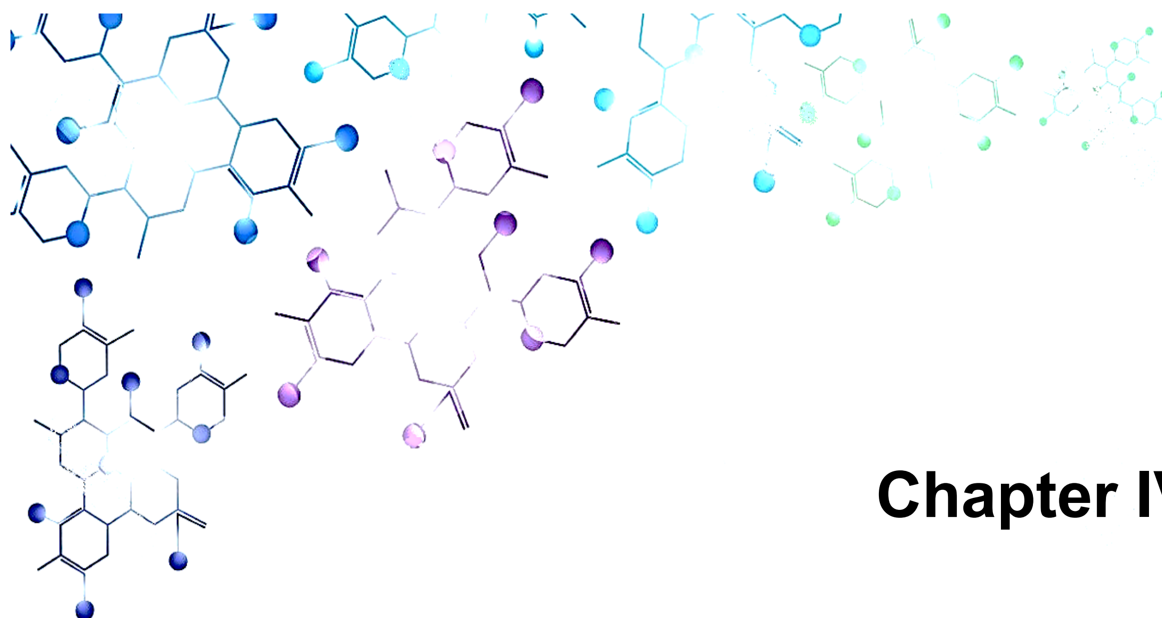
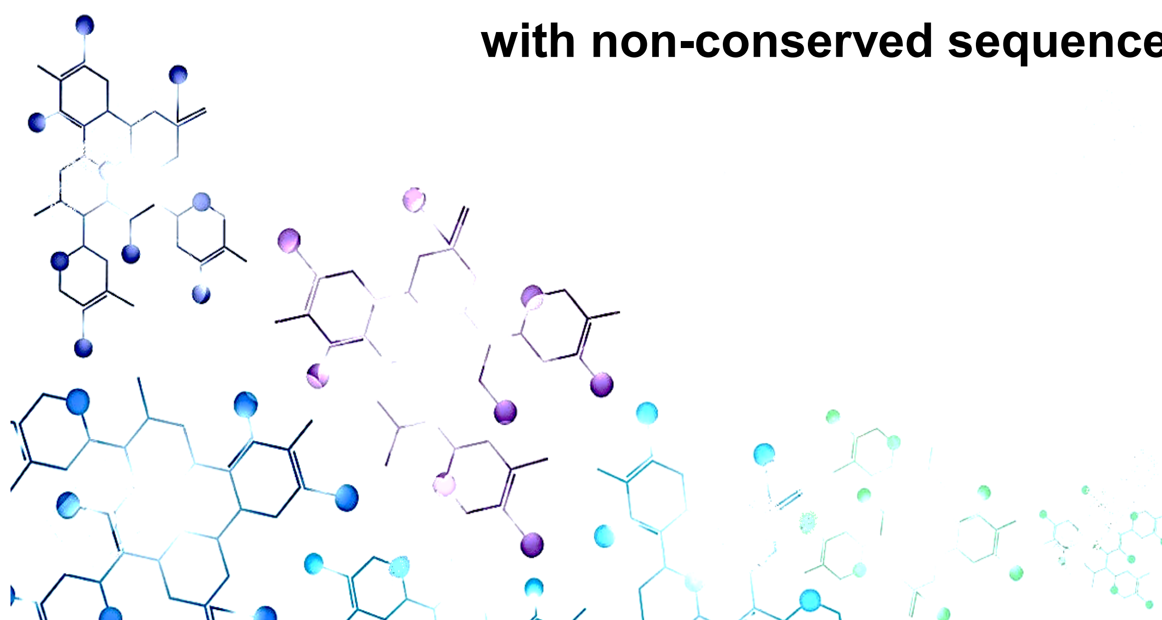


Chapter IV

Screening of fungi for the identification of FAE with non-conserved sequences



Chapter IV: Screening of fungi for the identification of FAE with non-conserved sequences

4.1 Introduction

As previously described in chapter 2 of this PhD thesis, the fungal kingdom represents an undeniable source of feruloyl esterases and glucuronoyl esterases.

Together to mining for new genes in known fungal genomes, functional screening of targeted fungal strains of unknown genome sequences could considerably expand the repertoire of available DNA sequences for feruloyl and glucuronoyl esterases.

This chapter describes the results concerning the screening of fungi for the identification of FAEs and GEs with non-corserved sequences. In detail, in sections 4.2.1, manuscript **"*Talaromyces borbonicus* sp. nov., a novel fungus from *Arundo donax* with potential abilities in lignocellulose conversion"** describes the identification of a new fungal species using a polyphasic approach and the sequencing and analysis of its genome.

In addition, a second *Talaromyces* species, *T. adpressus*, was selected for producing enzymes with potential synergistic actions on lignocellulose conversion and its genome was sequenced (see Appendix IV).

Microbiota from lignocellulosic source can be considered an important resource of fungal strains to upgrade lignocellulose conversion to 'greener' technologies. Around 1,000 microbial strains isolated from lignocellulosic biomasses during biodegradation under natural conditions by Department of Agriculture of University of Naples "Federico II" (Prof. Olimpia Pepe, Division of Microbiology, Portici, Naples, Italy) have been screened for the production of different enzymatic activities involved in lignocellulosic deconstruction. Their colony morphology was analysed observing the shape and colour, while the micromorphology of the conidia was analysed using an optic microscope. With the aim of evaluating the production of FAE activity, some selected fungi were chosen in this work for functional screening. They were firstly grown on solid medium containing indolyl ferulate or ethyl ferulate, as sole carbon source, showing to be able to grow on solid medium containing indolylferulate, but not to form blue halos of activity. In addition, the induction of FAE activity production was further studied in liquid culture media in the presence of de-starched wheat bran and wheat bran as sole carbon source. Furthermore, the influence of ferulic acid as inducer of FAE activity production was also explored, by adding it to untreated wheat bran in liquid growth medium. The best two FAE producing fungi were selected for the analysis of their genome and transcriptome to identify novel FAEs and GEs, as reported in this chapter.

4.2 Results

4.2.1 *Talaromyces borbonicus* sp. nov., a novel fungus from *Arundo donax* with potential abilities in lignocellulose conversion

(manuscript submitted to Mycologia)

Running head: *Talaromyces borbonicus* sp. nov., a novel fungus from *Arundo donax*

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Keywords: Fungi, genome sequence, *Talaromyces*, taxonomy

Abstract

A novel fungal species able to synthetize enzymes with potential synergistic actions in lignocellulose conversion was isolated from the biomass *Arundo donax* during biodegradation under natural conditions in the Gussone Park of the Royal Palace of Portici (Naples, Italy). In this work, this species was subjected to morphological and phylogenetic analyses. Its genome was sequenced and assembled into 27.05 Mb containing 9 744 predicted genes, among which 396 belonging to carbohydrate active enzyme (CAZyme) encoding genes. Here we describe and illustrate this previously unknown species, that was named *Talaromyces borbonicus*, by a polyphasic approach combining phenotypic, physiological and sequence data.

Introduction

Arundo donax (common name "giant reed") is a non-edible and perennial herbaceous rhizomatous crop belonging to the family of *Poaceae*, in the tribe of *Arundinaceae* (Mariani et al. 2010). It is widespread in the Mediterranean area and its adaptability to different soils and climatic conditions, high biomass productivity, annual harvesting period, make this crop an attractive lignocellulosic feedstock (Lewandowski et al. 2003, Corno et al. 2014). In the frame of use of renewable biomass as an alternative to fossil sources, *A. donax* has been used in the production of biogas, bio-ethanol and other biochemicals such as succinic acid by biological fermentation (Ask et al. 2012, Corno et al. 2015, Ventorino et al. 2017). Enzymatic treatment of lignocellulosic biomass for production of fermentable sugars represents the most prevailing alternative to chemical hydrolysis with the potential of higher selectivity, lower energy costs and milder operating conditions than chemical processes (Yang et al. 2011). In nature, lignocellulosic biomass is degraded due to the synergistic action of cellulolytic, hemicellulolytic, and ligninolytic enzymes within

complex enzymatic multistep processes (Voříšková and Baldrian 2013, Yang et al. 2014). These enzymes are produced by microbial communities that can be therefore used as a source of novel biocatalysts (Amore et al. 2013, Okeke et al. 2015, Daas et al. 2016) for improvement of lignocellulose conversion in green technologies.

During the screening of microbiota of *A. donax* biomass piles left to biodegrade under natural conditions in the Gussone Park of the Royal Palace of Portici (Naples, Italy), novel microorganisms were isolated and characterized for their activities in lignocellulose conversion (Ventorino et al. 2015, Varriale et al. 2016). In this study, we describe a novel *Talaromyces* species selected among these microorganisms for its ability to synthetize different enzymes with potentially synergistic actions in lignocellulose conversion, such as endo- and exo-cellulase, cellobiohydrolase, xylanase, pectinase and laccase (Ventorino et al. 2015, 2016). This strain was also able to produce feruloyl esterase (FAE) activity assayed towards *p*-nitrophenyl-ferulate. In addition to the description of the new species, we also report its draft genome sequence.

Materials and methods

Strain isolation and preservation

A *Talaromyces* strain (A-T2C-71X), belonging to the microbial collection of division of Microbiology Department of Agricultural Sciences of University of Naples Federico II, was isolated from lignocellulosic biomass of *A. donax* during biodegradation under natural conditions (Ventorino et al. 2015) in the Gussone Park of the Royal Palace of Portici, Naples, Italy (40°48’50.1"N, 14°20’48.2"E).

Cultures and media

Talaromyces sp. was cultivated in complete medium (de Vries et al. 2004). Mycelium was sampled after 48 h of growth and genomic DNA was extracted using a CTAB-based extraction buffer (Hildén et al. 2005). RNA was extracted and purified using RNeasy® Mini Kit (Qiagen, Hilden, Germany) from 6 and 9-day old cultures grown on wheat bran, wheat bran with ferulic acid, destarched wheat bran and glucose in minimal medium.

Morphological analysis

Macroscopic analysis of the strain was performed on creatine agar (CREA), Czapek yeast extract agar (CYA), CYA supplemented with 5 % NaCl (CYAS), dichloran 18% glycerol agar (DG18), malt extract agar (MEA, Oxoid), oatmeal agar (OA) and yeast extract sucrose agar (YES). All Petri dishes were incubated at 25°C for 7 days and additional CYA plates were incubated at 30 and 37 °C. Microscope preparations were made from 1 to 2 weeks old colonies grown on MEA and all media were used for observation on the presence of ascomata. Details on the inoculation of the agar media, Ehrlich reaction and the macro- and micromorphological analyses are given in Houbraken *et al*. and Yilmaz *et al*. (Houbraken et al. 2014, Yilmaz et al. 2014).

Phylogenetic analysis

The ß-tubulin (*BenA*), calmodulin (*CaM*), Internal Transcribed Spacer regions (including 5.8S rDNA) (ITS) and RNA polymerase II second largest subunit (*RPB2*) sequences of strain A-T2C-71X/CBS 141340 were extracted from the genome sequence and used in the phylogenetic analyses. The phylogenetic relationship of this strain with other section *Helici* members was studied using a maximum likelihood (ML) and Bayesian analysis (BI). A sequence alignment was made with the extracted sequences and the reference sequences of section *Helici* (Yilmaz et al. 2014, Chen et al. 2016) using MAFFT (Katoh et al. 2005), and the most optimal model was calculated in MEGA6 (Tamura et al. 2013). A maximum likelihood (ML) tree was inferred using MEGA6 and the Bayesian inference (BI) analysis was performed in

MrBayes v. 3.2.2 (Stamatakis 2014). Support of the nodes was calculated with 1 000 rapid bootstrap replicates for the ML analysis and in the Bayesian analysis, every 1 000 generations were sampled and the first 25 % of the samples were discarded. Bootstrap percentages and posterior probability values are presented at the nodes and values less than 70 % bootstrap support (bs) or 0.95 posterior probability (pp) are not shown. *Talaromyces adpressus* CBS 140620 was used as an outgroup.

<u>*Sequencing and data analysis*</u>

Concentration and quality of the samples were determined using the Life Technology Qubit and 0.6% agarose gel, respectively, while the RNA samples quality was checked using Fragment Analyser (Advanced Analytical Technologies). Genome and transcriptome sequencing were performed at GenomeScan. NEBNext® Ultra DNA Library Prep kit for Illumina (cat# NEB #E7370S/L) and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420S/L) were used according to manual for library preparation. Quality and yield after sample preparation was measured with Bioanalyzer (Agilent Technologies).

Clustering and DNA sequencing using the Illumina cBot and HiSeq 2500 was performed according manufacturer's protocols using concentration of 8.0 pM of DNA, standard Illumina primers and HiSeq control software HCS v2.2.58. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA v1.18.64 and Bcl2fastq v1.8.4. Reads were trimmed for adapter sequences and filtered for sequence quality using the in-house tool FASTQFilter v2.05. The short-read genome assembler Abyss v1.3.7 (Simpson et al. 2009) was used for assembly. An optimization for k-mer length was performed in previous fungi genome assemblies. A length of 64 was found to give the best results, optimized for the lowest amount of scaffolds with a longer average length. Scaffolds shorter than 500 bp, unlikely to contain complete coding sequences, were removed.

The HMM-based algorithm Glimmer (version 3.02) (Majoros et al. 2004) was trained for gene finding using the genome of *Talaromyces stipitatus* (Nierman et al. 2015). Furthermore, an evidence-based method of gene finding was performed using the CodingQuarry (Testa et al. 2015) software tool and the mapped mRNA-Seq reads. GC content was assessed by QUAST (Gurevich et al. 2013).

<u>*CAZymes analysis*</u>

The putative encoded protein sequences were first compared to the full-length sequences of the CAZy database (Lombard et al. 2014) using BLAST (Altschul et al. 1990) and query sequences that produced a e-value $>10^{-6}$ were discarded. Query sequences that had >50% identity with a sequence already classified in the CAZy database were automatically assigned to the same family as the subject sequence. The remaining query sequences were subjected to manual curation which involved BLAST searches against a library built with partial sequences corresponding to individual GH, PL, CE and CBM modules and examination of the conservation of specific family patterns and features such as catalytic residues (where known). Subfamily assignments were performed using a set of hidden Markov models (Rabiner and Juang 1986) built after each subfamily.

Results

<u>Phylogenetic analysis</u>

Phylogenetic analysis shows that *T. borbonicus* belongs to section *Helici (Fig. 1).* With the description of *T. borbonicus,* sect. *Helici* currently contains 10 species. Two main clades are present in section *Helici:* one clade includes *Talaromyces boninensis, Talaromyces borbonicus, Talaromyces helicus, Talaromyces reverso-olivaceus, Talaromyces varians,* and the other clade *Talaromyces. aerugineus,*

Talaromyces bohemicus, Talaromyces cinnabarinus, Talaromyces diversiformis and *Talaromyces ryukyuensis. T. borbonicus* CBS 141340 clusters in all phylogenies with URM 7624, an unidentified strain from inside nests of *Melipona scutellaris* bees in Brazil.

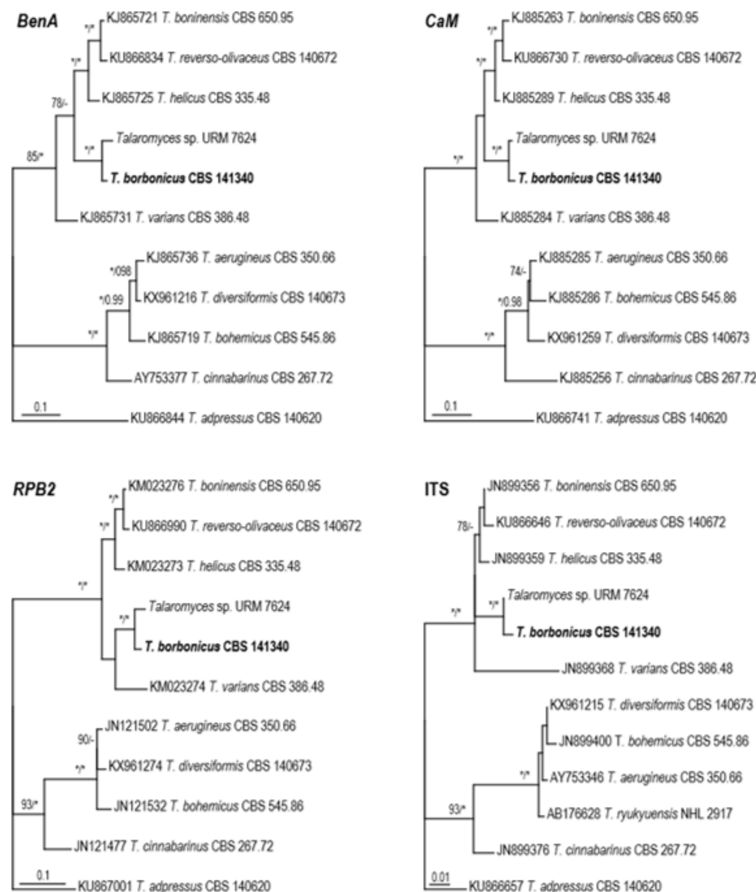

Figure 1: Phylogenetic trees showing the relationship among strains belonging to *Talaromyces* section *Helici*. Maximum likelihood bootstrap proportion (bs) and Bayesian posterior probability (pp) are appended to nodes; only bs > 70 % and pp >0.95 pp are shown, lower supports are indicated with a hyphen, whereas asterisks indicate full support (100 % bs or 1.00 pp). The bars indicate the number of substitutions per site. The phylogram is rooted with *Talaromyces adpressus* CBS 140620.

<u>*Genome features*</u>

Over 93.2 million reads were obtained after quality filtering and trimming, with average read length of 151 bp. The draft genome sequence of *T. borbonicus* has an estimated size of 27.05 Mb resulted from the assembly of 47 contigs (TABLE 1). The average genome coverage is 518x and the GC content of the assembly is 46.1 %.

The output from both Glimmer and CodingQuarry was combined into a single gene model of 9,744 genes.

The lengths of the datasets were 512 bp (*BenA*), 578 bp (*CaM*), 455 (ITS) and 851 bp (*RPB2*) and the best substitution models were HKY+G (*BenA*), TN93+G+I (*CaM*, *RPB2*) and T92+G+I (ITS).

<u>*CAZyme analysis*</u>

In order to identify putative genes and enzymes involved in the breakdown, biosynthesis or modification of carbohydrates, the total predicted ORFs in *T. borbonicus* genome were compared to the entries of the Carbohydrate-Active Enzymes (CAZymes) database (http://www.cazy.org/). A total of 396 putative CAZymes were identified in *T. borbonicus* genome (TABLE 2).

The most abundant predicted CAZymes was from the glycoside hydrolase (GH) class (approx. 63% of all CAZymes) followed by Glycosyltransferases (GT) with approx. 23% of the total predicted CAZymes. The amount of a Carbohydrate Esterases (CEs) (5%), Auxiliary Activities (AAs) (5%) and Polysaccharide Lyases (PLs) (0.5%) was significantly smaller. ORFs coding for putative Carbohydrate-binding modules (CBMs) having binding activity to carbohydrates corresponded to approx. 15% of the total CAZymes. In particular, 82% of the detected CBMs was in association with other CBMs or with GHs or CEs displaying a modular structure.

TABLE 1. Genome features of *T. borbonicus* sp. nov. A-T2C-71X (CBS 141340).

Features	
# of reads	93,219,198
Paired-end read length (bp)	151
Genome assembly size (Mb)	27.05
# of contigs	47
# of scaffolds	28
Contig N50	1,406,961
Scaffold N50 (bp)	2,198,792
# of exons per gene (average)	2.8
GC content (%)	46.1
# predicted genes	9,744

TABLE 2: CAZyme content of *T. borbonicus*.

	T. borbonicus	
Cazymes classification	ORFs	%
Glycosyltransferase (GTs)	91	23
Carbohydrate Esterase (CEs)	20	5,1
Glycoside Hydrolase (GHs)	250	63,1
Polysaccharide Lyase (PLs)	2	0,5
Auxiliary Activities (AAs)	19	4,8
Carbohydrate-Binding Module (CBMs)	60	15,2
Expansin (EXPs)	6	1,5
Total CAZymes *	396	

*The total numbers of CAZYmes is less than the sum (GTs + CEs + GHs +PLs+AAs + CBMs) due to the fact that some multimodular predicted proteins were detected.

TAXONOMY

Talaromyces borbonicus Houbraken, *sp. nov*. — MycoBank MB 821643. FIG. 2.

In: *Talaromyces* section *Helici*.

Etymology: Referring to dynasty of the Bourbons of Naples. The type specimen was collected in Gussone Park, which is part of the Royal Palace of Portici and formerly belonging to for the dynasty of the Bourbons of Naples.

Type specimen: ITALY, Naples, Gussone Park of the Royal Palace of Portici, *Arundo donax* biomass (after biodegradation under natural conditions), isolated and collected by O. Pepe, 2012 (holotype CBS H-22672, culture ex-type CBS 141340 = DTO 351-D3).

Diagnosis — *Talaromyces borbonicus* produces brown coloured funicles with short, brown pigmented conidiophores (10–40 × 2.5–3.5 (–4.5) μm) and small sized conidia

(1.5–2.0 µm). Furthermore, the species grows well on CYA incubated at 37 °C (25–32 mm, 7 d).

Colony characters — Colony diam, 7 d, in mm: CYA 20–55; CYA30°C 28–32; CYA37°C 25–32; CYAS 5–12; DG18 13–16; MEA 22–28; YES 20–28; creatine agar 12–17, poor growth, good acid and no base compounds produced. Optimum growth temperature on CYA approx. 28–30°C.

CYA, 25 °C, 7 d: Colonies centrally elevated, plane; sporulation poor, only in centre; colony texture floccose; mycelium white, pale brown in centre; exudate present as small droplets, clear to pale brown; soluble pigments absent; margin entire; conidia dull green; reverse concentric rings in different shades of brown. YES, 25 °C, 7 d: Colonies low, randomly sulcate; sporulation moderate; mycelium white; exudate droplets absent; soluble pigments absent; margin entire; conidia dull green in centre, brownish green at edges, reverse brown with dark brown centre. MEA, 25 °C, 7 d: Sporulation moderate to strong; colony texture floccose; mycelium white; exudate droplets absent; soluble pigments absent; conidia *en masse* dark green; reverse brownish yellow. DG18, 25 °C, 7 d: Colonies slightly elevated; sporulation moderate; mycelium white; reverse pale brown, becoming brown in centre. Ehrlich reaction negative.

Micromorphology: Ascomata and sclerotia absent. Conidiophores arising from dark brown pigmented funicles, predominantly biverticillate, occasionally with additional divergent branch; stipes smooth walled, pale brown pigmented, short, 10–40 × 2.5–3.5 (–4.5) µm; metulae pale brown pigmented, 3–8, 8.5–11 × 2.5–3.5 µm; phialides 2–7 per metula, pale brown pigmented, acerose, 9–11 × 2.0–3.0 µm; conidia broadly ellipsoidal or ellipsoidal, smooth walled, 1.5–2.0 µm.

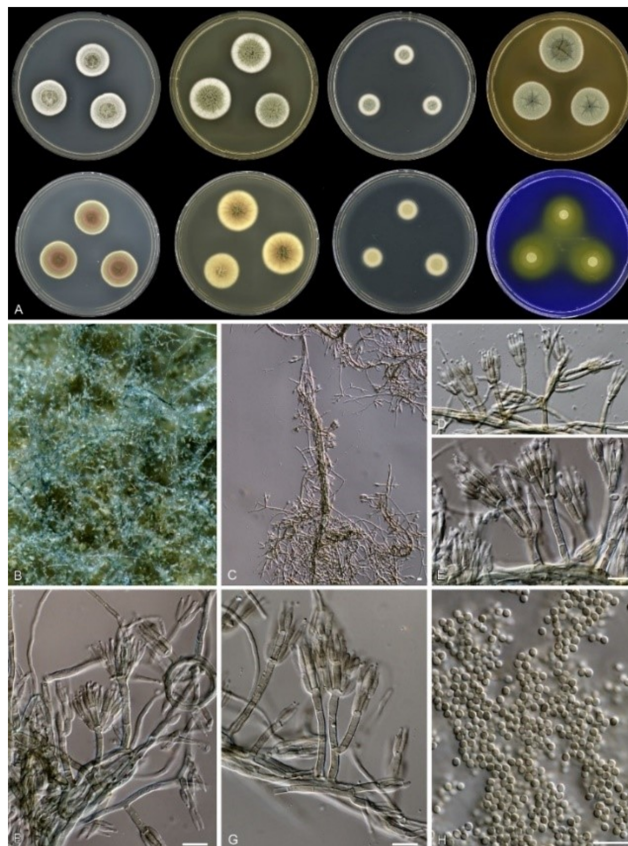

Figure 2: *Talaromyces borbonicus*, A. 7 d old cultures, 25 °C, left to right; first row, all obverse, CYA, YES, DG18, MEA; second row, CYA reverse, YES reverse, DG18 reverse and CREA obverse. B. Detail of colony on MEA. C–G. Conidiophores. H. Conidia. Scale bars = 10 µm.

DISCUSSION

The genus *Talaromyces* was initially described by Benjamin in 1955 as a sexual state of *Penicillium* producing yellow soft-walled ascomata covered by interwoven hyphae, with ovate to globose asci containing mostly spiny ascospores (Benjamin 1955). Due to the diverse characteristics of its members, the genus was divided into four sections based on the structure of the conidial state (Stolk and Samson 1972). However, Samson et al. and Yilmaz et al. (Samson et al. 2011, Yilmaz et al. 2014) redefined the taxonomy of *Talaromyces* by combining *Penicillium* subgenus *Biverticillium* into *Talaromyces* and proposing a new classification based on the ITS, BenA, RPB2 and CaM genes analysis together with phenotypic features including its morphology. The 88 accepted *Talaromyces* species were placed into seven sections, named *Bacillispori, Helici, Islandici, Purpurei, Subinflati, Talaromyces* and *Trachyspermi*.

Strains belonging to the *Talaromyces* genus have an enormous biotechnological potential, because of their ability to secrete a variety of small molecules and enzymes with different applications, such as xylanases for pulp bleaching (Maalej et al. 2009, Maalej-Achouri et al. 2009, Orencio-Trejo et al. 2016), cellulases for lignocellulosic biomass deconstruction (Inoue et al. 2014, Fujii et al. 2015, Schafhauser et al. 2015, Orencio-Trejo et al. 2016), feruloyl esterases for agro-food industries and tailored synthesis of pharmaceuticals (Crepin et al. 2003, Garcia-Conesa et al. 2004, Vafiadi et al. 2006, Mandalari et al. 2008, Watanabe et al. 2015) and pigments for the sustainable bioproduction of environmentally friendly dyes (Schafhauser et al. 2015). In this work, a *Talaromyces* isolate from the lignocellulosic biomass *A. donax* during biodegradation under natural conditions (Ventorino et al. 2015) in the Gussone Park of the Royal Palace of Portici, Naples, Italy was studied. Phylogenetic analysis has revealed that *T. borbonicus* belongs to section *Helici*, which currently comprises 10 species divided in two clades. *Talaromyces borbonicus* resides in a clade with *T. boninensis*, *T. helicus*, *T. reverso-olivaceus* and *T. varians*.

Yilmaz et al reported that section *Helici* members produce biverticillate conidiophores with stipes that are generally pigmented (Yilmaz et al. 2014). These features are shared by *T. borbonicus*. *T. borbonicus* is phylogenetically most closely related to *T. boninensis*, *T. helicus*, *T. reverso-olivaceus* and *T. varians*. *Talaromyces boninensis* and *T. helicus* produce ascomata, and this feature is not observed in *T. borbonicus.* Furthermore, *T. borbonicus* and *T. boninensis* produce acid compounds on CREA, while no growth and/or acid is produced by *T. helicus, T. varians* and *T. reverso-olivaceus*. The production of brown coloured funicles on MEA with short, brown pigmented conidiophores (10–40 × 2.5–3.5 (–4.5) µm) is unique for *T. borbonicus*.

The strain *T. borbonicus* A-T2C-71X (CBS 141340) studied in this work was selected for its ability to synthetize different enzymes having potentially synergistic actions on lignocellulose conversion, such as endo- and exo-cellulase, cellobiohydrolase, xylanase, pectinase, laccase and feruloyl esterase (Ventorino et al. 2015).

Sequencing of *T. borbonicus* genome revealed an estimated size of 27.05 Mb resulting from the assembly of 47 contigs containing 9,744 predicted genes. Genome analysis of *T. borbonicus* revealed the presence of 396 genes coding for enzymes devoted to degradation, modification, or creation of glycosidic bonds, corresponding to the 4% of the total predicted genes. There are several studies on *Talaromyces* species producing enzymes involved in plant biomass degradation (Inoue et al. 2014, Fujii et al. 2015, Schafhauser et al. 2015, Orencio-Trejo et al. 2016). An example is

Talaromyces cellulolyticus (formerly *Acremonium cellulolyticus*), which is one of the best-characterized cellulase-producing fungi (Fujii et al. 2015).

T. borbonicus revealed a number of CAZy genes analogous to other related species (TABLE 3, SUPPL. TABLE 1). In particular, genes belonging to families GH3, GH28, GH31, GH35, GH43 are the most abundant which include genes that encode enzymes involved in plant cell wall degradation. Moreover, the number of predicted CAZy genes in *T. borbonicus* genome is 40 - 50% higher than reported for *Trichophyton verrucosum* (Zhao et al. 2013) and *Thermomyces lanuginosus* (Winger et al. 2014), a unique cellulase-free fungus, producing high quantities of a GH11 xylanase. This presence of a broad set of genes falling into the class of plant biomass degrading enzymes reveals the potential of the novel fungal strain *T. borbonicus* A-T2C-71X/CBS 141340 to produce enzymes with biotechnological use in the deconstruction of the lignocellulosic biomass.

Accession number

This draft genome sequence of *T. borbonicus* sp. nov. A-T2C-71X/CBS 141340 has been deposited at DDBJ/ENA/GenBank under accession number NBSA00000000. The version described in this paper is version NBSA01000000. The BioProject in GenBank is PRJNA379116. The strain is available from the CBS culture collection (www.westerdijkinstitute.nl) housed at the Westerdijk Institute (Utrecht, the Netherlands). The genome is also available through the JGI fungal genome portal MycoCosm (Grigoriev et al. 2014).

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Table 3: CAZyme content of selected fungal genomes in number of genes per group. PBD = plant biomass degradation related genes, GHs = glycoside hydrolase, PLs = polysaccharide lyase, CEs = carbohydrate esterase, AAs = auxiliary activity, GTs = glycosyl transferase, CBMs = carbohydrate binding module, EXPs = expansin.

Species	Strain	Total CAZy	GHs		PLs		CEs		AAs		GTs	CBMs	EXPs
			Total	PBD	Total	PBD	Total	PBD	Total	PBD			
Talaromyces borbonicus	A-T2C-71X/ CBS 141340	396	251	144	2	0	20	17	14	4	91	67	6
Penicillium rubens	Wisconsin 54-1255	426	222	120	9	9	20	20	22	9	101	51	1
*Penicillium chrysogenum**	unknown	481	234	125	9	9	20	20	50	10	110	56	2
*Penicillium subrubescens**	CBS132785	719	410	241	9	9	38	38	63	16	107	85	7
*Talaromyces stipitatus**	ATCC 10500	514	271	136	2	0	17	18	47	8	105	65	7
Aspergillus niger	NRRL3	542	252	137	9	9	22	22	65	19	119	72	3
Aspergillus oryzae	RIB40	600	304	174	23	20	27	26	69	16	119	54	4
Aspergillus nidulans	FGSC A4	572	275	161	23	21	28	28	57	16	97	90	2
*Trichoderma reesei**	QM6a	410	200	77	5	0	16	15	32	6	92	58	7
Neurospora crassa	OR74A	416	182	77	4	3	22	22	51	27	86	68	3

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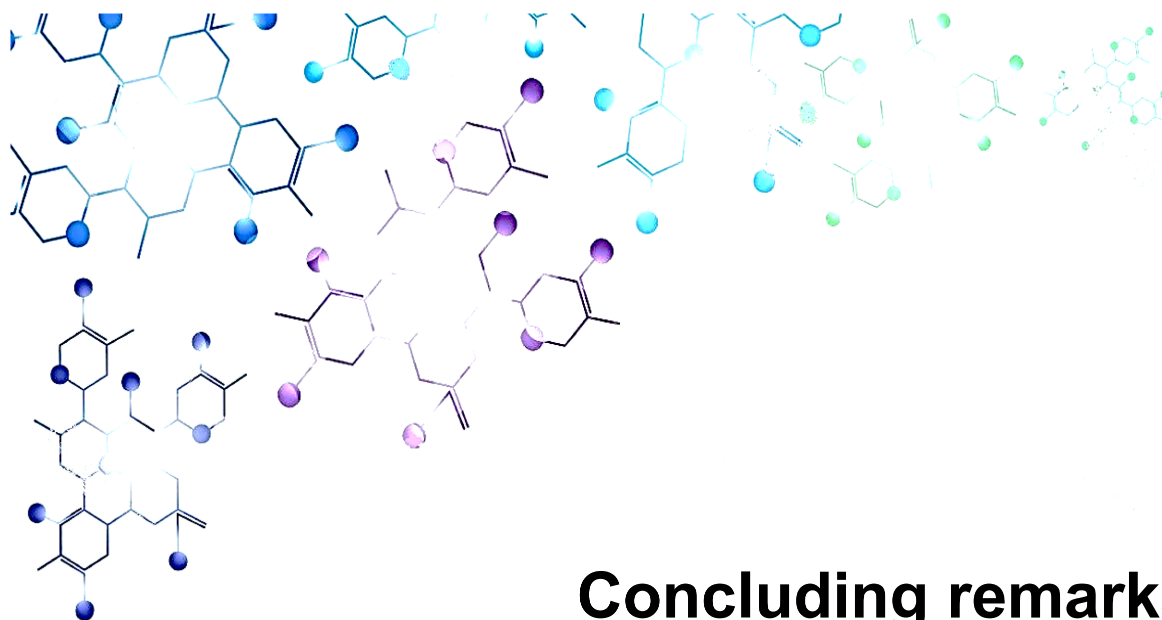
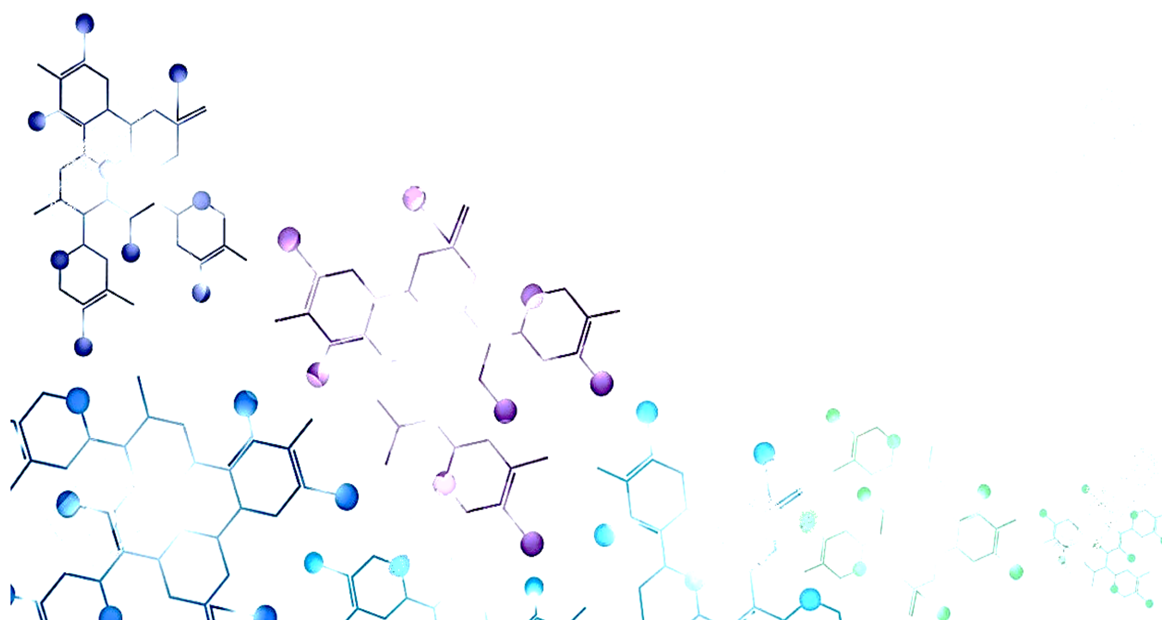
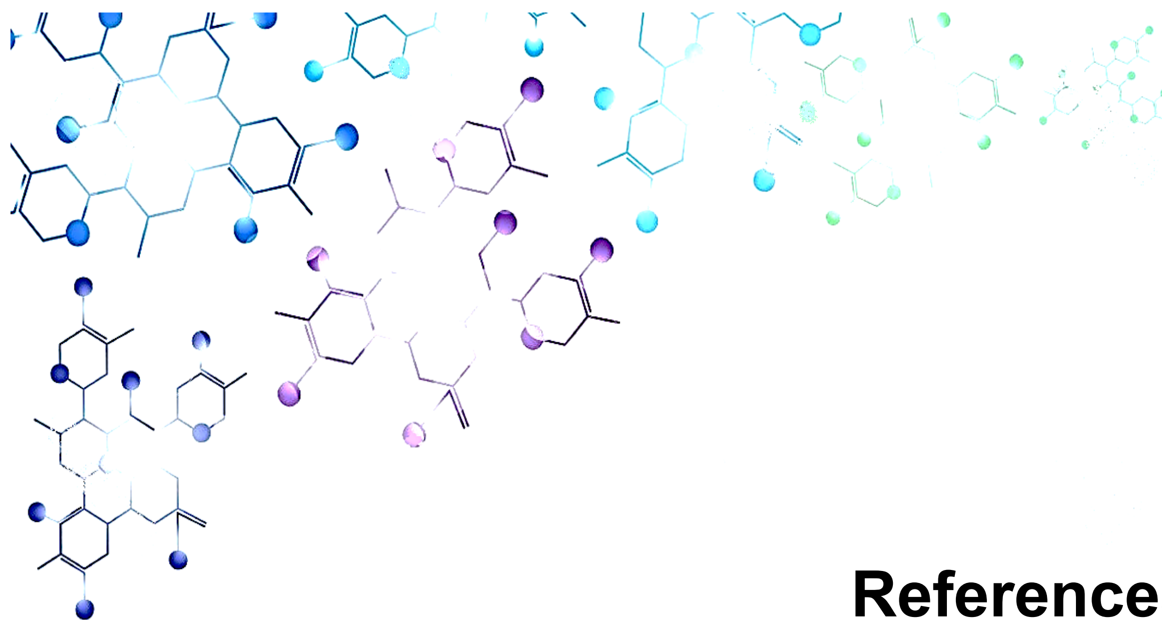


Concluding remarks



Concluding remarks

The overall aim of this PhD project was to develop improved biocatalysts based on FAEs and GEs for the production of compounds with antioxidant activity.
30 novel fungal FAEs and 20 GEs, identified through a bioinformatics approach from the analysis of 300 fungal genomes by the "Westerdijk Fungal Biodiversity Institute", were expressed in *P. pastoris* and characterized. This led to the selection of AwFaeG as the best enzyme to be subjected to site-directed mutagenesis. A homology model of this enzyme was developed and five site-directed variants were designed, expressed in *P. pastoris* and characterized assessing substrate specificity, solvent- and thermo- tolerance. N391V variant showed around ten-fold higher activity towards *p*NP-Fe than the wild type enzyme, whilst G470F variant showed an increase of around eight-fold in tolerance to 1 hour exposure at 55°C and tolerance after 25% acetone and 5% butanol exposure.
Directed evolution was applied to the already characterized FAEs FoFaeC and MtFae1a with the aim of improving their characteristics. Complete methodologies for the construction and the automated screening of evolved variants libraries were developed and applied to the generation of 30,000 mutants of each enzyme and their screening for selecting the variants with higher activity than the wild type enzymes. Randomly mutated variants of FoFaeC and MtFae1a were generated through ep-PCR and expressed in *Y. lipolytica* and *S. cerevisiae,* respectively. Screening for extracellular enzymatic activity towards chromogenic substrates, developed *ad hoc* for high-throughput assays of FAEs on solid and in liquid media, led to the selection of improved enzyme variants. Among the best FoFaeC mutants, L432I variant was the only variant bearing a single mutation taking place in the region of the substrate binding pocket, which reflected in an improvement of affinity towards all the methylated cinnamic acids (except for MSA). Although the screening strategy was based on the selection of evolved variants with improved hydrolytic activity, it was possible to obtain MtFae1a variants with enhanced synthetic activities. In particular, H105Y evolved variant exhibited twofold increased hydrolytic activity towards MFA, M*p*CA and *p*NP-Fe and a threefold increase towards MCA. Moreover, this variant showed enhanced abilities in GFA, PCA, PFA and BFA syntheses, with potential application in cosmetic industry.
With the aim of identifying novel FAEs with non-conserved sequences, different fungal strains isolated from lignocellulosic biomasses during biodegradation under natural conditions and belonging to the microbial collection of Department of Agriculture of University of Naples "Federico II" were screened for the production of different enzymes having potentially synergistic actions on lignocellulose conversion, such as endo- and exo-cellulase, cellobiohydrolase, xylanase, pectinase, laccase and feruloyl esterase. The fungus showing the highest FAE activity production towards *p*NP-Fe grown on different carbon sources was identified as a novel species named *Talaromyces borbonicus* and selected for genome and transcriptome sequencing. Genome analysis of *T. borbonicus* showed the presence of 396 genes coding for enzymes belonging to the CAZy family, revealing the potential of this novel strain to produce enzymes with biotechnological use in the deconstruction of the lignocellulosic biomass. Moreover, gene annotation revealed the presence of genes coding for a FAE, 4 putative FAEs and a glucuronoyl hydrolase.
The efforts of this PhD project support the actualization of competitive and eco-friendly bioconversions for the production of molecules with antioxidant activity, replacing the chemical processes currently used, through the development of improved biocatalysts based on FAEs and GEs.



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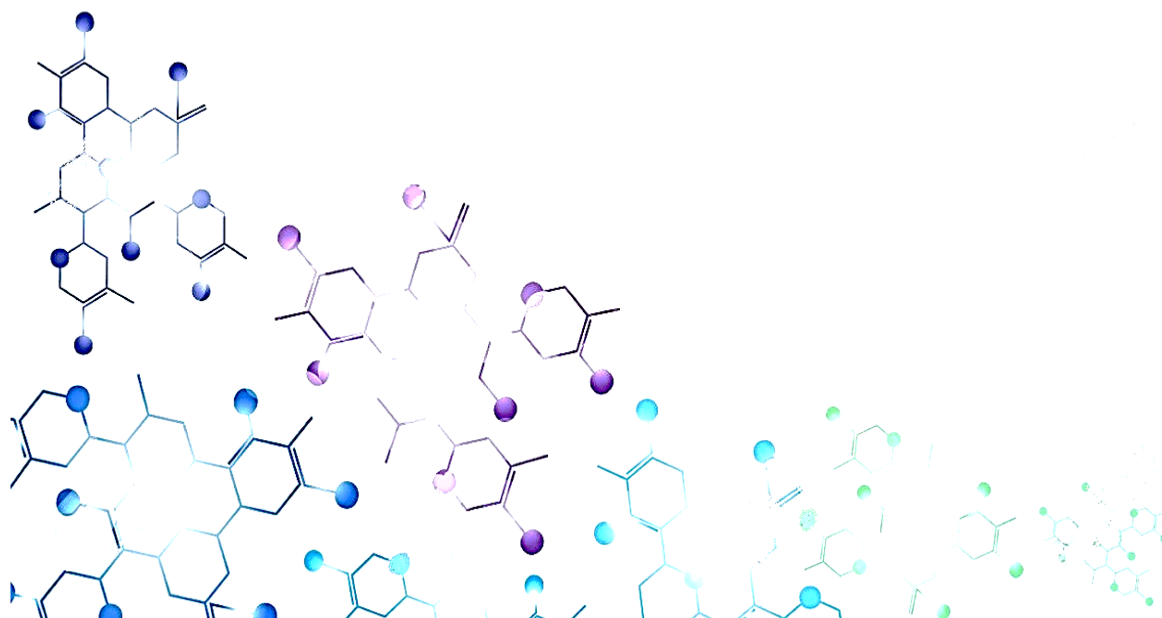

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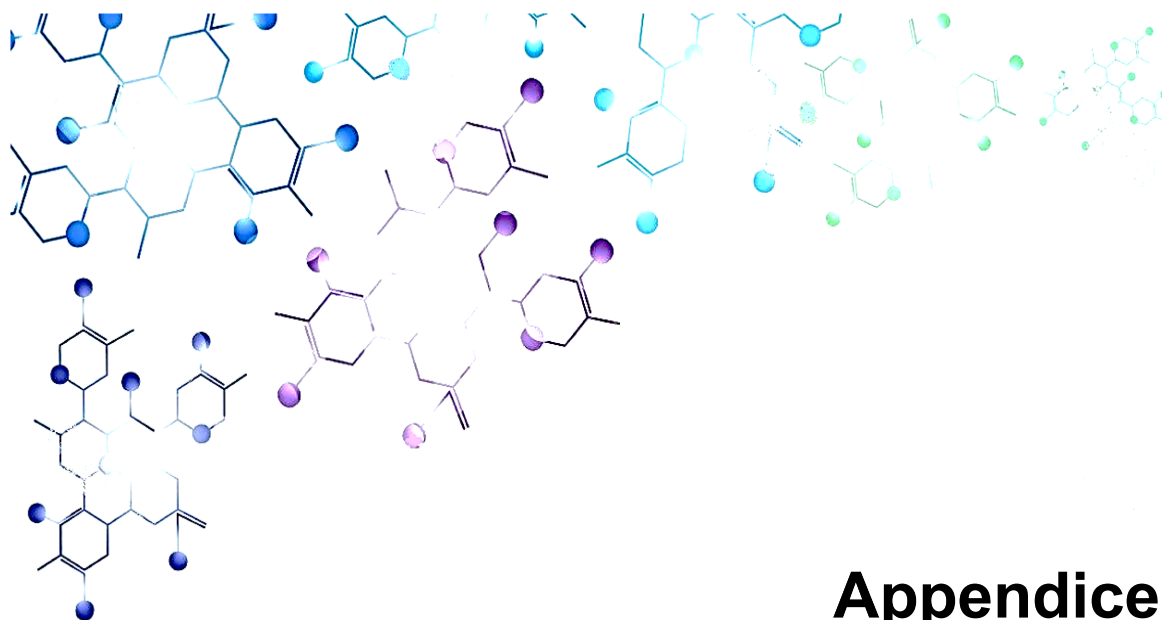
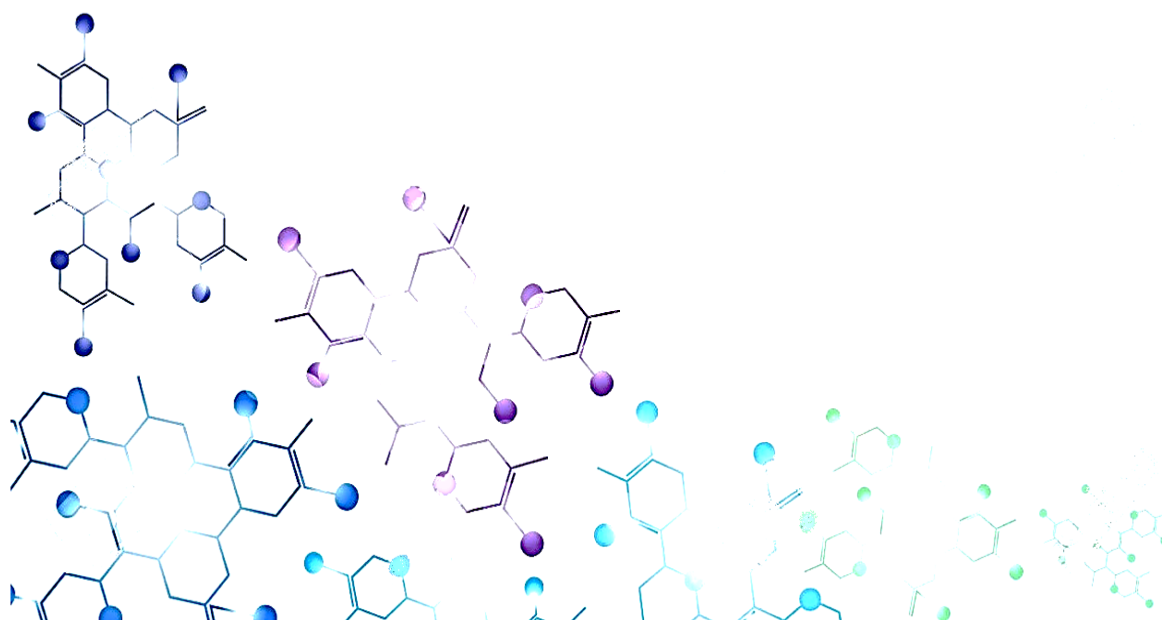
And thanks from the bottom of my heart to Colebemio, who keeps making every moment special. Meeting you was the best thing that could ever happen to me.



Appendices



Appendices

Appendix I: List of communications

- **Varriale, S.**, Cerullo, G., Ventorino, V., Pepe, O., Meijer, M., De Vries, R., & Faraco, V. (2016). Selection of new fungal strain producing feruloyl esterases for biotechnological applications. Poster session, book of abstract and abstract in New biotechnology. 17th European Congress on Biotechnology (ECB 2016) in Krakow, Poland.
- Cerullo G., **Varriale S**., Bras J., Fontes C.M.G.A., Faurè R., Piechot A., Jütten P., Faraco V. (2017): Development of improved variants of a fungal Feruloyl Esterase to replace conventional chemical reactions with eco-friendly bioconversions for cosmetic industries. Poster session and book of abstract. 13th International Symposium on Biocatalysis and Biotransformations (Biotrans 2017) in Budapest, Hungary.

Appendix II: List of publications

- Antonopoulou, I., **Varriale, S**., Topakas, E., Rova, U., Christakopoulos, P., Faraco, V. (2016). Enzymatic synthesis of bioactive compounds with high potential for cosmeceutical application. Applied microbiology and biotechnology, 100(15), 6519-6543. DOI: 10.1007/s00253-016-7647-9.
- Dilokpimol A[1], Mäkelä MR[1], Cerullo G[1], Zhou M, **Varriale S**, Gidijala L, Brás JLA, Jütten P, Piechot A, Verhaert R, Hilden KS, Faraco V, de Vries RP. (2018). Fungal glucuronoyl esterases: genome mining based enzyme discovery and biochemical characterization. New biotechnology, 40, 282-287. https://doi.org/10.1016/j.nbt.2017.10.003
- Dilokpimol A[1], Mäkelä MR[1], **Varriale S[1]**, Zhou M, Cerullo G, Gidijala L, Hinkka H, Brás JLA, Jütten P, Piechot A, Verhaert R, Hilden KS, Faraco V, de Vries RP. (2018). Fungal feruloyl esterases: Functional validation of genome mining based enzyme discovery including uncharacterized subfamilies. New Biotechnology, 41, 9–14. https://doi.org/10.1016/j.nbt.2017.11.004
- **Varriale S**, Houbraken J, Granchi Z, Pepe O, Cerullo G, Ventorino V, Woeng TCA, Meijer M, Riley R, Grigoriev IV, Henrissat B, Vries RP and Faraco V. *Talaromyces borbonicus* sp. nov., a novel fungus from *Arundo donax* with potential abilities in lignocellulose conversion. *(submitted to Mycologia)*
- Cerullo G[1], **Varriale S[1]**, Bozonnet S, Antonopoulou I, Christakopoulos P, Rova U, Gherbovet O, Faurè R, Piechot A, Jütten P, Brás JLA, Fontes CMGA, Faraco V. Directed evolution of the type C feruloyl esterase from *Fusarium oxysporum* FoFaeC and molecular docking analysis of its improved variants. *(manuscript submitted to New Biotechnology)*
- **Varriale S[1]**, Cerullo G[1], Antonopoulou I, Tron T, Faurè R, Piechot A, Jütten P, Brás JLA, Fontes CMGA, Rova U, Christakopoulos P, Faraco V. Evolution of the type B feruloyl esterase MtFae1a from *Myceliophtora thermophila* towards improved catalysts for antioxidants synthesis. *(manuscript submitted to Applied Microbiology and Biotechnology)*

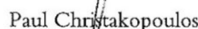
[1] Equal contribution

Appendix III: Experience in foreign laboratory

Stage at Biochemical Process Engineering Laboratory (Division of Chemical Engineering) of Luleå University of Technology (Luleå, Sweden) for the evaluation of synthetic abilities of selected feruloyl esterases in detergentless microemulsions and study of their structure-function relationship through docking simulations (March - June 2017) under the supervision of Prof. Dr. Paul Christakopoulos.

To whom it may concern,

In the frame of the OPTIBIOCAT project "Optimized esterase biocatalysts for cost-effective industrial production" supported by grant 613868 within the 7th European Framework Programme, coordinated by the University of Naples Federico II and leaded by professor Vincenza Faraco, Simona Varriale, PhD student in Biotechnology XXX cycle, carried out part of her PhD activities at our Biochemical Process Engineering Laboratory, Division of Chemical Engineering at Luleå University of Technology from March 27th to June 30th of the present year (2017).



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Appendix IV: Other publications

- Antonopoulou I, Hunt C, Cerullo G, **Varriale S**, Gerogianni A, Faraco V, Rova U, Christakopoulos P. Tailoring the specificity of the type C feruloyl esterase FoFaeC from *Fusarium oxysporum* towards methyl sinapate by rational redesign based on small molecule docking simulations. *(submitted to Plos One)*
- Cerullo G, Houbraken J, Granchi Z, Pepe O, **Varriale S**, Ventorino V, Woeng TCA, Meijer M, Riley R, Grigoriev IV, Henrissat B, Vries RP and Faraco V. Draft genome sequence of *Talaromyces adpressus.* Genome Announcements *(accepted, in press)*

Draft genome sequence of *Talaromyces adpressus*

(Genome Announcements, accepted, in press)

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Editor:	Section: Eukaryotes	Designation:

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Draft Genome Sequence of *Talaromyces adpressus*

AQ: au

Gabriella Cerullo,[a] Jos Houbraken,[b] Zoraide Granchi,[c] Olimpia Pepe,[d] Simona Varriale,[a] Valeria Ventorino,[d] Thomas Chin-A-Woeng,[c] Martin Meijer,[b] Ronald P. de Vries,[e] Vincenza Faraco[a]

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PROOF

ABSTRACT Here we present the draft genome sequence of the fungus *Talaromyces adpressus* A-T1C-84X (= CBS 142503). This strain was isolated from lignocellulosic biomass of *Arundo donax* during biodegradation under natural conditions in the Gussone Park of the Royal Palace of Portici, Naples, Italy.

Talaromyces adpressus, a recently described species belonging to section *Talaromyces*, was until now only known from the indoor environment in China (1). The strain sequenced here, CBS 142503, belonging to the microbial collection (strain number A-T1C-84X) of the Division of Microbiology, Department of Agricultural Sciences of the University of Naples Federico II, was isolated from lignocellulosic biomass of *Arundo donax* during biodegradation under natural conditions in the Gussone Park of the Royal Palace of Portici, Naples, Italy.

This strain was selected for its abilities to synthetize different enzymes having potentially synergistic actions on lignocellulose conversion, such as endo- and exocellulase, cellobiase, xylanase, pectinase, and laccase, assayed using specific solid media as previously described by Ventorino et al. (2), and to produce a feruloyl esterase (FAE) active against *p*-nitrophenyl-ferulate.

Talaromyces adpressus CBS 142503 was cultivated in complete medium (3). Mycelium was sampled after 48 h of growth and genomic DNA was extracted using a AQ: B cetyltrimethylammonium bromide (CTAB)-based extraction buffer (4). Concentration and quality of the samples were determined using the Life Technology Qubit and 0.6% agarose gel, respectively. Genome sequencing was performed at GenomeScan. The NEBNext Ultra DNA library preparation kit from Illumina (catalog number NEB E7370S/L) was used according to the manual for library preparation. Quality and yield after sample preparation were measured with Bioanalyzer (Agilent Technologies).

Clustering and DNA sequencing using the Illumina cBot and HiSeq 2500 were AQ: C performed according manufacturer's protocols using a concentration of 8.0 pM DNA, standard Illumina primers, and HiSeq control software HCS v2.2.58. Image analysis, base calling, and quality check were performed with the Illumina data analysis pipeline RTA v1.18.64 and Bcl2fastq v1.8.4. Reads were trimmed for adapter sequences and filtered for sequence quality using the in-house tool FASTQFilter v2.05. The short-read genome AQ: D assembler Abyss v1.3.7 (5) was used for assembly. An optimization for k-mer length was AQ: E performed in previous fungal genome assemblies. A length of 64 bp was found to give the best results, optimized for the smallest amount of scaffolds with longer average length. Scaffolds shorter than 500 bp, unlikely to contain complete coding sequences,

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jga-genome/jga99918/jga6158d18z | xppws | S=3 | 11/23/17 | 6:35 | ArtID: | DOI:10.1128/genomeA.01430-17 | CE: LS

were removed. The 36.12-Mb genome resulted from the assembly of 599 contigs. GC content was of 46.22% as assessed by QUAST (6).

Accession number(s). The draft genome sequence of *T. adpressus* A-T1C-84X (= CBS 142503) has been deposited at DDBJ/ENA/GenBank under accession number NHZS00000000. The version described in this paper is version NHZS01000000. The BioProject in GenBank is PRJNA381192. The strain is available from the CBS culture collection (http://www.westerdijkinstitute.nl) housed at the Westerdijk Institute (Utrecht, the Netherlands).

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AQ: F

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PROOF

Tailoring the specificity of the type C feruloyl esterase FoFaeC from Fusarium oxysporum towards methyl sinapate by rational redesign based on small molecule docking simulations

(submitted to Plos One)

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Abstract

The Type C FAE FoFaeC from *Fusarium oxysporum* is a novel enzyme with high potential for use in the hydrolysis of lignocellulosic biomass but it shows low activity towards sinapates. In this work, small molecule docking simulations were employed for identifying the catalytic mechanism behind the binding of the four model methyl esters of hydroxycinnamic acids, methyl ferulate/caffeate/sinapate/*p*-coumarate, on the structure of FoFaeC and subsequently rationally redesigning the enzyme' active site in order to improve its specificity towards methyl sinapate. A double mutation (F230H/T202V) was predicted to provide hydrophobic environment for stabilization of the methoxy substitution on sinapate and a larger binding pocket. Five mutant clones and the wild type were produced in *Pichia pastoris* and biochemically characterized. All clones showed improved activity, substrate affinity, catalytic efficiency and turnover rate than the wild type against methyl sinapate, with clone P13 showing 5-fold improvement. Although the affinity of all mutant clones was improved against the four model substrates, the catalytic efficiency and specificity decreased for the substrates containing a hydroxy substitution.

Keywords: protein engineering, feruloyl esterase, small molecule docking, rational protein redesign, enzyme specificity, enzyme activity, methyl sinapate

Introduction

Feruloyl esterases (EC 3.1.1.73, FAEs) are a subclass of carbohydrate esterases that are considered a biotechnological key for the degradation of lignocellulosic biomass, catalyzing the hydrolysis of the ester bond between hydroxycinnamic acids, such as ferulic acid (FA), caffeic acid (CA), sinapic acid (SA), *p*-coumaric acid (*p*CA) and sugars found in plant cell walls. Their application as accessory enzymes for hydrolysis as well as for the synthesis of bioactive compounds has been underlined during the past years [1-4]. The first and widely accepted system for the classification of FAEs is based on their specificity towards the hydrolysis of methyl esters of hydroxycinnamic acids: methyl ferulate (MFA), methyl caffeate (MCA), methyl sinapate (MSA) and methyl *p*-coumarate (M*p*CA).

The ABCD classification was initially based on the specificity of the two major FAEs from *Aspergillus niger*, AnFaeA and AnFaeB towards MSA and MCA, respectively, resulting to the categorization of two subclasses: type A and type B [5-7]. The system was later expanded into four subclasses [8]: Type A FAEs show preference on methoxy substituted substrates, such as MFA and MSA, are active on M*p*CA and

diferulates but not MCA, while Type B FAEs show preference on hydroxy substituted substrates, M*p*CA and MCA, are active on MFA, but not active against MSA and diferulates. Type C and D have a broader specificity towards all four substrates, but only type D FAEs are active towards diferulates. However, the increasing availability of fungal genome sequences led to the development of competitive classification systems based on phylogenetic analysis and functionality as the ABCD classification no longer reflected the wealth and diversity of putative FAEs [9-12].

An example of this diversity is the limited uniformity on the specificity profile of type C FAEs. To date, few type C FAEs have the specificity as it corresponds to the group such as TsFaeC from *Talaromyces stipitatus* [13], FaeC from *Aspergillus niger* [14] and two FAEs from *Aspergillus terreus* [15]. Others show a profile of type B FAEs with weak or no activity towards MSA, including AnFaeB from *A. niger*, AoFaeB from *Aspergillus oryzae* [16] and FoFaeC from *Fusarium oxysporum* [17]. The type C FAE from *Fusarium oxysporum* (FoFaeC) is a novel enzyme with broad pH stability and good synergistic action [17][18]. It belongs to the SF2 subfamily of phylogenetic classification [12] which is closely related to tannases, showing high similarity with AoFaeB from *A. oryzae* of known structure [16][19]. The structure of FoFaeC was recently determined (to be submitted to PDB).

Protein engineering is a young discipline employing powerful methods, from specific to random, for altering protein function and structure [20]. Engineering of the specificity of an enzyme can be done by rational re-design that is strongly dependent on the detailed understanding of the catalytic mechanisms and determinants of substrate specificity and their use for altering it in a predictable fashion. Several reports exist on rational redesign of different classes of enzymes such as oxidases, esterases, transferases [21-23]. Understanding the mechanisms behind the hydrolytic behavior of FAEs and its relation with the current classification systems is challenging, as FAEs are very diverse enzymes with broad specificity and little unifying sequence. Therefore, applying protein engineering techniques on the modification of the active site of the FAEs could provide insights on their catalytic mechanisms and specificity.

In this work, we used protein engineering techniques in order to rationally redesign the active site of the type C FoFaeC from *F. oxysporum* aiming to the increase of its activity towards MSA. We identified key residues that could possibly inhibit the binding of desired substrate on the enzymes' active site in a catalytic orientation and suggested substitutions that could benefit the binding via small molecule docking simulations and subsequently confirming the hypothesis by biochemical characterization. This is the first report of applying rational protein redesign on a FAE, opening the pathway for understanding the mechanisms behind FAE specificity towards model substrates and tailoring this diverse class of enzymes towards desired bioconversions.

Results and discussion

Docking of methyl esters of hydroxycinnamic acids on the FoFaeC structure

FoFaeC is a type C FAE that has been shown to have activity against M*p*CA, MCA, MFA and some activity against MSA [17]. Its activity towards MSA is determined significantly lower with a k_{cat}/K_m 50,000 times less than the next closest MCA. FoFaeC has high similarity (47% identity) with AoFaeB from *A. oryzae* and a root mean square deviation (RMSD) value of less than 4 Å calculated across the backbone. The binding pocket of AoFaeB is larger (93.87 Å) compared with that of FoFaeC (58.625 Å) while the catalytic triad of FoFaeC Ser-His-Asp and the formation

of disulfidic bonds are similar with AoFaeB. Thus, docking of ligands onto the FoFaeC active site was done according to the orientation of ligands on AoFaeB [16].

Docking of the four model substrates on FoFaeC resulted in a mean binding energy (MBE) for MCA and M*p*CA equal to -6.09 kcal mol^{-1} and -6.20 kcal mol^{-1}, respectively, with high proportion of elements and with the clusters accurately reflecting the high activity of the molecule on these substrates, comparing to MFA (-5.64 kcal mol^{-1}) (Table 1). The orientation of the binding of ligands is shown in Fig. 1. In the case of hydroxyl substituted esters, the hydroxyl group of the fourth position is hydrogen-bonded to glutamine 234 and aided by serine 237. MFA, as in the case of AoFaeB, is shifted to the right and downwards but at a lesser degree. The oxygen in the methoxy substitution is stabilized by the serine and the hydrophobic methyl group at residues 414 and 415. The distance between the catalytic serine S201 and the carbonyl carbon is approximately 3.5 Å in all cases. On the other hand, MSA appears to dock in the reversed orientation where the catalytic serine is within a functionally active distance of the carbon carbonyl. This may suggest that the low determined activity of FoFaeC on MSA is not a natural activity orientation of the enzyme on sinapates but an artefact of MSA as the small methyl group allows for this flipped orientation.

Table 1 Mean binding energy (MBE) and number of docked elements in cluster for the SMD of ligands against FoFaeC wild type. MBE is given in kcal mol^{-1} while maximum possible elements within an individual cluster are 20.

Ligand	MBE	N Clusters
MFA	-5.64	5
MSA	-[a]	-[a]
MCA	-6.20	17
M*p*CA	-6.09	19
FA	-5.12	4
SA	-[a]	-[a]
CA	-5.78	19
M*p*CA	-5.64	16

[a]: A more favorable MBE exists in the binding pocket but in a reversed direction

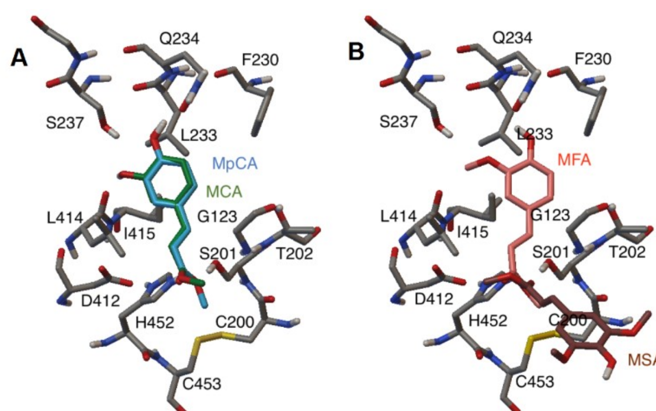

Figure 1 Position of ligands docked into FoFaeC wild type (A) MCA and M*p*CA (B) MFA and MSA. F349 and Y351 were removed for clarity

Identification of key residues for MSA activity

As was previously seen, docking of MSA resulted in a reversed orientation than that what was considered for activity as defined by Suzuki et al. [16] and the performed SMD for other methyl hydroxycinnamates in this work. Therefore, a synthetic MSA was prepared from the docking of MFA by reflecting the methoxy group perpendicular to the plane of the phenolic ring acquiring the "correct" orientation. Residues were identified to potentially prohibit binding of MSA based on the following assumptions: 1) Side-chains within 1.0 Å of the MSA residue are deemed to produce steric hindrance 2) The methyl group of the methoxy side group requires a hydrophobic environment 3) The oxygen in the methoxy side group should be stabilized by a hydrogen bond.

The resulting residues were deemed to be relevant as they existed within a 10.0 Å radius of MSA side group: Methionine 124 (M124), placed above the binding pocket, threonine 202 (T202) placed in the hydrophilic side chain and in close contact with the methyl group of the methoxy side chain of MSA, phenylalanine 230 (F230) which is hydrophobic and placed at the back of pocket near the oxygen of the methoxy side group of MSA, Tyrosine 168 (Y168) placed far right of the pocket providing small hydrophobic environment and Alanine 227 (A227) below the pocket (Fig. 2A,B).

Furthermore, analysis of the original methoxy side group for the binding of MFA highlights three important aspects. The distance between the methyl group and the nearest hydrophobic residues is 3.73 Å and 3.78 Å (a leucine and a phenylalanine, respectively) (Fig. 2C). Additionally the oxygen is stabilized by a hydrogen bond to a serine residue at a distance of 2.69 Å. Analysis of methoxy side group for the off-side reversed binding of MSA reveals that there is a distance of 2.19 Å between the methyl group and polar threonine, 3.16 Å to the polar tyrosine and 2.01 Å to the phenylalanine from the oxygen with this being non-polar/hydrophobic (Fig 2D). Of the three residues highlighted previously, two of them are also found in AoFaeB with the third tyrosine being substituted by a phenylalanine (Fig 3). As AoFaeB does not have activity on MSA and the low activity of FoFaeC could be considered an artefact, consensus is likely a good candidate for substitution. Threonine 202 does occur as part of the nucleophilic elbow GCSTGG but is not one of the consensus residues.

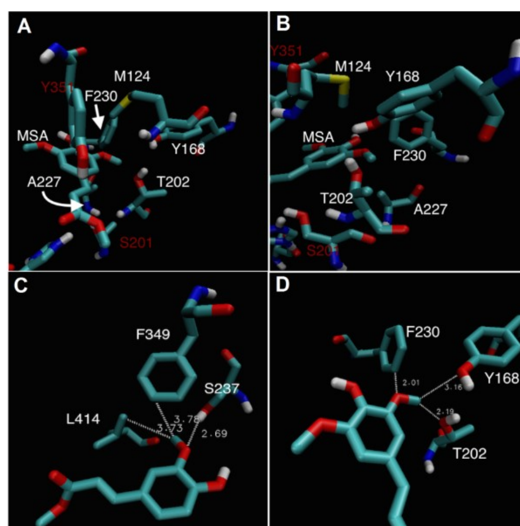

Figure 2 Relevant residues for determining binding of (A) (B) MSA to FoFaeC representing different angles with the tyrosine (Y351) and serine (S201) residues shown for orientation in red. Relevant residues involved in side group recognition of (C) MFA and (D) MSA in reversed orientation

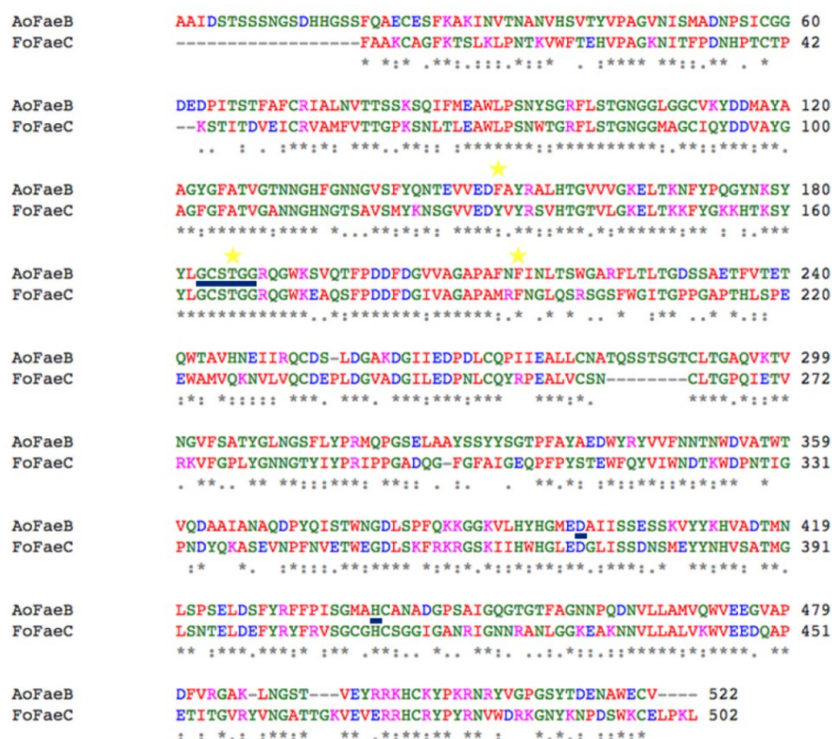

Figure 3 Sequence alignment between AoFaeB and FoFaeC with the catalytic residues underlined in blue and the potential MSA interacting residues underlined with a star

Prediction of mutations for increasing activity towards MSA

According to the previous observations, possible substitutions, based on the need of a polar group to support oxygen and a non-polar group to support the methyl group, are: Phenylalanine 230 to a bulky polar residue such as histidine (F230H), serine (F230S) or tyrosine (F230Y) in order to increase distance, threonine 202 to a hydrophobic valine (T202V) or alanine (T202A) and tyrosine 168 to a large hydrophobic residue such as phenylalanine (Y168F). Homology models of the six individual mutants were generated in order to identify the effect of mutation on the distance to the methoxy side group of MSA. In particular, F230H and F230Y increased the distance between the polar group on residue 230 and the oxygen to 2.98 Å and 3.33 Å, respectively. The mutation F230S increased the distance to 5.35 Å far beyond the needed for hydrogen bonding. The threonine mutations T202V and T202A increased the distance between the methyl group and the now hydrophobic side group to 3.28 Å and 3.48 Å, respectively. The single mutation Y168H increased the distance between the methyl group and the hydrophobic side chain of residue 168 to 4.31 Å. The effect of the single mutation on the distance was used to direct the creation of double or triple mutants. Two triple mutants where selected as the Y168F residue was deemed necessary along with the T202A mutation allowing more room within the binding pocket. Additionally, an alanine substitution was thought to provide better stability to the nucleophilic elbow. The two triple mutants F230H/T202A/Y168F and F230Y/T202A/Y168F were similarly modeled and all eight structures, five single mutants excluding F230S, the two double mutants and the wild type were used as the receptor for SMD with both MFA and MSA as ligands. A grid box was created according to the larger binding pocket of mutants, thus binding of MSA to wild type was achieved and MBE of MFA was differentiated.

The results were assessed in terms of MBE, orientation of binding and number of clusters represented in binding RMSD (Table 2). While the highest increase in MBE

was only 0.4 kcal mol^{-1}, the increase in the number of elements within the cluster is far more significant indicating that of the 20 genetic algorithm runs, 10 resulted in the desired orientation. The Y168F mutation appears to have little effect on the docking of MSA and thus could be omitted. Single mutation F230H appears to create a large number of positive clusters while the F230H triple mutant was the most successful. T202V was more suspenseful than T202A therefore one further double mutant F230H/T202V and a triple mutant F230H/T202V/Y168H were generated. As presented in Table 2, SMD revealed that T202V mutation on the triple mutant is more effective than the T202A decreasing the distance to the methyl group from the hydrophobic side chain and causing less torsion on the MSA. It also shows that the Y168F mutation is unnecessary and provided no additional stability to the hydrophobic nature of the pocket. From this observation the double mutant F230H/202V was recommended to increase activity of MSA. Fig. 4 shows the docking of MFA and MSA against the selected mutant and the wild type. Both mutations combined open up the right side of the pocket allowing the fitting of the methoxy group and subsequently the "correct" and catalytic binding of MSA. The distance between the catalytic serine and the carbonyl carbon is around 3.30 Å. The double mutant F230H/T202V increases the MBE to -5.50 kcal mol^{-1} and increases the number of runs in that cluster.

Table 2 Mean binding energy (MBE) and number in cluster for SMD of MFA and MSA to FoFaeC mutants and wild type. MBE is given in kcal mol^{-1} while the maximum possible elements within an individual cluster are 20.

Enzyme	MFA		MSA	
	MBE	N Clusters	MBE	Clusters
Wild type	-5.28	16	-5.03	2
F230H	-5.39	20	-5.30	13
F230Y	-5.35	17	-5.17	3
T202A	-5.27	12	-5.33	9
T202V	-5.28	16	-5.43	10
Y168F	-5.29	17	-4.99	2
F230H/T202A/Y168F	-5.40	18	-5.38	15
F230Y/T202A/Y168F	-5.27	14	-5.29	6
F230H/T202V	-5.28	19	-5.50	17
F230H/T202V/Y168H	-5.40	18	-5.51	13

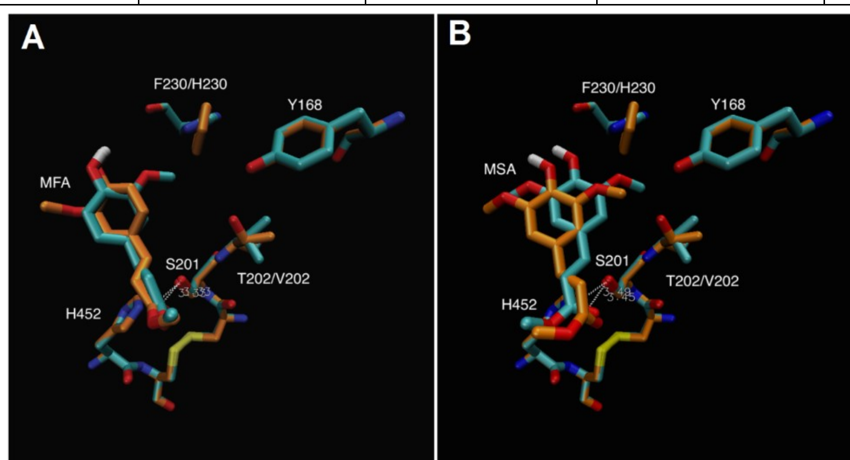

Figure 4 FoFaeC double mutant F230H/T202V (blue) compared with wild type (orange) with docked (A) MFA and (B) MSA

Screening of transformants in solid and liquid media

A synthetic gene was designed incorporating the most promising mutation (F230H/T202V) and was recombined in *P. pastoris* X33. Thirty colonies from *P. pastoris* X33 transformants were grown in micro-scale. After three days of incubation at 28°C, culture supernatants were recovered and spotted on solid media containing 4NTC-Fe. Twenty out of thirty clones were active showing activity halos (data not shown). Subsequently, the supernatants from fifteen transformants were analyzed for FAE activity in liquid medium towards *p*NP-Fe. Both assays were performed using wild-type strain as negative control and *P. pastoris* recombinant producing FoFaeC wild type as the positive sample. From these analyses, less than fifteen clones of thirty analyzed were active (data not shown). Based on these results, five transformants (P5, P12, P13, P14 and P15) were chosen to scale-up FAE production in 250 mL-flasks (Fig. 5). The cultures were incubated at 28°C for 3-5 days and after biomass removal, the supernatant was analyzed for the feruloyl esterase activity against *p*NP-Fe and MSA. The preliminary screening showed that FoFaeC wild type had activity towards *p*NP-Fe but no activity was detected towards MSA. However, the transformants showed activity for MSA while the activity towards *p*NP-Fe was more than halved. Moreover, no activity was detected in any case for non-transformed *P. pastoris* strain.

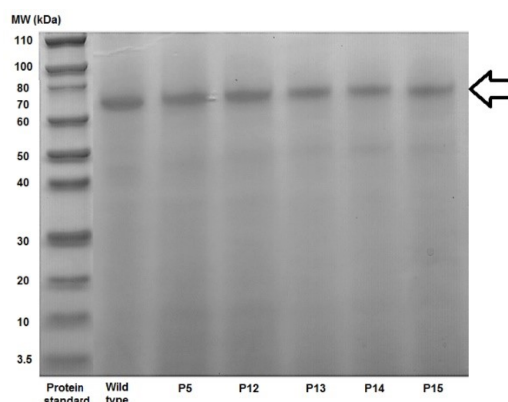

Figure 5 SDS-PAGE of mutant clones and wild type (20 µL protein load in each well)

Characterization of mutant clones and wild type

The five FoFaeC (P5, P12, P13, P14, P15) mutant clones carrying the double mutation F230H/T202V and the wild type were 4-fold concentrated and further characterized for their activity towards the four methyl esters of hydroxycinnamic acid (MFA, MSA, M*p*CA and MCA) using varying enzyme load (0-0.02 mg protein mL^{-1}). The wild type of FoFaeC showed highest activity in descending order against M*p*CA> MCA> MFA> MSA. In accordance with previous report [17], some activity towards MSA could be detected; however it was approximately 20 times lower than MFA. Validating our hypothesis, all mutant clones showed improved activity towards MSA compared with the wild type (Table 3). Mutant P13 showed highest activity towards MSA, approximately 5 times higher than the wild type, followed by mutant P15 and P12. Interestingly, the activity of mutant clones towards the other substrates was dramatically decreased but remained in the same order of magnitude with MSA. More specifically, the activity towards MFA was 5-fold decreased while towards hydroxyl substituted substrates, such as M*p*CA and MCA, was 10-fold decreased. The effect of enzyme load on the release of hydroxycinnamic acids is presented in Fig. 6.

Table 3 Relative specific activities of FoFaeC mutant clones and wild type against methyl esters of hydroxycinnamic acids (determined as U mg^{-1} FAE)

Enzyme	Relative specific activity (%)			
	MFA	MSA	MCA	M*p*CA
Wild type	54.2	2.8	85.1	100
P5	13.4	5.7	9.8	15.8
P12	17.9	7.1	15.6	15.0
P13	12.1	14.7	12.2	14.1
P14	7.9	4.7	7.4	8.2
P15	8.6	9.3	8.6	12.1

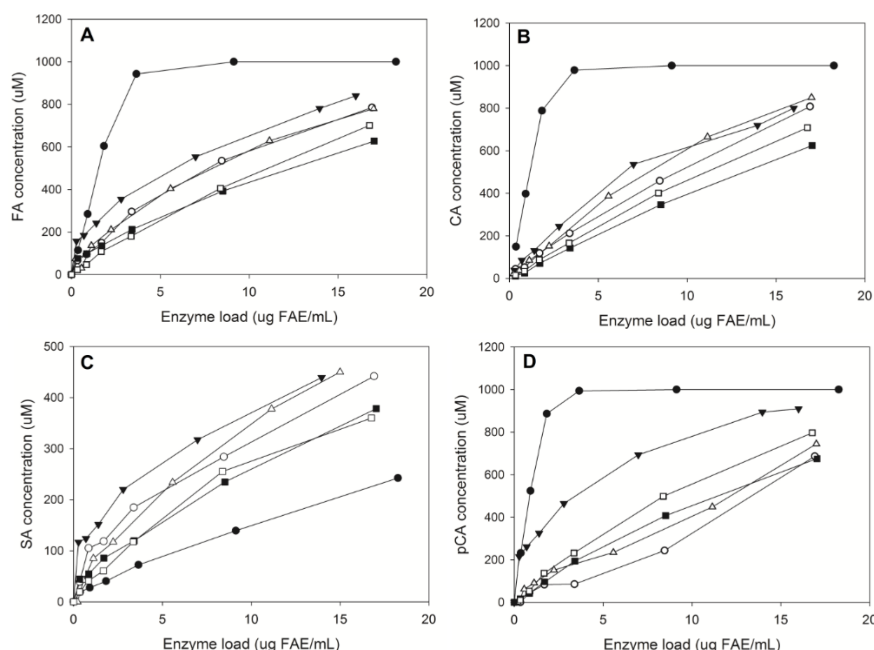

Figure 6 Effect of enzyme load on the release of hydroxycinnamic acid during hydrolysis of methyl esters (A) MFA (B) MCA (C) MSA (D) M*p*CA. Black circle: wild type; white circle: P5; black triangle: P12; white triangle: P13 black square: P14; white square: P15

Results on the effect of substrate concentration on the hydrolysis rate revealed that the FoFaeC wild type in this study has higher affinity (lower K_m) towards MFA> MSA> MCA>M*p*CA and higher turnover rate (higher k_{cat}) against M*p*CA> MCA> MFA> MSA (Table 4). Generally, all mutant clones showed improved substrate affinity against all esters compared to the wild type but, and in particular when MSA was used, the reaction rate was 1.5-fold increased. The catalytic efficiency (k_{cat}/K_m) of mutant P13 towards sinapate was 5-fold improved comparing to that of the wild type while the affinity was 2-fold increased. The effect of substrate concentration on the reaction rate is shown in Fig. 7. An explanation on the higher affinity of mutant clones towards the hydroxy substituted esters could be that the addition of histidine expands the binding pocket offering binding of substrates in the correct conformation (lower K_m). This is also predicted by the increased number of elements within a cluster for docking of MFA on the active site of mutant compared to the wild type (Table 2). However, the lower reaction rates (approximately 10-fold decrease) could be attributed to the small hydrophobic environment introduced by valine, which could be

opposing the hydroxyl group of substitution ester and resulting in a not so catalytically favorable orientation of the carbonyl carbon.

Table 4 Kinetic constants of FoFaeC mutant clones and wild type

Enzyme	K_m (mM)	v_{max} (µmol L^{-1} min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m
MFA				
Wild type	0.331 (0.102)	41.0 (3.6)	1392	4466
P5	0.141 (0.015)	7.4 (0.1)	271	1925
P12	0.276 (0.036)	7.6 (0.2)	338	1221
P13	0.155 (0.021)	7.0 (0.2)	389	2519
P14	0.153 (0.009)	5.7 (0.0)	208	1359
P15	0.133 (0.065)	6.5 (0.6)	240	1813
MSA				
Wild type	0.424 (0.063)	3.0 (0.1)	102	241
P5	0.201 (0.036)	4.9 (0.2)	180	894
P12	0.194 (0.030)	4.2 (0.1)	187	963
P13	0.189 (0.022)	4.2 (0.1)	234	1236
P14	0.245 (0.045)	3.6 (0.2)	131	535
P15	0.200 (0.047)	4.8 (0.2)	178	887
MCA				
Wild type	0.649 (0.183)	77.4 (7.4)	2628	4051
P5	0.075 (0.035)	6.9 (0.5)	253	3882
P12	0.118 (0.025)	5.9 (0.2)	262	2222
P13	0.218 (0.027)	9.3 (0.4)	517	2379
P14	0.177 (0.016)	6.1 (0.1)	222	1258
P15	0.074 (0.021)	6.1 (0.2)	226	3069
M*p*CA				
Wild type	0.799 (0.312)	96.4 (14.1)	3273	4094
P5	0.352 (0.036)	11.6 (0.3)	425	1209
P12	0.2746 (0.030)	8.1 (0.2)	360	1310
P13	0.719 (0.225)	14.6 (1.6)	812	1130
P14	1.122 (0.326)	13.3 (1.7)	484	431
P15	0.678 (0.202)	13.5 (1.4)	499	736

Numbers in parenthesis are the estimates of standard deviation

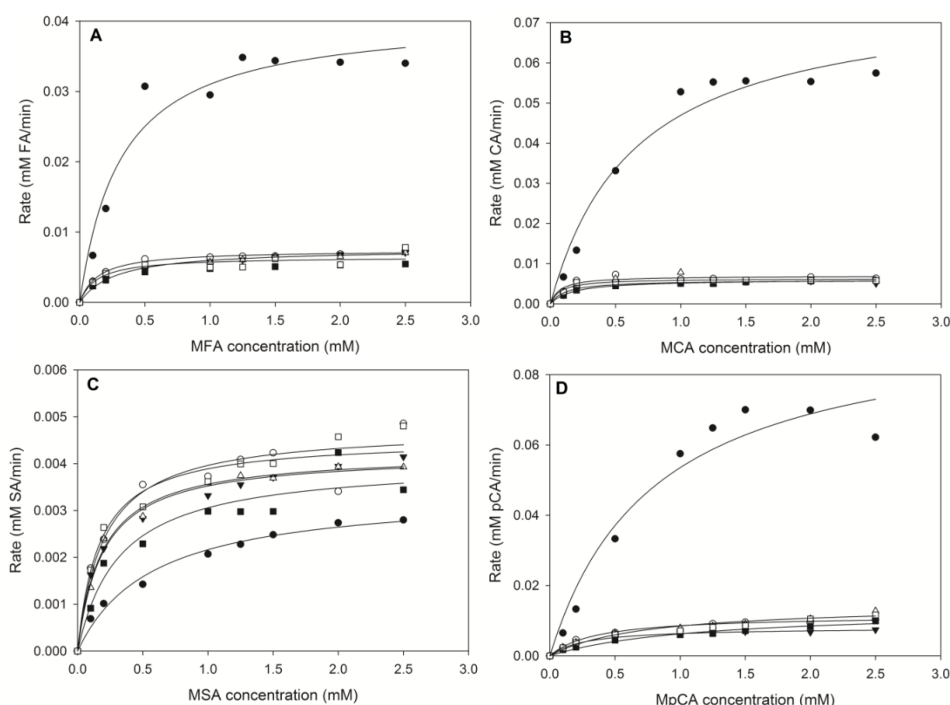

Figure 7 Effect of substrate concentration on the reaction rate during hydrolysis (A) MFA (B) MCA (C) MSA (D) M*p*CA. Black circle: wild type; white circle: P5; black triangle: P12; white triangle: P13 black square: P14; white square: P15

Conclusions

The rational redesign of the active site of type C FoFaeC provided an insight into the hydrolytic mechanisms of this enzyme and opens the way for a new approach on the exploitation of FAEs for use in novel bio catalytic processes by tailoring their specificity according to the desired reaction.

Materials and Methods

Substrates

MFA and MCA were purchased by Alfa Aesar (Karlsruhe, Germany) while MSA and M*p*CA from Apin Chemicals Ltd. (Abingdon, UK). *p*-Nitrophenyl ferulate (*p*NP-Fe) and 4-nitrocatechol-1-yl ferulate (4NTC-Fe) were provided by Taros Chemicals GmbH & Co. KG (Dortmund, Germany).

Small molecule docking simulations

Small molecule docking (SMD) and *in silico* mutational techniques were used to suggest possible mutations that would increase the activity of FoFaeC from *F. oxysporum* on MSA. SMD simulations of ligands were performed using Autodock [24] on the structure of FoFaeC (to be submitted to PDB). The pdb file was cleaned from water molecules and then converted to a pdbqt involving the addition of polar hydrogen and atom chargers. A grid box was generated around the active site of the enzyme at a spacing of 3 Å and large enough to cover the active site. A standard docking parameter file for each ligand was used for docking. Genetic algorithm was used with 20 runs and a maximum evaluation value of 25000000. Results were visualized using Autodock Tools. Homology models of mutants were generated using SWISS-MODEL [25] using the original FoFaeC structure as template for the construction of hybrid models. All acquired models passed both GMQE and QMEAN4 evaluation tests.

Strains, vectors and media

Escherichia coli TOP10F' strain was used for the amplification of the expression construct pPICZαC/FoFaecMut (Eurofins Genomics, Luxembourg) and the transformants were selected on Low Salt Luria-Bertani medium (1% Tryptone; 0.5% Yeast Extract; 0.5% NaCl

pH 7.5) by Zeocin™ resistance (25 μg mL^{-1}). The resistant transformants were grown overnight at 37°C under shaking and plasmid DNA was isolated by the Plasmid DNA Extraction Mini Prep Kit (Fisher Molecular Biology, Rome, Italy). The recombinant plasmids pPICZαC/FoFaecMut were linearized with SacI restriction enzyme (NEB, Ipswich, MA, USA) to transform *Pichia pastoris* X-33 (Invitrogen, Carlsbad, CA, USA). The transformation of yeast was performed with 5 μg pure recombinant vector by Electroporation protocol according to the EasySelect™ *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA). *P. pastoris* transformants were selected on YPDS agar (1% w/v yeast extract; 2% w/v peptone; 2% w/v dextrose; 1 M sorbitol; 2% w/v agar) containing Zeocin™ at final concentration of 100 μg mL^{-1}at 28°C. Selected transformants were grown in BMGY and BMMY (1% w/v yeast extract; 2% w/v peptone; 100 mM potassium phosphate, pH 6.0; 1.34% w/v YNB; 4 × 10-5% w/v biotin; 1% v/v glycerol or 0.5% v/v methanol) at 28°C.

Screening of FAE (+) by solid and liquid assays at micro-scale

Thirty colonies were inoculated in 900 μL of BMGY at micro-scale. After incubation at 28°C for 20 h, 1 OD mL^{-1} of pre-cultures were inoculated in 1 mL of BMMY medium following incubation for 3 days at 28°C and 700 rpm. Cultures were centrifuged (2500 g, 30 min) and the supernatant from each transformant was transferred to OmniTrays containing 75 μg mL^{-1} of 4NTC-Fe (0.2% v/v stock in DMSO), 50 mM sodium phosphate buffer pH 6.8, 1% w/v agarose and 0.5 mM ammonium iron citrate, necessary for the production of halos, following incubation at 37°C [26]. The supernatants of each transformant found positive in the solid screening assay were analyzed for FAE activity towards *p*NP-Fe according to [27], modifying the reaction volume and incubation time at 37°C for 60 min, and for activity towards MSA at 37°C for 15 min in 100 mM MOPS-NaOH pH 6.0 at a final volume of 1 mL.

Production of FAE recombinant clones

Enzyme production was performed in 250 mL flasks with 50 mL of induction medium (BMMY). The cultures were kept in a shaking incubator (180 rpm) at 28°C for 3-5 days with the addition of 0.5% v/v methanol once a day to maintain induction. Cultures were centrifuged (2500 g, 30 min) and the supernatant was collected and concentrated. The amount of protein production was detected by the Bradford method (Sigma, Saint-Louis, USA) and the homogeneity was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Blue. The FAE content (% w/w) was estimated by SDS-PAGE and subsequent quantification was done by a densitometric method using JustTLC software (Sweday, Sweden).

Characterization of FoFaeC mutant clones and wild type

Characterization experiments took place in a 2 mL Eppendorf thermomixer (Eppendorf, Hamburg, Germany). For the assessment of hydrolytic activity, a stock solution of substrate (50 mM; MFA, MSA, MCA and M*p*CA) was prepared in dimethyl sulfoxide (DMSO). The activity was assayed using 1 mM substrate in 100 mM MOPS-NaOH, pH 6.0 for 15 min at 37°C without agitation varying the enzyme load (0-0.02 mg protein mL^{-1}). One unit (1 U) is defined as the amount of enzyme (mg) releasing 1 μmol of hydroxycinnamic acid per minute under the defined conditions. The effect of substrate concentration on the reaction rate was assessed by incubation of enzyme at varying concentration of substrate (0-2.5 mM) in 100 mM MOPS-NaOH pH 6.0 for 15 min at 37°C. The kinetic constants (v_{max}, K_m) were determined by fitting the Michaelis-Menten equation on the data using non-linear regression ($p<0.0001$). Reactions were ended by incubating the reaction mixtures at 100°C for 5-10 min. All assays were carried out in duplicate at a final volume of 1 mL and were accompanied by appropriate blanks containing buffer instead of enzyme. There was no hydrolysis observed in the absence of esterase.

Quantitative analysis of hydroxycinnamates

Analysis was performed by HPLC on a 100-5 C18 Nucleosil column (250 x 4.6 mm) (Macherey Nagel, Düren, Germany). Elution was done with 7:3 v/v acetonitrile: water for 10 min at a flow rate of 0.6 mL min^{-1} and room temperature. Absorbance was measured at 300 nm with a PerkinElmer Flexar UV/Vis detector (Waltham, USA). Retention times for

hydrolyzates (FA, CA, SA, *p*CA) and substrates (MFA, MCA, MSA, M*p*CA) were 4.07-4.21 and 5.20-6.21 min, respectively. Calibration curves were prepared using standard solutions of hydroxycinnamates in acetonitrile (0.1-2 mM).

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