

Aromas flow: Eco-friendly, continuous, and scalable preparation of flavour esters

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Abstract

Flow-based biocatalysis offers advantages to perform multiphase reactions, including liquid–liquid reactions, due to intensified mass transfer, compartmentalization and high local concentration of the catalyst. Enzymatic immobilization leads to stable biocatalysts, with the possibility to incorporate them in continuous reactors. The combination between the two technologies allows for intensified process with high substrate concentration and high product recovery. The present paper is an excellent example of automated continuous biocatalytic process where a transferase from *Mycobacterium smegmatis* (MsAcT) was immobilized onto agarose beads and exploited for the preparation of a variety of flavour-esters, utilizing exclusively natural substrates, with excellent yields in 5-minute reaction times. The corresponding products can be labelled and commercialized of the corresponding products as natural too, thus increasing their market value.

Keywords: Flow biocatalysis, Multiphase reaction, MsAcT, Flavour-esters, Intensified processes

Article Highlights

- 1 Flavour-ester formation was performed in an aqueous medium using an immobilized transferase from *Mycobacterium smegmatis* (MsAcT).
- 2 Flow-based strategy leads to intensified processes with high substrate loading, yields and unprecedented reaction times.
- 3 Natural substrates processed *via* enzymatic reaction allow for the commercialization of the corresponding products as natural too.

1. Introduction

Biocatalysis is among the techniques used for sustainable processing in chemistry [1]. Biotransformations are used to prepare food and pharmaceutical ingredients due to the intrinsic selectivity of enzymatic catalysis; moreover, when starting from natural substrates, biocatalytic approaches guarantee the commercialization of the final products as natural, thus increasing their market value [2]. This last feature is particularly attractive in the manufacture of flavours and fragrances, where biotechnological production is highly preferred [3]. Flavour-active esters are commercially relevant agents in the aroma industry and can be obtained by lipase-catalyzed esterification or transesterification in organic solvents or in solvent-free environments [4]. Lipase-catalyzed reactions involve a mechanism with transitional formation of an acyl-enzyme intermediate, which further evolves through a nucleophile attack of the alcohol (leading to transesterification) or water (leading to hydrolysis) [5, 6]. The presence of water and its accessibility to the active site are therefore crucial for favouring ester formation [7, 8]; hydrophobic microenvironments, which disfavour the access of water to the active site, facilitate ester formation. Hydrophobic supports for lipase immobilization, such as the hydrophobic macroporous acrylic resin used for immobilizing the lipase B from *Candida antarctica* (the commercial preparation known as Novozym 435), result in favoured esterification [9, 10]. Alternatively, mycelium bound lipases, where the enzymes are linked to cellular hydrophobic membranes, can be efficiently employed for catalyzing direct esterification of different alcohols and carboxylic acids with high yields in a hydrophobic environment [11-13].

A less common situation is encountered in the case of the acyltransferase from *Mycobacterium smegmatis* (MsAcT), where the active site is located within a deep hydrophobic tunnel [14,15]; this enzyme was shown to be particularly suited for catalyzing ester [16-19] and amide formation in aqueous phases [20-22]. We have previously shown that MsAcT was able to catalyze the transesterification of different primary alcohols, allowing for the preparation of several flavour esters with good-to-high yields starting from alcohol concentrations up to 0.5 M [18]. Two major limitations were observed in batch biotransformations with the free enzyme: the large excess of acylating agent needed to favor the transesterification resulting in the formation of a biphasic system, and the production of ethanol as by-product, which partially inhibited the enzymatic activity [18].

The productivity of biotransformations can be additionally improved by running them in meso- or micro-flow reactors [23, 24]. Flow-based biocatalysis has rapidly developed as a system for predisposing sustainable and scalable processes; flow reactors seem particularly fit to promote reactions where the thermodynamic equilibrium plays a crucial role by quickly and continuously removing the products from the reaction mixture [25-30].

In this work, we have studied the continuous preparation of flavour esters in a flow reactor to improve the productivity, prolong biocatalyst longevity, and, finally, realize a simplified in-line work

up. Unlike previous studies, where non-natural activated acylating agents have also been used (*e.g.*, vinylacetate) to enhance the product conversion, here the overall process was optimized for the synthesis of aroma-compounds starting exclusively from natural, less reactive, substrates.

2. Results and discussion

A major problem experienced with the use of the free acyltransferase from *Mycobacterium smegmatis* (MsAcT) in catalyzing the transesterification between natural ethyl esters and alcohols was the negative effect of the produced EtOH on the enzyme stability [18], which strongly limited the re-use of the free catalyst. The acyltransferase from *Mycobacterium smegmatis* (MsAcT) was therefore immobilized onto agarose as reported before [22]. Taking into consideration that MsAcT is a large molecule, with an octameric structure, it is not surprising that the best results (>99% immobilization yield, 73% retained activity) were obtained with a low enzyme loading (1 mg/g_{agarose}), whereas higher MsAcT concentrations led to poor active biocatalysts. The stability of the free and immobilized enzyme was compared in the presence of different concentrations of EtOH (Figure 1).

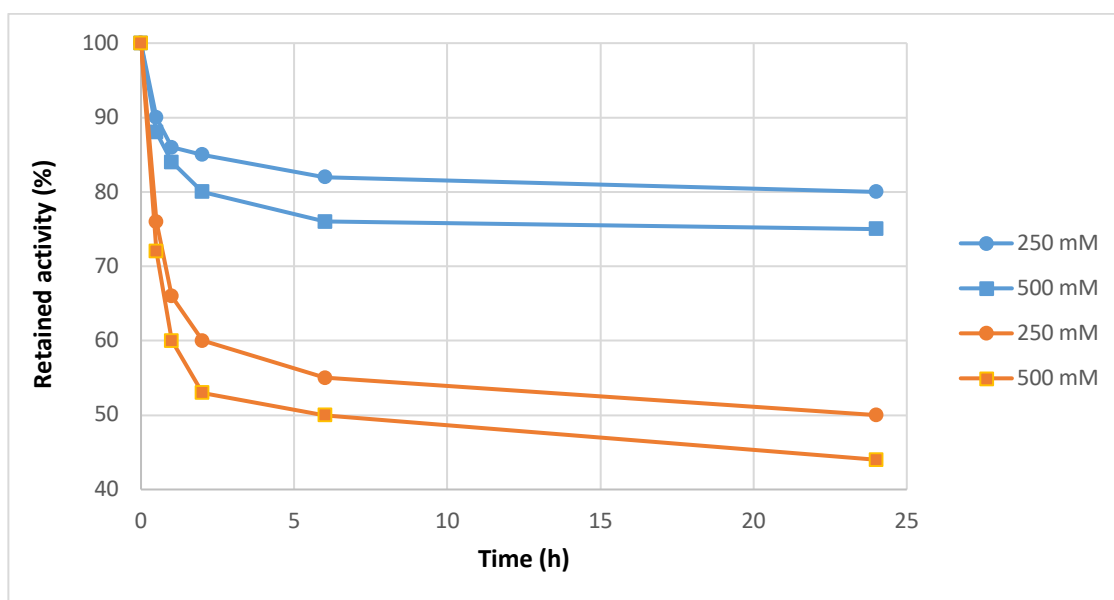


Fig. 1. Evaluation of the retained activity following batch incubation of the immobilized (blue line) and free (orange line) enzyme with different concentrations of EtOH. The activity assays were performed as previously reported [21,22].

Immobilization conferred higher stability to the enzyme, with a retained activity >75% after 24 h of incubation with EtOH, whereas the free enzyme lost more than 40% of the original activity after 2 h, independently of the EtOH concentration.

Immobilized MsAcT (enzyme loading 1 mg/g_{support}) was therefore assessed for the acetylation of primary alcohols, previously tested with the free enzyme. We chose 2-phenylethanol, cinnamyl

alcohol and *n*-hexanol were chosen as substrates, since their acetylation yields flavour esters of commercial importance (Table 1).

Alcohol	Molar conversion (%)	Time (h)
2-phenyl ethanol	75	1
cinnamyl alcohol	76	2
<i>n</i> -hexanol	95	0.5

Table 1. Acetylation of primary alcohols (0.25 M) with EtOAc (10% v/v) catalyzed by immobilized MsAcT (200 mg/mL, 1 mg/g_{agarose} enzyme loading) in 0.1 M phosphate buffer pH 8.0. Conversions were determined by GC.

The promising results obtained with immobilized MsAcT in batch mode (more than 75% of conversion in reasonable reaction times) led us to explore the possibility of carrying out continuous reactions to maximize the productivity of the biotransformation. Flow-based reactors may offer advantages when performing multiphase reactions, including gas-liquid or liquid-liquid reactions, due to facilitated mass transfer [31]; an increase of the interfacial area between heterogenous phases can be obtained by applying segmented liquid-liquid flow regime [32, 33] in flow chemistry, which is known to increase the interfacial area for exchange of chemical species.

A packed bed reactor (i.d. = 6.6. mm, reactor volume: 1.4 mL) with immobilized MsAcT (1.9 g with an enzyme loading of 1 mg/g_{agarose}) was employed in a flow-chemistry reactor (Figure 2). The inlet system was composed with an aqueous solution of 2-phenylethanol and an organic phase (pure EtOAc); the two phases were mixed in a T-piece to form a liquid heterogeneous segmented flow stream (buffer solution/EtOAc 9:1) before entering the column. A connected software was used to realize an automated process for the collection of the product at the steady state.

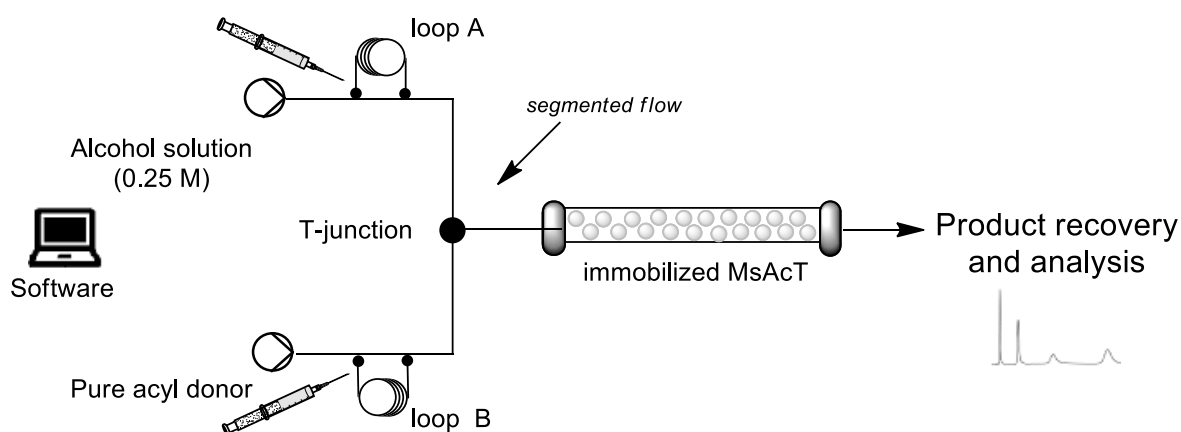


Fig. 2. Flow reactor set-up. A solution of alcohol (0.25 M in phosphate buffer 0.1 M pH 8.0 and 5% DMSO) and pure acyl donor (EtOAc) were pumped into the reactor column using injection loops. T = 28 °C; P= atm. Product was recovered at the steady state, and analyzed by GC.

An optimization regarding the residence time was carried out using 2-phenylethanol as substrate (Table 2).

Residence time (min)	Flow rate (mL min ⁻¹)	<i>r</i> (mmol min ⁻¹)	Conversion (%)
5	0.28	0.06	82
10	0.14	0.03	80
20	0.07	0.014	80

Table 2. Effect of residence time on the flow acetylation of 2-phenylethanol (0.25 M inlet solution) using pure EtOAc as acyl donor. The conversions were determined by GC.

The highest conversion was achieved after 5 minutes of residence time; a further increase of the residence time did not show any increase in the conversion.

To prove the versatility of the system, under optimized conditions (residence time: 5 min; T = 28 °C, P = atm), other flavour-esters were prepared starting from several primary alcohols and different acyl donors (Table 3); all the substrates used in this work are natural products.

Alcohol	Acyl donor	Conv. (%)	Ester main flavour property
2-Phenylethanol	HCOOEt	78	Floral-apricot
	CH ₃ CH ₂ COOEt	87	Floral-peach
	CH ₃ CH ₂ CH ₂ COOEt	76	Plum-pear
	(CH ₃) ₂ CHCOOEt	50	Floral-rose
	(CH ₃) ₂ CHCH ₂ COOEt	27	Fruity-berry
Cinnamyl alcohol	HCOOEt	93	Balsam-green
	CH ₃ COOEt	80	Floral-sweet
	CH ₃ CH ₂ COOEt	90	Fruity-spicy
	CH ₃ CH ₂ CH ₂ COOEt	72	Fruity-floral
	(CH ₃) ₂ CHCOOEt	65	Apple-banana
	(CH ₃) ₂ CHCH ₂ COOEt	30	Rose-apple
Geraniol	HCOOEt	88	Rose
	CH ₃ COOEt	70	Rose-lavender
	CH ₃ CH ₂ COOEt	65	Fruity
	CH ₃ CH ₂ CH ₂ COOEt	55	Fruity-apricot
	(CH ₃) ₂ CHCOOEt	53	Rose-apricot
	(CH ₃) ₂ CHCH ₂ COOEt	20	Rose-apple

<i>n</i> -Hexanol	HCOOEt	85	apple
	CH ₃ COOEt	83	Fruity-pear
	CH ₃ CH ₂ COOEt	80	Earthy-metallic
	CH ₃ CH ₂ CH ₂ COOEt	78	Apricot-pineapple
	(CH ₃) ₂ CHCOOEt	35	Green-sweet
	(CH ₃) ₂ CHCH ₂ COOEt	26	Green-fruity
Isoamyl alcohol	HCOOEt	84	Black currant
	CH ₃ COOEt	85	Banana-pear
	CH ₃ CH ₂ COOEt	80	Pineapple-apricot
	CH ₃ CH ₂ CH ₂ COOEt	70	Fruity
	(CH ₃) ₂ CHCOOEt	30	Sweet-fruity
	(CH ₃) ₂ CHCH ₂ COOEt	24	Sweet-fruity

Table 3. Flow-based acylation of primary alcohols for the preparation of flavour esters. Reaction conditions: alcohol concentration: 0.25 M in phosphate buffer 0.1 M pH 8.0 and 5% DMSO; residence time: 5 min; T = 28 °C, P = atm. The conversions were determined by GC. In the case of *n*-hexanol, the alcohol was solubilized in the organic phase.

Most of the esters were obtained with good molar conversions (65-93%); only in the case of branched acyl donors (ethyl isobutyrate and ethyl isovalerate) the conversions decreased to 20-53%. Noteworthy, the new formed flavour-esters were prepared in 5 min residence time, whereas biotransformations in batch mode needed prolonged time to take place. Remarkably, MsAcT is completely stable under these working conditions (liquid-liquid phase buffer/acyl donor) and the same packed bed reactor was used to perform all the experiments without any loss of the activity. Subsequently, the reactor was left to operate for one week for the production of phenethyl acetate, without any apparent decrease of the enzymatic activity. Immobilization coupled with continuous removal of the products (including the by-product ethanol) gave high stability to the biocatalyst under operating conditions (Table 4). It should be noted that hydrolysis of the product (phenethyl acetate), which occurs in batch reactions at prolonged time, was never observed. Moreover, the use of a flow environment associated with a two-liquid phase system helps also to avoid emulsions often present with the conventional stirring.

Residence time (min)	Conv. (%)	Isolated yield (%)	Space time yield (g/day)	Catalyst productivity (g _{product} /mg _{enzyme})	TTN (s ⁻¹)
5	87	82	13.6	7.2	12.6 MLN

Table 4. Continuous flow acetylation of 2-phenylethanol (0.25 M inlet solution) with immobilized MsAcT.

Continuous flow acetylation of 2-phenylethanol with ethyl acetate allowed for an impressive space time yield, whereas the longevity of the immobilized biocatalyst furnished a biocatalyst productivity (defined as amount of product formed per amount of biocatalyst) of 7.2 $\text{g}_{\text{product}}/\text{mg}_{\text{enzyme}}$.

Finally, an inlet of EtOAc and an in-line liquid/liquid extractor were introduced downstream of the process (Figure 3) for the separation of the organic/aqueous phase. The desired phenethyl acetate was obtained by flash chromatography (82% isolated yield).

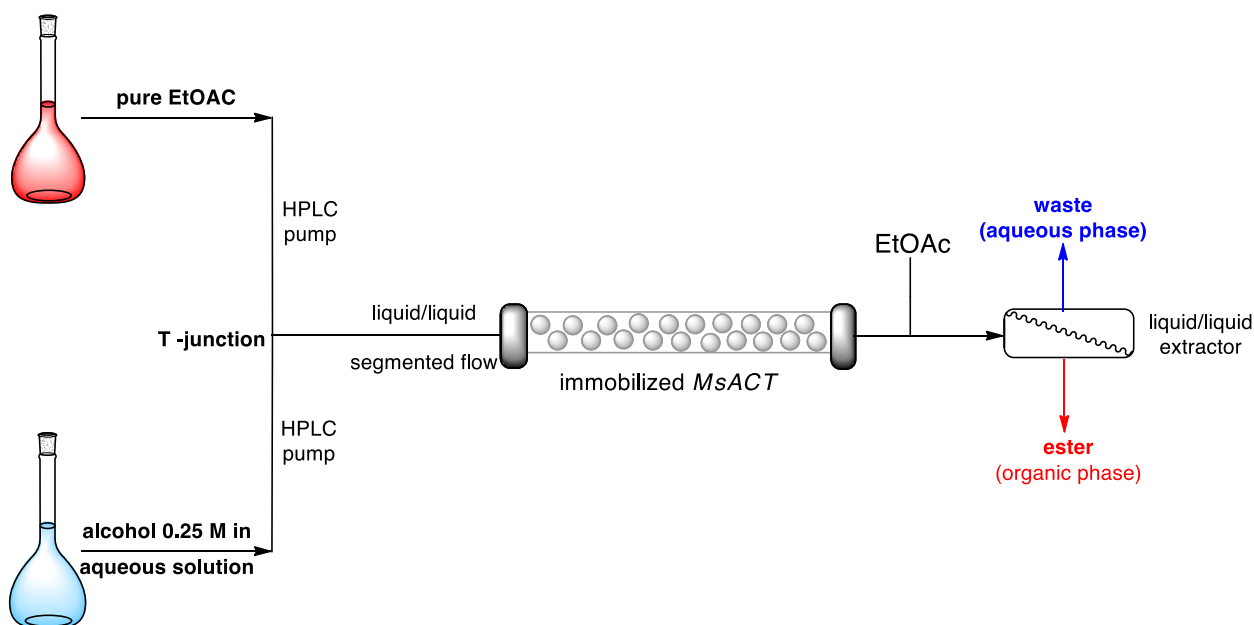


Fig. 3. Intensified process for obtaining phenethyl acetate. Solution A: pure EtOAc. Solution B: 0.25 M 2-phenylethanol, 5% DMSO in phosphate buffer 0.1 M pH 8.0. T = 28 °C, P = atm.

Conclusions

In the present paper, we described a versatile and fully automated platform for the production of a plethora of natural flavour-esters commonly used in food, pharmaceutical and cosmetic industry. MsAcT was further stabilized due to the immobilization onto agarose beads, while the use of flow system dramatically increased the overall production avoiding hydrolysis side-reactions and enzyme destabilization for the formation of ethanol by-product. The same packed bed reactor was used to perform all the experiments without any loss of activity. After one week of continuous production of phenethyl acetate no decreasing of the enzymatic activity was observed. Flow mode associated with a two-liquid phase system helps also to avoid emulsions often present with conventional stirring in batch reactions. The combination of biocatalysis and flow chemistry not only leads to unprecedented reaction times (5 min), but also established a sustainable strategy for the preparation of aromas and fragrances. Noteworthy, starting from natural substrates, biocatalytic approaches guarantee the commercialization of the final products as natural too.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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