Bioresource Technology 179 (2015) 573–584

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enhanced functionality and stabilization of a cold active laccase using nanotechnology based activation-immobilization

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HIGHLIGHTS

- A nanomaterial laccase (cold tolerant) system exhibited altered characteristics.
- Nano Cu (NP) enhanced activity and stability of laccase at 4 °C.
- Nanotube trapped laccase (+NP) stability at thermal extremes (4°, 80 °C).
- Multiple trap-release cycles retained laccase activity at the thermal extremes.
- Nanotube trapped laccase (+NP) maintained activity following repeated freeze–thaw.

ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form 18 December 2014
Accepted 19 December 2014
Available online 27 December 2014

Keywords:
Enzyme stability
Single wall carbon nanotubes
Immobilisation
Reusability
Kinetics determinants

ABSTRACT

A simple nanotechnology based immobilization technique for imparting psychrostability and enhanced activity to a psychrophilic laccase has been described here. Laccase from a psychrophile was supplemented with Copper oxide nanoparticles (NP) corresponding to copper (NP-laccase), the cationic activator of this enzyme and entrapped in single walled nanotube (SWNT). The activity and stability of laccase was enhanced both at temperatures as low as 4 °C and as high as 80 °C in presence of NP and SWNT. The enzyme could be released and re-trapped (in SWNT) multiple times while retaining significant activity. Laccase, immobilized in SWNT, retained its activity after repeated freezing and thawing. This unique capability of SWNT to activate and stabilize cold active enzymes at temperatures much lower or higher than their optimal range may be utilized for processes that require bio-conversion at low temperatures while allowing for shifts to higher temperature if so required.

1. Introduction

The use of enzymes is crucial in industrial applications and is based on critical determining properties such as specificity, reaction conditions, etc. Generally, the use of free enzymes poses problems in terms of stability and more importantly in the recovery process from the substrates and end products. Functionalized carbon nanotubes have been studied for many potential applications, including immobilization of proteins, to improve the physical stability (Gupta et al., 2002; Cang and Pastorin, 2009). Nano-materials exhibit properties such as large surface-to-volume ratios, high surface reaction activity, high catalytic efficiency, and strong adsorption ability (Mukhopadhyay et al., 2012). Enzyme adsorption on nanoparticles resulted in improved activity (Lynch and Dawson, 2008). Enhanced thermal stability of enzymes was observed after adsorption. Enzyme stability was maximized with nano-scaled supports, possibly as a result of modulation of the catalytic specificity (C.L. Wang et al., 2010). Though a number of studies on nanoparticles (NPs), nanofibers and carbon nanotubes (SWNT) as effectors of enzyme activity (Feng and Ji, 2011) exist, the use of SWNT has gained importance (Kim et al., 2006) owing to the large surface area, unique electronic properties and strong adsorptive ability of these nanoparticles. A significant concern in enzyme immobilization on SWNT (or on any other NPs) is the subsequent recovery of the enzyme from the reaction mixture. This severely limits the scope for repeated use of the enzyme. Here we have reported a single wall carbon nanotube (SWNT) enabled method in which pure laccase, was immobilized in lipid functionalized SWNT (using solid state chemistry). The basic principle of solid state functionalization at nanoscale had been first explored

Abbreviations: NP, Nanoparticle; Laccase (−NP), Untreated laccase; NP-laccase, Cu_2O nanoparticle treated laccase; SWNT, Single walled nanotubes; SWNT-NP-laccase, SWNT trapped NP-lac.

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http://dx.doi.org/10.1016/j.biortech.2014.12.070
0960-8524/

by Bhattacharyya et al. (2012). The theoretical insight of lipid functionalized SWNT self-assembly (nanorope design) which serve as a molecular machine has been reported previously by Bhattacharyya et al. (2013) for developing a reusable glucose sensor. This background has been utilized in our studies for developing and studying a SWNT-laccase system with value-added properties of wide thermal stability and re-usability.

Enzymes that can withstand harsh conditions during reaction are of potential industrial use. This usage has been accelerated by the discovery of novel enzymes from extremophilic microorganisms. In particular, enzymes from thermophilic organisms have found the most practical commercial use to date because of their overall inherent stability (Demirjian et al., 2001). On the other hand, several ecosystems are exposed to temperatures that are permanently below 5 °C. The microorganisms that have colonized these cold environments are referred to as psychrophiles. Such psychrophiles serve as the source for cold-adapted enzyme that have high activity at low temperatures, thereby providing for energy savings. Typically, the specific activity of cold-adapted enzymes is higher than that of their mesophilic counterparts at temperatures of approximately (0–30) °C. At higher temperatures, denaturation of the cold enzyme occurs (Gerday and Aittaleb, 2000). The two properties of cold-active enzymes that have the most obvious biotechnological application are their high catalytic activity at low temperatures and low thermostability at elevated temperatures. The biochemical properties (temperature optima, half-lives, melting temperatures, k_{cat}, V_{max}, K_m and activation energies) of numerous enzymes isolated from cold-adapted organisms are available.

Laccases (benzenediol:oxygen oxidoreductase, E1.10.3.2) catalyze the removal of a hydrogen atom from the hydroxyl group of ortho and para-substituted monophenolic and poly-phenolic substrates and from aromatic amines by one-electron abstraction, to form free radicals, capable of undergoing further depolymerization, repolymerization, demethylation or quinone formation (Gianfreda et al., 1999).

Laccase enzymes have enormous applications in the textile, dye and paper industries, in waste water treatment and bio-remediation. These enzymes have the capacity to degrade lignin (in ligno-cellulose) thereby enhancing its potential as raw material for industrial usage.

In recent work reported from our laboratory, the addition of co-factors (metal) in nano form greatly enhanced the activity and thermal stability of pectate lyase and laccase (Mukhopadhyay et al., 2012, 2013a,b). Such addition also restored the functional properties of a functionally impaired pectate lyase (Dutta et al., 2014).

In the present study, laccase, isolated from a psychrophilic bacteria obtained from Himalayan (Pindari glacier) soil was treated with nano Cu_2O. In general the enzyme became tolerant to a temperature (4 °C) significantly lower than it's optimum (of about 10 °C) while simultaneously exhibiting higher activity. This paper also describes a process in which cold-tolerant laccase from psychrophilic bacteria was, immobilized in lipid functionalized SWNTs. This system (SWNT-NP-laccase) could tolerate both high (upto 80 °C) and low temperatures (4 °C). Again SWNT-NP-laccase showed stability under repeated freeze–thaw cycles, indicating a chaperon-like role for the nano-particles.

2. Methods

2.1. Isolation of enzyme secreting bacterial strains

Soil samples were collected from a glacier soil near Pindari Glacier of Dwali (4000 m above sea level), Uttarakhand, India. Approximately 5 g of soil sample was added to sterile 0.9% saline water. The sample saline mix was incubated overnight at 10 °C and the saline supernatant was used as the bacterial source.

To identify laccase secreting bacteria 2% of the saline supernatant was inoculated into Luria Bertani (LB) broth (NaCl 0.5%, yeast extract 1.0%, tryptone 1.0% and cycloheximide 0.005%; pH 8.5) supplemented with 0.18 mM Cu^{2+} (copper sulfate). After 24 h of shaking at 20 °C the bacterial growth culture was spread on LB-agar plate. The plates were incubated at 20 °C for 24 h. After the formation of colonies, each bacterial colony from the plate was exposed to a few drops of 0.1% (w/v) syringaldazine to check laccase activity (C.L. Wang et al., 2010; Z.X. Wang et al., 2010). Laccase secreting bacterial colonies were identified by pink color formation. These colonies were streaked on new LB-Cu^{2+} plates for purification. The darkest pink coloration was produced by isolated AKPSYL. This colony was cultured in liquid LB without added Cu^{2+} medium for carrying out subsequent experiments for the laccase. This was done to eliminate the effect of Cu^{2+} in the subsequent assays.

2.2. Ribotyping

To identify the laccase secreting bacterial strain, 16S rDNA gene sequencing was carried out. Genomic DNA was prepared from AKPSYL by the sodium dodecyl sulfate proteinase K-cetyltrimethylammonium bromide (CTAB) method (Mukhopadhyay et al., 2012). Partial amplification of the 16S rRNA gene was performed with the thermal cycler ABI 9700 (ABI, Foster City, USA). The amplified and gel-eluted PCR fragments were sequenced with an ABI 3100 Genetic Analyzer. Sequencing reaction was performed by using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA). The nucleotide sequence was deposited in the GenBank.

2.3. Purification of laccase from Pseudomonas putida AKPSYL

Laccase was purified from 100-ml of the growth culture. Cell-free supernatant was fractionated with ammonium sulfate (0–30%; 30–80%), and the fraction containing the activity was used for further studies. The precipitate was dissolved in the minimum amount of Tris–HCl buffer (50 mM, pH 8.5) and dialysed against the same buffer. Then 5 ml of dialysed sample was loaded onto a CM-Sepharose column (10 ml bed volume), equilibrated with Tris–HCl buffer (50 mM, pH 8.5). Fractions (1 ml) showing laccase activity was concentrated using a Macrosep 10 K unit and loaded onto a glass column packed with Sephadex G-75 (bed volume 30 ml) and equilibrated with Tris–HCl buffer (as above). Elution of the proteins was done using the same buffer (with varying pH). SDS polyacrylamide gel electrophoresis (PAGE) in a 12% gel was performed. Protein markers and protein bands were stained by silver staining.

2.4. Activity assay of laccase

The activity of purified laccase was measured at 25 °C by the syringaldazine method (Mukhopadhyay et al., 2013a,b). The reaction mixture (1 ml) contained 0.25 mg/ml purified laccase in 0.3 ml, 0.5 ml of Tris–Cl buffer (25 mM, pH 8.5), and 0.2 ml of 1 mM of syringaldazine. The oxidation of syringaldazine by laccase was detected by measuring the absorbance increase at 525 nm. The systems also contained either 0.2 mM Cu^{2+} (as $CuSO_4$) or specified amounts of Cu nanoparticles. A concentration of 0.2 mM Cu^{2+} was found to be optimal for the activity of purified laccase.

2.5. CD spectroscopy

CD spectra over the range of 190–250 nm were obtained for the native laccase enzyme at 10 °C, enzymes at 4 °C, NP-enzyme and