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Enzymatic treatment of wastewater from the corn tortilla industry using chitosan as an adsorbent reduces the chemical oxygen demand and ferulic acid content

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ABSTRACT

The residual effluent from the corn tortilla industry, referred to as Nejayote in Mexico, causes serious pollution problems because of its high chemical oxygen demand (COD). However, as this effluent contains valuable corn phytochemicals, such as ferulic acid and its derivatives, it also represents an opportunity to produce high-added-value biocompounds. In this work, we addressed environmental and biosynthetic approaches by applying laccase oxidative treatment in the presence of chitosan to reduce the environmental impact of the effluents from the tortilla industry. A central composite design pattern was used to understand the relationship between several variables (pH, temperature, reaction time, and enzyme quantity) and two responses (removal of ferulic acid derivatives and COD decrease). The optimum conditions for maximizing both responses were pH 6, 35 °C, 24 h, and 0.3025 nmoles laccase, which resulted in 70 and 78% decreases in phenolics and COD, respectively. UV–vis, fluorescence and FTIR analyses revealed enzymatic grafting of chitosan with phenolics from the effluent. Grafted chitosan presented a higher capacity to scavenge DPPH radicals (EC_{50} 1.9 mg/mL) than neat chitosan (EC_{50} 2.5 mg/mL) and higher viscosity values. The obtained results indicate that a process involving a single enzymatic step could be adequate to decrease the effluent COD and to generate polymers with potential applications in the food and pharmaceutical industries.

1. Introduction

The processing of corn flour in the presence of $Ca(OH)_2$ at high temperatures produces the soft, clean corn paste used for tortilla production, which is known as nixtamalization in Mexico. This process is also applied in South and Central America and in many places in Asia, the United States and Europe to produce tortillas [1]. It is estimated that 3 L of alkaline water is needed to produce 1 kg of corn-processed pasta, generating an excess of wastewater. According to Salmerón-Alcocer et al. [2], a corn-processing plant with a capacity of 600 tons/day commonly generates between 1500 m^3 and 2000 m^3 of alkaline wastewater. An extrapolation of this value to the number of industrial plants in Mexico alone yields approximately 16 million m^3 wastewater/year. This alkaline effluent is considered to be an important contaminant because of its alkalinity (pH 7–11), its high chemical oxygen demand (COD) of 10,000–30,000 mg/L and its high temperature (up to 70 °C) [3,4]. However, in spite from being a serious contaminant, tortilla industry effluents are a source of potentially high-value products, including proteins, sugars, vitamins and phytochemicals such as antioxidants and carotenoids [5–9]. Among the antioxidant phytochemicals present this effluent contains significant amounts of ferulic acid (FAc) and its derivatives [7,8]. Ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid) is the major cinnamic acid derivative found in the cell walls of a wide variety of plants [10]. It has been reported to possess various physiological functions, including antioxidant, antimicrobial, and anti-cancer activities, among others [11].

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Therefore, tortilla industry effluents are an environmental pollutant; however, they could also be considered to represent a source of high-value biocompounds. In this work, both issues are addressed by applying enzymatic oxidative treatment in the presence of chitosan. This strategy has been applied to reduce the environmental impact of phenol-containing wastewaters, such as those from petroleum refineries, foundries and food processing [12–15], where the enzymatically oxidized phenols react with a polymer (chitosan or poly(ethylene)glycol), producing a precipitate that can be easily separated via filtration or sedimentation [16]. The COD and toxicity are significantly reduced [13–15], and a potentially useful grafted polymer is obtained as final product [15].

2. Materials and methods

2.1. Chemicals

Low molecular weight chitosan (75–85% deacetylated), 2,4,6-trinitrobenzene sulfonic acid (TNBSA), ferulic acid (FAc), glucosamine, and α,α-diphenylpicrylhydrazyl (DPPH) were purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA). Laccase solution (121 μM) from *Coriolopsis gallica* UAMH 8160 (MW 66 kDa via SDS page, pI 3.4, 21% carbohydrate content, purity index $A_{280}/A_{606} = 5$) was obtained as previously described [17] and provided by Altaenzymes. Buffer salts, isopropanol, and sodium hydroxide were procured from J.T. Baker.

Tortilla industry effluent (Nejayote) was kindly provided by a local tortilla-making industry located in Puebla, Mexico. The nixtamalization process was carried out as follows. In the first step, a total of 20 kg of Sinaloa corn variety was cooked during 38 min at 85 °C in a solution prepared with 800 g of calcium hydroxide dissolved in 200 L of water. Then, in the second step, corn kernels were rested during 12 h. After this time, 10 samples of 5 L of nejayote were drained, collected, cooled, and stored at −20 °C until analysis. The chemical oxygen demand (COD) of the effluent was 25,000 ppm, the ferulic acid content was 3.3 g/L and the total phenol concentration was 4.24 g of ferulic acid equivalents/L, determined by standard methods [18,19] (Figs. S1 and S2). Table S1 shows the physicochemical composition of the effluent.

2.2. Kinetic constants

2.2.1. Pure FAc oxidation

The enzymatic oxidation of FAc was carried out in a 1 mL reaction mixture containing 10% isopropanol and 90% acetate buffer (60 mM, pH 4.5), 75 μM FAc and 6 nM laccase. Measurements were carried out at 25 °C under constant stirring [20,21]. The progress of the reaction was monitored based on the decrease in substrate absorbance at λ = 314 nm ($\varepsilon = 13.4\ mM^{-1}\ cm^{-1}$). To determine the kinetic constants, the oxidation of different FAc concentrations, ranging from 10 μM to 150 μM, was assayed. The kinetic constants for effluent oxidation were determined as described for FAc using several effluent dilutions (10–50 μL and diluted to 1 mL reaction mixture), containing from 10 μM to 150 μM FAc equivalents, which is a suitable range for obtaining the rectangular hyperbola that describes Michaelis–Menten kinetics. The progress of the reaction was monitored based on the decrease in absorbance at 314 nm, and the results were transformed using the same extinction coefficient of pure FAc. Therefore, the data are reported as the reaction rates of the oxidation of ferulic acid equivalents in the effluent mixture. All assays were performed in triplicate to ensure repeatability.

All kinetic data were adjusted to the Michaelis–Menten equation by an iterative procedure following the Marquardt–Levenberg nonlinear least-squares algorithm using Origin 7.0 software.

2.2.2. Oxidation of tortilla industry effluent in the presence of chitosan

A total of 10 mL of wastewater from the corn tortilla industry was enzymatically oxidized by adding 0.3 nmoles laccase and 40 mL of phosphate buffer (60 mM, pH 4.5–6; final COD, 2500 ppm), for 10 min, and then 1 g of chitosan was added to the reaction mixture. The reaction was incubated for 24 h at 25 °C. Then, NaOH was added to precipitate chitosan (to a pH above 9), which was subsequently removed via centrifugation at 6000 rpm for 5 min. The COD in the supernatant was determined as described below. The obtained solid was washed ten times with a 50% isopropanol solution until no light absorption was detected in the UV–vis spectra. Finally, the product was dried in a vacuum chamber for further characterization [22]. For biopolymer characterization, controls for chitosan were produced by carrying out the whole process in the absence of the enzyme.

2.3. Operational stability

The total turnover number (TTN, total moles FAc oxidized per mole of laccase used) was calculated as a parameter of the operational stability of the enzyme [23]. To this end, a 50 mL reaction mixture containing 1 mM pure FAc and 6 nM laccase in 60 mM phosphate buffer pH 6 was incubated under stirring at 35 °C and the absorbance at 314 nm was monitored until no further substrate conversion was observed. The same experiment was carried out in the presence of 1 g of chitosan to determine its effect on TTN. Every 60 min, 100 μL of the reaction mixture was collected from the reactor and centrifuged at 5000 rpm, and the absorbance of the supernatant at 314 nm was determined. The absorbance at 314 nm was transformed into moles FAc using a molar absorptivity coefficient of $\varepsilon = 13.4\ mM^{-1}\ cm^{-1}$.

2.4. Quantification of amino groups of chitosan

The 2,4,6-trinitrobenzenesulfonic acid (TNBSA) assay was used to determine the quantity of free amino groups in chitosan before and after derivatization [24]. Primary amines form a highly chromogenic compound upon reaction with TNBS, which can be measured at 335 nm. The concentration of free NH_2 groups in control and modified chitosan samples was estimated by plotting a standard curve of absorbance as a function of glucosamine concentration, used as a model compound. The percentage of remaining free amino groups was determined using the following equation:

$$\%\,\text{Remaining-NH}_2 = \frac{A_g}{A_0} * 100 \quad (1)$$

where A_0 and A_g correspond to the quantity of free amino groups in neat and grafted chitosan, respectively. In all experiments, laccase was used as a blank.

2.5. Experimental design

2.5.1. Response surface methodology and central composite design

A central composite design (CCD) with four independent variables: enzyme amount (nanomoles, X1) reaction time (h, X2), pH (X3), and temperature (°C, X4), at three levels was applied to study the two response patterns, %COD decrease (Y1) and %ferulic acid removal (Y2). Table 1 shows the experimental values for the aforementioned variables. A total of 30 experiments were conducted according to the CCD (Table 2).

The experimental results were analyzed through Response Surface Methodology (RSM) using the Design-Expert software (trial version 7.0.1, USA). The responses were related to the selected variables via a second-order model.