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Water Activity Effects on Geranyl Acetate Synthesis Catalyzed by Novozym in Supercritical Ethane and in Supercritical Carbon Dioxide

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The esterification reaction of geraniol with acetic acid catalyzed by Novozym was studied in supercritical ethane (sc-ethane) and in supercritical carbon dioxide (sc-CO_2). Water activity (a_W) had a very strong effect on enzyme activity, with reaction rates increasing up to $a_W = 0.25$ and then decreasing for higher a_W. Salt hydrate pairs could not prevent changes in a_W during the course of reaction but were able to control a_W to some extent and had a beneficial effect on both initial rates of esterification and conversion in sc-ethane. The enzyme was more active in sc-ethane than in sc-CO_2, confirming the deleterious effect of the latter already observed with some enzymes. Temperatures between 40 and 60 °C did not have a strong effect on initial rates of esterification, although reaction progress declined considerably in that temperature range. For the mixture of 50 mM acetic acid plus 200 mM geraniol, 100% conversion was achieved at a reaction time of 10 h at 40 °C, 100 bar, an a_W of incubation of 0.25, and a Novozym concentration of 0.55 mg cm^{-3} in sc-ethane. Conversion was below 50% in sc-CO_2 at otherwise identical conditions. With an equimolar mixture of the two substrates (100 mM), 98% conversion was reached at 10 h of reaction in sc-ethane (73% conversion in sc-CO_2).

KEYWORDS: Novozym 435; *Candida antartica* lipase B; enzymatic esterification; supercritical fluids; geranyl acetate; geraniol; water activity

INTRODUCTION

Terpene alcohols and their esters are the major constituents of many natural fragrances. Of these, geranyl acetate is perhaps the most important (*1*). Traditional methods for obtaining fragrances include extraction from plant materials and fermentation processes, which exhibit low yields, and chemical synthesis. The production of esters via chemical synthesis normally involves catalysis by strong acids, which may lead to structural rearrangements in the case of terpenes. This adds to the interest of the enzymatic route as an alternative to conventional esterification (*1*). Here, the use of a nonaqueous solvent may be clearly advantageous by allowing easy manipulation of water activity and the shifting of thermodynamic equilibrium toward synthesis. In addition to being able to catalyze reactions that are difficult to carry out in water, in nonaqueous solvents enzymes become more stable and can exhibit altered selectivity (*2*). Lipases are the enzymes most commonly used for the production of geranyl esters in organic media, in particular, lipase B from *Candida antarctica* via direct esterification (*3*−*7*) or transesterification (*8*−*10*). The topic continues to receive a lot of attention, as revealed by the different enzymes and approaches utilized and described in the recent literature (*11*−*18*). Earlier references have been reviewed by Yahya et al. (*19*).

Concerted efforts toward green chemistry require that organic solvent residues be eliminated from reaction products. In this context, the choice of solvent must be addressed. Options today include performing reactions in supercritical fluids (sc-fluids), which can be completely eliminated (vented) from the reaction medium. Adjustable solvation ability and improved mass transfer relative to conventional liquid solvents are additional potential advantages of sc-fluids, also for biotransformations (*1*, *20*). Chulalaksananukul et al. (*21*) have studied the transesterification of geraniol catalyzed by *Mucor miehei* lipase in sc-CO_2. Here we report on the synthesis of geranyl esters catalyzed by *C. antarctica* lipase in sc-ethane and in sc-CO_2.

MATERIALS AND METHODS

Materials. Novozym 435 (*C. antarctica* lipase B immobilized on a macroporous acrylic resin), with a reported activity of 7000 PLU g^{-1}, was a gift from Novo Nordisk Bioindustrial. To investigate the existence of internal diffusion limitations, the enzyme preparation was sieved into different particle sizes. Geraniol (98% purity) was purchased from Sigma; acetic acid, Na_2HPO_4, $Na_2HPO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 7H_2O$, and $LiSO_4 \cdot H_2O$ were from Merck; decane (99% purity) and geranyl acetate (98% purity) were from Aldrich; and NaAc, $NaAc \cdot 3H_2O$, Hydranal Coulomat A, and C Karl Fisher reagents were from Riedel de Haën.

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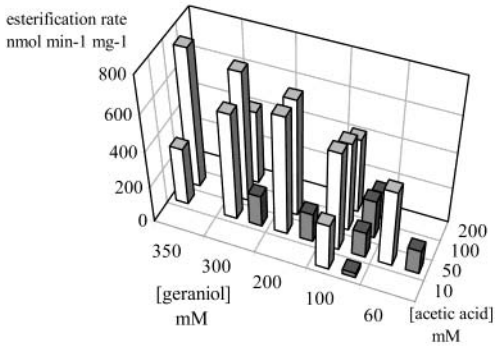

Figure 1. Dependence of the initial rate of esterification on substrate concentration: (white bars) sc-ethane; (shaded bars) sc-CO_2. $T = 40$ °C. $P = 100$ bar. Initial $a_W = 0.25$. [Novozym] = 0.15 mg cm^{-3} in sc-ethane, 0.60 mg cm^{-3} in sc-CO_2.

Geraniol and decane were dried over molecular sieves. Acetic acid was distilled instead. $LiSO_4$ was generated from $LiSO_4 \cdot H_2O$ by drying in an oven at 100 °C. Ethane, CO_2, and nitrogen were supplied by Air Liquide and guaranteed to have purities of >99.95 mol % (ethane) and >99.995 mol %.

Apparatus and Experimental Technique. Variable-volume stainless steel cells (reaction mixture volume of ~14 cm^3) equipped with a sapphire window and with loading and sampling valves were used. Details of the high-pressure apparatus and experimental technique have been given elsewhere (*22*). The reaction studied was the esterification of geraniol with acetic acid. Initial a_W was set by salt hydrate pairs in situ (1 g, usually 500 mg of each form): $LiSO_4 \cdot 1/0$, $Na_2HPO_4 \cdot 2/0$, $NaAc \cdot 3/0$, $Na_2HPO_4 \cdot 7/2$. a_W values were calculated by dividing the water concentration in the reaction mixture by the water concentration in the same mixture at saturation (*23*). Water concentration was measured by direct Karl Fischer titration. The enzyme and geraniol were allowed to equilibrate with the salt hydrate pair for 2 h to allow ample time for water transfer (*24*) before acetic acid was added to start the reaction. *n*-Decane (20 mM) was used as internal standard for GC analysis.

Analysis. GC analysis was performed with a Trace 2000 series Unicam gas chromatograph. GC conditions: 50 m × 0.32 mm i.d.; DB-Wax capillary column from J&W Scientific; oven temperature program, 50 °C for 5 min, 50−240 °C ramp at 6.5 °C min^{-1}, 240 °C for 3 min; injection temperature, 250 °C; flame ionization detection (FID) temperature, 250 °C; carrier gas (helium), 2.3 cm^3 min^{-1}; split ratio, 20:1. The data given are the average of at least two measurements.

RESULTS AND DISCUSSION

The availability of water in the reaction medium is a key parameter in nonaqueous enzymology because it affects both reaction rates and equilibrium conversions (*25*). The latter is particularly true in the case of esterification, in which water is a product. Water activity (a_W) is the most convenient parameter for correlating enzymatic activity in nonaqueous media (*25*). With the idea of controlling a_W during the course of esterification, pairs of salt hydrates were used in situ. **Figure 1** shows the effect of acetic acid and geraniol concentrations on the initial rates of esterification at fixed initial a_W, in sc-ethane and in sc-CO_2. Concentrations of acetic acid >100 mM caused Novozym inhibition in both sc-fluids, in agreement with the findings of Claon and Akoh (*3*, *4*) and Ikeda and Kurokawa (*6*). Both groups looked at the effect of geraniol, and although Claon and Akoh did not detect any inhibitory effect for concentrations up to 700 mM, Ikeda and Kurokawa remark that concentrations of this alcohol >500 mM already had a negative impact on reaction rates. As **Figure 1** shows, geraniol concentrations >200 mM were no longer beneficial in any of the sc-fluids for a 50 mM concentration of acetic acid, although for concentrations of acid above this value no inhibition by geraniol was detected.

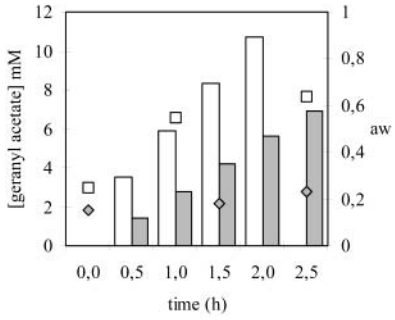

Figure 2. Change in a_W (symbols) during the period while progress for esterification is linear (bars) and initial reaction rates are measured: (white bars and symbols) sc-ethane; (shaded bars and symbols) sc-CO_2. The data given are for an initial a_W of 0.25 set by the salt hydrate pair $Na_2HPO_4 \cdot 2/0$. $T = 40$ °C. $P = 100$ bar. [Geraniol] = 200 mM. [Acetic acid] = 50 mM. [Novozym] = 0.15 mg cm^{-3} in sc-ethane, 0.60 mg cm^{-3} in sc-CO_2.

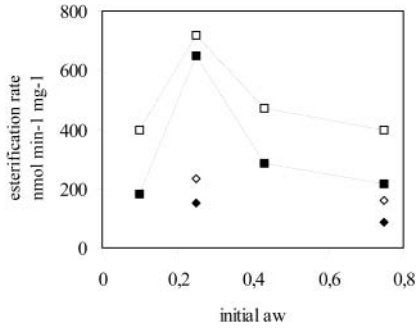

Figure 3. Dependence of the initial rate of esterification on initial a_W, solvent, and enzyme particle size: (□) sc-ethane, small particle sizes (100–300 μm); (■) sc-ethane, bulk enzyme preparation (particle sizes up to ≈900 μm); (◇) sc-CO_2, small particle sizes; (◆) sc-CO_2, bulk enzyme preparation. Initial a_W was set by pairs of salt hydrates in situ: $LiSO_4 \cdot 1/0$ ($a_W = 0.10$), $Na_2HPO_4 \cdot 2/0$, $NaAc \cdot 3/0$ ($a_W = 0.43$), $Na_2HPO_4 \cdot 7/2$ ($a_W = 0.75$). $T = 40$ °C. $P = 100$ bar. [Geraniol] = 200 mM. [Acetic acid] = 50 mM. [Novozym] = 0.15 mg cm^{-3} in sc-ethane, 0.60 mg cm^{-3} in sc-CO_2.

In the course of these studies, it was found that the measured a_W in sc-ethane increased abruptly during the early stages of reaction when initial rates were measured (exceptions will be mentioned later). This effect was much less pronounced in sc-CO_2, as illustrated in **Figure 2**. To try to overcome the problem in sc-ethane, the amount of salt hydrate pair used was increased but became excessive before a_W control proved to be effective. Our concern then was to verify whether enzyme activity responded to the a_W of incubation in the reaction medium, before addition of the second substrate to start the reaction. This is indeed the case. **Figure 3** clearly indicates that the initial rates of esterification showed a strong dependence on the initial a_W, even though in the time scale of data collection a_W was found to vary. Rates first increased up to an a_W of ~0.25 and then decreased for higher a_W. This is unlike what we observed for Novozym in a transesterification reaction (*26*), when rates varied little with a_W. However, bell-shaped curves such as the one shown in the figure for sc-ethane and, in general, a decrease in rates of esterification and transesterification at higher a_W are often reported for Novozym and other enzymes in the synthesis of geranyl esters (*6*, *7*, *13*, *21*, *27*, *28*).

In our earlier study with Novozym (*26*), reaction rates were found to depend on the size of the enzyme particles. Thus, here

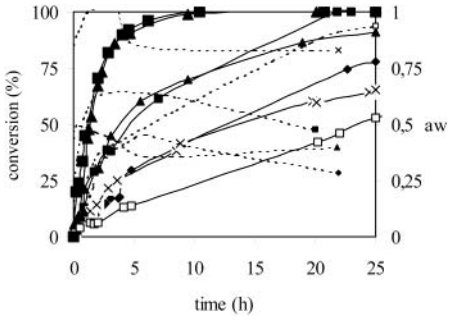

Figure 4. Dependence of the conversion of geraniol to geranyl acetate (symbols on full lines) on reaction time for the bulk enzyme preparation incubated at different initial a_W values, in sc-ethane. The larger black symbols are for [Novozym] = 0.55 and 1.10 mg cm^{-3} (data sets coincide), and the other symbols are for [Novozym] = 0.15 mg cm^{-3}. For the latter enzyme concentration, the dashed lines ending in small symbols show the actual a_W as a function of time. Initial a_W values were set by pairs of salt hydrates in situ: (◆) 0.10; (■) 0.25; (▲) 0.43; (×) 0.75; (□) blank (no salt hydrate). Actual a_W values >1 as obtained for (×) dashed line are artificial and indicate that the water concentrations measured were above saturation. T = 40 °C. P = 100 bar. [Geraniol] = 200 mM. [Acetic acid] = 50 mM.

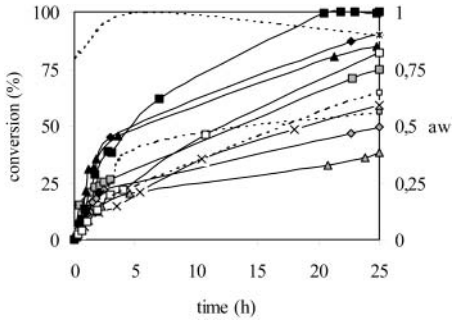

Figure 5. Dependence of conversion on reaction time at different temperatures: (black symbols) sc-ethane; (shaded symbols) sc-CO_2; (squares) T = 40 °C; (diamonds) T = 50 °C; (triangles) T = 60 °C. Reactions were performed in the presence of the pair Na_2HPO_4·2/0. Included in the figure are other results obtained at 40 °C in sc-CO_2: (□) blank; (×) initial a_W = 0.75. The dashed lines ending in small symbols show the actual a_W as a function of time for experiments in sc-CO_2 at 40 °C. P = 100 bar. [Geraniol] = 200 mM. [Acetic acid] = 50 mM. [Novozym] = 0.15 mg cm^{-3} in sc-ethane, 0.60 mg cm^{-3} in sc-CO_2.

reactions were done not only with the bulk enzyme preparation but also with the smaller enzyme particle sizes. **Figure 3** shows that reaction rates were higher for the smaller particle sizes, as anticipated, although particle size did not affect the rate versus a_W profile. At the time (*26*), we attributed the dependence of reaction rates on enzyme particle size to the existence of internal diffusion limitations. However, García-Alles and Gotor (*29*) have crushed small and large Novozym particles to comparable sizes and observed that the powder resulting from the smaller particles had higher activity and also higher protein content than the powder obtained from the larger enzyme particles. Thus, they attributed the dependence of reaction rates on enzyme particle size to a heterogeneous distribution of enzyme on the Novozym particles. For comparison, data points for sc-CO_2 have been included in this figure. These results again show that, at least for some enzymes, sc-CO_2 is less supportive of enzyme function than the alkanes, due to acid−base effects (*30*−*34*) and possibly carbamate formation (*31*). Enzyme activity maxima are thus comparatively less pronounced in sc-CO_2 than in sc-ethane (*32*). The latter solvent has the disadvantage of flammability, but this may not be so crucial for prospective smaller scale applications in biocatalysis.

As with initial rates, the a_W of incubation had a strong effect on conversion in sc-ethane despite the changes in a_W observed during the course of reaction, as seen in **Figure 4**. Initial a_W first had a positive effect on conversion and then a negative effect, in agreement with many accounts on geranyl ester synthesis via esterification and transesterification reactions found in the literature (*4*, *7*, *13*, *16*, *18*, *27*). Here the a_W that yielded a higher initial rate of esterification is also that which promoted a higher conversion. Vázquez Lima et al. (*27*) studied the esterification of geraniol with butyric acid catalyzed by Lipozyme and found that as the degree of hydration of the catalyst particles increased, the rate of uptake of each of the substrates decreased and particle aggregation occurred, as evidenced by electron microscopy. They considered water inhibition to be a predominantly physical effect.

Figure 4 also shows that as reaction progress slowed, the actual a_W decreased. This seems to indicate that at lower rates of water production, the salt hydrate pair was able to exert some sort of a_W control, although a_W never reached its initial value in the reaction times depicted in **Figure 4** (exceptions addressed below). In fact, a_W increased steadily for the blank, again less notoriously as reaction progress slowed. Selmi et al. (*35*) report a similar situation for a different esterification reaction in which the method selected for water removal was evaporation. These authors observed a drastic increase in the water content of the medium during the first 45 min of reaction, corresponding to an important production of water and a comparatively lower rate of water elimination. As the rate of water production decreased, the water content of the medium went down and stabilized. As mentioned above, there was one case in which a_W remained fairly constant, in particular, the salt hydrate pair NaAc·3/0 was used. The control of a_W that the pair NaAc·3/0 is apparently affording was also observed when the amount of water released into the medium was much higher, as was the case of experiments done with about 5 or 10 times more enzyme (larger symbols in **Figure 4**, for which actual a_W is not shown). Still, it was with Na_2HPO_4·2/0 that higher initial rates were obtained and reaction progress was faster, yielding 100% conversion at 10 h of reaction.

Whereas in sc-ethane it was clearly advantageous to provide some sort of a_W control, in sc-CO_2 the blank gave results similar to experiments performed at an initial a_W of 0.25, as seen in **Figure 5**. This is probably due to the similarity of the actual a_W profiles obtained in the two cases. The less pronounced increase in a_W must reflect the higher solubility of water in the medium, which is ∼5 times higher than in sc-ethane at the same temperature and pressure, as well as lower conversions/lower rates of water production in sc-CO_2. In the case of experiments done in the presence of the pair Na_2HPO_4·7/2, there was much more water in the reaction mixture at the start of reaction than found for the blank, and this must account for a less favorable reaction progress.

The impact of temperature on enzyme productivity can be seen in **Figure 5**. In both sc-fluids, the amount of geranyl acetate formed decreased as temperature increased above 40 °C, although temperature did not have a pronounced effect on initial rates of esterification in any of the solvents in the temperature range studied (550 nmol min^{-1} mg^{-1} in sc-ethane at both 40 and 60 °C, 125 and 175 nmol min^{-1} mg^{-1} in sc-CO_2 at 35 and 60 °C, respectively, for the bulk enzyme preparation). These results generally agree with the findings of Claon and Akoh (*4*), who reported an optimum temperature range between 35

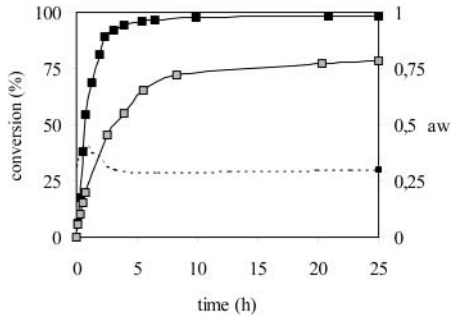

Figure 6. Conversion versus reaction time for the equimolar mixture (100 mM for both substrates). The data shown are for the bulk enzyme preparation incubated at an initial a_W of 0.25 in sc-ethane (black squares) and in sc-CO_2 (shaded squares). The dashed line shows the actual a_W as a function of time in sc-ethane. [Novozym] = 2.2 mg cm^{-3}. T = 40 °C. P = 100 bar.

and 40 °C for Novozym and the same reaction. Bartling et al. (*7*) obtained an increase in reaction rate and a slight reduction in conversion between 10 and 50 °C. Oguntimein et al. (*5*) indicate a sharp decrease in initial rates of formation of a different ester at temperatures >60 °C. The use of salt hydrates in the context of a_W control has already been discussed. In addition, the a_W set by the pair Na_2HPO_4·2/0 that was used in these experiments is considered to be too uncertain to quote at temperatures >40 °C (*36*). Even so, the presence of this salt hydrate pair kept a_W within certain boundaries (not shown) and was thus beneficial. It was particularly so in sc-ethane, again probably due to the higher conversions achieved in this solvent and a significantly lower solubility of water.

As pointed out by Bartling et al. (*7*), downstream processing would be much facilitated if the two substrates were completely consumed and only geranyl acetate remained at the end of reaction. These authors used an equimolar mixture of the two substrates (100 mM each) in *n*-hexane at 30 °C with a Novozym concentration of 2.2 mg cm^{-3} and obtained an equilibrium conversion of 94%. By applying membrane pervaporation, the authors were able to push conversion up to 100%. As shown in **Figure 6**, in sc-ethane it was not possible to go beyond 98% conversion at 10 h of reaction, a value that dropped to 73% in sc-CO_2. In this case, a_W remained fairly constant in sc-ethane throughout the reaction despite the high conversions obtained, a fact that may be due to a higher initial concentration of acetic acid and increased polarity of the medium.

A major focus of the present study has been the analysis of water activity effects. We avoided the use of zeolites as drying agents (*3*, *4*) because we have recently shown that these materials can also have very pronounced acid−base effects on enzyme activity (*23*). We are currently looking at how zeolites affect geranyl acetate synthesis by Novozym in sc-fluids.

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