Efficient Enzymatic Preparation of Flavor Esters in Water

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ABSTRACT: A straightforward biocatalytic method for the enzymatic preparation of different flavor esters starting from primary alcohols (e.g., isomyl, 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. 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. Therefore, new biotechnological approaches for obtaining flavors are highly demanded as long as they are efficient and sustainable. 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. The presence of water is critical for the equilibrium of lipase-catalyzed reactions in organic media, strongly limiting the overall yields. In fact, water can compete as a nucleophile with the alcohol in the attack of the acyl–enzyme intermediate, thus favoring hydrolysis over transesterification. Flavour 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. The potential of lipases for flavor-ester preparation; the enzyme was able to work in two-phase systems composed of the hydrophobic ester and water, accomplishing the reaction in the aqueous phase. The peculiar features of this enzyme make it also suitable for catalyzing transesterification reactions of primary and secondary alcohols with ethyl acetate in aqueous buffers, often with good enantioselectivity. The ratio between the catalytic rate constants of the hydrolysis and synthesis of benzyl acetate was compared, showing that MsAcT is indeed an effective acetyltransferase rather than a hydrolytic enzyme.

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 amidic synthesis in aqueous media.

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 different flavor esters derived from...
### 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**).
*E. 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. Recombinant *E. coli* was first grown at 37 °C in Luria–Bertani (LB) liquid medium in the presence of 25 μg/mL kanamycin, which was brought to an initial OD_{600} 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_{600} 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 g 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 114 °C). Cell debris were recovered by centrifugation (45 min, 36000 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 reaction times when high concentrations of substrates (up to 200 μM) were prepared in excellent yields with short reaction times.

#### Optimization of geranyl and cinnamyl acetate synthesis.

- **Geraniol acetate.** Acetylation of geraniol (250 mM) 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 0.5 M sulfuric acid and 0.5 M NaOH, 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.

- **Cinnamyl alcohol acetate.** Aliquots of the bio-transformation 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 cinnamyl esters and the residual quantity of alcohol substrates were determined by gas-chromatographic (GC) analysis. Geraniol, 160 cinnamyl alcohol, n-hexanol, benzyl alcohol, 2-phenylethanol, and their esters were analyzed by gas-GC analysis on a Carlo Erba 162 Fractovap GC equipped with a fused-silica capillary column MEGA-SE50 (100% methyl polysiloxane; 25 μM × 0.25 mm i.d.), with the detector and injector temperature at 200 °C. Oven temperatures ranged from 45 to 165 °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 at 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 1 °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, 15.8 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-phenethyl formate, 4.3 min; 2-phenylethyl acetate, 5.4 min; 2-phenethyl propionate, 6.6 min; 2-phenethyl propionate, 8.8 min.

#### 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

<table>
<thead>
<tr>
<th>geraniol (mM)</th>
<th>initial rate (μmol/mg/min)</th>
<th>maximum conversion (%)</th>
<th>maximum product yield (mg/mL)</th>
<th>time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>2.25</td>
<td>&gt;98</td>
<td>19.6</td>
<td>2</td>
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<td>200</td>
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<td>250</td>
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<td>98</td>
<td>48.1</td>
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<td>300</td>
<td>6.57</td>
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<td>55.9</td>
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</tr>
<tr>
<td>400</td>
<td>7.68</td>
<td>79</td>
<td>62.0</td>
<td>48</td>
</tr>
<tr>
<td>500</td>
<td>7.65</td>
<td>68</td>
<td>66.7</td>
<td>48</td>
</tr>
</tbody>
</table>

*As determined by gas chromatography. *Time refers to the maximum conversion observed.
Table 2. Acylation 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

<table>
<thead>
<tr>
<th>cinnamyl alcohol (mM)</th>
<th>initial rate (μmol/mg/min)</th>
<th>maximum conversion (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>maximum product yield (mg/mL)</th>
<th>time (h)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td>100</td>
<td>2.54</td>
<td>&gt;98</td>
<td>17.6</td>
<td>1</td>
</tr>
<tr>
<td>200</td>
<td>5.07</td>
<td>&gt;98</td>
<td>35.2</td>
<td>3</td>
</tr>
<tr>
<td>250</td>
<td>6.32</td>
<td>&gt;98</td>
<td>44.0</td>
<td>6</td>
</tr>
<tr>
<td>300</td>
<td>6.82</td>
<td>92</td>
<td>48.6</td>
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</tr>
<tr>
<td>400</td>
<td>8.53</td>
<td>84</td>
<td>59.2</td>
<td>24</td>
</tr>
<tr>
<td>500</td>
<td>8.65</td>
<td>79</td>
<td>69.6</td>
<td>24</td>
</tr>
</tbody>
</table>

<sup>a</sup>As determined by gas chromatography. <sup>b</sup>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

<table>
<thead>
<tr>
<th>alcohol</th>
<th>ester</th>
<th>ester main flavor property&lt;sup&gt;c&lt;/sup&gt;</th>
<th>initial rate (μmol/mg min)</th>
<th>conversion (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>time (h)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>geraniol</td>
<td>ethyl formate</td>
<td>rose</td>
<td>7.69</td>
<td>&gt;98</td>
<td>3</td>
</tr>
<tr>
<td>geraniol</td>
<td>ethyl propionate</td>
<td>fruity</td>
<td>1.51</td>
<td>86</td>
<td>48</td>
</tr>
<tr>
<td>geraniol</td>
<td>ethyl butyrate</td>
<td>fruity, apricot</td>
<td>1.15</td>
<td>62</td>
<td>48</td>
</tr>
<tr>
<td>geraniol</td>
<td>ethyl isobutyrate</td>
<td>rose, apricot</td>
<td>1.42</td>
<td>74</td>
<td>48</td>
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<tr>
<td>geraniol</td>
<td>ethyl isovalerate</td>
<td>rose, sweet apple</td>
<td>0.88</td>
<td>38</td>
<td>48</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>ethyl formate</td>
<td>fruity, apple</td>
<td>7.72</td>
<td>&gt;98</td>
<td>1</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>ethyl propionate</td>
<td>fruity, woody</td>
<td>2.13</td>
<td>&gt;98</td>
<td>3</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>ethyl butyrate</td>
<td>fruity, honey</td>
<td>1.87</td>
<td>90</td>
<td>24</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>ethyl isobutyrate</td>
<td>fruity, apple–banana</td>
<td>1.92</td>
<td>87</td>
<td>24</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>ethyl isovalerate</td>
<td>rose, apple</td>
<td>0.91</td>
<td>44</td>
<td>48</td>
</tr>
</tbody>
</table>

<sup>c</sup>As determined by gas chromatography. <sup>b</sup>Time refers to the maximum conversion observed.

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. 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<sup>2</sup> 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 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 alcohol.
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

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>initial alcohol (mM)</th>
<th>$R_2$</th>
<th>ester main flavor property $^1$</th>
<th>conversion (%)$^2$</th>
<th>time (h)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>100</td>
<td>H</td>
<td>black currant, currant</td>
<td>93</td>
<td>0.5</td>
</tr>
<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>250</td>
<td>H</td>
<td></td>
<td>93</td>
<td>0.5</td>
</tr>
<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>500</td>
<td>H</td>
<td></td>
<td>86</td>
<td>1</td>
</tr>
<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>100</td>
<td>CH$_3$</td>
<td>banana, pear</td>
<td>94</td>
<td>0.5</td>
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<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>250</td>
<td>CH$_3$</td>
<td></td>
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<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
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</tr>
<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>100</td>
<td>CH$_3$CH$_3$</td>
<td>pineapple–apricot, apricot</td>
<td>97</td>
<td>0.5</td>
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<tr>
<td>(CH$_2$)$_3$CHCH$_2$</td>
<td>250</td>
<td>CH$_3$CH$_3$</td>
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<td>1</td>
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<td>100</td>
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<td>fruity</td>
<td>94</td>
<td>24</td>
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<td>CH$_3$--</td>
<td>rose, raspberry</td>
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<td>H--</td>
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alcohol (Table 2). Cinnamyl acetate has a balsamic–floral odor and a pineapple flavor.

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 flavors 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/200 mL (geranyl acetate) and 41.0 mg/mL (cinnamyl acetate) products. Bioanalysis 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. 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, making it appealing at an industrial level.

### Table 4. continued

<table>
<thead>
<tr>
<th>R₁</th>
<th>initial alcohol (mM)</th>
<th>R₂</th>
<th>ester main flavor property ¹</th>
<th>conversion (%) ²</th>
<th>time (h) ³</th>
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<td>PhCH₂=CHCH₂=CH₂</td>
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<td>80</td>
<td>48</td>
</tr>
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</table>

¹As determined by gas chromatography. ²Time refers to the maximum conversion observed.

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**Notes**

The authors declare no competing financial interest.

**REFERENCES**


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