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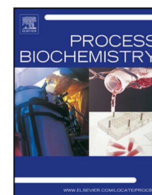

Improvement of synthetic activity and stability of a commercial lipase in a low-water system via immobilization of hydrated lipase aggregates

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ABSTRACT

Activation and immobilization of a commercial lipase from *Candida cylindracea* were investigated to improve its catalytic performance for esterification reaction between 1-octanol and three butyric acid derivatives in low-water solvent-free systems. Lipase powder was suspended in 1-octanol, which is the alcohol substrate of the esterification reaction, and then an appropriate amount of water (10% to 1-octanol in volume) was added to form hydrated lipase aggregates. Lipase exhibited high activity in this aggregated state, in contrast with the very low activity of nonhydrated lipase powder. We believe that activation using this hydration-aggregation pretreatment was based on interfacial activation at the 1-octanol-water interface. The hydrated lipase aggregates had paste-like morphology and could be immobilized on nonwoven fabrics by physical compression. Polypropylene exhibited the best specific activity among the tested nonwoven fabric materials. A stirred tank batch bioreactor was developed using lipase-immobilizing polypropylene nonwoven fabric sheets. This bioreactor stably maintained its reaction performance in repetitive-batch (maximum 30 times, for a total of 2000 h) esterification with three different acid substrates. The ester yields were over 90% throughout the all repetitive batches. During repeated reaction, the immobilized lipase maintained its water content at a constant level, thus exhibiting catalytic activity in nonaqueous reaction systems.

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1. Introduction

Lipase (E.C.3.1.1.3, triacylglycerol hydrolase) catalyzes both hydrolysis of esters, including oils and fats, and synthetic reactions to produce or modify various ester compounds. Therefore, lipase-catalyzed reactions have attracted much interest in the last two decades. Utilization of lipase for esterification, transesterification, and interesterification reactions in nonaqueous media is a promising alternative to organic synthesis, because of its regioselective and chiral selective reaction, environmentally friendly process, and safety. Therefore, application fields of lipases have recently been extended to various industries such as food, detergents, pharmaceuticals, fine chemicals, and bioenergy [1–4].

Low synthetic activity is problematic when using lipase in organic media with low water content. Many commercial lipases exhibit very low activity (or are inactive) in organic media without any pretreatment to activate them, mainly because of the interfacial properties of lipases [3,5]. Lipase is a well-known enzyme that exemplifies the interfacial activation phenomenon [6–9]. The three-dimensional structure of lipase determined by X-ray crystallography indicates that the active site in lipase molecules is covered with an α-helical fragment (known as the lid), suggesting that the lid is open at the oil–water interface [10].

Various techniques have been developed to improve the catalytic performance of lipases in low-water organic solvents. Previous studies for activating lipases included the use of immobilized lipases [3,11–16], biphasic systems [17], reversed micellar systems [18,19], surfactant-modified lipases [20–22], polyethylene glycol-modified lipases [23], and fatty acid-modified lipases [24–26]. Especially, the methods including enzyme immobilization is much advantageous for industrial application. Recently there have been various technique reported to activate and immobilize lipase using sophisticated materials such as hydrophobized gel particles [15,16,27,28], and nanomaterials including nanoscale fine particles [29,30]. Above techniques involve using chemical

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reagents, surfactants, polymers, or lipids to activate and stabilize lipases; thus, application is potentially limited because of the risk of chemical contamination by added reagents. In addition, some of these techniques require drying or lyophilization, which consumes more energy, to remove the solvent. Furthermore, recovery and reuse of activated lipase are not easy when lipase preparations are dispersed as fine particles or solubilized in organic solvents. Therefore, it is still impractical to obtain active, stable, and easy-to-reuse lipase preparations using energy-saving procedures.

In this study, we propose a novel method for activation and immobilization of a commercial lipase and for developing a highly stable bioreactor system involving activated lipase. Excellent immobilized preparations, such as Novozym® 435 from Novo Nordisk, are commercially available; however, less expensive biocatalysts are still desired from economical and practical perspectives. Therefore, activation and stabilization of commercial lipases are key technologies to develop efficient and feasible lipase-induced organic synthesis. With our method of activating lipases, we focused on the interfacial activation phenomenon of lipase via direct activation in an organic substrate by adding an adequate amount of water as well as controlling the water content of activated lipase preparations during long-term use. This method consists of two steps: (1) lipase powders are suspended in the liquid organic substrate and hydrated by adding water to form swollen aggregates, providing the organic substrate–water interface that is necessary to activate lipase and (2) the hydrated lipase aggregate is immobilized on nonwoven fabric and installed in a batch bioreactor. This procedure does not require any additives (e.g., surfactants, synthetic polymers, and chemical cross-linkers) that might cause chemical contamination of the reaction product. Evaporation and lyophilization of solvents are not included throughout the activation–immobilization process. Furthermore, during repeated esterification reactions, the hydration state of lipase was maintained adequately to exhibit high catalytic activity in the organic media. To our knowledge, any methods for preparing active, stable immobilized lipases that satisfied these criteria have not been reported. In this study, we evaluated the catalytic ability of the activated lipases and stability in long-term repetitive use of the immobilized lipase bioreactor to clarify the efficiency and usability of our technique. As model systems, we selected the condensation reaction of 1-octanol with different butyric acid derivatives. *n*-Octyl esters with various carboxylic acids are widely used in chemical, detergent, medical, and cosmetic industries. To study the effect of acyl donor substrate, we used 2-bromobutyric acid, *n*-butyric acid and 2-methylbutyric acid as model acids with different reactivity and polarity.

2. Experiment

2.1. Materials

Candida cylindracea (*Candida rugosa*) lipase, commercially designated as "Lipase OF," was obtained from Meito Sangyo Co., Ltd (Nagoya, Japan). This lipase has been established as an industrial lipase which is commercially available and has been widely used for food processing and synthesis of chemicals in many researches and industries. This white powdery enzyme product contained 44.9% of protein as determined using a protein assay kit (Bio-rad laboratory, Inc., Hercules, United States) with γ-globulin as standard protein, and was used without further purification. This lipase shows non-regioselectivity against triglyceride substrates and its specific activity values were determined as 360,000 and 1300 U/g for hydrolysis reaction of olive oil emulsion and *p*-nitrophenyl acetate, respectively (information from the supplier).

1-Octanol was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). D,L-2-bromobutyric acid, *n*-butyric acid, and D,L-2-methylbutyric acid were purchased from Tokyo Chemical Industry, Co., Ltd (Tokyo, Japan). They were used after removal of water using molecular sieves 3A (Wako Pure Chemical Industries, Ltd). All chemicals used were of extra pure or analytical grade.

Nonwoven fabric sheets made of polypropylene, polyacrylonitrile, wool, silk, nylon-6, vinylon, cotton, rayon (Japan Vilene Company Ltd, Tokyo, Japan), thick polyester (Kansai Felt Fabric Co., Ltd, Osaka, Japan), thin polyester, and polyester with active charcoal, aramide (Unitika Ltd, Osaka, Japan) were used as support materials for lipase immobilization.

2.2. Ester synthesis using free lipase powder

Lipase powder (4 g) was added to a mixture of 2-bromobutyric acid (11.8 mL, 111 mmol) and 1-octanol (88.2 mL, 555 mmol) that are placed in 300-mL reactor vessel (internal diameter 55 mm, actual working volume 100 mL). The water content of the reaction mixture was adjusted to 0–33% by adding 66.7 mM phosphate buffer (pH 7.7). The mixture was stirred using a turbine agitator with four flat blades (diameter 40 mm) at 250 rpm. The reaction temperature was kept at 37 °C using a water bath. Aliquots (1–3 mL) of the reaction mixture were withdrawn periodically and used as samples for analysis.

2.3. Ester synthesis using hydrated lipase aggregates immobilized on nonwoven fabric

Phosphate buffer (66.7 mM, 10 mL, pH 7.7) was added to the mixture of lipase powder (4 g) and 1-octanol (90 mL), and the

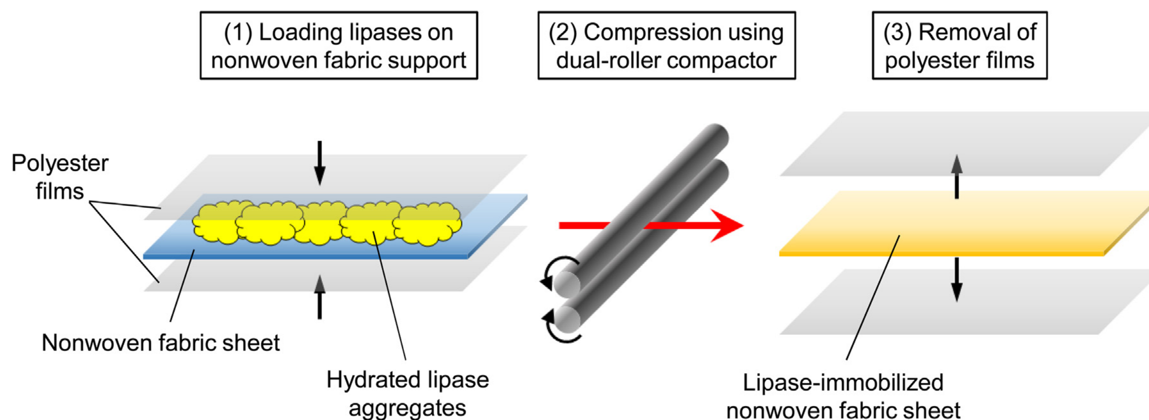

Fig. 1. Schematic illustration of the procedure for immobilizing the hydrated lipase aggregates on the nonwoven fabric supports.

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