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Quantitative Evaluation of the Effects of Moisture Distribution on Enzyme-Induced Acylation of Trehalose in Reduced-Moisture Organic Media

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Abstract

Enzymatic condensation of trehalose and myristic acid in organic media (2-methyl-2-butanol and acetone) with reduced moisture content was evaluated. Monomyristoyl trehalose was synthesized using immobilized lipase B from *Candida antarctica* (Novozym® 435). The product yield was significantly affected by process parameters, such as the initial moisture content in organic media, as well as in immobilized enzymes, and the added concentration of molecular sieves. Up to 25% yield of monomyristoyl trehalose could be attained, depending on the process. The experimental setup used in this study consisted of a multiphase component, i.e., Novozym® 435, an organic solvent, and molecular sieves. Moisture adsorbed either onto immobilized enzymes, or molecular sieves, and free moisture content in the organic solvent were characterized using individual experiments. The relationship between process parameters and the quantitative moisture distribution in the system was also investigated. The results presented in this paper indicates that a process design considering moisture distribution in the reaction system is important for understanding the effect of moisture on the reaction as well as for optimizing the process parameters.

Keywords: lipase, trehalose ester, multiphase reaction, moisture content, organic media, moisture adsorption isotherm

1. Introduction

Sugar-fatty acid esters, consisting of hydrophilic groups of mono-, di- or tri-saccharide and hydrophobic alkyl chain groups from fatty acids, have wide application fields such as food, cosmetic, pharmaceutical and chemical industries. Among them, trehalose-fatty acid esters have various potential industrial applications including foods, cosmetics, or medical supplies because of its surface activities (Chen, Kimura, & Adachi, 2005; Piao, Takase, & Adachi, 2007; Raku, Kitagawa, Shimakawa, & Tokiwa, 2003), physiological and biological activities (Okabe et al., 1999; Khan, Stocker, & Timmer, 2012; Hsieh et al., 2015) and biodegradability (Raku et al., 2003). For their intended uses, it is desirable to synthesize such esters enzymatically because they should be highly biocompatible. The lipase- catalyzed reactions to synthesize sugar-fatty acid esters by condensation with sugars and fatty acids in organic media with reduced moisture content have been widely studied (Plou et al., 2002; Adachi & Kobayashi, 2005; Kobayashi, 2011).

Water is a product from the condensation reaction and thus the moisture content in the reaction system affects the thermodynamic reaction equilibrium (Kobayashi & Adachi, 2004). As reported in the literature (Rupley, Gratton, & Careri, 1983; Klibanov, 2001), the small amount of water bound to enzyme molecules is called "essential water", which is critical for catalytic activity in organic media. Enzymatic reactions in organic media are often carried out in a multiphase system consisting of solid enzymes including those in immobilized forms with solid supports, organic solvent and desiccants such as molecular sieves. In such experiments, moisture has to be

distributed among each component in the experimental setup. Therefore, in order to elucidate the effects of moisture on the enzymatic reaction in the multiphase system, consideration of the quantitative moisture distribution among the enzymes, solvent and desiccant should be investigated.

In this study, we synthesized a trehalose-fatty acid ester in two organic media, 2-methyl-2-butanol and acetone with reduced moisture content, using immobilized lipase fraction B from *C. antarctica* (Novozym® 435). 2-Methyl-2-butanol is water-miscible, relatively hydrophobic solvent (log*P* = 1.3), while acetone is water-miscible, relatively hydrophilic solvent (log*P* = -0.23), where log*P* is octanol-water partition coefficient (Janssen et al., 1992). Both solvents have been widely used for lipase-catalyzed synthesis of sugar-based surfactants because they can partly dissolve a variety of sugars and fatty acids. Novozym® 435 has also been widely investigated as a catalyst for synthesis of sugar esters from di- and tri-saccharides and fatty acids (Chen et al., 2005; Piao et al., 2007; Woudenberg, van Rantwijk, & Sheldon, 1996; Ferrer, Cruces, Plou, Bernabe, & Ballesteros, 2000; Zhang et al., 2003). The authors investigated the effects of moisture in the experimental setup on the synthetic reaction of a trehalose-fatty acid ester using trehalose and myristic acid as substrates. The effects of moisture content in the organic media, and that of Novozym® 435, as well as the concentration of molecular sieves are reported here. Then, a comprehensive method for evaluating the effects of moisture on the yield of synthetic product is presented based on experimental results in relation to the quantitative moisture distribution amongst each component in the experimental setup. The results of this study should be applicable for various reaction systems containing multi-phase components for synthesis of sugar-based surfactants.

2. Materials and Methods

2.1 Materials

Novozym® 435 (a product of Novo Nordisk A/S, Bagsvaerd, Denmark) was obtained from Sigma Co. (St. Louis, MO). This was an immobilized lipase from *C. antarctica* fraction B (CALB), and a porous acrylic resin with a diameter of 0.3–0.9 mm was used as support material for the enzyme. Trehalose dihydrate, myristic acid, 2-methyl-2-butanol, acetone, methanol of HPLC analytical grade, and molecular sieves 3A were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Methyl-2-butanol and acetone were dried with 200 g/L of molecular sieves 3A for at least a week before use.

2.2 Enzymatic Acylation of Trehalose in Presence of Myristic Acid

Trehalose dihydrate (50 μmol), myristic acid (50 μmol), immobilized enzyme (100 mg), and molecular sieves 3A (0 – 400 mg) were placed in a 30-mL Erlenmeyer flask. Five milliliters of 2-methyl-2-butanol with moisture content of 0.058 – 46 g/L or acetone with moisture content of 0.040 – 2.5 g/L was added to the flask. The moisture contents of both solvents were adjusted by adding little moisture levels to the dried solvents and measured by Karl Fischer titration, using a Coulometer KF 737 (Metrohm, Switzerland) before use. The flask was sealed tightly with a screw cap and kept on incubation bath at 40 ˚C. After incubation for a given time (1-96 h), the solvent was evaporated from the reaction mixture, 15 mL of chloroform/methanol (2:1, v:v) were added to the solid residue, followed by removal of immobilized enzyme and molecular sieves from the mixture by filtration. After solvent evaporation, the solid residue was dried under reduced pressure and used for product analysis.

2.3 Purification of Reaction Products and Structural Analysis by Fourier-transform Infrared (FT-IR) Spectroscopy

The synthetic product in the enzymatic reaction mixture was purified by a column chromatography using a glass column containing octadecyl silica (Wakosil, Wako Pure Chemical Industries, Co., Ltd, Osaka, Japan). The mobile phase used consisted of methanol/water (85:15, v:v). Purified product was detected by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Silica gel 60 plate (Merck) was used for TLC analysis. The samples containing the synthetic products were spotted onto the TLC plate and then developed by using a mixture of chloroform/methanol (75:25, v:v). After drying the plate, the spots of hexose-containing compounds were visualized in purple color by heating at 110 ˚C with 20 wt% sulfuric acid containing 2 mg/mL of orcinol. HPLC analysis was also carried out to determine the purity of the reaction products. The operation conditions of HPLC were as described below. FT-IR spectra of myristic acid, trehalose, and the purified product were obtained by FT-IR spectrophotometer (FT/IR 5300, JASCO, Tokyo, Japan) using the KBr tablet method. Myristic acid and trehalose were used for analysis as they were purchased.

2.4 Determination of Product Yield

The yield of reaction product was determined using a HPLC system equipped with a COSMOSIL 5C18-MS-II column (ϕ 4.6 x 250 mm; Nacalai Tesque Inc., Kyoto, Japan) and a refractive index detector (Model RI-2031,

JASCO Co., Tokyo). Methanol/water (85:15, v: v) was used as the mobile phase at a flow rate of 1.0 mL/min. The column temperature was kept at 40 ˚C during analysis. Monomyristoyl trehalose was chemically synthesized according to a procedure previously reported (Ikemoto & Minamino, 1993) with a slight modification and used as a standard compound after purification. The synthetic yield was determined as the molar yield of monomyristoyl trehalose based on the amount of trehalose dihydrate used initially.

2.5 Isoterms of Moisture Adsorption onto Novozym® 435 and Molecular Sieves in Organic Solvents

Moisture adsorption equilibria on solid components in the experimental setup were investigated, basically by a procedure described previously (Kuroiwa et al., 2007). A specified amount of immobilized enzyme or molecular sieves 3A and 2-methyl-2-butanol or acetone with certain moisture content were incubated in a screw-capped glass vial at 40 ˚C. After a 100-h incubation period, the moisture content in the liquid phase was measured by the Karl Fischer titration. The amount of moisture adsorbed onto solids were calculated using equation (1), based on the moisture mass balance:

$$q_e = q_i + V(C_i - C_e)/m \quad (1)$$

where q_e and q_i are amounts of moisture adsorbed onto the solid components at the initial and equilibrium states, respectively, [g/g], C_i and C_e indicated the concentrations of free moisture in solvent at the initial and equilibrium states, respectively, [g/L], V is volume of solvent [L], and m is amount of solid component [g].

3. Results and Discussion

3.1 Synthesis of Monomyristoyl Trehalose by Immobilized Enzyme

We conducted the acylation of trehalose in presence of myristic acid using a commercial immobilized lipase, Novozym® 435, in organic media with reduced moisture content. Figure 1 shows the FTIR spectra of myristic acid, trehalose, and the purified synthetic product with scanning range from 4,000 to 1,000 cm^{-1}. The absorption band attributed to carboxyl groups (1,701 cm^{-1}) of myristic acid disappeared, and a new absorption band (1,734 cm^{-1}) assigned to ester bond appeared in the FTIR spectra of the purified product. The bands of hydroxyl groups and alkyl ether bonds (around 3,390 and 1,150-1,000 cm^{-1}) of trehalose moieties were also detected in the purified product. Furthermore, only one component was detected in the purified product by TLC and HPLC analyses, and its R_f value around 0.3 in TLC and retention time of 5.1 min in HPLC were identical to those of a standard monomyristoyl trehalose synthesized chemically. These results confirmed that monomyristoyl trehalose was successfully obtained by lipase-catalyzed condensation between trehalose and myristic acid. In the following studies, we determined the synthetic yield of the products from the area underneath the peak with a retention time of 5.1 min in HPLC analysis.

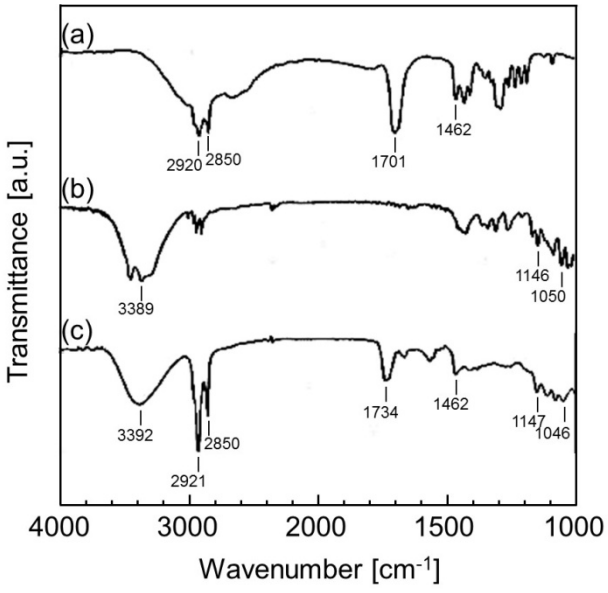

Figure 1. FT-IR spectra of (a) myristic acid, (b) trehalose and (c) purified synthetic product. The absorption band attributed to carboxyl groups (1,701 cm^{-1}), esters (1,734 cm^{-1}), methylene chains (2850 and 2920 cm^{-1}), methyl groups (1462 cm^{-1}), hydroxyl groups and alkyl ethers (around 3,390 and 1,150-1,000 cm^{-1})

Figure 2 shows the time courses of the synthetic yield of the monomyristoyl trehalose in 2-methyl-2-butanol with different initial moisture content. In both cases, the yield was increased as the reaction proceeded. However, the yields reached at the later stage of reaction (after 70 h) were considerably different each other. Therefore, the effects of operational factors on the yield of monomyristoyl trehalose in two different organic solvents, 2-methyl-2-butanol and acetone were further investigated.

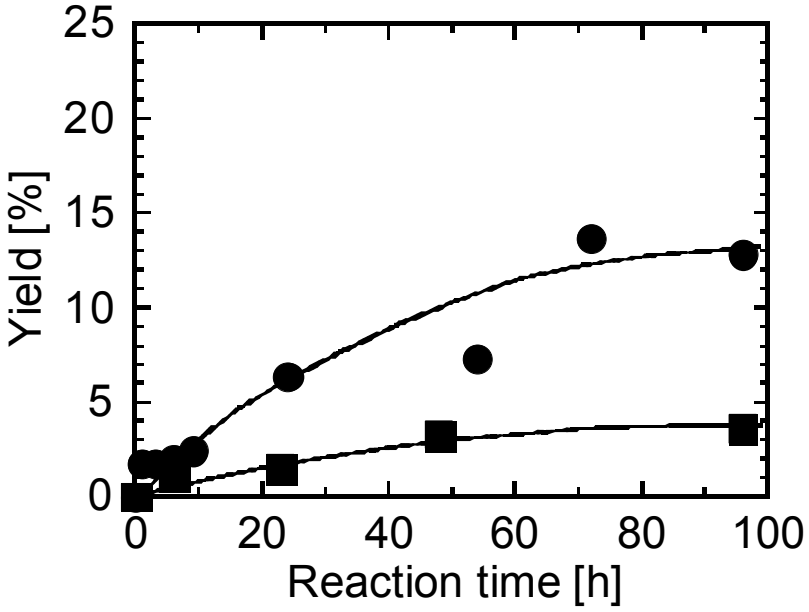

Figure 2. Time courses of the yield of monomyristoyl trehalose in 2-methyl-2-butanol with different initial moisture content of 0.42 g/L (●), 3.2 g/L (■). Amounts of added molecular sieves 3A and the immobilized enzyme moisture content were 40 g/L and 0.045 g-water/g-Novozym® 435, respectively

3.2 Effect of Process Parameters on the Synthetic Yield

Previous studies have reported that the moisture content of the solvent, and that of the solid catalyst, besides the amount of molecular sieves added to the system may affect enzyme-induced acylation of sugars and sugar moieties of natural compounds (Yahya, Anderson, & Moo-Young, 1998; Chamouleau, Coulon, Girardin, & Ghoul, 2001; Gayot, Santarelli, & Coulon, 2003; Zhang, Adachi, Watanabe, Kobayashi, & Matsuno, 2003). As shown in the previous section, reduced moisture content in the system also affected the synthesis of monomyristoyl trehalose using immobilized enzymes. Therefore, we studied the effect of process parameters associated to the moisture distribution in the system, on the product yield; namely, the moisture content of the organic solvent, and that of the solid enzymes immobilized on the solid support particles, as well as the amount of added molecular sieve were investigated.

Figure 3 shows the effect of the initial moisture content in two different solvents on the yield of monomyristoyl trehalose after 72 h of reaction. In both systems, the yield increased with the decrease of the initial moisture content. Low yields at high moisture levels can be explained by the reaction equilibrium, because water is a product in condensation of trehalose with myristic acid as well as monomyristoyl trehalose, and increased moisture leads to shift the reaction equilibrium toward hydrolysis. In the case of the 2-methyl-2-butanol system, the maximum yield appeared at about 0.5 g/L of moisture, and a lower yield was obtained in the region below the peak. However, a different water dependency was observed when immobilized enzymes with different moisture levels were used (Figure 2a, squares). These results indicate that the presence of moisture not only in the solvent but also in other solid components (immobilized enzymes and molecular sieves) affects the product yield, and the effect of moisture on different components are dependent on each other.

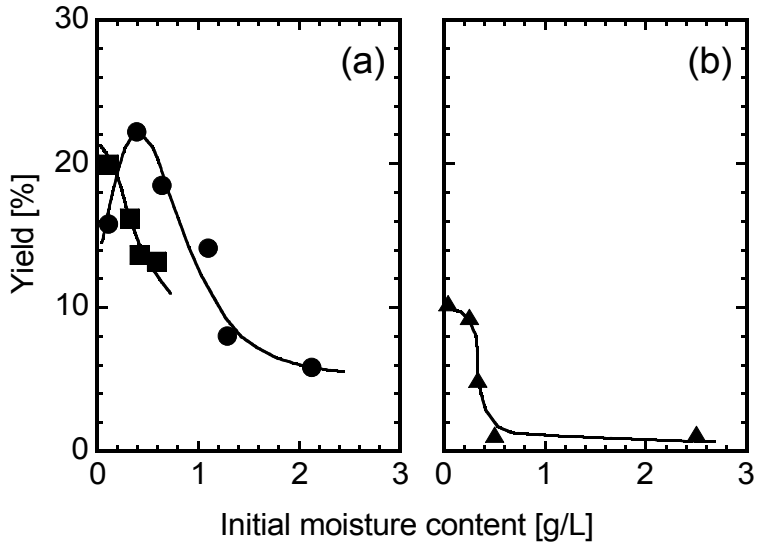

Figure 3. Effect of initial moisture content in organic solvent on the yield of monomyristoyl trehalose after 72 h reaction. Solvents used were: (a) 2-methyl-2-butanol containing 40 g/L of molecular sieves 3A; (b) acetone containing 20 g/L of molecular sieves 3A. Moisture contents of immobilized enzymes were 0.036 (●), 0.045 (■) and 0.028 (▲) g-water/g-Novozym® 435

Molecular sieves are often used for the enzymatic synthesis in organic solvent to keep the moisture content in the medium low, in order to suppress the undesirable reaction with water, i.e. hydrolysis of the products. Figure 4 shows the effect of addition of molecular sieve 3A to the reaction system on the product yield. The addition of the molecular sieve apparently affected the yield of the product at 72 h, as well as the initial moisture content in the solvents. The maximum yield was also obtained in 2-metyl-2-butanol system using the immobilized enzymes with a moisture content of 0.045 g-water/g-solid. Similar result is depicted in Figure 3a: the maximum yield was obtained at a certain initial moisture level in the solvent. However, these results suggest that the effects of above process parameters cannot be discussed separately; that is, a more comprehensive approach is needed to quantify moisture effect on the product yield. Therefore, the authors attempted to estimate the moisture distribution in the experimental setup.

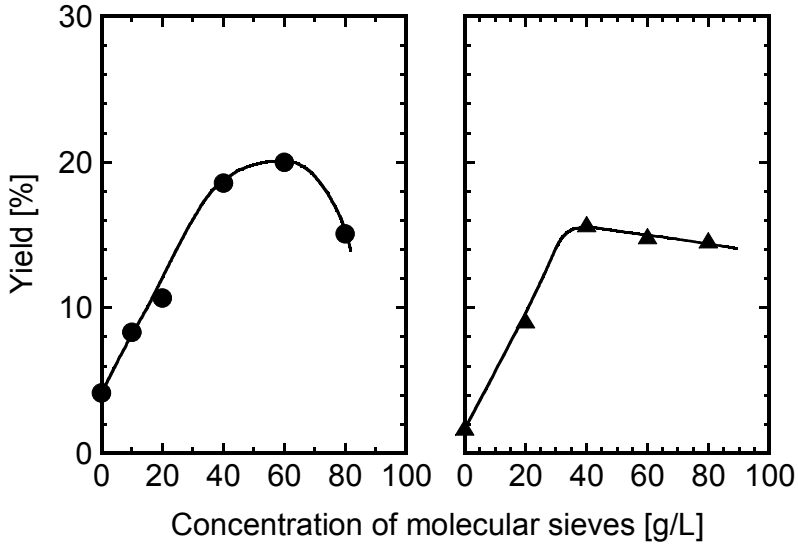

Figure 4. Effect of molecular sieves addition on the yield of monomyristoyl trehalose after 72 h reaction. Solvents used were (a) 2-methyl-2-butanol with initial moisture content of 0.11 g/L, and (b) acetone with initial moisture content of 0.044 g/L. As for the immobilized enzyme, 0.036 g-water/g- Novozym® 435 were used

3.3 Moisture Distribution in the System

The experimental setup in this study was a multicomponent system consisting of liquid and solid phases. Water in such a system has to be distributed among the individual components (Figure 5). Thus, in order to understand the effect of moisture on the product yield, it is necessary to quantify the distribution of moisture in the experimental setup.

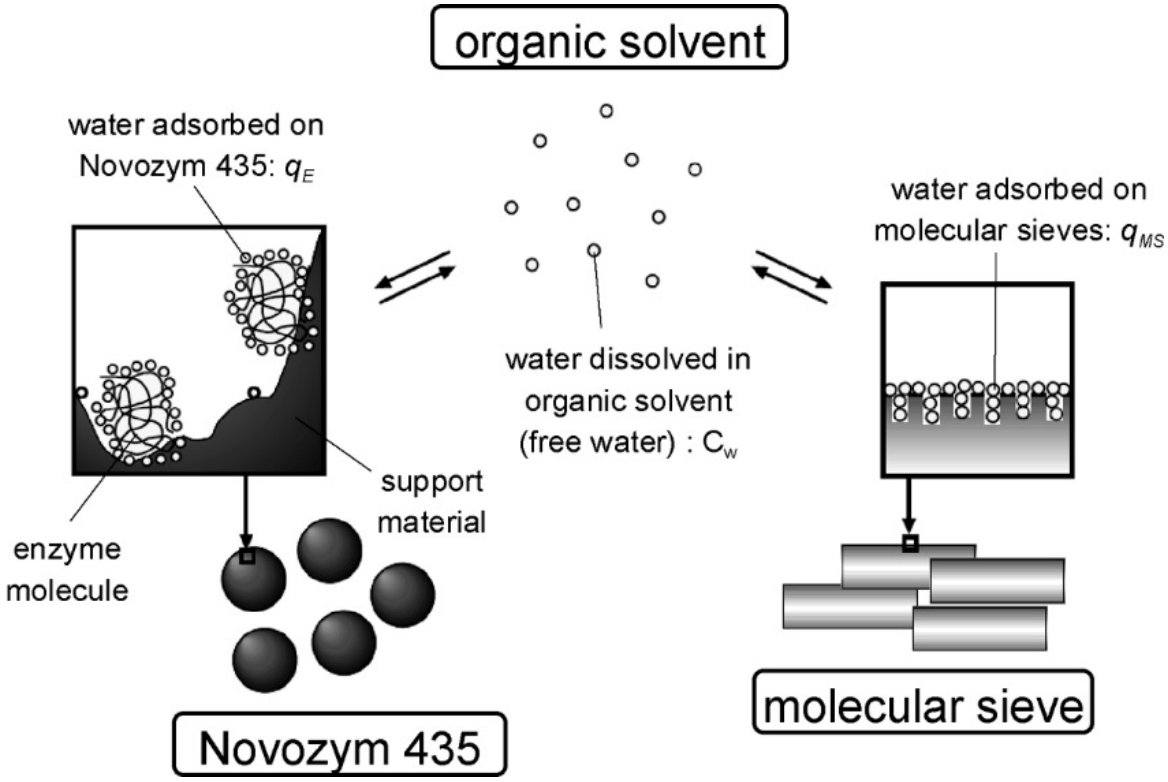

Figure 5. Schematic illustration of moisture distribution during immobilized lipase-induced reaction in a reduced-moisture organic solvent. Water in a multiphase system consisting of liquids containing solid components has to be distributed among the individual components such as a solvent, immobilized enzyme molecules, and molecular sieves

The water mass balance is given by the following equation:

$$V C_i + m_E q_{E,i} + m_{MS} q_{MS,i} + V C_r = V C_e + m_E q_{E,e} + m_{MS} q_{MS,e} \quad (2)$$

where C_r: is concentration of free water in solvent produced by synthetic reaction [g/L], m_E and m_{MS} are amounts of immobilized enzyme and molecular sieve 3A, respectively, [g], $q_{E,i}$ and $q_{E,e}$ are amounts of moisture adsorbed onto immobilized enzyme at the initial and equilibrium states, respectively, [g/g], and $q_{MS,e}$ and $q_{MS,i}$ are amounts of moisture adsorbed onto molecular sieves 3A at the initial and equilibrium states, respectively, [g/g].

In this study, the value of $q_{MS,i}$ can be set to zero because the molecular sieves were sufficiently dried before use. Therefore, Equation 2 can be rearranged as follows:

$$C_i + C_r = C_e + (m_E/V)(q_{E,e} - q_{E,i}) + (m_{MS}/V)q_{MS,e} \quad (3)$$

As shown later, the amount of moisture adsorbed onto the immobilized enzymes and molecular sieves at equilibrium, $q_{E,e}$ and $q_{MS,e}$, can be determined from moisture adsorption equilibria based on separate experimental results for each component. Thus, we can use Equation 3 to calculate the moisture distribution in the system at the equilibrium state — that is, moisture adsorbed onto the immobilized enzymes, moisture adsorbed onto the molecular sieves, and the moisture dissolved in the organic solvent.

Isotherms for moisture adsorption onto immobilized enzymes (Figure 6a) and onto molecular sieves 3A (Figure 6b) could be expressed by a Langmuir-type equation (Equation 4). The adsorption parameters q_s and K determined from Figure 5 are listed in Table 1.

$$q_e = q_s C_e/(K + C_e) \quad (4)$$

where q_s is saturated amount of moisture adsorbed onto the solid component [g/g] and K is adsorption equilibrium constant [g/L].

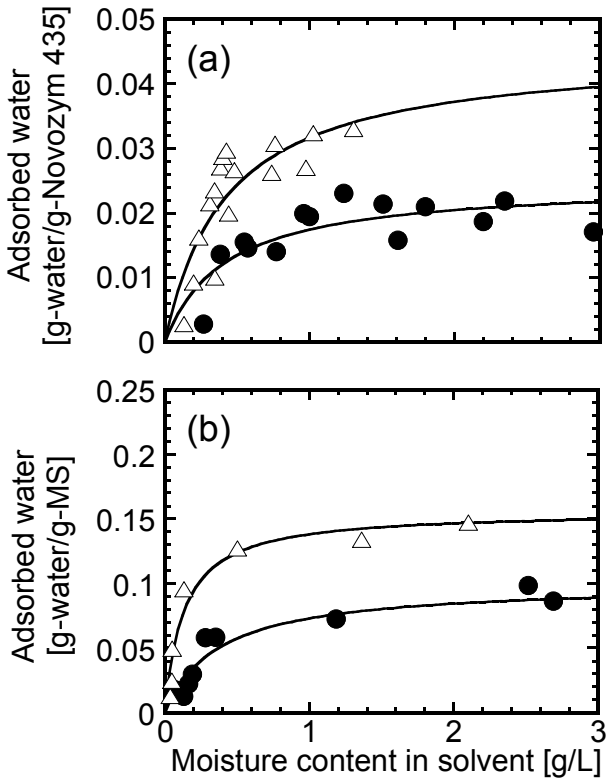

Figure 6. Isotherms for moisture adsorption onto (a) Immobilized enzyme and (b) molecular sieves 3A in 2-methyl-2-butanol (●) and acetone (△, data from Kuroiwa et al., 2007) at 40˚C

Table 1. Moisture adsorption parameters for Novozym 435 and Molecular sieve 3A in two different solvents

Solvent	Novozym 435		Molecular sieve 3A	
	q_{max} [g-water/g-solid]	K [g/L]	q_{max} [g-water/g-solid]	K [g/L]
2-Methyl-2-butanol	0.025	0.42	0.10	0.37
Acetone	0.045	0.42	0.16	0.13

3.4 Effect of Moisture Distribution on the Product Yield

The relationships between the yield of monomyristoyl trehalose and the initial (at 0 h reaction) and final (after 72 h reaction) moisture in 2-methyl-2-butanol and acetone were shown in Figure 7. The final moisture content in each solvent was calculated using Equation 3 with the initial process parameters for all experiments. As shown in Figures 7 (a) and (b), clear relationships between the yield and the initial moisture in both solvents could not be found, because the yield was affected by not only the initial moisture in the solvent but also the water content of immobilized enzymes and the concentration of added molecular sieves. On the other hand, the product yield was clearly correlated with the final moisture in each solvents (Figures 7(c) and (d)), which were calculated according to Equations 3 and 4; the resulting data are presented in Table 1. These results suggest that the effect of moisture in the experimental setup could be evaluated comprehensively by quantitative prediction of the state of moisture distribution to each component.

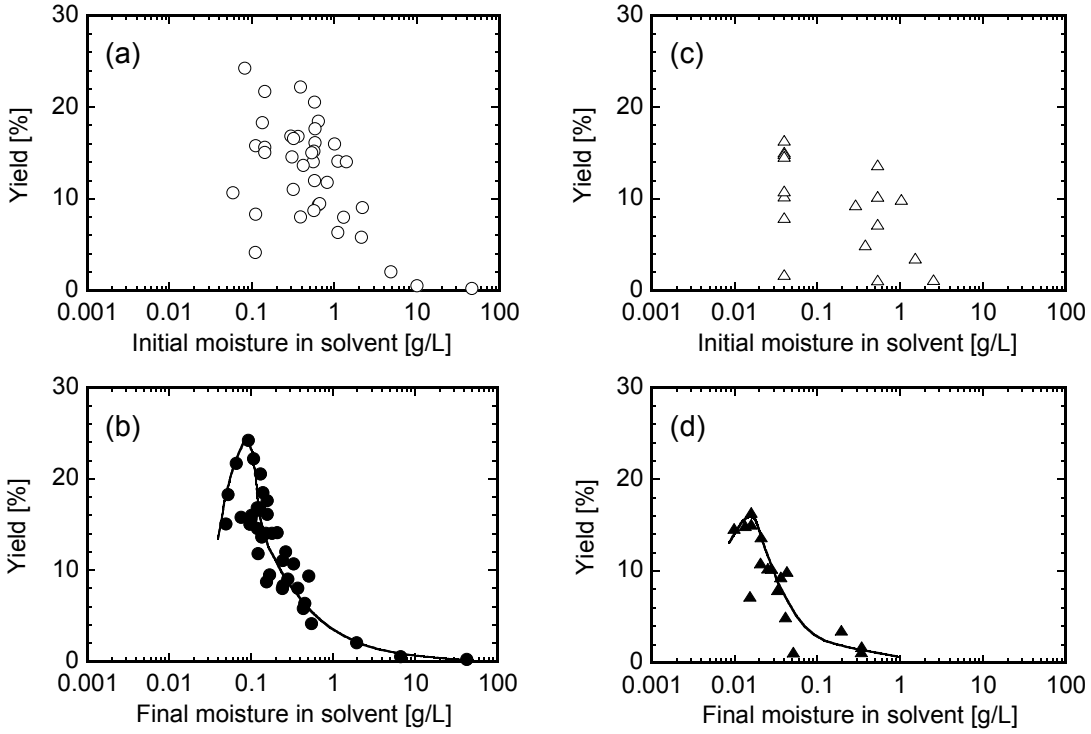

Figure 7. Relationships between free moisture at (a) (b) the initial (0 h) or (c) (d) final (after 72 h reaction) stages and the yield of monomyristoyl trehalose in (a) (c) 2-methyl-2-butanol, and (b) (d) acetone media. Values of final moisture in both solvents were calculated with equation (3) from each experimental condition

For both solvents, the product yield decreased with increasing free moisture when the final moisture in the solvent was high (> 0.1 g/L for 2-methyl-2-butanol, and > 0.02 g/L for acetone). Since water is a product of the condensation reaction, an increase in free moisture lowers the equilibrium concentrations of the condensed products. Therefore, the low yield obtained at high moisture content can be attributed to a shift in the reaction equilibrium towards hydrolysis, due to the existence of a large amount of free water molecules.

However, the product yield did not increase with the moisture decrease when it was very low (< 0.1 g/L for 2-methyl-2-butanol, and < 0.02 g/L for acetone); especially in the case of 2-methyl-2-butanol system, the yield decreased simultaneously with moisture. For synthetic reactions induced by immobilized preparations of CALBs, the initial catalytic activity and the conversion decreased at very low water content in previous studies (Kuroiwa et al., 2007; Gayot et al., 2003; Pepin & Lortie, 2001). These results cannot be explained in terms of reaction equilibrium. According to the literature, water molecules binding to enzyme molecules are critical for enzymatic activity in organic media; enzyme molecules have to be hydrated by a sufficient amount of water to maintain their active conformation and molecular flexibility (Rupley et al., 1983; Klibanov, 2001; Serdakowski & Dordick, 2007). The amount of such "essential" water for catalytic activity is around 0.4 g-water/g-enzyme, although the actual value depends on the enzyme (Rupley et al., 1983; Klibanov, 2001). The amount of such water approximately corresponds to the order of monolayer of water molecules on the surface of enzyme molecules, and in this state, several hundreds of water molecules bind to one enzyme molecule as previously calculated (Rupley et al., 1983). Based on these considerations, the conditions of low-moisture side from the peak of the product yield would correspond to the state that, at most, only several tens of water molecules are bound to one enzyme molecule with the assumption that Novozym® 435 contains 5 - 10% of protein (Gayot et al., 2003; Secundo, Carrea, Soregaroli, Varinelli, & Morrone, 2001). Therefore, the authors believe that the low product yield at low moisture content was due to the low catalytic activity of enzymes hydrated insufficiently.

4. Conclusions

The lipase-induced acylation of trehalose with a myristic acid was investigated, focusing on the effect of moisture in the experimental setup on the synthesis and yield of a trehalose-fatty acid ester. The acylation between trehalose and myristic acid by Novozym® 435 (a commercial lipolytic immobilized enzyme from *C.*

antarctica) in 2-methyl-2-butanol and acetone, so that synthesizing monomyristoyl trehalose. The yield of the reaction product was significantly affected by the moisture content of the solvent, the moisture content of the catalyst, and by the concentration of added molecular sieves. The effects of the operating conditions are discussed in relation to the quantitative distribution of moisture in the experimental setup, that is, moisture adsorbed either onto immobilized enzymes, or molecular sieves, and moisture content in the organic solvents. The changes in the yield of the synthetic product would be attributed to both the thermodynamic reaction equilibrium and the enzymatic activity in reduced-moisture organic solvents. In the present paper, we demonstrated that quantitative analysis of moisture in the system, as well as controlling process parameters are essential to elucidate the behavior of lipase-induced acylation in organic media with low moisture content. Actually, characteristics of the experimental setup, such as immobilized lipase, organic solvent and desiccant, are expected to play major roles on the enzymatic acylation. The technique and experimental results reported here may serve as basis for elucidating such multiphase systems, foreseeing the design of optimized processes for non-aqueous enzymatic catalysis. These considerations also should effective for designing continuous production process, such as packed-bed enzyme reactors feeding a non-aqueous substrate solution continuously.

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