

Review

## Reaction equilibrium for lipase-catalyzed condensation in organic solvent systems

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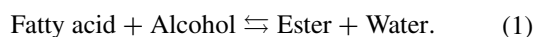
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### Abstract

Lipase-catalyzed condensation in an organic solvent is useful for the syntheses of esters. To reasonably design and optimize the reaction conditions, knowledge of the reaction equilibrium is required. The interaction of water with other reactants and the quantitative predictions for adsorption of water by a desiccant are discussed. The solvent effects on the reaction equilibrium are also elucidated in mixtures of nitrile and *tert*-alcohol.

### Introduction

Esters, such as fatty acid aliphatic alcohol esters and fatty acid polyol esters, have been used in many chemicals, medicines, cosmetics or foods by taking advantage of their particular properties. These esters can be synthesized by the condensation reaction (reverse hydrolysis) of a fatty acid and an alcohol (Equation (1)).



At present, many esters are industrially manufactured by chemical methods. Because chemical methods involve high temperature or high pressure, it is difficult in many cases to esterify unstable substances, such as polyunsaturated fatty acids, ascorbic acid, and polyols. Furthermore, the regio-specific acylation of polyols requires the protection and deprotection steps (Arcos *et al.* 1998). These steps cause a rise in manufacturing costs. There are other problems when the products are used in food processing. One of them is that the reagents which can be used in the process are restricted.

Recently, to overcome these drawbacks, enzyme-catalyzed condensations using lipases (E.C. 3.1.1.3)

have been developed. Lipase-catalyzed condensation has the following advantages: 1, mild reaction conditions; 2, one step synthesis without protection and deprotection steps; and 3, easy application to food processing.

A lipase catalyzes a reversible reaction and the direction and equilibrium of the reaction are determined by the activities of the substrates and products, temperature, and pressure. Although an enzyme-catalyzed reaction is usually performed in an aqueous solution, hydrolysis predominates to cause the production of desired product to fail when a lipase-catalyzed reaction is attempted in an aqueous solution. Thus, reduction of water in the reaction system would be effective for improvement in the conversion through the condensation reaction. Some lipases have catalytic activity even in the presence of little or a small amount of water (Kichner *et al.* 1985). For this reason, lipase-catalyzed condensation in a non-aqueous medium, such as organic solvents and ionic liquids (Sheldon 2001), has attracted much attention in this decade.

Based on these facts, many esters or lactones have been synthesized in organic solvents (Table 1). In these reactions, the reaction equilibrium constant, *K*, is used as a quantitative indicator of the degree of

Table 1. Synthesis of esters through lipase-catalyzed condensation in organic solvent systems.

Substrates	Product(s)	References
Ascorbic acid/fatty acid	Acyl ascorbate	Humeau <i>et al.</i> (1995, 1998a,b), Bradoo <i>et al.</i> (1999), Stamatis <i>et al.</i> (1999), Yan <i>et al.</i> (1999), Watanabe <i>et al.</i> (1999, 2000a), Kuwabara <i>et al.</i> (2003)
Polyol/fatty acid	Acyl polyol(s)	Arcos <i>et al.</i> (1998b), Adachi <i>et al.</i> (1999), Giacometti <i>et al.</i> (2001), Otero <i>et al.</i> (2001), Tewari & Bunk (2001), Piao <i>et al.</i> (2003), Castillo <i>et al.</i> (2003)
Saccharide/fatty acid	Acyl saccharide	Ikeda & Klibanov (1993), Arcos <i>et al.</i> (1998a), Bousquet <i>et al.</i> (1999), Degn <i>et al.</i> (1999), Ferrer <i>et al.</i> (1999), Gao <i>et al.</i> (1999), Watanabe <i>et al.</i> (2000b, 2001a,b), Chamouleau <i>et al.</i> (2001), Degn & Zimmermann (2001), Sereti <i>et al.</i> (2001), Soultani <i>et al.</i> (2001), Stamatis <i>et al.</i> (2001), Zhang <i>et al.</i> (2002)
Aliphatic alcohol/fatty acid	Fatty acid alkyl ester	Wu & Liu (2000), Flores <i>et al.</i> (2000), Krishna <i>et al.</i> (2001), He <i>et al.</i> (2002), Kobayashi <i>et al.</i> (2003b)
Kojic acid/fatty acid	Acyl kojic acid	Liu & Shaw (1998), Kobayashi <i>et al.</i> (2001), Chen <i>et al.</i> (2002)
Aliphatic alcohol/phenolic acid	Phenolic acid ester	Compton <i>et al.</i> (2000), Stamatis <i>et al.</i> (2001)
Hydroxyl fatty acid	Lactone	Gargouri <i>et al.</i> (2002)
Lactic acid	Polylactic acid	Sonwalkar <i>et al.</i> (2003)
Polyol/dicarboxylic acid	Polyester	Mahapatro <i>et al.</i> (2003)

condensation. The constant is, in principle, intrinsic for the reaction at constant temperature and pressure and can be expressed by the activities of reactants as follows:

$$K = \frac{a_P a_W}{a_F a_A}, \quad (2)$$

where  $a_i$  is the activity of reactant  $i$  at equilibrium, and F, A, P, and W represent a fatty acid, an alcohol, a product (an ester), and water, respectively. In some cases, the  $a$  value can be estimated by measuring some properties, such as vapor pressure or freezing point depression, or by prediction with the UNIFAC method (Fredenslund *et al.* 1975). However, in many cases, it is impossible or very difficult to evaluate all the  $a$  values of the reactants in the reaction system. Therefore, the apparent reaction equilibrium constant,  $K_C$ , based on the concentrations of the reactants is often used for convenience.  $K_C$  can be defined as follows:

$$K_C = \frac{C_P C_W}{C_F C_A}, \quad (3)$$

where  $C_i$  represents the concentration of reactant  $i$ , and  $K$  is correlated with  $K_C$  by

$$K = \frac{(\gamma_P C_P)(\gamma_W C_W)}{(\gamma_F C_F)(\gamma_A C_A)} = \frac{\gamma_P \gamma_W}{\gamma_F \gamma_A} K_C, \quad (4)$$

where  $\gamma_i$  is an activity coefficient of reactant  $i$ , which correlates the concentration with the activity. In this context, we will discuss the relationship between the  $K_C$  value and the natures of the solvent and substrates for the lipase-catalyzed condensation in organic solvent systems.

### Solvents and lipases used in the condensation reaction

Many types of solvents have been used in lipase-catalyzed condensation. They can be classified into water-miscible and immiscible solvents. Hexane, ethers, chloroform, and toluene are widely used as water-immiscible solvents. On the other hand, acetone, acetonitrile, and tertiary alcohols, such as *t*-butyl alcohol, are used as water-miscible solvents. The advantage of using water-miscible solvents is that the solvent can solubilize hydrophilic substrates such as saccharides to a certain degree without the addition of solubilizing reagents to facilitate the esterification of the hydrophilic substrates (Arcos *et al.* 1998a, Watanabe *et al.* 2000b, Degn & Zimmermann 2001, Otero *et al.* 2001, Castillo *et al.* 2003). However, a water-miscible solvent may remove water from a lipase molecule which is essential for catalytic activity (Krishna *et al.* 2001), and this removal may deactivate the lipase. For this reason, lipases available for condensation in water-miscible solvents are rather limited at present. Many researchers have been using the lipase from *Candida antarctica* fraction B (CALB) in an immobilized form. Therefore, we mainly deal with CALB-catalyzed condensation in water-miscible organic solvents.

### Substrate selectivity for the condensation

Lipase from *Candida antarctica* is a useful enzyme for condensation in a water-miscible solvent. However, there are cases where the reaction rate is rather low, e.g. synthesis of ferulic acid esters (Compton *et al.* 2000). This low reaction rate would be attributed to the electrical property of the carboxyl group in a ferulic acid molecule. Some reports have dealt with the electrical and steric effects of substrates (Char-ton 1987, Bevinakatti & Banerji 1988). However, the studies that numerically correlate these effects with substrate selectivity are still insufficient. Therefore, we performed a kinetic analysis of the lipase-catalyzed condensation of *p*-methoxyphenethyl alcohol and several carboxylic acids having various electrical or steric properties and correlated the rate constants with these properties (Kobayashi *et al.* 2003a). In the analysis, we used the projection area of the non-carboxylic region in a carboxylic acid molecule as a steric property, and the electron density of a carboxyl carbon as an electrical property.

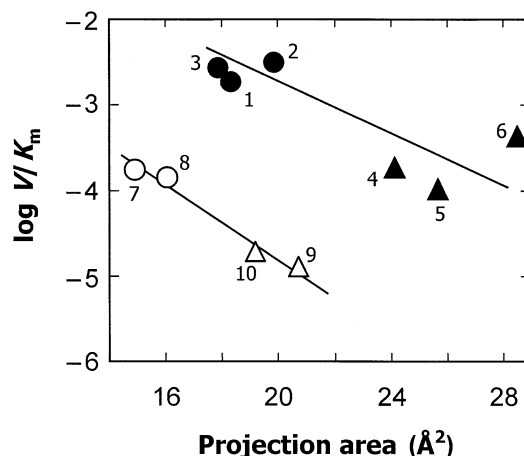


Fig. 1. Effect of the projection area of an acid molecule on the  $\log V/K_m$  value. The symbols, ● and ○, represent straight-chain acids, while ▲ and △ represent  $\alpha$ - or  $\beta$ -substituted acids. Closed and open symbols indicate the absence and presence of conjugation. Labels in the graph correspond to the substrate as follows: 1, propionic acid; 2, butyric acid; 3, vinylacetic acid; 4, isobutyric acid; 5, isovaleric acid; 6, cyclohexanecarboxylic acid; 7, acrylic acid; 8, crotonic acid; 9, methacrylic acid; 10, benzoic acid. (Kobayashi *et al.* 2003a).

Figure 1 shows the relationship between the  $\log V/K_m$  value and the projection area, where  $V$  is the maximum rate of reaction, and  $K_m$  is the Michaelis constant for carboxylic acid. In the presence of a substituent at the  $\alpha$ - or  $\beta$ -position of carboxylic acid, or in the presence of a conjugated double bond with a carboxyl double bond, the rate constant ( $V/K_m$ ) did change greatly. The two lines are almost parallel, indicating that the steric and electrical properties independently affect the  $V/K_m$ . In short, the presence of a conjugated double bond or a substituent decreases the rate constant in the order of 1, and the rate constant decreases in the order of 2 when both are present.

As mentioned above, some substances are poor substrates for the lipase-catalyzed condensation. Therefore, only the cases free from these two hindrances will be considered in the subsequent sections.

### Transesterification

Lipase-catalyzed transesterifications, which can be classified into alcoholysis, acidlysis, and interesterification, are other ways to synthesize esters. In this section, the lipase-catalyzed alcoholysis widely used in an organic solvent will be described.

In the lipase-catalyzed alcoholysis, methyl, ethyl, and vinyl esters have been widely used as substrates. Among the esters, vinyl ester has been most extensively used as a substrate because the by-product, vinyl alcohol, is almost irreversibly converted to acetaldehyde to increase the conversion (Ferrer *et al.* 1999, Athawale & Manjrekar 2000, Ferrer *et al.* 2000, Park *et al.* 2000, Li & Rethwisch 2002, Hazarika *et al.* 2003, Yadav & Trivedi 2003). The conversion in the transesterification using other esters as a substrate is also generally high compared to that in the condensation reaction (Compton *et al.* 2000, Maugard *et al.* 2000). However, there is a problem that acetaldehyde forms a Schiff base with a lipase (Berger & Faber 1991). Furthermore, the condensation reaction has the advantages that the free carboxylic acid can be directly esterified to reduce the cost and that the reaction affords only a harmless by-product, i.e. water, which will simplify the purification processes.

### Effects of water on the equilibrium conversion

In the condensation, water is produced as a by-product (Equation (1)). Although the water content in an organic solvent system is repressed in the initial stage of the reaction, the water content increases with the progress of the condensation. Because the water content is much lower in an organic solvent system than in an aqueous system, parameters not relevant in an aqueous solution, such as water concentration and the nature of the solvent, may greatly affect the reaction equilibrium. The effect of water content on the reaction equilibrium will be discussed.

#### *Removal of water by the addition of molecular sieve*

Water formed by condensation exists in solubilized form and acts as a substrate for the hydrolysis reaction when the reaction is performed in a water-miscible solvent. Therefore, elimination of water by a desiccant would be effective for increasing the conversion. Desiccants often employed for the lipase-catalyzed condensation are molecular sieves (Arcos *et al.* 1998a, Maugard *et al.* 2000, Chamouleau *et al.* 2001, Giacometti *et al.* 2001, Kuwabara *et al.* 2003) and silica gel (He *et al.* 2002, Sonwalkar *et al.* 2003). However, there is no criterion for the amount of desiccant to be added to the reaction system. The quantitative relationship between the equilibrium conversion and the concentration of a molecular sieve

was discussed using the adsorption isotherm of water onto molecular sieves, the apparent reaction constant, the solubility of a hydrophilic substrate in the solvent and the mass balance equation in terms of water into consideration to obtain the criterion.

The lipase-catalyzed synthesis of lauroyl mannose was carried out in acetonitrile, *t*-butyl alcohol and *t*-amyl alcohol in the presence of molecular sieve 3 Å or 4 Å (Zhang *et al.* 2003). In all cases, the equilibrium conversion improved with the increasing amount of the molecular sieve (Figure 2). The calculated results coincided well with the observed results for the case in *tert*-alcohols, indicating that the equilibrium conversion can be quantitatively predicted in the presence of a molecular sieve. However, in the case of acetonitrile, there is a discrepancy between the calculated and observed results at a high concentration of molecular sieves. This could be attributed to the formation of dilauroyl mannose, one of the negative effects of the addition of molecular sieves. Although the addition of a molecular sieve or silica gel usually improves the equilibrium conversion in many cases, negative effects such as the formation of diesters and degradation of unstable substrates have also been reported (Chamouleau *et al.* 2001, Kuwabara *et al.* 2003, Sonwalkar *et al.* 2003). Therefore, an appropriate combination of solvent and the amount of desiccant should exist for improvement of the equilibrium conversion in a lipase-catalyzed condensation.

#### *Interaction of water with other reactants*

Condensation in the absence of a desiccant has also been frequently performed. In such a case, water should play an important role in the reaction equilibrium. In the synthesis of a sugar ester (or a polyol ester) in an organic solvent, the unreacted sugar remains in the solid state. The sugar, which is more strongly hydrated, would further decrease the water activity in the system to shift the equilibrium toward synthesis. We correlated the  $\log K_C$  value for the syntheses of lauroyl sugars with the dynamic hydration number of the sugars (Watanabe *et al.* 2000b). A linear relationship between them could be obtained, indicating that the presence of the sugar greatly affected the water activity and that water activity played an important role in the condensation.

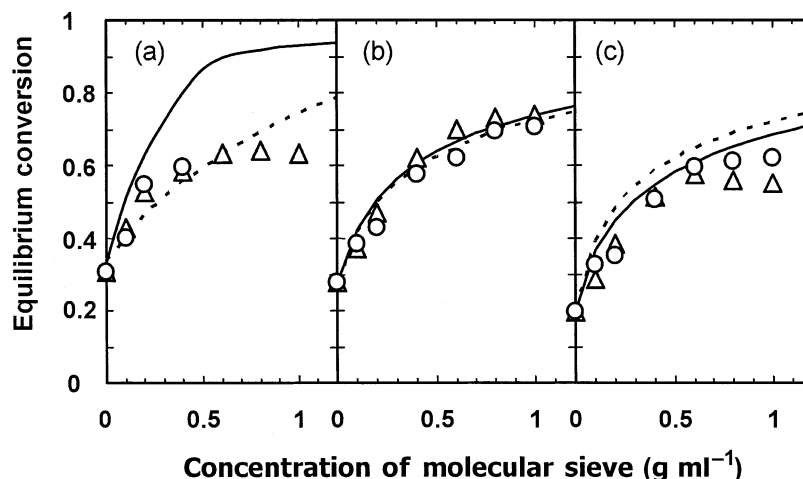


Fig. 2. Equilibrium conversion for the synthesis of lauroyl mannose in (a) acetonitrile, (b) *t*-butyl alcohol and (c) *t*-amyl alcohol at different amounts of molecular sieves 3Å (○) and 4Å (△). The solid and dotted curves are calculated results for molecular sieves 3Å and 4Å, respectively. (Zhang *et al.* 2003).

### Roles of the solvent for the equilibrium conversion

Although the organic solvent as a reaction medium greatly affects the equilibrium conversion, the reasons for this have not been clarified. Therefore, we tried to correlate the reaction equilibrium constant with the structure or property of an organic solvent.

#### *Equilibrium in a pure solvent*

The condensation reaction is extensively performed in a pure organic solvent. It has been known that the kind of solvent affects the equilibrium conversion even though the solvent provides only the field of the reaction. Castillo *et al.* (2003) reported that the diester (triester) formation was suppressed and that the monoester formation was preceded by changing the solvent from hexane to *tert*-alcohols for the synthesis of xylitol oleic acid esters. They discussed the differences in equilibrium conversion based on the polarity of the solvent. We also found that the equilibrium conversions for the syntheses of lauroyl mannose (Watanabe *et al.* 2001b) and vinylacetyl glucose (Zhang *et al.* 2002) in *tert*-alcohols were lower than those in acetonitrile. However, the reason for the difference has not been elucidated.

#### *Effect of solvent properties on the reaction equilibrium*

The kind of solvent affects the equilibrium conversion as shown above. We attempted to correlate the properties of a solvent and the reaction equilibrium constant

to elucidate the solvent effect on the equilibrium. When the relationship is obtained, the equilibrium conversion can be predicted with ease.

Each solvent has its inherent properties but mixing of two different solvents can adjust the values of the total properties between their inherent properties. Therefore, we used mixtures of two water-miscible solvents and evaluated the reaction equilibrium constant in the mixtures to determine the relationship.

We tried to find the properties that could clearly express the effects of a solvent. The most frequently employed property is a log P value (Tewari *et al.* 1999, Krishna *et al.* 2001), which is defined as a partition coefficient of a substance between 1-octanol and water phases. However, the log P value is a parameter defined for only a pure solvent and cannot, in principle, be used as a property of a mixed solvent. Therefore, the following parameters were used as properties of a mixed solvent: the dielectric constant,  $\epsilon_r$ , and the empirical parameter, the  $E_T(30)$  value, which was used as a parameter expressing the polarity of a solvent (Reichardt 1979).

Castillo *et al.* (2003) synthesized oleoyl xylitol in mixed solvents and found that the equilibrium conversion of the diester increased with a decreasing  $E_T(30)$  value, indicating an increase in the  $K_C$  value for the formation of the diester. In the case of lauroyl mannose synthesis, the  $K_C$  values could not be correlated with the  $E_T(30)$  values but with the dielectric constant of the solvent (Watanabe *et al.* 2001b). However, the dielectric constant also does not seem to be applicable

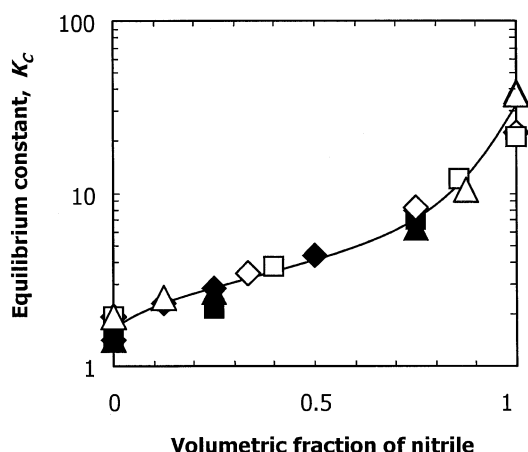


Fig. 3. The equilibrium constants for the formation of butyl decanoate in mixtures of alcohols and nitriles of various molar ratios at 50 °C. The open and closed symbols represent the mixtures of *t*-butyl alcohol and *t*-amyl alcohol, respectively, with (◇, ◆) acetonitrile; (□, ■) propionitrile; and (△, ▲) butyronitrile (Kobayashi *et al.* 2003b).

to the formation of butyl decanoate (Kobayashi *et al.* 2003b). Therefore, there is a possibility that a correlation between the  $K_C$  value and the dielectric constant obtained for lauroyl mannose synthesis is apparent.

One of the reasons for incompatibility may be attributed to these parameters being expressed as the overall properties of a solvent molecule. Thus, let us consider the solvent molecule as two parts; one is the polar region such as a hydroxyl group, and the other is the non-polar region such as an alkyl group. The  $K_C$  value increased with the increasing fraction of nitrile, although the alkyl chain length of the solvent had little effect on the  $K_C$  value (Figure 3, Kobayashi *et al.* 2003b). These results indicate that the polar region of a solvent molecule greatly affects the  $K_C$  value. This effect could be quantified by the IR spectrum. The IR absorption peak of C=O for a fatty acid shifted to a lower wavenumber with the increasing fraction of *tert*-alcohol, although the change in wavenumber for an ester was smaller than that for a fatty acid. This result can be explained by the difference in interaction between a *tert*-alcohol and the substrates: a fatty acid can interact well with a *tert*-alcohol, while an ester can only weakly interact.

Based on these results, the apparent equilibrium shifts to the right side of Equation (1) (favorable for condensation) when using a nitrile as a solvent, although the hydrolysis predominates in a *tert*-alcohol. The discrepancy between the observed and calculated yields for lauroyl mannose synthesis in acetonitrile

(Zhang *et al.* 2003) could be ascribed to the promotion of diester formation in the presence of a nitrile.

## Conclusions

Lipase-catalyzed condensation is a useful way to synthesize esters, and the equilibrium conversion is affected by several factors. Correlations have been obtained for the reaction equilibrium constant based on the concentrations of the substrates and products with the amount of desiccant and solvent properties. Further investigations seem to be required for better understanding of the phenomena occurring in the lipase-catalyzed synthesis of esters in water-miscible solvents and for reasonable design of the reaction.

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