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Liquid carbon dioxide as an effective solvent for immobilized *Candida antarctica* lipase B catalyzed transesterification

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ABSTRACT

The transesterification of alcohols catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435®) was found to be effectively enhanced using a liquid CO_2 medium when it was compared with that using organic solvents. The large-scale kinetic-resolution of secondary alcohol by the immobilized lipase was also successfully performed with a continuous packed-column reactor that stably afforded corresponding enantiopure products. Herein, liquid CO_2 was proved for the first time to be superior to conventional organic solvents for biotransformation.

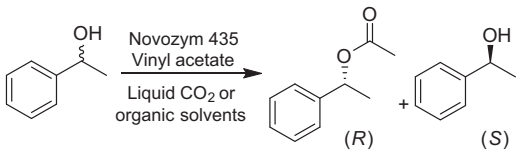
Introduction

The scope of biotransformation, particularly in asymmetric synthesis, has been extended through the use of enzymes in organic solvents[1–3] as well as in 'green' nonaqueous media, such as ionic liquids[4] and supercritical fluids.[5–7] Carbon dioxide, which has a number of positive impacts on green chemistry as a nonflammable, nontoxic, abundant, and generally chemically inert source, has been intensively studied in its supercritical phase as an alternative solvent for enzymatic reactions.[8] Some of the advantages of using supercritical CO_2 as a solvent include low viscosity, low surface tension, and ease of product recovery. At below critical temperature (31 °C), carbon dioxide can exist in a liquid phase, which can also be employed as a solvent for chemical reactions.[8,9] However, to the best of our knowledge, no reports exist on liquid CO_2 as a practical solvent for biocatalysts. Because carbon dioxide in a liquid phase is intrinsically different from its supercritical phase in many physical properties as a solvent, the enzymatic behaviors in liquid and supercritical CO_2 could be very different. The apparent benefit of using liquid CO_2 is that it can be maintained under relatively modest pressure (e.g., 4.5 MPa at 10 °C) that reduces the cost of specialized equipment for high-pressure (over 7.4 MPa) reaction in supercritical CO_2. Furthermore, it can be employed at low temperature, which has the potential to enhance the enatioselectivity by the low-temperature strategy.[10–12]

To exploit the potential of liquid CO_2 as a solvent for biotransformation, the transesterification of alcohols catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435®) was chosen as a model reaction. We found that the liquid CO_2 medium is superior to organic solvents. A liquid CO_2 fluid was also successfully employed for a flow system with a packed-column reactor for the continuous kinetic resolution of *rac*-1-phenylethanol and steadily afforded enantiopure products.

Results and discussion

First, the kinetic resolution of *rac*-1-phenylethanol catalyzed by Novozym 435® was performed in liquid CO_2 and in organic solvents using vinyl acetate as the acyl donor in a batch system (Scheme 1). As shown in Figure 1, as expected, the activity of the lipase is closely related to the solvent hydrophobicity (represented in log*P* value) where the more hydrophobic solvents generally showed higher yields. Interestingly, the lipase exhibited the highest transesterification activity with excellent enatioselectivity (ee >99%) in liquid CO_2, followed by hexane and toluene. Liquid CO_2



Scheme 1. Kinetic resolution of *rac*-1-phenylethanol.

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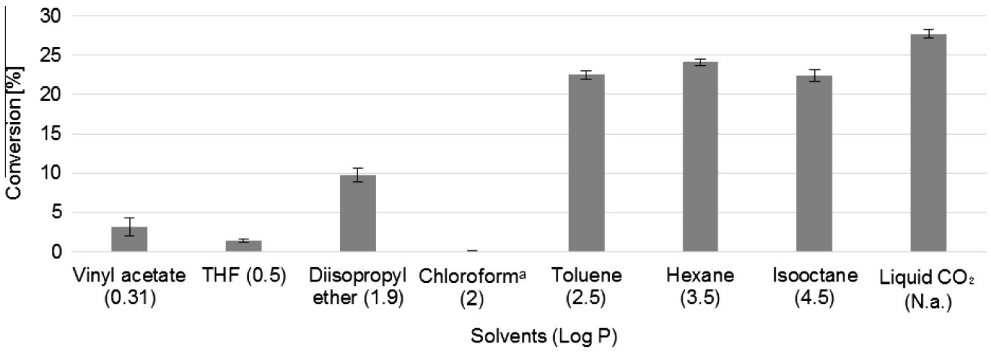
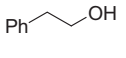
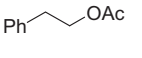
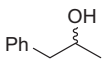
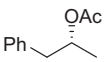
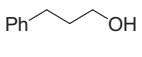
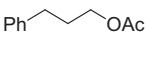
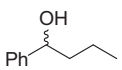
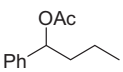
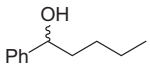
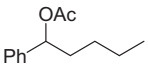
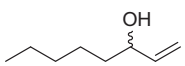
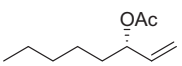
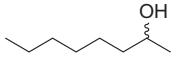
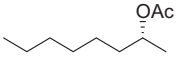


Figure 1. Effect of solvents on Novozym 435® catalyzed kinetic resolution of *rac*-1-phenylethanol. Reaction conditions:[15] Substrate 0.83 mmol, vinyl acetate 5.4 mmol, Novozym 435® 5 mg, solvent 10 ml, 20 °C, 2 h, pressure for liquid CO_2 6.5 MPa. Ee of the product (acetate) was found to be excellent (ee_p >99%) in all media. [a]No reaction occurred due to insufficient mixing of the enzyme and substrate since the density of the solvent greatly exceeds that of the immobilized enzyme. N.a.: not available. Dielectric constants of selected solvents: liquid CO_2 (20 °C, 6 MPa) 1.48, hexane 1.88, isooctane 1.94, toluene 2.38, diisopropyl ether 3.88, chloroform 4.81, THF 7.58.

was reported to have very similar behaviors with a hydrocarbon solvent with very low polarizability.[13] The high activity of the lipase in liquid CO_2 as well as in solvents with high log*P* values could be partly explained by the inability of hydrophobic solvents to strip off the essential water present on the outer layers of the enzyme.[14]

To further investigate the enhancement effect caused by liquid CO_2 using different substrates, the transesterification of various aromatic, aliphatic, and allylic primary and secondary alcohols was carried out in liquid CO_2 and in hexane (Table 1). The transesterification rates of 1-phenylethanol, 2-phenylethanol, and 1-octen-3-ol in liquid CO_2 were comparable with those observed in hexane (entries 1–3 and 10). Notably, with 1-, 2,- and 3-phenylpropanols and 2-octanol, liquid CO_2 promoted the reactions much more effectively than hexane (entries 4–7 and 11) with statistically significant differences (p <0.05). With substrates having longer side-chains, 1-phenylbutanol and 1-phenylpentanol, there was no reaction observed in hexane but some reactions in liquid CO_2 (entries 8 and 9). Our previous work[16] also found that the activity of Novozym 435® toward selected substrates was also enhanced in supercritical CO_2. This agreement supports the idea that the heterogeneous distribution of water and CO_2 molecules on certain regions of the lipase could efficiently facilitate the diffusion of certain substrates into the catalytic active site.[17] Furthermore, the difference was even more remarkable at lower temperature with the reaction with 1-phenyl-2-propanol (entries 5 and 6), but not in the

Table 1
Novozym 435®catalyzed transesterification of various alcohols in hexane or liquid CO_2 using a batch reactor[a]

Entry	Substrates	Solvent	Time (h)	Temp (°C)	Products	Yield (%)	ee_p[c] (%) (Config.)	*E*-Value
1		Hexane	1	20		31	>99 (*R*)	>200
		Liquid CO_2				32	>99 (*R*)	>200
2		Hexane	2	5		28	>99 (*R*)	>200
		Liquid CO_2				30	>99 (*R*)	>200
3[b]		Hexane	1	20		70	–	–
		Liquid CO_2				70	–	–
4		Hexane	18	20		4	>99 (*R*)	N.d.
		Liquid CO_2				25	>99 (*R*)	>200
5		Hexane	4	20		10	97 (*R*)	62
		Liquid CO_2				27	96 (*R*)	74
6		Hexane	18	5		7	97 (*R*)	70
		Liquid CO_2				34	96 (*R*)	80
7[b]		Hexane	0.5	20		82	–	–
		Liquid CO_2				93	–	–
8		Hexane	60	20		0		
		Liquid CO_2				6	N.d.	N.d.
9		Hexane	100	20		0		
		Liquid CO_2				4	N.d.	N.d.
10		Hexane	2	20		21	>99 (*S*)	>200
		Liquid CO_2				21	>99 (*S*)	>200
11		Hexane	1	20		33	>99 (*R*)	>200
		Liquid CO_2				40	>99 (*R*)	>200

[a] Reaction conditions:[15] alcohol (0.40 mmol) with vinyl acetate (2.2 mmol) and Novozym 435® (10 mg) in 10 ml hexane or liquid CO_2 (6.5 MPa) at 20 °C. Product yields and ee values are averages of at least three reaction runs determined by GC analysis. Absolute configurations were determined by comparing the optical rotation values with data from the literature.
[b] Novozym 435 (5 mg).
[c] ee of the products (acetates) N.d.: not determined due to the low conversion observed.

Table 2
Novozym 435® catalyzed kinetic resolution of *rac*-1-phenylethanol with a continuous flow of liquid CO_2 and substrates into a packed-column reactor[a]

Cycle No.[b]	In-flux rate[c] (mg/min)	Recovered[d] (g)	ee_s[e] (%)	ee_p[e] (%)	*c*[f] (%)	*E*-Value[g]	*E*-Factor[h]
1	18.72	7.08	>99	>99	49.9	>200	<0.3
2	18.75	7.27	>99	>99	50.0	>200	<0.3
3	18.62	6.85	>99	>99	49.9	>200	<0.3

[a] Reaction conditions[19]: immobilized enzyme 1.6 g, CO_2 flow rate 1.0 mL min^{-1}, substrate flow rate 0.020 ml min^{-1} (volume ratio of *rac*-1-phenylethanol/vinyl acetate = 2:1), 20 °C, 6.5 MPa.
[b] One cycle consists of four steps: pressurization and stabilization for 1 h; sampling for 8 h; washing with liquid CO_2 flow for 1 h; depressurization to 0.1 MPa and storing for 14 h at ambient conditions.
[c] The influx was monitored by a precision balance and measured by the amount of substrate mixture sent over time.
[d] Recovered outflux was collected at the stationary state for 8 h.
[e] ee_s of the substrate (alcohol) and ee_p of the product (acetate) were determined by chiral GC analysis.
[f] Conversion (c) was calculated from ee_s and ee_p (and confirmed by independent calculations from the ratio of the integral of the alcohol and the corresponding acetate signals in the ^1H NMR spectrum).
[g] Enantiomer selectivity (*E*-value) was calculated from ee_s and ee_p.
[h] The *E*-factor is equal to the kg waste (acetaldehyde and a small excess of vinyl acetate) divided by kg products (the enantiopure acetate and alcohol). Recyclable factors such as CO_2 gas and re-used immobilized enzyme are not included in the calculation.

case of 1-phenylethanol (entries 1 and 2), suggesting that the structure of the substrates mainly contributes the facilitation effect caused by the interaction of the solvent–enzyme–substrate.

Finally, to examine the potential of the industrial applicability of liquid CO_2 fluid for biocatalysis, the kinetic resolution of *rac*-1-phenylethanol with vinyl acetate by Novozym 435® was performed using a packed-column reactor with a continuous flow of liquid CO_2 and substrates (Table 2). The flow system was run for three operation cycles (24 h/cycle), to stably afford the corresponding enantiopure acetate and alcohol. For the green chemistry concept with a waste prevention goal, Sheldon's *E*-factor[18] has widely been adopted as a green chemistry metric for quickly assessing the environmental impact of manufacture processes. The ideal *E*-factor is zero, which means no waste was generated. Some chemical industry processes, such as fine chemicals and particularly pharmaceuticals, easily have *E*-factor >25.[18] Our system minimized waste with a very low *E*-factor (<0.3) using no organic solvent and a sufficient amount of vinyl acetate.

Conclusion

For the first time, liquid CO_2 was found to be superior to other conventional organic solvents for the lipase catalyzing the transesterification of alcohols. Large-scale asymmetric synthesis also successfully achieved waste minimization. On-going studies on liquid CO_2 as a 'green' medium for biocatalyzed synthesis continue in our laboratory.

Caution: All the reactions in liquid carbon dioxide involve high pressures and should only be conducted with appropriate equipment and proper precautions.

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15. *Batch reactions with liquid CO_2 were conducted as follows*: a mixture of alcohol and vinyl acetate, and enzyme was added and sealed in a high-pressure-resistant stainless-steel vessel (10 ml volume). The temperature was controlled by a thermostatic bath equipped with a recirculating chiller (Eyela, CCA-1111). CO_2 gas was sent into the vessel by a CO_2 pump (Jasco, PU-2080-CO_2 Plus) until the desired pressures were achieved. The vessel was then vigorously stirred with a magnetic bar. At the end of the reaction, the mixtures were collected by placing the vessel on ice and depressuring. The reaction mixture was collected in hexane (10 ml) and analyzed by GC. The reactants and products were all soluble under the reaction conditions that were confirmed by visual inspection of the reaction mixture in a reactor equipped with sapphire windows (2.5 cm in diameter).
The effect of supercritical CO_2 on the reaction was also investigated under the same reaction conditions but at 40 °C and compared with that of hexane at 40 °C. Supercritical CO_2 (conversion 35.7 ± 1.3%, ee_p >99%) showed a comparable effect with hexane (conversion 35.5 ± 2.2%, ee_p >99%).
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19. *The continuous flow apparatus* consisted of a CO_2 gas cylinder, a CO_2 pump (Jasco, PU-2080-CO_2 Plus), a substrate pump (Jasco, PU-2085 Plus), manometers (Taiatsu Techno, Osaka, 15 or 25 MPa), a packed-column reactor (3.94 ml, 28.5 cm long), stop valves (Swagelok, SS3NBS4), a turbulent mixer (a 0.5 ml tube packed with cotton), a thermostatic bath (Iuchi, TR1), a recirculation chiller (Eyela, CCA-1111), and a back pressure regulator (Jasco, BP-2080).