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# Application of a lipase-immobilized silica monolith bioreactor to the production of fatty acid methyl esters

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ABSTRACT

We developed a highly efficient bioreactor loaded with a lipase-immobilized non-shrinkable silica monolith by adopting a two-step sol–gel method, i.e., preparing a methyltrimethoxysilane (MTMS)-based silica monolith followed by coating of the latter with more hydrophobic alkyl-substituted silicates that entrapped lipase. We applied this type of bioreactor to the production of fatty acid methyl esters through methanolysis of rapeseed oil in *n*-hexane by *Rhizopus oryzae* lipase. As the result of screening alkyltrimethoxysilanes mixed with tetramethoxysilane (TMOS) during sol–gel coating, propyltrimethoxysilane (PTMS) gave the best performance, and the lipase immobilized therein exhibited ca. 10 times higher activity than non-immobilized lipase powder. The amount of the PTMS-based silicates with which the MTMS-based silica monolith was coated was optimized by adjusting the molar ratio of silanes (mixture of PTMS and TMOS at 4:1) to 100 mol of water. Conversion rate was highest at the molar ratio of 0.4 mol silanes to 100 mol of water, which was ca. 10 times higher than that with lipase deposited on the MTMS-based silica monolith. Conversion rate was approximately 1.5 times higher in the flow-through monolith bioreactor than in the batch slurry reactor.



## 1. Introduction

Sol–gel silica-based monolithic columns have been widely used as supports for chromatographic separation and immobilization of enzymes [1–4]. Monoliths can be formed by filling a column with sol, and then allowing gelation to occur inside the column. Resulting columns offer large through-pores and significant void volumes owing to open spaces between particles that are of the order of μm. This provides low-pressure drop, and enables a rapid flow-through of the solution and resultant accelerated mass transfer within the monolith.

Lipases are versatile enzymes for many organic syntheses. They are highly activated and stabilized when encapsulated in alkyl-substituted silicates by the sol–gel method [5–14]. We previously attempted to develop a lipase-immobilized silica monolith bioreactor to apply sol–gel encapsulated lipases to a continuous flow-through system [15]. A non-shrinkable silica monolith was formed from a 4:1 mixture of methyltrimethoxysilane (MTMS) and tetramethoxysilane (TMOS) [16]. It comprised an aggregate of fine spherical particles with diameters of a few micrometers [16]. These supports were coarse porous materials, and had interparticle gaps of a few micrometers that were a conduit for substrate solutions. Void volume amounted to >80% of the entire volume of the monolith. MTMS-based silicate did not lead to an optimal support material for lipase to express its highest activity. The latter was usually attained when lipases were entrapped into more hydrophobic silicates derived from a mixture of TMOS and alkyltrimethoxysilanes such as propyltrimethoxysilane (PTMS) and *n*-butyltrimethoxysilane (BTMS) [7]. Preparing silica monoliths from these hydrophobic precursors was not possible. These findings forced us to apply a two-step sol–gel method, i.e., formation of a silica monolith from the MTMS-based silicate, then coating it with more hydrophobic silicates to entrap lipase. We demonstrated that, in the transesterification between glycidol and vinyl *n*-butyrate in isooctane, the lipase immobilized onto the resultant silica monolith exhibited approximately 10 times higher activity than that deposited on the MTMS-based silica monolith [15]. The continuous operation led to increased conversion rates due to enhancement of liquid–solid contact by forced flow of substrate solution through the interparticle gaps of the monolith compared with a batch slurry reaction system in which particles of crushed monolith-immobilized lipase were suspended [15].

Biodiesel is increasingly perceived as an important component of solutions. This is primarily due to shortages of fossil fuel and environmental pollution. Lipases offer a promising approach to enzymatic production of biodiesel. Comprehensive reviews

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describing the enzymatic approach to biodiesel production have been published recently [17,18]. Few researchers have used sol–gel silica-immobilized lipase [19–22], let alone the continuous silica monolith bioreactor.

In the present study, we applied the silica monolith bioreactor to the production of fatty acid methyl esters through the methanolysis of triglycerides. The test reaction was the esterification of rapeseed oil (40 mM) with methanol (120 mM) in 10 mL *n*-hexane by *Rhizopus oryzae* lipase F-AP15. We investigated the feasibility of applying the lipase-immobilized silica monolith bioreactor to this important reaction.

## 2. Experimental

### 2.1. Materials

Lipase F-AP15 originating from *R. oryzae* was purchased from Amano Enzyme Inc. (Nagoya, Japan), and was used without further purification. The protein content was determined as 6.1 wt% by Bradford assay using a commercial Bio-Rad dye and bovine serum albumin as the protein standard. The specific activity of the lipase was measured to be 1.73 U $mg^{-1}$ solid sample, where 1 U was defined as the amount of lipase that liberated 1 μmol of butyric acid per minute from tributyrin at pH 7 and 40 °C. Rapeseed oil was obtained from Riken Nosan-Kako Co. (Fukuoka, Japan). The composition of fatty acids in this triglyceride was reported by the manufacturer to be 4.2% palmitic acid, 61.6% oleic acid, 19.6% linoleic acid, and 10.1% linolenic acid. The methyl esters of these four fatty acids were obtained from Sigma–Aldrich Japan Co. (Tokyo, Japan), and used to determine the calibration curves of a gas chromatograph. TMOS, MTMS, PTMS, and other chemicals were reagent grade, and were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Ethyltrimethoxysilane (ETMS), *n*-butyltrimethoxysilane, and *n*-hexyltrimethoxysilane (HTMS) were kindly supplied by Dow Corning Toray Co. (Ichihara, Japan). Poly(ether ether ketone) (PEEK) tubes with an inside diameter of 1.6 mm were obtained from GL Sciences Inc. (Tokyo, Japan).

### 2.2. Preparation of a silica monolith derived from a mixture of MTMS and TMOS

At room temperature, a mixture of 3.44 mL MTMS and 890 μL TMOS (molar ratio of MTMS to TMOS, 4:1), 1.05 mL distilled water, and 45 μL 40 mM HCl were mixed in a test tube to form a homogeneous sol. After cooling the mixture to 4 °C, 9.85 mL 100 mM phosphate buffer (pH 7.5) was added. The molar ratio of silanes to water was 5:100 under this condition. PEEK tubes (1–4) of length 5 cm were placed into a test tube and filled with sol mixture. Gelation was allowed to proceed at room temperature for 1 day. The hydrogel formed was lyophilized for 1 day. This provided a non-shrinkable xerogel in the test tube and PEEK tubes.

### 2.3. Preparation of a lipase-immobilized silica monolith coated with alkyl-substituted silicates

A 10-mL sol solution comprising a mixture of alkyltrimethoxysilane and TMOS at a fixed molar ratio of 4:1 was prepared in the presence of lipase using the same procedure as mentioned above. Lipase solution (4 mL and pH 7.5) was added after 0.55 mL homogeneous sol was mixed with 5.45 mL 100 mM phosphate buffer solution (pH 7.5). In changing the molar ratio of total silanes to 100 mol of water in the range of 0:100 to 3.2:100, an increase in the volume of the total silanes was compensated by a decrease in the volume of 100 mM phosphate buffer solution. A mixture containing 0.6 or 1.2 g lipase was slowly poured into the MTMS-based silica monolith formed in the test tube, in which the silica monolith containing PEEK tubes were also fixed. The liquid–solid mixture was degassed under reduced pressure for 10 min to permeate sol solution into the interstices of the monolith. Gelation was allowed to proceed at room temperature for 1 day. The lipase-immobilizate coated with alkyl-substituted silicates was lyophilized for 1 day. The PEEK tube was retrieved, and the gel outside the PEEK tube (inside the test tube) was collected and crushed in a mortar. The mass of gels inside and outside the PEEK tube was measured, and the mass of lipase on each side calculated by assuming its uniform distribution in the gel. The silica monolith PEEK tube was used for flow-through experiments, and the crushed silica particles used for batchwise experiments.

### 2.4. Reaction experiments

Alcoholysis of 40 mM rapeseed oil with 120 mM methanol in *n*-hexane containing 0.01% (v/v) water was used as the test reaction.

The PEEK tube loaded with the silica monolith-immobilized lipase was provided with end fittings, attached to a semi-micro-HPLC pump (PU610, GL Sciences) and immersed in a constant temperature bath maintained at 35 °C. Substrate solution was fed at a volumetric flow rate of 0.02–0.4 mL $min^{-1}$ using the HPLC pump. Steady state at each flow rate was confirmed when the exit concentration of the fatty acid methyl esters produced became constant independent of the process time. Inactivation of lipase was not observed within the range of the process time measured.

Batchwise reaction experiments were carried out at 35 °C in 20-mL screw-capped vials on a shaking incubator maintained at 1600 $min^{-1}$. Reaction mixture comprised 40 mM rapeseed oil, 120 mM methanol, and 490–760 mg crushed silica monolith immobilizing 200 mg crude lipase suspended in 10 mL *n*-hexane containing 0.01% (v/v) water.

Organic samples were analyzed using a gas chromatograph (Shimadzu GC-14 B) equipped with a FSS ULBON HR-20 M capillary column. Concentrations of the four fatty acid methyl esters produced were quantified. Total ester concentrations were plotted against the ratio of the mass of crude lipase to the volumetric flow rate of the substrate solution, $W/v$, with the flow-through silica monolith bioreactor, and the product of the crude lipase concentration and the reaction time, $(w/V)t$, with the batch slurry reactor [15,16]. Activities of immobilized lipases and performance of the two reactors were compared by determining the initial reaction rates based on the unit mass of lipase calculated from initial slopes of the total ester concentrations vs. $W/v$ or $(w/V)t$ [15,16].

## 3. Results and discussion

We previously proposed a two-step sol–gel method, consisting of formation of a methyl-substituted silica monolith followed by coating of the latter with more hydrophobic alkyl-substituted silicates that entrapped lipase [15]. This methodology resulted in a high-performance silica monolith bioreactor that immobilized highly activated lipase.

We investigated the effect of alkyl groups in the second-step sol–gel silicates on the methanolysis activity of immobilized lipase. Fig. 1 compares the initial rates of formation of methyl esters with lipase immobilized in the silicates derived from a 4:1 mixture of each silane and TMOS. The molar ratio of the mixed silanes to water was 0.33:100, and activity measurements were carried out using a batch slurry reactor in which particles of the crushed silica monolith were suspended. Activities of non-immobilized lipase powder and lipase immobilized in inorganic silica derived from TMOS only are shown for comparison. Methanolysis activity increased with increase in chain length of alkyl groups. Maximum activity was

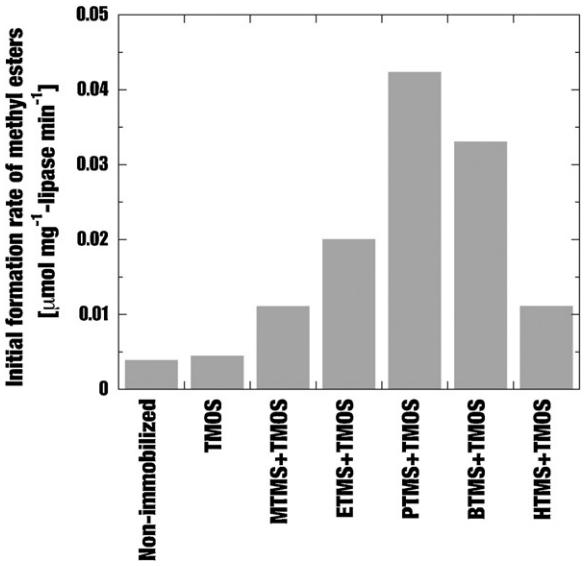


**Fig. 1.** Effect of alkyl groups in the second-step sol–gel silicates coated onto the MTMS-based silica monolith on the initial rate of formation of methyl esters. Molar ratio of each alkyltrimethoxysilane to TMOS, 4:1; molar ratio of the mixed silanes to water, 1/3:100. Particles (490–570 mg) of the crushed silica monolith containing 200 mg of crude lipase were suspended in a 10-mL reaction mixture. "Non-immobilized" means crude lipase powder.

attained with PTMS, giving approximately 10 times higher activity than that of non-immobilized lipase powder. This result was consistent with many reports evaluating the effect of chain length of alkyl groups [5–14]. In general, the propyl or butyl group seems to exert the most favorable hydrophobic interactions toward the active center of enzyme molecules such as lipases [5,7,10–14], protease [23], and cutinase [24] in organic solvents. PTMS was used as an organic silane precursor in subsequent experiments.

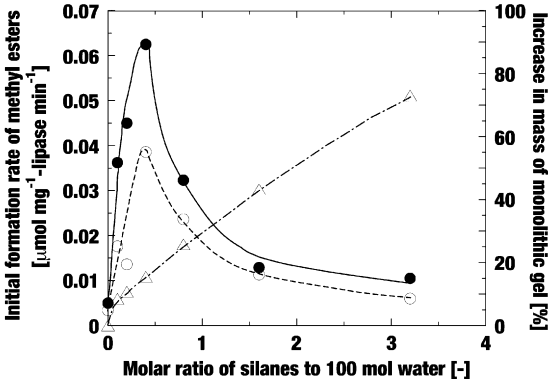


**Fig. 2.** Effect of molar ratio of the 4:1 mixture of PTMS and TMOS to water in the sol–gel coating of the silica monolith on the initial rate of formation of methyl esters. Closed circles: flow-through silica monolith bioreactor; size of PEEK tube, 1.6 mm inside diameter and 10 cm length; mass of crude lipase, 15.6–16.8 mg; mass of entire monolith, 45–52 mg; and volumetric flow rate of substrate solution, 0.028–0.166 mL min$^{-1}$. Open circles: batch slurry reactor; mass of crude lipase, 200 mg; mass of crushed monolith, 554–667 mg; and liquid volume, 10 mL. Open triangles: increase in the mass of entire monolith caused by the sol–gel coating with PTMS-based silicates.

The amount of the propyl-substituted silicates entrapping lipase formed onto the MTMS-based silica monolith was optimized. This was investigated by changing the molar ratio of the 4:1 mixture of PTMS and TMOS to water. That is, using different amounts of silanes; 0 (lipase deposited onto the MTMS-based silica monolith), 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mol per 100 mol of water. Fig. 2 shows the effect of molar ratio on initial rates of formation of methyl esters, based on the unit mass of lipase, in the silica monolith bioreactor and the batch slurry reactor. The increase in mass of the entire monolith caused by the second-step sol–gel coating is also stated. Reaction rates in both reactors were drastically increased with an increase in the amount of silanes, and reached a max-

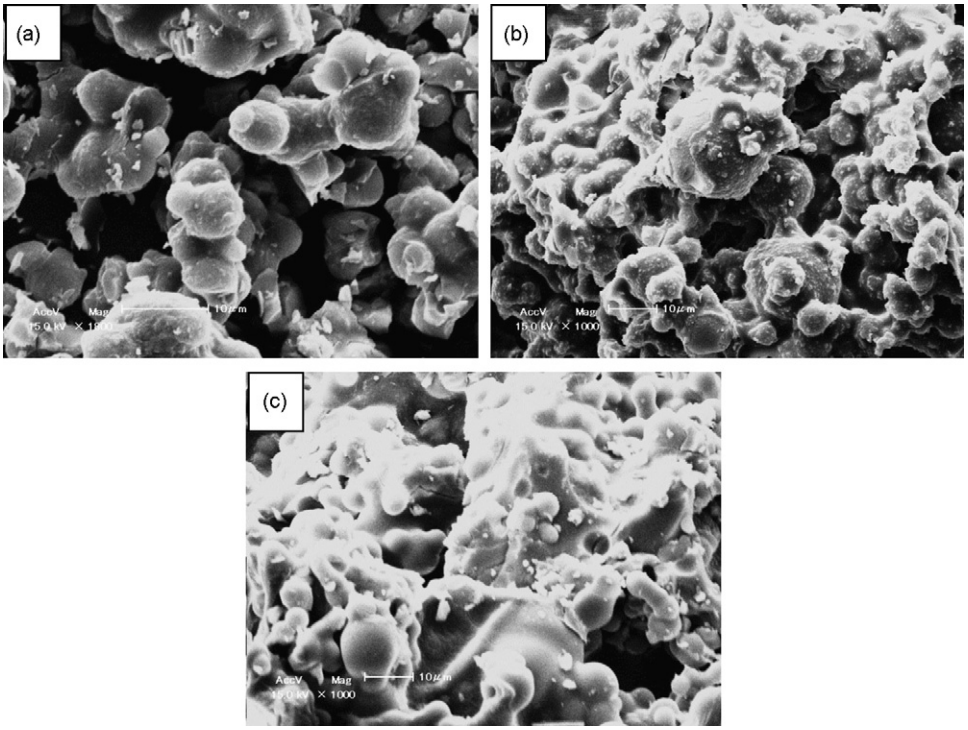


**Fig. 3.** Scanning electron micrographs of cross-sections through the silica monoliths coated with PTMS-based silicates entrapping lipase. Molar ratio of a 4:1 mixture of PTMS and TMOS to 100 mol of water: (a) 0.1 mol, (b) 0.4 mol, and (c) 3.2 mol.

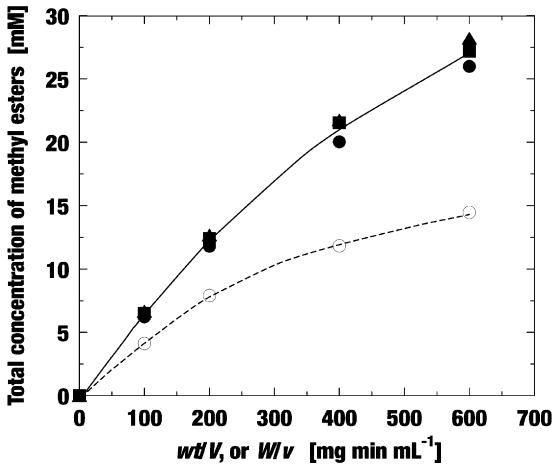


**Fig. 4.** Effects of reactor types (flow-through monolith bioreactor vs. batch slurry reactor) and length of monolith bioreactor on the production of methyl esters as a function of $W/v$ or $(w/V)t$. Closed circles, closed triangles, and closed squares: 5, 10, and 20 cm length of monolith bioreactors, respectively; mass of crude lipase, 9.5–38.8 mg; mass of entire monolith, 23–96 mg; and volumetric flow rate of substrate solution, 0.015–0.388 mL min$^{-1}$. Open circles: batch slurry reactor; mass of crude lipase, 200 mg; mass of crushed monolith, 493 mg; and liquid volume, 10 mL.

imum at the molar ratio of 0.4 mol silanes to 100 mol of water (corresponding to an increase of 15% in the mass of the entire monolith). Maximum activity was approximately 10 times higher than that of lipase deposited on the MTMS-based silica monolith (silanes:water, 0:100). This is consistent with a previous result [15], and may be ascribed to optimal hydrophobic interaction between lipase molecules and the alkyl groups of silanes. Further increase in the amount of silanes caused a significant decrease in reaction rates. This may be due to the embedding of lipase molecules and/or stoppage of the monolith void space by an excess formation of PTMS-based silicates. Fig. 3 shows scanning electron micrographs of the cross-section of the silica monolith covered with silicates prepared at the molar ratios of (a) 0.1 mol, (b) 0.4 mol, and (c) 3.2 mol silanes to 100 mol of water. A moderate amount of PTMS-based silicates covered the surfaces of the spherical particles that constituted the silica monolith (Fig. 3b), whereas an excess amount of silicates closed some of the interparticle gaps of the monolith (Fig. 3c). The sol–gel coating using 1.6 mol silanes to 100 mol of water caused an increase of 43% in the mass of the entire monolith (Fig. 2).

In almost all cases, reaction rate was higher in the flow-through silica monolith bioreactor than in the batch slurry reactor (Fig. 2). This is clearly shown in the time-dependent profiles of the production of methyl esters (Fig. 4). The most probable reason for the enhanced reaction rate would be efficient liquid–solid contact by a forced flow of substrate solution through the interparticle gaps of the monolith. Fig. 4 also shows the effect of tube length of the silica monolith bioreactor on the conversion vs. $W/v$. Conversions at a fixed value of $W/v$ were less influenced by an increase in reactor length, or an increase in the mass of enzyme, $W$. This means that conversions were independent of the flow rate of substrate solution, $v$, at a fixed value of $W/v$, so flow-through of substrate solution brought efficient liquid–solid contact and rapid mass transfer within the monolith.

## 4. Conclusions

We applied the silica monolith bioreactor immobilizing *R. oryzae* lipase to the enzymatic production of fatty acid methyl esters through methanolysis of rapeseed oil. The lipase exhibited the highest activity when immobilized in the PTMS-based silicates formed on the MTMS-based silica monolith, which corresponded to ca. 10 times higher conversion rates than those deposited on the MTMS-based silica monolith. The conversion rate was approximately 1.5 times higher in the flow-through monolith bioreactor than in the batch slurry reactor, probably due to forced flow-through the interparticle gaps of the monolith. It was demonstrated that the lipase-immobilized silica monolith bioreactor is highly efficient for bioconversions in organic media. Production of biodiesel from *jatropha* oil as non-edible oil stock in a solvent-free system is in progress.

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