JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Efficient Enzymatic Preparation of Flavor Esters in Water

Igor Chiarelli Perdomo,[†] Stefania Gianolio,[†] Andrea Pinto,[†] Diego Romano,[†] Martina Letizia Contente,[†,‡] Francesca Paradisi,[‡] and Francesco Molinari*[,†]

[†]Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via Mangiagalli 25, 20133 Milan, Italy

[‡]School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

ABSTRACT: A straightforward biocatalytic method for the enzymatic preparation of different flavor esters starting from primary alcohols (e.g., isoamyl, *n*-hexyl, geranyl, cinnamyl, 2-phenethyl, and benzyl alcohols) and naturally available ethyl esters (e.g., formate, acetate, propionate, and butyrate) was developed. The biotransformations are catalyzed by an acyltransferase from *Mycobacterium smegmatis* (MsAcT) and proceeded with excellent yields (80−97%) and short reaction times (30−120 min), even when high substrate concentrations (up to 0.5 M) were used. This enzymatic strategy represents an efficient alternative to the application of lipases in organic solvents and a significant improvement compared with already known methods in terms of reduced use of organic solvents, paving the way to sustainable and efficient preparation of natural flavoring agents.

KEYWORDS: *flavor esters, enzymatic acylation, biocatalysis, acyltransferase, Mycobacterium smegmatis (MsAcT)*

INTRODUCTION

Many esters are components of natural flavors and are used as flavor and fragrance in food, drinks, and cosmetics.[1] Their preparation starting from natural substrates and using bioprocesses (e.g., fermentation or enzymatic reactions) is appealing, because the final product can be labeled and commercialized in the European Union and the United States as natural.[2,3] Therefore, new biotechnological approaches for obtaining flavors are highly demanded as long as they are efficient and sustainable.[4,5] Many flavor and fragrance esters can be enzymatically obtained using lipases that catalyze esterification, transesterification, or interesterification reactions in media (e.g., organic solvents) characterized by low water activity.[6−8] The presence of water is critical for the equilibrium of lipase-catalyzed reactions in organic media, strongly limiting the overall yields. In fact, water may compete as a nucleophile with the alcohol in the attack of the acyl−enzyme intermediate, thus favoring hydrolysis over transesterification,[9−11] and for this reason, water must be avoided or removed during the reaction to achieve high yields.[12,13] Flavor-ester biosynthesis in an aqueous system composed of coconut cream and fusel oil was achieved with limited conversion (14.25 mg/g, based on cream weight), after optimization of the process catalyzed by the lipase Palatase.[14] The potential of lipases for flavor-ester production has also been exploited using immobilized enzymes[15] and whole microbial cells;[16−18] these systems have been especially employed for setting up robust processes and facilitating product recovery. Acetate esters are among the most valuable flavor esters, and few of them have been produced in hydrophobic solvents (e.g., *n*-pentane, *n*-hexane, or *n*-heptane) using different immobilized lipases;[19] in a large scale production of flavor esters, Novozym 435 (35 g/L) was able to give yields ranging between 91 and 95% after 24 h, starting from an alcohol concentration of 1.0 M and an excess of acetic acid.[12] Alternatively, the use of *Candida rugosa* lipase, immobilized in calcium alginate gel, allowed for a productivity of 1600−2200 μmol/h per gram of biocatalyst for the preparation of isoamyl and butyryl acetate.[20]

An interesting enzymatic alternative for preparing esters is the use of the acyltransferase from *Mycobacterium smegmatis* (MsAcT).[21] MsAcT is characterized by a hydrophobic tunnel leading to the active site, where water access is disfavored;[21,22] moreover, MsAcT is functionally active as a closely aggregated octamer in aqueous solution.[21] This overall architecture means that MsAcT is able to favor transesterification reactions with respect to hydrolysis even in water, unlike lipases that catalyze transesterification only under conditions of low water activity.[22] For these structural reasons, MsAcT has been used for catalyzing transesterification reactions of primary and secondary alcohols with ethyl acetate in aqueous buffers, often with good enantioselectivity.[22−24] The ratio between the catalytic rate constants of the hydrolysis and synthesis of benzyl acetate were compared, showing that MsAcT is indeed an effective acetyltransferase rather than a hydrolytic enzyme.[24] At reasonably high concentrations of the ester, the reaction mixtures of these biotransformations are actually two-liquid-phase systems composed of the hydrophobic ester and water, and the reaction occurs in the aqueous phase. The peculiar features of this enzyme make it also suitable for catalyzing amide synthesis in aqueous media.[25,26]

In this work, we have explored the potential of MsAcT for flavor-ester preparation; the enzyme was able to work in two-liquid-phase systems composed of sparingly water-soluble esters and water, accomplishing the reaction in the aqueous phase. Several (24) different flavor esters derived from

Received: March 21, 2019
Revised: May 15, 2019
Accepted: May 17, 2019
Published: May 17, 2019

DOI: 10.1021/acs.jafc.9b01790
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 1. Acetylation of Geraniol at Different Initial Concentrations Using MsAcT (1.0 mg/mL, 110 U/mL) in the Presence of EtOAc (10%, v/v) in Phosphate Buffer (0.1 M, pH 8.0) at 25 °C

geraniol (mM)	initial rate (μmol/mg/min)	maximum conversion (%)[a]	maximum product yield (mg/mL)	time (h)[b]
100	2.25	>98	19.6	2
200	4.56	>98	39.2	24
250	6.36	98	48.1	24
300	6.57	95	55.9	24
400	7.68	79	62.0	48
500	7.65	68	66.7	48

[a]As determined by gas chromatography. [b]Time refers to the maximum conversion observed.

transesterification of primary alcohols (e.g., isoamyl, *n*-hexyl, geranyl, benzyl, 2-phenylethyl, and cinnamyl) with ethyl esters bearing short-chain acyl groups (e.g., formyl, acetyl, propionyl, and butyryl) were prepared in excellent yields with short reaction times when high concentrations of substrates (up to 500 mM) were loaded, making the process interesting at industrial level.

MATERIALS AND METHODS

Chemicals. All reagents and solvents were obtained from commercial suppliers and were used without further purification.

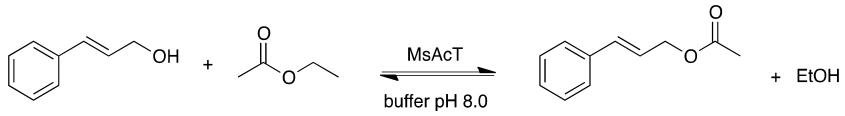
Preparation of the Acetyltransferase from *Mycobacterium smegmatis* (MsAcT). *Escherichia coli* BL21 star (DE3) was used as the host for the production of the recombinant protein; cloning was carried out as described before, starting from a synthetic gene encoding for MsAcT (GenBank accession: ABK70783) from *M. smegmatis* strain MC2 155.[26] Recombinant *E. coli* was first grown at 37 °C in Luria−Bertani (LB) liquid medium in the presence of 25 μg/mL kanamycin for 16 h; this starting culture was used to inoculate the cultivation medium (Terrific Broth: 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 g/L glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 25 μg/mL kanamycin), which was brought to an initial OD_{600nm} of 0.1. Cultivation was carried out at 37 °C in orbital shakers with an agitation speed 110 rpm; cells were grown until an OD_{600nm} of 0.5−0.6. The expression of MsAcT was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The culture was further incubated for 16 h at 25 °C. Wet cells (2.0 g) were then harvested by centrifugation (20 min, 9000 rcf at 4 °C), washed with 20 mM phosphate buffer (pH 8.0), resuspended in 10 mL of buffer (100 mM phosphate buffer, pH 8.0; 6 mM imidazole; 100 mM NaCl), and sonicated (5 cycles of 1 min on and 1 min off at 4 °C). Cell debris were recovered by centrifugation (45 min, 36 000 rcf at 4 °C). The enzyme was purified by affinity chromatography with HIS-Select Nickel Affinity Gel. The fractions showing activity were pooled and dialyzed against phosphate buffer (100 mM, pH 8.0) and stored at 4 °C; 96 mg of pure protein was obtained. Activity was spectrophotometrically evaluated at 400 nm by determining the release of *p*-nitrophenol after hydrolysis of *p*-nitrophenylacetate at 25 °C in a half-microcuvette (volume of 1 mL) for 2 min. One unit (U) of activity is defined as the amount of enzyme that catalyzes the consumption of 1 μmol of *p*-nitrophenylacetate per minute. Reference conditions were 0.1 mg/mL *p*-nitrophenylacetate, 0.1% (v/v) EtOH, and different amounts of MsAcT in 100 mM phosphate buffer (pH 8.0). The specific activity of the purified enzyme was 110 U/mg.

Preparation of Flavor Esters. Standard transesterification reactions were carried out in 10 mL screw cap tubes: alcohols (250 mM) and ethyl esters (10%, v/v) were added to 2.5 mL of phosphate buffer (100 mM, in the range of pH 8.0) containing MsAcT as purified enzyme (0.1−1.5 mg/mL; the reaction was left under magnetic stirring at 25 °C. The reactions were stopped after 24 h and extracted with EtOAc (2 × 8 mL); the organic phases were collected, dried over Na_2SO_4 and evaporated. Products were purified by flash chromatography (eluent: *n*-hexane/EtOAc), and their structures and purities were determined via ^1H NMR analysis by comparison with commercially available samples. Optimization of geranyl and cinnamyl acetate synthesis was carried out using a 1 mL reaction mixture in phosphate buffer (100 mM, in the range of pH between 5.0 and 8.0) containing geraniol or cinnamyl alcohol (25−500 mM), MsAcT (0.1−1.5 mg/mL) and a fixed amount of ethyl acetate (10%, v/v); the solution was left under magnetic stirring at different temperatures (20−40 °C). Aliquots (50 μL) were withdrawn at different reaction times and extracted twice with EtOAc (2 × 100 μL), and the collected organic phase was dried over Na_2SO_4. Preparative biotransformations at the multimilligram scale aimed at synthesizing geranyl acetate and cinnamyl acetate were carried out using geraniol (250 mM) and cinnamyl alcohol (250 mM) dissolved in 9 mL of phosphate buffer (0.1 M, pH 8.0) in the presence of 10 mg of MsAcT and 1 mL of EtOAc at 25 °C. The reactions were stopped after 24 h and extracted with EtOAc (2 × 8 mL); the organic phases were collected, dried over Na_2SO_4, and evaporated. Products were purified by flash chromatography; 442 mg (90% yield) of geranyl acetate (eluent: *n*-hexane/EtOAc, 98:2; chemical purity >98%) and 410 mg (93% yield) of pure cinnamyl acetate (eluent: *n*-hexane/EtOAc, 95:5; chemical purity >98%) were obtained. Purity of the product was confirmed by ^1H NMR and gas-chromatography analysis.

Analysis of the Biotransformations. Aliquots of the biotransformation medium (100 μL) were withdrawn at different reaction times, quenched with 5 μL of 0.5 M sulfuric acid and extracted with 100 μL of ethyl acetate. The amounts of produced flavor esters and the residual quantity of alcohol substrates were determined by gas-chromatographic (GC) analysis. Geraniol, cinnamyl alcohol, *n*-hexanol, benzyl alcohol, 2-phenylethanol, and their esters were analyzed by gas-GC analysis on a Carlo Erba Fractovap GC equipped with a fused-silica capillary column MEGA-SE30 (100% methyl polysiloxane; 25 m × 0.25 mm i.d.), with the injector temperature at 200 °C. Oven temperatures ranged from 45 to 180 °C. The retention times for geraniol and its esters (temperature gradient: from 80 to 180 °C at 5 °C/min) were as follows: geraniol, 7.3 min; geranyl formate, 10.2 min; geranyl acetate, 10.2 min; geranyl propionate, 12.5; and geranyl butyrate, 14.5 min. The retention times for cinnamyl alcohol and its esters (temperature gradient: 80 °C for 2 min then from 80 to 180 °C with a 5 °C/min gradient) were as follows: cinnamyl alcohol, 9.9 min; cinnamyl formate, 10.7 min; cinnamyl acetate, 13.2 min; cinnamyl propionate, 15.5 min; and cinnamyl butyrate 17.8 min. The retention times for *n*-hexanol and its esters (temperature gradient: 50 °C for 10 min then from 50 to 150 °C with a 10 °C/min gradient) were as follows: *n*-hexanol, 5.3 min; *n*-hexyl formate, 7.7 min; *n*-hexyl acetate, 8.8 min; *n*-hexyl propionate, 15.2 min; and *n*-hexyl butyrate, 16.8 min. The retention times for benzyl alcohol, 2-phenylethanol, and their esters (temperature gradient: from 80 to 180 °C with a 10 °C/min gradient) were as follows: benzyl alcohol, 3.0 min; benzyl formate, 3.4 min; benzyl acetate, 4.2 min; benzyl propionate, 5.0 min; benzyl butyrate, 6.5 min; 2-phenylethanol, 3.7 min; 2-phenylethyl formate, 4.3 min; 2-phenylethyl acetate, 5.4 min; 2-phenylethyl propionate, 6.6 min;

DOI: 10.1021/acs.jafc.9b01790
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

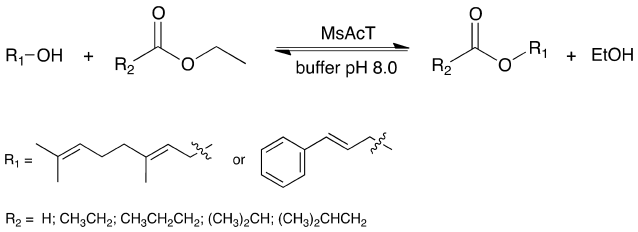
Table 2. Acetylation of Cinnamyl Alcohol at Different Initial Concentrations Using MsAcT (1.0 mg/mL, 110 U/mL) in the Presence of EtOAc (10%, v/v) in Phosphate Buffer (0.1 M, pH 8.0) at 25 °C



cinnamyl alcohol (mM)	initial rate (μmol/mg/min)	maximum conversion (%)[a]	maximum product yield (mg/mL)	time (h)[b]
100	2.54	>98	17.6	1
200	5.07	>98	35.2	3
250	6.32	>98	44.0	6
300	6.82	92	48.6	24
400	8.53	84	59.2	24
500	8.65	79	69.6	24

[a]As determined by gas chromatography. [b]Time refers to the maximum conversion observed.

Table 3. Acylation of Geraniol (250 mM) and Cinnamyl Alcohol (250 mM) Using MsAcT (1.0 mg/mL, 110 U/mL) in the Presence of Different Ethyl Esters (10%, v/v) in Phosphate Buffer (0.1 M, pH 8.0) at 25 °C



alcohol	ester	ester main flavor property[1]	initial rate (μmol/mg min)	conversion (%)[a]	time (h)[b]
geraniol	ethyl formate	rose	7.69	>98	3
geraniol	ethyl propionate	fruity	1.51	86	48
geraniol	ethyl butyrate	fruity, apricot	1.15	62	48
geraniol	ethyl isobutyrate	rose, apricot	1.42	74	48
geraniol	ethyl isovalerate	rose, sweet apple	0.88	38	48
cinnamyl alcohol	ethyl formate	fruity, apple	7.72	>98	1
cinnamyl alcohol	ethyl propionate	fruity, woody	2.13	>98	3
cinnamyl alcohol	ethyl butyrate	fruity, honey	1.87	90	24
cinnamyl alcohol	ethyl isobutyrate	fruity, apple−banana	1.92	87	24
cinnamyl alcohol	ethyl isovalerate	rose, apple	0.91	44	48

[a]As determined by gas chromatography. [b]Time refers to the maximum conversion observed.

and 2-phenylethyl butyrate, 7.8 min. Conversions of isoamyl alcohol into its esters were determined by GC analysis on a Carlo Erba Fractovap GC equipped with a fused-silica capillary column MEGA-DEX DMP-Beta (dimethyl pentyl-β-cyclodextrin, 25 m × 0.25 mm i.d.), with the injector temperature at 200 °C. The retention times (temperature gradient: from 40 to 180 °C at 5 °C/min) were as follows: isoamyl alcohol, 6.7 min; isoamyl formate, 5.8 min; isoamyl acetate, 7.1 min; isoamyl propionate, 9.4 min; and isoamyl butyrate, 11.5 min. Initial rates were defined as the amount (μmol) of ester produced after 30 min per amount of enzyme.

RESULTS AND DISCUSSION

The reaction between geraniol and ethyl acetate (EtOAc) using the acyltransferase from *M. smegmatis* (MsAcT) as biocatalyst was studied in a two-liquid-phase system composed of water and EtOAc; the product (geranyl acetate) presents a green-type flavor and a floral-type odor.[1] Different parameters (pH, temperature, and enzyme concentration) were optimized, while the amount of geraniol (0.065 mM) and the phase ratio between water and EtOAc (9:1) were kept fixed; a Multi-simplex optimization design was employed[27] using initial rate and molar conversions after 30 min as response variables. The best results (corresponding to conversions >98% after 30 min) were obtained using 1.0 mg/mL MsAcT at pH 8.0 and 25 °C; under these conditions, the production of the ester was studied using different initial geraniol concentrations (Table 1).

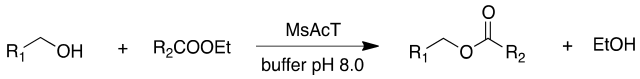
The highest rates of geranyl acetate synthesis were achieved at geraniol concentrations between 400 and 500 mM, whereas almost quantitative yields were obtained only at alcohol concentrations lower than 300 mM. Possible product inhibition was evaluated by assessing the activity of MsAcT in the presence of increasing concentrations of EtOH and geranyl acetate (0−500 M); the activity of the enzyme was assayed (with *p*-nitrophenyl acetate) in the presence of concentrations of EtOH between 250 and 500 mM and showed that the enzyme was 40−50% inhibited, whereas no significant effects were observed with geranyl acetate.

The best compromise between rates and conversion was observed starting from 250 mM geraniol, allowing for 98% conversion (48.1 mg/mL analytical yield) after 24 h. Notably, good conversion (68%) after 48 h was achieved even when the biotransformation was carried out starting from 500 mM geraniol, allowing for the accumulation of 66.7 mg/mL geranyl acetate.

A similar evaluation of the effect of substrate concentration was carried out in the case of the acetylation of cinnamyl

DOI: 10.1021/acs.jafc.9b01790
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 4. Acylation of Different Primary Alcohols (100−500 mM Starting Concentrations) with Short-Chain Ethyl Esters Using MsAcT (1.0 mg/mL, 110 U/mL) in the Presence of Different Ethyl Esters (10%, v/v) in Phosphate Buffer (100 mM, pH 8.0) at 25 °C



R_1	initial alcohol (mM)	R_2	ester main flavor property[1]	conversion (%)[a]	time (h)[b]
$(CH_3)_2CHCH_2-$	100	H−	black currant, currant	93	0.5
$(CH_3)_2CHCH_2-$	250	H−		93	0.5
$(CH_3)_2CHCH_2-$	500	H−		86	1
$(CH_3)_2CHCH_2-$	100	CH_3-	banana, pear	94	0.5
$(CH_3)_2CHCH_2-$	250	CH_3-		95	0.5
$(CH_3)_2CHCH_2-$	500	CH_3-		88	0.5
$(CH_3)_2CHCH_2-$	100	CH_2CH_3-	pineapple−apricot, apricot	97	0.5
$(CH_3)_2CHCH_2-$	250	CH_2CH_3-		97	1
$(CH_3)_2CHCH_2-$	500	CH_2CH_3-		95	1
$(CH_3)_2CHCH_2-$	100	$CH_2CH_2CH_3-$	fruity	94	24
$(CH_3)_2CHCH_2-$	250	$CH_2CH_2CH_3-$		93	24
$(CH_3)_2CHCH_2-$	500	$CH_2CH_2CH_3-$		92	24
$CH_3(CH_2)_4-$	100	H−	apple	97	0.5
$CH_3(CH_2)_4-$	250	H−		97	1
$CH_3(CH_2)_4-$	500	H−		88	3
$CH_3(CH_2)_4-$	100	CH_3-	fruity, pear	97	0.5
$CH_3(CH_2)_4-$	250	CH_3-		95	1
$CH_3(CH_2)_4-$	500	CH_3-		90	3
$CH_3(CH_2)_4-$	100	CH_2CH_3-	earthy, metallic−fruity	72	1
$CH_3(CH_2)_4-$	250	CH_2CH_3-		50	1
$CH_3(CH_2)_4-$	250	CH_2CH_3-		35	2
$CH_3(CH_2)_4-$	500	CH_2CH_3-		30	1
$CH_3(CH_2)_4-$	500	CH_2CH_3-		24	2
$CH_3(CH_2)_4-$	100	$CH_2CH_2CH_3-$	apricot, pineapple	94	2
$CH_3(CH_2)_4-$	250	$CH_2CH_2CH_3-$		96	6
$CH_3(CH_2)_4-$	500	$CH_2CH_2CH_3-$		95	24
Ph−	100	H−	floral−fruity, apricot	92	0.5
Ph−	250	H−		84	1
Ph−	500	H−		86	3
Ph−	100	CH_3-	jasmine	92	0.5
Ph−	250	CH_3-		90	1
Ph−	500	CH_3-		87	3
Ph−	100	CH_2CH_3-	floral−fruity, peach	96	0.5
Ph−	250	CH_2CH_3-		95	1
Ph−	500	CH_2CH_3-		89	3
Ph−	100	$CH_2CH_2CH_3-$	plum, pear	95	8
Ph−	250	$CH_2CH_2CH_3-$		92	8
Ph−	500	$CH_2CH_2CH_3-$		95	24
$PhCH_2-$	100	H−	hyacinth	95	0.5
$PhCH_2-$	250	H−		88	2
$PhCH_2-$	500	H−		86	3
$PhCH_2-$	100	CH_3-	rose, raspberry	96	0.5
$PhCH_2-$	250	CH_3-		91	0.5
$PhCH_2-$	500	CH_3-		82	0.5
$PhCH_2-$	100	CH_2CH_3-	red rose, strawberry	97	0.5
$PhCH_2-$	250	CH_2CH_3-		97	0.5
$PhCH_2-$	500	CH_2CH_3-		96	0.5
$PhCH_2-$	100	$CH_2CH_2CH_3-$	rose, honey	97	4
$PhCH_2-$	250	$CH_2CH_2CH_3-$		97	8
$PhCH_2-$	500	$CH_2CH_2CH_3-$		90	24
$PhCH{=}CHCH_2-$	100	H−	fruity, apple	>98	0.5
$PhCH{=}CHCH_2-$	250	H−		>98	4
$PhCH{=}CHCH_2-$	500	H−		82	24
$PhCH{=}CHCH_2-$	100	CH_3-	floral, pineapple	>98	1
$PhCH{=}CHCH_2-$	100	CH_3-		>98	4
$PhCH{=}CHCH_2-$	250	CH_3-		96	24

DOI: 10.1021/acs.jafc.9b01790
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 4. continued

R_1	initial alcohol (mM)	R_2	ester main flavor property[1]	conversion (%)[a]	time (h)[b]
PhCH=CHCH$_2$–	100	CH_3CH_2–	fruity, woody	>98	1
PhCH=CHCH$_2$–	250	CH_3CH_2–		>98	5
PhCH=CHCH$_2$–	500	CH_3CH_2–		97	24
PhCH=CHCH$_2$–	100	$CH_3CH_2CH_2$–	fruity, honey	>98	16
PhCH=CHCH$_2$–	250	$CH_3CH_2CH_2$–		92	24
PhCH=CHCH$_2$–	500	$CH_3CH_2CH_2$–		80	48

[a]As determined by gas chromatography. [b]Time refers to the maximum conversion observed.

alcohol (Table 2). Cinnamyl acetate has a balsamic−floral odor and a pineapple flavor.[1]

Enzymatic acetylation of cinnamyl alcohol occurred with generally better rates and yields than those observed with geraniol, allowing for almost complete conversion of the alcohol up to 250 mM initial concentration after 6 h. In the case of the reaction carried out with the highest initial concentration of alcohol (500 mM), 79% molar conversion was obtained after 48 h.

Acetylation of geraniol and cinnamyl alcohol was performed on semipreparative scale (10 mL) starting from 250 mM alcohol concentration; the reactions were stopped and worked up when conversions reached the maximum yield, allowing for the recovery of 442 mg of geranyl acetate (90% recovered yield) and 410 mg of cinnamyl acetate (93% yield), respectively.

Acylation of geraniol and cinnamyl alcohol was then studied with other acyl donors that varied in acyl-chain length; the results of the biotransformations carried out under optimized conditions (alcohol, 250 mM; acyl donor, 10%, v/v; enzyme, 1.0 mg/mL; buffer, pH 8.0; 25 °C) are summarized in Table 3.

The ability to act as an acyl donor decreased as the acyl-chain length increased; geranyl formate was obtained in quantitative yield after 1−3 h, whereas with geranyl propionate and butyrate, the maximum yields (ranging between 65 and 98%) were reached only after 1−2 days.

Enzymatic acylation was then studied with different primary alcohols and different acyl donors; all the reactions studied were aimed at preparing esters used as flavor or fragrance components (Table 4). Biotransformations were carried out starting from 100, 250, and 500 mM alcohol concentrations.

Formylation and acetylation of all the tested primary alcohols allowed for quantitative or almost quantitative conversions with high rates (≥90% within 30−60 min) when the initial concentration of alcohols was kept between 100 and 250 mM. When the biotransformation was carried out with higher alcohol concentration (500 mM), still high molar conversions of the flavor-esters were observed (from 80 up to >98%), albeit with longer reaction times. Slower reactions were generally found with acyl donors of increased chain length (propionyl- and butyryl-). The only biotransformation occurring with relatively low yields (30−70%, depending on the initial concentration) was the propionylation of *n*-hexanol to give *n*-hexyl propionate; interestingly, in this case, hydrolysis of the formed ester was predominant after reaching its maximum accumulation, as noticeable from an increase in the alcohol concentration (data not shown).

In conclusion, a highly efficient preparation of different flavor-esters was achieved using the acyltransferase from *M. smegmatis* (MsAcT), which catalyzes the acylation of different primary alcohols in aqueous systems. The results collected so far indicate that the biotransformation can not only be applied to the production of acetate esters but can also be expanded to a range of other acyl donors (e.g., ethyl formate, propionate, butyrate) useful for the formation of different flavor and fragrance esters, making this process strongly versatile. Notably, all the primary alcohols tested were accepted as substrates, even at higher concentrations (up to 500 mM); preparation of geranyl acetate and cinnamyl acetate was carried out on a semipreparative scale (10 mL) starting from 250 mM alcohol concentration, allowing for the recovery of 44.2 mg/mL (geranyl acetate) and 41.0 mg/mL (cinnamyl acetate) products. Biocatalysis is sometimes perceived to be inefficient compared with conventional chemical processes because of the low productivity, mostly because of substrate or product inhibition of the enzymatic activity, which often occurs at somewhat low concentrations. To become an industrially attractive technology, a biocatalytic process must be engineered to improve space−time yields.[28] In this work, we have described that the use of relatively low amounts of MsAcT allowed the conversion of remarkably high substrate concentrations with good yields. The proposed enzymatic method may pave the way for an efficient and environmentally sustainable preparation of natural esters; the biotransformation may be further intensified by immobilizing the enzyme and carrying out continuous processes in suited reactors,[29] thus making it appealing at an industrial level.

AUTHOR INFORMATION

Corresponding Author

*E-mail: francesco.molinari@unimi.it.

ORCID

Andrea Pinto: 0000-0002-2501-3348

Francesca Paradisi: 0000-0003-1704-0642

Francesco Molinari: 0000-0003-1958-423X

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Burdock, G. A. *Fenaroli's handbook of flavor ingredients*, 6th ed.; CRC Press: Boca Raton, 2010.

(2) Berger, R. G. Biotechnology of flavours - the next generation. *Biotechnol. Lett.* **2009**, *31*, 1651−1659.

(3) Sabisch, M.; Smith, D. The complex regulatory landscape for natural flavor ingredients. *Sigma-Aldrich*. https://www.sigmaaldrich.com/technical-documents/articles/white-papers/flavors-and-fragrances/natural-flavor-ingredients-regulations.html (accessed 2018).

(4) Hofmann, T.; Krautwurst, D.; Schieberle, P. Current status and future perspectives in flavor research: highlights of the 11th Wartburg symposium on flavor chemistry & biology. *J. Agric. Food Chem.* **2018**, *66*, 2197−2203.

(5) Fraatz, M. A.; Goldmann, M.; Geissler, T.; Gross, E.; Backes, M.; Hilmer, J. M.; Ley, J.; Rost, J.; Francke, A.; Zorn, H. Biotechnological

DOI: 10.1021/acs.jafc.9b01790
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

production of methyl-branched aldehydes. *J. Agric. Food Chem.* **2018**, *66*, 2387−2392.
(6) Dhake, K. P.; Thakare, D. D.; Bhanage, B. M. Lipase: A potential biocatalyst for the synthesis of valuable flavour and fragrance ester compounds. *Flavour Fragrance J.* **2013**, *28*, 71−83.
(7) Nikolaivits, E.; Makris, G.; Topakas, E. Immobilization of a cutinase from *Fusarium oxysporum* and application in pineapple flavor synthesis. *J. Agric. Food Chem.* **2017**, *65*, 3505−3511.
(8) Sà, A. G. A.; de Meneses, A. C.; de Arauújo, P. H. H.; de Oliveira, D. A review on enzymatic synthesis of aromatic esters used as flavor ingredients for food, cosmetics and pharmaceuticals industries. *Trends Food Sci. Technol.* **2017**, *69*, 95−105.
(9) Halling, P. J. Solvent selection for biocatalysis in mainly organic systems: Predictions of effects on equilibrium position. *Biotechnol. Bioeng.* **1990**, *35*, 691−701.
(10) Klibanov, A. M. Improving enzymes by using them in organic solvents. *Nature* **2001**, *409*, 241−246.
(11) Spizzo, P.; Basso, A.; Ebert, C.; Gardossi, L.; Ferrario, V.; Romano, D.; Molinari, F. Resolution of (R,S)-flurbiprofen catalysed by dry mycelia in organic solvent. *Tetrahedron* **2007**, *63*, 11005−11010.
(12) Gubicza, L.; Kabiri-Badr, A.; Keoves, E.; Belafi-Bako, K. Large-scale enzymatic production of natural flavour esters in organic solvent with continuous water removal. *J. Biotechnol.* **2000**, *84*, 193−196.
(13) Tamborini, L.; Romano, D.; Pinto, A.; Contente, M.; Iannuzzi, M. C.; Conti, P.; Molinari, F. Biotransformation with whole microbial systems in a continuous flow reactor: resolution of (RS)-flurbiprofen using *Aspergillus oryzae* by direct esterification with ethanol in organic solvent. *Tetrahedron Lett.* **2013**, *54*, 6090−6093.
(14) Sun, J.; Yu, B.; Curran, P.; Liu, S. Q. Optimisation of flavour ester biosynthesis in an aqueous system of coconut cream and fusel oil catalysed by lipase. *Food Chem.* **2012**, *135*, 2714−2720.
(15) Rodriguez-Nogales, J. M.; Roura, E.; Contreras, E. Biosynthesis of ethyl butyrate using immobilized lipase: a statistical approach. *Process Biochem.* **2005**, *40*, 63−68.
(16) Gandolfi, R.; Converti, A.; Pirozzi, D.; Molinari, F. Efficient and selective microbial esterification with dry mycelium of *Rhizopus oryzae*. *J. Biotechnol.* **2001**, *92*, 21−26.
(17) Converti, A.; Del Borghi, A.; Gandolfi, R.; Lodi, A.; Molinari, F.; Palazzi, E. Reactivity and stability of mycelium-bound carboxylesterase from *Aspergillus oryzae*. *Biotechnol. Bioeng.* **2002**, *77*, 232−237.
(18) Brault, G.; Shareck, F.; Hurtubise, Y.; Lepine, F.; Doucet, N. Short-chain flavor ester synthesis in organic media by an *E. coli* whole-cell biocatalyst expressing a newly characterized heterologous lipase. *PLoS One* **2014**, *9*, No. e91872.
(19) Ozyilmaz, G.; Gezer, E. Production of aroma esters by immobilized Candida rugosa and porcine pancreatic lipase into calcium alginate gel. *J. Mol. Catal. B: Enzym.* **2010**, *64*, 140−145.
(20) Mathews, I.; Soltis, M.; Saldajeno, M.; Ganshaw, G.; Sala, R.; Weyler, W.; Cervin, M. A.; Whited, G.; Bott, R. Structure of a novel enzyme that catalyzes acyl transfer to alcohols in aqueous conditions. *Biochemistry* **2007**, *46*, 8969−8979.
(21) Kazemi, M.; Sheng, X.; Kroutil, W.; Himo, F. Computational study of *Mycobacterium smegmatis* acyl transferase reaction mechanism and specificity. *ACS Catal.* **2018**, *8*, 10698−10706.
(22) Wiermans, L.; Hofzumahaus, S.; Schotten, C.; Weigand, L.; Schallmey, M.; Schallmey, A.; Domínguez de María, P. Trans-esterifications and peracid-assisted oxidations in aqueous media catalyzed by *Mycobacterium smegmatis* acyl transferase. *ChemCatChem* **2013**, *5*, 3719−3724.
(23) de Leeuw, N.; Torrelo, G.; Bisterfeld, C.; Resch, V.; Mestrom, L.; Straulino, E.; van der Weel, L.; Hanefeld, U. Ester synthesis in water: *Mycobacterium smegmatis* acyl transferase for kinetic resolutions. *Adv. Synth. Catal.* **2018**, *360*, 242−249.
(24) Mestrom, L.; Claessen, J. G. R.; Hanefeld, U. Enzyme-catalyzed synthesis of esters in water. *ChemCatChem* **2019**, *11*, 2004−2010.
(25) Land, H.; Hendil-Forssell, P.; Martinelle, M.; Berglund, P. One-pot biocatalytic amine transaminase/acyl transferase cascade for aqueous formation of amides from aldehydes or ketones. *Catal. Sci. Technol.* **2016**, *6*, 2897−2900.
(26) Contente, M. L.; Pinto, A.; Molinari, F.; Paradisi, F. Biocatalytic *N*-acylation of amines in water using an acyltransferase from *Mycobacterium smegmatis*. *Adv. Synth. Catal.* **2018**, *360*, 4814−4819.
(27) Romano, D.; Gandolfi, R.; Guglielmetti, S.; Molinari, F. Enzymatic hydrolysis of capsaicins for the production of vanillylamine using ECB deacylase from Actinoplanes utahensis. *Food Chem.* **2011**, *124*, 1096−1098.
(28) Sheldon, R. A.; Pereira, P. C. Biocatalysis engineering: the big picture. *Chem. Soc. Rev.* **2017**, *46*, 2678−2691.
(29) Tamborini, L.; Fernandes, P.; Paradisi, F.; Molinari, F. Flow bioreactors as complementary tools for biocatalytic process ntensification. *Trends Biotechnol.* **2018**, *36*, 73−88.

DOI: 10.1021/acs.jafc.9b01790
J. Agric. Food Chem. XXXX, XXX, XXX−XXX