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Biocatalytic production of lactose ester catalysed by mica-based immobilised lipase

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ABSTRACT

Enzyme immobilisation technology is an effective means to improve sugar ester production through the employment of biocatalysts. In the present study, immobilisation of *Candida rugosa* (CRL) lipase onto amino-activated mica is performed *via* covalent bonding (namely Amino-CRL) and the cross-linking of lipases into nano-reactors through physical adsorption (namely NER-CRL). Free and immobilised lipases were tested for their esterification activities. Specific activities for Amino-CRL and NER-CRL increased by 2.4 and 2.6-fold, respectively, upon immobilisation. Extending this work, immobilised lipases have novel capabilities in the synthesis of sugar esters. The optimised conditions for sugar fatty acid ester syntheses are 48 h at 2:1 of molar ratio of lactose sugar to capric acid at 55 °C. Furthermore, a high operational stability with half-lives of over 13 and 10 runs was achieved for NER-CRL and Amino-CRL, respectively, indicating the efficiency of the immobilisation process.

1. Introduction

Sugar fatty acid esters were approved as food additives 40 years ago. Their initial application was as a foaming agent for cakes, leading to a great demand. Subsequent applications were developed, such as emulsifiers for oil and fat and processed milk products, including whipped cream and coffee whiteners (Watanabe, 1999).

Sugar fatty acid esters are odourless, non-toxic and are biodegradable; they also possess a wide range of hydrophilic–lipophilic balance (HLB) values, which extend their applications as emulsifiers, food additives, low calorific sweeteners and antimicrobial agents in the food and pharmaceutical industries (Adamopoulos, 2006; Ferrer et al., 2005; Ganske & Bornscheuer, 2005; Habulin, Sabeder, & Knez, 2008; Kennedy et al., 2006).

The synthesis of sugar fatty acid esters through enzyme-catalysed processes is notably more selective than using chemical catalysts (Plou et al., 2002). Today, lipases stand amongst the most important biocatalysts, used for novel reactions in both aqueous and non-aqueous media (Shah, Solanki, & Gupta, 2007). Owing to their ability to utilise a wide spectrum of substrates and high stability in extreme temperatures, pH and organic solvents, lipases find immense applications in various industries such as food and flavours, detergents, cosmetics, oil and fat processing and pharmaceuticals (Blanco, Terreros, Fernandez-Perez, Otero, & Diaz-Gonzalez, 2004; Schmid & Verger, 1998). In order to use them more economically and efficiently in aqueous and non-aqueous solvents, their activity, selectivity and operational stability can be modified by immobilisation. Furthermore, the extent of stabilisation depends on the enzyme structure, the choice of support and the immobilisation method.

Enzyme immobilisation on solid supports offers advantages over the free enzymes, due to the possibility of rapid termination of reactions, controlled product formation, ease of enzyme–product separation and reusability. Among the various immobilisation methods, physical adsorption is still the most frequently used method because it is easy to perform and inexpensive. Functionalisation of carriers is used as a strategy to increase and improve the adsorption of protein *via* adsorption through hydrophobic interactions between the support and lipases. In the presence of a hydrophobic interface, adsorption of lipase onto a hydrophobic interface promotes conformational changes on the lipase, leading to an 'open' form of the lipase. This unique characteristic is called 'interfacial activation', where lipases can be hyperactivated at hydrophobic interfaces of a matrix by removing the lid covering their active sites, which is necessary to enable free access of substrates to their active centres (Vaidya, Ingavle, Ponrathnam, Kulkarni, & Nene, 2008). However, covalent attachments through surface functionalisation

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and cross-linking of the lipases are still the most commonly used methods of immobilisation (Fernandez et al., 2005; Shah, Sridevi, Prabhune, & Ramaswamy, 2008).

Lipases have been adsorbed physically on numerous supports, including porous chitosan beads (Magnin, Dumitriu, & Chornet, 2003), anion exchange resins (Yesiloglu, 2005), $CaCO_3$ and layered double hydroxides (Abdul Rahman et al., 2004, 2008). Adsorption of lipase was also studied in three different phyllosilicates of sepiolite, palygorskite and montmorillonite by De Fuentes, Viseras, Ubiali, Terreni, and Alcantara (2001). In addition, the clay mineral of phyllosilicate, known as mica, was first used in our previous work (Zaidan et al., 2010). Accordingly, we have found that mica with almost zero cost, exhibits potential as a support in enzyme immobilisation in bioorganic synthesis. Enzymatic synthesis of fatty acid esters (Abdul Rahman et al., 2004, 2005) and palm-based esters (Gunawan, Basri, Abdul Rahman, Salleh, & Abd Rahman, 2005; Keng et al., 2009) using immobilised lipases has been extensively studied owing to their many practical advantages for industrial applications.

Enzymatic synthesis of sugar esters is achieved by the coupling of sugars with fatty acids in the presence of immobilised lipases under ambient temperatures. Due to the high cost of commercially available immobilised lipases, many attempts have been made to immobilise lipase onto low-cost supports for use as biocatalysts. However, the use of low-cost mica as a lipase support in the enzymatic synthesis of sugar fatty acid esters has never been reported elsewhere. The rationales of this work were to immobilise lipase onto mica through the different immobilisation approaches and to investigate their abilities and operational stabilities in catalysing the sugar esters syntheses.

2. Materials and experimental

2.1. Materials

Lipase from *Candida rugosa* (EC3.1.1.3) (1150 U/mg solid, with olive oil hydrolysis at pH 7.2), glutaraldehyde, aminopropyltriethoxysilane (Amino), lauric acid, capric acid, propanol and lactose monohydrate were purchased from Sigma–Aldrich. Other solvents used were of analytical grade (AR). Mica samples were collected from the region of Tanah Putih, Gua Musang, Kelantan (Malaysia). The mica (received as flakes) was milled in a planetary ball mill for 3 h to pass 100 μm mesh and was calcined at 120 °C for 4 h prior to use. Acid-activation of mica was conducted by stirring the sample in a solution of HCl for 60 min at 50 °C. Excess acid was removed by repeated water rinsing and the acid-activated mica was dried at 110 °C.

2.2. Lipase immobilisation procedures

2.2.1. Preparation of lipase solution

The commercial crude lipase from *C. rugosa* was partially purified through water extraction prior to immobilisation. Purification was carried out by dissolving 1.5 g of crude commercial lipase in 15.0 mL of distilled water followed by stirring for 30 min. The mixture was then centrifuged at 10,000 rpm for 15 min at 4 °C. The undissolved solid suspension was discarded after centrifugation while the supernatant was used as partially purified lipase prior to immobilisation.

2.2.2. Preparation of Amino-CRL

Activation of mica with amino-silane group was carried out *via* silylation, as reported in a previous work (Zaidan et al., 2010), prior to immobilisation. Accordingly, preparation of the Amino-CRL was as follows: 2.0 g of amino-activated mica were dispersed in 15.0 mL of *C. rugosa* lipase (CRL) solution at room temperature by continuous agitation at 100 rpm for 1 h. The possible adsorption of lipase-support at this stage was performed by covalent bonds between the enzyme carboxylate groups and the amino groups of the activated mica. The immobilised lipase was separated by filtration through Whatman No.1 filter paper before being dried and lyophilised.

2.2.3. Preparation of NER-CRL

Lipase solution was absorbed onto 2.0 g of acid-activated mica at room temperature by continuous agitation at 100 rpm for 1 h. At the end of the reaction, the lipase-loaded support solution was treated with glutaraldehyde (5% aqueous solution) and stirred for 17 h. The immobilised lipase was separated by filtration through Whatman No. 1 filter paper before being dried and lyophilised.

2.3. Protein assay

Protein determination was carried out according to the Bradford method, using bovine serum albumin as a standard (Bradford, 1976). The amount of bound protein was indirectly determined by comparing the difference between the amount of total protein (21.5 mg protein) introduced into the supports and the amount of protein both in the filtrate and in the washing solutions after immobilisation. The efficiency of protein immobilisation was investigated as follows (Eq. (1)):

$$\text{Protein immobilisation } (\%) = (\text{Amount of protein introduced}/m) \times 100\% \quad (1)$$

2.4. Esterification activity assay

This enzymatic esterification system consisted of lauric acid (2.0 mmol), propanol (4.0 mmol) and lipase preparations (containing 2.14 mg protein in 0.3 g of free lipase) in hexane (10.0 mL). A control containing the same amount of substrates and solvent, but without the addition of a biocatalyst, was simultaneously prepared. The reaction mixture was incubated at 40 °C for 2.5 h, with continuous shaking at 150 rpm in a horizontal water-bath shaker. The reaction was terminated by the addition of 5.0 mL ethanol:acetone (1:1, v/v) mixture and the biocatalyst was filtered out. The remaining free fatty acid in the reaction mixture was determined by titration with 0.15 M NaOH. The specific activity was expressed as μmol of ester formed per min per mg protein, calculated using the following formula (Eq. (2)):

$$\text{Esterification activity} = [(V_{\text{control}} - V_{\text{sample}}) \times M/V_{\text{reaction}}]/t \times W \quad (2)$$

where V_{control} = average titration volume of control (mL), V_{sample} = average titration volume of sample (mL), M = molarity of NaOH, V_{reaction} = volume of reaction (mL), t = time (min) and W = amount of protein (mg).

2.5. Synthesis of sugar fatty acid esters

2.5.1. Effects of various saccharides

The enzymatic esterification reaction consisting of sugar and capric acid (molar ratio of 1:1), molecular sieve (0.3 g) and lipase preparations (containing 2.14 mg protein content) in acetone (10.0 mL) incubated at 50 °C for 48 h with a shaking speed of 250 rpm. Saccharides used were lactose, trehalose, ribose and mannose. The conversion percentage was calculated from the remaining free fatty acid in the reaction mixture, as determined by titration with 0.15 M NaOH.