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Application of agriculture waste as a support for lipase immobilization

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ABSTRACT

Agricultural production contributes considerable waste generating green house gases and impacting environmental quality. This study describes a use for agricultural waste residues as a support for immobilization of *Acinetobacter baylyi* lipase (ABL), a solvent-stable lipase. Six agricultural waste residues were evaluated for their abundances, morphological characteristics, and immobilization efficiency. *Salacca wallichiana* stem had the highest immobilization efficiency. Immobilized ABL had a lower optimum temperature compared to the suspended enzyme and showed remarkable solvent stability. Reusability for palm oil hydrolysis of immobilized ABL was comparable to that for commercial lipase, Novozyme 435. Interestingly, ABL immobilized on *S. wallichiana* stem showed improved storage stability at room temperature (25–32 °C).

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1. Introduction

Thailand has approximately 21 million hectares or 41% of the country's total area for agriculture from which several hundred million tons of waste is produced annually (Office of Agricultural Economics, 2004; Prasertsan and Sajjakulnukit, 2006). Some waste is recycled into fertilizer with the remainder unused and, in many instances, posing a disposal problem. Waste generated by harvest and post-harvest operations from agricultural industries is usually burned or used as landfill. Waste disposal is a major problem. In addition, leakage and improper storage of agricultural waste can produce ammonia and methane, both contributors to global greenhouse gas emissions (European Environmental Agency, 2006). One solution is to recycle these wastes as alternative useful materials.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that are typically used in biocatalysis and organic chemistry (Hasan et al., 2006; Reetz, 2002). Industrial applications of lipolytic enzymes include the production of optically active compounds for the agrochemical and pharmaceutical industries, and biodiesel (Fang et al., 2006; Hasan et al., 2006). Lipases such as those produced by extremophilic bacteria (Gupta and Khare, 2009; Sardessai and Bhosle, 2002) are tolerant of organic solvents, thermally stable and catalyze a variety of important reactions. We recently isolated a benzene-tolerant bacterium, *Acinetobacter baylyi*, from marine sludge in Thailand that secretes a solvent-stable lipase capable of catalyzing transesterification of palm oil to fatty acid methyl esters (Uttatree et al., 2010). A suspended form of enzymes usually exhibits lower stability and is resistant to enzyme recovery and re-use, severely restricting its practicality (Wang et al., 2006). The use of immobilized enzymes may overcome many of these issues within industrial applications, as enzyme immobilization can lead to enhanced enzyme stability, activity and recovery, leading to significant economic benefits for the process (Salis et al., 2003). High cost of commercial support is encouraging searches for cheaper substitutes. In this study, agricultural waste residues were evaluated for their immobilization of lipase from *A. baylyi* (ABL) by simple adsorption. Lipolytic activity and stability of the immobilized ABL were investigated in comparison to reported values for the suspended ABL (Uttatree et al., 2010) and commercial lipase, Novozyme 435.

2. Experimental

2.1. Materials

Agricultural waste residues (corn cob, rice hulls, banana stalk, *Wodyetia bifurcata* A.K. Irvine leaves, coconut husks and *Salacca*

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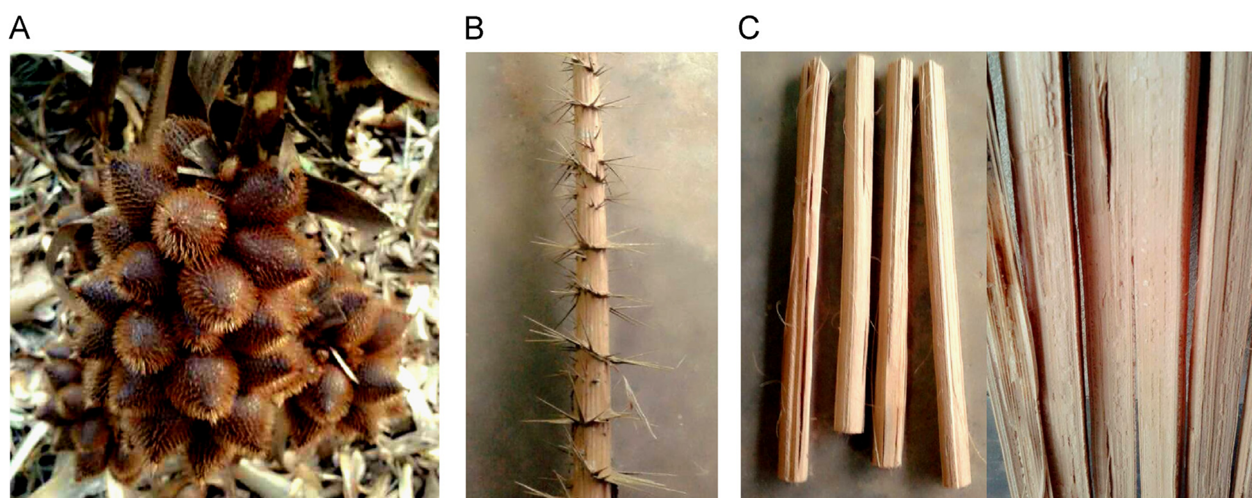

Fig. 1. (A) Fruits and (B) stem of *Salacca wallichiana* used in this study. (C) *Salacca wallichiana* stem after removal of the spines and inside surface.

wallichiana stem, Fig. 1) were obtained from fruit gardens in Chanthaburi, Thailand. *Candida antartica* lipase B immobilized on macroporous acrylic resin (Novozyme 435) was purchased from Sigma-Aldrich (Tokyo, Japan) and classified as belonging to the triacylglycerol hydrolases (EC 3.1.3.3), with a declared activity of ≥ 10,000 U/g (propyl laurate units per gram). All other chemicals were of analytical grade.

2.2. Bacterial strain and culture condition

The bacterial strain used in this study, *A. baylyi*, was isolated from marine sludge in Thailand (Uttatree et al., 2010). Lipase was produced in 1-l of 0.2 × Luria-Bertani (LB) (Sambrook et al., 1989) medium supplementing 0.8% (v/v) of Tween80 as an inducer (Uttatree and Charoenpanich, 2011) at 25 °C and 150 rpm for 15 h. When cell growth reached late exponential phase, the culture broth was collected for centrifugation at 4 °C and 9000 rpm for 20 min. The supernatant containing extracellular lipase was purified as described previously (Uttatree et al., 2010).

2.3. Lipase activity assay and protein determination

Hydrolytic activities of suspended and immobilized lipase were related to spectrophotometric absorption (410 nm) promoted by the hydrolysis of pNPP (Sigma, Germany) (Pencreac'h and Baratii, 1996). One unit (U) of enzyme was equated to the amount releasing 1 μmol of p-nitrophenol (pNP) per minute under assay conditions. Amount of pNP was calculated from a standard curve (Sigma, Germany). Also, hydrolysis towards palm oil was determined as described previously using 50 mM NaOH and phenolphthalein as titrant and indicator, respectively (Yan et al., 2007). One unit of lipase activity was defined as the amount associated with the release of 1 μmol of free fatty acid per minute under test conditions. Enzyme activity of immobilized lipase is expressed as enzyme units/g of support.

Total protein concentration was determined spectrophotometrically according to Bradford (1976) using Bio-Rad assay reagents (Hercules, USA). The protein standard was bovine serum albumin. Lipase loading efficiency was defined as the percentage of enzymes on the supporter based on protein concentration before and after immobilization.

2.4. Preparation of immobilized lipase from A. baylyi

Agricultural waste residues (10 g) were immersed in 3 l of 0.8% (w/v) NaCl for 30 min followed by washing with an equal volume of deionized water (3 times) and sterilized in an autoclave (15 min). Then, residues were dehydrated at 65 °C for 7 days, blended and sieved to retain particles between 0.1 and 0.5 mm. Retained particles (5 g) were pretreated with 2 N HCl (500 ml) and neutralized by washing 3 times with equal volume of phosphate buffer (pH 7.0). Thereafter, supports were dried at 60 °C for 24 h. A specified amount of support (1 g) was suspended in 100 ml of ABL solution (0.5 mg/ml) prepared in 20 mM phosphate buffer (pH 7.5) and stirred (350 rpm) for 6 h at room temperature. Immobilized supports were separated by filtration and washed thoroughly with 20 mM phosphate buffer (pH 7.5) and distilled water (100 ml each). Supernatants and washings were assayed for lipase activity and protein. Protein bound on supports was calculated as the difference between that loaded and free in supernatants and washings. Immobilized supports designated as immobilized ABL were dried in a desiccator at room temperature and finally, activities were assayed from hydrolysis of palm oil. During immobilization with the selected possible support, parameters were optimized (speed 100–400 rpm, pH 3.0–11.0, ionic strength 10–50 mM, enzyme loading 0.1–0.5 mg/ml, time 1–4 h, and temperature 10–50 °C). Morphological characterization of the supports was done by scanning electron microscopy (SEM) with Leo1450VP and energy dispersive spectroscopy (EDS) (Leo, Eindhoven, Netherlands). The analysis parameters were pressure 1.3×10^{-2} Pa and resolution < 10 nm at 15 kV.

2.5. Characterization of immobilized lipase

Hydrolytic activity of immobilized ABL was analyzed using palm oil as a substrate over pH and temperature ranges of 3.0–13.0 (37 °C) and 20–80 °C (pH 9.0), respectively, to identify favorable conditions for the lipase reaction. Buffer systems were acetate (pH 3.0–5.0), phosphate (pH 6.0–7.0), Tris–HCl (pH 7.0–9.0) and carbonate (pH 9.0–13.0). For solvent stability, immobilized ABL was mixed with an equal volume of each selected organic solvent to prepare the 50% organic solution. Mixtures were shaken and incubated at 37 °C for 6 h at 150 rpm. The solvent contained in the mixture was partially eliminated by evaporation at 37 °C for 5 min. Residual lipase activity was measured at 37 °C and pH 9.0 and compared to that of the control (no solvent).

2.6. Reusability and storage stability of immobilized lipase

Hydrolysis activity towards palm oil by immobilized ABL was monitored as described above. After the reaction, the immobilized ABL was collected by filtration, washed 3 times with cold-acetone

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