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Delignification and fruit juice clarification properties of alginate-chitosan-immobilized ligninolytic cocktail

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ABSTRACT

In the present study, an in-house extracted ligninolytic enzyme cocktail from *Ganoderma lucidum* IBL-05 was effectively immobilized on alginate-chitosan beads using glutaraldehyde. The firm quality stable alginate-chitosan beads developed using optimum condition of 3.0: 0.5% (w/v), alginate: chitosan ratio; 1.0% (v/v) glutaraldehyde concentration and 0.5 mg/mL of protein concentration registered the maximum immobilization yield. The functional characteristics and surface morphology of the control and ligninolytic enzyme conjugated alginate-chitosan beads were characterized using Fourier-transform infrared and scanning electron microscopy, respectively. Evidently, after enzymatic treatment, a significant reduction in turbidities up to 84.02%, 57.84%, 86.14%, and 82.13% was recorded for apple, grape, orange and pomegranate juice, respectively. The enzymes were subsequently used for the delignification of various agro-industrial materials. The immobilized consortium caused the marked reduction in lignin content of all the plant residues with a maximum delignification of 57.3% in sorghum stover after 15 h. In conclusion, the study suggests the potential of the immobilized ligninolytic enzyme, as a cost-effective industrially desirable green catalyst, for biotechnological at large and industrial in particular, especially for delignification, food, and beverage applications.

1. Introduction

Contemporary research in enzyme biotechnology enabled the chemical or biological modification of enzymes to attain the improved performance and catalytic stabilization against unfavorable industrial conditions that otherwise inactivated the enzyme (Bilal, Asgher, Iqbal, Hu, & Zhang, 2016; Gassara-Chatti et al., 2013). In case, if enzymes are intended to be applying in larger-scale processes, it is crucial to trim-down the industrial biocatalyst costs. Enzyme immobilization on calcium alginate hydrogel beads is economically feasible, and the process can be easily carried out under extremely mild experimental conditions, so, therefore, could be considered for industrial exploitation (Lu, Xu, Jiang, Yuan, & Wang, 2005; Mørch, Donati, Strand, & Skjåk-Bræk, 2006; Matto & Husain, 2009). However, high biomolecule leaching, low mechanical strength, and greater pore size are the inadequacies frequently associated with this immobilizing biopolymer (Konsoula & Liakopoulou-Kyriakides, 2006; Elnashar, Danial, & Awad, 2009). Poly-cations, such as chitosan considerably reduce the porosity of the alginate gel, increase the immobilization efficiency as well as restrict the enzyme leakage out from the gel matrix (Li & Li, 2010). Indeed, chitosan is a naturally occurring non-toxic linear polysaccharide molecule with a positive charge at low pH and has been employed at its lower concentration for coating the alginate beads (Gandomi, Abbaszadeh, Misaghi, Bokaie, & Noori, 2016; Kumar, Dwevedi, & Kayastha, 2009).

The key objectives of the juice clarification are eliminating phenolic compounds and reduce the astringency of the resulting product (Alper & Acar, 2004; Bilal et al., 2016). Among various techniques, ultrafiltration is commonly employed to stabilize fruit juices. However, it represents an inability to remove reactive

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phenolic compounds efficiently, and as a consequence, ultrafiltered juices do not always remain stable and tend to generate marked haze/cloudiness. Comparatively, an enzyme-based treatment of raw juices has been demonstrated as a potential alternative (Gassara-Chatti et al., 2013). In the industrial arena, ligninolytic enzymes, secreted by white rot basidiomycetes refers to the mixture of primarily three different extracellular enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). These enzymes display great perspective for numerous technological applications in different industries, such as paper and pulp delignification, biofuel production, dye discoloration, wastewater treatment, food juice extraction-clarification, and biosensors (Neifar et al., 2011). In the present study, the ligninolytic cocktail was immobilized on alginate-chitosan beads. The immobilized ligninolytic cocktail was exploited for the delignification and fruit juice clarification purposes.

2. Materials and methods

2.1. Chemicals, lignocellulosic materials, and fungal strain

All the chemicals used in this study were of analytical laboratory grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). The lignocellulosic-based materials including corn stover (CS), cotton stalk (CoS), sorghum stover (SS), sugarcane bagasse (SCB) and wheat bran (WB) were collected from the research farm center of the University of Agriculture Faisalabad, Pakistan and the local (Ghulam Abad) fruit market in Faisalabad, Pakistan. The substrates mentioned above were separately crushed into small pieces and washed three times with 30 min interval using warm water to minimize/avoid the dust contamination. Each of the washed substrates was first sun-dried for 3 h and then oven-dried at 60 °C for 72 h. The fully dried materials were ground to a 40 mm-mesh particle size and stored in air-tight plastic jars to avoid the absorption of free atmospheric moisture until they were used for the subsequent experimental work as required. The indigenously isolated local fungal strain i.e. *Ganoderma lucidum* IBL-05 was collected from the fungal culture unit of the Industrial Biotechnology Lab at the Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. The *G. lucidum* IBL-05 was used as a fermentative organism for ligninolytic enzymes production under previously optimized growth conditions (Bilal, Asgher, & Ramzan, 2015).

2.2. Production and extraction of ligninolytic enzyme

Ligninolytic enzymes were produced in solid-state bioprocessing of rice straw by *G. lucidum* IBL-05. The SSF was carried out in sterilized cotton-plugged triplicate production flasks (250 mL capacity) containing 5.0 g of rice straw moisturized with Kirk's basal salts medium (66% w/v) without glucose. After adjusting the medium pH to 4.5, the flasks were inoculated with 5.0 mL homogeneous mycelium suspension and subjected to fermentation in a controlled incubation at 30 ± 0.5 °C for 120 h. After designated time duration, the extracellular ligninolytic enzymes were extracted from the fermented matter by adding 100 mL of distilled water. The extracts in the flasks were shaken at 150 rpm for 20 min followed by centrifugation at 4000×*g* for 10 min. The clear aqueous filtrate was used as crude enzyme extract for activity assay purposes (Iqbal, Asgher, & Bhatti, 2011).

2.3. Determination of ligninolytic enzyme activities total proteins contents

The enzymatic activities of all the oxidoreductases were measured spectrophotometrically (HALO DB-20S, UV/Visible double beam spectrophotometer, Dynamica). The activity of LiP was assayed following the H_2O_2-dependent oxidative transformation of veratryl alcohol (VA) to veratraldehyde at 25 ± 1.0 °C. The reaction mixture contained 1.0 mL of tartrate buffer (100 mM, pH 3.0), 1.0 mL of VA (4.0 mM), 500 μL of H_2O_2 and 100 μL of enzyme aliquots. MnP activity was assessed by determining the formation of manganic-malonate complexes at 270 nm, as reported earlier (Bilal et al., 2015). Assay mixture comprised of 1.0 mL of $MnSO_4$ (1.0 mM), 1.0 mL of Na-malonate buffer (50 mM, pH 4.5), 500 μL of H_2O_2 and 100 μL of crude enzyme solution. Laccase was examined by monitoring 2, 2 azinobis (3-ethylbenzthiazoline)-6 sulphonate (ABTS) oxidation in Na-malonate buffer at 436 nm (Iqbal et al., 2011). The reaction mixture contained 1.0 mL of Na-malonate buffer (50 mM, pH 4.0), 1.0 mL of ABTS and 100 μL of enzyme solution. Blank samples contained all the reagents along with 100 μL of distilled water in replacement of enzyme solution. The enzymatic activities were expressed in units per gram of dry substrate (U/gds), where, one catalytic unit (U) corresponds to the enzyme quantity catalyzing the formation of 1 μmol product per min under standard assay environment. Total proteins were quantified from standard curve standardized with bovine serum albumin (BSA) as a calibration standard. Ten μL of ligninolytic enzyme extract was mixed with 1.0 mL of the Bradford reagent and allowed to incubate at 37 °C for 15 min (Bradford, 1976). After agitation, the optical density was recorded using a double beam UV/Vis spectrophotometer at 595 nm.

2.4. Immobilization of ligninolytic enzyme extract

A previously reported protocol of Li and Li (2010) was adopted for ligninolytic enzymes immobilization. Briefly, 3.0% (w/v) Na-alginate was well dissolved in water, and the equivalent volume of enzyme extract was added to the Na-alginate solution under constant agitation. A 0.5% (w/v) chitosan was dispersed ultrasonically (SB-5200DTDN Ultrasonic Bath, Scientz) in 5.0% (w/v) acetic acid solution for 30 min followed by the addition of 2.0% (w/v) $CaCl_2$ solution. Alginate mixture containing ligninolytic enzymes was extruded drop by drop into 50 mL chitosan/$CaCl_2$ dispersion through a syringe needle to generate spherical beads of uniform size i.e. 2.0 mm diameter and allowed to harden in the same solution for 6.0 h. After 6.0 h, the beads were rinsed with Na-malonate buffer (50 mM, pH 4.5) and then activated with 0.5% (w/v) GA solution for another 2.0 h. The GA-activated beads were washed three times with distilled water and used for the enzyme activity measurements. Immobilization yield (IY) was evaluated using the relation indicated in Equation (1).

$$IY\,(\%) = 100 \times \frac{\textit{Total activity of immobilized ligninolytic enzyme extract}}{\textit{Total activity of free ligninolytic enzyme extract}} \quad (1)$$

2.5. Fourier-transform infrared spectral analysis

Fourier-transform infrared (FT-IR) spectra were recorded using a Thermo Nicolet 6700 spectrophotometer (Thermo Fisher Scientific). The alginate-chitosan beads before and after immobilization of ligninolytic enzyme extract were grounded with potassium bromide, and the resulting powder was pressed into pellets using a hydraulic press. The infrared absorption spectra were recorded from the wavelength region of 4000–400 cm^{-1}. The FT-IR spectra were collected with 64 scans at a resolution of 4.0 cm^{-1} and