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SHORT COMMUNICATIONS

Influence of Metal Ions on the Immobilization of β-Glucosidase Through Protein-Inorganic Hybrids

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Abstract Immobilization of enzymes through metal-based system is demonstrated as a promising approach to enhance its properties. In this study, the influence of metals ions, including copper, cobalt and zinc (Zn) on the immobilization of β-glucosidase (BGL) through the synthesis of protein-inorganic hybrid was evaluated at 4 °C. Among these metal ions-based hybrids, Zn showed the highest encapsulation yield and relative activity of 87.5 and 207%, respectively. Immobilized BGL exhibited higher pH and temperature stability compared to free form. Thermal stability of hybrid improved up to 26-fold at 60 °C. After 10 cycles of reuse, immobilized enzyme retained 93.8% of residual activity. These results suggested that metal ions played a significant role in the enzyme immobilization as a protein-inorganic hybrid. Overall, this strategy can be potentially applied to enhance the properties of enzymes though effective encapsulation for the broad biotechnological applications.

Keywords β-Glucosidase · Immobilization · Protein-inorganic hybrid · Reusability · Stability

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The biocatalytic transformation reactions success is largely influenced by the properties of the enzyme. Primarily, the use of costly free form of enzyme has resulted in low process efficiency due to their lower stability and limitation for subsequent reuses. In order to solve these problems, immobilization of enzymes has been demonstrated using different methods, including adsorption, covalent, cross-linking or encapsulation [1–8]. The enzyme properties such as residual activity and stability are significantly influenced by the immobilization strategies [2, 7]. From past few years, the protein and metal ions-based enzyme immobilization through protein-inorganic hybrid as a nanoflowers has been shown as a potential approach [9–13]. The synthesis of protein-inorganic hybrids occurs through the three steps mechanism of nucleation, aggregation and anisotropic growth. Overall, the synthesis conditions, types of metal and enzyme played a key role in the efficient immobilization [9, 12, 13]. In addition, the structural properties such as size and morphology of synthesized hybrid vary a lot with the modifications in conditions for synthesis and enzymes [9, 13]. The nano-flower based-system has shown major advantages such as retention of higher efficiency and improved stability of the enzyme over their immobilization through typical approaches [1, 10, 14]. It is due to the effective confinement of enzymes and high surface area of the synthesized hybrid. In contrast, the long incubation periods required for the synthesis and low structural stability during repeated uses of the synthesized nano-flowers are the critical disadvantages [9, 13].

β-glucosidase (BGL) is an industrially important enzyme for the broad applications, including production of biofuels from agricultural wastes, food processing, wine or beverage industry and synthesis of glucosides [15, 16]. Still, immobilization of BGL as protein-inorganic hybrid

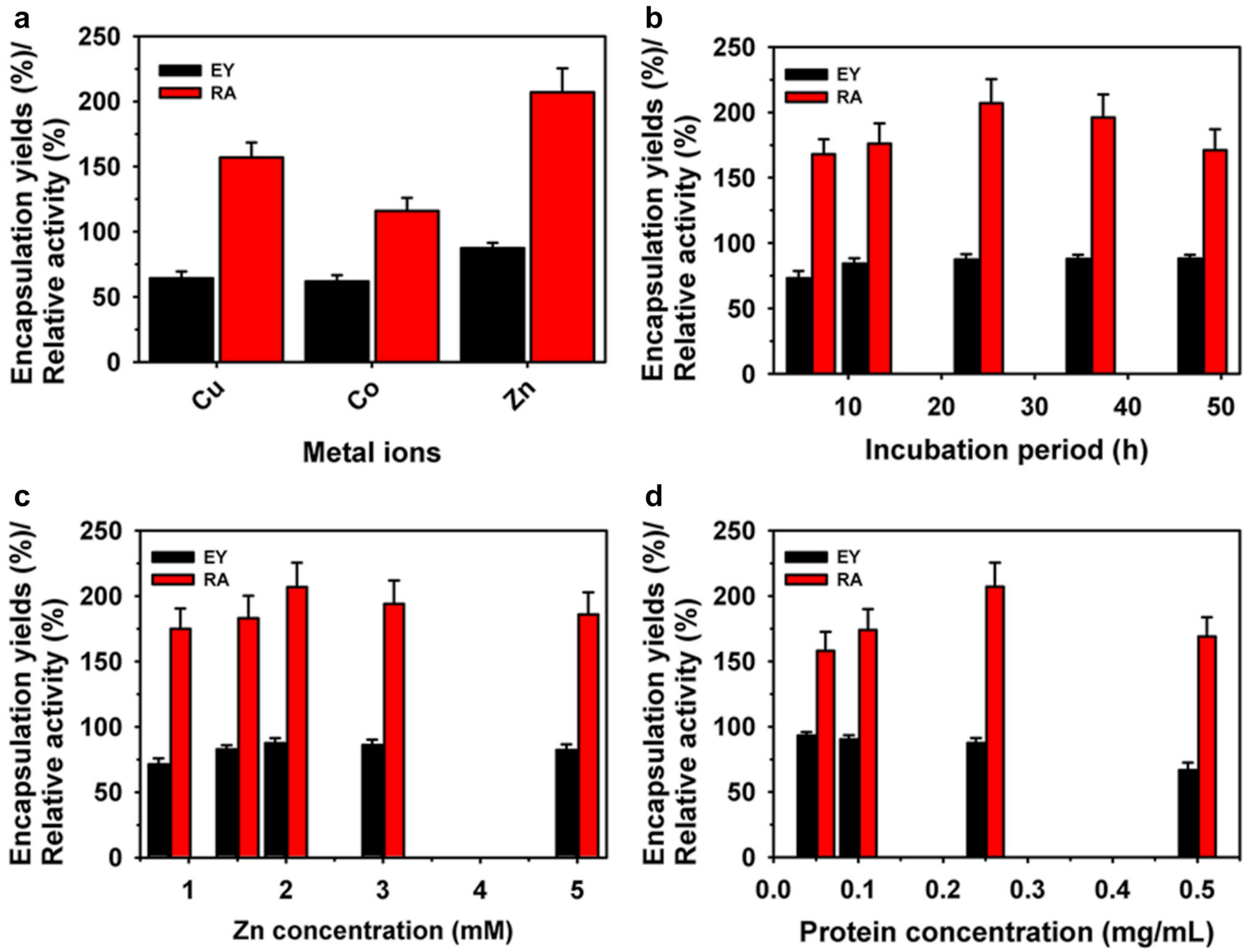

Fig. 1 Immobilization of BGL as protein-inorganic hybrid using different metal ions at a concentration of 2 mM (**a**). Effect of incubation period (**b**), protein (**c**) and metal ions (**d**) concentrations on the immobilization of BGL using Zn metal ions

has not been reported. Therefore, immobilization of BGL using different metal ions copper (Cu), cobalt (Co) and zinc (Zn) as protein-inorganic hybrid has been investigated in this study. The synthesis of BGL hybrids was performed using metal ions (Cu, Co or Zn) and enzyme in the phosphate buffer saline (PBS) incubated for 24 h at 4 °C, as reported previously [12]. The synthesized hybrids exhibited encapsulation yield (EY) and relative activity (RA) in the range of 61.9–87.5 and 116–207%, respectively (Fig. 1a). Here, Zn metal ions-based hybrid exhibited both higher EY and RA as compared with Cu and Co. Here, variations in these properties might be associated with differential influence of metals ions on BGL. Remarkably, synthesized BGL-$Zn_3(PO_4)_2$ hybrid showed high RA of 207% compared to those of free enzyme. Further, the optimization of the synthesis conditions, including incubation period (4–48 h), metal (0.8–5.0 mM) and protein (0.05-0.50 mg/mL) concentrations were evaluated to improve the immobilization of enzyme using Zn as a metal ion in the PBS (pH, 7.4) (Fig. 1b–d). The optimum incubation period, metal ions and protein concentrations were observed to be 24 h, 2.0 mM and 0.25 mg/mL for effective immobilization of BGL to achieve EY and RA of 89.2 and 207%, respectively. Overall, protein-inorganic hybrid of BGL showed significantly higher efficiency of 207% compared to those due to immobilization of BGL using solid supports [17–19]. In addition, the synthesis of BGL required shorter incubation period in comparison to 3 days of incubation used for immobilization of enzymes thorough protein-inorganic hybrid using different metal ions and enzymes [9, 11, 20]. In contrast, BGL immobilized on solid supports including silica and magnetic nanoparticles exhibited significantly lower RA in the range of 19.6-41.4% [18, 19].

The synthesized BGL-$Zn_3(PO_4)_2$ hybrid using 0.25 mg/mL of protein and 2.0 mM of Zn metal ions were characterized to evaluate the properties of hybrids (Fig. 2). The flower like morphology of BGL-$Zn_3(PO_4)_2$ hybrids was

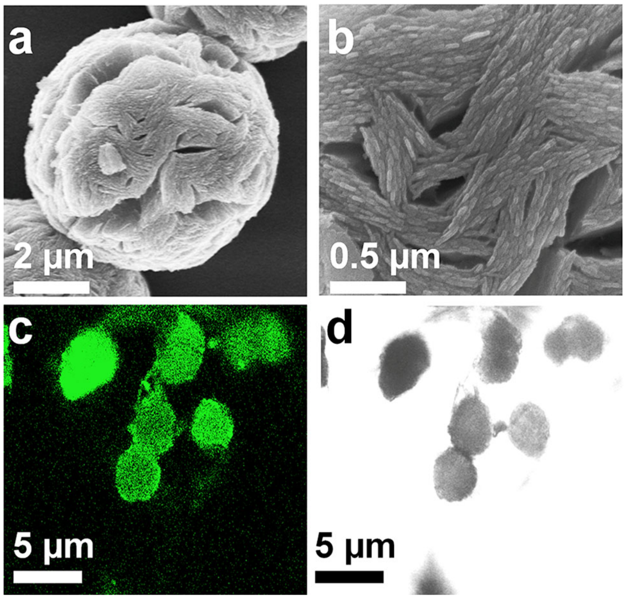

Fig. 2 Characterization of synthesized BGL-$Zn_3(PO_4)_2$ hybrid. Field emission scanning electron microscopy image (**a**), in high resolution (**b**), confocal microscopy image of synthesized hybrid using FITC-labelled BGL in green channel (**c**) and bright channel (**d**)

confirmed through field emission scanning electron microscopy analysis. The average size of hybrids was observed to be ~ 6 µm. The presence of C, P and Zn in the energy dispersive spectrometry analysis confirmed the synthesis of hybrid (Table S1). Further, the green fluorescence of fluorescein isothiocyanate labelled BGL in the confocal laser scanning analysis confirmed the immobilization of enzyme as reported previously [12].

In order to characterize the immobilized BGL, the properties of BGL-$Zn_3(PO_4)_2$ hybrid nanoflower were compared with the free form of enzyme (Fig. 3). A shift in the higher optimum pH activity of BGL nanoflower was observed to 5.5 as compared to free enzyme of 5.0. Whereas, a similar optimum temperature of 60 °C was recorded for both free and immobilized BGL. Remarkably, BGL nanoflower exhibited higher residual activities over broad ranges of pH and temperature. In contrast, BGL immobilized through zeolitic imidazolate framework-8 (ZIF-8) showed lower residual activity at higher pH and temperature to those of free enzyme [18]. The thermal stability was measured at 60 °C for an incubation period of 4 h. The nanoflowers hybrid retained significantly higher residual activity of 85.6% as compared to 2.2% residual activity of free enzyme. Here, half-life value of nanoflower was found to improve 26-fold. These results suggested better suitability of immobilized enzyme over free form for application at higher temperatures. Primarily, the significant reduction in residual activity of immobilized enzymes during reusability is a major limiting factor despite of efficient immobilization. However, the reusability of BGL-$Zn_3(PO_4)_2$ hybrid nanoflower was assessed up to 10 cycles of reuses under standard assay conditions. BGL nanoflower retained residual activity of 98.6 and 93.8% after five and 10 cycles of reuse, respectively. Previous studies have shown that, BGL immobilized on the silica and magnetic particles retained residual activity in the range of 38.0–67.0% [18, 19]. In contrast, a significant reduction up to 80% residual activity was reported for BGL immobilized through ZIF-8 [18].

In summary, the immobilization of BGL was reported through the protein-inorganic hybrids using different metal ions, including Cu, Co and Zn. The metals ions significantly influenced the EY and RA of immobilized BGL. Interestingly, Zn showed very efficient immobilization of BGL to achieve high EY and RA. BGL nanoflowers exhibited flower like morphology and showed improved pH and temperature profiles, and stability compared to those of free enzyme. In addition, BGL nanoflowers exhibited significantly higher reusability than those previously reported on the various solid supports and ZIF-8 hybrids. Therefore, synthesis of efficient protein-inorganic hybrid using different metals ions may be employed to improve the properties of industrially important enzymes for biotechnological applications.

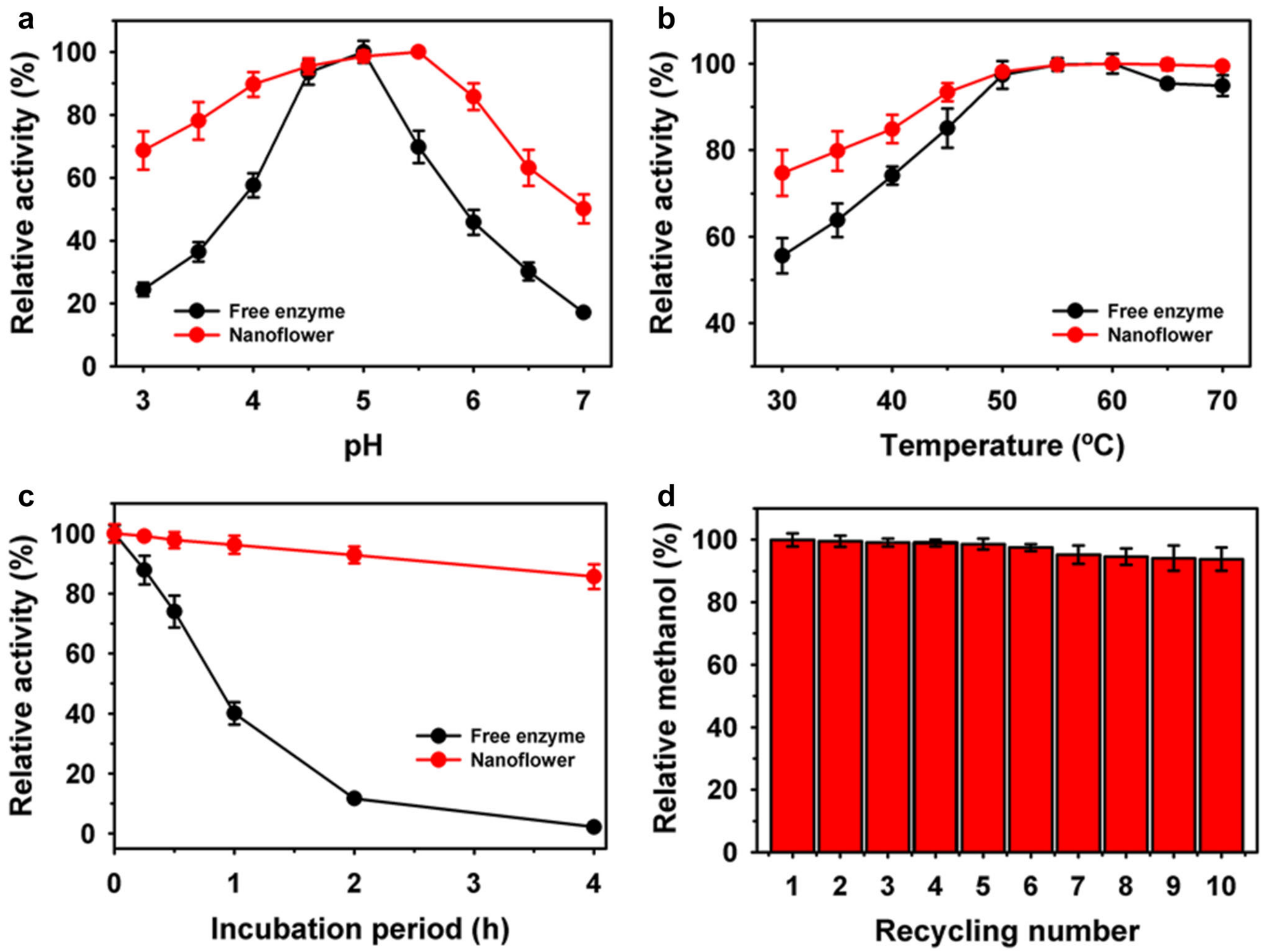

Fig. 3 Activity profile of immobilized BGL-$Zn_3(PO_4)_2$ hybrid at different pH (**a**), and temperature (**b**). Thermal stability at 60 °C (**c**), and reusability (**d**)

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest among the authors.

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