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Lipase in biphasic alginate beads as a biocatalyst for esterification of butyric acid and butanol in aqueous media

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ABSTRACT

Esterification of organic acids and alcohols in aqueous media is very inefficient due to thermodynamic constraints. However, fermentation processes used to produce organic acids and alcohols are often conducted in aqueous media. To produce esters in aqueous media, biphasic alginate beads with immobilized lipase are developed for in situ esterification of butanol and butyric acid. The biphasic beads contain a solid matrix of calcium alginate and hexadecane together with 5 mg/mL of lipase as the biocatalyst. Hexadecane in the biphasic beads serves as an organic phase to facilitate the esterification reaction. Under optimized conditions, the beads are able to catalyze the production of 0.16 mmol of butyl butyrate from 0.5 mmol of butyric acid and 1.5 mmol of butanol. In contrast, when monophasic beads (without hexadecane) are used, only trace amount of butyl butyrate is produced. One main application of biphasic beads is in simultaneous fermentation and esterification (SFE) because the organic phase inside the beads is very stable and does not leach out into the culture medium. SFE is successfully conducted with an esterification yield of 6.32% using biphasic beads containing iso-octane even though the solvent is proven toxic to the butanol-producing *Clostridium* spp.

1. Introduction

Butyl butyrate (BB) is an ester which is commonly used as food flavor and flagrance due to its pineapple-like scent [1]. Recently, BB is also considered as an aviation fuel due to its unique fuel properties [2–4]. Traditionally, BB is synthesized by using Fisher–Speier esterification in which butanol and butyric acid are reacted in the presence of acid catalysts [5]. However, product degradation usually occurs due to the elevated reaction temperature (200–250 °C). The catalysts also need to be recovered before pure BB can be obtained [1,6]. In contrast, biocatalytic esterification can be conducted at 30 °C with a high yield (>90%) [7,8] and stereoselectivity [9]. Among all biocatalysts, lipase (triacylglycerol hydrolase, EC 3.1.1.3) is the most commonly used [10]. For instance, Borzeix et al. [11] reported that BB can be produced from 250 mM of butanol and butyric acid in hexane by using 0.7 g/L of lipase from *Mucor miehei*. The reaction was completed within 20 h at 40 °C, and the final yield was around 86%. Similarly, Welsh and Williams [12] employed lipase from *Candida cylindracea* to react 500 mM of butanol and 260 mM of butyric acid in hexane. After 24 h at 50 °C, around 217 mM of BB was produced from the reaction. Both works demonstrate that BB can be produced efficiently by using lipases from various sources.

One common element in the reactions mentioned above is that they were both conducted in organic media. This is because esterification in aqueous media is innately an equilibrium process, and excess water drives the equilibrium towards the hydrolysis rather than the synthesis of esters [7]. In addition, the presence of an organic solvent is also crucial to lipase activity. This is because most lipases have a polypeptide chain called 'lid'. When the lid is closed, the lipase active site is shielded from the substrates and this renders the lipase inactive. This explains why the formation of ester is negligible in pure aqueous media [13]. In contrast, when an organic phase is present, the lid will be opened for esterification [14].

However, performing esterification in aqueous solutions is attractive because some bacteria are able to produce both organic acids and alcohols during fermentation. For example, Mermelstein et al. [15] reported that *Clostridium acetobutylicum* ATCC 824 cultured with 90 g/L of glucose produced 9.5 g/L butanol and 6.7 g/L butyric acid. However, to recover butanol and butyric acid from the culture medium, separation processes such as distillation, gas stripping or extraction are needed [16–19]. Therefore, it would be interesting if butanol and butyric acid can be further reacted in the culture medium to produce BB, which has very low water sol-

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ubility (0.56 g/L at 30 °C) and can be recovered more easily from the culture medium [20]. Such idea has been explored by van den Berg et al. [4] recently. They showed that BB can be produced from butanol and butyric acid in a biphasic system consisting of hexadecane and an active growing *C. acetobutylicum* culture. At the end of the reaction, around 0.51 g/L of BB was concentrated in the organic phase. One major constraint here is the requirement of a separate organic phase. The solvent added has to be biocompatible since it is in direct contact with the growing cultures. Another restriction of the biphasic system is the limited interfacial area which slows down the reaction [21]. One way to increase the interfacial area is through emulsification [7]. However, emulsifying a growing bacterial culture is impractical because vegetative cells of *C. acetobutylicum* are unable to survive the harsh emulsification process [22].

In this study, a new biphasic system with an organic solvent and lipase entrapped in a solid matrix is investigated. Unlike the traditional biphasic system proposed by van den Berg et al., the organic solvent will not be in direct contact with the growing cultures. In addition, the interfacial area can be increased by dispersing the solvent within the matrix without affecting the bacterial growth. Herein, calcium alginate was chosen as the solid matrix because it is non-toxic towards bacterial cells and is often used in enzyme immobilization [8]. Unlike conventional alginate beads, this new type of biphasic beads contain uniformly dispersed organic solvent droplets within the alginate matrix. On the other hand, synthesis of BB is possible because the hydrophilic nature of alginate beads allows the diffusion of substrates (butyric acid and butanol) into the organic phase inside the beads.

2. Materials and methods

2.1. Materials and reagents

Candida rugosa type VII lipase (1179 U/mg) from Sigma–Aldrich (U.S.A.) was used throughout this study without further purification. Hexane (95%) and hexadecane (99%) were provided by Tedia (U.S.A) and Acros (U.S.A), respectively. Iso-octane (99%) was purchased from QRec (New Zealand). Ethylenediaminetetraacetic acid (EDTA) was provided by Bio-Rad (U.S.A.). Reinforced clostridia medium (Oxoid, U.K.) was used as the culture medium for fermentation. All other chemicals were obtained from Sigma–Aldrich (U.S.A.) unless stated otherwise.

2.2. Fabrication of calcium alginate beads

To prepare monophasic beads (without hexadecane), an aqueous solution containing 2 wt% of sodium alginate and 5 mg/mL of lipase was stirred for 30 min. The mixed solution was filled into a syringe, and dripped from a needle (21G) into a calcium chloride solution (5 wt%) for gelling. The beads were kept under agitation for 1 h to prevent agglomeration. Then, the beads were stored at 4 °C for setting. Before use, the beads were washed with copious amount of deionized water and dried at ambient conditions.

To prepare biphasic beads, 2 mL of hexadecane (equivalent to 10% v/v of the final alginate mixture) and 16 mL of 1% v/v of Triton X-100 solution were mixed together. Subsequently, the solution was homogenized with an ultrasonic processor (Vibra-Cell, Sonics, U.S.A.) until an emulsified solution was obtained. Sodium alginate and lipase were added into the emulsified solution to a final concentration of 2 wt% and 5 mg/mL, respectively. The same syringe-dripping method (described above) was used to prepare biphasic beads.

Scanning electron microscopy (SEM) was used to observe both the surface morphology and cross-sectional structure of the beads. First, the beads were sliced into half and dried in a freeze-drier (Christ, Germany) for 4 h. Subsequently, they were coated with a layer of platinum using a metal coating machine (JFC-1300, JEOL, Japan) for 1 min with a current of 20 mA. The coated beads were observed under a scanning electron microscope (JSM 5600LV, JEOL, Japan) with a silicon drift detector (INCA-x-act, Oxford Instruments, U.K.).

2.3. Standard esterification conditions for butyric acid and butanol

A standard reaction was carried out in 5 mL of aqueous solution containing 100 mM of butanol and butyric acid with 1 g of biphasic beads (~10,000 U lipase) at 35 °C under agitation (150 rpm). The initial pH of the solution was adjusted to 3 if necessary. In kinetic studies, the scale of the reaction volume was tripled due to frequent sampling. At the end of the reaction, the yield of BB was determined as follows:

$$\text{Yield} \quad (\%) = \frac{C_1 + C_2}{C_i} \times 100\% \tag{1}$$

where C_1 and C_2 are the amount of BB (mol) recovered from the aqueous solution and biphasic beads, respectively. C_i is the theoretical amount of BB produced (mol) if butanol is fully converted to BB.

For reactions in an emulsion system, 1 mL of hexadecane was added into an aqueous solution containing butanol and butyric acid (both in 100 mM) and 0.15% v/v of Triton X-100. An emulsion was created by using an ultrasonic processor. Finally, lipase solution (~10,000 U) was added to initiate the reaction.

2.4. Quantification of BB

To detect BB in an aqueous solution, 2 mL of hexane was added to the solution to extract BB, and the hexane was analyzed by using gas chromatography with a flame ionization detector (GC-FID). The GC-FID was equipped with a DB-WAXETR capillary column (length: 30 m, diameter: 0.32 mm), manufactured by Agilent (U.S.A.). Helium was used as a carrier gas. To detect BB trapped inside the alginate beads, the beads were first washed with deionized water and dissolved in 0.3 M EDTA solution [23]. After degrading the beads completely, BB was extracted with hexane and quantified by using GC-FID. To detect BB formed in an emulsified solution, the emulsion was disrupted by using centrifugation (14,000 × *g*). The top hexadecane layer was diluted and analyzed using GC-FID. All experiments were carried out in triplicates and the mean values were reported. The corresponding standard deviation values were indicated as error bars in the figures.

2.5. Optimization of biphasic beads

To improve the performance of biphasic beads, optimization was conducted by varying one parameter at a time. To optimize the standard protocol, the concentration of hexadecane was varied between 10 and 50%. Next, the lipase concentration in the beads was increased from 2.5 to 15 mg/mL. Esterification reactions were carried out in triplicates and the amount of BB produced were compared. A biphasic bead formulation (30% hexadecane and 5 mg/mL lipase) which gave the largest amount of BB was used to further optimize the pH values (between 2 and 9) and initial butanol concentrations (between 10 and 500 mM). Initial butyric acid concentrations were maintained at 100 mM in all experiments.