Mycobacterium smegmatis acyltransferase: the big new player in biocatalysis

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Abstract

After several decades during which proteases and after lipases took the biotransformation world scene as the predominant biocatalysts, a new, promising enzyme was discovered and characterized. The acyltransferase from *Mycobacterium smegmatis* (MsAcT) has in fact an extraordinary activity for a wide array of reactions, such as trans-esterification, amidation, trans-amidation and perhydrolysis, both in water and solvent media, giving rise to a series of interesting compounds including APIs (*i.e.,* active pharmaceutical ingredients), natural flavors and fragrances, monomers for polymer synthesis, and peracids employed as disinfectants or antimicrobials. Although the most used acylating agent has been ethyl acetate (EtOAc), depending on the reaction type also acetamide, dimethyl carbonate and a variety of other esters, have been reported. The best yields were reached using very reactive donors such as vinyl or isopropenyl esters (almost complete conversion in rapid reaction times and water media for condensation reactions). In this review article the most innovative scientific advances on MsAcT, its mechanism and engineering were summarized, putting a particular focus on the different kind of processes (batch and flow) that it is possible to carry out using this enzyme as free or immobilized form. In conclusion, the author personal view on the unexplored reaction possibilities using MsAcT was reported as a window on the future of the topic.

Keywords: *Mycobacterium smegmatis* acyltransferase (MsAcT), perhydrolysis reaction, condensation reaction, flow chemistry, enzyme engineering, enzyme immobilization, green and sustainable chemistry

List of abbreviations

APIs	Active pharmaceutical ingredients
EtOAc	Ethyl acetate
imm-MsAcT	immobilized *Mycobacterium smegmatis* acyltransferase
INT	Tetrahedral intermediate
MsAcT	*Mycobacterium smegmatis* acyltransferase
TS	Transition state

VinylAc	Vinyl acetate
CAL B	lipase B from *Candida Antarctica*
m.c.	molar conversion
e.e.	enantiomeric excess

1. Introduction

Since 1930, with the pioneer work of Ernest A. Sym (Sym, 1930) later followed by Zaks and Klibanov in the 1980s (Zaks and Klibanov, 1985), the possibility of using enzymes as catalysts for alcoholysis and perhydrolysis reactions attracted scientist attention. In these early days, the most suitable candidates for such transformations have been found to be proteases and lipases, enzymes which belong to the α/β hydrolase superfamily and have similar catalytic triads (acid-base-nucleophile triad as the common motif for generating a nucleophilic residue for covalent catalysis) (Berg et al., 1988).

Lipases are now widely used for stereospecific esterification and trans-esterification reactions, in both academic and industrial sectors. Among the various lipases studied over the years, the lipase B from *Candida antarctica* (CAL B) is nowadays the election biocatalyst for reactions such as aminolysis (Torre et al., 2005), condensation (Fabbri et al., 2021) and kinetic resolutions (Lund et al., 2016) due to its high enantioselectivity, temperature/organic media stability and efficiency.

Since the early 2000, cutinases, another subfamily of the α/β hydrolase superfamily, was more intensively investigated and reported. The utilization of enzyme sub-classes was mainly directed to the selective hydrolysis of polyesters to their A-B type constituting monomers (Herrero Acero et al., 2011) as well as to the surface functionalization of such materials for creating grafting points on the polymer surface (Pellis et al., 2015a). The use of cutinases was also exploited for synthetic applications related mainly to polymer biotechnology with resulting aliphatic and aromatic polyesters (Pellis et al., 2016) and polyamides (Stavila et al., 2013) being successfully produced.

Despite the success of the mentioned enzymes, it is important to highlight how all these synthetic reactions -when not carried out in bulk- were run in various apolar petrol-based anhydrous solvents. These conditions were of course chosen by the researchers to promote the desired condensation reaction while preventing the hydrolysis of the target product. For this reason, over the years a wide collection of scientific articles were published on the topic helping the present-day user to select the correct biocatalyst, immobilization technique, medium, reaction conditions, substrate specificity, etc (Mathews et al., 2007).

When analyzing such reactions from today-green-chemistry-perspective, it is possible to evince some limitations due to the use of potentially hazardous organic solvents as reaction media, stability of the biocatalyst in the operational conditions, as well as the related process upscaling that is, in most cases, too expensive to become of industrial interest (Pellis et al., 2015b). The ability of catalyzing condensation reactions in a more sustainable way (*e.g.,* water-based medium, room temperature, etc..) was in fact a goal of biocatalysis that was achieved only in the last decade. Such reactions eliminating the need for protection and deprotection steps dramatically reduce the environmental impact as well as the overall process-related costs (Mathews et al., 2007). The first steps in this direction were made by the use of alternative, potentially greener solvents such as ionic liquids as the reaction media (De Diego et al., 2009), the implementation of recirculating

membrane reactors operating in supercritical CO_2 (Hernández et al., 2006) and the application of innovative enzyme immobilization techniques such as the one on cationic lignin nanospheres for the synthesis of short esters in classical biphasic water-solvent systems (Sipponen et al., 2018). All these ingenious strategies were based on known hydrolases and only from 2007-on a new outstanding trend from the enzymatic point of view started. In these years, interest in enzymatic synthesis in water boomed, with several research groups worldwide that published the use of a novel enzyme, the acyltransferase from *Mycobacterium smegmatis* (MsAcT).

In fact, using water as medium and MsAcT as catalyst, synthetic reactions such as esterification and amidation can be directly carried out in the fermentation broth without the need for expensive and time-consuming purification steps (de Leeuw et al., 2018). The chance to add a new catalyst with promiscuous acyltransferase/hydrolase (AT/H) activity to the portfolio of enzymes able to catalyze synthetic reactions in aqueous media open different applications from asymmetric synthesis by coupling multi-step biotransformations to further possibilities in downstream processing, by changing the physicochemical properties of products *(i.e.,* hydrophobicity), making them more easily extractable. On the other hand, performing water synthesis of peracids from carboxylic acids or esters with hydrogen peroxide (Dinu et al., 2012, 2010; Wiermans et al., 2013), a variety of interesting industrial applications can be identified employing MsAcT as free or immobilized form such as decolorizing systems for dyes, teeth whitening methods (using low concentrations of peracetic acid), surface disinfection as well as detoxification of contaminated water streams (Domínguez de María, 2020). The high impact that MsAcT-mediated synthetic reactions in aqueous environments can have on the industrial world is also demonstrated by the numerous patents filed in the last decade (Barnett, 2008; Barnett and Sala, 2010; Bott and Cervin, 2008; Concar et al., 2007).

It is important to mention that enzymes with promiscuous AT/H activity have the potential to revolutionize the application of biotransformations for the water synthesis of an endless series of valuable molecules containing ester, thioester, amide, carbonate, and carbamate functionalities. Apart from MsAcT, family VIII carboxylesterases presenting high AT/H ratio and a not very hydrophobic active site are noteworthy. Among them, EstCE1 has been highlighted as able to catalyze irreversible amidation and carbamoylation of amines in water media enabling the preparation of APIs such as the antidepressant drug moclobemide (m.c. 20%) (Müller et al., 2021).

This review, going through all the research articles published until now on MsAcT, aims to shed the light on the unusual architecture of this biocatalyst and the mechanistic basis that favor condensation over hydrolysis in aqueous media. All known applications will be showcased, demonstrating the versatility and efficiency of this enzyme for a wide array of biotransformations at industrial interest (Fig. 1). As conclusion, the author personal perspective on future works that could be carried out using MsAcT will be given.

2. MsAcT structural features

To understand how the unusual architecture of MsAcT provides a structural basis for its catalytic mechanism in water, Matthews et al. solved the MsAcT crystal structure at 1.5 Å resolution (GenBank accession: ABK70783) (Mathews et al., 2007).

The refined MsAcT octamer model was found to contain 1720 residues (216 amino acids per subunit), 8 sulfate ions, 8 glycerol molecules, and 1608 H_2O molecules. The octamer having dimensions of 72×72×60 Å (Fig. 2) is characterized by a big hydrophobic channel in the center running from the “top” to the “bottom” (Structural data from PDB: 2Q0S). Each monomer presents a five-stranded parallel ß-sheet structure sandwiched by R-helices on either side (Fig. 2), a common structural motif found in other SGNH hydrolases (Mathews et al., 2007).

The catalytic triad is composed of Ser11 responsible for the nucleophilic attack to the acyl donors (see below) and positioned in a short helical segment following the first ß-strand, Asp192 and His195 mainly involved in the catalytic system stabilization and placed in the loop before the C-terminal helix. The sulfate ion bound at the active site is involved in hydrogen bonding with both Ser11 and His195 as well as with the amide nitrogen of Ala55 and the side chain of Asn94 (conserved GXND in block III motif). Unlike the conserved asparagine, Asn94, MsAcT deviates from the SGNH hydrolases by having an alanine rather than glycine at position 55, which represents the block II motif. The oxyanion hole is made of the backbones of Ser11 and Ala55, together with the side chain of Asn94.

The substrate-binding site consists of a small cavity (formed by Leu12, Thr93, and Ile194) and a large one (composed of Asp10, Trp16, Ala55, Ser54, Asn94, Lys97, Val125, Phe150, Ile153, Phe154, and Phe174). During the formation of the acylated enzyme intermediate, the small cavity is occupied by the acyl group bound to Ser11, whereas the large cavity will accommodate the substrate as acyl acceptor. The catalytic site ingress channel is made of three adjacent subunits and exhibits a particular hydrophobic character, which has been suggested to be the main reason why condensation reactions are favored over hydrolysis also in water media (de Leeuw et al., 2018; Mathews et al., 2007).

The overall oligomerization state, resulting in a highly restrictive reactive channel, was the most important structural difference between MsAcT-like enzymes and other belonging to SGNH superfamily contributing also to MsAcT greater stability (no protein size change after incubation in 2 M urea, 50 °C for 48 h) (Mathews et al., 2007).

2.1 Reaction mechanism

As previously described for esterases and lipases, MsAcT follows a 2-step mechanism (Fig. 3), in which the enzyme is acylated by an acyl donor in the first half-reaction and the acyl group is then transferred to the substrate in the second half-reaction (Kazemi et al., 2018). It was shown through DFT calculations and free energy perturbation simulations that the first half part occurred rapidly and did not affect the overall kinetics of the reaction (Grimme et al., 2011; Kazemi et al., 2019). The second half reaction involves two other steps. After the substrate binding to the acylated enzyme, the first one corresponds to the formation of a negatively charged tetrahedral intermediate (INT). The transition state (TS1) of this step involves the nucleophilic attack performed by Ser 11 to the substrate and a simultaneous proton transfer involving His195 (Kazemi et al., 2018). Stabilization of the tetrahedral intermediate occurs through hydrogen bonds with the oxyanion hole. In the second step, INT collapses to generate the condensation product *via* another transition state (TS2), which involves C-O bond cleavage with concurrent proton transfer from His195 to Ser11.

Calculations of the energy profile suggest that the rate-limiting step for both hydrolysis and condensation reactions is the decomposition of the negatively charged tetrahedral intermediate in the second half-reaction (Kazemi et al., 2018).
Unlike many cofactor-dependent acyltransferases, which often show virtually no hydrolase activity, MsAcT is able to hydrolyze both the acyl donor and the final product. In 2018, Kazemi and coworkers described the competition between hydrolysis and trans-esterification employing water and benzyl alcohol as final acyl acceptors. The calculated energy difference between their rate-limiting transition states is 4.1 kcal/mol, thus indicating that MsAcT favors condensation over hydrolysis, which is in agreement with the experimental observations. As said above, the main reasons are the hydrophobic microenvironment formed by its oligomeric structure as well as the hydrophobic tunnel leading to the active site which contributing in a more favored binding of organic nucleophiles instead of water (Kazemi et al., 2018; Mathews et al., 2007). Based on this observation, studies regarding lipase A from *Candida antarctica* and CpLIP2 from *Candida parapsiolosis* revealed that the substitution of active site residues with more hydrophobic ones improves the acyltransferase activity (Jan Deniau et al., 2018; Subileau et al., 2015).
More recently Müller and colleagues proposed the use of the hydrophobicity score, which accurately reflects active-site hydrophobicity based on aminoacidic sequence, for the prediction of promiscuous acyltransferase activity within the hydrolase-enzyme family (Müller et al., 2020). On this scale, whereas hydrophobic residues have positive values, polar and charged aminoacids present negative ones and are considered as penalties. Consequently, hydrophobic pockets are expected to have high scores than hydrophilic ones.
Notably, even when MsAcT favors acylation over hydrolysis, the enzyme displays hydrolysis activity towards the newly generated product as well as the acyl donor, resulting in the formation acids as by-products (Szymańska et al., 2016). The hydrolytic reaction not only reduces the efficiency of the trans-esterification, but also inactivates the enzyme due to its lower activity at strongly acid pHs (de Leeuw et al., 2018).

2.2 Enantioselectivity

Several computational approaches have been used to explore the catalytic mechanism and selectivity of lipases (de Leeuw et al., 2018). Even though MsAcT did not show any stereopreference for some molecules such as (*S*)- or (*R*)-methylphenethylamine (Contente et al., 2018), it was noticed a stereopreference for a wide range of other substrates, making this enzyme an even more attractive candidate for biocatalytic applications.
The second half-reaction and consequently how the active-site residues interact during the transition states are key points for understanding MsAcT enantioselectivity. In this context, Kazemi et al. showed that chiral substrates in the transition states are oriented with the CH of the chiral carbon pointing toward the oxyanion hole (*i.e.,* Ala55 and Asn94), resulting in fewer steric clashes. This orientation dictates the MsAcT enantioselectivity defining how the substituents of the substrate are positioned in the active site (Kazemi et al., 2019).
The indications gained by the calculations of this model can be further generalized providing a way to rationalize and predict the enantiopreference of MsAcT wild-type enzyme. These insights can also be exploited to rationally re-design the enzyme structure for better biocatalytic applicability, improving selectivity properties or studying mutations to alter the MsAcT stereoprefence for specific substrates.

3. Batch Reactions

3.1 Alcohol trans-esterification

After the MsAcT biochemical characterization and the first report on alcohol MsAcT-mediated acetylation in water solution (Mathews et al., 2007) a further investigation was conducted by Wiermans et al. (Wiermans et al., 2013) rapidly revealing the distinctiveness of MsAcT compared to other hydrolases. In particular, the authors observed how the enzyme in synthetic direction (*i.e.,* condensation reactions), could accept a variety of different alcohols in a broad pH range (4-11), demonstrating not only the wide substrate scope but also the high stability of this biocatalyst. Considering diols as starting material, the trans-acylation of neopentylglycol (NPG) to the corresponding monoester was preferred to diester formation, particularly in aqueous systems with low catalyst concentration (95% *v/v* buffer solution, 5% *v/v* acyl donor, 0.0025 mg/mL catalyst concentration) (Fig. 4). Remarkably, in these same reaction conditions other lipases such as CAL B did not show any synthetic activity.

According to the Mathews assumption (Mathews et al., 2007), Wiermans and colleagues concluded that the MsAcT peculiar capability was conductible to the highly hydrophobic MsAcT architecture, especially in the active site where the apolar microenvironment disfavors the water entrance. As a result, condensation is favored over hydrolysis. In agreement with the published data reported so far, primary alcohol acetylation with ethyl or vinyl acetate (EtOAc, VinylAc) is the most rapid reaction catalyzed by MsAcT. From an in-depth substrate screening performed by de Leeuw and colleagues (de Leeuw et al., 2018) it was observed that the complete trans-esterification of high concentrations (up to 100 mM) of small sized achiral primary alcohols with EtOAc/VinylAc are usually concluded in less than 2 h. Employing secondary alcohols the enzymatic efficiency is drastically decreased (7-21% m.c., 2-5 h) and a considerable *S* stereopreference was observed just for one substrate (1-phenylpropan-2-ol, e.e. 81%, E value: 10). With tertiary ones no transformation into the corresponding esters has ever been observed. A similar behavior was described employing alkynols and cyanohydrins: an increased acetylation activity has been demonstrated for less sterically hindered substrates, while employing more bulky acceptors such as aromatic alcohols, the enzymatic activity dramatically decreased. Considering that highly-reacting donors such as VinylAc can spontaneously react with aliphatic cyanohydrins at high pH giving the corresponding acetate, pH 7.5 has been selected for enzymatic reactions. EtOAc, the most used acyl donor for batch transformations, is usually employed in large excess compared to the acyl acceptor (5-10 eq.) to push the reaction in the synthetic direction, trying to avoid reverse hydrolysis of the newly generated products which typically takes place at prolonged reaction times. The final ratio between condensation and hydrolysis is regulated by the thermodynamic stability of the system. Indeed employing high enzyme concentration (>100 ng/µL) and equimolar amount of substrates, a rapid reverse hydrolysis of the desired products is often observed (Mestrom et al., 2019). Generally speaking, more reactive acyl donors such as VinylAc result in higher conversion rates.

Wiermans et al. performing (*R)*- or *(S*)-2-octanol acetylation in water medium (97.5% *v/v* buffer and 2.5% *v/v* EtOAc) confirmed the MsAcT *S* stereopreference obtaining eightfold kinetic selectivity for the (*S)*-enantiomer over the (*R)*-one, although the (*R)*-

compound was accepted by the enzyme (Wiermans et al., 2013). In the presence of cyanohydrins characterized by polar nitrile groups, as already reported for other lipases (Gedey et al., 2001), MsAcT stereoselectivity shifts from *S* stereopreference to the opposite one (de Leeuw et al., 2018). This is not related to a change in the enzyme/substrate binding but just to a switch in the nomenclature rule priority. Aside from the investigation of different acyl acceptors, researchers have also studied the possibility of using different acyl donors for alcohol acetylation. An increased enantioselectivity toward (*S)*-enantiomers was observed employing donors with sterically demanding leaving groups such as phenyl acetate. In fact, it was hypothesized that non polar, bulky leaving groups can interact with the hydrophobic residues in the MsAcT active site, thus reducing its size and improving enantiopreference.

Moreover, considering the possibility to change the physicochemical properties of different compounds by combining the MsAcT condensation capabilities with other reactions, molecules such as the valuable biomass-derived 5-hydroxymethylfurfural (HMF) can be esterified to produce more hydrophobic derivatives, thus allowing for a straightforward recovery and valorization. The reaction was carried out in water medium (98% *v/v* buffer, 2% *v/v* EtOAc) reaching a conversion between 20-25% in 24 h, using 2 mg/mL of the catalyst (Fig. 5a). For sure the combination of immobilized enzyme technology with "*in continuous*" strategies, as reported in the next chapter, could allow for better and faster reaction outcome.

A part from trans-esterification reaction, adding diluted H_2O_2, MsAcT is able to *in situ* form peracids. By exploiting perhydrolysis reaction, furfural has been oxidized to furoic acid (Fig. 5b). This latter will be better discussed in the dedicated paragraph.

In a recent work by Chiarelli and coworkers MsAcT abilities were exploited for the generation of a variety of different natural esters typically employed in the pharmaceutical, food and cosmetic sectors as flavors and fragrances (Chiarelli Perdomo et al., 2019). Different natural substrates among primary alcohols (*e.g.,* isoamyl, *n*-hexyl, geranyl, cinnamyl, 2-phenethyl, and benzyl alcohols) and acyl donors (*e.g.,* ethyl formate, acetate, propionate, and butyrate) have been utilized, demonstrating the strong versatility of the system (Fig. 6). While all the assayed substrates have been accepted at high concentration (up to 500 mM) producing the corresponding esters in good yields (30->99%), the preparation of both geranyl acetate and cinnamyl acetate was carried out on a semipreparative scale (10 mL). The proposed enzymatic strategy paved the way for the development, few years later, of an intensified and sustainable platform for the automated preparation of natural aroma-compounds (Martina Letizia Contente et al., 2020).

3.2 *N*-Acylation of amines and trans-amidation

Amide bond formation is one of the most important reaction in organic chemistry, as it represents a core linkage in many biologically active compounds (Pattabiraman and Bode, 2011). The typical methodologies for amide preparation involving both chemical and enzymatic approaches, are usually considered inefficient and unsustainable, so that "*amide formation avoiding poor atom economy*" became a top challenge for the researchers in this field in the last years (Constable et al., 2007). Land and coworkers, exploiting the condensation ability of MsAcT demonstrated the potential of this enzyme also in the amide synthesis in aqueous solutions (Land et al., 2016) performing a transaminase/acyltransferase cascade for the formation of amides from the corresponding aldehydes. This will be better described in the

dedicated paragraph. A further investigation was subsequently performed by Contente and colleagues (Contente et al., 2018). Firstly, *N*-acetylation in buffer medium using (*E*)-cinnamylamine as model substrate (50 mM), a broad list of acetyl donors (10% *v/v*, 20 eq.) and MsAcT 1 mg/mL was performed, identifying isopropenyl acetate and VinylAc as the most promising donors (82%, 92% m.c and 30, 20 min reaction times, respectively). Subsequently an in-depth study employing a variety of primary amines and EtOAc or VinylAc was carried out. With substrates such as *para*-aminophenol and vanillylamine MsAcT chemoselectivity for amines against phenols was observed. Acylation reaction was also investigated employing (*E*)-cinnamylamine or 2-phenethylamine as substrates and obtaining the corresponding amides in good-to-excellent yields and variable reaction times (11->99% m.c., 30 min-24 h) (Fig. 7a). Among them, the preparation of *N*-formyl amides without any particular formylating agents and tedious purification steps is noteworthy. Finally, the challenging trans-amidation reaction between acetamide and model primary amines (*i.e.,* (*E*)-cinnamylamine and 2-phenethyalmine) in aqueous environment has been efficiently carried out (60-70% m.c., 1 h-30 min), demonstrating this strategy as a greener and useful alternative for the most known chemical syntheses or lipase-mediated amidation reactions (Fig. 7b).

Despite the main MsAcT drawback regarding its hydrolytic activity towards the newly formed products and the acyl donors employed in the reactions, above already discussed, this enzyme has been demonstrated to be a very efficient biocatalyst. In fact, by simply optimizing the reaction conditions in terms of enzymatic loading, substrate and acyl donor concentrations it is possible to obtain rapid, high-yielding and sustainable syntheses of esters and amides in water media.

3.3 The exploration of promiscuous MsAcT activity: perhydrolysis reaction

Already in the earlier investigations on MsAcT crystal structure a secondary but very promising activity was reported. Similarly with other hydrolases carrying the so called "α/β hydrolase" fold, MsAcT displays the peculiar capability to catalyze the formation of peracids from aliphatic esters in presence of hydrogen peroxide (Mathews et al., 2007). In this case as in the one of condensation reactions, the uniqueness of MsAcT is represented by the fact that, differently from other hydrolases, the perhydrolysis efficiently takes place in aqueous solutions. Moreover, it has been observed that in presence of diluted solution of hydrogen peroxide, MsAcT perhydrolysis is 50-times higher than common commercially available lipases (Mathews et al., 2007). As reported for alcohol acylation, MsAcT is able to accept different acyl donors since the formation of peracids was observed starting from acetate esters (*i.e.,* EtOAc or glyceryl triacetate) (Jia et al., 2021) and dimethyl carbonate (Dinu et al., 2012, 2010; Wiermans et al., 2013). To the best to our knowledge, no other donors have been studied so far (Fig. 5b).

The potential of this reaction is very appealing in numerous industrial applications. Peracids are strong chemical oxidants which find application for wastewater and biomass pretreatments, as bleaching or antimicrobial agents. The chemical synthesis of those compounds involves hazardous reagents and the storage of peracids is often dangerous as highly concentrated peracid solutions may be explosive while low concentrated ones are extremely instable. Thus, their *in situ* generation, in a sustainable and inexpensive manner represents a great potential from an industrial perspective. Again the above mentioned proof-of-concept opened the path to the development of cascade biotransformations in which MsAcT-

mediated straightforward peracid formation can be coupled with other enzymes to obtain new chemical entities.

3.4 Expanding synthetic potential of MsAcT: chemo-enzymatic synthesis and enzymatic cascade biotransformation

Enzymatic cascades employ reaction products as intermediates and substrates of the following reaction steps. The careful design of chemo-enzymatic systems offers several advantages in terms of operational feasibility, stability of the whole system and enhancement of the overall kinetic constants. A typical application of multi-step synthesis in batch mode is the removal of the reaction products to avoid any enzymatic inhibition. In the case of perhydrolysis catalyzed by MsAcT, the reaction products (*i.e.,* aliphatic peracids) can be a problem for their multiple interactions with amino acid residues, such as catalytic cysteine or other acidic functionalities (Kuban-Jankowska et al., 2015) rapidly causing enzyme inactivation. A straightforward way to overcome this issue is the introduction in the reaction mixture of organic compounds highly prone to oxidation which easily react with peracids. Significant examples have been reported by Wiermans and coworkers with the oxidation of furfural to furoic acid after the *in situ* MsAcT-mediated generation of peracids (Wiermans et al., 2013) (Fig. 5b) and the chemo-enzymatic Baeyer-Villiger oxidation of cyclic ketones into lactones described by Drozdz et al. (Drozdz et al., 2016) (Fig. 8a). In the first case the best reaction conditions for the preparation of furoic acid in water environment (m.c. 90%) were realized using 15 eq. of EtOAc, 8 eq. of H_2O_2, 100 mM substrate, 2.5 mg/mL of enzyme after 3 h of reaction time at 40 °C. Lactonization of different cyclic ketones (84->99% m.c., 2-120 h) was obtained employing 0.25 mmol of the starting material, 2 eq. of H_2O_2, 24 eq. of EtOAc and 4 mg/mL of the enzyme at 35 °C.

Even if it has been demonstrated that peracids are more powerful enzymatic inhibitors than hydrogen peroxide (Kuban-Jankowska et al., 2015), the possibility to limit the amount of highly reactive hydrogen peroxide, employed as cosubstrate in large excess in MsAcT-catalyzed perhydrolysis is desirable. To do so, a multi-enzymatic cascade system has been proposed coupling a hydrogen peroxide-producing glucose oxidase (GOx) with MsAcT, and ISCO (*in situ* chemical oxidation) (Jia et al., 2021). The designed multi-enzymatic system was tested for melanin decolorization and after an in-depth optimization of the reaction parameters, the cascade was proposed as an alternative skin whitening technology (Fig. 8b), which represents a widespread cosmetic strategy, especially in Asia. Although the traditional cosmetic procedure relies on the use of different natural or synthetic compounds for the inhibition of tyrosinase, it may result in metabolic disorders due to the importance role of this enzyme in human metabolism (Lee et al., 2016). Recently, some enzymes such as lignin peroxidase and laccase, were added in cosmetic formulations to directly destroy melanin, which is the responsible for pigmentation. Decolorization processes mediated by enzymes seem to be moderate and progressive, and rarely caused adverse reactions (Sung et al., 2019). In this context the GOx-MsAcT-ISCO cascade proposed by Jia et al. can display an excellent application prospect. According to the authors, MsAcT high specific activity pushes the overall thermodynamic equilibrium of the GOx-MsAcT cascade to the perhydrolysis, dramatically enhancing previous glucose oxidation enzymatic step (Jia et al., 2021). The best reaction conditions for melanin decolorization (87% m.c.) were found to be 50:1 Gox/MsAcT activity unit ratio, buffer pH 7.0, 50 mM glucose and 15

mM glyceryl triacetate as acyl donor. Considering that GOx-MsAcT-ISCO cascade reaction system would be used for skin whitening, the reaction temperature was directly performed at 37 °C. The case of MsAcT perhydrolysis is a clear example of how coupling different enzymatic and/or chemical approaches represents an improved catalytic system compared to the distinct reaction steps.

Another interesting application of MsAcT in cascade multi-enzymatic system was reported coupling a transaminase for the formation of amines and their transformation into amides (Land et al., 2016) (Fig. 9). An efficient multi-step 20 mM scale biotransformation was set up using *Silicibacter pomeroyi* amine transferase (*Sp*-ATA, 3 U/mL), MsAcT (1.6 U/mL), and L-alanine (0.5 M) as amino donor for aromatic and aliphatic carbonyl transamination. In the cascade, methyl acetate and methyl methoxyacetate were employed as acyl donors for MsAcT-mediated biotransformations, while the newly formed amines as nucleophiles. After an optimization of the reaction conditions the authors identified 2% *v/v* methyl methoxyacetate as the best acyl donor. In this case the authors point out that MsAcT was able to significatively push the equilibrium of the transamination step thanks to the high MsAcT specific activity and because of the irreversibility of newly synthetized amides in the proposed conditions (buffer medium pH 10, 90 min-24 h reaction times).

4. MsAcT engineering

Although the wilde-type MsAcT (WT MsAcT) is an outstanding catalyst, it also presents some limitations especially in terms of acyl donor acceptance, poor enantioselectivity and unwanted substrate and product hydrolysis. These undesirable factors limit its use in industrial biocatalysis, thus in recent years several attempts have been made to boost the applicability of MsAcT by employing advanced molecular and computational tools (Subileau et al., 2015). Since the structure and the mechanism of MsAcT is known, *in silico* models are used to predict the effect of a mutation on the geometry of the enzyme and explain interactions of a substrate in the active site. Based on structural and dynamic models, rational design is used to select alterations that can give a desired effect but also to keep intact some strategic bonds essential for enzymatic functionality (Tiwari et al., 2012).

Punctual and systematic investigations have been launched to identify a MsAcT variant that is competitive with other industrially applied hydrolases while maintaining all the benefits related to a solvent and cofactor free reaction performed in water.

Recently, a method to expand the acyl donor specificity has been developed by using two immobilized MsAcT variants (L12A and T93A/ F154A) (Finnveden et al., 2019). Unlike other esterases such as CAL B, the L12A variant was found to be selective for mono-trans-esterification of divinyl adipate (DVA), yielding over 95% conversion with 98% of the product identified as the mono-substituted product. The MsAcT L12A model shows a different orientation of the acyl donor (DVA) during the deacylation with 1-octanol due to a deeper binding-site behind the side chain. The mono-substituted product is too long to fit into the restricted space generated from mutating leucin to alanine, thus the unreacted ester will not fit productively in the active site to achieve the di-substituted product (Finnveden et al., 2019). Based on Kazemi computational studies, first Godehard et al.(Godehard et al., 2020) and then Jost et al.(Jost et al., 2020), selected some critical residues and developed libraries of single and double variants of MsAcT with different specificities and selectivities (the most relevant mutations are listed in Table 1). By re-shaping the acyl enzyme

intermediate networks as predicted by computational simulations, the most mutated enzymes presented higher condensation-to-hydrolysis ratio than the wild-type, a desirable characteristic for synthetic applications. One of the best variants tested by Godehard et al., K97R/F150I, showed more than 4-fold increased condensation activity at 2 to 20 mM benzyl alcohol, while hydrolase activity was drastically decreased.

Residues T93 and F154 seem to be of particular importance for acceptance of larger acyl donors, another crucial aspect for MsAcT applicability. As reported by Finnveden et al., the double mutation T93A/F154A generated a new space that enables to higher degree of flexibility for the substrates and allows longer acyl donors to bend out towards the entrance of the active site. In addition, the double mutant T93S/F154A is highly selective (96% e.e.) for the (*R*)-enantiomer of 1-phenylethanol (Godehard et al., 2020).

Within the library developed by Jost et al., (Jost et al., 2020) the phenylalanine residues at 174 and 154 positions seemed to play a role in both enantioselectivity and conversion. All beneficial variants (F174A, F174V , F154A, F154V, and F154L) have a substitution with decreased bulk in the F-direction (toward Phe150), which opens the active site and increases both the activity and the preference for the (*R*)-enantiomer of substrate 1-phenylethanol. When tested in buffer on other substrates such as phenyl alkanols, aliphatic alcohols, and alkynols at differently hindered and barely converted by the WT, at least one variant showed improvements in enantioselectivity and conversion. Thanks to a new cavity in the active site that lodges the phenyl group of the chiral carbon of the (*R*)-configured substrate, the majority of aliphatic and phenylalkanols were accepted by the F154A variant displaying high *R*-enantioselectivity (56-99% e.e.). The double mutations F154V/F174V and F150V/F154V confirmed to be superior to the single mutants in both enantioselectivity and activity, as predicted by computational models (Jost et al., 2020).

In both libraries, variants with inversed enantioselectivity toward phenylethanol with respect to the wild type enzyme were discovered (W16A, N94A/F150, F154A/I194V) (Godehard et al., 2020; Jost et al., 2020). Interestingly, dynamic calculations of transition state of W16A show the chiral carbon in *cis* to the methyl group of the acetyl, favoring the relief of steric repulsion between the phenyl group of the substrate and Trp16 in the complex with the (*S*)-enantiomer. It was proved that decreasing the steric hindrance in the Trp16 direction (called W-direction) leads to a clear switch in preference toward (*S*)-1-phenylethanol (Jost et al., 2020).

In addition, manipulations of catalytic residues were designed to modulate the formation of the acyl enzyme intermediate. Three mutants of the catalytic aspartate (D192A, D192E, and D192N) and two mutants of the catalytic histidine (H195N and H195D) were tested by Godehard et al., resulting all in a weak total activity. Nevertheless, most variants showed enhanced acyltransferase properties (Godehard et al., 2020). Regarding the catalytic serine, a remarkable rational single point mutation (S11C) dramatically changing the impact of MsAcT was proposed by Contente et al. The strategic Ser/Cys exchange expanding the MsAcT S11C synthetic capability, yields a biocatalyst able to efficiently catalyze the water formation of thioesters and tertiary amides on preparative scale (250 mM) employing a variety of thiols and secondary amines as substrates while vinyl esters as acylating agents. The large substrate-to-catalyst ratio (250 mM/0.04 mM) made this process a cost-effective, sustainable procedure. Thanks to favorable binding energies, S11C retains high activity towards alcohols and primary amines. By computational and

experimental studies, it was also demonstrated that S11C can efficiently transform CoA into acetyl-CoA (100 mM, 80% conversion) with VinylAc as the donor. The absence of hydrolytic side reaction, rapid reaction time and excellent conversion make S11C MsAcT a green and efficient tool for acetyl-CoA and its analogues synthesis, expanding applications to *ex vivo* cellular metabolism simulation (Martina L. Contente et al., 2020).

5. Intensification process through MsAcT-immobilization and in continuous processing

For years the innovation driving force of chemical processes has been represented by cost-efficiency, but nowadays, other factors have been taken under consideration. In the last decade public awareness on the importance to minimize the environmental impact of our ever-growing product demand has reached a turning point, consequently process sustainability became a priority. In the last few years, biocatalysis has been recognized as one of the key techniques for a greener way to operate in chemistry (Sheldon and Brady, 2019; Sheldon and Woodley, 2018) and the combination of biocatalysts with flow facilities has lately come up as a powerful tool to enhance process selectivity, productivity and sustainability (Boodhoo et al., 2022; Contente and Paradisi, 2018; Garcia-Verdugo et al., 2020; Santi et al., 2021) In this context, enzymatic immobilization has been at the forefront of applied biocatalysis as it enables convenient catalyst isolation and reuse when the target reaction is performed in batch, and it has opened up significant opportunities to carry out biocatalytic processes under continuous conditions (Benítez-Mateos et al., 2021; Romero-Fernández and Paradisi, 2020; Sheldon and Brady, 2021; Tamborini et al., 2018).

This part of our review is dedicated to the MsAcT-mediated biotransformations in continuous mode with emphasis on MsAcT immobilization strategies for its applications in flow single step reactions as well as multi- and chemo-enzymatic cascade systems to address more complex chemical functionalities.

The first attempts to immobilize MsAcT have been performed by Dordick group, which employed the immobilized catalyst for the propylene glycol diacetate (PGD) perhydrolysis generating peracetic acid (PAA) (Fig. 10), a potent disinfectant against a broad spectrum of bacteria and fungi species (Dinu et al., 2012, 2010).

PAA on the market is typically obtained under drastic conditions by reacting acetic acid with H_2O_2 through sulfuric acid catalysis. This reaction not only requires several days for yielding a sufficient amount of PAA but also generates a lot of waste. In the current paper, MsAcT perhydrolase activity, demonstrated to be 50-times higher than the most known lipase CAL B*,* has been fulfilled as a greener alternative to PAA chemical synthesis. Two covalent immobilization strategies based on the interaction of MsAcT with multi-walled carbon nanotubes (MWNTs) have been considered (Fig. 10). In the first case MWNTs previously functionalized *via* acid treatment have been used as water-soluble support followed by a covalent attachment of MsAcT through EDC/NHS chemistry (Jiang et al., 2004). Due to the large dimension of the enzyme (octameric structure, 72 x 72 x 60 Å) and the strong non-specific hydrophobic interactions between the protein and the carrier, MsAcT molecules presented limited flexibility and reduced substrate accessibility (7% of the native MsAcT activity was retained). To prevent this, a bi-functional amino-dPEG$_{12}$-acid linker was added between the support and protein, increasing the hydrophilicity of the system and enhancing the retained specific activity to 24% with respect to the free enzyme. The

final aim of this work was the incorporation of the resulting conjugates into polymers such as poly(methyl methacrylate) (PMMA) and poly(vinyl acetate) (PVAc) as well as latex-based paints. These coatings provided of decontaminant activity (conjugate loading 0.16 w/v, 11 mM PAA produced in 20 min starting from PDG) may be useful in the field of environmental remediation as well as for medical applications where effective killing of a variety of infectious organisms is critical. A further improvement in the post-immobilization MsAcT activity and stability have been reported 2 years later by the same group (Dinu et al., 2012). Using the above mentioned immobilization technique, but selecting single-walled carbon nanotubes (SWNTs) as carrier a greater retained activity was obtained (>40% with respect to the free enzyme). In fact, SWNTs presenting higher surface curvature than MWNTs allowed for a reduction of the lateral interactions between adjacent enzymatic molecules with better catalyst performance in the production of PAA from PDG. By crosslinking MsAcT with aldehyde dextran, followed by a covalent bonding with the newly selected support also the operational stability of the conjugates was dramatically enhanced. Finally, the obtained conjugates incorporated into paints were tested for decontamination activity against *Bacillus cereus* spores.

After demonstrating the MsAcT capability of catalyzing condensation reactions over hydrolysis in water media due to its particular protein structure (Mathews et al., 2007; Wiermans et al., 2013), Szymańska and collaborators were the first researchers assessing MsAcT-mediated continuous biotransformations (Szymańska et al., 2016). From an application perspective, high-performance continuous-flow systems are desirable as they offer a new handle on reaction engineering, while enhancing the process sustainability. The previous reports on batch MsAcT-catalyzed trans-esterification of neopentylglycol (NPG) both in buffer and solvent media (Wiermans et al., 2013) provided a good model reaction to compare the enzyme performance after immobilization onto silica monoliths and its employment in a microchannel reactor. While NPG, is a symmetric diol particularly suitable for this study as it allows to follow both its mono- and di-esterification, the authors decided to use a hydrophilic immobilization support in order to avoid any possible non-specific interaction between the biocatalyst active site and the surface of the carrier. Two different immobilization strategies have been investigated: the first one based on a covalent bond between the protein and the carrier to obtain a very stable catalyst (Fig. 11a), while the second one through specific adsorption onto the Ni/Co-modified support *via* the His-tag, since it is far away from the active site (Fig. 11b).

In both cases the results were very good in terms of flow-monoester preparation (complete conversion, 30 s of residence time, 100 mM of starting material), while longer residence times (12 min) were necessary to reach 50-60% of the diester. Thus, the rate of the imm-MsAcT-mediated reaction appeared to be deeply enhanced under continuous conditions when compared to batch mode using the free enzyme. In the latter system, in fact, full substrate conversion was never achieved and the mono-to-diester biotransformation was much lower (Wiermans et al., 2013). Both the microreactors with MsAcT coupled by His-tag adsorption and by covalent binding demonstrated very good stability with no decreasing activity after 50 h of continuous operation.

Integration of biocatalytic methods and continuous flow reactors (either micro- or meso-reactors) is typically designed for the intensification of the overall process overcoming low productivity, which is one of the most common limitation of biocatalysis. Although continuous-flow microchannel reactors above presented, demonstrated to be efficient, the synthesis and functionalization of silica monoliths is

anything but eco-friendly (*e.g.,* requirement of HNO_3, high temperature, inert atmosphere, long reaction times), moreover just a single biotransformation as a proof-of-concept has been reported. An in-depth immobilization study based on the covalent binding between MsAcT and various hydrophilic supports (*i.e.,* agarose, cellulose, 3-aminopropylic silica and epoxy resins) has been subsequently carried out by Contente and coworkers (Contente et al., 2019). The final author aim was the development of a robust and durable catalyst to be used specifically for fast syntheses and high flow rates. Same supports with different pore diameter (10-60 nm) as well as spacer size have been taken into consideration. Different enzymatic concentrations were assayed (1-5 mg/g_{matrix}) for each carrier and the best results in terms of recovered activity and operational stability under flow conditions were obtained with activated glyoxyl agarose as support and low MsAcT loading (73% retained activity, 1 mg/g_{matrix}, 100 cycles without losing the initial performance). Scanning electron microscopy (SEM) experiments to detect any change in the surface before and after the immobilization strategies have been performed. Additionally, spatial distribution of the fluorophore-labelled MsAcT was investigated, demonstrating the enzymatic localization across the porous surface of the agarose beads favoring an intimate contact with the substrates, as well as the stable spatial enzyme organization given by the covalent bond between the protein and the matrix. The newly developed immobilized MsAcT has been applied for the "*in continuous*" preparation of melatonin analogues (Fig. 12) as well as a variety of different natural aroma-compounds (Contente et al., 2019; Martina Letizia Contente et al., 2020) (Fig. 13).

In the first case using less than 2 mg of the catalyst, high concentration of different tryptamine derivatives (0.25-0.5 M, 30-60 g/L) have been biotransformed in 5 min of residence time, producing 19-30 g/day of pure amides respectively from EtOAc or the more reactive VinylAc employed as acyl donors. By simply adding an in-line extraction downstream the process, both the unreacted starting material and EtOAc were recovered and recirculated into the system, giving rise to a virtually zero waste reaction, while increasing the system automation.

In the second case, a flow-based platform was optimized for the preparation of esters typically employed as flavors and fragrances starting exclusively from natural substrates and using loop automated injections. Flow mode associated with biphasic biotransformations dramatically increased the overall production avoiding hydrolysis side-reactions, enzyme destabilization due to the accumulation of ethanol by-product and emulsions typical of batch reactions.

Not only single step biotransformations have been carried out exploiting the immobilized MsAcT under flow conditions, but also multi-enzymatic and chemo-enzymatic cascades. A very good example of a fully biocatalytic multi-step reaction has been recently reported by Annunziata and coworkers where the acetate metabolites of tyrosol (Ty) and hydroxytyrosol (HT) have been produced (Annunziata et al., 2021). The commercially available tyrosinase from *Agaricus bisporus* was employed as free-form for the oxidation of Ty to firstly obtain HT. Tyrosol in fact not only is readily accessible but also 10-times cheaper than the corresponding HT. The enzyme with ascorbic acid used to avoid over-oxidation reactions was recovered after an in-line extraction with EtOAc and reused for 3 cycles. A catch-and-release strategy involving supported boronic acid able to selectively trap HT through the formation of a cyclic borate with the catechol group, leaves the unreacted Ty in the exiting flow stream. HT was then released using an acidic solution. In the second step both the unreacted starting material and the newly generated HT were

acetylated using MsAcT immobilized as previously described by Contente et. al (Contente et al., 2019) and EtOAc as acetyl donor (Fig. 14). The regioselective acetylation of both Ty and HT, increasing their lipophilicity and modifying their bioavailability make these molecules appealing for cosmetic and food formulation.

Among the continuous chemo-enzymatic syntheses, merging together the advantages of flow reactors (*i.e.,* better parameter control, higher mass and heat transfer, modularity), the flexibility of chemical transformations and the selectivity of biocatalysts (*i.e.,* chemo-, regio-, and stereoselctivity), good examples have been reported involving imm-MsAcT on the preparation of APIs (active pharmaceutical ingredients) (Annunziata et al., 2020; Pinna et al., 2022). In the described methodologies an in-batch preparation of vinyl esters used as reactive acyl donors was performed through $Pd(OAc)_2$ catalysis. In the first example procaine and butacaine, two local anesthetics as well as the antiarrhythmic procainamide have been prepared through MsAcT-mediated condensation followed by a flow hydrogenation using a 10% Pd/C cartridge. In the second one, a series of nature-inspired vanillamides have been synthesized showing an enhanced antimicrobial activity with respect to the vanillic acid precursor, especially against the Gram negative bacterium *Pseudomonas aeruginosa*. In both cases the use in pure toluene let to overcome any solubilization problem of the starting material giving rise to more productive protocols while demonstrating high stability and reusability of the immobilized enzyme. A further implementation of this process was obtained by Contente and coworkers (Padrosa and Contente, 2021), which, to increase the overall process sustainability submitted the Pd(II)-mediated trans-vinylation to a flow switch (Fig. 15).

Using immobilized $Pd(OAc)_2$ filtration steps necessary in batch mode to remove metal traces were avoided, and the process related costs dramatically reduced as the catalyst was easily recycled. In the biocatalyzed part imm-MsAcT has been efficiently employed for the flow preparation of cinnamoyl tryptamines, emerging for their cosmetic potential as hyperpigmentation-correcting ingredients. The final 2-step robust synthetic methodology allowing for a fast preparation (15 min) of cinnamoyl tryptamines in large quantity (0.1 M) represents a major leap forward in validating their benefits in cosmetic formulations as well as their potential biological properties (*e.g.,* UV protecting, antioxidant, antimicrobial, and anti-inflammatory).

6. Future perspectives

As we saw in the previous sections, the acyltransferase from *Mycobacterium smegmatis* (MsAcT) is a very efficient and versatile catalyst that keeps well its activity when immobilized (on a wide variety of solid supports) and can be implemented in various reaction set ups (batch/flow, aqueous/solvent, etc.). Since all the most relevant research carried out in the last few years was already discussed in detail pointing out advantages, limitations, and mechanistic insights relative to this extraordinary enzyme, in the concluding remarks of this review the authors would like to give their personal perspective on research that could be carried out using MsAcT in the near future (Fig. 16).

First of all, novel MsAcT mutants will be produced in order to catalyze different reactions than the wild type enzyme. The brightest example is probable the recent publication of Contente et al. in 2020 that changing the catalytic serine with a cysteine, unlocked the MsAcT-mediated synthesis of thioesters and tertiary amides that was, until that point, never reported before (Martina L. Contente et al., 2020).

Moreover, the utilization of alternatives to the classically used vinyl esters should also be investigated since these compounds are expensive and rather difficult to find on the market.

Looking at all the various publications on MsAcT, it is also clear that researchers focused on the use of MsAcT for the conversion of small molecules using trans-esterification, *N*-acylation and perhydrolysis reactions but very little work was carried out on macromolecules. In fact, on polymer biotechnology, the only publication we could find touching the topic is the one from Finnveden et al. that in 2019 reported the possibility of synthesizing mono- and di-substituted diesters (starting from symmetric, aliphatic dicarboxylic acids) using the selectivity of MsAcT variants (Finnveden et al., 2019). One of the biggest advantages in this direction could in fact be the possibility of synthesizing novel bio-based polymers starting directly from fermentation products that do not need to be extracted from the aqueous-based media, deeply impacting on the process time and costs. Connected to that, the possibility of synthesizing lactones using a Baeyer-Villiger oxidation of cyclic ketones was also reported (Drozdz et al., 2016). We believe that the utilization of MsAcT for polymers synthesis (both using trans-esterifications and ring opening polymerization reactions) could be a new frontier for the exploitation of this biocatalyst in a field where no reports of its potential are yet available. All this can be inserted in a more general "*green context*" that involves the use of bulk reactions in which the acyl donor acts as the solvent or the employment of bio-based solvent alternatives to the traditional media such as toluene and hexane as recently demonstrated for lipases (Pellis et al., 2019).

Areas like biorefineries and downstream processing could certainly benefit from reactions carried out in batch or flow with immobilized MsAcT helping industries in detoxifying aqueous effluents by making some selected molecules more extractable or converting API precursors to the desired products (*e.g.,* containing esters, amides, thioesters etc.) using an environmentally friendly catalytic system. Moreover, more work could also be done on the family VIII carboxylesterases, therefore expanding the assortment of enzymes able to perform synthetic reactions in water, especially thanks to EstCE1 solved crystal structure and the individuated amino acid motif important for promiscuous acyltransfesare activity (Müller et al., 2020).

Moreover, due to MsAcT ability to perform condensation and perhydrolysis reactions in water media a high compatibility with different other biocatalysts is expected, opening the possibility to realize a variety of cascade reactions.

Last but not least, another unreported set of reactions using MsAcT but that is well known using proteases, lipases and especially cutinases are hydrolytic processes. Also in this case there are plenty of possibilities for the engineering of MsAcT and its application for the selective hydrolysis of triacylglycerols, fatty acids esters and eventually also polymers to their constituent monomers using an environmentally friendly process that would implement the possibility of re-synthesizing the molecule therefore allowing the compound circularity.

Authors contributions

All authors wrote the manuscript and contributed to the preparation of the original figures. A.P. and M.L.C. conceptualized the work, corrected, and revised the manuscript.

Acknowledgements

Pietro Cannazza is grateful to Cariplo Foundation for funding (Grant: CIRCLE – CItrus waste ReCycLing for added value products; CAR_RIC21DROMA_01).

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Figure 1. Biotransformations previously reported using the acyltransferase from *Mycobacterium smegmatis* (MsAcT) as the catalyst.

Figure 2. *Mycobacterium smegmatis* acyltransferase (MsAcT) crystal structure from RCSB PDB number 2Q0S https://www.rcsb.org/3d-view/2Q0S/1 a. octamer; b. monomer; c. catalytic triad.

Figure 3. 2-step mechanism of MsAcT divided in two half-reactions.

Figure 4. Trans-esterification of neopentylglycol to mono- and di-ester catalyzed by MsAcT.

Figure 5. a. MsAcT-biocatalyzed transformation towards HMF; b. Oxidation of furfural to furoic acid. Both the biotransformations were carried out in buffer media.

Figure 6. Esters as flavor and fragrances synthesized by MsAcT starting from natural substrates. m.c. = molar conversion.

Figure 7. a. *N*-acylation catalyzed by MsAcT; b. trans-amidation of (*E*)-cinnamylamine and 2-phenethylamine with acetamide. m.c. = molar conversion.

Figure 8. a. Example of chemo-enzymatic synthesis of lactones catalyzed by MsAcT coupled with Baeyer-Villiger oxidation of 2-methylcyclohexanone by peracids; b. melanine decolorization catalyzed by GOx-MsAcT-ISCO cascade.

Figure 9. Amide biocatalytic synthesis catalyzed by *Sp*ATA-MsAcT cascade.

Figure 10. a. MsAcT-catalyzed perhydrolysis of PDG; b. MsAcT immobilization strategies.

Figure 11. Silica carrier functionalization a. through covalent bonding; b. His-tag-mediated adsorption on Ni/Co sites.

Figure 12. Flow melatonin-analogues production.

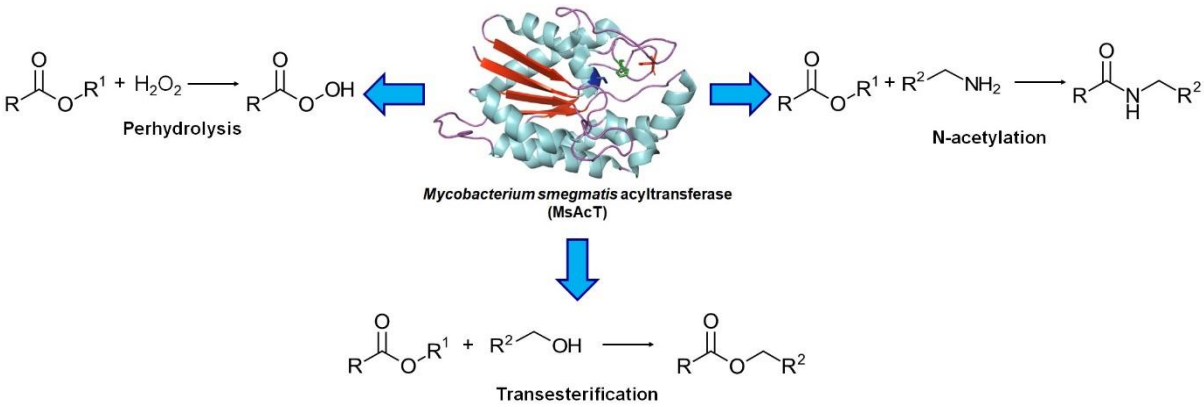
Figure 13. Flow natural aroma-compounds preparation.

Figure 14. Flow-biocatalyzed synthesis of tyrosol and hydroxytyrosol acetate.

Figure 15. Chemo enzymatic synthesis of cinnamoyl tryptamines.

Figure 16. Future perspectives on the biotransformations that could be investigated using the acyltransferase from *Mycobacterium smegmatis* (MsAcT) as the catalyst.

1049



1050
1051
1052
1053 Figure 1
1054

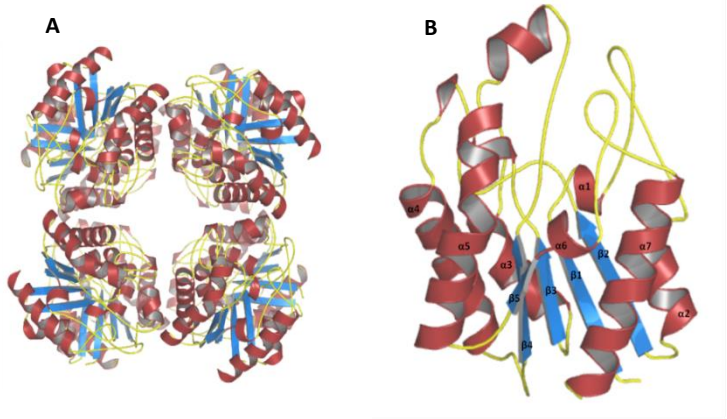


Figure 2.

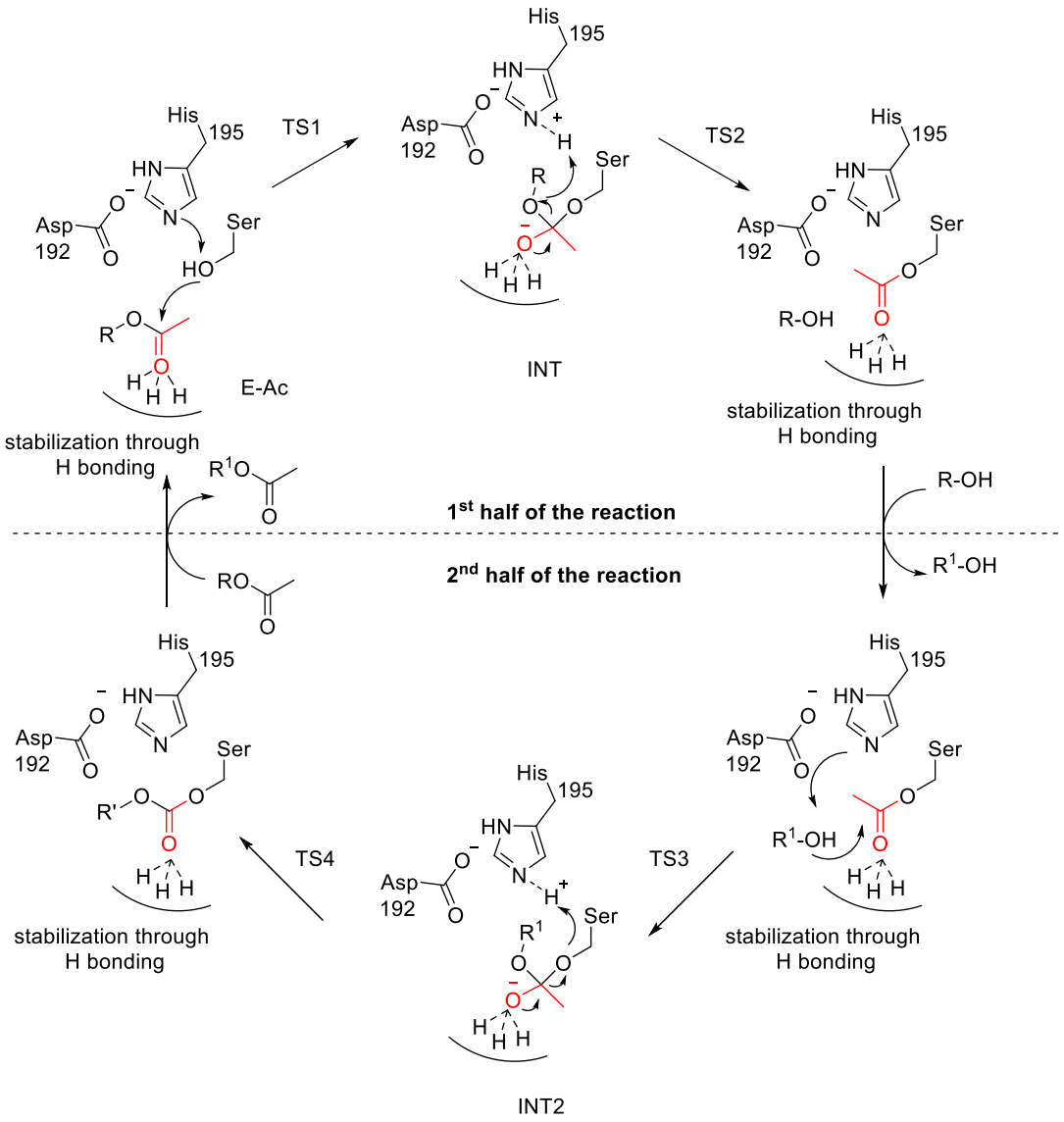

His
195
HN
Asp
192
O
Ser
HO
TS1
INT
TS2
R-OH
E-Ac
stabilization through
H bonding
1st half of the reaction
2nd half of the reaction
R1O
RO
R1-OH
TS3
TS4
INT2

Figure 3.

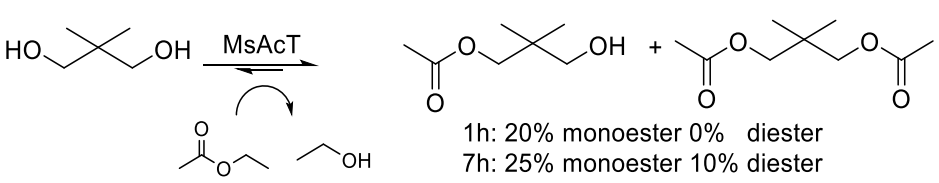

HO
OH
MsAcT
O
O
OH
OH
O
O
O
O
1h: 20% monoester 0% diester
7h: 25% monoester 10% diester

Figure 4.

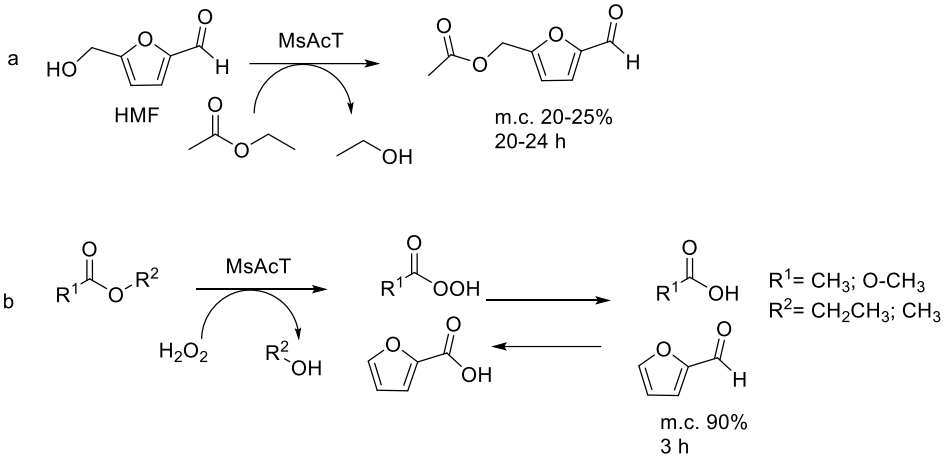

a
HO
O
H
HMF
MsAcT
O
O
OH
O
O
O
H
m.c. 20-25%
20-24 h
b
R1
O
O
R2
MsAcT
H2O2
R2
OH
R1
OOH
O
OH
R1
OH
O
H
m.c. 90%
3 h
R1= CH3; O-CH3
R2= CH2CH3; CH3

Figure 5.

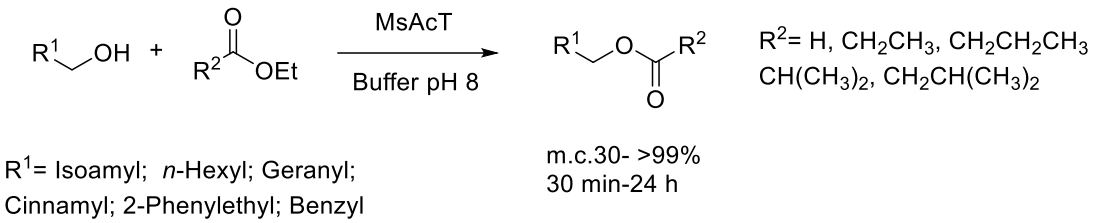

R1 OH + R2 O OEt
MsAcT
Buffer pH 8
R1 O R2 O
R2= H, CH2CH3, CH2CH2CH3
CH(CH3)2, CH2CH(CH3)2
R1= Isoamyl; n-Hexyl; Geranyl;
Cinnamyl; 2-Phenylethyl; Benzyl
m.c.30- >99%
30 min-24 h

Figure 6.

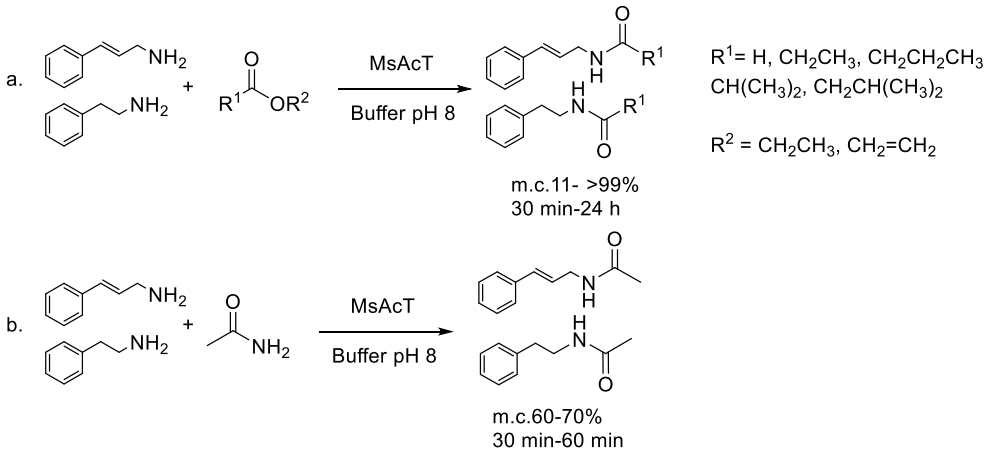

a.
MsAcT
Buffer pH 8
m.c.11- >99%
30 min-24 h
R1= H, CH2CH3, CH2CH2CH3
CH(CH3)2, CH2CH(CH3)2
R2 = CH2CH3, CH2=CH2
b.
MsAcT
Buffer pH 8
m.c.60-70%
30 min-60 min

Figure 7.

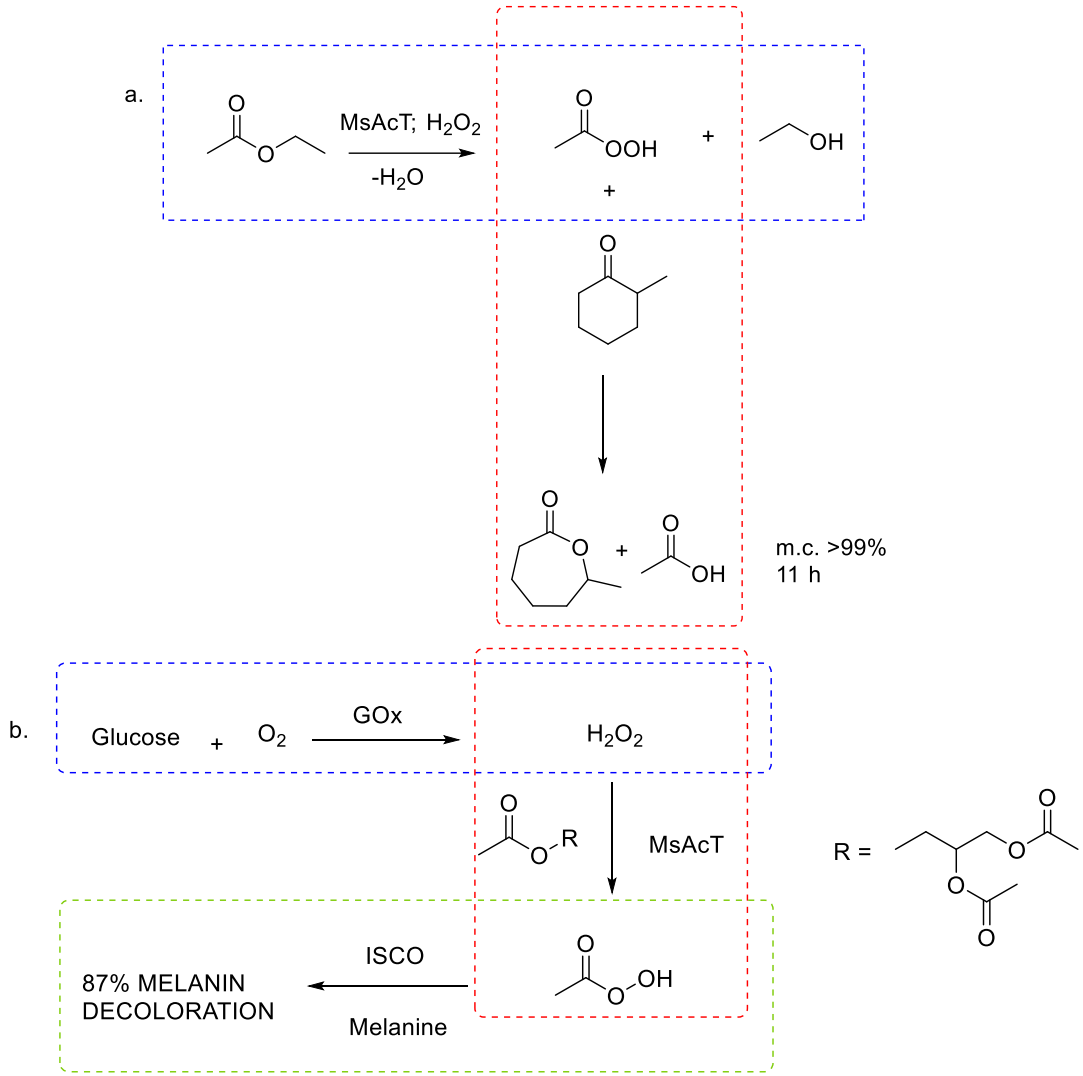

a.
MsAcT; H_2O_2
$-H_2O$
OOH
OH
m.c. >99%
11 h
b.
Glucose
O_2
GOx
H_2O_2
MsAcT
R =
ISCO
87% MELANIN
DECOLORATION
Melanine

Figure 8.

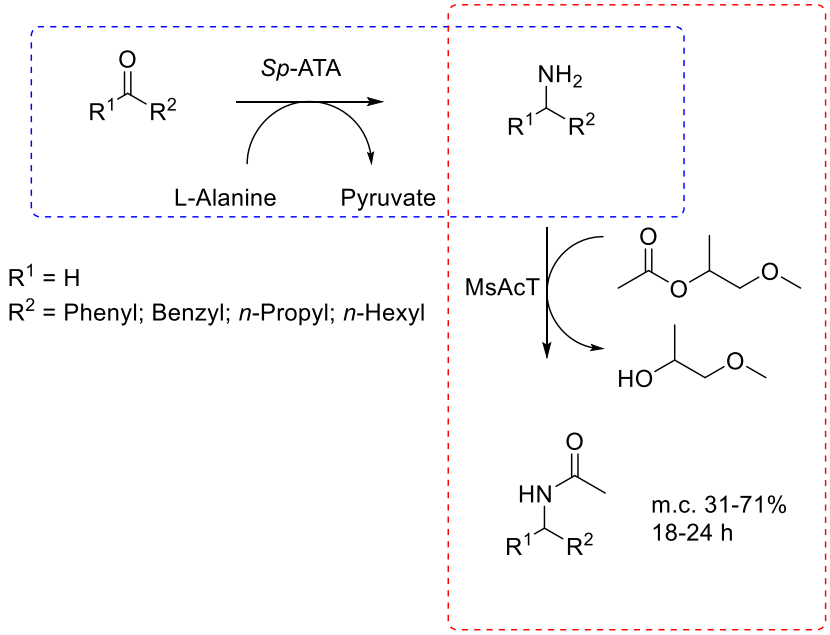

Sp-ATA
L-Alanine
Pyruvate
R1 = H
R2 = Phenyl; Benzyl; n-Propyl; n-Hexyl
MsAcT
m.c. 31-71%
18-24 h

Figure 9.

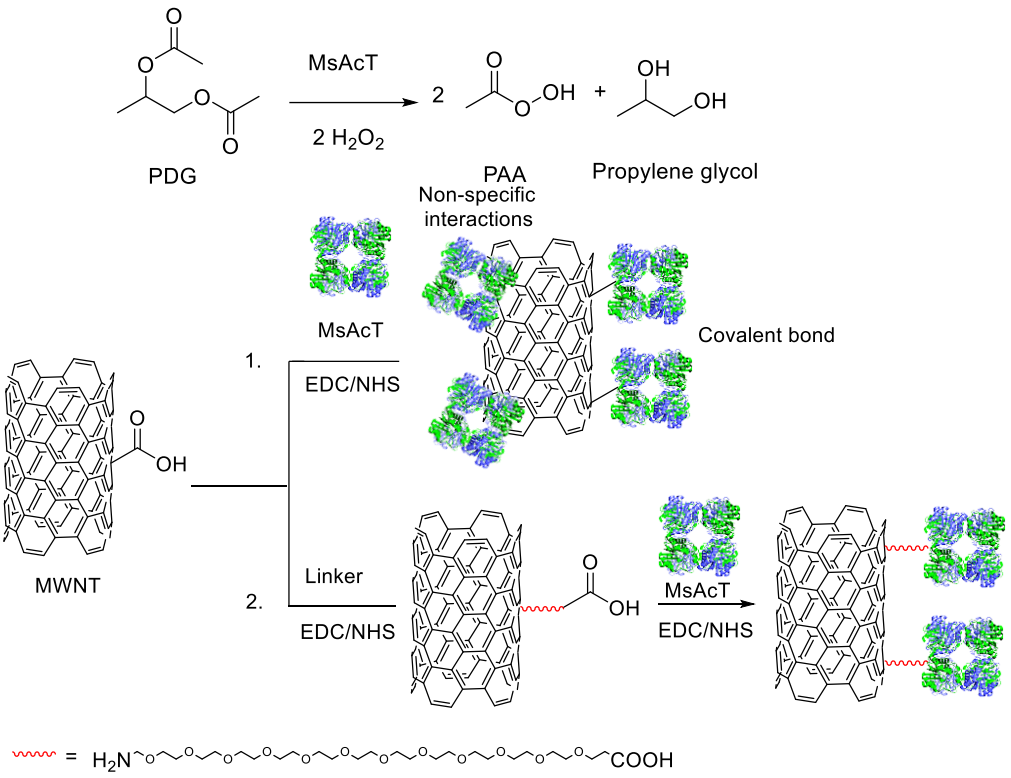

MsAcT
2 H2O2
2
OH
OH
PDG
PAA
Propylene glycol
Non-specific
interactions
MsAcT
1.
EDC/NHS
Covalent bond
OH
MWNT
2.
Linker
EDC/NHS
OH
MsAcT
EDC/NHS
= H2N
COOH

Figure 10.

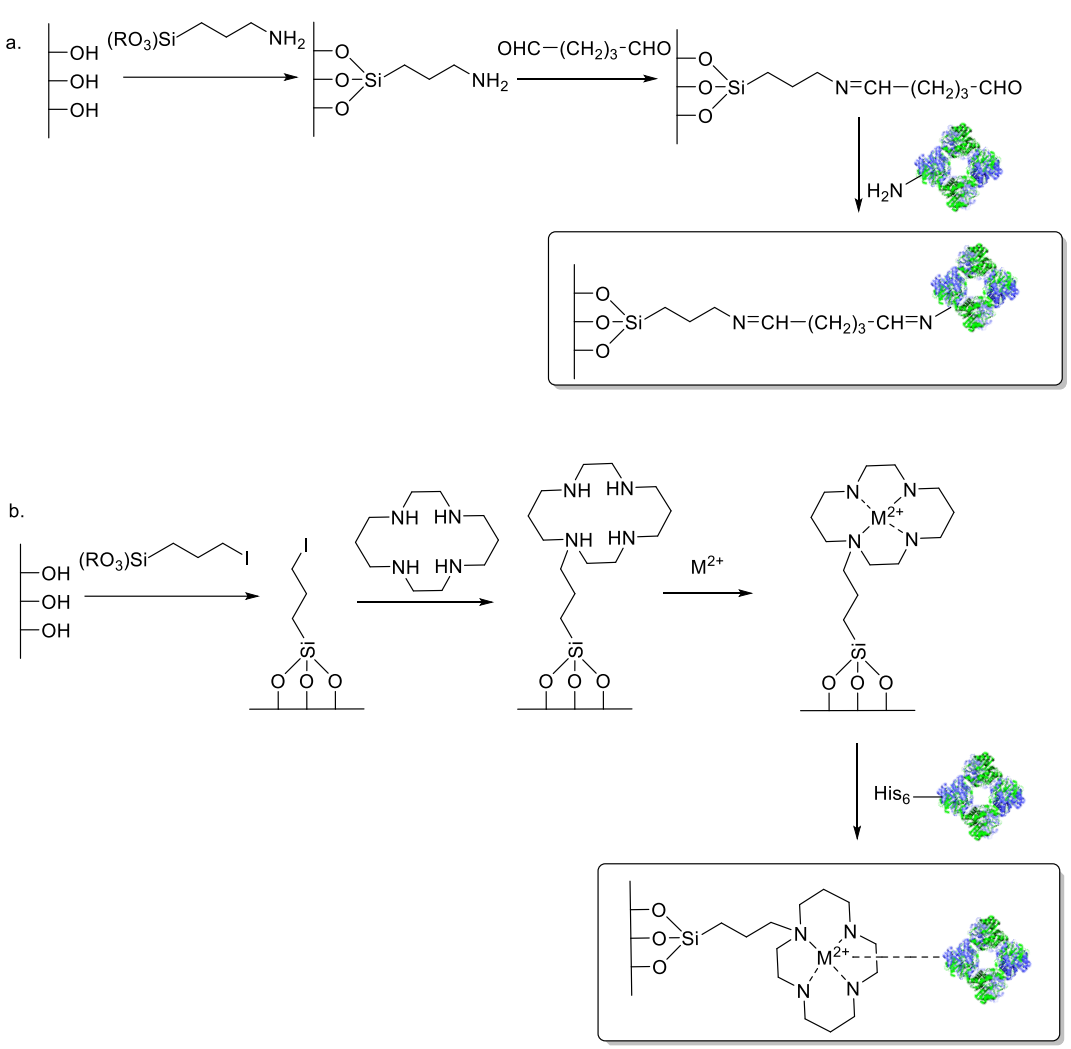

a.
OH
OH
OH
(RO3)Si
NH2
O
O
Si
O
NH2
OHC—(CH2)3-CHO
N=CH—(CH2)3-CHO
H2N
N=CH—(CH2)3-CH=N
b.
I
NH HN
NH HN
M2+
His6

Figure 11.

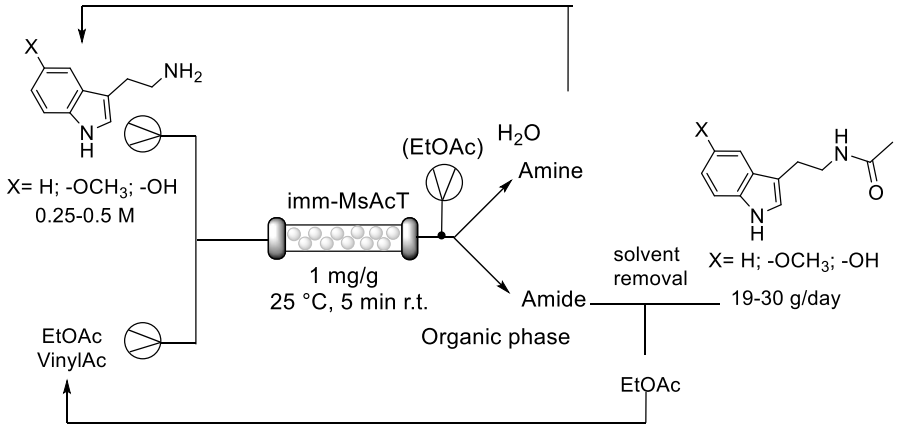

X
NH2
N
H
X= H; -OCH3; -OH
0.25-0.5 M
EtOAc
VinylAc
imm-MsAcT
1 mg/g
25 °C, 5 min r.t.
(EtOAc)
H2O
Amine
Amide
Organic phase
solvent
removal
EtOAc
X
H
N
O
N
H
X= H; -OCH3; -OH
19-30 g/day

Figure 12.

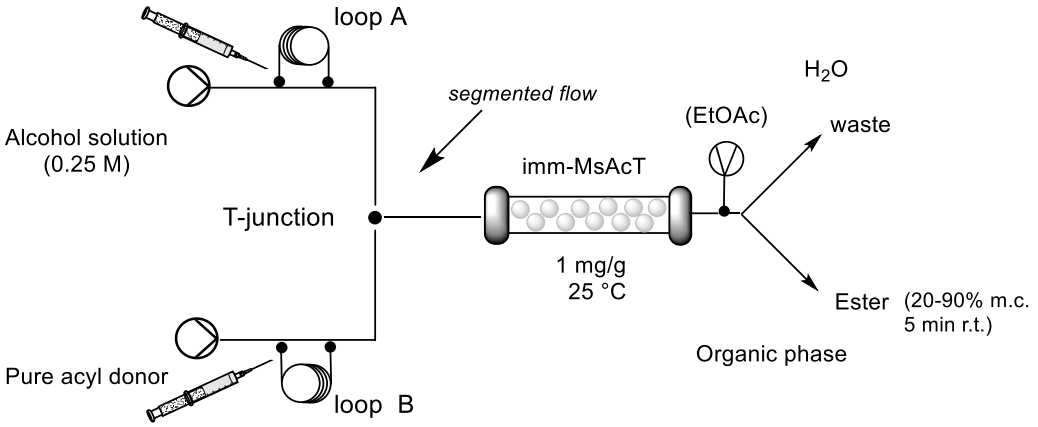

loop A
segmented flow
H_2O
(EtOAc)
waste
Alcohol solution
(0.25 M)
imm-MsAcT
T-junction
1 mg/g
25 °C
Ester (20-90% m.c.
5 min r.t.)
Organic phase
Pure acyl donor
loop B

Figure 13.

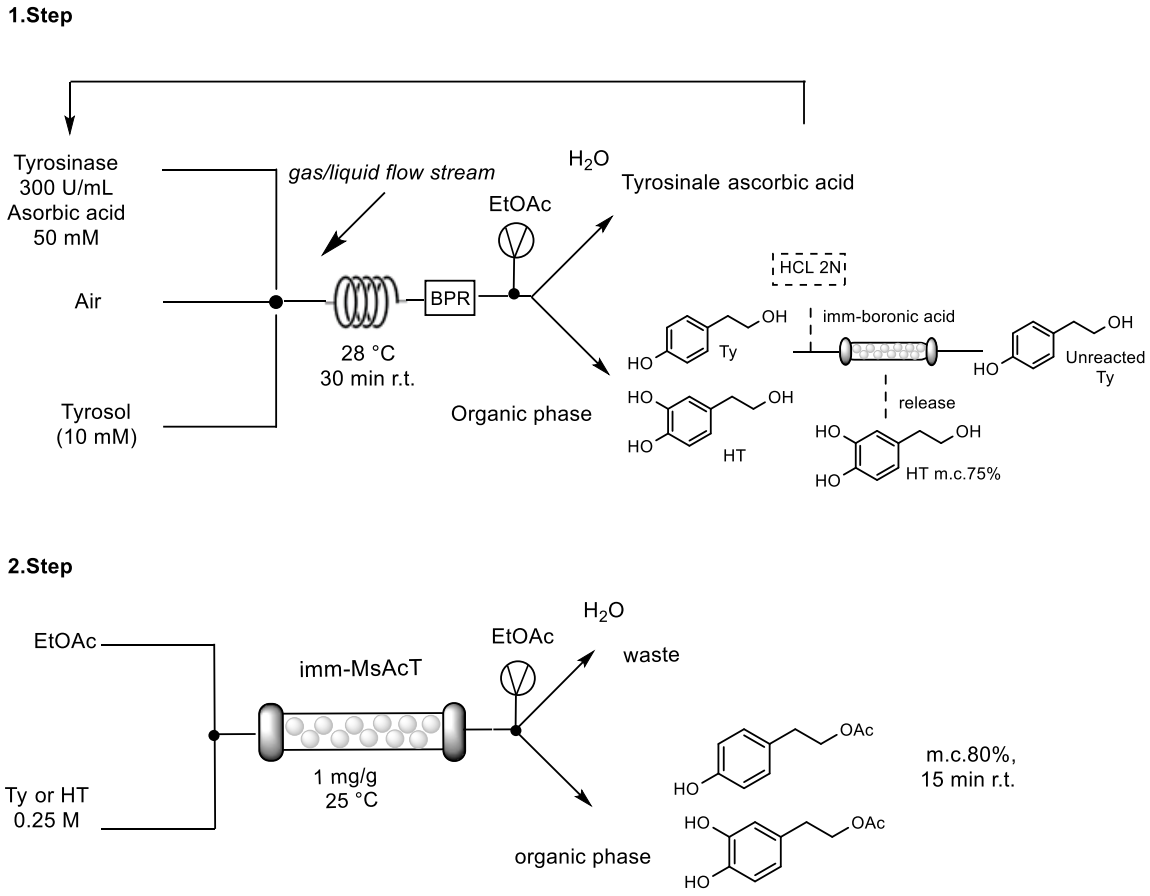

1.Step
Tyrosinase
300 U/mL
Asorbic acid
50 mM
Air
Tyrosol
(10 mM)
gas/liquid flow stream
28 °C
30 min r.t.
BPR
EtOAc
H2O
Tyrosinale ascorbic acid
Organic phase
HCL 2N
Ty
imm-boronic acid
Unreacted
Ty
release
HT
HT m.c.75%
2.Step
EtOAc
Ty or HT
0.25 M
imm-MsAcT
1 mg/g
25 °C
EtOAc
H2O
waste
organic phase
m.c.80%,
15 min r.t.

Figure 14.

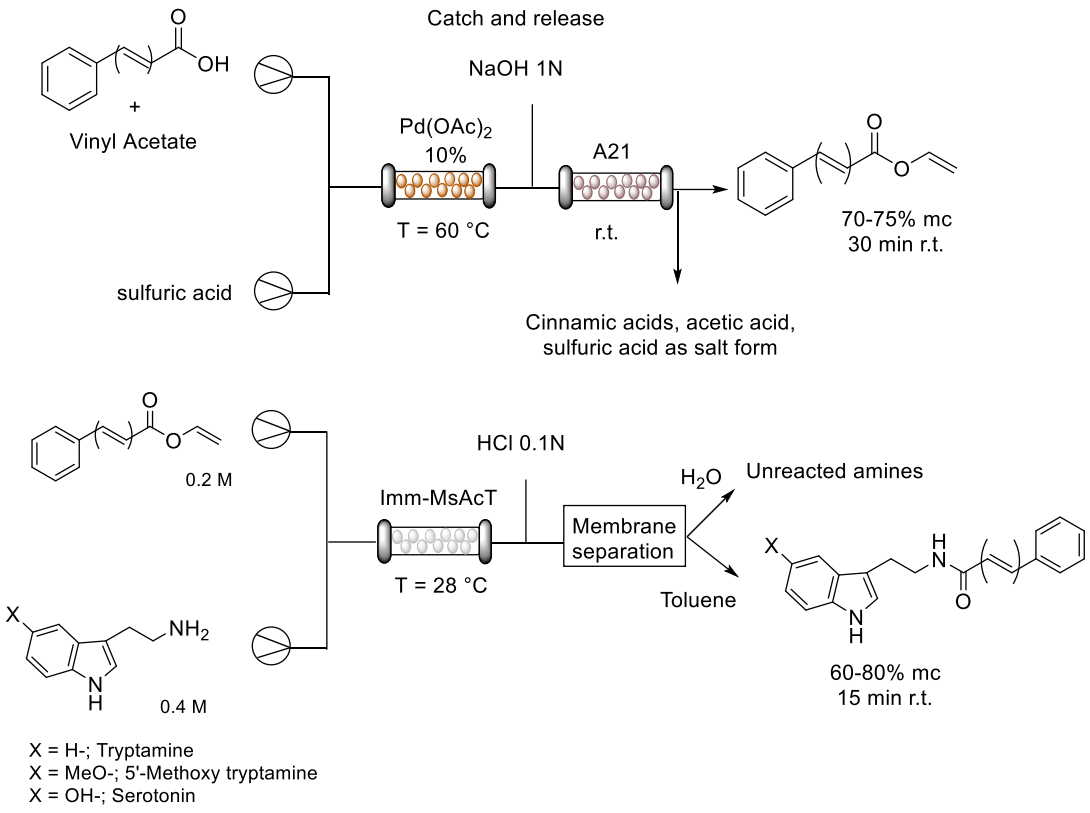

Catch and release
NaOH 1N
Vinyl Acetate
Pd(OAc)2
10%
A21
T = 60 °C
r.t.
70-75% mc
30 min r.t.
sulfuric acid
Cinnamic acids, acetic acid,
sulfuric acid as salt form
0.2 M
HCl 0.1N
Imm-MsAcT
H2O
Unreacted amines
Membrane
separation
T = 28 °C
Toluene
0.4 M
60-80% mc
15 min r.t.
X = H-; Tryptamine
X = MeO-; 5'-Methoxy tryptamine
X = OH-; Serotonin

Figure 15.

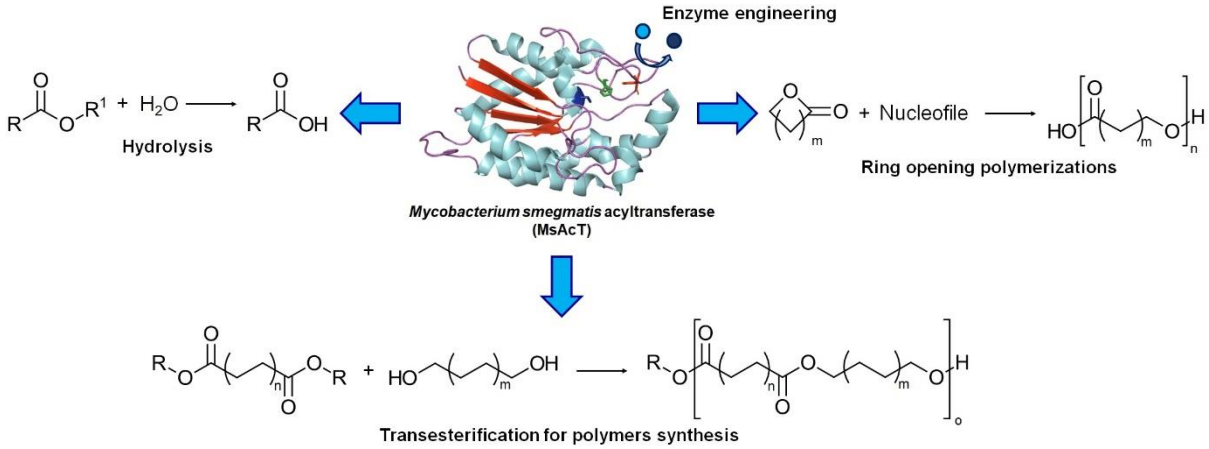

Enzyme engineering
Hydrolysis
Ring opening polymerizations
Nucleofile
Mycobacterium smegmatis acyltransferase
(MsAcT)
Transesterification for polymers synthesis

Figure 16.

Table 1. Summary of all MsAcT reported mutations and their relative effect on the enzyme activity.

Mutation	Effect	Reference
K97R/F150I	Increased AT and reduced H	Godehard et al, 2020
K97A	Increased AT and reduced H	Godehard et al, 2020
K97R	Increased AT and reduced H	Godehard et al, 2020
N94A	Increased AT and reduced H (*S*)-enantiopreference	Godehard et al, 2020
R56K	Increased AT and reduced H	Godehard et al, 2020
H195D	Lower total activity; high AT:H ratio	Godehard et al, 2020
H195N	Lower total activity; high AT:H ratio	Godehard et al, 2020
D192A	Lower total activity; high AT:H ratio	Godehard et al, 2020
D192E	Lower total activity; high AT:H ratio	Godehard et al, 2020
D192N	Lower total activity; high AT:H ratio	Godehard et al, 2020
L12A	DVA monotrans-esterification	Finnveden et al., 2019
T93A/F154A	DVA di-substitution	Finnveden et al., 2019
T93S	Acceptance of hindered acyl donors (R)-enantiopreference	Godehard et al, 2020
T93S/T154A	Acceptance of hindered acyl donors (*R*)-enantiopreference	Godehard et al, 2020
F154A	Acceptance of hindered acyl donors (*R*)-enantiopreference	Godehard et al, 2020
F154A/I194V	Acceptance of hindered acyl donors (*R*)-enantiopreference	Godehard et al, 2020
F153V	Increased AT (*R*)-enantiopreference	Jost et al., 2020
F154L	Increased AT (*R*)-enantiopreference	Jost et al., 2020
F174L	Increased AT (*R*)-enantiopreference	Jost et al., 2020
F154V/F174V	Increased AT (*R*)-enantiopreference	Jost et al., 2020
F150V/F154V	Increased AT (*R*)-enantiopreference	Jost et al., 2020
W16A	(*S*)-enantiopreference	Jost et al., 2020
N94A/F150I	(*S*)-enantiopreference	Godehard et al, 2020
W16F/N94S	(*S*)-enantiopreference	Godehard et al, 2020
S11C	Acceptance of thiols and secondary amines	Contente et al, 2020

*AT: Acyltrasferase activity H: Hydrolysis