

Characterization and Engineering of Basidiomycota-derived Lipases: Structural Insights and Optimization of hydrolytic Selectivity and Stability

Cumulative Dissertation

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Abstract

In the production of piquant cheese varieties, such as Feta and Provolone, pregastric esterases (PGEs) are commonly used to generate characteristic piquant flavor profiles. These enzymes hydrolyze triglycerides present in milk fat during cheese ripening, releasing volatile free fatty acids that contribute to the distinctive aroma of these cheeses. These enzymes are traditionally derived from animal origin, and their use conflicts with the requirements for foods labeled as vegetarian, kosher, or halal. This interference raises the demand for enzymes with similar activity but of different origin. A lipase from the basidiomycete *Pleurotus citrinopileatus* (PCI_Lip) has been identified as a promising fungal substitute. To further adapt its catalytic properties to those of PGEs, a semi-rational protein engineering approach was applied to modify the chain length specificity for cheese production.

Key residues influencing substrate specificity were identified *in silico* based on an AlphaFold3 model of PCI_Lip. In three rounds of mutagenesis, a small but smart library was generated. The library was screened for mutants with increased release of short to medium chain fatty acids and reduced release of long chain fatty acids. Alteration of substrate specificity was proven by changes in kinetic values compared to the wild type (WT) enzyme. Mutants with improved hydrolysis profiles were used for cheese production. The analysis of volatile free fatty acids and sensory evaluation showed that some mutants are suitable substitutes for PGEs.

For a comprehensive characterization, stability, effects of immobilization, and regioselectivity of PCI_Lip were examined. The lipase was found to be highly thermostable with the potential to further increase its stability through suitable buffers and additives, as well as by immobilization. In the hydrolysis of triglycerides, the enzyme prefers the outer ester positions, therefore being characterized as an sn 1,3-selective lipase.

Investigations on further Basidiomycota-derived lipases gave deeper insights into the structure and function of these enzymes. The characterization of a *Pleurotus sapidus* lipase (PSA_Lip) showed its thermostability and alkali tolerance. Besides activity on fatty acid esters, PSA_Lip was also active on ferulic acid esters. Sequence alignments with other hydrolases revealed that it does not belong to the big group of the α/β -hydrolases but displays a unique SGNH-like motif in the active site of the enzyme. The comparison of PCI_Lip with a lipase from *Phlebia centrifuga* (PCE_Lip) emphasized the role of the lid domain. A lack of this domain could be connected to reduced solubility and activity.

The present work provides a comprehensive characterization of PCI_Lip and demonstrates strategies to optimize its catalytic properties and stability for various industrial applications. Through a combination of structural analysis, protein engineering, and immobilization techniques, the enzyme can be adapted to different parameters such as substrate selectivity

and thermostability, making it applicable in various biotechnological applications. Comparative results with other Basidiomycota-derived lipases contribute to a better understanding of fungal hydrolases.

Zusammenfassung

In der Herstellung pikanter Käsesorten, wie Feta oder Provolone, werden traditionell prägastrische Esterasen (PGEs) eingesetzt, um das komplexe und pikante Aromaprofil dieser Sorten zu erzielen. Diese Enzyme setzen während des Reifeprozesses volatile Fettsäuren aus Triglyceriden im Milchfett frei, welche zu dem spezifischen Käsearoma beitragen. Traditionell werden PGEs aus tierischen Quellen gewonnen. Dies steht jedoch im Konflikt mit Anforderungen an vegetarische, koschere oder halal-konforme Ernährungsweisen. Dieser Zwiespalt fordert eine Alternative mit ähnlichen Eigenschaften, aber anderer Herkunft. In dieser Arbeit wurde eine Lipase aus dem Basidiomyceten *Pleurotus citrinopileatus* als vielversprechender Ersatz identifiziert. Um deren katalytische Eigenschaften weiter an diejenigen von PGEs anzupassen, wurde ein semi-rationaler Protein engineering Ansatz zur Anpassung der Kettenlängenspezifität gewählt.

Basierend auf einem AlphaFold3-Modell wurden Schlüsselreste, welche die Kettenlängenspezifität beeinflussen, *in silico* identifiziert. In drei Mutageneserunden wurde eine kleine, aber intelligente Bibliothek erstellt. Die Bibliothek wurde nach Varianten mit effizienter Hydrolyse kurz- bis mittelkettiger Fettsäuren und verringerter Freisetzung langkettiger Fettsäuren gefiltert. Verschiebungen der Substratspezifität wurden durch Änderungen kinetischer Parameter im Vergleich zum Wildtyp-Enzym (WT) belegt. Varianten mit verbessertem Hydrolyseprofil wurden in der Käseherstellung eingesetzt. Analysen volatiler freier Fettsäuren und sensorische Beurteilungen zeigten, dass einzelne Varianten als adäquater Ersatz für PGEs verwendet werden können.

Für eine umfassende Charakterisierung von PCI_Lip wurden deren Stabilität, Auswirkungen von Immobilisierung und Regioselektivität untersucht. Die Lipase zeigte eine hohe Thermostabilität, welche durch geeignete Puffer, Additive und Immobilisierung weiter erhöht werden konnte. Bei der Hydrolyse von Triglyceriden bevorzugt das Enzym die äußeren Esterbindungen und kann daher als sn-1,3-selektive Lipase eingestuft werden.

Untersuchungen weiterer Lipasen aus Basidiomycota ergaben tieferen Aufschluss über Struktur und Funktion dieser Enzyme. Die Charakterisierung einer Lipase aus *Pleurotus sapidus* (PSA_Lip) zeigte deren Thermostabilität und Toleranz gegenüber alkalischen pH-Werten. Neben der Aktivität gegenüber Fettsäuren zeigte diese auch Aktivität gegenüber Ferulasäure-Derivaten. Der Sequenzvergleich mit anderen Hydrolasen enthüllte, dass diese nicht zur großen Gruppe der α/β -Hydrolasen gehört, sondern ein besonderes SGNH-Motiv im aktiven Zentrum aufweist. Der Vergleich von PCI_Lip mit einer Lipase aus *Phlebia centrifuga* (PCE_Lip) unterstrich die Rolle der Liddomäne in PCI_Lip, welche den Substratkanal vom

Medium abschirmt. Das Fehlen dieser Domäne konnte mit reduzierter Löslichkeit und Aktivität in Verbindung gebracht werden.

Die vorliegende Arbeit stellt eine umfassende Charakterisierung von PCI_Lip dar und zeigt Strategien auf, deren katalytische Eigenschaften und Stabilität für unterschiedliche industrielle Anwendungen zu optimieren. Durch die Kombination von Strukturanalysen, Protein engineering und Immobilisierung kann das Enzym an unterschiedliche Parameter angepasst werden, wodurch es Anwendung in verschiedenen biotechnologischen Verfahren finden kann. Vergleichende Ergebnisse mit anderen Lipasen aus Basidiomycota tragen zu einem besseren Verständnis pilzlicher Hydrolasen bei.

List of publications

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List of abbreviations

AI	artificial intelligence
CALA/CALB	<i>Candida antarctica</i> Lipase A/B
CAZymes	carbohydrate active enzymes
DE	directed evolution
DG	diglyceride
EMC	enzyme modified cheese
(ep)PCR	(error prone) polymerase chain reaction
FAME	fatty acid methyl esters
GH	glycosyl hydrolase
GLOX	glyoxal oxidase
GMC	glucose-methanol-choline
GRAS	Generally Recognized as Safe
HTP	high throughput
IDP	intrinsically disordered protein
ITCHY	Incremental Truncation for the creation of hybrid enzymes
LC-HR-ESI-MS/MS	liquid chromatography high resolution tandem mass spectrometry
LiP	lignin peroxidase
LPMO	lytic polysaccharide monooxygenase
MG	monoglyceride
ML	machine learning
MnP	manganese peroxidase
MSA	multiple sequence alignment
PCE_Lip	<i>Phlebia centrifuga</i> lipase
PCI_Lip	<i>Pleurotus citrinopileatus</i> lipase
PE	protein engineering
PET	poly(ethylene terephthalate)
PGE	pregastric esterase
PLM	protein language model
pNP	<i>para</i> -nitrophenol
pNPA	<i>para</i> -nitrophenyl acetate
pNPO	<i>para</i> -nitrophenyl octanoate
pNPP	<i>para</i> -nitrophenyl palmitate
PSA_Lip	<i>Pleurotus sapidus</i> lipase

QPS	Qualified Presumption of Safety
RACHITT	random chimeragenesis of transient templates
RD	rational design
ROS	reactive oxygen species
SDS-PAGE	sodium dodecylsulfate - polyacrylamide gel electrophoresis
SHIPREC	sequence homology independent protein recombination
SISDC	sequence independent site directed chimeragenesis
SRD	semi-rational design
SSM	site saturation mutagenesis
TG	triglyceride
(v)FFA	(volatile) free fatty acid
VP	versatile peroxidase
WT	wild type

Introduction

Basidiomycota

In the phylogenetic tree, fungi represent one of four kingdoms in the domain of eukaryotes. Species within this very diverse branch populate any ecosystem on our planet. The number of species within the kingdom of fungi is still discussed in the literature. Estimations reach from 1.5 to 5.1 or even up to 12 million species belonging to the kingdom of fungi (Bhunjun et al., 2022, Blackwell, 2011, Hawksworth, Lücking, 2017, Li et al., 2021, Wu et al., 2019). The vast range arises from different extrapolation techniques to estimate the number of species. More recent DNA sequencing techniques further boosted these estimations. Extrapolations are not only made based on a molecular level, but also on the publication rate of new taxa, plant-to-fungus ratios, and ecological approaches, which result in a range of nearly one order of magnitude (Hawksworth, Lücking, 2017). This huge kingdom is grouped into six subkingdoms, with the Dikarya being the most abundant one (James, Rokas, 2025). Dikarya are divided into the two phyla of Ascomycota and Basidiomycota. Both phyla share a filamentous network, also referred to as dikaryotic hyphae (Cittadino et al., 2023). Species in the phylum of Basidiomycota are able to form the eponymous basidia during the sexual stage of their life cycle. Each basidium can produce four haploid basidiospores by meiosis (Berger, Ersoy, 2022, He et al., 2019).

Currently, over 40000 species of Basidiomycota are known. These can be categorized into four subphyla, namely Agaricomycotina, Pucciniomycotina, Ustilaginomycotina, and Wallemiomycotina (He et al., 2019, He et al., 2022). Further characteristics that differentiate Basidiomycota from Ascomycota are a guanidine-cytosine content of over 50% in their DNA, the ability to form ureases, and a deviant ubiquinone system (He et al., 2019). Of the four subphyla, Wallemiomycotina are the smallest group, consisting of xerophilic microfungi (He et al., 2024). Species belonging to the Pucciniomycotina, also known as rust fungi, are mostly plant but also insect-associated pathogens. This subphylum holds species with the biggest genomes among fungi, comparable in base pair and gene number to the human genome. Simultaneously, one of the smallest genomes of filamentous fungi is also assigned to this subphylum (Berger, Ersoy, 2022, Spatafora et al., 2017). Ustilaginomycotina, the smut fungi, are plant pathogens with rather small genomes compared to other Basidiomycota (Spatafora et al., 2017). The subphylum of Agaricomycotina represents the most species, with roughly two-thirds grouped here. It is divided into four classes, with the Agaricomycetes being the most prominent one. This class holds 22 orders and, up to now, more than 21000 species. A characteristic of this class is the formation of fruiting bodies in a variety of

shapes, often called mushrooms (Berger, Ersoy, 2022, Naranjo-Ortiz, Gabaldón, 2019, Spatafora et al., 2017). About 1000 species in this class are edible and internationally valued for their nutritional value and aroma (Berger, Ersoy, 2022). These fungi have conquered many different ecological niches due to their metabolic flexibility. While being able to adapt to various carbon and nitrogen sources, they produce a huge variety of biomolecules. Some of them are highly sought-after in the pharmaceutical and fragrance industries today. Their unique metabolism enables them to use wood as a source of nutrients. Wood decay is performed by two types of fungi. Hemicellulose and lignin are preferentially degraded by white-rot fungi. Besides Agaricomycetes, this group also comprises some Ascomycetes. Cellulose and hemicellulose are preferentially degraded by brown-rot fungi, which consist only of Basidiomycota (Berger, Ersoy, 2022, Bouws et al., 2008, Riley et al., 2014).

The genus *Pleurotus* ssp. belongs to the group of white-rot fungi, featuring a vast set of extracellular oxidoreductases to degrade their lignocellulosic substrate (İnci et al., 2023, Parmar et al., 2024). One of these species is *Pleurotus citrinopileatus*, the golden oyster mushroom. The fruiting body is funnel-shaped with a rather fragile stipe and a yellow pileus (Chen et al., 2025, Rosnina et al., 2016). The edible mushroom originates from East Asia and is cultivated worldwide. However, on a commercial scale, it is mainly cultivated in India, China, Korea, and Japan (Chen et al., 2025, İnci et al., 2023, Rosnina et al., 2016). In nature, this fungus grows on dead wood, but recent studies have shown that it can grow on different lignocellulosic substrates, e.g., food side streams as well (İnci et al., 2023). The aroma of the fresh fruiting body is ascribed to the typically mushroom-like flavors induced by oxylipins such as oct-1-en-3-ol, oct-1-en-3-one, and octan-3-on (Chen et al., 2025, Liu et al., 2023). However, when the fruiting body is cooked crispy, it develops a cashew-like flavor (Rosnina et al., 2016). Besides its nutritional values as being high in protein and fiber, *P. citrinopileatus* is rich in bioactive substances with potentially positive physiological effects. The current focus of research is the identification of various polysaccharides produced by this fungus, which may have antitumor, anti-inflammatory, and anti-obesity effects (Diamantis et al., 2024, Qian et al., 2025, Tao et al., 2025, Wang et al., 2021).

Enzymes from Basidiomycota

The evolutionary success of Basidiomycota is attributed to their ability to adapt to different ecological conditions. Their ability to adapt their metabolism to different substrates is one factor in the evolutionary success of Basidiomycota. Another driving force is the possibility to degrade complex and persistent biopolymers empowered by their large set of extracellular enzymes, which can be referred to as the secretome (Bouws et al., 2008). Of special interest and the most frequent topic in research on Basidiomycota-derived enzymes is their ability to degrade the major components of wood. This complex lignocellulosic biomass requires particular enzymes for valorization (McGregor et al., 2022). It consists of partly crystalline cellulose (30-50%) being β -1,4-glycosidic linked β -D-glucose units, hemicellulose (15-40%), a complex mixture of β -xylans, β -mannans, and β -glucans, and lignin (10-30%), a heteropolymer out of cross-linked polyphenols (Arntzen et al., 2020, McGregor et al., 2022, Shankar et al., 2022).

For the degradation of lignin, these fungi upregulate the production of oxidoreductases (Arntzen et al., 2020). White-rot fungi secrete oxidative enzymes such as phenol oxidases to oxidize and degrade lignin. Oxidative enzymes for lignin degradation are grouped in class II peroxidases. The most important representatives are lignin peroxidases (LiP; EC 1.11.1.14), versatile peroxidases (VP; EC 1.11.1.16), and manganese peroxidases (MnP; EC 1.11.1.13). All three enzyme groups are heme-peroxidases. MnP holds a Mn^{2+} -ion in the active center, whereas LiP and VP utilize tryptophane radicals in their active centers. Another important class of enzymes for lignin degradation are laccases (EC 1.10.3.2). These copper-containing enzymes catalyze the oxidation of phenolic compounds (Alfaro et al., 2014, Mattila et al., 2022). To perform extracellular redox reactions, reactive oxygen species (ROS) are required. Hydrogen peroxide and superoxide ions serve as electron acceptors under acidic conditions. For the extracellular supply of ROS, white-rot fungi secrete glucose-methanol-choline (GMC) oxidoreductases and glyoxal oxidases (GLOX) (Mattila et al., 2022). Lytic polysaccharide monooxygenases (LPMO) act on polysaccharides by the oxidative cleavage of glycosidic bonds. Therefore, the active site Cu^{2+} is reduced to Cu^{+} by a reductant such as gallic acid. With the help of ROS, the enzyme can perform an oxidative cleavage of glycosidic bonds (Qin et al., 2023). Current research in the field of secretomes from white-rot fungi mainly targets the regulation of enzymes responsible for lignocellulosic degradation. Even though the extent depends on the fungal species and substrate, there seems to be a co-occurrence of H_2O_2 -producing and consuming enzymes in white-rot species. For the genus *Pycnoporus*, an enzymatic synergy of LPMO and cellobiose dehydrogenase was proven. The oxidative enzymes targeted lignin and crystalline cellulose structures, and the dehydrogenase was active on the polysaccharide

structures (Miyauchi et al., 2020). Nevertheless, lignin degradation within the group of white-rot fungi can follow different strategies. Alfaro et al. found that *Phanerochaete chrysosporium* contained LiP and MnP, but did not produce phenol oxidases. For lignin degradation, this fungus mainly secreted glycosyl hydrolases (GH) and a lower number of oxidoreductases. Based on these findings, the authors pointed out that enzyme production and secretion are influenced by environmental conditions and substrate (Alfaro et al., 2014).

The group of brown-rot fungi mainly decomposes hemicellulose and cellulose to satisfy their nutrient demand. White and brown-rot fungi are mostly distinguished by the absence or strongly reduced number of class II peroxidases and the presence of cellulose-degrading enzymes (Figueiredo et al., 2021). Brown-rot fungi utilize an oxidative and hydrolytic mechanism to degrade the partially crystalline substrate. The extracellular oxidative mechanism is not solely enzyme-driven and therefore not selective. In the Fenton reaction, a hydroxyl radical is generated by the oxidation of a Fe^{2+} -ion and the use of H_2O_2 . The supply of H_2O_2 is covered by extracellular GMC oxidases and GLOX (Mattila et al., 2022). The hydrolytic part of degradation is covered by carbohydrate active enzymes (CAZymes), which are responsible for the degradation and modification of oligo- and polysaccharides (Shankar et al., 2022). As the oxidative mechanism is non-selective, it cannot proceed simultaneously with the hydrolytic mechanism. Therefore, most brown-rot species developed a staggered mechanism, where the organisms primarily excrete enzymes for the oxidative mechanism at the beginning of the degradation and, in later stages, the secretion of hydrolytic enzymes increases (Mattila et al., 2022, Presley, Schilling, 2017). The group of CAZymes is composed of a plethora of enzymes that can be divided into cellulases and hemicellulases, depending on the substrate on which they are active. Many CAZymes are classified as hydrolases (EC 3) (Alfaro et al., 2014, Metreveli et al., 2021). The production of CAZymes was shown to be inducible, with the best results when crystalline cellulose was used as the only carbon source. Metreveli and co-workers proved that substrates rich in cellulose induced the production of cellulases and xylanases in *Irpex lacteus*. However, the availability of easily accessible carbon sources, such as saccharose, suppressed the expression of these enzymes. The authors postulated that both types of enzyme are regulated under a common control mechanism (Metreveli et al., 2021). A staggered mechanism was also found in the two brown-rot fungi *Serpula lacrymans* and *Gloeophyllum trabeum*. The hydrolytic part of the degradation strongly distinguishes both fungi, as *S. lacrymans* displayed high mannanase activity, while *G. trabeum* displayed major xylanase activity. The differences in their secretomes were explained by the different habitats of these species, where one prefers to grow on soft wood, the other on hard wood. Also, the amount of secreted proteins differs between both species, which was attributed to different growth rates and translocation efficiencies, describing the

ability of a fungus to transport nutrients through its mycelium (Presley, Schilling, 2017). Biochemical variations in the group of brown-rot fungi can be found in many of these species. Figueiredo and co-workers described a *Laetiporus sulphureus* strain that, besides cellobiohydrolases, which are typical for brown-rot fungi, also expressed class II peroxidases. Furthermore, this study pointed out that depending on the type of analysis, the secretome of *L. sulphureus* can be evaluated differently. Transcriptomic data showed that mainly oxidative enzymes were upregulated, while the secretome displayed mainly hydrolytic enzymes (Figueiredo et al., 2021). The described fungal metabolic pathways and enzymes are highly interesting for industrial applications, as the valorization of wooden biomass otherwise requires elaborate and costly pretreatments. Research on fungal wood degradation will further improve commercial enzyme preparations for the saccharification of polysaccharides (Alfaro et al., 2014, Qin et al., 2023).

The high metabolic flexibility and production of various small molecules by Basidiomycota make them an interesting source for novel enzymes (Berger, Ersoy, 2022, Bouws et al., 2008). Tyrosinases (EC 1.14.18.1) catalyze the hydroxylation of mono- and diphenols to quinones. In fungi, they are involved in the synthesis of melanin and show a broad substrate spectrum. Due to its high industrial potential, a tyrosinase from *Phlebia brevispora* was characterized by Schröder et. al. This species offered high production and secretion levels under optimized conditions (Schröder et al., 2025). As the depletion of oxalate favors nutrient availability, Basidiomycota-derived oxalate oxidases (EC 1.2.3.4) are a rising topic in Basidiomycota research. Oxalate oxidase activity was proven in 23 Basidiomycota species. Here, the enzymes were involved in lignocellulose degradation. This study showed that oxalate metabolism can be facilitated via different metabolic routes, as it can be metabolized by oxalate oxidases or oxalate decarboxylases (Grąz et al., 2022). Another group of enzymes derived from Basidiomycota are terpene synthases, mostly belonging to the enzyme class of carbon-oxygen lyases, which act on phosphates (EC 4.2.3.X). Terpenes and terpenoids present the largest group of secondary fungal metabolites, and especially their broad structural and functional diversity places them in the focus of current research. In a study of Hoberg and coworkers, a bifunctional terpene synthase was identified in *Cyclocybe aegerita*, capable of synthesizing sesqui- and diterpenes (Hoberg et al., 2024). Lipolytic enzymes can also be produced by Basidiomycota. With the rising demand for environmentally friendly processes, Basidiomycota become a promising source for lipolytic enzymes with new functionalities and improved stability in biotechnology (Berger, Ersoy, 2022).

Basidiomycota-derived lipases

Lipases (EC 3.1.1.3) are defined as carboxylesterases that act on long-chain triglycerides (TG). They typically display an α/β -hydrolase fold and belong to the family of serine hydrolases. This class can be subdivided into three classes according to their oxyanion pattern (Kumar et al., 2023, Mahfoudhi et al., 2022, Verger, 1997). There are many biotechnological applications for lipases, making these enzymes interesting for industry and research. Application of lipases happens in food, detergent, cosmetic, and pharmaceutical industries, including the production of tailored lipids, the generation of flavor compounds, and transesterification (Berger, Ersoy, 2022, Mahfoudhi et al., 2022, Robles-Machuca et al., 2025). For biotechnical applications, mainly microbial lipases are utilized. The large-scale production of microbial lipases is superior to plant or animal-derived sources, as the microbial production is faster and cheaper (Cesário et al., 2021). Furthermore, the utilization of fungal lipases is advantageous over the use of bacterial ones. Fungi produce extracellular enzymes, including lipases. This makes them easier to extract and to purify, also reducing their cost (Kumar et al., 2023).

Most industrially applied lipases originate from fungi belonging to the phylum of Ascomycota or yeasts. Still, investigations on basidiomycetous lipases are scarce in the literature. Some reports conjecture that this is the case because lipases from the Basidiomycete *Moesziomyces antarcticus* (formerly *Candida antarctica*) already fulfill most of industrial needs up to now (Berger, Ersoy, 2022, Mahfoudhi et al., 2022, Vishnupriya et al., 2026). Lipases A and B from this species (CALA, CALB) are among the most intensely studied and utilized lipases in research and industry. Their popularity is attributed to their exceptional catalytic versatility, enantio- and regioselectivity, and the broad reaction spectrum they cover (Robles-Machuca et al., 2025, Vishnupriya et al., 2026). This model lipase has been used in protein engineering to optimize diverse attributes ranging from catalytic capabilities to selectivity to stability. Immobilized CALB is marketed under the trade name Novozyme 435 (Vishnupriya et al., 2026). This enzyme also plays a major role in biodiesel synthesis, which is currently the most trending field of application for fungal lipases. Biodiesel as a renewable fuel is generated by the esterification of long-chain fatty acids with mostly linear short-chain alcohols. Alkaline catalysis is the most important reaction for biodiesel production. Enzymatic catalysis of the transesterification is the more desirable one, as fewer side products are produced, less waste is generated, and the process can be conducted under mild conditions. However, the present market cost of the required biocatalysts is the limiting factor in large-scale production (Puppe et al., 2015). As mentioned above, there is still a limited number of reports on lipases from Basidiomycota, except for *C. antarctica* lipases. It is to mention that in some of them, there is no specific differentiation between carboxylesterase and lipase activity. Roughly, these reports can be split into two categories.

The larger group of reports attests lipase activity by screening several Basidiomycota, and the second group characterizes a distinct lipase from a basidiomycetous species. Mondego et al. investigated the genome of *Moniliophthora perniciosa*, a plant pathogenic basidiomycete. The identified 41 enzymes belong to the carboxylesterase/lipase family. Strikingly, the amount of these enzymes is twice as high as in most other Basidiomycota. The authors propose that this is due to the hemibiotrophic lifestyle of this species, as these enzymes are induced during cell wall degradation but also in the phase of carbon and nitrogen starvation (Mondego et al., 2008). In a screening of fifty Basidiomycota species from South India, Goud et al. used a plate screening technique to identify lipase activity in the tested species. Seventeen Basidiomycota exhibited lipase activity, with *Gomophus clavitus* and *Trametes pubescens* being the best lipase producers (Goud et al., 2009).

Besides lipase activity in the order of Agaricomycetes, which is the most frequently researched group of fungi concerning enzyme activity, the literature is poor on lipolytic activity of Basidiomycetes from other subphyla. The secretomes of twelve smut fungi belonging to the order of Ustilaginales were compared. The investigated smut fungi were all biotrophic plant pathogens. All of them only had a small number of cell wall degrading enzymes, which was constituted by the need to keep their hosts alive. In this investigation, *Microtyrum lychnidis-dioicae* was identified to secrete a large number of lipases, an attribute missing in the other investigated fungi. The high secretion level of lipases and also the presence of unique putatively cell wall degrading enzymes are attributed to a peculiar lifestyle, where it replaces the pollen of infected plants with its own spores (Schuster et al., 2018). With the screening of cholinesterase activity in 45 Basidiomycota, their lipase activity was also examined. Cholinesterases and lipases belong to the group of carboxylesterases (EC 3.1.1.X) and share the conserved Ser-Glu-His catalytic triad. Fungi were investigated with the intention of finding cholinesterase activity, because it has neuronal functions in vertebrates. While all of the investigated 45 Basidiomycota exhibited lipase activity, cholinesterase activity was observed in ten of them. Moreover, the lipase activity did not correlate with the cholinesterase activity (Sepčić et al., 2019). For a screening of lipase activity, 64 wild Basidiomycota species were collected from the Slovenian forests. Lipase activity was observed in thirty species, of which this activity was described for 24 for the first time. The highest lipolytic activities were observed in *Flammulina velutipes*, *Daedaleopsis confragosa*, *Cuphophyllus fornicates*, *Polyporus ciliates*, and *Lactarius torminosus* (Popošek et al., 2025).

The characterization of enzymatic properties from particular Basidiomycota-derived lipases started off in 2005 with a lipase from *Pleurotus sapidus* that was investigated for xanthophyll ester

hydrolyzing activity. The culture broth of this fungus was tested for lipolytic activity over the cultivation period. On the day of highest lipolytic activity, the supernatant was subjected to ion exchange chromatography and subsequently to size exclusion chromatography. After gaining information on the primary sequence by Edman sequencing, genomic data were evaluated, and the gene of this particular carboxylesterase from *P. sapidus* was identified. The bioinformatic comparison of the identified lipase to already described lipases, such as *Candida rugosa* lipase, gave further structural information about the enzyme (Zorn et al., 2005). Later on, the cDNA coding for this lipase was transformed into *Escherichia coli* for heterologous expression. Only parts of the enzyme were expressed in soluble form, while the rest remained in the periplasmic space. The recombinant lipase could be purified via a His₆-tag. Lipolytic activity was measured using *p*-nitrophenyl (pNP) palmitate as substrate, resulting in a specific activity of 30 mU · mg⁻¹ (Krügener et al., 2009, Zelena et al., 2009). An extracellular lipase from the Basidiomycete *Antrodia cinnamomea* was purified from the culture supernatant. The purified enzyme exhibited a specific activity of 187 U · mg⁻¹. Closer characterization revealed that the enzyme had a molecular mass of approximately 60 kDa, being alkali-tolerant and stable in activity in a range of 25 to 60 °C (Shu et al., 2006). Later on, a lipase in the secretome of the basidiomycete *Bjerkandera adusta* was partially purified from the culture broth. The lipase was used in free and immobilized form to investigate optimum operation parameters. Both variants were highly thermostable, exhibiting activity up to 60 °C. Free lipase showed maximum activity at 45 °C, and the immobilized variant had a temperature optimum at 50 °C, while both were most active at pH 7.0. An important feature of the free lipase was its superior stability in *n*-hexane compared to aqueous solutions. This makes the enzyme interesting for industrial applications that often require organic solvents (Bancerz, Ginalska, 2007). *Coprinopsis cinerea* represents a model organism within fungi. In the cDNA of this fungus, an extracellular carbohydrate esterase encoding sequence was identified, which was associated with hemicellulose degradation of *C. cinerea*. For closer characterization of this enzyme, the 996 bp long gene was transformed into *Komagatella phaffii* for heterologous expression. The enzyme was predicted to have a molecular mass of 33.5 kDa, whereas sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) suggested a molecular mass of 46.4 kDa. The authors of this study concluded that the enzyme contained posttranslational *N*- and *O*-glycosylations. The activity of the recombinant enzyme against methyl esters of ferulic, *p*-coumaric, and caffeic acids, α -naphthyl acetate, and *p*-nitrophenyl acetate (pNPA) as esterase substrate was tested. The enzyme was active against all of these substrates, exhibiting the highest activity against pNPA. The characterization of this *C. cinerea* lipase revealed a very versatile hydrolase with unique lipase activity (Hashimoto et al., 2010). A lipase from *Malassezia globosa* received special attention as it belongs to a very small group of lipases that are

only active on di- and monoglycerides (DG/MG) but not on TG. As such, lipases are involved in neuronal communication. They are of special interest for the design of new drugs. Besides that, they can act in the synthesis of high purity DG, biodiesel production, and food emulsifiers. The elucidation of its crystal structure paved the way for a rational design approach to alter the substrate spectrum of *M. globosa*. Two mutations appeared to alter the selectivity of the wild type (WT) enzyme. Mutant Y56A was only active on MG. Q282L displayed the desired activity on TG, even though it was less active than the WT. This mutant was less stable than the WT and also preferred shorter-chain fatty acids than the WT. A closer analysis of Q282L revealed that not steric hindrance but altered hydrophobicity was the reason for the extended substrate spectrum. During the reaction, one fatty acid of a TG is in contact with the amino acid in position 282. The hydrophobic substrate cannot interact with this residue when it is occupied by a polar amino acid residue. The exchange to the non-polar leucine allowed hydrophobic interaction between the substrate and this position (Lan et al., 2015). Another lipase from the basidiomycete *Schizophyllum commune* was characterized as a thermo- and alkali-tolerant enzyme, which also showed high stability in the presence of organic solvents. The lipase was produced by solid state fermentation and used for the production of fatty acid methyl esters (FAME). *S. commune* lipase showed an activity of $1581 \text{ U} \cdot \text{g}^{-1}$, reaching 94% conversion yield in the first round of FAME production, keeping the yields over 90% over several cycles (Singh et al., 2015). In a genome mining approach, 53 putative lipases and 34 putative carboxyl esterases were identified in the genome of *Pleurotus ostreatus*. Four lipases were expressed under the given conditions, and two were selected for recombinant production and closer characterization. Despite a high sequence similarity, the lipases differed in activity, substrate specificity, and stability. One *P. ostreatus* lipase could be identified as a true lipase, being active on a broad range of fatty acid chain lengths. The other *P. ostreatus* lipase was predominantly active on esters of decanoic acid, which was attributed to an extra loop in the substrate channel (Piscitelli et al., 2017). Further investigations on lipases from *P. ostreatus* used the culture broth of this fungus to purify a lipase. The authors chose this approach as they noted that posttranslational modifications in recombinant expression may affect the enzyme activity. The purified lipase had a molecular mass of 27 kDa with the pH optimum at 5.0 and a temperature optimum of 30 °C. Substrate selectivity was similar to that described for the first lipase of the previous study. Here, the lipase was most active on fatty acids with ten or more carbons (Nakagame et al., 2023). The corn-infectious fungus *Ustilago maydis* has ten hypothetical lipase-encoding genes in its genome. The two lipases UMLA and UMLB showcase 67% and 66% sequence identity with CALA and CALB, respectively. For a closer comparison, UMLB and CALB were recombinantly expressed in *K. phaffii*. In this host, the expression level of CALB was 13 times higher than that of UMLB. Both lipases had similar pH optima

and preferred short- to medium-chain TGs as substrates. UMLB showed activity at cold temperatures and appeared highly halotolerant. However, the thermostability of UMLB was lower than that of CALB. The authors concluded that this was due to the flexible loop as a thermosensitive structure. Compared to CALB, this lipase originates from an organism living in mild conditions (Robles-Machuca et al., 2025).

With the intention to find an alternative for animal-derived enzymes that are used for flavor generation in cheese making, previous to the work presented here, 31 Basidiomycota strains were screened for lipase activity. The lipolytic activity in the secretome of *Pleurotus citrinopileatus* attracted special attention. Transcriptomic data combined with liquid chromatography high resolution tandem mass spectrometry (LC-HR-ESI-MS/MS) led to the identification of the gene encoding the respective lipase. The sequence was transformed into *Escherichia coli* as an expression host. The recombinant lipase from *P. citrinopileatus* was purified and biochemically characterized. The selectivity of PCI_Lip was evaluated by using pNP fatty acid esters of different chain lengths. Here, PCI_Lip displayed the highest activity against octanoate and palmitate. The temperature optimum was at 60 °C and the pH optimum at 8.0. Kinetic characterization underscored the selectivity results of PCI_Lip with the highest efficiencies for pNP octanoate (pNPO) and palmitate (pNPP). With the idea to substitute pregastric esterases (PGEs) in cheese production, PCI_Lip was applied in the production of Feta-type brine cheese. Sensory evaluation, as well as the analysis of volatile free fatty acids (vFFA) by solid phase micro extraction gas chromatography mass spectrometry (SPME-GC/MS), revealed that PCI_Lip could be a potential substitute for PGEs, but yet gave a slightly soapy off-flavor, which was attributed to the release of long-chain fatty acids compared to PGEs (Sowa et al., 2022).

Protein engineering

For more than a century, humankind has been utilizing biocatalysis in different fields of application. Biocatalysis describes the harnessing of natural catalysts for purposes they originally were not meant for, especially in synthetic chemistry (Bornscheuer et al., 2012, Romero-Rivera et al., 2016). The first wave of biocatalysis was mainly promoted by the application of microbes and components of living cells, which were used as catalysts in chemical transformations. In the late 1980s, biocatalysis leaped as initial protein engineering (PE) techniques came up (Bornscheuer et al., 2012). PE describes the process of altering the structure of existing proteins to improve their properties (Kazlauskas, Bornscheuer, 2009). As the potential of enzymes is limited in industrial processes due to inefficient catalysis of non-cognate substrates and weak stability under given reaction conditions,

PE gives the chance to customize enzymes to maximize their performance for selected applications (Ndochinwa et al., 2024, Sellés Vidal et al., 2023). Naturally, enzymes regulate metabolic pathways and cellular functions under physiological conditions. This implies that many of the currently industrially relevant chemical transformations do not match to naturally available enzymes (Ndochinwa et al., 2024, Romero-Rivera et al., 2016). The increasing emphasis of green chemistry in many sectors of the chemical industry raises the demand for appropriate enzymes as biocatalysts (Chowdhury, Maranas, 2020).

To tackle this request, PE makes use of the natural capability of enzymes to adapt to new functions and environments (Romero, Arnold, 2009). Therefore, PE makes use of an artificially increased genetic diversification by modifying existing genes or creating new ones to extend known protein functions, adapt them to new environments, or even create new functions (Romero, Arnold, 2009, Sellés Vidal et al., 2023, Soni, 2022). In particular for industrial purposes, PE enables scientists to tailor enzymes precisely for the operating conditions of the respective industrial processes. Enzymatic attributes often considered for engineering are their efficiency, improvement of temperature or pH stability, tolerance towards organic solvents, increased specific activity, alteration of substrate specificity and binding, as well as changes in enantio-, regio-, and stereoselectivity (Ndochinwa et al., 2024).

Any attempt to engineer an enzyme starts with defining the engineering goals (Bornscheuer et al., 2012). After that, the procedure follows three common steps. Firstly, an appropriate target protein as a starting point is selected. Simultaneously, the engineering strategy is defined, which sets the placement of mutation points. The second step is the mutagenesis to create a so-called library of enzyme variants. Depending on the chosen strategy, this library can contain a variable number of variants. This number can be a small one-digit to up to 10^7 . The final step is the selection of an enzyme with improved characteristics (Kazlauskas, Bornscheuer, 2009, Romero-Rivera et al., 2016).

There are two strategies to identify positive variants in a library. The choice of selection strategy depends on various factors, such as library size, target of engineering, and technical setup in the lab. A screening evaluates the target property across the variant library. It requires a separation of variants, and each one is assessed individually. For selection, there must be a link between the evolved property and the recovery or an increased survival rate. Selection automatically discards variants with undesired features, therefore facilitating a higher throughput. However, it is less generally applicable due to a broad substrate range (Sellés Vidal et al., 2023). Even though PE enables the adaptation of enzymes to various factors, it remains challenging to reach the set goals because there is still a lack of understanding of enzyme catalysis (Romero-Rivera et al., 2016).

The fitness landscape is an important term in this respect. This term describes the protein sequence space displaying the genotype in relation to the phenotype. In PE, the fitness of the enzyme is defined by the experimenter in advance, with the challenge to find highly functional sequences in the fitness landscape (Romero, Arnold, 2009, Wang et al., 2023). The fitness landscape is a vast space. Depending on the engineering goals, it can be rough to overview. A desirable shape of this landscape is the so-called Fujiyama landscape. It exhibits one optimum with many smooth paths leading uphill. The more local optima this landscape shows, the rougher it is to climb. These local optima create traps that are hard to escape without making bigger jumps. Further challenges when moving on the fitness landscape are the uneven distribution of functional sequences and the robustness of some proteins to mutations, where many mutants retain their fold and function (Romero, Arnold, 2009). During the endeavor of engineering a protein, experimenters often come across the trade-off between catalytic activity and stability, constituting another obstacle in creating improved variants (Hu et al., 2025). While a stepwise movement on the fitness landscape relates to natural evolution, simultaneously, this strategy can miss cooperative interactions of miscellaneous mutations. On the other hand, the simultaneous substitution of more than one amino acid generates larger libraries with a higher number of inactive variants (Kazlauskas, Bornscheuer, 2009). The larger the changes of the properties of the selected enzymes are supposed to be, the larger changes in the free energy are required (Bornscheuer et al., 2012). It is the task of the protein engineer to select the best strategy to achieve the set goals. With the fitness landscape in mind, it is their task to determine the way to explore this landscape. This way should be chosen to navigate more or less directly to the engineering goals, using minimal resources and effort.

Strategies

PE can be conducted according to two different mutagenesis strategies. The applied techniques can be distinguished into random and rational mutagenesis approaches (Sellés Vidal et al., 2023). The choice of strategy will mainly be guided by the amount of information available on the target protein. In the beginning, PE was based on random mutagenesis strategies that were experimental approximations of natural evolution on a much shorter time scale (Ranbhor et al., 2025).

The random mutagenesis approach is suitable if there is not much information available about the enzyme structure and the catalytic mechanism (Romero, Arnold, 2009). Further, this strategy of not targeting a specific sequence is chosen when the property that shall be evolved cannot be attributed to specific positions (Sellés Vidal et al., 2023). Random mutagenesis creates a library of mutants,

which has to be screened by high-throughput (HTP) screening for desirable candidates (Chowdhury, Maranas, 2020). In this case, the improvement within a single generation is limited, and depending on how far the engineering goal is apart from the characteristics of the WT, several rounds of random mutagenesis are necessary to achieve it. Further, random mutations tend to have harsh effects on the enzyme, decreasing or even destroying its activity. As 30% to 50% of mutations are deleterious, 50% to 70% do not affect the enzyme properties, and only 0,01% to 1% are beneficial, the choice of appropriate library size and evaluation strategy is vital for a successful PE (Romero, Arnold, 2009).

The increasing popularity of PE, the bottleneck of identifying good mutants in large libraries, and growing computational capacities gave rise to the rational mutagenesis approach. It is based on computational tools to generate a small library with mutants that contain substitutions at a limited but targeted number of positions (Sellés Vidal et al., 2023). However, a rational strategy requires a huge amount of information about the target enzyme. A close understanding of the three-dimensional structure of the protein is essential for this approach. This requires the availability of crystal structures of high resolution. The biological function and catalytic mechanism of the enzyme have to be well understood to identify specific residues that influence the targeted property (Diao et al., 2025).

Both strategies can be conducted *in vitro* and *in vivo*. In the beginning, *in vitro* techniques deploying chemical or physical mutagenesis were used. The first techniques showed a bias towards certain mutations. Further advances in this field solved this problem and make *in vitro* techniques still the most popular way of mutagenesis. Later on, *in vivo* techniques have been developed. It facilitates the mutation directly in the host cell, being time-saving in comparison to *in vitro* techniques. The host cells belong to special mutator strains that are characterized by an increased mutation rate due to the inactivation of DNA repair mechanisms. The *in vivo* mutation is scarcely applied because mutator strains show several disadvantages, such as low mutation rates, biased mutagenesis spectrum, and slow growth (Sellés Vidal et al., 2023). Another important point for choosing the best mutagenesis strategy is to determine which type of variants shall be created. Either random or rational mutagenesis can be applied to the whole protein or only to specific regions. Here, a rational approach is more suitable when specific regions are targeted, and the random approach can better cover the whole protein, but this is not exclusive. Also, the engineering goal is important in deciding for the best strategy. Enantio- and diastereoselectivity can be improved best by mutating amino acids close to the active site. However, mutation spots to improve catalytic activity are mostly scattered throughout the whole protein (Kazlauskas, Bornscheuer, 2009).

Random and rational mutagenesis strategies should not be considered harshly separated but can complement each other to find the best path on the fitness landscape (Mazurenko et al., 2020). The

random approach is mainly governed by Directed Evolution (DE), and the rational approach is covered by Rational Design (RD), while a combination of both is merged as Semi-Rational Design (SRD). The rising field of artificial intelligence (AI), together with machine learning (ML), has recently expanded the opportunities of PE. Computational biology will complement both strategies in the future and make PE more effective (Hu et al., 2025).

Directed evolution

To evolve an enzyme for which there is little to no structural and catalytic information and a lack of understanding of key influencing factors of the property that is to evolve, DE is the strategy of choice for improvement (Diao et al., 2025, Romero-Rivera et al., 2016). DE artificially mimics natural evolution by introducing random mutations in the target gene sequence. No specific mutations or mutation sites are defined beforehand, instead, genetic diversification is used as a promoter on the way to an improved enzyme (Hu et al., 2025, Sellés Vidal et al., 2023). The genetic diversification is represented by a mutant library that has to be screened for variants with the desired properties (Mazurenko et al., 2020).

Contingent on the starting point and aims that shall be achieved by DE, iterative rounds of mutagenesis and subsequent selection may be required to create an enzyme with the requested attributes. This gives a guideline for the procedure of any DE attempt. The first step has to be the identification of an appropriate starting sequence, followed by its mutation to create a library. This process is repeated until the desired protein characteristics are achieved (Romero, Arnold, 2009). For the selection of the best variants, HTP assays are required (Wang et al., 2023). The success of DE depends on the genetic diversification compiled in the mutant library. Therefore, this library can hold 10^4 to 10^7 variants. Even though nowadays many HTP methods are available, the identification of variants of interest remains a bottleneck in this system (Romero-Rivera et al., 2016, Sellés Vidal et al., 2023, Soni, 2022).

The most common techniques in DE are error-prone polymerase chain reaction (epPCR), site saturation mutagenesis (SSM), and DNA shuffling (Diao et al., 2025, Hu et al., 2025). For epPCR, a low-fidelity polymerase is used, such as the one from *Thermus aquaticus*, which incorporates one error per 10^5 nucleotides. The error rate can be increased by the addition of an unbalanced dNTP concentration or the addition of magnesium or manganese ions (Lee, Fried, 2021). In SSM, a single amino acid in the target sequence is substituted with any other of the 19 proteinogenic amino acids. This method is less biased compared to epPCR, and the influence of specific positions and residues can be investigated more closely (Siloto, Weselake, 2012). DNA shuffling is a recombination

technique. Homologous DNA sequences of a target gene are fragmented. These fragments from different parental sequences are then recombined randomly in a PCR reaction (Schweiggert et al., 2024). By this technique, combinatorial libraries are created out of segments coding for functional proteins. It requires a pool of closely related sequences, but the chimeric variants usually contain a higher percentage of functional ones. In the meantime, further recombination techniques for DE have been developed, such as Random Chimeragenesis of transient Templates (RACHITT), Incremental Truncation for the creation of hybrid enzymes (ITCHY), Sequence Homology Independent Protein Recombination (SHIPREC), Sequence independent site directed Chimeragenesis (SISDC), and an *in vivo* technique called Mutagenic Organized Recombination Process by Homologous *in vivo* grouping (MORPHING) (Sellés Vidal et al., 2023). The generation of genetic diversity makes DE the more efficient PE strategy for the discovery of novel enzymes (Ndochinwa et al., 2024). As for DE, no structural information is required, a drawback is that not much knowledge can be gained on how mutations lead to improved proficiency (Romero-Rivera et al., 2016).

The implementation of AI tools in DE helped to generate more powerful enzyme design methods. With these tools in hand, the evaluation of rules deduced from DE boosted PE (Romero-Rivera et al., 2016). A current research topic in DE is to get away from prokaryotic and yeast-based systems and the establishment of stable mammalian systems. Existing mammalian systems for DE often face limitations due to safety concerns, low mutation rates, and a lack of functionality. Cole and co-workers recently developed a platform enabling broad DE implementation in mammalian cells. PROTEin Evolution Using Selection (PROTEUS) uses chimeric virus-like vesicles to create genetic diversity. They applied this system in the optimization of a nanobody for a biosensor to detect DNA damage response in mammalian cells (Cole et al., 2025). DE also finds application in the investigation of diseases and in the development of therapeutics. A current study on intrinsically disordered proteins (IDP) used DE to gain a deeper understanding of these proteins, as knowledge on their sequence-function relationship is still limited. IDPs modulate cellular functions, and closer investigation of the sequence-function relationship aids in drug development (Ma et al., 2026). DE also made its way into the trending topic of plastic degradation. A breakthrough in tackling the worldwide plastic crisis was the development of poly(ethylene terephthalate) (PET) hydrolases. These enzymes were mainly developed in rational and semi-rational approaches. To overcome the low activity, expression, and thermostability, current studies use DE approaches coupled with newly developed HTP assays to overcome these hurdles (Groseclose et al., 2025). The most skyrocketing topic in DE is the incorporation of AI tools. These tools can help range the fitness landscape and reduce the experimental load in the lab. Recently, EVOLVEpro was developed by combining a protein language model (PLM) and regression models. This tool has been shown to predict a small

set of variants with multiple improved characteristics for each round of mutagenesis. It learns from the data collected in each cycle. The effective machine learning DE was demonstrated on enzymes involved in RNA production and genome editing, as well as on antibodies (Jiang et al., 2025a).

Rational design

The second strategy in PE is RD. In contrast to a DE approach, RD requires extensive information and knowledge of the target protein. Therefore, the three-dimensional structure of the enzyme has to be known at a high resolution (Mazurenko et al., 2020). Nowadays, advances in X-ray, cryo-electron microscopy, and nuclear magnetic resonance (NMR) provide a growing number of high-quality structural data that can be used for RD (Mohammed et al., 2025). Further, there has to be detailed knowledge of the biological function and catalytic mechanism of the specific enzyme. To successfully apply this strategy, multiple sequence alignments (MSA) to related sequences and biochemical data of considerable size have to be available (Sellés Vidal et al., 2023). RD requires considerable computational capabilities, which could be provided at the end of the 1990s. In general, the target protein is examined and designed *in silico* before empirical evaluation (Ranbhor et al., 2025). Based on the prior knowledge, a limited number of positions in the target sequence is determined for mutagenesis. The site-directed mutagenesis gives small libraries with highly successful variants, which reduces the evaluation effort (Romero-Rivera et al., 2016, Soni, 2022). To alter substrate selectivity or activity, mostly positions in the active site or binding pocket are targeted (Sellés Vidal et al., 2023). Therefore, amino acids at selected positions are altered and their physicochemical properties and interactions with the environment are simulated. The drawback is the need for intensive calculations in the construction of the required models (Mazurenko et al., 2020).

The rational approach in PE depends on various computational tools to understand and rationalize the impact of distinct mutations. Especially molecular dynamics (MD) simulations are essential to identify suitable mutation spots (Romero-Rivera et al., 2016). MD simulates the movement of the atoms in the protein over time. This provides information on structural changes in response to factors such as pH value and temperature. These results help to elucidate folding pathways and to evaluate protein stability. In terms of altering activity, docking studies can reveal crucial positions that interact with the substrate in terms of chemo-, regio-, and stereoselectivity (Mohammed et al., 2025, Romero-Rivera et al., 2016). Energy and scoring functions also play an important role in RD. Energy functions attempt to capture all forces that are responsible for the protein to fold into a certain three-dimensional structure as low energetic state. Scoring functions expand these calculations with

statistical terms that depend on the protein sequence (Albanese et al., 2025). Advances in computational biology gave the chance to elevate RD. *De novo* design of proteins describes the creation of proteins not known in nature and putatively with new to nature functions. In the *de novo* design, an active site is conceptualized that is capable of catalyzing the desired reaction. This active site is embedded into a suitable backbone (Chen et al., 2022c).

RD is used in current research topics to tackle the present challenges in biocatalysis. For a more efficient biodiesel production, a lipase from *Geobacillus stearothermophilus* was subjected to PE, aiming for better methanol stability. To achieve this, Gihaz and co-workers used a widespread RD approach. The structure of the lipase was investigated *in silico* for putative positions for the introduction of disulfide bridges. The analysis predicted 13 double mutants. One of these mutants displayed superior stability and activity in the biodiesel production process (Gihaz et al., 2020). A study by Lu and co-workers represents a state-of-the-art research field combining RD with AI tools. A lipase from *Paenibacillus larvae* was characterized for its activity and stability properties. AlphaFold2 was used as an AI tool to predict the structure of this lipase. The lipase model was then used to identify putative mutation spots in the enzyme. Based on these findings, mutants with two additional disulfide bonds were generated. One mutant was found to exhibit increased thermostability. MD simulations revealed that the disulfide bridges stabilize a rather flexible loop region, making the enzyme sturdier. Another mutant displayed increased specific activity without compromising stability. Here, MD simulations showed that these mutations enlarged the binding pocket for better access of the substrate (Lu et al., 2024). A similar RD approach combined with AI was used to improve the enantioselectivity of *Geobacillus thermoleovorans* CCR11 lipase. Also in this study, a model of the lipase was generated by AlphaFold2. RD was used to identify six amino acids in the substrate pocket that influenced the enzyme's enantioselectivity. One mutant was capable of only producing (*R*)-dimethyl malate (Bustos-Baena et al., 2025). Enzymatic degradation of plastics, besides PET, challenges protein engineers to *de novo* design enzymes for other types of plastics. Holst and co-workers were successful in developing a polycarbonate hydrolase that does not consist of a α/β -hydrolase scaffold. They first designed an active site capable of catalyzing the hydrolysis of polycarbonate and integrated it into a thermostable scaffold. After heterologous expression, the enzyme proved to be active on that plastic (Holst et al., 2023). In another *de novo* design approach, a medium-chain-length selective lipase was designed based on the backbones of other hydrolytic enzymes with similar selectivity (Huang et al., 2023).

SRD constitutes a combination of both random and rational approaches to PE. It can be applied to proteins with minor structural information. Computational tools are similarly important as in RD. Both

prior information and computational tools are used to preselect promising sequences (Lutz, 2010). Mostly, enzyme models are used to identify sequences that potentially influence the targeted characteristic. Then, mutagenesis is conducted either in a site-directed or random manner. Thereof, small but smart libraries are generated holding a high amount of functional mutants (Ndochinwa et al., 2024, Soni, 2022).

SRD allows a PE approach that is hypothesis-driven and less reliant on random discovery of improved variants as DE does. It gives the chance of drastically reducing the screening effort, even though structural and mechanistic information is not fully given (Lutz, 2010). A key feature in SRD is MSA. Several protein, DNA, or RNA sequences are aligned to compare similarities and differences. This helps to unravel evolutionary relationships by spotting point mutations, insertions, and deletions, but is also useful to identify targets for PE (Zhang et al., 2024).

There are different approaches in which random and rational approaches are combined. In a targeted randomization based on structural information, positions are defined for mutagenesis that are in contact with the substrate or near the active site. The choice of amino acid substitution is random and therefore offers the chance to generate synergistic effects (Chica et al., 2005, Mildvan, 2004). An early study from Santoro and Schultz successfully applied this strategy to alter the specificity of a recombinase from a bacteriophage (Santoro, Schultz, 2002). Another strategy in SRD is the combination of random and SSM. This strategy was used to enhance the catalytic efficiency of a methyl parathion hydrolase from *Pseudomonas* sp. First, random mutagenesis was applied to identify promising positions. In a second round, SSM was applied to find the best amino acid for substitution. With this strategy, a mutation away from the active site was identified that increased the catalytic efficiency by more than threefold (Li et al., 2018).

The implementation of computational approaches also boosts SRD. *In silico* screening of variants reduces the workload of creating and screening mutant libraries in wet lab trials (Chica et al., 2005). Recent research focusing on the engineering of lipases mainly deals with aspects of nutrition and biodiesel production. The substrate selectivity of a lipase from *G. thermoleovorans* was shifted towards the preferred hydrolysis of ω -3 fatty acids. *In silico* docking experiments were conducted to spot amino acids interacting with the substrate in the active site. These positions were then changed by SSM. The generated library contained promising mutants that preferentially released unsaturated fatty acids (Moharana, Rao, 2020). Tian and co-workers used an SRD approach to improve the activity and methanol tolerance of a *Rhizomucor miehei* lipase. Further, *N*-glycosylation sites were inserted in the protein sequence. After expression and posttranslational glycosylation, two mutants stood out. One showed much higher methanol tolerance, and the other displayed a conversion yield

of 93% in biodiesel production (Tian et al., 2022). A similar study was undertaken more recently using a lipase from *Thermomyces lanuginosus*. As this lipase is also applied in biodiesel production, a semi-rational approach was used to increase its thermostability and methanol resistance. Site-directed mutagenesis introduced amino acid substitutions in the lid domain. Further, a mutant with a deleted lid domain was investigated. Simultaneously, potential *N*-glycosylation sites were introduced. Finally, a double mutant was identified with strongly increased thermostability and methanol resistance. In a one-step biodiesel synthesis approach, this mutant yielded over 90% conversion (Chang et al., 2025). Besides the currently trending application of engineered lipases in biodiesel production, these enzymes are also utilized in the feed sector. Lipases aid in improved feed efficiency. To extend the shelf life of feed, in some cases, glucose oxidases are added. The produced hydrogen peroxide is supposed to inhibit microbial growth. For the effective utilization of lipase A from *Bacillus subtilis* in feed, it was engineered for improved hydrogen peroxide resistance. The changes in molecular mass of the native and oxidized forms of the lipase were employed to identify positions prone to oxidation. These positions were mutated, and a small library with a high number of more resistant variants was generated. One double mutant exhibited a nine-fold higher hydrogen peroxide tolerance (Jiang et al., 2020).

Artificial intelligence in protein engineering

AI is gaining worldwide attention in many different fields, ranging from business applications to personal space. ML is an AI tool especially valued in the field of PE and synthetic biology (Wang et al., 2025a). ML is a data-driven method deploying algorithms to deduce rules directly from data (Kouba et al., 2023). The algorithms learn patterns from the given data. As they do not rely on hard rules, it enables them to predict new data with similar properties as the input (Kouba et al., 2023, Ndochinwa et al., 2024, Qiu, Wei, 2023). Even though AI needs remarkable computational power, training is at a low cost (Dou et al., 2023). Once the algorithm is trained on a specific input, predictions are generated very quickly (Ndochinwa et al., 2024). In PE, these tools are used to predict protein structures or various characteristics such as stability, solubility, or substrate specificity. It can be used on its own to create new, high-quality variants or in combination with DE or RD (Wang et al., 2025b). At this point, it is important to differentiate AI approaches from RD. ML is data-based, while RD is model-driven. In fact, an AI approach to create an engineered variant of an enzyme with certain improved characteristics uses available data to forecast appropriate mutations. In RD, a new model needs to be generated, which is based on physical functions and requires a lot of calculation (Mazurenko et al., 2020, Ndochinwa et al., 2024).

For PE, mostly unsupervised learning is used. In this case, the algorithm is supposed to find patterns in unlabeled data (Kouba et al., 2023). The quality of an ML algorithm depends on the quality and amount of data it has been trained on (Dou et al., 2023). Here, a current problem in many cases is an insufficient data set in combination with consistency and comparability issues (Mazurenko et al., 2020). For appropriate training, the available data are split into three groups. The first one is called a training set. The algorithm uses this data to learn underlying patterns. Next, a validation set is used to evaluate progress. Finally, the trained algorithm predicts data from the test set to evaluate the model's performance (Herrera-Rocha et al., 2025, Rao et al., 2019, Xu et al., 2020). If the model or the data are not good enough, over- or underfitting can occur. If the model fails to identify the underlying pattern in the data, it is underfit. Overfitting describes the lack of pattern recognition because the model focused on learning too many details (Ghojogh, Crowley, 2019).

The implementation of AI in PE was rewarded with a shared Nobel Prize in chemistry in 2024. David Baker received it for advances in computational protein design. Demis Hassabis and John Jumper were rewarded for the development of AlphaFold. It is considered the first highly reliable algorithm to predict the three-dimensional structure of a protein based on the amino acid sequence. AlphaFold was trained on more than 200000 experimentally solved protein structures and combines physical and biological principles for learning (Buller et al., 2025).

Even though AI can reduce the workload that comes with DE or RD, it requires thoughtfulness in a protein improvement project. There are four key points that have to be considered. Firstly, the property to evolve has to be defined, and an AI model for prediction has to be chosen. As the sequence space is too big to be considered overall, it needs to be narrowed down. Here, only single or double mutants or a restricted number of mutation sites can be considered. Next, an acquisition function evaluates the output of the model to suggest the best output for experimental measurements (Hie, Yang, 2022). AI-aided PE is present in the major biocatalytic tasks of today. Enzymatic PET degradation offers the chance to tackle the global plastic waste problem. As PET hydrolases often lack pH and temperature robustness and display low reaction rates, these issues need to be targeted by PE. Using a structure-based deep learning neural network, Lu and co-workers generated a variant with five mutations that exhibited higher activity in combination with improved stability. Both objectives could not be reached in prior DE or RD approaches (Lu et al., 2022). An example of combining traditional DE with AI tools has been demonstrated by Thomas and co-workers to improve the activity of a nuclease. Data from a DE approach were utilized to train an ML framework to efficiently navigate the protein sequence space, finding improved variants (Thomas et al., 2025). A current application of ML in lipase engineering has been reported by Yoshida and co-workers. They

enhanced the thermostability of the commercialized lipase PS from *Burkholderia cepacia* in a PE approach combining DE and ML. First, three consecutive positions were mutated randomly in twelve loops. The library of 214 variants was screened for their thermostability. One loop was identified to have a significant impact on thermostability. As screening all possible triple mutants in that spot would comprise more than 8000 mutants, an ML approach was chosen to narrow down the screening effort in the lab. An ML model was trained on the previously determined data to virtually screen this library for the best variants. The 20 best variants were assayed for their thermostability, revealing one triple mutant with much better thermostability than the WT (Yoshida et al., 2021). Another aspect of ML applications for PE is the development of models that can reliably predict certain characteristics of proteins. A novel model to predict the thermostability of single mutants was evaluated in comparison to established models. Evaluation was performed on single mutants of CALB. The novel model used sequence-based information without the need for structural information. In application to CALB single mutants, it outperformed other models with higher prediction efficiency and accuracy (Jiang et al., 2025b). Still, AI has its limitations, which were shown in a study on lipase A of *Pseudomonas aeruginosa*. The lipase was engineered to enhance the hydrolysis of an industrially relevant, non-natural substrate. In a library, some variants with increased activity were identified. The attempt to rank these variants employing deep-learning models failed. The authors assume that small energetic shifts are responsible for the changes in activity, but these cannot yet be captured by current models (Gardiner et al., 2025).

Lipolytic enzymes in cheese production

Pregastric esterases

The specific flavor of different cheese varieties develops during the ripening process, where flavor- and taste-active compounds are released to create each cheese's unique profile (Tekin, Hayaloglu, 2023). Lipolysis plays a vital role in the release of flavor-active molecules. This process involves the enzymatic release of fatty acids from TG, DG, and MG as primary events (Ardö, 2021). Lipolytic enzymes in traditional cheese production originate either from milk when raw milk is used or from rennet paste (Moschopoulou, 2011, Thierry et al., 2017). The paste contains lipolytic enzymes from the sublingual glands of young ruminants such as goat kids and lambs, also referred to as PGEs (O'Connor et al., 1996). Depending on the variety of the cheeses, lipolytic enzymes from the microflora and exogenous sources play a minor but not negligible role in the flavor formation (Collins

et al., 2003). PGEs preferentially hydrolyze fatty acids from sn 3-position of the TG. In milk fat, the fatty acids at this position commonly are short-chain fatty acids, which directly contribute to the flavor of the cheese when released (Wilkinson et al., 2011).

In a second stage of flavor formation, the products from lipolysis are metabolized to further flavor compounds (McSweeney, 2004). If hydroxylated fatty acids are released, γ - and δ -lactones can form spontaneously via intramolecular esterification (Chen et al., 2022a, Chen et al., 2022b). These hydroxylated fatty acids originate from the mammary gland or from the lipoxygenation of unsaturated fatty acids (McSweeney, Sousa, 2000). Furthermore, free fatty acids (FFA) react with alcohols, most commonly ethanol, to form different esters. Both lactones and esters are associated with fruity and floral perceptions, but also contribute to the overall flavor of several cheese varieties (Sutay Kocabaş et al., 2022).

The complex flavor profile derived through many different biochemical events during ripening is demanded for a broad spectrum of cheese varieties. However, replicating this flavor using natural cheese is limited by factors such as cost, production time, and intensity (Kendirici et al., 2020). Enzyme-modified cheese (EMC) is a product widely used to overcome this obstacle in cheese-flavored groceries, such as sauces, dips, and snacks (Bolat et al., 2025, Salum et al., 2022). EMC is considered a natural cheese concentrate in which the formation of flavor-active compounds is reinforced by enhancing the biochemical pathways that contribute to flavor formation (Fan et al., 2025). Intensified flavor is achieved by adding exogenous peptidases and lipases to fresh cheese curd (Zhang et al., 2023). The release of fatty acids and the products of further metabolism are increased by a factor of 10 to 100 (Salum et al., 2019). The controlled and optimized conditions of lipolysis enable the producers to accelerate the ripening and to tailor the flavor of EMC to meet consumer preferences effectively (Ali et al., 2019).

The primary challenge in lipolysis involves achieving a proper balance between short- and long-chain FFAs. An imbalance leads to soapy and rancid off-flavors, mainly caused by the release of long-chain FFAs (Erbay et al., 2021). To produce the flavors of different kinds of cheese, a wide range of commercial PGEs and lipase preparations are available. While some of these enzymes are derived from animal origin, microbial lipases dominate the market due to their convenience of purification and suitability for vegetarian, kosher, and halal certifications (Dekker, 2019, Sutay Kocabaş et al., 2022).

PCI_Lip as potential substitute

A recent study dealt with the search for a proper substitution for PGEs. Basidiomycota, with their rich secretomes, were employed to find lipolytic enzymes primarily active on short-chain fatty acids. The secretomes of 31 Basidiomycota strains were screened for their lipolytic activity. Among them, the Golden Oyster mushroom, *Pleurotus citrinopileatus*, exhibited promising enzymatic activity. By combining transcriptomic data with LC-HR-ESI-MS/MS, a lipase responsible for the lipolytic activity was identified. Furthermore, the lipase was recombinantly expressed in *Escherichia coli* using chaperones, ensuring proper folding and functionality (Sowa et al., 2022).

In a further study, the hydrolysis profile of PCI_Lip was further optimized to meet the demands required for cheese flavor formation. Broel and co-workers used an SRD approach and *in silico* docking experiments to minimize the release of long-chain FFAs while enhancing the release of short- to medium-chain FFAs. Out of a small but smart library of 24 single mutants, the ones with the most promising lipolytic activities were used in Feta-type cheese applications. The comparison of volatile FFAs, as well as the sensory evaluation, depicted that the selected mutants of PCI_Lip were able to create a similar flavor to PGEs in Feta-type cheese and thereby could potentially make their way to industrial applications (Broel et al., 2022).

After engineering PCI_Lip to have the desirable catalytic characteristics, sufficient expression yields remained an obstacle. Heterologous expression in *E. coli* BL21 (DE3) yielded insufficient amounts of soluble enzyme, even with the support of chaperones. While the prokaryotic expression system offers the advantages of easy handling and comparatively fast expression, which is needed to build up a library of mutants, its limitations in post-translational modifications can hinder proper enzyme folding and solubility (Krupodorova et al., 2014, Pouresmaeil, Azizi-Dargahlou, 2023). To address this challenge, a study by Wang and co-workers aimed to use a eukaryotic, yeast expression host with QPS and GRAS status. Both are safety assessment systems regulating the use of microorganisms in food. The Qualified Presumption of Safety (QPS) concept is applied in the EU to evaluate the safety of microorganisms applied in food and feed. For getting the QPS status, the taxonomy, knowledge on the respective organism, non-pathogenicity, and application in food systems are considered. For food prepared with microorganisms carrying QPS status, no individual safety assessment is necessary. In the US, food ingredients, including microorganisms, are on a list containing substances that are Generally Recognized as Safe (GRAS) and can be applied in food (Heo et al., 2022).

For this purpose, the PCI_Lip gene was transformed into the yeast *Komagataella phaffii*. This expression host is capable of posttranslational modifications such as the formation of disulfide bonds, glycosylation, and phosphorylation. The posttranslational modifications support the correct folding and hence solubility of heterologously expressed enzymes (Valero, 2012). PCI_Lip was successfully transformed into *K. phaffii*. Expression was performed in a 5 L fermenter reaching an activity of $5.94 \times 10^5 \text{ U} \cdot \text{g}^{-1}$. The increased activity and solubility could be attributed to the posttranslational glycosylation of PCI_Lip (Wang et al., 2025c).

Objectives and outlook

The present work addressed the characterization of Basidiomycota-derived lipases. Lipolytic enzymes from the fungi *P. citrinopileatus*, *Pleurotus sapidus*, and *Phlebia centrifuga* should be heterologously expressed and purified. The lipases should be investigated for their activity against fatty acid esters and TG. The lipase from *P. sapidus* (PSA_Lip) should be investigated concerning substrate selectivity. Sequence alignments with other lipases should give information about similarities and differences to previously characterized hydrolases. To further elucidate the potential of this lipase, optimal temperature and pH conditions should be examined. Future investigations on substrate selectivity and PE could optimize this lipase for specific applications. A closer look at the novel structural motif revealed additional information on the catalysis mechanism and could help in the characterization of other fungal lipases.

The focus of this research should be on PCI_Lip, trying to further adapt this lipase for cheese production as a potential substitute for PGEs. In three rounds of mutagenesis, the hydrolysis profile of the lipase should be shifted to fortify the release of short- to medium-chain fatty acids, while simultaneously the hydrolytic release of long-chain fatty acids should be reduced. The adaptation should be realized in a semi-rational PE approach. In three rounds of mutagenesis, a small but smart library should be generated. Mutants with impaired characteristics should be analyzed to prove the aptitude of the underlying AlphaFold3 model. Evaluation of the selectivity of all mutants should give further insights into the structure-function relationship of this enzyme. Finally, an application of PCI_Lip in cheese production should test its performance under realistic conditions. In the future, PCI_Lip may be utilized to produce cheese for vegetarian, kosher, or halal diets. With appropriate TG present, this lipase could also enrich the market of vegan cheese alternatives. A comparison to a lipase from *P. centrifuga* (PCE_Lip) with high sequence similarity should give insights into the role of the lid domain in fungal lipases. Structural comparison and investigations of the activity of both lipases should pave the way for a deeper understanding of fungal lipases.

To increase the understanding on PCI_Lip, the stability and regioselectivity of the enzyme should be characterized in combination with enzyme immobilization. The thermodynamic and kinetic stability of PCI_Lip should be investigated. The effects of different buffers, additives, and mutations should be studied. Effects of immobilization should also be examined. A variety of different support materials should be tested to identify the most appropriate one with high binding capacity, low leakage, and activity retention over several cycles of use. Investigations on regioselectivity should reveal if PCI_Lip displays any positional selectivity in the hydrolysis of TG. These findings should increase

the potential field of application of PCI_Lip. Concluding studies should take a closer look at its applicability, especially in the production of biodiesel and tailored lipids, also considering further substrates to broaden the scale of industrial applications of PCI_Lip.

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Characterization of PSA_Lip with unique SGNH-motif

Characterization of a GDS(L)-like hydrolase from *Pleurotus sapidus* with an unusual SGNH motif

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ORIGINAL ARTICLE

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Characterization of a GDS(L)-like hydrolase from *Pleurotus sapidus* with an unusual SGNH motif

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Abstract

The GDS(L)-like lipase from the Basidiomycota *Pleurotus sapidus* (PSA_Lip) was heterologously expressed using *Trichoderma reesei* with an activity of 350 U L⁻¹. The isoelectric point of 5.0 was determined by isoelectric focusing. The novel PSA_Lip showed only 23.8–25.1%, 25.5%, 26.6% and 28.4% identity to the previously characterized GDSL-like enzymes phospholipase, plant lipase, acetylcholinesterase and acetylxyloxy esterase, from the carbohydrate esterase family 16, respectively. Therefore, the enzyme was purified from the culture supernatant and the catalytic properties and the substrate specificity of the enzyme were investigated using different assays to reveal its potential function. While no phospholipase, acetylcholinesterase and acetylxyloxy esterase activities were detected, studies on the hydrolysis of ferulic acid methyl ester (~8.3%) and feruloylated carbohydrate 5-*O*-transferuloyl-arabino-furanose (~0.8%) showed low conversions of these substrates. By investigating the hydrolytic activity towards *p*-nitrophenyl-(pNP)-esters with various chain-lengths, the highest activity was determined for medium chain-length pNP-octanoate at 65 °C and a pH value of 8, while almost no activity was detected for pNP-hexanoate. The enzyme is highly stable when stored at pH 10 and 4 °C for at least 7 days. Moreover, using consensus sequence analysis and homology modeling, we could demonstrate that the PSA_Lip does not contain the usual SGNH residues in the active site, which are usually present in GDS(L)-like enzymes.

Keywords *Pleurotus sapidus*, GDSL, SGNH hydrolase, Heterologous expression, *Trichoderma reesei*, Enzyme characterization

Introduction

The use of residual biomass for the production of daily products, food and feed is the key in the circular economy (European Commission 2021), and simultaneously partially fulfills the UN Sustainable Development Goals (El-Chichakli et al. 2016). However, the depolymerization of renewable agro-industrial residues is a challenge that can only be overcome by efficient biomass hydrolysis (Ahorsu et al. 2018). A crucial step for the leap to technological applications is the production and use of efficient enzymes, which evolution has already tailored to degrade and modify biopolymers efficiently. Especially

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fungi, as natural cell factories with their unique enzymes, can degrade organic lignocellulosic biomass into biogenic raw materials, which are then available as feedstocks (Filiatrault-Chastel et al. 2021). The great biodiversity of the enzymes of the phylum Basidiomycota, the “pillar” fungi, often referred to as “higher fungi”, has not yet been sufficiently characterized, and only a few Basidiomycota-derived enzymes have been commercialized thus far (Schmidt-Dannert 2016). *Pleurotus sapidus* (oyster mushroom), a white-rot fungi of the phylum Basidiomycota, is an efficient lignin decomposer and able to fully degrade complex polymers (Croan 2003; Patrick et al. 2014; Ahlborn et al. 2019). Depending on the substrate, numerous enzymes are produced by this fungus to decompose lignocellulosic biomass efficiently (Zorn et al. 2005a, b). Basidiomycota produce pronounced oxidative enzymes, comprising *inter alia* laccases and peroxidases/peroxygenases as well as an extended hydrolytic system, with both of them being necessary to fully utilize renewable biomasses (Filiatrault-Chastel et al. 2021). Within the hydrolytic system, cellulases, hemicellulases, peptidases, and lipases/esterases are the main representatives of the secreted enzymes of these fungi (Filiatrault-Chastel et al. 2021). Esterases act on ester bonds (E.C.3.1) and are intensively used in various applications, such as in the synthesis of optically pure chemicals (Bornscheuer 2002) and foods (Mehta et al. 2021). In nature, a great variety of different structural folds of esterases have been reported. The best studied structural group are the enzymes with α/β -hydrolase fold (Ollis et al. 1992), which have a unique arrangement of α -helices and β -sheets, that is found in a variety of enzymes such as acetylcholinesterases, acyltransferases, dehalogenases, hydroxynitrile lyases, proteases, thioesterases, PETases, MHETase and others (Bauer et al. 2019). However, especially in the group of carbohydrate esterases (CEs) of the carbohydrate-active enzymes (CAZymes) (Drula et al. 2021), different hydrolase folds are present. Beside the α/β -hydrolase fold, right-handed β -helix fold, (β/α)-barrel fold, two-layer-sandwich fold and three-layer $\alpha/\beta/\alpha$ -structure fold, also known as SGNH fold, with or without additional domains, exist (Nakamura et al. 2017). SGNH hydrolases, as a subgroup of the GDSL hydrolases, contain the amino acid motif of Ser, Gly, Asn, and His, which are not directly consecutive in the amino acid sequence (Akoh et al. 2004). Typically, Gly and Asn donate protons to the oxyanion hole with Ser at the active site (Upton and Buckley 1995). Inside the GDSL(L) family, named after the amino acids motif “Gly, Asp, Ser”, often followed by Leu, in sequential order, different enzymatic activities such as thioesterase, peptidase, arylesterase, phospholipase activity, and acetylcholine esterase activity have been reported (Akoh et al. 2004). In CE family proteins, the SGNH motif can be found in CE2, CE3, CE6, CE12, CE16 and CE17 groups

(Urbániková 2021). As a result of their wide range of applications, ester hydrolyzing enzymes are interesting tools for biotechnological processes and especially for food production (Raveendran et al. 2018). For example, a lipase from *Pleurotus citrinopileatus* (PCI_Lip), is used to produce special flavors in brine cheese as an alternative to pre-gastric esterases (Sowa et al. 2022). In this study, the cDNA sequence encoding for a GDS(L)-like hydrolase from *P. sapidus* (PSA_Lip) was cloned in different vectors and subsequently used for heterologous expression of the protein using *Trichoderma reesei*. To our knowledge, this is the first GDS(L)-like hydrolase from Basidiomycota produced recombinantly. Homology studies and bioinformatics analysis allow the prediction of a SGGI motif as an alternative to the SGNH in the PSA_Lip, while different enzymatic assays provided hints to unravel its potential function.

Materials and methods

Chemicals

Chemicals and reagents used were obtained from Carl Roth (Karlsruhe, Germany), Alfa Aesar (Karlsruhe, Germany), Applichem, (Darmstadt, Germany), Sigma Aldrich (Schnelldorf, Germany), Th. Geyer (Renningen, Germany) or Merck (Darmstadt, Germany). Chemicals and materials for electrophoresis were from Serva (Heidelberg, Germany) and Bio-Rad (Düsseldorf, Germany).

Strains and culture methods

Pleurotus sapidus (DSM 8266) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was cultivated in standard nutrition solution (SNS, 30 g L⁻¹ D-(+)-glucose H₂O, 4.5 g L⁻¹ L-asparagine H₂O, 1.5 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgSO₄ H₂O, 3.0 g L⁻¹ yeast extract, 1 mL L⁻¹ trace element solution: 5 mg L⁻¹ CuSO₄ 5 H₂O, 80 mg L⁻¹ FeCl₃ 6 H₂O, 90 mg L⁻¹ ZnSO₄ 7 H₂O, 30 mg L⁻¹ MnSO₄ H₂O, and 0.4 g L⁻¹ EDTA; pH 6.0) for 8 days (24 °C, 150 rpm, 25 mm shaking diameter). For induction of lipase production, either 4% v/v Tween® 80 or 1% v/v corn oil were added to the main culture, replacing glucose as the main carbon source. *Escherichia coli* (TOP10) was obtained from Invitrogen (Karlsruhe, Germany) and was used for vector propagation. Chemically competent cells were transformed by heat shock treatment (2 min, 42 °C) according to standard protocols. Recombinant cells were cultivated in sterile LB medium (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl) with 150 mg L⁻¹ ampicillin used as selection marker (37 °C, 225 rpm).

Secretome analysis

Secretome analysis of *P. sapidus* was performed at Protagen Protein Services GmbH (Dortmund, Germany)

by 2D electrophoresis and subsequent MALDI-MS/MS analysis. Sequence homologies of the obtained fragments were analyzed using BLAST (Altschul et al. 1990) and Pfam (Sonnhammer et al. 1997) database.

cDNA-synthesis

To isolate RNA, the mycelium of a submersed culture containing SNS+Tween® 80 was harvested on day 5 and 100 mg was ground in liquid nitrogen with mortar and pestle. Isolation of total RNA was performed with RNeasy™ Plant Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of the RNA was verified by an 1% (w/v) agarose electrophoresis and ethidium bromide staining. A cDNA library of *P. sapidus* was produced using the isolated RNA as template with the SMART™ PCR cDNA Synthesis Kit (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France) for 21 to 24 cycles (Additional file 1: Fig. S1). Amplification of a coding GDSL-like hydrolase sequence of *P. sapidus* from the cDNA library was performed by PCR. The primers were derived from a homologous *P. ostreatus* putative GDSL type lipase sequence (Genbank accession number: KAF7436219.1, Additional file 1: Table S1). Amplification of the specific cDNA was performed in an Alpha SC PCR Thermocycler (Analytik Jena, Jena, Germany). The following PCR protocol was used: 50 ng template, 5×PCR-Puffer including dNTP's (Qiagen), forward and reverse primer each 50 pmol, 1.25 U HotStar HiFidelity DNA-Polymerase (Qiagen), ddH₂O ad 50 µL, 95 °C 5 min, −95 °C 1 min, 53 °C 1 min, 72 °C 90 s—for 40 cycles 72 °C 5 min. PCR products were separated electrophoretically (1.2% (w/v) agarose gel, (Additional file 1: Fig. S2), subsequently isolated from the gel using NucleoSpin Extract II Kit (Macherey–Nagel, Düren, Germany) and finally ligated (Topo TA-Cloning® Kit, Invitrogen) into the vector pCR2.1–TOPO® (Invitrogen). The plasmid DNA was replicated in *E. coli* TOP10 cells (Invitrogen), isolated from the cells and purified (NucleoSpin® Plasmid DNA Purification, Macherey–Nagel). Sequencing of the cloned cDNA was performed by Eurofins Genomics GmbH (Ebersberg, Germany). The codon-optimized PSA_Lip sequence can be found in the supplementary file.

Enzyme production

For recombinant production of the PSA_Lip, the codon usage of the gene was adapted to the host organism *T. reesei* (a derivative of RUT C30) (Peterson and Nevalainen 2012) and an expression cassette was constructed from the adapted sequence. The expression of lipase was investigated with three different constructs. pAB500-LipPS, in which the lipase gene with its signal sequence was under control of the *cbhI* (cellobiohydrolase I) promoter and the *cbhI* terminator of *T. reesei*, was used to

generate strain RH32919. pAB510-LipPS, in which the lipase gene was under control of the same *cbhI* promoter and *cbhI* terminator and secretion was mediated by the *cbhI* signal sequence was used to generate strains, which are the parallel transformants from same transformation, RH32924, RH32926 and RH32927; pAB600-LipPS, in which the lipase gene without signal sequence was fused with a *cbhII* (cellobiohydrolase II) carrier was used to generate the strains RH32928 and RH32929. The fermentation process was investigated in batch and fed-batch mode. Efficient production of the lipase was achieved by optimization of the fermentation process used batch conditions (pH 5.5, 28 °C, 161 h) in monosaccharide medium D5 (5% monosaccharides, 0.7% (NH₄)₂SO₄, 0.3% KH₂PO₄) (Additional file 1: Fig. S3). The supernatants were used for determination of enzyme activity (Additional file 1: Fig. S4).

Determination of enzyme activity

Enzyme activity was determined photometrically using a temperature controlled multi-mode plate reader (Synergy™ 2, BioTek Instruments GmbH, Bad Friedrichshall, Germany) by performing esterase and lipase activity assays with various *p*-nitrophenol (pNP) esters (Purdy and Kolattukudy 1973; Winkler and Stuckmann 1979). For the hydrolysis of pNP-acetate (pNPA), pNP-butyrate (pNPB), pNP-valerate (pNPV), pNP-hexanoate (pNPH), and pNP-octanoate (pNPO), an esterase assay was performed using the following conditions: 130 µL of potassium phosphate buffer (80 mM, pH 7.0) supplemented with 0.5% v/v Triton X-100 (without Triton X-100 for pNPA) was mixed with 20 µL of enzyme solution in a 96-well microplate, starting the reaction by adding 50 µL of substrate solution (3.5 mM of one pNP ester). The reaction was monitored for 10 min at 405 nm and 30 °C. To determine lipase activity, 50 µL of the enzyme solution was mixed with 200 µL of the substrate solution: 0.4 mM pNPP in potassium phosphate buffer (50 mM, pH 8.0), which was supplemented with 2.2 g L^{−1} sodium deoxycholate and 1.1 g L^{−1} gum arabic. The activity was measured at 37 °C for 10 min. For both assays, one unit of enzymatic activity refers to the amount of enzyme that hydrolyzes 1 µmol of pNP ester per minute under the respective conditions (esterase assay: $\epsilon_{\text{p-nitrophenol}} = 0.00985 \text{ L } \mu\text{mol}^{-1} \text{ cm}^{-1}$ (Purdy and Kolattukudy 1973), lipase assay: $\epsilon_{\text{p-nitrophenol}} = 0.0183 \text{ L } \mu\text{mol}^{-1} \text{ cm}^{-1}$ (Winkler and Stuckmann 1979)). Besides the pNP-esters, glyceryl trioctanoate (TriC8:0) (Merck, 0.12 mol L^{−1} stock solution dissolved in DMSO) as a triacylglyceride was used as substrate. The activity was measured in 5 mM HEPES buffer (pH 8.0) with a non-esterified free fatty acids (NEFA) colorimetric assay kit (Elabscience Bionovation Inc., Houston, TX, USA) according to the manufacturer's protocol after 1, 2 and 4 h of reaction at 30 °C,

950 rpm. Phospholipase A activity was examined with the EnzChek® phospholipase A2 assay kit (Thermo Fisher Scientific, Dreieich, Germany) according to the manufacturer's manual. PSA_Lip was also tested for choline esterase activity with an acetylcholinesterase activity assay kit (Sigma-Aldrich) following the manufacturer's instructions. Feruloyl esterase activity was examined by two methods, a photometric assay, modified from Ralet et al. (1994) and an HPLC method as demonstrated by Linke et al. (2013). The photometric assay is based on a shift of absorption maximum from 322 nm, corresponding to methyl ferulate, to 293 nm, which indicates formation of free ferulic acid at pH 6.0. The assay mixture was composed of 50 µL enzyme solution mixed with 940 µL of 1.3 mM ferulic acid methyl ester, 1 mM sodium azide and 2% (v/v) ethanol in 100 mM MOPS buffer (pH 6.0). Heat inactivated enzyme (incubated 10 min at 95 °C) served as control. After incubation at 37 °C and 200 rpm for 72 h, a UV/Vis spectrum from 280 to 340 nm was recorded. To detect feruloyl esterase activity via HPLC–DAD (LC-20 with Degaser DG-20A5R, quaternary pump LC20AD, Autosampler SIL-20A8HT, column oven CTO-20AC, Controller CBM-20A and PDA-Detector SPD-M20A, Shimadzu, Kyoto, Japan) at 325 nm, 125 µL enzyme solution was mixed with 375 µL substrate solution (100 mM tartrate–succinate buffer pH 6.0, 3% (v/v) DMSO, 2 mM 5-O-transferuloyl-arabino-furanose), incubated at 37 °C for 17 h, cooled to 4 °C, and supplemented with 500 µL acetonitrile. Heat-inactivated enzyme served as a control. 1 mM sodium azide was used to inhibit laccases. Prior to analysis, the samples were filtered through 10 kDa MWCO centrifugal filter units (Merck). 10 µL of filtrate was injected into HPLC–DAD for separation of substrate and free ferulic acid using a 250/4 Nucleosil 100–5-column (Macherey–Nagel, Düren, Germany). The mobile phase consisted of 0.05% (v/v) formic acid in water (A) and 0.05% (v/v) formic acid in acetonitrile (B). The following gradient was run: 90% A (1 min), linear gradient from 90 A to 0% A over 10 min, 0% A (5 min), linear gradient from 0 to 90% A over 1 min, 90% A (2 min) at 1.5 mL min^{−1} flow (Additional file 1: Fig. S5).

The acetylxyylan esterase activity of PSA_Lip (0.3 U based on pNPO activity) towards D-xylofuranose tetraacetate (4 mM) and α-D-(+)-glucose pentaacetate (4 mM, in 10% ethanol) dissolved in potassium phosphate buffer 80 mM pH 8 was determined by TLC using pre-coated plates (Silica Gel 60 F₂₅₄, 0.25 mm; Merck) with water/methanol/ethyl acetate, 1:2:7 (v/v), as the mobile phase. Sugars were visualized with a solution containing 120 g of (NH₄)₂MoO₇·24H₂O and 5 g of (NH₄)₂Ce(NO₃)₆ in 800 mL of 10% H₂SO₄. The fully deacetylated sugars were used as control (4 mM). A reaction with an acetylxyylan esterase from *Orpinomyces* sp. (5 U, Megazyme, Bray, Ireland) was used as positive control. The reactions were carried

out at 30 °C and 600 rpm (HLC Cooling-ThermoMixer MKR 23, DITABIS - Digital Biomedical Imaging Systems AG, Pforzheim, Germany) and monitored over 24 h, while samples were taken at different time points (0.25 h, 4 h, 24 h) (Additional file 1: Fig. S6).

Protein purification and mass spectrometry-based protein identification

The *T. reesei* supernatant containing recombinant PSA_Lip was purified using size-exclusion chromatography (HiLoad 16/60 Superdex 200, Cytiva, Uppsala, Sweden) with 0.6 mL min^{−1} flow with potassium phosphate buffer (80 mM, pH 7.0) as mobile phase. The fractions with the highest hydrolytic activity against pNPO were collected for gel electrophoresis and subsequent mass spectrometric analyses. The fractions were concentrated via ultrafiltration using a centrifugal filter unit (10 kDa MWCO, Merck). The purified and filtered fractions were mixed with semi-native loading buffer and semi-native sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) (semi-native refers to: no heat denaturation, no mercaptoethanol, 50% SDS concentration in all buffers) was performed adapted from Laemmli using a 4% stacking and 12% resolving gel and Coomassie Brilliant Blue R250 staining (Additional file 1: Fig. S7) (Laemmli 1970). Respective proteins were cut from the gel and mixed with RapiGest™ SF solution (Waters GmbH, Eschborn, Germany), approx. 10 µL per 15 µg protein, and incubated for 20 min at 80 °C. After cooling, 5 µL 100 mM dithiothreitol (DTT) was added and the mixture was heated to 60 °C for 20 min. Then 5 µL of 200 mM 2-iodoacetamide (IAA) was added and the mixture was incubated for 30 min in the dark at room temperature. Afterwards, the solution was mixed with 1.5 µL trypsin (1 µg L^{−1}, Promega GmbH, Mannheim, Germany) and incubated overnight at 37 °C. The samples were purified using Zip-Tip® filter tips as described in the instruction manual (Merck, Darmstadt, Germany). After vacuum drying, the samples were resuspended in 3% acetonitrile/0.1% formic acid (v/v in H₂O). The resulting peptides were separated by UltiMate 3000 RSLC UHPLC system (Thermo Fisher Scientific) equipped with a Kinetix C18 column (Phenomenex, 2.6 µm, 100 Å, inner diameter 2.1 mm, 100 mm length) coupled with a Q Exactive HF-X Orbitrap (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer. Chromatographic analysis was performed at a flow rate of 250 µL min^{−1} with water/0.1% formic acid (mobile phase A) and acetonitrile/0.1% formic acid (mobile phase B). The gradient elution of 60 min was performed as follows: 3% B for 5 min, followed by 3–40% B over 40 min, 40–50% B over 5 min, 50–98% B over 5 min, and re-equilibration in 3% B. The mass spectrometer was operated in data-dependent acquisition mode (top-15 DDA) with the following parameters in full MS scans: a

mass range of m/z 350–1800, mass resolution of 120,000, automatic gain control (AGC) target of 3×10^6 , injection time (IT) of 50 ms, and MS/MS scans with mass resolution of 30,000, AGC target of 1×10^5 , IT of 120 ms, isolation window m/z 1.3, and dynamic exclusion of 60 s. The raw files were processed using Proteome Discoverer version 2.4 (Thermo Fisher Scientific) with SEQUEST and MS Amanda search engines against the PSA_Lip databases (Additional file 1: Fig. S8).

Biochemical characterization

For detailed characterization of *P. sapidus* lipase (PSA_Lip), photometric esterase and lipase assays (see: Determination of enzymatic activity) were performed. For determination of temperature and pH optima, as well as stability, pNPO was used as model substrate. The optimum reaction temperature was determined by incubation of assay solutions and using the heater of the micro plate reader at the respective temperature. To evaluate the temperature stability, portions of 100 μ L enzyme solution were incubated for 60 min at the respective temperature. The graphical analysis of measured activities in relation to the temperature formed the typical sigmoid curve. Its turning point can be calculated by fitting the Y-values and is equivalent to the T_{50}^{60} value of PSA_Lip, which indicates a decrease of enzymatic activity by 50% after incubation at this temperature for 60 min. To determine the pH optimum, the esterase assay was performed using 100 mM Davies buffer (Davies 1959), which was set to the desired pH. PSA_Lip was incubated for 60 min at different pH values. The data obtained with different concentrations of each substrate for the Michaelis–Menten kinetics were analyzed with OriginPro® 2021 (category: enzyme kinetics, function: Michaelis–Menten, iteration algorithm: Levenberg Marquardt, without weighting). Equation for the Michaelis–Menten fit:

$$y = \frac{v_{max} \cdot x}{K_M + x}$$

Protein concentrations were determined by Bradford assay, ROTI® Nanoquant (by Carl Roth), following the manufacture's manual.

Bioinformatic and phylogenetic analysis

For better understanding of the potential classification of PSA_Lip, a PSI-BLAST (Altschul et al. 1990) search with the non-redundant protein database was conducted, where the scoring matrix BLOSUM62 and gap cost of 11 for existence and 1 of extensions with a PSI-BLAST threshold of 0.005 were used. The twelve most similar sequences to the PSA_Lip, 27 GDSL lipases from Akoh et al. (2004), and seven CE16 family proteins from <http://www.cazy.org/> as well as seven α/β -lipases from

Basidiomycota and yeast were used to perform a multiple sequence alignment with ClustalW (Thompson et al. 1994). The phylogenetic tree generated using Geneious v.9.1 (Biomatters, Ltd., Auckland, New Zealand), was inferred under the maximum likelihood (ML) (Guindon et al. 2010) by using the PhyML plugin, substitution model (Le Gascuel) LG with 100 bootstraps, all other parameters were set as default (Guindon et al. 2010). The calculation of identities and similarities of the PSA_Lip was done with the EMBOSS Stretcher online tool (ebc.ac.uk/Tools/psa/emboss_stretcher) (Myers and Miller 1988) and the consensus sequence was visualized with WebLogo v.3 (Crooks et al. 2004).

A hybrid homology model was built based on the PSA_Lip sequence using the automatic homology modelling script of YASARA v.17.1.28 (Krieger et al. 2002). This script automatically performs sequence alignments, loop building, side chain modelling, and energy minimization (Krieger et al. 2002). Herein, the script ran three PSI-BLAST (Altschul et al. 1997) iterations to build a total of 25 models based on five template structures (PDB codes: 6UQV, 6JLZ, 5XTU, 3KVN and 4HYQ). Out of the 25 models, the best parts were automatically chosen to construct a hybrid model. Structural alignments using the homology model and structures from the protein database were performed with Pymol™ v.2.3.4. The ExPASy compute pI/Mw tool (Gasteiger et al. 2005) was used to calculate molecular mass and isoelectric point of the protein. Potential homologies to carbohydrate-active enzymes were analyzed by searching the dbCAN meta server (Zhang et al. 2018) based on the PSA_Lip sequence.

Results

Identification of a *Pleurotus sapidus* lipase

In previous work, the secretome of *P. sapidus* has been mined for different enzyme activities, which for example led to the identification of a dye-decolorizing peroxidase (Lauber et al. 2017). Since there are few reports on basidiomycetous lipases (Zorn et al. 2005a, b; Bancarz and Ginalska 2007; Singh et al. 2014; Sowa et al. 2022), *P. sapidus* culture supernatants were also screened for respective activities using *para*-nitrophenol palmitate (pNPP) as model substrate. Monitoring of a *P. sapidus* culture in SNS medium over 8 days revealed a maximum activity of 2 U L⁻¹ at day 8. To enhance lipase production, potential lipase substrates, either 1% v/v corn germ oil (Mazola®, Peter Kölln GmbH & Co. KgaA, Elmshorn, Germany) or 0.4% v/v Tween® 80 (Boekema et al. 2007) were added to the medium, replacing glucose as sole carbon source. While there was no significant difference with corn germ oil, Tween® 80 did indeed induce lipase formation and a maximum activity of 25.8 U L⁻¹, which was observed after five days of cultivation (Fig. 1A).

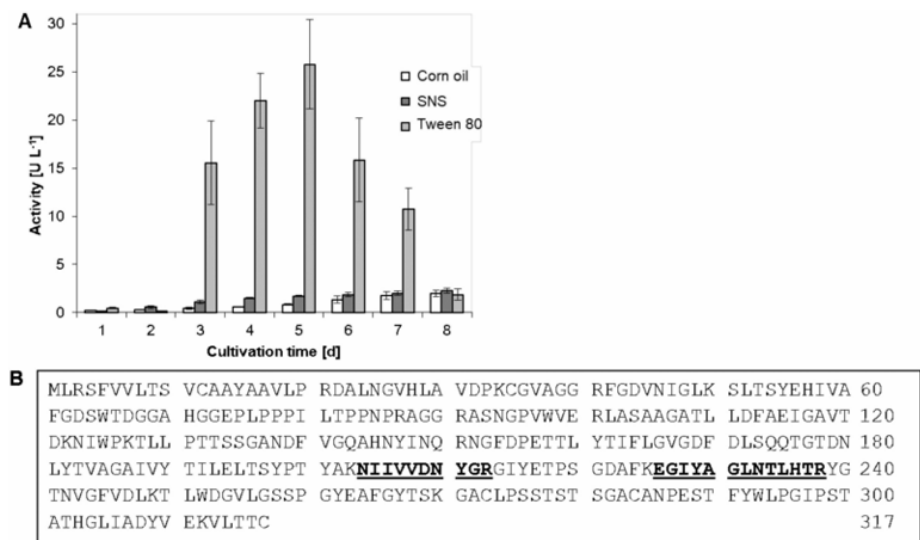


Fig. 1 **A** Comparison of extracellular lipase activity of *Pleurotus sapidus* cultures in three different media (SNS, and SNS supplemented with either 1% (v/v) corn oil or 0.4% (v/v) Tween® 80) against pNPP on different days of cultivation. **B** Sequence of hypothetical GDSL lipase from *Pleurotus ostreatus* (Genbank accession number: KAF7436219.1). Peptide fragments identified after MALDI-MS/MS analysis of *P. sapidus* secretome are highlighted

Consequently, a *P. sapidus* mycelium sample was taken from culture day 5 in Tween® 80 medium to isolate total RNA and to synthesize cDNA libraries (Additional file 1: Fig. S1), aiming to identify the lipase sequence. *Pleurotus sapidus* lipase specific primers for the respective gene were derived from the DNA sequence of a putative GDS(L) type lipase from *P. ostreatus* (Genbank acc. No.: KAF7436219.1) and amplified a 954 bp gene sequence (Additional file 1: Fig. S2) coding for a 317 amino acid protein, the putative *P. sapidus* lipase (PSA_Lip, Fig. 1B) with a calculated molecular mass of 33.5 kDa and an iso-electric point of 4.9.

Bioinformatic analysis of PSA_Lip

For identifying similar enzymes, a PSI-BLAST (Altschul et al. 1997) similarity search was performed, which identified only experimentally uncharacterized enzymes from different Basidiomycota. However, some of them were predicted to fall into the CE 16 family of enzymes with acetylsterase activity, based on the BLAST entries. Encouraged by that, the dbCAN meta server (Zhang et al. 2018) was used with the PSA_Lip sequence as query yielding no result for any potential CAZymes, neither with the HMMER search nor with the DIAMOND algorithm. Then, seven different CE 16 family protein sequences and as well as 27 different GDSL lipases (Akoh et al. 2004), and, as outliers known α/β -lipases from Basidiomycota and yeast, were downloaded to analyze their phylogenetic relations (Fig. 2). As expected, the most similar proteins to the PSA_Lip group clearly clustered together (upper red box in Fig. 2), while the GDSL

lipase from Akoh et al. used in this study clearly group in three groups, which can be defined as plant-based group (green box in Fig. 2) and two bacterial groups (orange boxes in Fig. 2). The CE 16 family proteins from the CAZyme database are not grouping together. This indicates that the PSA_Lip and similar enzymes are different from the currently classified GDSL enzymes and especially from the known CE16 family proteins. The consensus sequence block analysis yielded clear differences in the sequences (Fig. 3).

To gain more insight into its structure-to-function relationship, 3D homology models of PSA_Lip were built. The closest PSI-BLAST hit and thus the underlying template structures were a choline esterase from *Pseudomonas aeruginosa* with PDB code 6UQV, and a phospholipase A from *Vibrio vulnificus* (PDB code: 6JKZ), though sequence homologies (Myers and Miller 1988) to PSA_Lip of 36–40% were in a medium range. Sequence identities were determined to be 26.6% to 6UQV and 23.8% to 6JKZ using EMBOSS Stretcher online tool. Therefore, the quality of the resulting model was relatively poor, with a Z-score of – 2.120. All of the template structures are also part of the SGNH hydrolase family and hence contain a GDS(L)-like motif, so the structures and, specifically, active sites were compared to the homology model. As shown in Fig. 4, the overall structure of PSA_Lip (Fig. 4A) is more similar to a *P. aeruginosa* cholinesterase (Fig. 4B) than to the *V. vulnificus* phospholipase, (Fig. 4C), which possesses a β -sheet fold in the upper right part that the other two structures are missing. The active site region, which is located on the lower

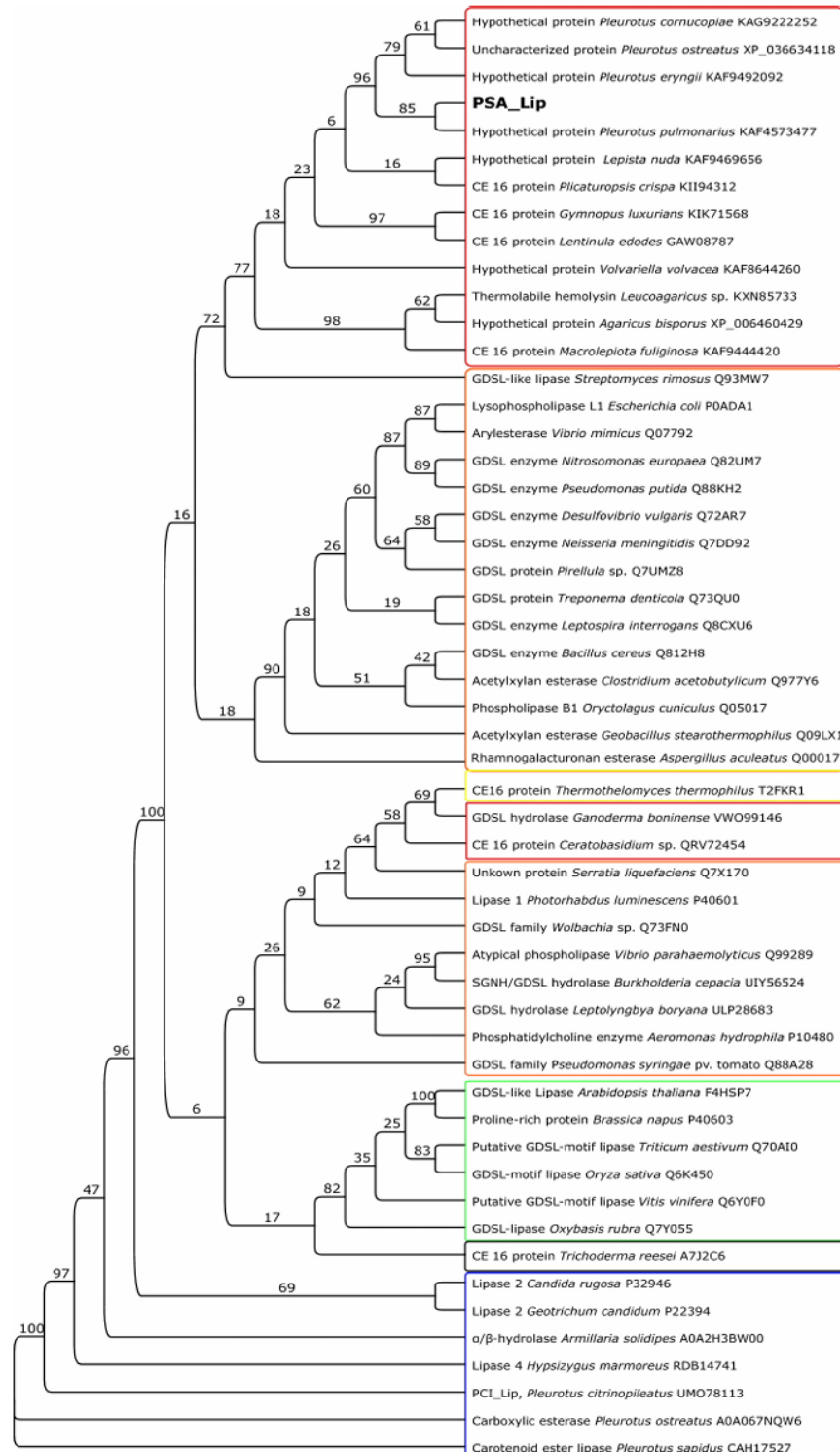


Fig. 2 Phylogenetic protein tree based on 54 different hydrolases. The alignment was done with ClustalW. The tree was inferred under the maximum likelihood (ML) by using the PhyML plugin of Geneious v.9.1. Red frame indicates Basidiomycota origin, yellow: animal origin, black: Ascomycota origin, green: Planta origin, blue: α/β -Hydrolases, orange: Bacteria origin



Fig. 3 **A** Sequence of GDS(L) lipase PSA_Lip (Genbank accession number: CFW94144.1). Peptide fragments identified after LC-ESI-HR-MS/MS analysis of purified *Trichoderma reesei* supernatants are highlighted in bold and underlining. Blue font indicates the GDS(L)W motif, whilst S64 also is part of the SGGI motif (corresponding to SGNH in other hydrolases), which is highlighted in red, green blocks indicate the five consensus blocks from plant GDSL lipase. **B** Sequence alignment with ClustalW of the PSA_Lip with a GDSL lipase from *Arabidopsis thaliana*, two bacterial phospholipases (PDBs: 6JJKZ and 4HYQ), one acetylcholinesterase (PDB: 6UQV) and one CE16 acetylcholinesterase of *T. reesei*, demonstrate the large differences. **C** Consensus analysis of the five blocks from plant GDSL lipases, **D** consensus analysis of the five blocks from CE16 GDSL hydrolyses from the cazy.org, **E** consensus analysis of the five blocks of PSA_Lip-like GDSL hydrolyses. Red squares indicated the SGNH motif, and the red arrows the catalytic S, D and H triad from the usual GDSL hydrolyses, x = no consensus amino acid at this position, no amino acids (in Block 4 of E) = part is missing in the PSA_Lip-like hydrolyses

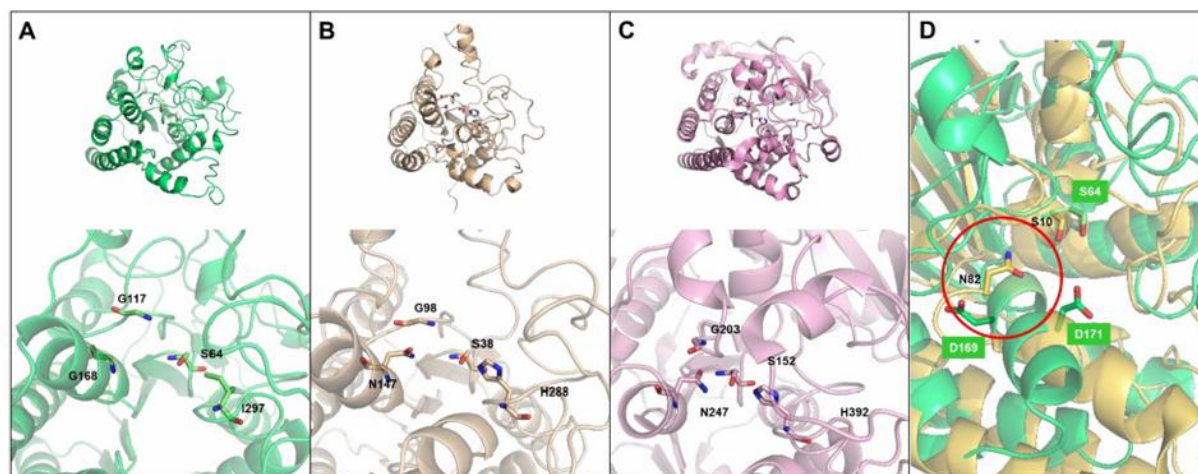


Fig. 4 **A** Homology model of PSA_Lip and enlarged detail of the putative active site with labeled SGGI-residues. **B** Structure of a bacterial acetylcholinesterase from *Pseudomonas aeruginosa* (PDB: 6UQV) and enlarged detail of the active site with labeled SGNH-residues. **C** Structure of a bacterial phospholipase A2 from *Vibrio vulnificus* (PDB: 6JJKZ) and enlarged detail of the active site with labeled SGNH-residues. **D** Structural alignment of the extracellular lipase from *Streptomyces rimosus* (PDB: 4HYQ) in yellow with the PSA_Lip in green, residues marked in white for the PSA_Lip or black for the 4HYQ, the red circle marks the shorted helix in the 4HYQ compared to the homology model of the PSA_Lip

left part of the overall structures and framed by α -helices, seems to have a similar geometry for all three enzymes. When focusing on the active side residues, the typical SGNH motif, consisting of S38-G98-N147-H288 and S152-G203-N247-H392 in 6UQV and 6JKZ respectively, is obvious (Fig. 4B, C). In contrast, the same motif cannot be found in PSA_Lip. The putative catalytic nucleophile S64 and G117 are roughly in the same position, but no asparagine and histidine or residues, which can fulfil the same role, could be found within a close distance to those residues in the generated model. When comparing the residue positions, PSA_Lip appears to rather have a SGGI motif, consisting of S64-G117-G118-I297, then the expected SGNH motif.

Heterologous expression and purification

The sequence coding for PSA_Lip was transferred to AB Enzymes GmbH (Darmstadt, Germany) to establish heterologous expression in *T. reesei*. In total, six lipase-positive strains were cultivated in 0.5-L bioreactors (Additional file 1: Fig. S3). The strain RH32919, containing the construct pAB500-LipPS, reached maximum lipase activity of 360 U L⁻¹ against pNPP after 161 h in medium D5. With RH32939 (construct pAB510)

and RH32924 (pAB600-LipPS), maximum activity was observed in medium D5 after 137 h with 340 U L⁻¹ or 280 U L⁻¹, respectively (Additional file 1: Fig. S4). During a second expression in strain RH32919, samples of the supernatant were taken after 88.2 h, 115.5 h, 137 h and 161 h. The hydrolysis profile towards different pNP-esters was determined for each sample (Fig. 5A). In all samples, the maximum activities were reached after 161 h, except for lipase activity against pNPP, which slightly decreased after 137 h. Overall, the activities towards pNPA (729 U L⁻¹), pNPB (350 U L⁻¹) and pNPO (540 U L⁻¹) were the highest at 161 h. The same samples were also used to examine feruloyl esterase activity of PSA_Lip, but only very low conversions were detected via photometric assays and HPLC–DAD (Fig. S5). Hence, these samples were used to purify PSA_Lip from *T. reesei* supernatant to further investigate the enzyme. Size-exclusion chromatography was performed, and the eluted fractions were then merged into three major fractions (F1, F2 and F3) based on the UV chromatogram (5B). After ultrafiltration, hydrolysis profiles were determined for the three samples (Fig. 5C). Whereas F2 did not display significant esterase activity, F1 and F3 displayed distinct differences. Whilst F1 showed highest activities against pNPB

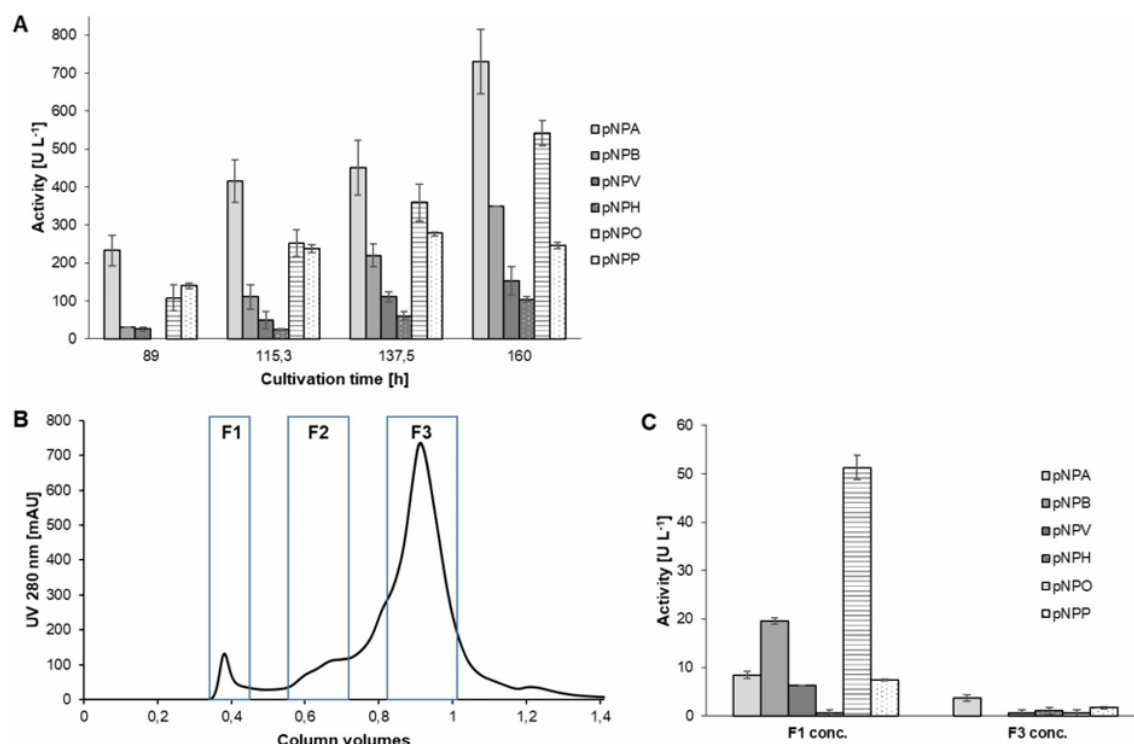


Fig. 5 **A** Hydrolysis profiles of *T. reesei* culture supernatants samples during PSA_Lip expression at different time points of the cultivation. **B** Size exclusion chromatography for purification of PSA_Lip using HiLoad 16/60 Superdex 200 column. For the framed sections the fractions were pooled to samples F1, F2 and F3 for further analyses. **C** Hydrolysis profile of SEC purified samples F1 and F3 after ultrafiltration and concentration (10 kDa MWCO). F2 did not display significant hydrolytic activities

(19.6 U L⁻¹) and pNPO (51.3 U L⁻¹), the best substrate for F3 was pNPA (3.7 U L⁻¹), indicating that PSA_Lip is contained in F1. The purification factor of F1 was determined to be 36.7 (Table 1).

A subsequent semi-native SDS-PAGE (Additional file 1: Fig. S7B) did not show a protein band at the expected size of ca. 33.5 kDa in F1. Therefore, the two bands in F1 (F1_1 with ~118 kDa and F1_2 with >212 kDa) and one band in F3 (at the expected size) were cut, and after tryptic digestion subjected to liquid-chromatography high-resolution tandem mass spectrometry (LC-HR-MS/MS) analysis to confirm the presence of PSA_Lip. As shown in Figs. S7 and S8, the five peptide fragments of the F1_1 band showed PSA-Lip sequence similarity with a sequence coverage of 24.6% (Fig. 3A, Fig S7–8).

Biochemical characterization

Samples of PSA_Lip culture supernatant were also used for electrophoretic analyses to explore more preliminary characteristics of PSA_Lip, by isoelectric focusing, followed by activity staining with α -naphthyl acetate, again combined with Coomassie staining, an estimated pI of 5.0 was determined (Additional file 1: Fig. S9), which also supported the in silico result of pI 4.9. To characterize the purified PSA_Lip, the effects of pH and temperature were investigated using pNPO as a model substrate, as the activity towards this substrate was the highest. The optimum pH value was determined to be pH 8 (Fig. 6A). Storing the enzyme at 4 °C in buffer with different pH values revealed high pH stability. When stored at a range from pH 5 to pH 10, PSA_Lip retained at least 53% of activity, even after seven days of storage. At pH 10, the enzyme was most stable for at least 24 h and, after seven days, still 73% of the initial activity was detected (Fig. 6B). The optimum reaction temperature was at 65 °C (Fig. 6C) and the T₅₀⁶⁰ value of PSA_Lip was determined to be 48.5 °C. After 60 min of incubation at 65 °C, no residual activity could be detected (Fig. 6D). Kinetic parameters of PSA_Lip were examined for the model substrates pNPB, pNPO and pNPP. With the substrates pNPB and pNPO, Michaelis–Menten kinetics were observed by outlining saturation curves for the substrates (Table 2). The catalytic efficiency (k_{cat} K_M⁻¹) towards pNPO with 6556 s⁻¹ mol⁻¹ L was roughly a quadruple of the respective parameters for pNPB (1607 s⁻¹ mol⁻¹ L), which corresponds well to the hydrolysis profile (Fig. 6E, F).

Interestingly, it was not possible to fit the data for pNPP according to the Michaelis–Menten equation, so no kinetic data could be calculated (Additional file 1: Fig. S10). With regards to the bioinformatic analyses, it was examined whether the enzyme has (acetyl-)cholinesterase or phospholipase A2 activity, though no significant activity was detected with the respective assays. Moreover, no acetylxylose or acetylglucose esterase activity for the PSA_Lip was found (Additional file 1: Fig. S9), while low activity for ferulic acid methyl ester (~8.3% conversion) and the feruloylated carbohydrate 5-O-transferuloyl-arabino-furanose (~0.8% conversion) was found. The PSA_Lip released octanoate for the entire investigated time, while the highest activity of 11.2 ± 1.6 mU mg⁻¹ was present after 1 h (Table S2).

Discussion

The identified PSA_Lip hydrolytic enzyme from *P. sapindus* was expressed under induced conditions using Tween® 80, as the gene could be amplified from the cDNA or the protein was found by MS (Fig. S2). It was already demonstrated by several groups that the secretion of fungal and in particular basidiomyceteous enzymes is highly dependent on the culture conditions as the organisms have to adapt to the challenging environmental conditions (Filiatrault-Chastel et al. 2021; Gressler et al. 2021). However, normally large amounts of different enzymes are induced (Xie et al. 2016) and some of them have catalytic promiscuity to a certain degree (Leveson-Gower et al. 2019), so that the function and the mechanism of those enzyme are often predicted but not totally solved. Moreover, basidiomyceteous enzymes often do not have simple counterparts in the better studied Ascomycota (Liers et al. 2011; Brack et al. 2022). Therefore, the information on basidiomyceteous enzymes in databases is still underrepresented, making the predictions even more tedious. In regard of this, a function mining of the PSA_Lip was done, using database information about GDS(L) hydrolases, which were discovered since 1995 (Upton and Buckley 1995) and a vast amount of already known functions exists (Akoh et al. 2004). While using different kinds of predictions, only moderate identities to previously characterized GDSL enzymes phospholipase, acetylcholinesterase and acetylxyloxyan esterase, from the carbohydrate esterase family 16, with 23.8–25.1%, 26.6% and 28.4% respectively, were found, giving no clear information about the function of the enzyme. In the literature, no distinct threshold for a precise identification is denoted. In general >50% identity (Espadaler et al. 2008) is suggested, however sometimes lower identities may be sufficient, as e.g. shown for imine reductases with 29% identity (Gand et al. 2014). In contrast, even a high identity did not provide a clear distinction of function, as shown for two enzymes, a fatty acid isomerase

Table 1 Specific activities towards pNPO and purification factors for PSA_Lip

Sample	Specific activity [U mg ⁻¹]	Purification factor
<i>T. reesei</i> supernatant (161 h)	0.076	1
SEC F1	2.797	36.7
SEC F3	0.059	0.6

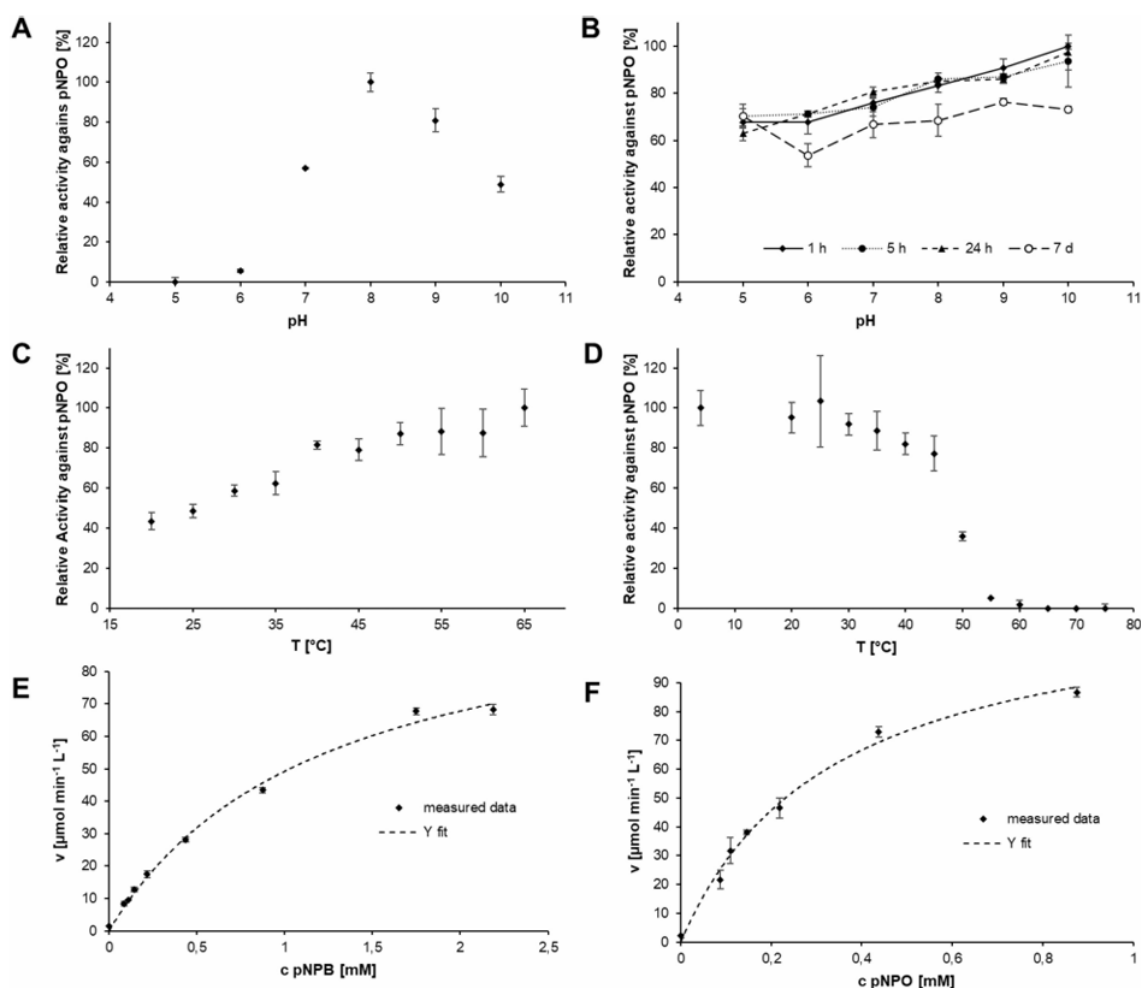


Fig. 6 **A** Optimum pH of PSA_Lip. **B** pH stability of PSA_Lip after 1 h, 5 h, 24 h and 7 d of incubation at the respective pH and 4 °C. **C** Effect of temperature on PSA_Lip activity. **D** Temperature stability of PSA_Lip after incubation of 1 h at the respective temperatures. All four characteristics have been determined by measuring activity towards pNPO. **E** Saturation curves of PSA_Lip toward pNPB and **F** pNPO. Activity data were fitted via OriginPro® 2021 based on the Michaelis–Menten kinetics model and equation

Table 2 Apparent kinetic constants of the purified recombinant lipase from *P. sapidus*

Substrate	$v_{\max}$ [$\mu\text{mol min}^{-1} \text{L}^{-1}$]	K_M [mmol L ⁻¹]	k_{cat} [s ⁻¹]	$k_{\text{cat}} K_M^{-1}$ [s ⁻¹ mol ⁻¹ L]
pNPB	108.98	1.22	1.95	1607
pNPO	122.27	0.33	2.19	6556

and a fatty acid hydratase, which share 99.5% identity (Fibinger et al. 2016). If only the total sequences are compared, the resulting phylogenetic tree looks irregular for the known CE16 proteins, as they did not cluster into one particular clade (Fig. 2), but differences are known for bacterial and eukaryotic enzymes in the same CAZyme families (Chettri et al. 2020), especially for the CE16 family (Urbániková 2021). However, the great differences of

the PSA_Lip-like proteins from the described enzymes is clearly obvious. To depict the differences even more, a motif analysis with (plant) GDSL hydrolases and a selection of CE16 GDSL(L) type hydrolases was done. According to Akoh et al. (plant) GDSL hydrolases have five distinct sequence motif blocks (Akoh et al. 2004). Those motifs were investigated showing huge differences from the PSA_Lip-like enzymes from Basidiomycota (Fig. 3E) to known plant GDSL hydrolases (Fig. 3C), while higher similarities were found for CE16 GDSL(L) type hydrolases (Fig. 3D). These findings fit very well to the in silico analysis of Urbániková, who identified five conserved blocks as well, while that work was focussed on all acylesterases annotated in the CAZy database (Urbániková 2021). It was clearly shown that fungal CE16

have not a GDSL but a GDS(Y/W/F) motif, while GDSY is the dominating form of the motif (Fig. 3E, block 1). In block 3 the “conserved” GxND (position 6–9 in Fig. 3C, E, Block 3) is different from the PSA_Lip-like enzymes where asparagine is predominantly exchanged to glycine. In block 5, the already described enzymes exhibit a D/NxxH motif, including the deposited basidiomycetous CE16 enzymes in the CAZY database. However, the PSA_Lip-like enzymes are clearly different here, having neither a histidine nor an aspartate or an asparagine. If the 3D model of the PSA_Lip is compared with the moderately similar enzymes, no other acidic or alkaline or polar residues except for D63, as part of the GDS(L) motif, (position 3 in the block 1 in Fig. 3C–E) are in a distance of <5 Å. As no crystal structure of a CE16 enzyme is deposited in the PDB database, the PSA_Lip model is a computational prediction and the relevant motifs should, therefore, only be considered as a structural aid for the observed results. Interestingly, the extracellular GDSL lipase from *Streptomyces rimosus* (PDB: 4HYQ) has one short helix compared to the PSA_Lip (Fig. 4D, red circle), which allows N82 pointing to the catalytically active S10 in this enzyme. This residue is involved in oxyanion hole formation, while this enzyme still has the histidine in suitable distance to form a catalytic dyad (Lešćić Ašler et al. 2017). A potential option for the PSA_Lip is that a “close” residue in the PSA_Lip can take over the function of the catalytic active histidine. Therefore, the distances of the S64 were analyzed (data not shown), but only D169 and D171 with 12 or 9.7 Å were identified. Those residues may work as a bridge to fix a water molecule, which hydrolyzes the enzyme–substrate complex, as they are in a relevant distance, especially if taken into account that the helix in which they are located could be shortened as in the *S. rimosus* lipase. Moreover, an induced fit mechanism for the GDSL lipases is known, which makes a precise analysis of potential residues in a homology model complicated, but at the same time underlines the potential involvement of nearby amino acid residues such as the mentioned D169 and D171. Therefore, potentially relevant residues should be investigated in more detail via structural determination and molecular dynamics analysis.

The expression of the PSA_Lip using different constructs yielded high activity against pNPO using the strain RH32919, where the *cbhI* promoter and *cbhI* terminator of *T. reesei* were used. An easy one-step SEC purification yielded a high purification factor of 36, and LC-ESI-HR-MS/MS confirmed the identity of the enzyme. In the industry, *T. reesei* is used as one of the working horses of the commercial production of different enzymes because of its generally high yields (Jørgensen et al. 2014).

The temperature and pH-optima of PSA_Lip were similar to those of enzymes with acetyl xylan esterase activity from *Schizophyllum commune* (Halgasová et al. 1994), *Streptomyces lividans* (Dupont et al. 1996) and *Bacillus pumilus* (Degrassi et al. 1998). Interestingly, it was not possible to fit a hyperbolic curve to the data for pNPP according to the Michaelis–Menten equation, so no kinetic data could be calculated, which raised the question, whether the natural functionality of PSA_Lip actually is typical lipase activity or an acylesterase of acetylated carbohydrates (either from short oligomers or from polymeric carbohydrates such as xylan). One potential reason could be that the used substrate concentrations for pNPP were insufficient to reach the saturation state, however, higher substrate concentrations could not be measured in the used assay due to solubility issues. Beside hyperbolic fits, linear and sigmoidal fits were tested, resulting in an unreasonable K_M value of $25 \pm 197 \text{ mmol L}^{-1}$ and low determination coefficients (R^2) of <0.92. Additionally, the activity for the hydrolysis of a real lipid (trioctanoate) compared to the corresponding *para*-nitrophenol ester analog (pNPO) with a good leaving group was roughly 250 times lower, providing another indication that the actual substrates may not be triglycerides. As no activity of the PSA_Lip was found for the acetylated monosaccharides, no carbohydrate deacetylase activity could be claimed, but not completely excluded. Until now, at least two types of enzymes able to deacetylate the polymer xylan have been described: (I) acetyl esterases (AE; E.C. 3.1.1.6), which remove acetyl groups from short xylooligosaccharides (XOS) (Poutanen et al. 1990; Biely et al. 2011) and (II) acetyl xylan esterases (AXE; E.C. 3.1.1.72), which are able to deacetylate polymeric xylan and in addition XOS (Biely 1985; Poutanen et al. 1990). The data generated indicate that more research in that field has to be done to better understand the enzymes involved in the degradation of lignocellulosic biomass by Basidiomycota.

The information presented in this study is useful for identifying potential functions of the PSA_Lip and similar basidiomycetous enzymes due to their unusual SGGI motif. Their exact mechanism has to be further characterized with the help of structural determination and molecular dynamics and mutagenesis of different amino acid residues in the near future. Maybe, these enzymes will form a new CE family and will be applied at slight alkaline conditions due to their high stability at this pH range.

Abbreviations

CAZymes	Carbohydrate active enzymes
CE	Carbohydrate esterases
d	Days
DTT	Dithiothreitol
EMBOSS	European Molecular Biology Open Software Suite
ExPASy	Expert protein analysis system

HPLC–DAD	High performance liquid chromatography diode-array detection
LB	Lysogeny broth
min	Minutes
MALDI-MS/MS	Matrix-assisted laser desorption/ionization mass spectrometry
MOPS	3-(N-Morpholino)propanesulfonic acid
ML	Maximum likelihood
MWCO	Molecular weight cut-off
PDB	Protein data bank
pNPA	p-Nitrophenyl acetate
pNPB	p-Nitrophenyl butyrate
pNPO	p-Nitrophenyl octanoate
pNPP	p-Nitrophenyl palmitate
PSA_Lip	<i>Pleurotus sapidus</i> lipase
PSI-BLAST	Position-specific iterative basic local alignment search tool
rpm	Rounds per minute
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SNS	Standard nutrient solution
TLC	Thin layer chromatography
T ₅₀ ⁶⁰ value	Temperature at which enzymatic activity is reduced to 50% after 60 min
U	Units (μmol min ⁻¹)
v/v	Volume per volume
XOS	Xylooligosaccharides

Supplementary Information

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Additional file1

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Author contributions

MAF and LH performed the experimental work on enzyme purification and its biochemical characterization and wrote the manuscript. CL performed the cultivation of the PSA fungus and work on enzyme purification. DK and NB performed the experiments for the TLC, PG and BS provided and performed LC–MS/MS analysis, TS and KL were responsible for the cloning and heterologous expression of the lipase, HZ applied for funding and discussed the results with MAG, who designed the study. All authors read and approved the final manuscript.

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Availability of data and materials

We conducted experiments and data were generated. All data is shown in Figures and Tables within the article.

Declarations

Ethics approval and consent to participate

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

Consent of publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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A semi-rational design approach to alter chain-length selectivity of PCI_Lip

Computational and experimental engineering of a *Pleurotus citrinopileatus* lipase: Structural insights and functional optimization to adapt the hydrolytic profile for cheese applications

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Computational and experimental engineering of a *Pleurotus citrinopileatus* lipases: Structural insights and functional optimization to adapt the hydrolytic profile for cheese applications

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ABSTRACT

Mutants of a golden oyster mushroom lipase (*Pleurotus citrinopileatus*; PCI_Lip), were engineered to enhance hydrolysis profiles for cheese production. Key residues affecting activity were identified by SMME (Structure model based on AlphaFold3 prediction, followed by Molecular docking, Molecular dynamics, and Experimental validation) in three rounds of mutagenesis. Double mutants S163M + L302G and L302G + L305A showed significant improvements in photometric assays. The pNPH/pNPP ratios of 11.6 ± 1.1 and 10.4 ± 1.1 of both mutants, respectively, were improved compared to the wild type's 0.4 ± 0.1 . Cheese made with S163M + L302G had a taste similar to the one prepared with a commercial enzyme in a descriptive testing, and both mutants demonstrated enhanced catalytic efficiency for short- to medium-chain fatty acids. These findings could be further confirmed by SPME-GC-MS analysis of the cheese samples. A structurally similar lipase from *Phlebia centrifuga* was investigated due to structural similarity with RMSD of 0.444 Å. The comparison of both lipases highlighted the key role of the lid domain in substrate specificity and affinity. This work advances the understanding of fungal lipases and their potential in food biotechnology.

1. Introduction

Fungi represent a vast but largely untapped source of enzymes, particularly within the phylum Basidiomycota. Their secretome, which comprises all secreted enzymes, adapts dynamically to environmental conditions such as carbon and nitrogen availability, making them highly versatile biocatalysts (Bouws et al., 2008; Filiatrault-Chastel et al., 2021). As the shift toward renewable biological resources accelerates, fungal enzymes are gaining importance in white biotechnology, particularly for biopolymer degradation and fine chemical synthesis. Currently, most applied enzymes from fungal origin in white biotechnology are derived from Ascomycota (El-Gendi et al., 2021) while Basidiomycota, comprising more than 30,000 species remain underexplored (Berger & Ersoy, 2022). There is special interest of industry in esterases that are capable of catalyzing the hydrolysis of triglycerides into glycerol and free fatty acids (FFA) and the formation of mono- and

diglycerides, commonly named lipases (EC 3.1.1.3). Those enzymes are the key players in enzyme-modified cheese (EMC), which is widely produced by the food industry to efficiently enhance cheese flavor in a cost-effective manner. By accelerating the natural ripening process through enzymatic application, EMC achieves more intense flavors by releasing free fatty acids (FFAs). Short-chain FFAs are particularly important for imparting the sharp, tangy, and savory notes characteristic of cheese types such as Provolone and Feta. Medium-chain FFAs contribute to creamy and slightly fatty flavors, while long-chain FFAs on the one hand enhance the buttery mouthfeel and add a waxy texture, enriching the overall sensory complexity of cheese, while on the other hand if their concentration is too high causing soapy off-flavors (Ebba et al., 2012; Efthymiou, 1967; Hanselman et al., 2021; Yoo & Lee, 2024). Still the most commonly used traditional lipolytic enzymes originate from various animal sources, including native milk enzymes, rennet paste, and pregastric esterases (PGEs) (Collins et al., 2003; Wilkinson,

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Doolan, & Kilcawley, 2011). PGEs, secreted by the sublingual glands of milk-drinking animals like goat kids and lambs, preferentially hydrolyze short to medium chain fatty acids (C4:0 – C10:0), which give a savory flavor in the final product (Fox & Wallace, 1997)(Moschopoulou, 2011). Most commercially available lipases, such as Piccantase A from *Rhizomucor miehei*, exhibit broad substrate specificity, hydrolyzing triacylglycerides indiscriminately. This enhances the overall cheese flavor, but due to the non-selective release of FFAs, ultimately affecting the balance and refinement of the cheese's sensory.

Traditional animal-derived lipase preparations are unsuitable for vegetarian diets and may not comply with kosher or halal dietary requirements, limiting their marketability amid the rising demand for ethical and dietary-compliant alternatives. This could be partially demonstrated by the global market value of 65 billion USD of halal ingredients was increased with 6% compound annual growth rate from 2019 to 2025 (Markets and Markets, 2019). Mushrooms from the Basidiomycota phylum naturally meet these dietary requirements and have demonstrated promising enzymatic activities, as revealed by fungal secretome studies over the past decades. A promising candidate was identified in a study by Sowa et al., where the purified lipase from the golden oyster mushroom, *Pleurotus citrinopileatus* (PCI_Lip), was used to produce cheese with sensory properties resembling those made with the PGE.

However, a slightly soapy aftertaste remained (Sowa et al., 2022). This aftertaste attributed to an enhanced release of long chain fatty acids (LCFA) is considered as an off-flavor in cheese. Therefore, the amount of LCFA should be kept low. Depending on the cheese variety the ratio of short chain fatty acids (SCFA) to LCFA should be between 0.4 and 0.7 (Delgado et al., 2011; Rani & Jagtap, 2019). To address this issue, Broel et al. successfully applied a semi-rational design strategy, supported by biomolecular simulations, to engineer site-directed mutants with improved selectivity. Their work led to enhancements in the hydrolysis activity and substrate selectivity of certain variants, contributing to a more refined cheese flavor profile. Nevertheless, a substantial amount of long-chain fatty acids was released by hydrolysis, contributing to the undesirable soapy aftertaste. Additionally, the low expression levels of PCI_Lip further limited the enzyme's potential for broader application (Broel et al., 2022).

To overcome the limitations of site-directed point mutations in improving substrate specificity, a multiple-site mutation approach, similarity like the SMME, was explored in this work. Unlike single-point mutations, which often yield limited effects on enzymatic selectivity, multiple-site mutations by combining mutations allow for a more comprehensive modification of the enzyme's active site, efficiently enhancing the specificity (Huang et al., 2023; Sandström et al., 2012). By simultaneously introducing mutations at key residues, researchers can fine-tune the enzyme's interaction with substrate molecules, reducing the hydrolysis of long-chain fatty acids while maintaining or even improving the hydrolysis of short and medium-chain fatty acids (Yang et al., 2002) essential for cheese flavor development.

This study aimed to optimize PCI_Lip for industrial applications by refining its substrate selectivity, and improving its functional properties. With the multiple site mutation approach mutants of PCI_Lip with superior lipolytic characteristics shall be identified. These mutants can help to fill the lack of non-animal derived lipolytic enzymes in dairy industries. Molecular dynamics simulations were employed to systematically analyze its structure-function relationship, providing a foundation for targeted modifications. Based on this information, we designed and generated a mutant library comprising three rounds of mutagenesis. After recombinant expression and subsequent purification, the library was tested on substrate selectivity to identify mutants with altered chain length selectivity. Through multiple-site mutations, we aimed to effectively minimize the hydrolysis of long-chain fatty acids while maintaining activity toward short to medium-chain fatty acids. Kinetic analysis was further performed to confirmed the improved hydrolytic efficiency and specificity of the engineered variants. Finally, we applied

these optimized mutants in cheese production. Sensory evaluation combined with SPME-GC-MS analysis were applied to evaluate improved substrate specificity regarding a positive impact on the taste of the cheese, highlighting the potential as superior alternatives to traditional lipases in cheese manufacturing.

2. Materials and methods

2.1. Cloning

The GenBank file for PCI_Lip can be found under accession number OL364849. For the design of the primers for PCI_Lip without lid Geneious v.9.1.8 software (Biomatters, Auckland, New Zealand) was used to create the primers with overlaps of the particular fragments. Primers were received from Biomers.net GmbH (Ulm, Germany). The respective fragments were amplified in a polymerase chain reaction (PCR). The PCR preparation contained 2.5 µL of each primer, 1 µL dNTPs (Thermo Fisher, Kandel Germany), 10 µL 5× Phusion HF buffer (Thermo Fisher GmbH), 0.5 µL Phusion polymerase (Thermo Fisher) and 0.5 µL of template and was filled up with water to a total volume of 50 µL. To apply different annealing temperatures, aliquots of 10 µL were prepared. The reaction was started by an initial denaturation step at 98 °C for 120 s followed by denaturation at 98 °C for 30 s, annealing at 47–51 °C for 30 s, elongation at 72 °C for 150 s repeated in 35 cycles and a final elongation at 72 °C for 300 s. For all PCRs T100™ thermal cycler (Bio-Rad Laboratories, Munich, Germany) was used. After the reaction the aliquots were pooled, 1 µL of *DpnI* (Thermo Fisher) was added and incubated at 37 °C for 2 h. The fragment was checked in an agarose gel electrophoresis. Therefore 9 µL of sample were mixed with 1 µL of Midori Green Direct (Biozym Scientific, Hessisch Oldendorf, Germany) and loaded on a 1% agarose gel. Electrophoresis was performed at 100 V and 1000 mA for 1 h. For evaluation the gel was exposed to UV light. After confirmation the fragment was joint using Gibson-Assembly (New England BioLabs, Frankfurt a. M., Germany). 2 µL of PCR products were added to 10 µL of 2× Gibson Assembly Master Mix and filled up with water to a total volume of 20 µL. The preparation was incubated for 15 min at 50 °C. The product of Gibson-Assembly was used for the transformation of chemo competent *Escherichia coli* 10 β (New England BioLabs). Overnight cultures in lysogeny broth (LB) medium and kanamycin (Carl Roth, Karlsruhe, Germany) as selection marker were incubated at 37 °C, 180 rpm. After incubation the cells were harvested by centrifugation and used for plasmid isolation using the NucleoSpin® Mini kit for plasmid DNA (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Isolated plasmids were sequenced by Eurofins Genomics (Ebersberg, Germany) and sequencing results were analyzed using Geneious 9.1.8 software. Plasmids with positive results were transformed into *E. coli* BL21 (DE3) Gold (New England BioLabs), for recombinant expression.

2.2. Protein engineering

Mutants of PCI_Lip were prepared according to Broel et al. The primer design for site-directed mutagenesis was based on the QuikChange™ protocol (Stratagene, La Jolla, CA, USA). For multiple variants the template plasmids contained the respective mutation. For the double mutant L302G + L305A, a single pair of primer incorporating both mutations were used due to close mutation sites. All used primers are shown in Table S1. For the mixture of PCR and PCR reaction conditions see Broel et al. After PCR reaction the aliquots were pooled and *DpnI*-digestion was performed by adding 1 µL of *DpnI* (Thermo Fisher) and incubation at 37 °C for 2 h. Firstly, the digested PCR products were transformed into chemocompetent *E. coli* 10 β (New England BioLabs). Positive transformants were selected on LB-kanamycin (Carl Roth) agar plates and used to prepare overnight cultures with adjacent plasmid isolation as described above (Broel et al., 2022). Isolated plasmids were sequenced by Eurofins Genomics (Ebersberg, Germany) and sequencing

results were analyzed using Geneious v.9.1.8 software (Biomatters). In a second transformation approved plasmids were transformed into *E. coli* BL21 (DE3) (New England Biolabs) with chaperone plasmid pG-KJE8 (TaKaRa, Kusatsu, Japan) for recombinant expression.

2.3. Enzyme production and purification

The cultivation was done according to Broel et al. *E. coli* pellets from 400 mL of culture were resuspended in 12 mL of 50 mM sodium phosphate (Carl Roth) buffer, pH 7.5, 0.3 M NaCl (Carl Roth). Cultivations of mutants were conducted once, and mutants applied in cheese production were cultivated in triplicates. For lysis sonication was applied three times for 2.5 min, 5 cycles and 50% amplitude (sonifier MS72, microtip diameter 5 mm, Bandelin Electronic, Berlin, Germany). The lysate was centrifuged at 14,000 rpm and 4 °C for 15 min. For purification immobilized metal ion affinity chromatography (IMAC) and desalting was performed according to Broel et al. Expression was checked by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Broel et al., 2022; Laemmli, 1970).

For PCI_Lip without lid which was expressed as inclusion bodies application note 18-1134-37 AC by GE Healthcare (Düsseldorf, Germany) for refolding was followed (GE Healthcare Bio-Sciences AB, 2020) (Supplementary material S1.1 Protein refolding). Subsequent desalting and SDS-PAGE were conducted as described above. The purity of all mutants was determined by densitometry (Table S3) using ImageJ v.1.54r.

2.4. Determination of enzyme activity

Lipolytic activity of the enzymes was tested in photometric assays using *para*-nitro phenyl (pNP) fatty acid esters of different chain length as model substrates. For PCI_Lip mutants pNP-acetate (pNPA) (Sigma Aldrich), pNP-butyrate (pNPB) (Th. Geyer, Renning, Germany), pNP-valerate (pNPV) (Sigma Aldrich), pNP-hexanoate (pNPH) (TCI Europe, Heuchelheim, Germany), pNP-octanoate (pNPO) (Alfa Aesar, Karlsruhe, Germany) and pNP-palmitate (pNPP) (Sigma Aldrich) were deployed. For the substrates pNPA (Sigma Aldrich), pNPB (Th. Geyer), pNPV (Sigma Aldrich), pNPH (TCI Europe) and pNPO (Alfa Aesar) an esterase assay was conducted at 30 °C with an optical path of 0.64 cm, $\epsilon_{\text{pNP}} = 0.00985 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and Triton X-100 as surfactant according to Broel et al. A lipase assay was performed with the substrate pNPP (Sigma Aldrich) at 37 °C with an optical path of 0.8 cm, $\epsilon_{\text{pNP}} = 0.0183 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and deoxycholate and gum arabic as surfactants according to Broel et al. In both assays blanks were measured substituting the lipase by potassium phosphate buffer (PPB). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of pNP in one minute in the respective assays. For the calculation of specific activity, protein concentration of the samples was determined according to the method of Bradford (Bradford, 1976; Broel et al., 2022). Briefly, an external calibration with bovine serum albumin (Carl Roth) dissolved in water between $3.125 \mu\text{g} \cdot \text{mL}^{-1}$ and $200 \mu\text{g} \cdot \text{mL}^{-1}$ was prepared. 25 μL of samples and standards were mixed with 200 μL of ROTInanoquant (Carl Roth) in a 96-well microplate. The absorbance was measured at 450 nm and 590 nm. Statistic evaluation was performed by one way ANOVA with a significance level of 0.05 in OriginPro 2023 (OriginLab, Northampton, USA).

2.5. Biochemical characterization

For the PCI_Lip mutants S163M + L302G, I245F + L302G, L302G + L305A and L302G + I529D Michaelis-Menten kinetics were determined for the substrates pNPB, pNPH and pNPO. To determine the kinetics different concentrations of the respective substrates were applied in esterase assays conducted as described above. Every measurement was taken in triplicates. The data were subjected to OriginPro 2023 for further analysis. Enzyme activities were plotted against the substrate

concentrations using a non-linear fit (category: enzyme kinetics; function: Michaelis-Menten; iteration algorithm: Levenberg-Marquardt; Eq. 1).

$$y = \frac{v_{\max} \cdot x}{K_M + x} \quad (1)$$

2.6. Production of feta-type brine cheese using PCI_Lip mutants

To examine the effects of different PCI_Lip mutants on the cheese flavor mutants F91L, S163M, I245F, L302G, L305A, I529D, F91L + I245F, F91L + L305A, S163M + L302G, I245F + L302G, L302G + L305A and L302G + I529D as well as the WT were applied in the production of Feta-type brine cheese. Cheese production was conducted according to Broel et al. Briefly, for each preparation 3 L of milk were used. Milk fat content was 4.2% adjusted with cream. Lactic acid culture (Lyofast M036L), calcium (0.3 mL of 34% *m/v* CaCl_2) and the lipase were added and incubated at 33 °C for 45 min. For comparison one cheese sample was prepared without the addition of lipolytic enzymes. As a reference the commercially available PGE opti-zym z10uc (0.35 g $\bullet$ (10L)⁻¹, optiferm, Oy-Mittelberg, Germany) was added to one cheese preparation. One U of each mutant was added to the cheese samples, except for mutants L302G + L305A only 0.5 U were added due to low amount of purified enzyme for this mutant. The amount of lipase used was based on the activity of the respective mutant to pNPO in the esterase assays which corresponds to 2.0 to 9.5 mL of purified enzyme. Then, 0.75 mL microbial rennet (opti-ren micro, 220 IMCU; optiferm,) was added. The mixture was set to rest for 1 h. After formation of cheese curd, it was cut into cubes and transferred to molds, where the whey could drain. After resting for 24 h, the loafs were submerged brine (20% *m/v* NaCl, pH 5.2) for 50 min. After 30 days of ripening at 13 °C the edges of the samples were removed to get rid of the preservative natamycin. The samples were subjected to analysis of volatile free fatty acids (vFFA) by solid phase micro extraction gas chromatography-mass spectrometry (SPME-GC-MS).

2.7. Sensory evaluation and vFFA analysis of feta-type brine cheese samples

After 30 days of ripening the cheeses prepared with different mutants were used for analysis. All cheese samples were prepared in cubes of $1 \times 1 \times 1 \text{ cm}^3$ and labeled with a random three-digit code. The panel consisted of four female and four male people, two of which were smokers. The age of the panel reached from 24 to 44 years of age. Evaluation was based on defined attributes (smell: piquant, sour, goat-like, soapy, dairy, intensity; while for the taste: piquant, sour, goat-like, soapy, dairy, bitter, intensity are used), which were rated on a scale from 0 to 5 with increasing perception. Meanwhile, the profile of vFFAs in cheese samples were analyzed by SPME-GC-MS (Supplementary material S1.2, Broel et al., 2022.). Statistic evaluation was performed by one way ANOVA with a significance level of 0.05 in OriginPro 2023.

2.8. Molecular dynamics (MD) simulation

The structure of PCI_Lip was generated by AlphaFold3. The enzymatic reaction system was constructed using Packmol (Martínez et al., 2009), and molecular dynamics (MD) simulations were performed using NAMD (Phillips et al., 2020). Details of the MD simulation protocol follow those described in our previous work (Zhang et al., 2024). The lipase protein conformation was centrally positioned within a cubic water box measuring $90 \times 90 \times 90 \text{ Å}^3$, with 10 triglyceride molecules incorporated into each system. Four distinct model triglycerides, differing in fatty acid chain length (C4:0, C10:0, C16:0, C18:0, and C18:1) were used, leading to the construction of four separate simulation units. To optimize the system and eliminate unfavorable atomic contacts, energy minimization was first conducted using a 5000-step

conjugate gradient method. Subsequently, MD simulations were initiated, gradually increasing the temperature from 0 K to 303 K under NPT conditions over 200 ps. Three independent simulations were executed for each system under identical conditions, each lasting 200 ns, with a time step of 2 fs to generate the PCI_Lip lipase trajectory files. For all MD processes, energy minimization was performed using the conjugate gradient method, while system equilibration was achieved via Langevin dynamics. Finally, the simulation data were analyzed using VMD software (Nakkala et al., 2022).

2.9. Molecular docking

Molecular docking was conducted using AutoDock Vina v.1.2.3, developed by Arthur J. Olson at the Scripps Research Institute (La Jolla, CA, USA), employing the Broyden-Fletcher-Goldfarb-Shanno optimization algorithm. Ligand and receptor preparation was carried out using the AutoDock Tools package. A semi-flexible docking approach was utilized, with the active site residue S213 designated as the docking center. All other docking parameters remained at their default settings. Four model triglycerides with distinct fatty acid chain lengths (C4:0, C10:0, C16:0, C18:0, and C18:1) were used in the simulations.

3. Results and discussion

3.1. Analysis of the active site

PCI_Lip displays a typical interfacial-activated lipase with a highly conserved catalytic center (S213, H449, E339; Fig. 1). To modify its hydrolytic selectivity, it was essential to identify the effective triglyceride-binding pocket. Therefore, MD simulations were used to investigate the hydrolytic selectivity of PCI_Lip toward four triglycerides with different carbon chain lengths, which were the main fatty acids in milk (Fig. S1) (Broel et al., 2022; Wang et al., 2025). As shown in Fig. 2A, the WT PCI_Lip exhibited the longest binding time for C16:0 indicating a higher selectivity for this fatty acid. During triglyceride binding, the crucial lid and binding pocket must undergo structural changes to accommodate the sterically hindered triglyceride molecules

entering the active pocket. Thus, the RMSD of the carbon backbone was calculated for each residue of PCI_Lip during the simulation of the four substrates (Figs. 2B and S2). The flexible region was highlighted in Fig. 2C, with special attention paid to the green flexible region as it is orientated in close proximity to the active center. The most flexible region was the interfacial-activated lid, which covers the residues A69–F91. Fig. 2D illustrates the frame in which the distance between C16:0 and the active site S213 (not shown) was at its minimum. In contrast, the other three triglyceride substrates failed to fully enter the active pocket. This may be attributed to the higher steric hindrance of C18:0 and C18:1, as well as interference caused by multiple substrate molecules present simultaneously in the reaction system. For C4:0, instability might result from its small size and high flexibility, making it difficult to position them in a potential active conformation within the pocket. Meanwhile, the smaller C10:0 substrate's failure to enter effectively could be due to relatively low affinity for the PCI_Lip's for this substrate. The MD simulation revealed that, in addition to the lid containing F91, significant structural changes occurred in the loops or α -helices where F129, S163, I245, L300, L302, L305, and I529 were located, allowing adaptation to triglyceride entry into the active center. As shown in Fig. 2E, the initial conformation of PCI_Lip formed a complete tunnel-shaped binding pocket by the residues F91, F129, S163, I245, L300, L302, L305, and I529. This tunnel shape could lead to an easy penetration of the esterified fatty acid chains of the triglycerides. Considering the substantial steric hindrance of triglyceride molecules, as well as solvation effects and intermolecular interactions, it was nearly impossible for the total substrates to perfectly enter the active center solely through MD simulations. To further validate this finding, molecular docking using S213 as the center all four triglyceride molecules were performed (Fig. 3). The results demonstrated that all four triglycerides could fit perfectly into the binding pocket (blue mesh), further supporting the above-mentioned hypothesis.

To underline the importance of the residues valuable data on mutations in the binding pocket of PCI_Lip, as shown in Fig. 4 and S7, were provided by Broel et al., which produced and partially characterized 26 variants at seven of the eight relevant substrate binding amino acids in the tunnel. Among these mutants, F91L stood out, displaying a

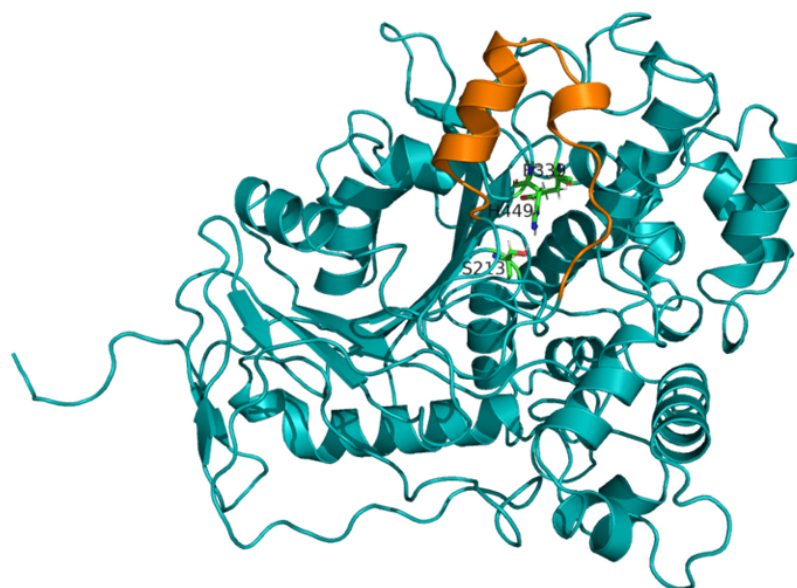


Fig. 1. AlphaFold 3 model of PCI_Lip. The lid region (A69–F91) is marked in orange. The amino acid residues of the catalytic triad (S213, H449, E339) are shown in green and labeled with the respective one letter code. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

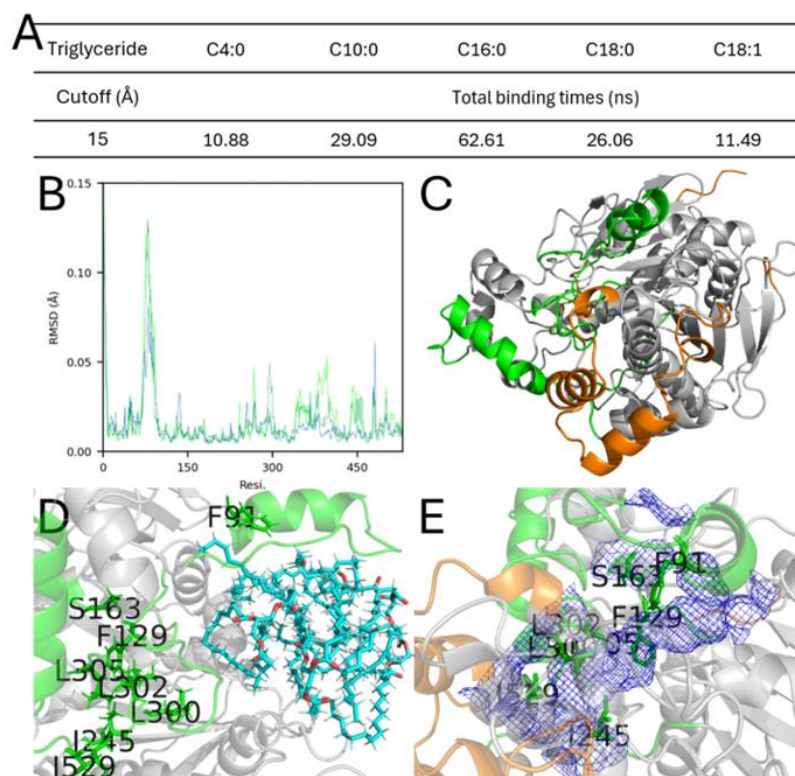


Fig. 2. Molecular docking and simulation analysis of the PCI_Lip. (A) The total binding time of the substrates entering the active center, calculated as the sum of three parallel simulations. It represents the total duration during the three carbonyl carbon atoms of the triglyceride enter the active center. (B) RMSD of each residue when C16:0 was used. (C) 3D structure of PCI_Lip. Orange: flexible region distant from the active pocket. Green: flexible region forming part of the binding pocket. (D) Snapshot of C16:0 binding to the active pocket. The frame was selected at the point of minimum distance. C16:0 was shown as cyan sticks. (E) The binding pocket of PCI_Lip shown as blue mesh. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrolysis profile similar to that of the WT. The activity of this mutant toward pNPA, pNPB, pNPV, pNPH, and pNPO remained nearly unchanged, whereas its activity against pNPP was reduced by more than 50%. This reduction in activity might be attributed to the location of F91 at the entrance of the binding tunnel. When F91 was mutated to a smaller amino acid leucine (L), it might be more flexible and rotate to block the larger substrate pNPP to access the active center, thereby reducing the hydrolysis. Furthermore, when mutations performed at I245, L300, L302, L305, and I529, significant changes in the enzyme's catalytic performance (activity and selectivity) were observed, indicating the sensitivity of these sites. This suggests that these residues play a direct role in substrate binding, thereby directly influencing hydrolytic selectivity. For example, when L302 was mutated to either glycine (with lower steric hindrance) or proline (with greater rigidity), the hydrolysis profile underwent a significant change. Although the overall activity decreased, the selectivity for medium-chain fatty acids was markedly enhanced. Although in the study by Broel et al., mutants at position F129 either exhibited almost complete loss of activity or failed to express. Additionally, since S163 is located at a highly critical position (Figs. 2E and 3), it was still included as a potential mutation site. Therefore, our selected potential mutation sites are F91, F129, S163, I245, L300, L302, L305, and I529.

3.2. Comparison of the PCI_Lip with another mushroom-derived lipase without lid

Another lipase derived from the Basidiomycota *Phlebia centrifuga*

(PCE_Lip) has been identified using Enzyme Miner (Hon et al., 2020) searching for similar lipases to PCI_Lip. With a putatively improved solubility index of 0.6771 and a sequence similarity of 66.5% as this enzyme was 2022 the only entry with high similarity (>65%) with improved solubility index compared to the PCI_Lip with an index of 0.451. The alignment of AlphaFold3 models of both PCI_Lip and PCE_Lip revealed a high structural similarity of the lipases. The RMSD was only 0.444 Å basing on their whole structures. Considering the predicted superior solubility of PCE_Lip, the recombinant expression was attempted with *E. coli* BL21 (DE3) Gold and *E. coli* BL21 (DE3) with five different chaperones provided by TaKaRa Bio Inc. No soluble expression of PCE_Lip was found in any of the different attempts. PCE_Lip could only be expressed as insoluble inclusion bodies (Fig. S10). The inclusion bodies were subjected to an on-column refolding to get soluble enzyme for determining the activity. The activity was measured photometrically using pNP fatty acid esters as model substrates. The specific activity of PCE_Lip against any pNP fatty acid ester was close to the detection limit, which is an indication that the catalytic behavior of the PCE_Lip is different to the PCI_Lip. Comparing the two lipase models the most significant difference is the N-terminal region of PCI_Lip (Fig. 5). The region M1 to V90 in PCI_Lip is assumed to be the putative lid area and not present in PCE_Lip (Fig. S8). Lids are mobile domains exposing the substrate channel when there is contact to the non-polar substrate. This interfacial activation is often seen as another criterion for the classification as a lipase. However, lipases can also have immobile elements covering the substrate channel and restricting access to the substrates, which are referred to as caps (Bauer et al., 2020). As the PCE_Lip does

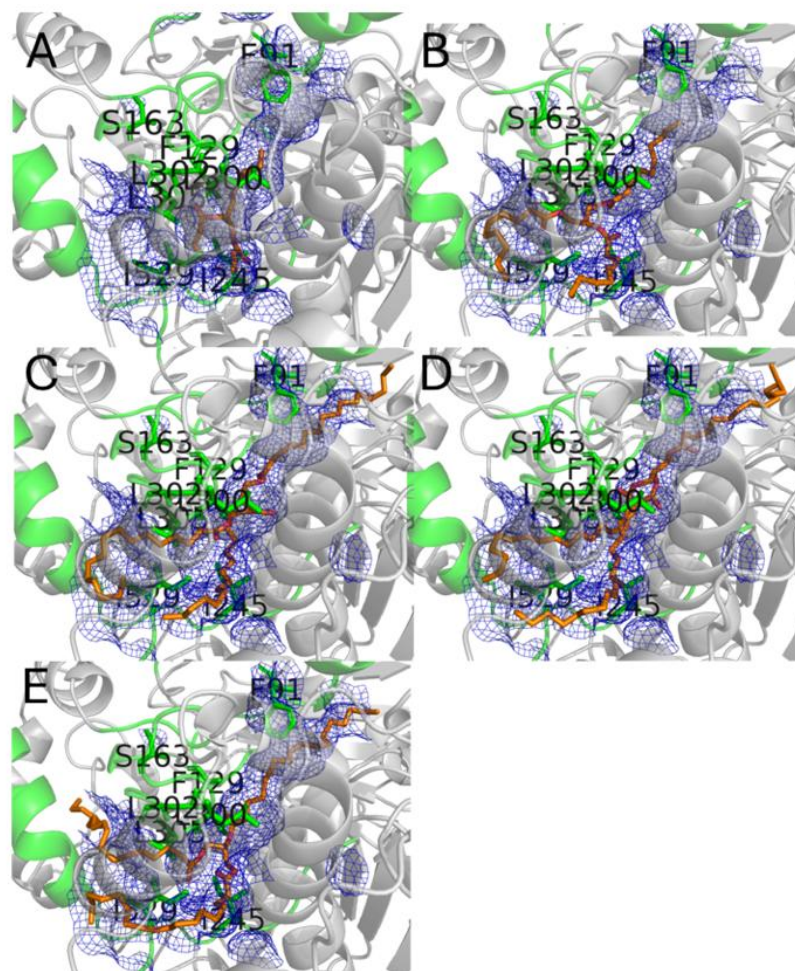


Fig. 3. Docking of C10:0 (A), C16:0 (B), C18:0 (C), and C18:1 (D) in the binding pocket of PCI_Lip (blue mesh). Substrates were shown as orange sticks. Green: flexible region forming part of the binding pocket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

not possess a lid, it can still be categorized as a lipase as a lid is no mandatory structural element. The resolution of the crystal structure of lipase A from *Bacillus subtilis* is another example for a microbial lipase without lid domain. This lipase is showing a minimal α/β -hydrolase fold with compact globular structure lacking a lid or cap domain (Van Pouderoyen, Eggert, Jaeger and Dijkstra, 2001).

Figs. S3–S7 illustrate the kinetic behaviors of five triglycerides interacting with PCI_Lip. Despite variations in effective diffusion trajectories, a common feature among all cases was the necessity for triglycerides to pass through the critical lid region (A69–F91) before reaching the active site for hydrolysis (Figs. S3A–S7A). Structural alignment analysis revealed that most of the protein maintains its rigidity due to stable secondary structures, the lid region undergoes significant conformational changes due to its flexible loops and small α -helix (Figs. S3B–S7B). These structural dynamics play a key role in regulating substrate access, while each substrate exhibited distinct binding behaviors. MD simulations revealed complex and individual movements of the lid domain depending on the different substrates. The individual changes of the lid domain are affected by the different diffusional channels of different substrates (Fig. S3C + D–7C + D). C10:0 showed notable trajectory aggregation at the back side of the active

pocket, though it could not directly pass the α -helix structures due to steric hindrance. Instead, it accessed the active site through adsorption-desorption events or lateral movement along the protein surface, ultimately crossing the lid (Fig. S4C). C16:0 displayed a higher trajectory density on the protein surface and a larger opening angle, facilitating its passage through the lid. Residue-specific affinity analysis identified the lid as the primary binding site for C16:0 (Fig. S5D), making it the most effective substrate for activating the lid. For C18:0, an additional aggregation site appeared around another α -helix and a β -strand (Fig. S6D), increasing overall hydrophobic interactions but reducing regional selectivity. Its smaller opening angle and steric resistance limited entry into the active site, decreasing binding efficiencies. Meanwhile, C18:1 exhibited random trajectory scattering across the reaction microunit (Fig. S7A), with minimal aggregation in the active pocket, suggesting weak and unstable binding. Despite lid activation, the opening angle remained relatively small, further hindering stable substrate binding. As a lid domain can strongly impact the overall characteristics of a lipase in this study it was decided to add the putative lid domain of PCI_Lip to the structurally similar PCE_Lip (Fig. 5).

To add the lid of PCI_Lip to PCE_Lip the nucleotides coding for amino acids M1 to L85 in PCI_Lip were integrated into the pET28a(+) carrying

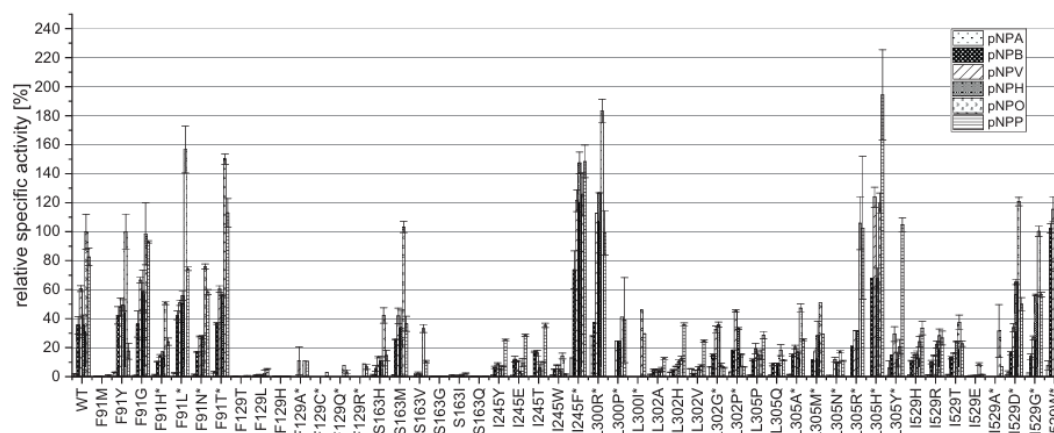


Fig. 4. Relative specific activity [%] of PCI_Lip single mutants against six *para*-nitrophenyl (pNP) fatty acid esters of different chain length as model substrates. All specific activities are compared to the specific activity of the WT against pNPO, which was set to 100%. All photometric assays were performed in triplicates. Standard deviation of the measurements is described by the error bars. Mutants marked with * have been characterized by Broel et al. For statistical evaluation see Fig. S19.

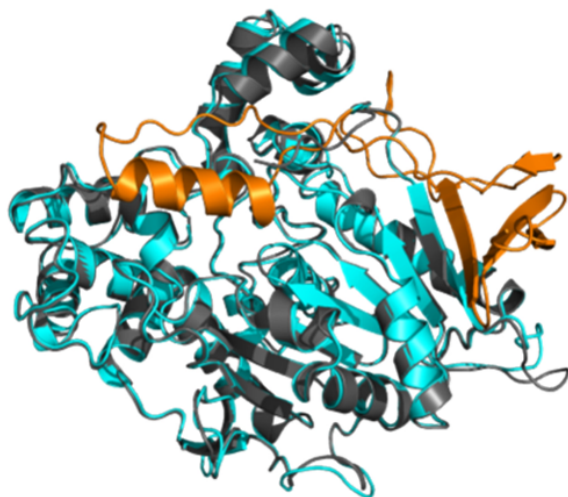


Fig. 5. Structure alignment PCI_Lip (cyan; lid orange) and PCE_Lip (black). The additional structure in PCI_Lip compared to PCE_Lip is highlighted in orange, which includes the crucial lid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the PCE_Lip gene. Recombinant expression in *E. coli* BL21 (DE3) with the chaperones DnaK-DnaJ-GrpE and GroES-GroEL led to the formation of inclusion bodies. To get soluble enzyme for further analysis an on-column refolding was performed. The activity directly after the refolding was comparable to the one of PCE_Lip without a lid, providing insights that the addition of a lid barely improved the activity. This gives reason to consider other substrate as native to this enzyme that may be less lipophilic as the enzyme does not require a lid domain.

To further investigate the role of the putative lid structure in PCI_Lip another mutant was created coding for PCI_Lip without lid domain (Fig. 5). Recombinant expression using the same expression host as before also led to the formation of insoluble inclusion bodies and on-column refolding has been conducted to investigate the influence of the lid on the activity of this lipase. In comparison to the WT the activity is drastically reduced by more than 100-times. The drastic changes in the

activity and solubility indicated that the putative lid domain in PCI_Lip is an essential structural part of this enzyme.

3.3. Tailoring the PCI_Lip via several rounds of mutagenesis

For mutations the positions F91 in the putative lid domain, S163 near the substrate channel and positions F129, I245F, L302, L305 and I529 in the substrate channel were mutated *in silico* with the 19 other amino acids (Broel et al., 2022). In total 19 out of 21 predicted plasmids containing mutations could be constructed, and the mutants could be expressed and purified (Fig. S9). Overall, most of those mutants exhibited quite low activity, in the range of 1 to 40% (Fig. 4). Mutant F91Y is standing out of this set as it displays a similar hydrolysis profile as the WT. Comparing the hydrolysis profiles of the three F91 mutants in these experiments to the four single mutants from Broel et al. the exchanges led mainly to mutants with similar profiles as the WT but with reduced activities. The position is located in a flexible domain of the putative lid area (Fig. 3). Furthermore, all mutants at positions F129 and S163 have lost almost all activity. F129 seems to be a crucial position for the enzyme activity as the single mutants predicted to have a positive impact also barely show activity. All mutations at I245, L302, L305 and I529 lead to low but still active mutants exhibiting high activities toward pNPP. The I245 mutants all display the highest affinity toward pNPP with similar hydrolysis profiles. Mutating L302 to G or P to generate improved mutants preferentially hydrolyzing pNPV and pNPH. Exchanging L for A or V reduced the activity to pNPV and pNPH but enhanced affinity for long chain fatty acids. All involved amino acids carry hydrophobic residues only differing in steric matters. Interestingly, small residues seem to support the hydrolysis of short chain substrates, while bigger residues promote hydrolysis of long chain substrates. Mutating L302 to a polar H led to a higher affinity toward pNPP. The exchange to a polar residue might hinder the short chain substrates in fully entering the substrate channel. The effects of different amino acid residues are quantitatively reflected by the selectivity ratios (Table 1). In case of position L302, a change to a smaller residue such as G markedly increased the pNPH/pNPP ratio whereas substitutions to moderate bulky residues give ratios similar to the WT. Similar effects appear at position L305. Substitutions to smaller residues increase the selectivity ratio while larger and polar residues decrease it. At the same time mutation may support the slightly different binding mode described for pNPP (Broel et al., 2022). Overall, the photometric characterization of mutants with impaired characteristics is in accordance

Table 1

List of single, double and triple mutants in the mutant library of PCI_Lip. Mutants marked with * have been characterized by Broel et al., 2022. For the overview the positions of the respective mutations inside the protein structure are listed. The ratios of pNPO/pNPP and pNPH/pNPP are calculated from the specific activities of the mutants against pNPO or pNPH in relation to the specific activities against pNPP, respectively (n.a. – not applicable).

Mutant	Position of mutation	Ratio pNPO/pNPP	Ratio pNPH/pNPP	Protein concentration [$\mu\text{g mL}^{-1}$]
WT	–	1.2	0.4	511.7 $\pm$ 23.4
F91M	lid	n.a.	59.8	315.8 $\pm$ 12.7
F91Y	lid	5.7	n.a.	313.7 $\pm$ 5.4
F91G	lid	1.1	0.6	1075.0 $\pm$ 12.5
F91H*	lid	2.1	0.7	303.9 $\pm$ 19.2
F91L*	lid	2.1	1.2	244.4 $\pm$ 49.5
F91N*	lid	1.3	0.5	338.9 $\pm$ 16.3
F91T*	lid	1.3	0.5	212.2 $\pm$ 4.4
F129T	substrate channel	0.1	67.4	426.8 $\pm$ 17.3
F129L	substrate channel	0.4	n.a.	313.8 $\pm$ 14.7
F129H	substrate channel	n.a.	3.7	439.1 $\pm$ 11.8
F129A*	substrate channel	1.0	n.a.	76.2 $\pm$ 2.4
F129C*	substrate channel	n.a.	n.a.	94.2 $\pm$ 16.9
F129Q*	substrate channel	2.3	n.a.	108.4 $\pm$ 2.6
F129R*	substrate channel	1.3	n.a.	93.9 $\pm$ 2.3
S163H	near substrate channel	2.9	0.7	266.5 $\pm$ 14.2
S163M	near substrate channel	2.8	0.9	289.8 $\pm$ 41.8
S163V	near substrate channel	3.2	0.2	231.4 $\pm$ 12.0
S163G	near substrate channel	0.4	0.6	895.7 $\pm$ 14.7
S163I	near substrate channel	0.4	0.4	197.3 $\pm$ 3.4
S163Q	near substrate channel	n.a.	0.2	421.9 $\pm$ 13.3
I245Y	substrate channel	0.3	0.2	553.9 $\pm$ 7.6
I245E	substrate channel	0.3	0.1	376.0 $\pm$ 16.9
I245T	substrate channel	0.3	0.2	417.9 $\pm$ 18.9
I245W	substrate channel	8.2	2.5	526.7 $\pm$ 72.9
I245F*	substrate channel	0.8	1.0	96.4 $\pm$ 4.8
L300R*	substrate channel	1.8	1.3	89.0 $\pm$ 1.3
L300P*	substrate channel	1.1	0.6	50.8 $\pm$ 4.8
L300I*	substrate channel	1.5	n.a.	18.2 $\pm$ 3.8
L302A	substrate channel	0.4	0.3	518.4 $\pm$ 22.7
L302H	substrate channel	0.3	0.3	316.4 $\pm$ 13.3
L302V	substrate channel	0.3	0.2	498.5 $\pm$ 3.2
L302G*	substrate channel	2.3	10.0	170.8 $\pm$ 11.9
L302P*	substrate channel	2.3	4.9	173.0 $\pm$ 10.7
L305P	substrate channel	0.5	0.5	344.6 $\pm$ 49.7
L305Q	substrate channel	1.6	0.8	344.6 $\pm$ 49.7
L305A*	substrate channel	1.9	1.4	170.1 $\pm$ 3.0
L305M*	substrate channel	1.7	1.0	73.6 $\pm$ 1.5
L305N*	substrate channel	1.6	0.9	135.7 $\pm$ 4.5
L305R*	substrate channel	1.0	0.3	39.5 $\pm$ 0.4
L305H*	substrate channel	0.6	0.3	104.7 $\pm$ 3.4
L305Y*	substrate channel	0.2	0.1	142.5 $\pm$ 1.9
I529H	substrate channel	0.7	0.5	516.7 $\pm$ 11.4
I529R	substrate channel	1.1	0.8	532.9 $\pm$ 22.9
I529T	substrate channel	1.7	1.1	511.7 $\pm$ 23.4
I529E	substrate channel	5.7	0.5	916.5 $\pm$ 176.4
I529A*	substrate channel	4.6	n.a.	39.8 $\pm$ 2.0
I529D*	substrate channel	2.4	1.3	182.1 $\pm$ 26.5
I529G*	substrate channel	1.8	1.0	240.7 $\pm$ 30.2
I529W*	substrate channel	1.1	0.5	112.2 $\pm$ 1.1
F91L + S163M	lid - near substrate channel	1.2	0.5	155.4 $\pm$ 19.7
F91L + I245F	lid - substrate channel	4.0	2.2	238.3 $\pm$ 1.3
F91L + L302G	lid - substrate channel	9.2	25.7	125.3 $\pm$ 4.9
F91L + L305A	lid - substrate channel	3.4	1.7	517.3 $\pm$ 11.0
F91L + I529D	lid - substrate channel	4.0	2.5	261.8 $\pm$ 57.8

Table 1 (continued)

Mutant	Position of mutation	Ratio pNPO/pNPP	Ratio pNPH/pNPP	Protein concentration [$\mu\text{g mL}^{-1}$]
S163M + I245F	near substrate channel - substrate channel	0.4	0.4	221.5 $\pm$ 41.7
S163M + L302G	near substrate channel - substrate channel	2.6	11.6	142.4 $\pm$ 26.3
S163M + L305A	near substrate channel - substrate channel	0.6	0.3	201.2 $\pm$ 26.9
S163M + I529D	near substrate channel - substrate channel	0.7	0.5	204.7 $\pm$ 22.6
I245F + L302G	substrate channel - substrate channel	1.3	16.8	292.3 $\pm$ 6.3
I245F + L305A	substrate channel - substrate channel	1.8	4.6	240.7 $\pm$ 12.1
I245F + I529D	substrate channel - substrate channel	1.7	0.3	255.3 $\pm$ 8.8
L302G + L305A	substrate channel - substrate channel	1.0	10.4	168.2 $\pm$ 32.7
L302G + I529D	substrate channel - substrate channel	2.7	2.2	255.2 $\pm$ 9.6
L305A + I529D	substrate channel - substrate channel	2.5	1.2	277.9 $\pm$ 10.9
F91L + I245F + L302G	lid - substrate channel - substrate channel	1.3	8.0	378.2 $\pm$ 15.7
F91L + I245F + L305A	lid - substrate channel - substrate channel	2.0	2.3	218.1 $\pm$ 22.1
F91L + L302G + L305A	lid - substrate channel - substrate channel	0.5	3.0	160.8 $\pm$ 28.9
F91L + L302G + I529D	lid - substrate channel - substrate channel	1.4	5.2	219.3 $\pm$ 17.3
F91L + L305A + I529D	lid - substrate channel - substrate channel	2.1	1.0	189.7 $\pm$ 14.6
I245F + L302G + L305A	substrate channel - substrate channel - substrate channel	0.7	6.3	219.5 $\pm$ 15.9
L302G + L305A + I529D	substrate channel - substrate channel - substrate channel	0.4	0.5	179.8 $\pm$ 13.0

with the predictions from MD simulations underlining that the model is appropriate.

In total, 15 gene constructs for double mutants have been created (Table 1 and Table S1) by combining promising single mutations. All 15 double mutants were successfully expressed and purified (Figs. S12 and S13). The hydrolysis profiles of the double mutants are shown in Fig. 6. Except for F91L + S163M all double mutants show lower relative specific activities than the WT. The desired decrease in the hydrolysis to yield palmitic acid is observed in ten double mutants which show better pNPH/pNPP or pNPO/pNPP ratios than the WT (Table 1). For all these double mutants either the ratios pNPO/pNPP or pNPH/pNPP could be improved. In most cases both ratios improved compared to the WT. In the WT enzyme the highest activity is against pNPO. The double mutants F91L + L302G, S163M + L302G, I245F + L302G, I245F + L305A and L302G + L305A primarily show highest activity to pNPH and activity to pNPO is drastically restricted. The activity toward pNPH of I245F + L305A is four times higher than in the WT. The four other mutants mentioned stand out with some of the highest ratios pNPH/pNPP between 10 and 25 within all the examined mutants. Considering both ratios, double mutant F91L + L302G strikes by the highest ratio of pNPO/pNPP of 9.2 being the highest within the whole library as well as a ratio pNPH/pNPP of 25.7 being third highest within the library. The same change of affinity has been observed for the single mutant L302G,

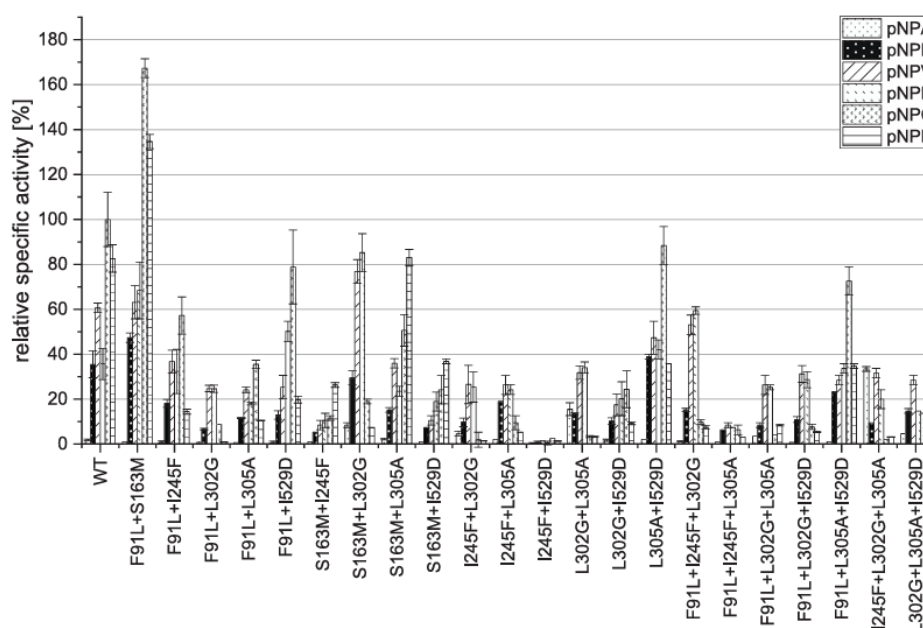


Fig. 6. Relative specific activity [%] of PCI_Lip 15 double and nine triple mutants against six *para*-nitrophenyl (pNP) fatty acid esters of different chain length as model substrates. All specific activities are compared to the specific activity of the WT against pNPO, which was set to 100%. All photometric assays were performed in triplicates. Standard deviation of the measurements is described by the error bars. For statistical evaluation see Fig. S20.

also seen in a ten-fold higher affinity against pNPH than pNPP as the WT. These results underline the assumption that the position L302 is crucial for PCI_Lip chain length specificity (Broel et al., 2022). The overall increase of hydrolytic activity elicited by the mutation I245F could not be transferred to its double mutants as these generally exhibit rather low overall activity. After two rounds of mutagenesis three mutants still exhibited a hydrolysis profile similar to the WT but with lower affinity toward pNP palmitic acid. The reduced affinity against pNPP while simultaneously keeping a comparable hydrolysis profile as the WT is resembled in improved ratios of pNPO/pNPP and pNPH/pNPP. These results go along with the hydrolysis profile of the single variant L305A and emphasize the importance of the position L305 for chain length specificity notably affecting its affinity to palmitic acid. The *in silico* analysis revealed that the ester bond of substrates with a long acyl chains is situated more toward the solvent accessible part of the substrate channel (Broel et al., 2022). The mutation L305A seems to emphasize this situation making access for long chain fatty acids more difficult. For cheese making applications the hydrolysis profile ideally should shift to primarily hydrolyzing butyrate, hexanoate and octanoate while minimizing palmitic hydrolysis (Sowa et al., 2022). The hydrolysis profiles of F91L + L302G, S163M + L302G, I245F + L302G, I245F + L305A and L302G + L305A comply with these requirements very well. Additionally, the comparison of both ratios shows that double mutants that are considered best as substitutes have considerably higher values for the pNPH/pNPP than for the pNPO/pNPP ratio. Most of these double mutants display a relative specific activity of 20% to 40% compared to the pNPO activity of the WT, which is comparable to the activity of the single mutant L302G with a similar hydrolysis profile. Combining L302G with the exchange S163M kept the desirable hydrolysis profile, while simultaneously boosting activity to over 80% compared to the WT for pNPH. Combining favorable single mutants did not necessarily show additive or synergistic effects in the second round of mutagenesis. However, the comparison of selectivity ratios of single and double mutants underlined non-linear coupling effects rather than single-site effects. As the examined residues shape the substrate pocket of PCI_Lip we

propose cooperative mechanisms of gating effects caused by F91L in the lid domain and a remodeling of the channel geometry by the other mutations leading to restricted access for long chain substrates, while maintaining activity against short to medium chain substrates. Rokytá et al. describe similar observation in microbial and viral systems. Combining beneficial single mutants can result in a mutant that shows less beneficial properties than the single mutants. Even though the single mutants show beneficial properties double mutants can be a step away from the optimum in the fitness landscape (Rokytá et al., 2011).

The most interesting double mutants according to their hydrolysis profiles as well as the pNPH/pNPP and pNPO/pNPP ratios S163M + L302G, I245F + L302G, L302G + L305A and L302G + I529D have been further biochemically characterized by means of Michaelis-Menten kinetics. Therefore, the substrates pNPB, pNPH and pNPO have been chosen as these fatty acids are important for the flavor of piquant cheese varieties. The values obtained for maximal velocity (v_{max}), Michaelis-Menten constant (K_M), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_M) are listed in Table 2. The differences of these parameters within the four mutants showcase the changes in hydrolytic activities caused by the amino acid exchanges (Figs. S15–S18). The maximal velocity is highest for pNPH for all the double mutants. Michaelis-Menten constants increase with decreasing chain length of the substrates. Turn over numbers and catalytic efficiencies go along with the hydrolysis profiles obtained in the photometric assays (Fig. 6). Both values are low for pNPO in the analysis of double mutants I245F + L302G and L302G + L305A which displayed very low activity against this substrate in their hydrolysis profiles. In comparison to the catalytic efficiencies of the WT (Broel et al., 2022) the ones of the double mutants are lower for the three substrates. This correlates with the overall lower hydrolytic activity of the double mutants compared to the WT. All catalytic efficiencies and especially the turnover numbers confirm that we were able to change the specificity of PCI_Lip within this second round of mutagenesis rather toward short chain fatty acids. The second round of mutagenesis offered some mutants that exhibited altered chain length specificity with distinguishable changes in pNPH/pNPP and pNPO/pNPP ratios.

Table 2

Kinetic data of PCI_Lip double mutants S163M + L302G, I245F + L302G, L302G + L305A and L302G + I529D for the substrates pNPB, pNPH and pNPO with confidence intervals (95% CI) and determination coefficient (R^2).

	Protein concentration [$\mu\text{mol} \cdot \text{L}^{-1}$]	Substrate	v_{max} [$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L}^{-1}$]	lower CI v_{max} [$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L}^{-1}$]	upper CI v_{max} [$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L}^{-1}$]	K_M [mmol $\cdot \text{L}^{-1}$]	lower CI K_M [mmol $\cdot \text{L}^{-1}$]	upper CI K_M [mmol $\cdot \text{L}^{-1}$]	k_{cat} [s^{-1}]	k_{cat}/K_M [$\text{s}^{-1} \cdot \text{mol}^{-1} \cdot \text{L}$]	R^2
S163M + L302G	2.20 $\pm$ 0.21	pNPB	40.83	25.51	56.16	1.31	0.45	2.18	0.309	236	0.95
		pNPH	91.88	74.40	109.35	0.65	0.40	0.89	0.696	1071	0.95
		pNPO	13.46	9.10	17.81	0.07	-0.04	0.18	0.102	1456	0.50
I245F + L302G	2.12 $\pm$ 0.06	pNPB	42.07	25.69	58.44	1.81	0.70	2.94	0.331	183	0.97
		pNPH	95.43	74.11	116.75	0.75	0.41	1.10	0.750	1000	0.97
		pNPO	11.18	8.42	13.47	0.15	0.05	0.25	0.088	586	0.81
L302G + L305A	1.46 $\pm$ 0.03	pNPB	23.00	13.94	32.06	1.75	0.65	2.86	0.262	150	0.96
		pNPH	30.36	24.44	36.27	0.42	0.19	0.65	0.346	823	0.96
		pNPO	6.53	5.16	7.90	0.13	0.02	0.23	0.074	572	0.84
L302G + I529D	2.35 $\pm$ 0.10	pNPB	26.50	18.85	34.14	1.41	0.67	2.16	0.188	133	0.96
		pNPH	50.45	44.17	56.74	0.50	0.36	0.63	0.358	715	0.96
		pNPO	45.15	39.26	51.05	0.39	0.29	0.50	0.320	821	0.97

Additionally, the importance of the positions L302 and L305 for the enzymes chain length specificity have been underlined.

Taking the protein engineering of PCI_Lip a step further a third round of mutagenesis has been accomplished. Here the mutation sides F91L, I245F, L302G, L305A and I529D have been considered. Seven triple mutants have also been successfully expressed and purified (Fig. S14). The relative specific activity of all triple mutants is lower than the WT activity (Fig. 6). The highest activity is shown by F91L + L305A + I529D with over 70% against the substrate pNPO. Secondly mutant F91L + I245F + L302G displays more than 60% of activity in comparison to the WT against pNPH. The other five mutants show rather low activities from 30% and lower. The library of mutants in this study underlines the discrepancy of better fitness due to diversity but simultaneously loss of activity (Ostermeier, 2003). Moreover, the hydrolysis profiles of the triple mutants are more deviate from the profile of the WT as mutants from the first two rounds. Here, the preferred affinity is predominantly shifted from pNPO to pNPH. Therefore, the significance of position L302 falls into place as five out of seven mutants contain L302G. These mutants resemble the hydrolysis profile of the single mutant. This underlines the postulation by Broel et al. for this side to be crucial for chain length specificity. Interestingly, F91L + I245F + L302G shows a synergistic effect in terms of its hydrolysis profile. The distribution of relative specific activities is mainly influenced by L302G and very similar to the profile of double mutant F91L + L302G. The combination with I245F, which was found to increase the overall activity led to an increase to nearly 60% relative specific activity indicating an additive effect of these mutations (Broel et al., 2022). Especially the third round of mutagenesis demonstrates that small steps in the fitness landscape of a protein can result in different effects as additivity as well as positive or negative coupling. Additivity is seen in F91L + I245F + L302G with its hydrolysis profile being the sum of the individual changes the double and single variants displayed (Lunzer et al., 2005). Positive coupling effects can be observed in all triple mutants except F91L + L305A + I529D and L302G + L305A + I529D as combinations led to the desired reduction in activity to pNPP. But also negative coupling effects are observed for example in the considerable decline in the overall activity particularly noticeable for F91L + I245F + L305A. Coupling is caused by altered interactions of the amino acid residues and can cause beneficial such as stabilizing effects as well as negative effects for example due to van der Waals conflicts (Voigt et al., 2000). The third round of mutagenesis revealed mutants with hydrolysis profiles shifted to mainly hydrolyze pNPH as a result of additive effects of individual mutations. However, overall activity was considerably lower than in the previous rounds, which means the combination of more active variants did not lead to improved variants indicating a valley in the fitness landscape.

Hence the third round of mutagenesis is a good example for better fitness of the enzyme due to increased diversity but simultaneously substantial loss of activity (Ostermeier, 2003).

3.4. Evaluation of vFFA and sensory of feta-type brine cheese samples

Mutants have been deemed to have a positive impact, when the hydrolysis profile of the WT was shifted to an enhanced hydrolysis of short to medium chain fatty acids. Also, a lowered affinity toward pNPP resembling a long chain substrate was desirable. Other criteria to evaluate the effect of the mutations are the ratios of relative specific activity of pNPH and pNPO to pNPP (Table 1). Due to rather low overall activity of mutants from the third round those have not been applied in the cheese making experiments. A mutant exhibiting an appealing hydrolysis profile, was considered the most effective candidate to substitute for PGE. Mutants matching these criteria best were F91L, S163M, I245F, L302G, L305A, I529D, F91L + I245F, F91L + L305A, S163M + L302G, I245F + L302G, L302G + L305A and L302G + I529D. Therefore, one unit of each PCI_Lip mutant compared to the activity against pNPO was subjected to the making process of Feta-type brine cheese. To compare the impacts of the different mutants, samples were prepared without the addition of lipolytic enzymes, with the addition of opti-zym z10uc a commercially available PGE, and PCI_Lip WT. The results of vFFA analysis (Fig. 8) display that all applied mutants had an impact on the release of vFFA as the peak areas for C4:0 to C10:0 are higher than these in the sample without addition of a lipolytic enzyme. Even though mutants S163M and I245F exhibited significant differences in the photometrically determined hydrolysis profile, their vFFA profiles were comparable to the that of the WT. In the sensory evaluation, the addition of both mutants was perceived as more intense, sour and goat-like for both smell and taste and taste-wise the samples were also described as soapier than the one prepared with the WT. Four single and three double mutants show overall lower lipolytic activity within cheese preparation than the WT as well as the commercial PGE. The reduced activity could be seen for some of the mutants beforehand in the photometric assays, however, the overall lipolytic activity of F91L, I529D, F91L + I245F and S163M + L302G had not been seen to have strongly reduced in the prior experiments. For the double mutants L302G + L305A and L302G + I529D the overall activity in the photometric assays was lower in comparison to the WT. For the vFFA analysis the overall activity is in a similar range as the WT. The vFFA profiles of all applied mutants and the WT display the highest peak areas for hexanoic acid while the major peak area in the cheese prepared with the PGE is butyric acid. Barely any octanoic acid is released by opti-zym z10uc whereas it resembles the second highest peak area of vFFA in all cheese samples prepared with a

mutant except for the one with L302G + L305A. Four single mutants seemed to be less active on butyric acid in comparison to the WT according to the peak areas. This is in contradiction to the MD simulations and is not in accordance with the photometric results. However, it could be caused by the fatty acid volatility during SPME-GC-MS detection.

The sensory evaluations of cheeses are shown in Fig. 7. The smell of the cheese samples prepared with the addition of single mutants is still very divergent from the one described for the cheeses prepared with PGE. The samples are much lower in the piquant perception but also are described to have a soapier smell. The samples prepared with the addition of I245F and L305A are standing out in this group as their smell has been rated highest in overall intensity, sourness and most goat-like. The sample prepared with I245F had an overall more intense smell and exhibited larger peak areas in the vFFA analysis consistent with enhanced activity in photometric assays. The amount of vFFA, which majorly contribute to the piquant and goat-like flavor, for the samples prepared with F91L, L302G, L305A and I529D was much lower, which also had a milder smell than in the samples prepared with S163M and I245F. These were consistent with both the vFFA analysis and sensory evaluation, where the last two samples scored highest in all taste attributes except creaminess. The taste of the samples prepared with the other single mutants was perceived similar to the sample prepared with PGE. The soapy off-flavor of PCI_Lip due to the fortified release of long chain fatty acids could be reduced by the utilization of the engineered single mutants. All the samples except for the one prepared with I245F were rated lower in soapiness than the one with the WT. Samples prepared with four single mutants were even lower rated in soapiness than the sample prepared with PGE. Overall, the taste profile of the sample prepared with F91L was very similar to the one prepared with PGE. Here it is to point out that the addition of all single mutants except I245F as a mutant with generally increased activity led to a lower rating in soapiness than the WT. This implies that these mutants release less long chain fatty acids which was the aim of engineering PCI_Lip. This facilitates the

results of MD simulations and experiments with model substrates.

While in the photometric experiments the overall hydrolytic activity of double mutants was lower than the one of the WT the analysis of vFFA showed that the release of vFFA of S163M + L302G, L302G + L305A and L302G + I529D in the cheese samples was in the same range as in the sample prepared with the WT. Samples prepared with double mutants are barely comparable to the smell of the sample prepared with PGE. The addition of two double mutants evoked a sourer smell than perceived in the sample prepared with PGE. These two samples were also rated highest in goat-like smell and overall intensity while simultaneously creaminess was rated lowest of all samples. In comparison with the vFFA profiles of the samples, they stand out as both exhibit a considerable release of octanoic acid. The odor perception of octanoic acid is described as rancid and goat-like (Miyazawa et al., 2009). For the sample with the addition of F91L + L305A the sensory evaluation resembles the results from the vFFA analysis. There the peak area was beyond the lowest of all samples while simultaneously the smell was described as faintly for all given attributes. Generally, the evaluation of the taste of the samples with the addition of double mutants is more similar to the sample prepared with PGE analogue to the results for the samples prepared with single mutants. Contrary to the evaluation of the smell, the taste of all samples except the one with the addition of F91L + L305A has been assessed as more piquant than the sample prepared with PGE. Compared to the sample prepared with the WT, the addition of all double mutants led to a reduction in the perception of soapiness. Also, the soapy sensation was lower than the sample prepared with PGE was rated.

The application of the second round of mutants in cheese production revealed some promising candidates to replace PGE. The addition of L302G + L305A resulted in a cheese sample with a taste which has been evaluated similar to the one prepared with PGE with even lower rating of the attributes bitter and soapy which are rather undesired in cheese. Further the double mutants S163M + L302G, I245F + L302G and

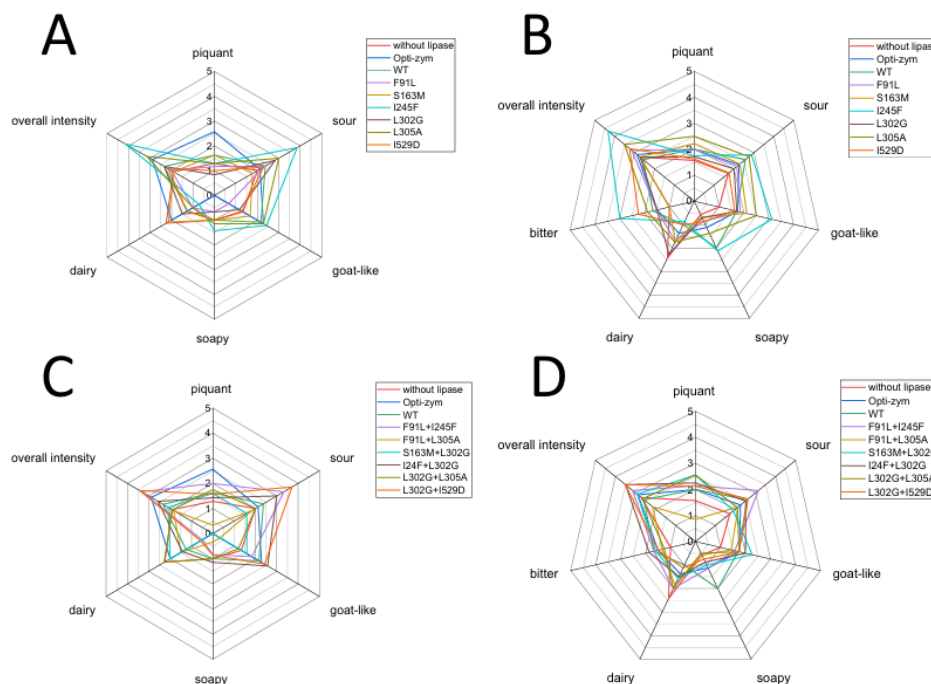


Fig. 7. Sensory evaluation of Feta-type brine cheese produced with PCI_Lip mutants. The evaluation is based on the rating of eight panelists from 0 (no perception) to 5 (strong perception) on the given attributes, while (A) is the smell of cheese prepared with single mutants and (B) the taste of cheese prepared with single mutants, (C) is the smell of cheese prepared with double mutants and (D) the taste of cheese prepared with double mutants. For statistical evaluation see Fig. S21.

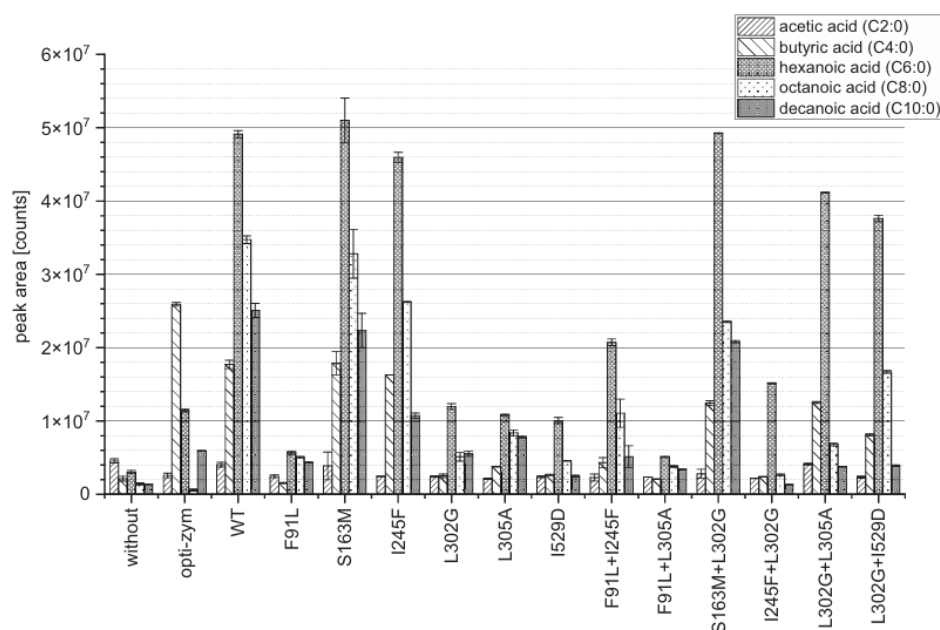


Fig. 8. Peak areas of volatile free fatty acids measured in Feta-type brine cheese samples by SPME-GC-MS. The cheeses were produced with the addition of different lipase mutants. As a reference PGE opti-zym z10uc was used. During cheese making 1 U of lipase mutant was added to each approach, for L302G + L305A 0.5 U of lipase was added. Measurements were performed in duplicates, error bars correspond to the mean deviation. For statistical evaluation see Fig. S22.

L302G + I529D promote a more piquant, goat-like and intense flavor than the PGE opti-zym z10uc. As initially observed in the photometric assays, the mutation L302G seems to have a positive impact on the hydrolytic activity of PCI_Lip. This impact is transferred to the application trials as well. The combinatorial approach in mutagenesis could improve especially the taste profile of the cheese samples prepared with different double mutants, all of which carried the mutation L302G.

4. Conclusion

In three rounds of mutagenesis, the selectivity of the Basidiomycota derived lipase PCI_Lip was adapted to fit the requirements of animal derived PGE used in cheese production. Most mutants showed the predicted shift in the hydrolysis profiles with enhanced release of short to medium chain fatty acids and reduced hydrolysis of long chain fatty acids. Selected single and double mutants were evaluated as potential PGE substitutes in the production of Feta-type brine cheese. A vFFA analysis showed that the addition of four double mutants increased the release of short to medium chain fatty acids. Sensory evaluation confirmed their high potential as substitutes of PGE, producing a flavor comparable in piquancy to the reference cheese while markedly reducing the soapy off-flavor observed in the cheese prepared with the WT. For the production of Feta-type cheese the mutant L302G + L305A seems to be the most appropriate substitute for PGE. Cheese varieties that use PGE in the production process with a more piquant flavor such as Parmigiano Reggiano mutants S163M + L302G or L302G + I529D may be the best choice, as both provide a highly intense, piquant flavor. A dosage range of 0.5 to 1 U gave good sensory results warranting a good start for product development. Three double mutants when used in cheese with improved goat-like attributes could be identified, while the variant S163M + L302G stands out for the best overall taste, high catalytic activity toward pNPO and pNPH and improved selectivity. Kinetic characterization of these mutants underline that the substrate specificity of PCI_Lip was changed in this *in silico* guided approach for application in dairy industries. Studies on impaired mutants further validated the

PCI_Lip. The importance of the lid domain for PCI_Lip-like enzymes for activity and solubility was demonstrated. These results demonstrate the potential of Basidiomycota derived lipases, especially if they are tailored. The protein engineering results combined with first application trials provide a foundation for scale up and further fine tuning of process parameters in future product development.

CCRediT authorship contribution statement

Lea Henrich: Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Niklas Broel:** Data curation. **Jonathan Schüler:** Data curation. **Marius Lang:** Data curation. **Binglin Li:** Writing – review & editing. **Martin Gand:** Writing – review & editing, Supervision, Conceptualization.

Ethical statement

The authors certify that this study was conducted in strict accordance with the ethical principles outlined in the World Medical Association's Declaration of Helsinki for research involving human participants. In the context of sensory evaluation, national regulations do not require formal ethical approval, and no established ethics committee or formal documentation process is available for such studies. Despite this, the authors implemented rigorous protocols to safe-guard the rights and privacy of all participants. These protocols included ensuring voluntary participation without coercion, providing comprehensive information about the study's aims, procedures, and potential risks, obtaining verbally informed consent from each participant, ensuring that no participant data was disclosed without prior consent, and allowing participants the freedom to withdraw from the study at any point without prejudice.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2026.103639>.

Data availability

No data was used for the research described in the article.

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Thermostability and regioselectivity of free and immobilized PCI_Lip

Investigation of regioselectivity and thermostability of free and immobilized *Pleurotus citrinopileatus* lipase

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RESEARCH



Investigation of regioselectivity and thermostability of free and immobilized *Pleurotus citrinopileatus* lipase

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Abstract

A fungal lipase from the Basidiomycete *Pleurotus citrinopileatus* (PCI_Lip) has been investigated for its regioselectivity. Therefore, the conversion products of a triglyceride were analyzed using ¹H-NMR to determine the position of hydrolysis within the substrate. PCI_Lip is a 1,3-selective lipase, as it preferentially hydrolyzes the fatty acids at the outer positions of the triglyceride. Furthermore, this study aimed to gain deeper insights into the stability of the lipase. The melting point of the enzyme was determined to be at 63 °C in ultrapure water, which could be increased to 71 °C by dissolving PCI_Lip in a 400 mM citrate buffer pH 6.0 with the addition of glucose and trehalose. To optimize the stability of the enzyme, PCI_Lip was immobilized via its affinity tag. Twenty-one supports were tested, with IB-HIS-19 being chosen as the most appropriate for PCI_Lip immobilization due to its high binding capacity and low leakage. This immobilized lipase displayed only minor loss of activity over 13 cycles of reuse. Immobilization of the wild-type preserved activity and selectivity, whereas it induced notable changes in the case of the double mutants. The increased stability of the immobilized PCI_Lip was further proven in a thermal inactivation experiment. Double mutants of PCI_Lip displayed even higher inactivation temperatures, while the immobilization raised this temperature by 5 °C. Overall, in this study, the 1,3-regioselective PCI_Lip identified from our group has been further characterized, focusing on the improvement of its thermal and kinetic stability. A major boost in stability can be achieved via immobilization of the enzyme to an appropriate support.

Key points

- IB-HIS-19 had the highest binding capacity and kept up enzyme activity over 13 cycles of the screened immobilization materials
- Immobilization increased the stability of PCI_Lip by 5 °C, protein engineering increased the stability of two double mutants up to 4 °C, and thermostability could be increased by 9 °C by using high molarity buffers
- PCI_Lip was found to be a 1,3-regioselective lipase by analyzing its products by ¹H-NMR

Keywords Fungal lipase · Immobilization · Thermostability · Regioselectivity · Protein engineering

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Introduction

Various industries such as food, pharmaceutical, and chemical industry are shifting towards more sustainable production systems and in this effort, enzymes have been applied as biocatalysts in different production processes. Biotechnological processes operate under mild reaction conditions in terms of temperature and pressure (Iyer and Ananthanarayan 2008; Silva et al. 2018; Stepankova et al. 2013). For these applications, recombinant protein expression is used to efficiently produce large quantities of enzymes with up to 50% of the host's protein metabolism dedicated to heterologous expression (Zhao et al. 2024). However, overexpression

bears the risk of low solubility of the protein, loss of activity due to conformational changes, enzyme aggregation, or even precipitation (Boivin et al. 2013; Silva et al. 2018). Selecting appropriate buffer components can affect enzyme solubility and stability and help prevent the formation of inclusion bodies by supporting proper folding of the enzyme. While kosmotropic ions support enzyme stabilization, chaotropic ions destabilize the dissolved enzymes (Ugwu and Apte 2004). The addition of neutral salts can help with stabilization as they interact with the charges on the protein surface and thus support hydrophobic interactions within the macromolecule. Stabilizing as well as destabilizing effects of neutral salts are closely related to the Hofmeister series (Silva et al. 2018). Moreover, the thermostability can be further enhanced by the addition of sugars and polyols. These substances form a layer around the macromolecule that can only be passed by water molecules, which results in an improved hydration (Silva et al. 2018).

However, optimization of the storage buffer in most cases is insufficient to adapt enzymes to the requirements of industrial processes. Most enzymes do not meet the requirements for direct application in industrial operations, as these reactions do not occur under physiological conditions. Enzymes applied in industry often face challenges such as high temperatures, extreme pH conditions, high salinity, and also organic solvents which lead to denaturation (Iyer and Ananthanarayan 2008). Immobilization offers stabilization of the enzyme in such environments. Immobilized enzymes can also be separated from the reaction mixture, enabling the accurate control of the process. Another advantage is their reusability, as immobilized enzymes can be applied in several reaction cycles. The chance to remove the enzyme from the products makes this technique particularly interesting for applications in the food industry, as no listing as a food ingredient is required (Yushkova et al. 2019; Sheldon 2007). Enhanced stability of immobilized enzymes is attributed to the increased rigidity of the biocatalyst (Stepankova et al. 2013). Adsorption of the enzyme to the support is the easiest way of immobilization, though binding is not strong and leakage is high. Covalent binding using bifunctional spacers attaches the enzyme sturdier to the support. It offers good immobilization yields with low leakage, but bears the risk of loss of activity when the biocatalyst is immobilized in a way that the substrate cannot enter the active site (Homaei et al. 2013; Maghraby et al. 2023; Silva et al. 2018; Stepankova et al. 2013; Sheldon 2007). Encapsulating the enzyme into a polymer matrix and cross-linking of enzymes are further methods to immobilize biocatalysts (Homaei et al. 2013; Sheldon 2007). A specific way to immobilize enzymes onto a support is affinity interaction. This strategy makes use of tags originally fused to the enzyme as purification tags. The commonly used histidine

tag is used to bind to a chelate-bound transition metal. For this immobilization method, the support is functionalized with chelating moieties (Homaei et al. 2013).

Hydrolases, particularly lipases (EC 3.1.1.3), are of major interest for industry. Their chemo-, regio-, and enantioselectivity and also their wide substrate spectrum make them a sustainable substitute in chemical production processes. Depending on the process conditions, these enzymes can catalyze hydrolysis, esterifications, transesterifications, acidolysis, or aminations. Another benefit is that hydrolases do not require a cofactor (Abellanas-Perez et al. 2024; Stepankova et al. 2013). In this work, we used a lipase from the Basidiomycete *Pleurotus citrinopileatus* (PCI_Lip). In a previous work of our group, the lipase has been used as a substitute for pregastric esterases, enzymes used in cheese production to generate flavor by the release of volatile free fatty acids (Sowa et al. 2022). As the amount of long-chain fatty acids released by the enzyme caused a slightly soapy off-flavor in the prepared cheese samples, a mutant library has been generated. In a semi-rational design approach, optimization of the hydrolysis profile of the enzyme was conducted (Broel et al. 2022). In this study, we combine the Basidiomycota-derived lipase with affinity immobilization to enhance the stability of PCI_Lip for putative industrial applications. Another aspect was the investigation of the regioselectivity of PCI_Lip. Natural triglycerides often carry different fatty acid moieties at the three ester positions. These triglycerides are chiral molecules with a stereo-specific numbering of *sn* 1–3 for the different positions of the fatty acid esters. Lipases are categorized into nonspecific lipases, hydrolyzing any ester bond within a triglyceride, and regiospecific lipases only active on the two outer ester bonds (Park and Park 2022). All in all, this study gives insights into improved conditions and reactivity for a fungal lipase for an expanded field of applications in industrial processes.

Material and methods

Enzyme production and purification

Heterologous expression of PCI_Lip wildtype (WT) and the two double mutants S163M+L302G and I245F+L302G was performed according to Henrich et al. (2026). The gene of PCI_Lip originates from *P. citrinopileatus* (DSM number 5341, GenBank file OL364849). Briefly, *Escherichia coli* pellets from 200 mL culture were lysed by sonication, purified by immobilized metal ion affinity chromatography (IMAC), and desalted. Purified lipase was stored at 4 °C and expression was checked via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Broel et al. 2022; Laemmli 1970).

Hydrolytic activity

Lipolytic activity of free and immobilized PCI_Lip was tested in photometric assays using *para*-nitro phenyl (*p*NP) fatty acid esters according to Broel et al. (2022). The model substrates were *p*NP-acetate (*p*NPA) (Sigma-Aldrich, Taufkirchen, Germany), *p*NP-butyrate (*p*NPB) (Th. Geyer, Renning, Germany), *p*NP-valerate (*p*NPV) (Sigma-Aldrich), *p*NP-hexanoate (*p*NPH) (TCI Europe, Heuchelheim, Germany), *p*NP-octanoate (*p*NPO) (Alfa Aesar, Karlsruhe, Germany), and *p*NP-palmitate (*p*NPP) (Sigma-Aldrich). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μ mol of *p*NP in 1 min in the respective assays. Protein concentration was determined according to the method of Bradford (Bradford 1976; Broel et al. 2022). To examine the hydrolytic activity of immobilized enzyme, Triton X-100 was substituted with the same amount of dimethyl sulfoxide (DMSO) (Honeywell, Seelze, Germany) with $\epsilon_{pNP} = 0.0076 \text{ L} \cdot \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$. Statistical evaluation in this and the following experiments was performed by one-way analysis of variance (ANOVA) with a significance level of 0.05 in OriginPro 2023 (OriginLab, Northampton, USA).

Enzyme immobilization

For enzyme immobilization, 17 IB-HIS carriers (ChiralVision, Den Hoorn, Netherlands) (Supplementary Table S1) and four carriers from Purolite (Ratingen, Germany) were tested. Adsorptive immobilization was performed by adding 1 mL of enzyme solution to 5 mg of carrier. Immobilization was performed at 800 rpm, 4 °C for 22 h. For covalent immobilization, 125 mg of carrier was mixed with 1 mL of 0.125% glutardialdehyde (Fisher Scientific, Schwerte, Germany) solution and incubated at 650 rpm, 25 °C for 2 h. The supernatant was discarded, and the carrier was washed with 1 mL 50 mM potassium phosphate (Carl Roth, Karlsruhe, Germany) buffer (PPB) at 650 rpm, 25 °C for 30 min. Supernatant was discarded again. Five milligrams of the activated carrier was then incubated with 1 mL of enzyme solution at 800 rpm, 4 °C for 22 h. The resulting immobilized enzyme is hereafter called “immobilisate.”

Binding capacity

Binding capacity of the different carriers was determined by removing the enzyme solution after immobilization and measuring its protein concentration according to Bradford (1976). The amount of immobilized protein has been calculated according to Formula 1 with *K* representing the binding capacity in percent. Here, *m* represents the protein concentration of the enzyme solution after immobilization and β the protein concentration of the enzyme solution before immobilization.

$$K = \left(1 - \left(\frac{m}{\beta} \right) \right) \cdot 100 \quad (1)$$

Leakage

The leakage of the bound enzyme was determined in two cycles after immobilization. Therefore, 1 mL of 80 mM PPB was added to the loaded carriers. The carriers were incubated at 800 rpm, 4 °C for 2 h. Afterward, the washing solution was removed, and its protein content was determined according to Bradford (1976).

Enzyme recycling

Kinetic measurements for immobilized enzymes were conducted for four min as this time reflects the linear part of the saturation curves in photometric assays. For the reaction, 20 μ L of enzyme solution have been used for immobilization; *p*NPO was used as a substrate. The mixture was incubated at 30 °C, 800 rpm for 4 min. Two hundred microliters of supernatant was used to measure the absorbance at 405 nm. After each reaction cycle, 1 mL of PPB has been added to the immobilized enzymes. Washing has been conducted for 15 min, 800 rpm at 4 °C with 1 mL of PPB, and the supernatant was removed with a syringe. The activity of each carrier has been documented over 13 or 14 cycles.

Enzyme characterization

Thermofluor assays

For Thermofluor assays, free PCI_Lip or mutants were dissolved in water. Each preparation was composed of 21 μ L buffer (including additives), 2 μ L of enzyme, and 2 μ L 62.5 $\times$ SYPRO® Orange Protein Gel Stain (Sigma-Aldrich) (see Supplementary Tables S2 and S3 for all buffers and additives tested). For the determination of the melting curves, a real-time PCR-cycler (Applied Biosystems, Darmstadt, Germany) with excitation at 470 nm and detecting emission at 520 nm was used. Samples were conditioned at 15 °C for 5 min. Subsequently, melting curves were measured from 15 to 95 °C applying a temperature gradient of 1 °C $\cdot$ min^{−1}.

Enzyme stability

The T_{50}^{60} values of free and immobilized PCI_Lip and its mutants were investigated according to Broel et al. (2022).

Storage stability

Storage stability was examined under various buffer conditions. Therefore, free and immobilized PCI_Lip and mutants were stored over a period of 28 days at 4 °C. Activity was measured photometrically after storage (1, 10, and 28 days) using *p*NPO as substrate for WT and *p*NPH as substrate for the mutants.

Determination of regioselectivity

Conversion of triglycerides

Triotanoate (Sigma-Aldrich) was dissolved in DMSO to a final concentration of 0.12 M as substrate. Nine hundred microliters of enzyme solution, 450 μ L of 5 mM HEPES (Carl Roth) buffer pH 8.0, and 450 μ L of substrate were incubated at 30 °C, 900 rpm for 30 min and 2 h. For a blank, the enzyme solution was substituted by 80 mM PPB pH 7.0. All conversions were conducted as duplicates.

Analysis of hydrolysis products by ^1H -NMR

After the conversion, the samples were frozen at -80°C and subsequently lyophilized at -59°C and 1.5 mbar overnight. The freeze-dried samples were dissolved in 0.8 mL acetone- D_6 (VWR Chemicals, Darmstadt, Germany) by mixing for 1 min. Samples were transferred to 5 mm nuclear magnetic resonance (NMR) tubes and subjected to ^1H -NMR

analysis using standard instrument software. The measurements were performed on an Advance II 400 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) at room temperature. To ensure the correct quantification of samples, the zg30 pulse sequence was used for proton NMR spectra acquisition utilizing a 30° pulse angle, 16 scans, and a relaxation delay of 5.1 s. NMR spectra processing and quantification were performed using TopSpin 4.0 (Bruker Biospin). The spectra were referenced to the residual proton peak of acetone- D_6 at 2.05 ppm.

Results

Immobilization of PCI_Lip

PCI_Lip was successfully immobilized to 21 carriers, while 17 used the affinity immobilization His₆-tag of PCI_Lip. These carriers vary in polarity due to different matrices and functional groups (Supplementary Table S1). Adsorptive immobilization was conducted on two carriers (ECR 1090 M, ECR 8806 M), and covalent immobilization was conducted on another two carriers (ECR 8309 M, ECR 8409 M). The binding capacity and leakage data are given in percent and in total for better comparison (Fig. 1A). Binding capacities vary from $3.7 \pm 0.7 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ to $19.2 \pm 0.7 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$. The carriers IB-HIS-2, 4, 8, 19, and 20 showed the highest binding capacities from $18.1 \pm 0.9 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ to $19.2 \pm 0.7 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$.

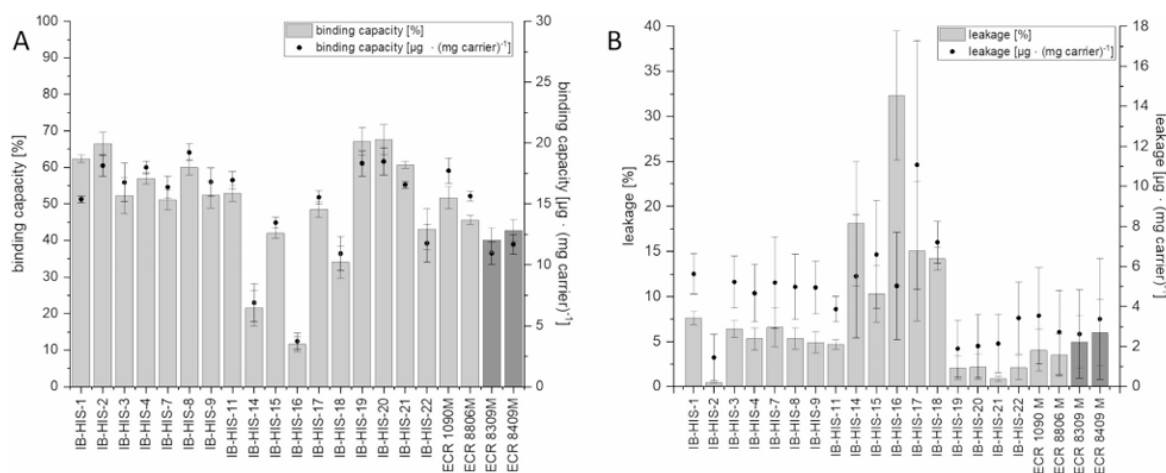


Fig. 1 **A** Binding capacities of PCI_Lip on various carriers. The left axis displays the percental binding capacity, while the right axis shows the total binding capacity. Light gray bars represent carrier materials for adsorptive or affinity immobilization, and dark gray bars denote supports for covalent binding. Measurements were conducted in duplicates, with error bars representing the half span. The difference in mean values at significance level of 0.05 was not significant with an *F*-value of 0.3. **B** Leakage of PCI_Lip from different carriers.

The left axis displays the percental leakage, and the right axis shows the total leakage. These carriers are distinguished by color: light gray for adsorptive or affinity carrier materials and dark gray for covalent binding carriers. Measurements were conducted in duplicates, with error bars indicating the half span. The difference in mean values at significance level of 0.05 was not significant with an *F*-value of 1.6.

Generally, polarity did not seem to impact the binding capacity of PCI_Lip to the different carriers, as for the best ones it is indicated from very hydrophobic to very hydrophilic. Adsorptive immobilization led to binding capacities of $17.7 \pm 1.0 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ for ECR 1090 M and $15.6 \pm 0.4 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ for ECR 886 M. Binding capacities of the covalent carriers with $11 \pm 1 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ were rather low. To further characterize the aptitude of these 21 different carriers for immobilization of PCI_Lip, the enzyme leakage after two h was determined (Fig. 1B). IB-HIS-2 showed the lowest leakage with less than $1.5 \pm 1.2 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$. IB-HIS-19, 20, and 21 also scored well in leakage, with $1.9 \pm 1.4 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ to $2.1 \pm 1.5 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$. From the set of 21 carriers, five IB-HIS carriers and the two covalent carriers were chosen to be investigated in a recycling experiment (Fig. 2). The activity of these specific immobilisates was compared to that of free PCI_Lip, which displayed an activity of $565.9 \text{ U} \cdot \text{L}^{-1}$. IB-HIS-1, 2, 8, 19, and 20 were selected because of their comparatively high binding capacity, all in a similar range between 15.3 and $19.2 \mu\text{g protein} \cdot (\text{mg carrier})^{-1}$ and rather low leakage. Simultaneously, these support materials cover a broad polarity range from very hydrophobic to very hydrophilic. At the beginning of the recycling experiment, IB-HIS-1, 2, 8, and 20 started with a relative activity of $25 \pm 1\%$ to $31 \pm 8\%$. The course of relative activity within these carriers was similar, starting to increase in the first couple of cycles and then decreasing to $18 \pm 1\%$ to $29 \pm 1\%$. The initial relative activity of IB-HIS-19 of $65 \pm 5\%$ was comparable to the two covalent carriers with $65 \pm 7\%$ and

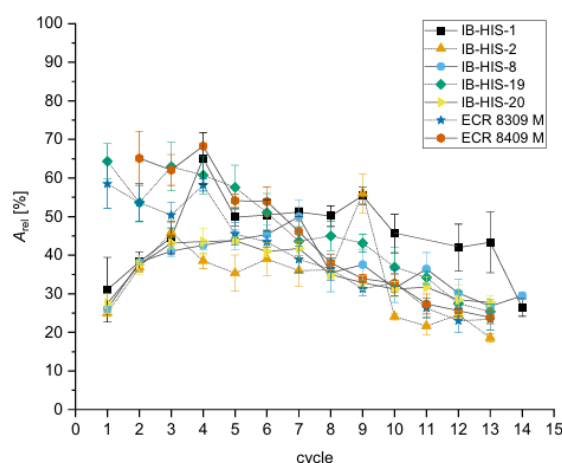


Fig. 2 Relative activity of immobilized PCI_Lip on seven selected affinity and covalent carriers compared to the free enzyme over several reaction cycles. Activity was measured photometrically in triplicates using *p*NPO as model substrate. Error bars represent the standard deviation. The difference in mean values at a significance level of 0.05 was significant with an *F*-value of 2.8

$58 \pm 6\%$. These three carriers exhibited an ongoing decrease of relative activity during the cycles, with final relative activities between $23 \pm 3\%$ and $25 \pm 2\%$. The relative activity of IB-HIS-2 and 20 increased within the first three cycles to over 40% and then started to decline. For IB-HIS-1, the highest relative activity of $65 \pm 7\%$ is reached after four cycles. Next, the relative activity drops to below 50% but remains at this level for the following eight cycles, just dropping to $26 \pm 2\%$ in the final cycle. IB-HIS-8 reached the maximal relative activity of $50 \pm 4\%$ after seven cycles and continuously decreased afterward. To further investigate immobilized PCI_Lip and its mutants, IB-HIS-19 was selected because of its high binding capacity and low leakage combined with the highest relative activity in several cycles of recycling.

Next, the hydrolysis profiles of PCI_Lip WT and the two double mutants S163M+L302G and I245F+L302G, which exhibit an altered selectivity towards short- and medium-chain fatty acids (Henrich et al. 2026), immobilized to IB-HIS-19, were examined to see how immobilization affects activity and selectivity (Fig. 3). The activity was normalized to *p*NPO of the free WT, while for the free mutants it was normalized to *p*NPH as they are not active on *p*NPO. The immobilized WT showed slightly higher activity towards short-chain substrates. Activity towards medium- and long-chain substrates was reduced by 10–20%. Immobilization of the WT did not cause major changes in the activity and selectivity of the enzyme (Fig. 3A). As mutants S163M+L302G and I245F+L302G have altered selectivities, *p*NPO is no longer the preferential substrate, but selectivity was shifted more towards short-chain fatty acids. Both mutants are strongly reduced in hydrolytic activity against *p*NPO; therefore, relative specific activity is calculated for *p*NPH. The hydrolysis profile of immobilized S163M+L302G did not change the enzyme selectivity. However, immobilization boosted the activity against *p*NPV and *p*NPH. These substrates were converted with 1.5- or 3-fold increase compared to the free enzyme (Fig. 3B). The immobilization of I245F+L302G affected both activity and selectivity. The free mutants predominantly hydrolyzed *p*NPV and *p*NPH among the tested esters. Immobilization reduced the specific activity for two substrates by more than half of the activity in comparison to the free enzyme. Besides the lower activity, selectivity has been changed to more intensely hydrolyze short-chain fatty acids such as *p*NPA but also long-chain fatty acids such as *p*NPP (Fig. 3C).

Stabilization of PCI_Lip

The melting temperature T_M of PCI_Lip WT in ultrapure water was determined to be $63 \pm 0^\circ\text{C}$. First, the most suitable buffer, concentration, and pH value for improved enzyme stability were investigated (Fig. 4). The melting temperatures showed that PCI_Lip WT is most stable at slightly acidic

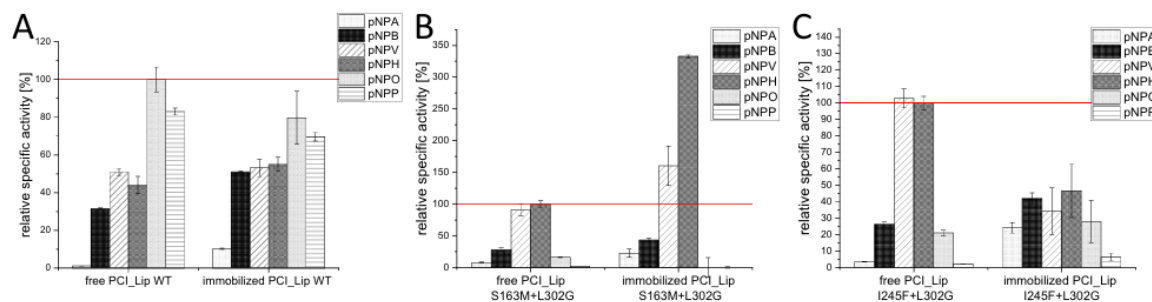


Fig. 3 Hydrolysis profiles of PCI_Lip WT and the two mutants (S163M+L302G and I245F+L302G). **A** Relative specific activities of six different pNP esters of the free and immobilized PCI_Lip WT compared to the activity of the free WT to pNPO (100%, red line). **B** Relative specific activities of six different pNP esters of the free and immobilized PCI_Lip S163M+L302G compared to the activity

of free S163M+L302G to pNPH (100%, red line). **C** Relative specific activities of six different pNP esters of the free and immobilized PCI_Lip I245F+L302G compared to the activity of free I245F+L302G to pNPH (100%, red line). The difference in mean values at significance level of 0.05 was significant with an *F*-value of 52.0

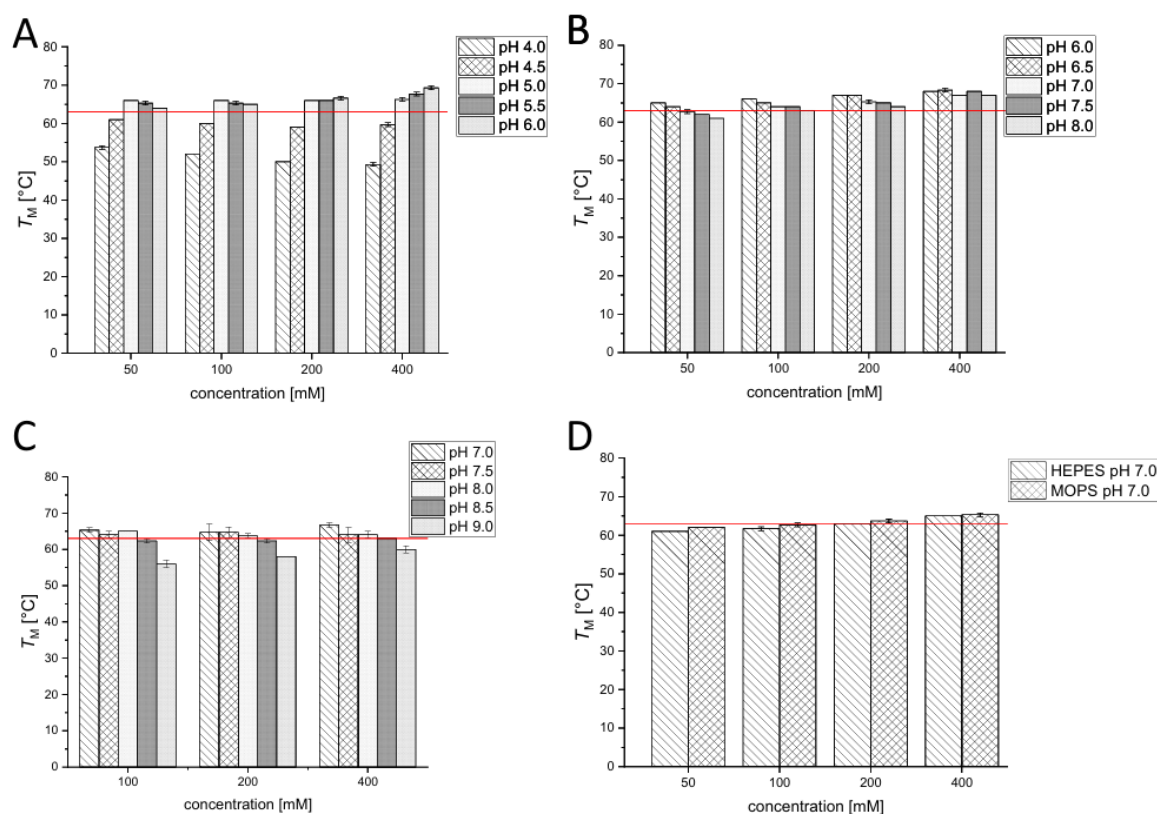


Fig. 4 **A** Melting temperature of PCI_Lip WT using various concentrations and pH values of citrate buffer. The red reference line indicates the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was significant with an *F*-value of 2.8. **B** Melting temperature of PCI_Lip WT using different concentrations and pH values of potassium phosphate buffer. The red reference line shows the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was not significant with an *F*-value of 1.2. **C** Melting temperature of

PCI_Lip WT using different concentrations and pH values of TRIS buffer. The red reference line represents the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was significant with an *F*-value of 22.3. **D** Melting temperature of PCI_Lip WT using different concentrations of HEPES and MOPS buffers, each at pH 7.0. The red reference line denotes the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was not significant with an *F*-value of 0.4

to neutral pH values. From pH 5.0 to 8.0 almost all melting points with citrate buffer, PPB and TRIS buffer displayed higher melting temperatures compared to the reference. More acidic and alkaline pH values led to a destabilization of PCI_Lip and T_M dropped below 60 °C. Within the optimum pH range a higher concentration of the buffer supported the stability of the lipase. This effect was especially visible for HEPES and MOPS buffers. There was no major difference between both buffers. For both buffers, the lowest concentration was destabilizing, but with increasing concentration, T_M rose above the reference. As a conclusion from the buffer screening, citrate buffer pH 5.5 and 6.0 as well as PPB pH 6.0 and 7.0 each 400 mM were the best for stabilizing PCI_Lip WT increasing T_M up to 6 °C. Further additives were screened to analyze their effects on enzyme stability (Fig. 5). Most of the screened salts stabilized the enzyme. The stabilizing effect increased with higher concentration for all alkaline chlorides, while it was most prominent for KCl. For $MgCl_2$, stability decreased with increasing concentration. The addition of

$CaCl_2$ did not show major impact on the melting temperature. Higher concentrations of ammonium sulfate enhanced the thermostability at higher concentrations. Potassium iodide stabilized PCI_Lip better than sodium iodide. Among the sodium halogenides, iodide had the highest increase and bromide the least increase of T_M ; however, all of them increased the melting temperature with increasing concentration. The effect of increasing stability with concentration was also identified for glycerol. DMSO and polyethylene glycol reduced T_M of PCI_Lip. Also, the addition of different amino acids lowered T_M . Adding sugars to PCI_Lip increased its thermodynamic stability slightly but no effect of the concentration was observed. Furthermore, the two double mutants S163M+L302G and I245F+L302G were investigated on their thermodynamic stability (Fig. 6A, B). Melting temperatures were determined in the four best buffers identified in the buffer screening for the WT. The mutations S163M+L302G did not affect the thermodynamic stability as melting temperatures are the same as PCI_Lip WT in these buffers. The mutations I245F+L302G

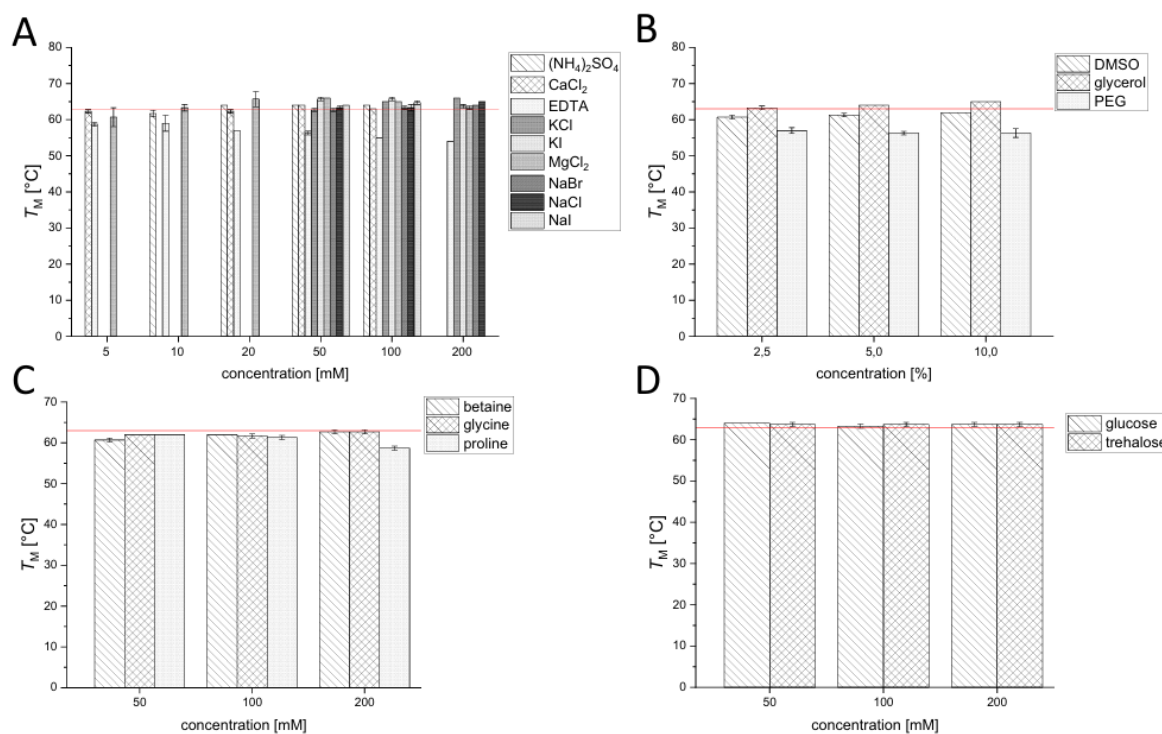


Fig. 5 **A** Melting temperature of PCI_Lip WT using various concentrations of selected salts. Different salts were tested at different concentrations according to literature references. The red reference line indicates the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was significant with an F -value of 14.7. **B** Melting temperature of PCI_Lip WT using different concentrations of polyols and DMSO. The red reference line shows the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was sig-

nificant with an F -value of 100.2. **C** Melting temperature of PCI_Lip WT using different concentrations of amino acids. The red reference line represents the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was not significant with an F -value of 1.2. **D** Melting temperature of PCI_Lip WT using different concentrations of sugars. The red reference line denotes the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was not significant with an F -value of 0.2

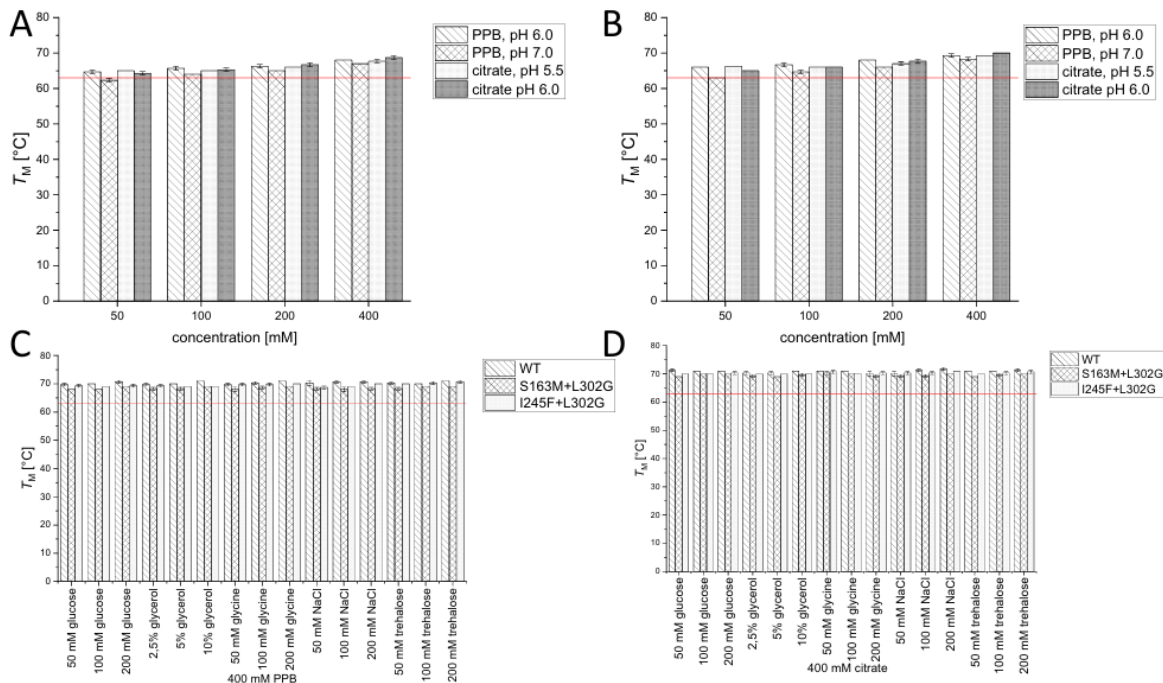


Fig. 6 **A** Melting temperature of PCI_Lip S163M+L302G using potassium phosphate buffer (PPB) and citrate buffer at various concentrations and the pH values that performed best in previous measurements with PCI_Lip WT. The difference in mean values at significance level of 0.05 was not significant with an F -value of 0.9. **B** Melting temperature of PCI_Lip I245F+L302G using PPB and citrate buffer at different concentrations and the pH values that performed best in previous measurements with PCI_Lip WT. The difference in mean values at significance level of 0.05 was not significant with an

F -value of 0.9. **C** Melting temperature of PCI_Lip WT and the two double mutants using 400 mM PPB at pH 7.0 and different additives. The difference in mean values at significance level of 0.05 was significant with an F -value of 36.2. **D** Melting temperature of PCI_Lip WT and the two double mutants using 400 mM citrate buffer at pH 6.0 and different additives. The red reference line indicates the melting temperature of PCI_Lip WT in water. The difference in mean values at significance level of 0.05 was significant with an F -value of 16.8

had a positive effect on the thermodynamic stability compared to the PCI_Lip WT as T_m for each buffer composition was increased by 1 ± 0.3 °C. Finally, the thermodynamic stability of PCI_Lip and both double mutants in 400 mM citrate buffer pH 6.0 and 400 mM PPB pH 7.0 with a variety of additives was investigated (Fig. 6C, D). Overall, combining the most stabilizing buffers with favorable additives further increased the stability of PCI_Lip. No negative effects were observed by combining the buffers with the additives and only a few combinations did not change T_m . Within this set, PCI_Lip WT in 400 mM PPB pH 7.0 with 200 mM glycine and 200 mM trehalose respectively showed the highest rise in T_m with 4 ± 0.5 °C compared to the melting temperature in this buffer. All in all, the highest melting temperatures of 71 ± 0 °C were reached by the WT in 400 mM citrate buffer pH 6.0 with the addition of high concentrations of glucose and trehalose. In summary, the most effective additives to elevate thermodynamic stability were 400 mM citrate buffer pH 6.0 or PPB pH 7.0 combined with 10% glycerol or 200 mM of NaCl, glucose or trehalose. Besides the thermodynamic stability,

kinetic stability was examined by monitoring the activity of the lipase after incubation for 60 min at different temperatures. T_{50}^{60} values were determined for the free and immobilized mutants (Fig. 7). The T_{50}^{60} values help to evaluate changes in the kinetic stability of an enzyme by representing the temperature where the enzyme activity is reduced by 50% after 1 h of incubation. For the free WT, T_{50}^{60} is calculated to be 29.7 ± 2.8 °C; for mutant I245F+L302G, T_{50}^{60} is calculated to be 34.4 ± 0.3 °C; and for S163M+L302G, T_{50}^{60} is calculated to be 38.1 ± 1.2 °C. The immobilized WT had a T_{50}^{60} value of 37.1 ± 0.7 °C, while for S163M+L302G T_{50}^{60} is 43.0 ± 0.4 °C and for I245F+L302G it is 39.6 ± 1.6 °C.

Further the storage stability of free and immobilized PCI_Lip WT and mutants was investigated over a period of 28 days. Therefore, the activity of the lipase after 1, 10, and 28 days of storage at 4 °C was measured (Fig. 8). The highest specific activities were measured with PCI_Lip WT within the first 10 days stored in PPB of any concentration. The WT activity increased within the first 10 days before a decrease after 28 days was noted. During the

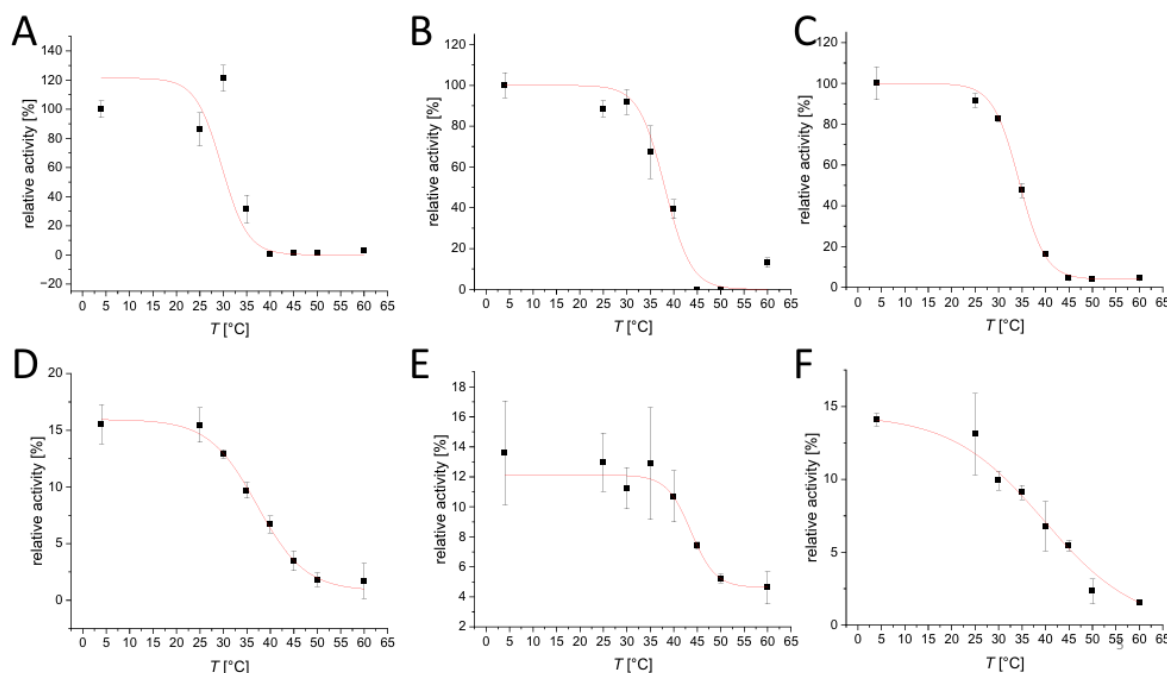


Fig. 7 Top row: Activities of free PCI_Lip WT (A), S163M+L302G (B), and I245F+L302G (C) after incubation at different temperatures measured photometrically using *p*NPO for the WT and *p*NPH for the mutants as substrate. All samples were measured in triplicates, error bars represent standard deviation. The activity data were fitted in OriginPro® 2023 using Boltzman fit. The turning point of the curve constitutes the T_{50}^{60} value of this mutant. Bottom row: Activities compared to the respective free enzyme activity of immobilized

PCI_Lip WT (D), S163M+L302G (E), and I245F+L302G (F) after incubation at different temperatures measured photometrically using *p*NPO for WT and *p*NPH for the mutants as substrate. All samples were measured in triplicates, error bars represent standard deviation. The activity data were fitted in OriginPro® 2023 using Boltzman fit. The turning point of the curve constitutes the T_{50}^{60} value of this mutant. For confidence intervals and determination coefficients, see Supplementary Table S4

storage of S163M+L302G, the activity remained almost the same over 28 days. Within the first 10 days of storage of I254+L302G, activity increased and stayed almost the same up to 28 days. The initial specific activities of immobilized PCI_Lip WT stored in PPB were slightly lower than the activity of the free lipase. The activity of PCI_Lip WT decreased within the first 10 days and then stayed almost similar over the remaining storage time. The specific activity of immobilized PCI_Lip S163M+L302G was similar beyond the different concentrations of storage buffer. In this case, activity increased over the whole storage period. In the case of immobilized PCI_Lip I245F+L302G the initial activity of the lipase stored in 200 and 400 mM PPB is comparable to the initial specific activities of the free mutant. Preparations stored in 80 and 100 mM PPB displayed incipiently a specific activity twice as high as the ones of the free mutant. Within the first 10 days, the activity increased in all preparations. Over the remaining storage time activity stayed the same except for immobilized I245F+L302G stored in 400 mM PPB where it further increased.

Regioselectivity of PCI_Lip WT

After the conversion of trioctanoate for 30 min and 2 h in aqueous buffer at 30 °C, the hydrolysis products were investigated by ^1H -NMR analysis (Supplementary Figure S1). The respective compounds and the associated proton shifts are listed in Supplementary Table S5. In the ^1H -NMR spectra of the hydrolysis mixture, one can distinguish peaks of protons from the glycerol group of the triglyceride (TG) initially added as substrate and peaks that can be assigned to di- and monoglycerides. Evaluation was conducted with the help of NMR spectra of standard glycerides, also reported in the Supplementary Figures S2–S5. The results of the quantification of all glycerides in the ^1H -NMR spectra of hydrolysis mixtures in duplicate are reported in Supplementary Table S6, along with blank experiments. 1,2-Diglycerides (DG) and 1-monoglycerides (MG) are the most important products of TG hydrolysis already at 30 min, and their amount is even higher at 2 h. In conversion experiments using 1,2- and 1,3-DGs without lipase, an acyl shift was observed for both types of DGs after 30 min in aqueous buffer at 30 °C. As shown in Supplementary Figure S6, 60% of 1,2-DG was transformed to

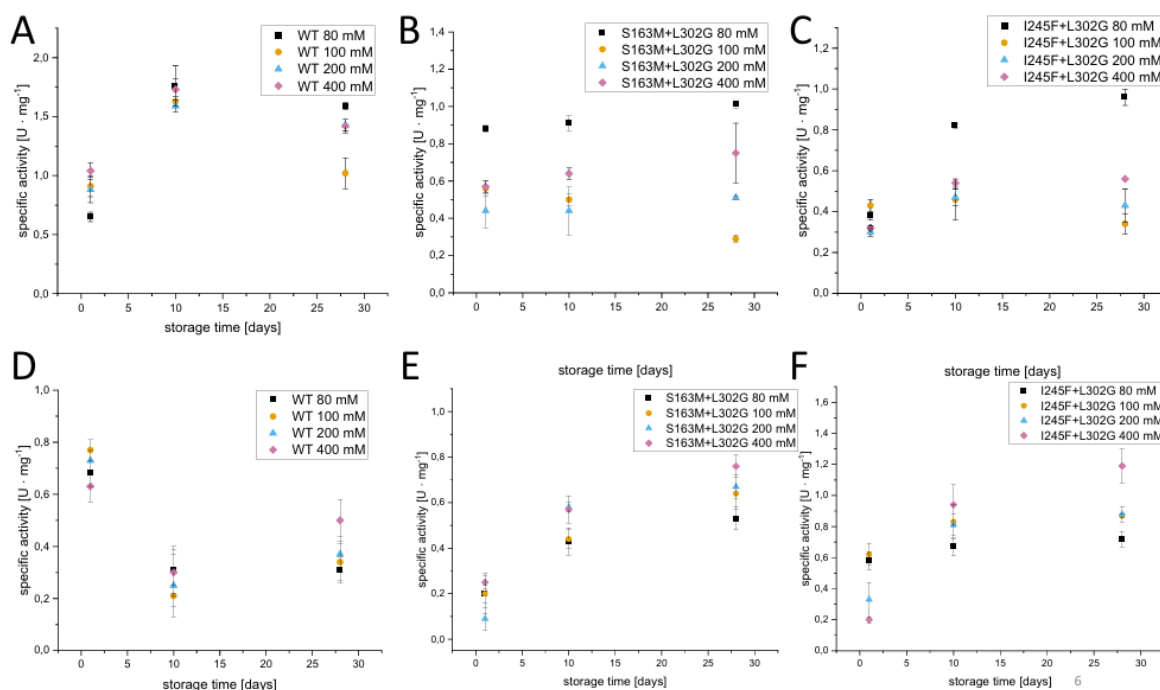


Fig. 8 Storage stability of PCI_Lip WT and variants over a period of 28 days in different potassium phosphate buffers (PPB), evaluated by specific activity: **A** PCI_Lip WT, **B** PCI_Lip S163M+L302G, **C** PCI_Lip I245F+L302G, **D** immobilized PCI_Lip WT, **E**

immobilized PCI_Lip S163M+L302G, **F** immobilized PCI_Lip I245F+L302G. The difference in mean values at significance level of 0.05 was significant with an *F*-value of 13.8

1,3-DG in 30 min, while only 20% of 1,3-DG was isomerized to 1,2-DG in the same time. This indicates a much stronger acyl shift for 1,2-DGs. The strong acyl shift for 1,2-DGs implied that the initial amounts of 1,2-DGs produced were even higher than those observed in the NMR spectra. Some of it was isomerized directly to 1,2-DGs, indicating a strong preference of the enzyme for the outer positions of TGs. The presence of high quantities of 1-MG also points to this direction, as 1,3-DGs only have outer ester groups that the enzyme hydrolyzed to produce 1-MGs. From these findings, we concluded that PCI_Lip WT displayed a preferential regioselectivity for fatty acids esterified in the outer positions of a TG in the first stage of hydrolysis at the tested conditions.

Discussion

Immobilization

Binding capacity and leakage of different carriers

Generally, the best immobilization rates were achieved on the IB-HIS carriers with affinity binding of the lipase via a His₆-tag.

However, no conclusion can be drawn concerning the functionalization of the carriers or polarity that promoted binding of PCI_Lip. IB-HIS-1 is classified as very hydrophobic due to the high amount of butyl groups and low amount of Ni-IDA. It displays a similar binding capacity as IB-HIS-20 functionalized with ammonia groups and Ni-NTA, which is classified as very hydrophilic. The particle size reached from 150 to 710 μm, while the supplier offers no information on the pore size. Beyond all tested IB-HIS carriers, the particle size is mostly similar, only IB-HIS-15 with 250–400 μm, IB-HIS-16 with 20–40 μm, and IB-HIS-17 with 100–250 μm deviate from the average of 150–710 μm. Studies on immobilized lipases from *Mucor miehei* and *Rhizopus oryzae* revealed that the particle size of the support has no influence on the binding capacity. The main factor concerning binding capacity was found to be the pore size of the support material, which may also influence the activity of the immobilized enzyme (Gustafsson et al. 2012). The advantage of these supports is immobilization by affinity using the His₆-tag fused to PCI_Lip to immobilize the enzyme. Compared to other immobilization techniques such as adsorptive or covalent binding methods, affinity immobilization uses highly selective interactions between the support and enzyme. This offers a controlled orientation of the enzyme avoiding inactivation by blocking the

active site. Further, the mostly peripheral position of affinity tags reduces conformational changes of the enzyme due to immobilization (Anboo et al. 2022). The relative specific activity and hydrolysis profiles of free and immobilized PCI_Lip WT are very similar, which rules out major conformational changes and supports the minimal impact of this immobilization technique. The polar cellulose matrix of IB-HIS-16 might be one reason for its overall low binding capacity. Mostly hydrophobic support surfaces are described as more suitable for effective immobilization of lipases due to their interfacial activation (Deon et al. 2020). Even though, this affinity binding is described as rather weak (Homaei et al. 2013), for PCI_Lip immobilized on the IB-HIS carriers also other factors seemed to have an impact on the binding strength. Putatively, there might be further non-covalent interactions of the lipase with the surface of the support besides the binding of the His₆-tag to the Ni²⁺-ion. The hydrophobic, mesoporous polystyrene support ECR 1090 M was marginally better than the hydrophilic amino C₂-derivatized methacrylate support ECR 8806 M, even though the latter had a much lower surface area and smaller pore size. Adsorption is the most favorable immobilization method for industrial applications because of its low cost and simplicity (Ishak et al. 2025). Though the two supports investigated in this study do not seem to be the optimal supports for PCI_Lip WT. Some studies report an increased specific activity of immobilized lipases, which is attributed to the interfacial activation of the lipase being immobilized in its open conformation (Mokhtar et al. 2020). The low amount of lipase immobilized to the adsorptive carriers may influence specific activity of PCI_Lip positively, as the enzyme carries a putative lid domain responsible for interfacial activation (Broel et al. 2022). Both covalent carriers were used in the recycling experiment with glutaraldehyde as the linker and spacer between enzyme and support. This was reported to have positive effects on the activity of immobilized enzymes. By using a spacer in covalent immobilization, the enzyme remains more flexible and steric hindrance is avoided (Zhang et al. 2013). Further, the activity of PCI_Lip after a harsher immobilization treatment should be investigated. As covalent immobilization is described to have a reduced loss of enzyme (Homaei et al. 2013), it is possible that besides the covalent immobilization of PCI_Lip, some of the enzyme was absorptively attached to the support and was washed off during the cycle.

Recycling of immobilized PCI_Lip

During the recycling, seven immobilisates could be divided into two groups. The activity of the first group exhibits a similar development as the activity observed for *Candida antarctica* lipase B (Cal B) immobilized on bagasse for the production of biodiesel (Cui and Di Cai 2018). A minor increase during the first cycles may be due to a rearrangement of the immobilized lipase into a superior conformation or position. This could be observed especially when

there was a longer storage period up to the following measurement (Gustafsson et al. 2012). The microenvironment around the immobilized PCI_Lip keeps it stable to retain a relative activity above 60% in the beginning. Accessibility of the active site does not seem to be heavily impacted by immobilization to these support materials. However, the stabilization of the enzyme by immobilization can be caused by increased rigidity of the enzyme, hindering conformational changes during the catalysis (Stepankova et al. 2013). This may cause lower initial activity than the free PCI_Lip. But, immobilization to these support materials seemed to result in smaller conformational changes with little impact on the activity than the other four immobilisates. The second group of immobilized enzymes may face restricted active site accessibility or mass transfer limitations, causing the relatively low activity compared to the free enzyme (Robescu and Bavaro 2025). The increase within the first cycles was probably caused by diffusional limitations of the substrate and product. For the recycling, the activity of immobilized PCI_Lip was measured photometrically, using pNPO as substrate. Even though the supernatant has been totally removed after each cycle and the carriers have been washed, a slight yellow coloring of the carriers remained, indicating that at least pNP could not be removed completely. The increased activity could be attributed to the potential retention of substrate in the immobilisate matrix from previous cycles and elution in the further cycles. Mass transfer limitations are common constraints in enzyme immobilization. For efficient use of immobilized enzymes, free diffusion of substrate and product is necessary. The mobility of both can be restricted by the functionalization of the carrier surface and the pore size of the support. Diffusional limitations can severely impact enzyme activity by reducing the reaction rate, fortifying substrate inhibition and the formation of pH gradients (Hanefeld et al. 2009; Zdarta et al. 2018). In the case of these four IB-HIS carriers, the pore size does not seem to be suitable for the immobilization of PCI_Lip, as the product was held back. Generally, a smaller pore size leads to a larger surface area, which can increase enzyme loading, but it can inhibit mass transfer. Therefore, the ideal support material has to be evaluated for every enzyme immobilization individually (Chen et al. 2019). Selective individual increases in relative activity during the recycling can be described through longer storage periods overnight between the cycles. Storage was conducted in the standard buffer, which seemed to regenerate inactivated, immobilized lipase. These increases in activity are probably assigned to a combination of restricted mass transfer and enzyme regeneration. Chen and Wu describe the regeneration of an immobilized lipase in biodiesel production. The washing removed substrate molecules that stuck to the immobilized enzyme, causing its inactivation (Chen and Wu 2003). In our case, longer storage may have removed perturbing molecules from PCI_Lip.

After the recycling experiment, IB-HIS-19 was chosen as the best carrier for further investigations of the immobilized PCI_Lip. The affinity binding of PCI_Lip offered a simple way of immobilization where no hazardous chemicals were needed. This carrier had already displayed a high binding capacity in combination with low leakage. In the recycling experiments, it stood out with high initial activity, which was kept up for five cycles with a slow decline afterward.

Characterization of immobilized PCI_Lip

For PCI_Lip WT the advantage of affinity immobilization becomes clear. The immobilization on the peripheral affinity tag of PCI_Lip did not cause severe conformational changes or hinder the flexibility of the enzyme. The affinity tag of PCI_Lip ensures controlled orientation of the enzyme without the risk of activity loss caused by blocking the active site. The attachment of the enzyme on the His₆-tag causes only minor conformational changes as opposed to other immobilization techniques. Either adsorption or covalent binding always affects the surface of the enzyme, sometimes even at multiple points. This enhances the stability of the enzyme by making it more rigid. However, the increased rigidity may cause reduction or even loss of activity (Anboo et al. 2022). Besides acting as the chelating agent of the divalent nickel ion the nitrilotriacetic acid is a spacer between the carrier surface and the enzyme. This enlarges the orientation space of the enzyme further reducing conformational restrictions. Also the higher flexibility of the enzyme leads to a stronger binding (Alács et al. 2024). The slight decrease of activity of immobilized PCI_Lip WT is caused by the interaction of the lipase with the carrier surface. Besides the attachment via the His₆-tag parts of the enzyme surface can interact with functional groups of the carrier surface by means of hydrophobic and van-der-Waals interactions as well as hydrogen bonds (Alács et al. 2024; Tzialla et al. 2009). The additional adsorptive interactions further stabilized the immobilized PCI_Lip. This goes along with the increased T_{50}^{60} values of the immobilized enzyme compared to the free one. However, the increased stability engendered a slight reduction of the activity. Further hydrolysis profiles of the two immobilized double mutants S163M+L302G and I245F+L302G have been compared to their free versions. These mutants have been created to shift the selectivity of PCI_Lip to an increased hydrolysis of short- to medium-chain fatty acids and also reduce the hydrolysis of long-chain fatty acids. Therefore, amino acids mainly in the binding pocket of PCI_Lip have been substituted (Henrich et al. 2026). For S163M+L302G the activity for its preferential substrates *p*NPV and *p*NPH is strongly increased. Several studies describe an increased activity of lipases when immobilized (Cui and Di Cai 2018; Deon et al. 2020; Dong et al. 2021; Tzialla et al. 2009; Zhao et al. 2008). This is often explained by the immobilization of the lipases

in their most active state. Most lipases including PCI_Lip share the common feature of a lid domain that covers the active site and opens up, when in contact to a hydrophobic interphase. Especially when using adsorptive immobilization on hydrophobic supports the lipase can be immobilized in its open conformation showing the highest activity. The permanent open conformation of a lipase enables free access of the substrate to the active site and causes considerably higher activity often called hyperactivation (Blanco et al. 2004; Deon et al. 2020). Even though the amino acid exchanges of S163M+L302G are located near and inside the substrate channel, they seem to affect the overall conformation of PCI_Lip. The implementation of methionine and glycine may have changed the conformation of the lipase. This might be in a way that adsorptive interactions are enhanced that favor the open conformation and cause hyperactivation. As mutation L302G is present in both investigated mutants, the hyperactivation seems to be associated with mutation S163M. This one is positioned a little aside the substrate channel and has previously been shown to alter the chain length selectivity towards short- to medium-chain fatty acids (Henrich et al. 2026). The mutation is located further away from the active site, near the substrate channel and the lid domain. It may therefore influence the conformation of the lid domain without noticing beforehand but refining the mutants' properties when immobilized. The free form of PCI_Lip mutant I245F+L302G is most active against *p*NPV and *p*NPH, but already exhibits reduced activity compared to the WT (Henrich et al. 2026). Immobilization of this mutant reduced its activity by almost half and simultaneously shifted its selectivity. Both substitutions are located in the substrate channel but replacing isoleucine with phenylalanine and leucine with glycine seems to impact on the overall enzyme conformation, leading to a diminished enzyme activity when immobilized. The combination of amino acid substitutions and immobilization lead to a reduced activity in this case. These minor changes can also impact enzyme selectivity. Several factors have been described to putatively influence selectivity when an enzyme is immobilized. The outcome is hard to predict and has to be tested for each enzyme/carrier combination individually. Potential reasons for an alters selectivity in a specific enzyme/support combination may be diffusional limitations, the micro-environment, structural modifications, or a changed rigidity (Rodrigues et al. 2013). In this case, the substitution of isoleucine to the bulkier phenylalanine in combination with the immobilization may downgrade the free diffusion of the substrate molecules into the active site leading to the reduction of activity. A similar change of selectivity has been observed for a lipase from *R. miehei* when immobilized to a resin. This immobilized lipase became more active on short-chain fatty acids as well (Lee and Parkin 2001). The examination of the hydrolysis profiles of PCI_Lip WT and two double mutants underlined that every enzyme/

support combination accounts for different results. Immobilization of WT to HB-HIS-19 displayed only minor changes in activity, while immobilization of S163M+L302G showed a hyperactivation and I245F+L302G exhibited reduced activity combined with altered selectivity.

Among other aspects, the aim of this study was to improve the stability of PCI_Lip. Therefore, thermodynamic stability and kinetic stability of the lipase have been investigated. Thermodynamic stability refers to the enzyme conformation and its tendency to denature, while kinetic stability deals with irreversible inactivation and the loss of activity (Iyer and Ananthanarayan 2008). The introduction of two other amino acids in the free double mutants led to an increase in the T_{50}^{60} value compared to the WT with a T_{50}^{60} value of 29.6 ± 2.8 °C. Exchanging leucine for glycine at position 302 raised the semi-inactivation temperature for 1.5 °C (Broel et al. 2022). Both double mutants carry this mutation, which has been shown to have a positive effect on the T_{50}^{60} value. The increase of kinetic stability in the free double mutants compared to the WT is likely a side effect of mutating selected amino acids in or near the substrate channel. Xie et al. have chosen a similar approach to stabilize Cal B. They chose against established approaches that target increased structural rigidity, restricted conformational flexibility or boost the interactions of unstable domains. Since the active site of an enzyme is a rather fragile part, nevertheless vital to catalytic activity, they aimed to stabilize this part of the enzyme by exchanging amino acids near the active site. This strategy led to a mutant with a semi-inactivation temperature 12 °C above that of the WT in the Cal B (Xie et al. 2014). The mutations in the substrate channel stabilized the active site of PCI_Lip, which led to an increased kinetic stability of the free double mutants. The combination of amino acid exchanges seemed to have increased the semi-inactivation temperature even more. Similar effects have been observed in a creatinase, where the combination of mutations led to an increase in T_{50} (Bian et al. 2024). Immobilization of PCI_Lip further enhanced the kinetic stability of the enzyme. The semi-inactivation temperature of the WT and both mutants was increased by about 6–7 °C through immobilization. As the increase is similar in all three variants it can be attributed to immobilization, and the individual differences have no major impact here. The enhanced thermal stability was a result of the rigidification of PCI_Lip through immobilization. A more rigid enzyme can resist the denaturing effects of higher temperatures better than the free enzyme with high flexibility (Khan 2021). Our findings go along with literature data showing that immobilization of lipases leads to an enhanced thermostability of the respective enzymes (Wilson et al. 2006; Xie et al. 2014; Costantini and Califano 2021). The enhanced stability of PCI_Lip through immobilization that simultaneously keeps its activity offers the chance for industrial applications. Immobilized PCI_Lip could be

implemented in the biotechnological production of biodiesel. Therefore, the current findings could be combined with previous findings, showing that chain length selectivity of PCI_Lip can be altered by protein engineering (Henrich et al. 2026). This can offer a chance to find a variant suitable to hydrolysis of long-chain fatty acids with improved stability when immobilized to a support material such as IB-HIS-19.

Thermodynamic stability of PCI_Lip and its mutants

Thermofluor assays provide the possibility to examine the structural stability of a protein and the influence of its environment. While for the kinetic stability the hydrolytic activity of PCI_Lip was monitored, the thermodynamic stability monitored the structural denaturation. The melting temperature observed in the Thermofluor assays refers to the temperature at which half of the enzyme is denatured (Iyer and Ananthanarayan 2008). The comparison of kinetic and thermodynamic stability of PCI_Lip displays that the enzyme structures that are essential for the hydrolysis unfolded before the whole enzyme scaffold denatured. Compared to Cal B with T_M of 54.5 °C, which is often used for industrial purposes, PCI_Lip offers a higher thermostability (Kim et al. 2010). The elevated T_M brings advantages for putative industrial applications. In industrial processes, higher temperatures are used as reaction rates are increased and substrate solubility is better. In this case, lipases from thermophile organisms offer much higher thermostability; for example, a lipase from *Thermomyces lanuginosus* has a T_M of 93 °C (Xiang et al. 2023). However, microbial lipases mainly catalyze the hydrolysis of long-chain fatty acids (Chandra et al. 2020). The advantage of PCI_Lip and especially the two double mutants is their selectivity for the hydrolysis of medium-chain fatty acids and its mutants offer a selectivity for short-chain fatty acids (Wang et al. 2025; Henrich et al. 2026). The Thermofluor method applied in this study offered the chance to improve the thermostability of this lipase with special selectivity for short- to medium-chain fatty acids to meet the requirements for an industrial application. An increase in T_M with higher buffer concentration has also been observed for other proteins. Higher buffer strength leads to a higher concentration of ions in the hydration shell of the enzyme and increases water activity on the protein surface. Both give a better structural order and reduce conformational flexibility, resulting in a more thermostable enzyme (Boivin et al. 2013; Kalisz et al. 1997). The chelating agent EDTA can be added to an enzyme solution in order to avoid oxidation of SH-moieties (Boivin et al. 2013). For PCI_Lip, the addition of EDTA did not help to stabilize the enzyme. The addition of salts is a common tool to elevate enzyme stability. Generally, the stabilizing effect of neutral salts is ascribed to ions binding to charged groups on the protein

surface and a competition for solubilization in water. Both enhance the hydrophobic interactions of the protein, boosting its stability. While some ions with chaotropic effects rather destabilize enzymes, ions with kosmotropic effects support stabilization. The effects of each ion on enzyme stability are described by the Hofmeister series (Silva et al. 2018). In this study, chloride, bromide, and iodide as anions were tested on the stability of PCI_Lip. Bromide has a less stabilizing effect on the lipase than chloride, but iodide has a more stabilizing effect. Within the Hofmeister series, chloride is claimed to have no effect on the water structure; bromide is described as an anion with slightly chaotropic effect (Lavelle and Fresco 2003). This chaotropic influence of bromide in comparison to chloride is displayed by PCI_Lip with a lower T_M . According to the Hofmeister series, iodide should be more destabilizing. At this point, the series is not in accordance with our results. By screening the three most common sodium halogenides, iodide displayed the highest increase in T_M . In the case of Hofmeister series for cations, larger ones with lower charge are considered to salt out proteins, while smaller ones with a higher charge, salt proteins (Kherb et al. 2012). According to the Hofmeister series, in this study, Mg^{2+} should stabilize the protein best and K^+ least good. However, the melting temperatures of PCI_Lip WT were highest with the highest concentration of KCl. With increasing concentration of Mg^{2+} , T_M decreased. Calcium, as another divalent ion, barely affected the stability of PCI_Lip even though it is in similar position as magnesium in the Hofmeister series. This discordance also goes along with the results of magnesium. Ammonium sulfate combines two ions that are supposed to have a positive impact on the enzyme stability (Silva et al. 2018). At this point, it is to mention that the Hofmeister series acts as a general guideline with some conformity to stabilizing ions for PCI_Lip. Effects of ions on enzyme stability are highly individual. This is reflected by the deviance from this theoretical series in our data. For PCI_Lip, an increase in T_M appears at higher concentrations, but other salts at the respective concentrations enhance stability even more. Polyols in general are known to stabilize proteins. Even though the mechanism behind this effect has not yet been fully elucidated, it is traced back to the promotion of hydrophobic interactions within the enzymes, making them more rigid, which increases thermostability (Balcão and Vila 2015). The addition of glucose, trehalose, and glycerol as polyols increased the thermostability of PCI_Lip. The highest increase in T_M was observed with the addition of 10% glycerol by 2 °C. Research is ambivalent about the effects of DMSO on protein structures and stability. On the one hand, it is described to have positive effects on protein stability, being a popular and widely used cosolvent in bioassays. On the other hand, in some studies, its destabilizing effect on proteins even at low level concentrations is shown

(Cubrilovic and Zenobi 2013; Landreh et al. 2014). PCI_Lip seems to be another example for DMSO having destabilizing effects on the enzyme. Still, this does not mean that DMSO generally affects protein stability in a negative way, as Chan et al. found that the impact of DMSO on enzyme stability is highly protein-dependent. In this case, the individual interactions with charged states and electrostatic repulsions caused destabilization of avidin at 4% DMSO but boosted the stability of a bacterial cytochrome at a concentration of 40% (Chan et al. 2017). PEG had a destabilizing effect on PCI_Lip while high molecular weight, PEG 20k, was used. Most literature describes PEG as crowding agents with positive effects on stability. However, recently, some studies showcased some negative effects. It has become clear that, similar to DMSO, effects are protein-dependent and also the size of PEG matters. Conformational changes caused by high molecular PEG have been proven in a tRNA-synthetase from *E. coli* (Liebau et al. 2024). As high molecular PEG has an amphiphilic character, this might affect hydrophobic parts of the lipase causing destabilization and eventually a lower T_M . Some amino acids have been reported to inhibit protein aggregation and support renaturation (Boivin et al. 2013). Therefore, the impact on T_M of PCI_Lip by adding betaine, glycine, and proline respectively has been investigated. In this case, none of the amino acids had a positive impact on thermal stability. Even though the improvement of thermostability has not been the initial aim of the protein engineering approach, we decided to investigate the two mutants with the best changes in the hydrolysis profiles in this study. These investigations gave deeper insights in the stability of these mutants for putative industrial applications of a microbial lipase highly selective for short- to medium-chain fatty acids. We suggest that the mutation I245F is causing enhanced stability as the mutation L302G appears in both of the tested mutants. Even though the enhancement of stability was not the initial intention of the protein engineering, the results are in line with other studies aiming to improve thermostability by the exchange of amino acids. The mechanisms underlying the stabilization of an enzyme by the introduction of other amino acids are versatile. In this case, it seems likely that the increased stability could be attributed to the formation of new π - π interactions with F165 stabilizing this rather flexible region (Bai et al. 2020; Bian et al. 2024). This mutation is located in the substrate channel. Xie and co-workers followed a similar strategy to improve the kinetic stability of Cal B by mutating amino acids in flexible regions near the active site. This increased the rigidity of the enzyme in an area vital for its activity, increasing its half-life time 13-fold (Xie et al. 2014). Finally, the thermostability of PCI_Lip WT and both double mutants was investigated using the best buffer systems with the best additives. The results show that the improvement of the composition of the enzyme solution is a good tool to improve the properties of

an enzyme for a potential industrial application. Glycerol, glycine, and trehalose were most effective in increasing T_M in comparison to the pure buffer. All in all, citrate buffer with glucose, glycerol, glycine, NaCl, and trehalose could increase T_M to $71 \pm 0^\circ\text{C}$ and PPB with glycerol, glycine, and trehalose reached the same T_M . The improvement of thermodynamic stability of PCI_Lip by selection of appropriate buffer systems gives rise to a broad use of this lipase in biotechnological processes. The high stability makes the lipase a good candidate for a biocatalyst in the production of chemical or pharmaceutical compound, as these reactions are often conducted under elevated temperatures.

Storage stability of free and immobilized PCI_Lip

An increase of activity of free PCI_Lip WT within the first days after purification has been observed beforehand. We assume that this is attributed to an ongoing refolding of the enzyme during the first days of storage as heterologous expression also leads to the formation of inclusion bodies besides some amount of soluble lipase. Spontaneous refolding is also known for other proteins like different fibronectin domains (Shah et al. 2017). A decrease of activity over a long storage period is commonly observed for various enzymes. Another factor is the storage temperature that can affect stability over a longer storage period. However, over the course of 28 days, several denaturing effects such as pH changes, proteolytic activity, and microbial contamination can lead to enzyme denaturation (Dos Santos et al. 2018). For S163M+L302G, 80 mM PPB seemed to be the most appropriate buffer showing considerably higher activities than the other concentrations over the whole storage time. For this mutant, the use of PPB in appropriate concentration can help to stabilize the enzyme over a longer storage time even supporting refolding which results in an increase in activity. Over the 28-day storage period, the concentration of the buffer has the most impact on the enzyme activity of I245F+L302G. The activity of the preparation in 80 mM PPB increased further indicating that this buffer further favors refolding. Overall, this experiment showed that free PCI_Lip exhibits good storage properties when the most suitable buffer concentration is used. Mostly, the use of 80 mM PPB led to the highest activities of the mutants during storage. Higher stability in the presence of low phosphate buffer concentration has been reported for fibroblast protein before. Here it is assumed that substrate effects are most important in the interaction of enzyme and substrate (Ugwu and Apte 2004; Min Won et al. 1998). However, the application of 100 mM PPB appears to be no good choice for the activity and storage stability of free PCI_Lip. These findings go along with the results reported by Kornecki et al (2020). They showed that 100 mM phosphate buffer pH 7.0 had a negative effect on the stability of different lipases.

Also, they were able to show that this effect changes with the use of different buffers and pH values. They assume that the reason is within the lipase structure and not the ionization degree of the phosphate (Kornecki et al. 2020). In accordance with these previous results, we could show that the concentration of the phosphate buffer also influences lipase stability under the given conditions. Generally, this experiment underlines the importance of screening for the appropriate buffer concentration as this affects enzyme activity and stability. The initial activity of immobilized PCI_Lip WT is a little lower than that of the free lipase, which is in accordance with the results of the comparison of the hydrolysis profiles. Within the first 10 days of storage, the activity of all immobilisates decreased strongly, yet it remained at this level for the residual storage time. The comparison of specific activity of immobilisates and free WT suggested that it would be best to store PCI_Lip as free enzyme, which increased activity. Immobilization should be performed immediately before the use of the immobilisates. In the case of the mutants, immobilization supported storage stability, even leading to an increase of activity. In both cases, activity further increased for any buffer concentration, while the activity of the free enzymes remained the same or even decreased. The improved storage stability by immobilization has been reported by several researchers before. Compared to the free enzyme a catalase immobilized on a chitosan/ZnO support the decrease in activity over a period of two month was much lower than for the free enzyme (El-Shishtawy et al. 2021). Al Angari et al. demonstrated that a *Candida rugosa* lipase lost its activity after 30 days of storage while immobilization helped to keep 80% of the initial activity after 60 days of storage (Al Angari et al. 2023). Restricted flexibility and higher rigidity due to structural modifications reducing the effects of denaturation caused by immobilization are discussed as the reasons for the improved storage stability (Atiroğlu 2020). In the case of PCI_Lip, we tried to keep up a higher degree of flexibility by choosing for affinity immobilization via the His₆-tag. Still, this type of immobilization seemed to affect the conformation of the enzyme in such manner that it improved storage stability, being less prone to denaturation. Both immobilized double mutants displayed an increasing activity over the whole storage period in all buffer concentrations. We assume that the refolding process described for the free enzyme before is expanded over a longer time in the immobilized variants. Probably the refolding of free PCI_Lip can take place faster, which is reflected in an activity increase within the first 10 days as there is no restriction of flexibility. Immobilization diminished the conformational flexibility and, therefore, also the chance to refold. As immobilization prevents the enzyme from denaturation and causes a slower refolding process, an increase in activity has been observed over the whole storage period.

Investigation of regioselectivity

Substrate selectivity is a key factor in the application of lipases, especially in the industrial field. Substrate selectivity of these hydrolases can be further distinguished into typosselectivity, stereoselectivity, and regioselectivity. The typosselectivity of PCI_Lip, which covers the preference of the enzyme for a certain type of fatty acid, has been investigated in detail beforehand (Park and Park 2022). In several studies, PCI_Lip has been found to be most active on medium- to long-chain fatty acids, especially capric and palmitic acids. Also, the typosselectivity has been adapted towards short- to medium-chain fatty acids via protein engineering (Broel et al. 2022; Sowa et al. 2022; Wang et al. 2025). For an industrial application, knowledge about the regioselectivity is essential to achieve the desired products. Most TG consist of different fatty acids esterified to the three hydroxy groups of the glycerol backbone. Depending on the source of the TG, fatty acids of different chain length are esterified at defined positions (*sn* 1–3) of the glyceryl moiety. As there are differences in the fatty acids esterified in the *sn* 1 and 3 position compared to the *sn* 2 position, regioselectivity of a lipase is an important factor considering the substrate and desired products. In milk fats, for example, short-chain fatty acids are predominantly esterified in the outer positions of a TG. Differently, in palm oil, palmitic acid is mainly esterified in these positions. Fatty acid distribution is a very important topic when it comes to human breast milk. Here, it is key to the nutritional value for the baby, but still most infant formulas show considerable differences to breast milk in that aspect (Qi et al. 2018; Wei et al. 2019; Yener et al. 2021). Within the group of lipases, some almost exclusively hydrolyze fatty acids at the outer positions, *sn* 1 and 3, producing 1,2- or 2,3-DGs. The other part of lipases is summarized under the term non-regioselective lipases hydrolyzing fatty acids at any position of the TG (Lee et al. 2024). In this study, we choose to analyze the products of the conversions of homogeneous TGs by ¹H-NMR. By using a homogeneous TG, possible influences caused by a preferred hydrolysis of a certain fatty acid are avoided (Rodrigues et al. 2021). The evaluation of the resulting spectra suggests that PCI_Lip WT is *sn* 1,3-regioselective lipase. Besides the peaks assigned to the remaining substrate, the biggest peaks in the spectra could be assigned to 1,2-DG, indicating that these are the main products of the first stage of hydrolysis. Besides 1,2-DG, there also were peaks that were assigned to 1,3-DG. In additional experiments, under the same conditions but without lipase, especially 1,2-DG showed acyl migration. Acyl migration is a thermodynamically driven process describing the migration of fatty acids in a di- or monoglyceride (MG) to another position at the glycerol backbone (Da Silva et al. 2012). Considering the quantitative NMR results of glyceride distribution in the

hydrolysis experiments reported in Supplementary Table S6 as well as the results from the migration experiments, we concluded that PCI_Lip is 1,3-regioselective. The small amounts of 1,3-DG probably result from acyl migration. Similar findings concerning acyl migration in diglycerides have been identified when the regioselectivity of immobilized lipozyme RM IM was investigated (Meng et al. 2014). The setup of our experiment combines some factors promoting acyl migration. Acyl migration is a thermodynamically driven process being favored by elevated temperatures. Depending on the reaction conditions, previous studies showed that a reaction equilibrium can be reached within 1 to 2 h (Kodali et al. 1990; Yang et al. 2005). Further, the deployed substrate trioctanoate is reported to enhance acyl migration. The shorter chain length reduces steric hindrance and acyl migration is fostered compared to long-chain fatty acids (Zhang et al. 2024). Depending on the reaction conditions, the water content in the reaction mixture affects acyl migration in different ways. In our solvent-free system, the high water content seems to enhance acyl migration as observed in a study investigating the enzymatic production of structured phospholipids (Vikbjerg et al. 2005). As MGs were also identified in the spectra, it is evident that some of the DGs are further hydrolyzed by PCI_Lip to produce MGs. With PCI_Lip acting as a putative 1,3-selective lipase, one would expect 2-MG to be the most abundant form of monoglycerides produced from the second stage of hydrolysis. The opposite was observed in our experiments, with 1-MGs being the dominant form of monoglycerides identified in the hydrolysis products (Supplementary Table S6). There are two more theories to explain the presence of 1-MG besides the chance to be a hydrolysis product of the lipase from 1,2-DG, its main product in the first stage of hydrolysis. Firstly, 1-MG might have been a product of the enzymatic hydrolysis of 1,3-DG arising from acyl migration. Secondly, 1-MGs might have originated from 2-MG after acyl migration. Due to the large acyl migration measured under our experimental conditions for 1,2-DGs, the first hypothesis is far stronger, although the contribution of 2-MG acyl migration cannot be ruled out. In the literature, especially 1,2-DG and 2-MG are described to be prone to acyl migration. Therefore, a nucleophilic attack of primary hydroxyl oxygen to the secondary acyl carbon group is proposed. This leads to the formation of an intramolecular ring intermediate, which opens up to the thermodynamically more stable 1,3-DG or 1-MG, respectively (Laszlo et al. 2008). Overall, the investigations on the regioselectivity of PCI_Lip revealed that the enzyme favors the hydrolysis of the outer (*sn* 1,3) esterified fatty acids in the first stage of hydrolysis of a TG. This selectivity makes PCI_Lip an interesting enzyme for the industrial production of tailored lipids, which can be found in infant nutrition. Nevertheless, acyl migration was found to play a major role in this specific setup.

Conclusion

The optimal support for PCI_Lip immobilization was identified through a comprehensive screening of support materials. Remarkably, five His₆-tag affinity immobilisates and two adsorption immobilisates exhibited superior binding capacities (15–19 µg protein · (mg carrier)⁻¹) with minimal leakage (< 2 µg protein · (mg carrier)⁻¹). Recycling studies highlighted IB-HIS-19 as the most suitable support, maintaining 65% of the free lipase's activity. Consequently, IB-HIS-19 was chosen for in-depth characterization. Intriguingly, immobilization preserved PCI_Lip's activity and selectivity, whereas immobilization of two double mutants induced notable changes. The double mutant S163M+L302G demonstrated a striking enhancement in activity against medium-chain fatty acids, likely attributable to hyperactivation. Notably, immobilization bolstered the enzyme's kinetic stability by 5 °C. Further the thermodynamic stability of PCI_Lip was assessed, showing that the lipase has a *T_M* of 63 °C. Careful optimization of buffer conditions, concentrations, pH values, and additives unveiled the most stabilizing environments. Citrate buffer and PPB (pH 5.5–7.0) emerged as the most effective stabilizers, with higher concentrations further augmenting thermostability. Salts and sugars were particularly effective in increasing *T_M*. A combinatorial approach, utilizing 400 mM citrate buffer (pH 6.0) or PPB (pH 7.0) with glycerol, NaCl, glucose, or trehalose, achieved a remarkable increase in *T_M* to over 70 °C. Regioselectivity studies revealed that PCI_Lip preferentially targets outer fatty acids in TGs, as evidenced by the predominant formation of 1,2-DG during trioctanoate conversion, as confirmed by ¹H-NMR analysis.

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Author contributions MG and IP conceived and designed the research. LH, JP and MN conducted the experiments. LH, JP, MN and AS analyzed the data. FB and AS contributed new methods and analytical tools. LH wrote the manuscript. BL reviewed and revised the manuscript.

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Data availability Data and materials will be made available on request.

Declarations

Ethical approval Research does not involve human participants (including data/samples) or animal subjects.

Competing interests The authors declare no competing interests.

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