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Statistical optimization and operational stability of *Rhizomucor miehei* lipase supported on magnetic chitosan/chitin nanoparticles for synthesis of pentyl valerate

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ABSTRACT

The chemical-catalyzed transesterification process to produce biofuels i.e. pentyl valerate (PeVa) is environmentally unfriendly, energy-intensive with tedious downstream treatment. The present work reports the use of *Rhizomucor miehei* lipase (RML) crosslinked onto magnetic chitosan/chitin nanoparticles (RML-CS/CH/MNPs). The approach used to immobilize RML onto the CS/CH/MNPs yielded RML-CS/CH/MNPs with an immobilized protein loading and specific activity of 7.6 mg/g and 5.0 $U \cdot g^{-1}$, respectively. This was confirmed by assessing data of field emission scanning electron microscopy, X-ray diffraction, thermal gravimetric analysis and Fourier transform infrared spectroscopy. A three-level-four-factor Box-Behnken design (incubation time, temperature, substrate molar ratio, and enzyme loading) was used to optimize the RML-CS/CH/MNP-catalyzed esterification synthesis of PeVa. Under optimum condition, the maximum yield of PeVa (97.8%) can be achieved in 5 h at 50 °C using molar ratio valeric acid:pentanol (1:2) and an enzyme load of 2 mg/mL. Consequently, operational stability experiments showed that the protocol adopted to prepare the CS/CH/MNP nanoparticles had increased the durability of RML. The RML-CS/CH/MNP could catalyze up to eight successive esterification cycles to produce PeVa. The study also demonstrated the functionality of CS/CH/MNP nanoparticles as an eco-friendly support matrix for improving enzymatic activity and operational stability of RML to produce PeVa.

1. Introduction

The increasing energy demand coupled with the aggressive and irresponsible use of non-renewable energy resources (i.e. gas and oil) have resulted in perilous consequences in the energy race. The unwarranted emission of carbon dioxide [1] over the past few decades has negatively impacted the environment and instigated significant global climatic changes [2]. Hence, carbon-lean energy resources e.g. biofuels, ethanol produced from fermentation of sugars [3] and hydrolysis of vegetable oils [4] were introduced to lessen the impact of fossil fuels on the environment. Certain countries have begun using valeric biofuels viz. butyl and pentyl valerate (PeVa) as additives to fossil fuels. PeVa is fully compatible with gasoline and diesel compression ignition of existing engines, vehicles and infrastructure, with practically no changes [6,7]. However, the switch to biofuels remains controversial due to concerns related to competition with food consumption and the high cost of edible oil [5]. The chemically-catalyzed production of PeVa also has drawbacks viz. requiring large quantities of renewable starting materials [8], energy-intensive and non-ecofriendly [8].

Enzymatic production of biofuels i.e. PeVa using lipases (triacylglycerol acylhydrolyases, EC 3.1.1.3) offers a greener and energy efficient manufacturing route. Nonetheless, reducing the cost of lipase and the overall economics of the process are major hurdles to overcome before large-scale enzymatic production of PeVa can be accepted [5]. This has led to the development of robust immobilized enzymes i.e. lipases to catalyze the enzymatic process for PeVa production, as the biocatalyst is reusable while requiring only mild reaction conditions [6,9]. *Rhizomucor miehei lipase* (*RML*) *is* commercial lipases for this purpose because of its specificity, reproducibility under ambient conditions [10–12], and ability to catalyze esterification, transesterification and hydrolysis reactions [10]. *RML immobilized onto solid surfaces renders easy recovery* [13] *along with improved enzymatic productivity* [6,14–16]. *A* well-controlled protocol for immobilizing RML can effectively activate the lipase by the adoption of an active-form (open conformation) via

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interfacial activation [17,18]. Such an aspect has always been one of the key considerations for preparing immobilized enzymes. Moreover, there are only few reports in the literature concerning the enzymatic esterification to produce PeVa.

In this study, we immobilized free RML by crosslinking the lipase onto ternary blend nanoparticles that consisted of chitosan (CS), chitin nanowhiskers (CH) and magnetite (MNP), and the obtained RML-CS/CH/MNPs were used to catalyze the enzymatic synthesis of PeVa. CS and chitin nanowhiskers CH were chosen following their well-reported biocompatibility, non-toxicity and mechanical sturdiness, as well as having surfaces high in concentrations of amino groups to form cross-linkages with RML [19–21]. CH when used as a nanofiller can reduce the internal over-flexibility and improve the mechanical strength of CS. In this work, we encapsulated CS/CH to magnetite (MNPs) or Fe_3O_4, prior to the covalent immobilization of RML to allow facile and rapid separation of the biocatalysts from reaction mixture by magnetic force [21,22]. So far, CS-MNP hybrid nanoparticles have been applied in the treatment of wastewater to remove a myriad of contaminants viz. mercury, toxic metal ions, as well as other types of trace pollutants [22,23].

The study would like to note that this is the first report detailing the immobilization of RML onto ternary blend CS/CH/MNP nanoparticles. It is hypothesized that the RML covalently bound to CS/CH/MNPs can improve the lipase activity of RML over its free form. Consequently, the RML-CS/CH/MNPs-catalyzed esterification synthesis of PeVa was statistically optimized by the technique of response surface methodology using a three-level-four-factor Box-Behnken design (BBD) for variables temperature, molar ratio, enzyme loading and time. RSM can establish the best reaction conditions for the highest conversion of PeVa by merging the experimental designs with interpolation by first or second-order polynomial equations in a sequential testing procedure [18].

2. Material and method

2.1. Materials

Pentanol (99%), valeric acid (pentanoic acid) and RML (≥20,000 U·g) were purchased from Sigma-Aldrich (St, Louis, USA), while 2-morpholinoethanesulfonic acid (MES) was obtained from TCI Chemicals (India) Pvt. Ltd. *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), *N*-hydroxysuccinimide (NHS), chitosan (CS) (course ground flakes and powder) deacetylation chitin and chitin (CH) from shrimp shells were all acquired from Sigma-Aldrich (St, Louis, USA). Similarly, sodium hydroxide (NaOH) pellets and phenolphthalein, as well as glacial acetic acid, Tween 80, liquid paraffin, and glutaraldehyde, (25 wt% solution in water), toluene, cyclohexane, iron (II) chloride 4-hydrate ($FeCl_2.4H_2O_6$) and iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) were also purchased from Sigma-Aldrich (St, Louis, USA). Milli-Q Type 1 (Millipore Sigma, Darmstadt, Germany) ultrapure water was used without treatment.

2.2. Experimental

2.2.1. Development of CS/chitin/magnetite (CS/CH/MNPs)

The CS/CH/MNPs were prepared using the method proposed by previous researchers [24] with several modifications. CS (0.5 g) was dissolved in 5% of acetic acid and stirred overnight until a clear solution was obtained. The CH (30%, w/w) was added and left to stir for 1 h before the resultant CS/CH mixture was sonicated for 15 mins. The mixture was transferred into a solution that consisted of mineral oil (50 mL), Tween 80 (0.5 mL) and MNPs (200 mg). The mixture was stirred *v*igorously for 1 h prior to the addition of glutaraldehyde (3 mL, 25% v/v), and was stirred further before it was poured into the solution of CS/CH/MNPs. The mixture was continuously stirred for 1 h at 250 rpm at 40 °C. NaOH (1 M) was added into the mixture until a pH 10 was reached and the mixture was stirred for another 2 h at 70 °C. The formed CS/CH/MNPs were washed several times with acetone and ultrapure water till neutrality and air-dried overnight.

2.2.2. Immobilization of RML onto CS/CH/MNPs

The immobilization protocol for covalent immobilization of RML onto the nanoparticles was adapted from a method described by previous researchers [25] with minor modifications. The CS/CH/MNPs (1.5 g) were rinsed in MES buffer (pH 6.1) and filtered before adding into EDAC (7 mL, 40 mg/mL) and stirred for 1 h at 200 rpm. The mixture was decanted to remove the unbound EDAC and an aliquot of NHS (7 mL, 48 mg/mL) was then added. The mixture was stirred at 200 rpm for a further 1 h to activate the CS/CH/MNPs. The solution was decanted, and the product was repeatedly rinsed with MES buffer to remove unbound EDAC.

The study performed slight modifications to the method used by Raghavendra and co-workers [26]. The activated CS/CH/MNPs were added to a solution of RML (10 mg/mL) prepared in a 20 mL of MES buffer supplemented with toluene (20%, v/v) and Tween 80 (30 μL). Toluene (10%, v/v) and Tween 80 (10%, v/v) were simultaneously incorporated into the immobilization solution whereby both functioned as activating agents for RML. It was to ensure that RML was interfacially activated and hence, immobilized in its open (active) form [18] onto the surface of CS/CH/MNPs. The mixture was left to stir at room temperature overnight at 200 rpm. The RML-CS/CH/MNPs were collected by an external magnetic field and was repeatedly rinsed with MES buffer to remove unbound RML. RML-CS/CH/MNPs were activated by stirring in phosphate buffer (pH 7.0) supplemented with toluene (20%, v/v) for 1 h, left to dry overnight at room temperature and stored at 4 °C. In this study, immobilization of RML onto CS/CH/MNPs yielded 7.6 mg/g RML-CS/CH/MNPs which corresponded to a specific activity of 5.0 $U \cdot g^{-1}$.

2.2.3. Synthesis of pentyl valerate

Esterification synthesis of PeVa was done in a 15 mL screw-capped bottle that contained pentanol (Sigma Aldrich, 99%) and valeric acid (Sigma Aldrich, ≥ 99%) (molar ratio alcohol to acid; 1–3 mmol) with the presence of cyclohexane (Sigma Aldrich, ≥ 99%). Free RML or RML-CS/CH/MNPs (1–3 mg/mL) was added to initiate the reaction and each mixture was magnetically stirred in an oil bath at 200 rpm for up to 6 h under various temperatures (30 °C–70 °C). Molecular sieves were added to the reaction mixture after the second hour to absorb excess water produced during the esterification process. An aliquot of 300 μL of the reaction mixture was sampled and the mixture was quenched with addition of ethanol (2 mL) followed by titration with NaOH (100 mM) using phenolphthalein as the indicator. Percentage conversion of PeVa was calculated using the following Eq. (1):

$$\text{PeVa conversion } (\%) = \frac{V_o - V_t}{V_o} \times 100 \quad (1)$$

where:

V_o: Volume of NaOH at initial time ($t = 0$) and V_t: Volume of NaOH at each hour ($t = t_1, t_2, t_3 \ldots$). It is pertinent to indicate here that the reaction profile was monitored over the course of 6 h. The optimum condition that yielded the highest conversion of PeVa was then used in the subsequent investigations for other parameters.

2.2.4. Characterization of RML-CS/CH/MNPs

The CS, CS/CH, CS/CH/MNP and RML-CS/CH/MNP biocatalysts were examined using Field Emission Scanning Electron Microscope (FESEM) (JEOL JEM–6700F) operating at an accelerating voltage of 5 kV and electric current of 10 μA. Prior to examination, a sample was mounted on the surface of a silicon wafer and sputter-coated with a thin film of gold to avoid charging under the electron beam.

The crystalline structure and phase purity of the MNPs as well as CS/CH/MNPs were investigated using an X-ray diffraction measurement (X-Ray Diffractometer Smartlab Thin Film, Rigaku). The diffractograms