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Characterization, optimization and stability studies on *Candida rugosa* lipase supported on nanocellulose reinforced chitosan prepared from oil palm biomass

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ABSTRACT

The contribution of chitosan/nanocellulose (CS-NC) to the enzymatic activity of *Candida rugosa* lipase covalently bound on the surface of CS-NC (CRL/CS-NC) was investigated. Cellulosic material from oil palm frond leaves (OPFL) were bleached, alkaline treated and acid hydrolyzed to obtain the purified NC and used as nano-fillers in CS. XRD, Raman spectroscopy and optical fluorescence microscopic analyses revealed existence of strong hydrogen bonds between CS and the NC nanofillers. The CRLs were successfully conjugated to the surface of the CS-NC supports *via* imine bonds that occurred through a Schiff's based mechanism. Process parameters for the immobilization of CRL were assessed for factors temperature, concentration of glutaraldehyde and pH, to afford the highest enzyme activity to achieve maximum conversion of butyl butyrate within 3 h of incubation. Conversion as high as 88% was reached under an optimized condition of 25 °C, 0.3% glutaraldehyde concentration and buffer at pH 7. Thermal stability of CRL/CS-NCs was 1.5-fold greater than that of free CRL, with biocatalysts reusability for up to 8 successive esterification cycles. This research provides a promising approach for expanding the use of NC from OPFL for enhancing enzyme activity in favour of an alternative eco-friendly means to synthesize butyl butyrate.

1. Introduction

The current practice of passive dumping and open burning of unwanted matured oil palm fronds in large plantations are not only aesthetically displeasing [1,2], but contributes to degraded regional air quality along with increased health problems [3]. Such methods for disposing large quantities of agricultural biomass are environmentally challenging [4] as well as unsustainable in the long run. Moreover, the full benefits of oil palm biomass (OPF) are not fully explored as the palm oil fronds leaves (OPFL) is a good source of renewable cellulosic materials *viz.* cellulose, hemicelluloses and lignin [5,6]. In recent years, these materials have found commercial biotechnological importance due to their biocompatibility, biodegradability, low cost [5,7] and low carbon dioxide release [8]. In view of the major drawbacks in current methods for disposing OPF biomass, development of new technological applications which expands the use of OPFL merits attention of the scientific community. Such move also fulfils the "Zero Waste" initiative outlined by the Malaysian Palm Oil Board [9] and transforms this broadly available unwanted biomass into a functional material that has potential industrial applications.

Herein, we report the development of biocompatible and eco-friendly immobilization support that uses nanocellulose (NC) extracted from the cellulosic materials of OPFL as nanofillers in the CS biopolymer (CS-NC). The influence of CS-NC support to the enzymatic activity of covalently bound *Candida rugosa* lipase (CRL) for improved enzymatic esterification of butyl butyrate was investigated. The irreversibility of covalent binding is favorable for biocatalysts preparation due to the formation of stronger linkages between the enzyme and support material which prevents the leaching of enzymes activity [10]. Enzyme inactivation associated with conformational change can be averted which eventually improve the enzyme activity. Compared to inorganic polymers, the polymers fabricated from naturally source macromolecules are more biocompatible [11]. Correspondingly, incorporation of NC into the CS polymer network was hypothesized to reduce the internal mobility of the β–(1,4)-glycosidic bonded D-glucopyranose subunits in CS. The resultant CS-NC bi-polymer blend would be less fragile than the

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homopolymeric CS due to additional intermolecular network of hydrogen bonds that formed between the CS and NC [12]. The operational stability and recyclability of CRL covalently bound to the surface of CS-NC can prospectively be extended over the free lipases. This has been linked to the immobilization protocol that covalently binds the lipase to hydrophobic surfaces. This in turn, leads to rigidification of the CRL protein structure attributable to intense cross-linking of the enzyme structure to the support surface. By immobilization onto a suitable support, the native structure and catalytic properties of lipase is better preserved even under drastic conditions [13,14]. Moreover, the hydrophobic lid that extends over the active site of CRL is triggered to open only when there are interactions with a suitable hydrophobic surface [15]. Furthermore, studies that explore the use of CS-NC for enzyme immobilization remained unreported and the feasibility of CS-NC to improve the catalytic efficiency of CRL remains unknown.

In continuation of our research interest in esterification reactions [16–19] in favour of non-toxic, reusable and facile recovery of the biocatalysts, we hereby describe a greener protocol to produce butyl butyrate, an additive in biodiesel. Current commercial Fisher-Speier method produces low yields of the ester 50% [20,21] and is environmentally unfriendly. Other flaws associated are the need of a high reaction temperature (250 °C), the use of corrosive acid catalysts, liberation of large amounts of harmful by-products and tedious downstream processes [16–19]. Several strategies have been explored to improve the enzymatic synthesis of butyl butyrate. Nonetheless, the results are far from satisfactory [20–22] and there is still much room for improvements. Considering these issues, the homogeneous acid catalysts were substituted with CRL/CS-NC to carry out the esterification to synthesize butyl butyrate. CRL has numerous advantages in comparison to other enzymes to synthesize butyl butyrate, showing high activity and broad specificity in reaction medium [17]. The enzyme specifically catalyzes reactions *viz.* hydrolysis, transesterification, esterification and interesterification [17]. Utilization of CRL/CS-NC would facilitate biocatalysts recovery and reusability, and prospectively reduce downstream processing [23].

Availability of efficient and cost-effective biocatalysts, as well as the associated protocol of preparation is key aspects for the competitiveness of the enzyme-assisted process. In this frame, it was therefore necessary to optimize the protocol for immobilizing CRL onto the CS-NC support. The study aimed to improve the activity and the operational stability of the immobilized CRL over those of the free forms. Optimization of immobilization parameters was performed for temperature of immobilization, concentration of crosslinker *i.e.* glutaraldehyde and pH of the immobilization buffer for maximum yield of butyl butyrate in the shortest duration. Glutaraldehyde was selected for this study in view of its versatility and wide utilization as crosslinker for immobilizing different kinds of enzymes [24]. The preferential use of glutaraldehyde for such application is related to its low cost, commercial availability, high reactivity, rapidly reacts with amine groups at neutral pH as well as being more thermally and chemically stable than other aldehydes [25]. A well though-out decision on enzyme-glutaraldehyde ratio and their concentrations is vital to ensure that insolubilization of the enzyme will avert distortion of the enzyme structure while retaining its catalytic activity [24,25]. In this study, we discovered that the use of CS-NC as support resulted in significant improvements in catalytic activity of the lipase, hence maximizing yield of butyl butyrate. A high yield of the ester was achieved in 3 h using CRL/CS-NC as the biocatalysts.

2. Materials and methods

2.1. Chemicals and materials

The oil palm frond leaves (OPFL) were collected from an oil palm plantation within the grounds of Universiti Teknologi Malaysia (UTM). Analytical grade chemicals *viz.* butanol, butyric acid, *n*-heptane, cyclohexane, methanol, acetic acid, sodium chlorite and glutaraldehyde were purchased from Qrec Chemicals (New Zealand). *Candida rugosa* lipase (CRL) Type VII (≥700 U/mg), chitosan (CS) flakes and phenolphthalein were acquired from Sigma-Aldrich (St, Louis, USA). Distilled water was used without further purification.

2.2. Isolation of nanocellulose, beads production and covalent crosslinking of CRL onto the beads

OPFL were bleached, alkaline treated and acid-hydrolyzed to obtain the purified nanocellulose (NC). The suitability of extracted cellulose as nano-fillers in the preparation of chitosan/nanocellulose (CS-NC) supports was tested to immobilize *Candida rugosa* lipase (CRL) to produce the CRL/CS-NC biocatalysts. The procedure of nanocellulose isolation from oil palm biomass, the beads production and immobilization process was conducted following the method describe in our previous study [26].

2.3. Characterization of CRL/CS-NC

2.3.1. X-ray diffraction (XRD)

The X-ray diffraction analysis patterns were measured for samples of CS, NC and CS-NC beads using a powder X-ray diffractometer (D/max 2200, Rigaku, Japan) equipped with Cu K_α radiation source (λ = 0.154 nm) operating at 40 kV and 30 mA. The XRD patterns were recorded over the angular range $2\theta = 10°–40°$ at an increment of $10° \text{ min}^{-1}$. The crystallinity index was calculated from the heights of the 002 peak (I_{002}) and the intensity minimum between the 002 and 101 peaks (I_{am}) using the Segal method [27] (Eq. (1)). I_{002} represents both the crystalline and amorphous material, whereas I_{am} signifies only the amorphous material.

$$\text{Crystallinity index} = \frac{I_{002} - Iam}{I_{002}} \times 100 \quad (1)$$

2.3.2. Raman spectroscopy

CS, CS-NC, free CRL and CRL/CS-NC samples were examined using a Raman LabRAM HR Evolution (Horiba Scientific, UK) in the 200–1800 cm^{-1} region. A 633 nm solid state laser (12 mW) was focused over a membrane circle area using a Raman microprobe equipped with a 10× eyepiece. The spectrophotometer dispersed the scattered light while the charge-coupled devices (CCD) chamber detected the light with a spectral resolution of 3 cm^{-1}. The system was calibrated using Si spectra at 520 cm^{-1} before and after measurement. All Raman spectra were recorded at room temperature.

2.3.3. Fluorescence optical microscopy

To visualize the distribution of NC within the CS-NC bi-polymer matrix and for observing CRL molecules on the surface of CS-NC, a fluorescence optical microscopy (Olympus BX53) was used. The signals from the CRL proteins and NC were distinguished by tagging the NC with Rhodamine B (λ_{em} = 570 nm) and the CRL with fluorescein isothiocyanate (FITC) (λ_{em} = 490 nm), before exposure to a Fluorescence Illuminator with an Hg Lamp 100 W to excite the fluorophore. The stained lipase was then used for the subsequent immobilization process. Tagging of the CRL with FITC used dry fluorescein (0.2) mg that was directly added into the purified CRL solution (10 mg/mL). The mixture was left to stand for 4 h to allow the FITC to react with the amino groups on the CRL. The tagging process was carried out at in a chiller at 4 °C to minimize hydrolysis of the dye. Unbound FITC and CRL were removed by repeated rinsing with copious amounts of phosphate buffer (0.05 M, pH 7), before filtering through a Whatman No. 1 filter paper to separate the CRL/CS-NC beads. The tagged CRL/CS-NCs were left to dry in a desiccator overnight at room temperature. In all fluorescence optical