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Towards a resource friendly circular cotton processing: From carbohydrate rich wastewaters to hydrogen peroxide using carbohydrate oxidases

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ABSTRACT

One of the main concerns in the textile cotton industry is the substantial amount of contaminated effluent generated from various stages, including desizing and pre-wash. The wastewater, rich in carbohydrates from starch hydrolysis, presents a promising feedstock for hydrogen peroxide (H_2O_2) production, a crucial component in cotton fabric bleaching. this study, comprehensively characterized various cotton processing wastewater, evaluating their total sugar content and carbohydrate profile. Based on the results, wastewater from the pre-washer emerged as the most suitable for enzymatic H_2O_2 production. Given the variability in carbohydrate profile of wastewater, two strategies based on the carbohydrate oxidase promiscuity were developed: N-acetylglucosamine oxidase (NagOx), specific for glucose, and Cellobiose oxidase (COX), displaying a high carbohydrate promiscuity. A pretreatment with glucoamylases was employed to increase glucose levels for NagOX, while COX was directly applied towards untreated wastewater. pH control was assessed for both systems. Results indicated NagOX's performance was enhanced by pH control, unlike COX. The robustness and flexibility of both proposed strategies were validated using diverse batches industrial wastewaters samples, confirming the efficacy of the proposed enzymatic-based hydrogen peroxide production processes. Biobleaching tests were conducted, revealing an impact of wastewater color on the bleaching process. However, the use of a hydrogen peroxide activator (TAED) emerged as a promising approach to implement the proposed strategies.

1. Introduction

Cotton fibers, along with others plant-based fibers, have been used for thousands of years, primarily to produce fabric for domestic applications. Nevertheless, in recent decades, cotton fibers have begun to be used in several different areas, including the alimentary industry, cosmetics, pharmaceuticals and the production of plastic [13,28,49]

The global textile cotton industry has a market value of $3 trillion, with an annual production of 25 million tons per year of textile goods, demonstrating consistent growth. However, the highly intensive processes involved in cotton textile fabrication are responsible for the consumption of 98 million tons of non-renewable resources and an estimated 20 % contribution of the world's water pollution [18,32,45,57]. Indeed, the production of one ton of cotton fabric requires the utilization of 80,000 liters of tap water and 73 kg of chemicals (including hydrogen peroxide), 240 kWh of electricity and 5400 MJ of heat. Furthermore, the entire process results in the generation of 80 cubic meters of wastewater, containing 46 kg of chemical oxygen demand (COD) (Data provided by Novonesis).

In the initial stages of the cotton textile processing (Fig. 1), it is necessary to protect the cotton fiber from the mechanical and thermal abrasive conditions it may encounter during the weaving and singeing stages [24,34]. Typically, a solution comprising starch, wax, and polyvinyl alcohol (PVA) is applied during the sizing stage. Subsequently, once the weaving of the fabric is complete, the protective layer is often removed using a starch-degrading enzyme such as amylase, a stage referred to as desizing. This process generates carbohydrate-rich effluents [2].

The presence of carbohydrates with different degrees of polymerization (mono-, di-, tri-saccharides), resulting from starch hydrolysis, contributes to elevated levels of COD/BOD_5. Furthermore, in conjunction with physical characteristics such as color and pH, these factors have a detrimental impact on the environment, reducing the availability of oxygen in water bodies and reducing the fertility of adjacent land

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[26]. Consequently, approximately 20 % of the total wastewater generated in the cotton processing industry is produced as a result of the desizing of fabrics and pre-wash processes (data provided by Novonesis).

To reduce the environmental impact of these types of wastewaters, various physicochemical and biotechnological strategies have been described [7]. Physicochemical methods include using metal salts as flocculating agent, active charcoal for color reduction [23], and filtration techniques such as ultra-, micro-, and nano-filtration to reduce the COD/BOD_5 and color [1]. Biotechnological strategies involve using the carbohydrates in the wastewater as a carbon source for biogas and biomass production via fermentation [50] and employing diverse consortia of microorganism to remove organic matter and color [27,33]. However, utilizing anaerobic fermentation is the most common strategy in the treatment of wastewater, and in various cases is employed to produce hydrogen as an energetic fuel [3,53].

The utilization of enzymes for the treatment of industrial and recalcitrant waste materials offers several advantages over alternative methods [8,11]. These include high specificity, low energy consumption, and shorter processing times (L. [30,35,48]). Various biotechnological approaches employ diverse enzymes to reduce organic pollutants in effluents while also producing value-added products [20,25,52]. In this context, carbohydrate oxidases are a subject of extensive research and application in this field due to their broad substrate recognition capacity. This characteristic is influenced by the enzyme's source and its role in specific metabolic pathways [59].

While most of the report focused on the removal or degradation of natural or synthetic dyes from wastewater using enzymes, primarily laccases, only a few authors have investigated the potential valorization of cotton wastewater, specifically from the desizing stage, due to the significant amount of carbohydrates [6,17,40].

Carbohydrate-rich wastewaters from cotton processing can be utilized as a glucose source to produce hydrogen peroxide for the bleaching step using carbohydrate oxidases. This approach offers a promising alternative to the traditional bleaching method, reducing the need for fresh water. Additionally, the gluconic acid generated as a by-product can be employed as a sequestering agent for metal ions that may interfere with the bleaching process due to its chelating properties. This has the potential to reduce the use of chemicals [5,38,39,42–44,56]. However, most reported studies have focused on the use of glucose oxidases, which requires a preliminary step of glucose release.

The present study involves an in-deep characterization of wastewater generated during various stages of cotton textile processing. The availability of the effluents, their carbohydrate profile, and physiochemical parameters were crucial in selecting the feedstock. Furthermore, two different enzymatic approaches for hydrogen peroxide generation were developed and evaluated depending on enzyme promiscuity. First, a two-step approach using a heterologous bacterial N-acetylglucosamine oxidase (NagOX) mutant L251R from *Ralstonia solanacearum* with high glucose specificity and an uncommon pH and temperature profile was employed [10]. A previous hydrolysis of carbohydrates with glucoamylases was applied to achieve high glucose concentrations, which was subsequently oxidized by NagOX.

As a second approach, a one-step strategy using commercially available cellobiose oxidase (COX) from *Microdochium nivale,* provided by Novonesis, was employed. Kulys et al. [29,41,47] The high promiscuity of COX allows the implementation of the strategy directly on the polluted effluent under mild conditions.

The objective of the work was to establish the foundations for an environmentally friendly industrial bio-bleaching process. To achieve this, operational conditions were set based on the exhaustive characterization of both carbohydrate oxidases. This included an evaluation of the implementation of a pH control system and assessing the variation of parameters of the wastewater used as a feedstock. Finally, the robustness and flexibility of both proposed approaches were analyzed using various batches of real industrial wastewaters samples.

2. Material and methods

2.1. Reagents and materials

All reagents were procured from Sigma Aldrich® (St. Louis, MO, USA) and from Thermo Scientific™ (Waltham, MA, USA). Carbohydrate oxidase from *Microdochium nivale* (Cellobiose oxidase), glucoamylase blends were kindly donated by Novozymes (Bagsvaerd, Denmark). The plasmid encoding N-acetylglucosamine oxidase mutant L251R was kindly donated by Marco Fraaije from the University of Groningen (Groningen, Netherlands). The heterologous enzyme was produced and purified in accordance with the methodology presented in the supplementary material. Wastewater samples from desizing and pre-wash stages of cotton processing were provided by Zorluteks Textile and Industry Inc. (Kırklareli, Turkey).

2.2. Activity determination

The specific oxidative activity was determined by continuously measuring the appearance of H_2O_2 at wavelength of 515 nm (ε_{515} = 28 mM^{-1} cm^{-1}), in accordance with the protocol established by Ferrari [16]. The oxidative activity of carbohydrate oxidases was determined by coupling reaction of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid carried out carried out by horseradish peroxidase, resulting in a pink/purple product. This reaction allows the measurement of the capacity of the oxidases to generate 1 μmol of H_2O_2 per 1 min, which is defined as their oxidative activity. The reaction medium is composed of 50 mM Britton-Robinson buffer, with 0.1 mM of 4-aminoantipyrine, 1 mM of 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4.0 IU of peroxidase of horseradish. The reaction conditions were as follows: 30 °C, pH 6.0 and 20 mM cellobiose as a substrate for Cellobiose oxidase (COX) and 50 °C, pH 9.0 and 50 mM glucose as substrate for N-acetylglucosamine oxidase (NagOX). The activity measurements were performed in 96-microwell plates using a photometer SPECTROstar Nano (BMG LABTECH) with 200 μL of volume reaction volume, conducted in triplicate.

2.3. Carbohydrate quantification by HPLC-IR method

The determination of different carbohydrates in wastewaters samples was conducted using an IR-HPLC (Agilent Technologies 1200) equipped with Luna Omega (250 mm×4.6 mm×3 μm) column supplied by Phenomenex. The mobile phase employed was acetonitrile (65 % v/v) at a flow rate of 1 mL/min at 30 °C.

2.4. Total carbohydrates analysis

The total amount of reducing sugars was quantified using Benedict's method, whereby a copper sulfate solution was reduced at alkali and high temperature conditions. The bivalent copper is reduced to copper oxide and the remaining bivalent copper is reacted with potassium

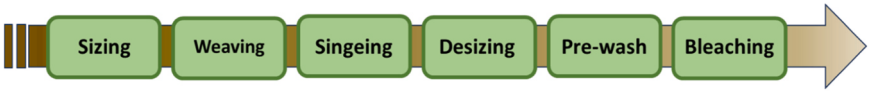

Fig. 1. Stages of industrial cotton textile processing.

iodide. The iodine formed then titrated back with sodium thiosulfate [22].

2.5. Chemical oxygen demand (COD) quantification

The determination of COD of the wastewater was conducted using HACH® kit (COD 150 – 1000 mg/L). Each kit tube was homogenized before adding 2 mL of sample. Once the sample was mixed, was incubated at 148 °C for 2 h After the incubation, the tubes were cooled at room temperature to then be analyzed by spectrophotometer HACH® LANGE DR3900.

2.6. Hydrogen peroxide quantification

The quantification of hydrogen peroxide is based on the immediate reaction with vanadium pentoxide (V_2O_5) in the presence of sulfuric acid, forming a peroxovanadate complex that exhibited an absorption maximum at 454 nm [62]. The analysis was performed contacting 1 mL of sample with 0.2 mL of H_2SO_4 at 0.5 M, followed by centrifugation at 8000 rpm for 5 min. Subsequently, 0.5 mL of supernatant was extracted and combined with 0.5 mL of a color indicator solution (0.2 g of V_2O_5 dissolved in 100 mL of 0.5 M of H_2SO_4). The absorption of the resulting solutions was quantified at 454 nm using photometer SPECTROstar Nano (BMG LABTECH) in microplates with 200 μL of volume, in triplicate.

2.7. Effect of pH and temperature on carbohydrate oxidases activity

The effect of the pH on the oxidative activity of COX and NagOX was measured using a peroxidase-coupled reaction in the presence of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid in 50 mM Britton-Robinson buffer (pH 4.0 – 11.0). The measurements were carried out in triplicate at 30 °C and 50 °C for COX and NagOX, respectively. To assess the effect of temperature on the oxidative activity of the enzymes, measurements were taken at a range of temperatures (20 – 80 °C) for both enzymes in a 50 mM Britton-Robinson buffer at pH 6.0 and 9.0 for COX and NagOX respectively. The measurements were also performed in triplicate.

2.8. Effect of pH on carbohydrates oxidases stability

The pH stability of Cellobiose oxidase (COX) and N-acetylglucosamine oxidase (NagOX) was evaluated at the previously determined optimal temperature (30 °C for COX and 50 °C for NagOX), under non-reactive conditions. 1 mL of each enzyme was incubated in 50 mM Britton-Robinson buffer at a range of pH values between 4.0 and 9.0 (11.0 in the case of NagOX) Ki, at 1000 rpm. The inactivation profiles for all cases were well described by a one-stage first-order kinetic model with no residual activity [21]:

$$\frac{e}{e_0} = \exp(-k_D * t) \quad (1)$$

Where e_0 and e are the initial and residual enzyme activity after time t respectively, and k_D is the first order kinetic rate constant of inactivation. Half-time life ($t_{1/2}$) is defined as the time when the enzyme activity has been reduced to 50 % of its initial value.

2.9. Determination of catalytic potential (CP)

The catalytic potential (CP) is defined as the amount of activity that a defined mass of biocatalyst is capable of providing during a defined time in specified conditions of incubation [58]. The determination of the catalytic potential is calculated using the following expression:

$$CP = \int_0^t A(t)dt = \int_0^t e_0 \bullet \exp(-k_D * t)dt \quad (2)$$

Where CP is the catalytic potential ($AU/mg_{protein}$), t is the time established for the biocatalyst replacement (h) and A is the activity, expressed as $AU/mg_{protein}$ as a function of time present for the biocatalyst in specific conditions of incubation which is the expression obtained by the inactivation model (Eq.1). To compare all the biocatalyst at the different conditions, the time established for the biocatalyst replacement selected was the Half-time life.

2.10. Enzymatic pretreatment of wastewaters

A defined volume of wastewater was putting treated with the glucoamylase enzyme mixture from Novozymes (Glucoamylase/Pullulanase/Acid-Amylase/Lysophospholipase preparation), to obtain higher concentrations of glucose. The pH of the wastewater was adjusted to 5.5 and incubated at 65 °C for 2 h with 0.5 % of the total volume of the glucoamylase preparation, following the protocol described in the supplementary material. The concentration of glucose was quantified using a YSI 2950D Biochemistry Analyzer (YSI, Yellow Springs, OH, USA), with a range of 40–45 mM of glucose.

2.11. Hydrogen peroxide production on wastewater from cotton processing

In order to assess the efficacy of hydrogen peroxide production, different amounts of activity for COX and NagOX were added to a defined volume of wastewater derived cotton processing. In the case of COX, the pH of the wastewater was adjusted to 6.0 using HCl 6 M, while in the case of NagOX the pretreated wastewater, the pH was adjusted to 8.0 using NaOH 6 M.

In the case of the reactions conducted with a pH-controlled system, the pH was set to the specified values (6.0 for COX and 8.0 for NagOX) using a 0.5 M NaOH solution. The monitoring and addition of NaOH were prosecuted using a pH-meter Ti Touch 916 from Metrohm (Herisau, Switzerland). Obtained wastewater were treated with 5 % of active charcoal (50 g per litre) for color remotion for subsequent biobleaching application. The solution was incubated in rotative agitation for 30 min at room temperature, to then remove the insoluble fraction by centrifugation at 8000 g for 20 min.

The concentration of hydrogen peroxide was determined using the vanadium pentoxide (V_2O_5) method, previously described. Samples were collected at regular intervals throughout the entire reaction process, with each sample being analyzed in triplicate.

2.12. Bio-bleaching of cotton fabric

10 cm× 10 cm cotton fabric samples were bleached in 150 mL of media at 90 °C, pH 8.0, and 20 mM H_2O_2 for 1 h in a rotating Julabo SW22 water bath. Three conditions were tested: (1) distilled water with commercial H_2O_2, (2) wastewater with enzymatically generated H_2O_2, (3) wastewater with enzymatically generated H_2O_2 and 5 g/L TAED (tetraacetylethylenediamine) and (4) wastewater with enzymatically generated H_2O_2, decolored with 5 % active charcoal, and 5 g/L TAED (tetraacetylethylenediamine). Whiteness degree (%WI) was determined by folding the fabric 2 times, and the measurements were performed on quadruplicates using a reflectance spectrophotometer Minolta CM-3600d, measurement geometry of d/8, illuminant D65, spotter 10° with specular component exclusion.

3. Results

3.1. Characterization of wastewaters from cotton processing

Aiming to develop an enzymatic system to produce hydrogen peroxide from cotton textile effluents, an in-depth characterization is necessary. The carbohydrate profile and the total sugar content of the

effluents from different processes stages need to be considered when developing and implementing the enzymatic system. Therefore, a quantification of the total sugar content and an HPLC-IR analysis of individual carbohydrate concentrations were performed on wastewaters generated during desizing and pre-wash steps, which are the wastewaters obtained after amylase treatment (Fig. 1). Additionally, COD levels of both types of wastewaters were quantified, as this is an important parameter for environmental impact assessment. Table 1 presents the different amounts of carbohydrates and their concentrations in two distinct stages of the wet processing, as well as the COD levels.

The wastewater from the desizing chamber, where the fabric is exposed to an amylase solution, exhibited a notable glucose content. In contrast, the wastewater produced in the pre-washer chambers presented a diverse range of sugars. The observed difference between wastewater composition can be attributed to the enzymatic hydrolysis of starch by α-amylase, which results in the release of simpler carbohydrates, such as tri- and di-saccharides. However, the rate of glucose production is relatively low, as saccharification is not a primary application of α-amylase preparations [4].

It is noteworthy that several investigations have demonstrated the utilization of wastewater from cotton processing to produce hydrogen peroxide [4,5,38,42]. This suggests the potential for achieving elevated glucose concentrations when subjected to glucoamylase pretreatment. Nevertheless, there are no available reports on wastewater characterization regarding the type and concentration of carbohydrates, which are crucial factors for the successful development of hydrogen peroxide production.

Considering the total amount of carbohydrates, the broad range of their content, and the availability of the industrial wastewaters, the stream from the pre-washer chamber is considered as the most suitable for the application of carbohydrate oxidases.

The pH and COD levels were analyzed in the selected wastewater. The pH of the stream from the pre-washer is slightly acid, with a value between 6.3 and 6.9, which is consistent with the previous addition of amylases (optimum pH of 6) [26,50]. In terms of COD, the analysis shows values consistent to those reported in the literature, which vary from 15000 to 60000 mg/L [31,33,44].

3.2. Enzyme characterization

Given that Cellobiose oxidase and N-acetylglucosamine oxidase were selected to produce hydrogen peroxide utilizing wastewater from cotton processing, a thorough characterization is imperative. Both enzymes were subject to a study in terms of screening substrate, pH and temperature profile, along with stability assessments, to determine the optimal conditions for efficient hydrogen peroxide production.

Table 1
Total sugar content and carbohydrate profile from desizing and pre-washer stage of cotton industrial processing.

	Concentration (g/L)	
Parameter	**Desizing chamber**	**Pre-washer chamber**
Total sugar content	9.2	5.8
Glucose	3.5	0.3
Fructose	0.2	0.9
Manose	< 0.2	< 0.2
Galactose	< 0.2	< 0.2
Xylose	< 0.2	< 0.2
Maltose	< 0.2	0.6
Cellobiose	0.3	0.3
Saccharose	< 0.2	2.2
Lactose	< 0.2	< 0.2
Maltotriose	< 0.2	0.8
COD (mg/L)	33440 ± 1640	36640 ± 1830

3.2.1. Screening substrate

Considering the diverse carbohydrate composition of cotton processing wastewater, particularly the abundance of starch in the initial stages of the process, it was crucial to assess the substrate specificity of the studied carbohydrate oxidases. While cellobiose oxidase primarily utilizes cellobiose as a substrate, native NagOX exhibits activity against N-acetylated sugars. A modified variant of NagOX (L251R) with improved recognition of smaller carbohydrates. Broad substrate recognition is key for efficient hydrogen peroxide production. To this end, a panel of mono-, di-, tri-saccharides, as well as more complex carbohydrates, were evaluated (Table 2). The maximum oxidative activities, used as 100 % reference, were 22.0 AU/mg_{enzyme} for Cellobiose oxidase (measured with 20 mM cellobiose) and 69.5 AU/mg_{enzyme} for N-acetylglucosamine oxidase L251R (measured with 50 mM glucose), respectively.

By analyzing the substrate specificity of both enzymes, it becomes evident that COX is a more promiscuous enzyme, recognizing a diverse array of carbohydrates as substrates, which could consequently lead to the production of hydrogen peroxide. The results demonstrate that the enzyme exhibits high activity towards cellobiose, the natural substrate of the enzyme, as well as significant activity towards other monosaccharides (glucose, galactose, xylose), disaccharides (maltose and saccharose) and trisaccharide (maltotriose). This is consistent with previous reports on the promiscuity of cellobiose oxidase towards different sugars [15,29,60] as well as others glucooligosaccharide oxidases from fungal sources [36,51].

In comparison, NagOX demonstrates notable activity towards glucose and galactose, while exhibiting relatively lower activity towards other carbohydrates such as cellobiose, mannose and maltose. For more complex carbohydrates, the activity was essentially null. NagOX displays significant activity towards N-acetylated sugars like N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, which are their main substrate, however, the introduction of a modification at the active site (Leu251Arg) enables the alteration of the recognition to monosaccharides, including glucose and galactose. Similar results were obtained previously with the same modification in Chitooligosaccharide oxidase (ChitO) [9].

3.2.2. Activity towards pH and T

Regarding the behavior of the enzymes at different pH values (Fig. 2A), it can be observed that COX exhibits a profile similar to other carbohydrate oxidases derived from fungal sources, including glucose and galactose oxidases [14,51]. The enzyme exhibits a maximum activity peak at pH 6.0, although it also displays a relative activity above 60 % over a broad range of pH values. Conversely, NagOX shows a more alkaline pH profile, reaching a maximum activity at pH 9.0, which corroborates previously described results for this enzyme [9]. Furthermore, NagOX still exhibited a significant amount of oxidative activity at higher pH, showing 75 % of the maximum activity.

In terms of temperature profile (Fig. 2B), COX presents a relatively mild temperature profile, with an optimal temperature value of 30 °C. An increase in temperature results in a reduction of the activity towards cellobiose between 60 % and 80 %. In contrast, NagOX is more active at higher temperatures than COX, with an optimum temperature value of 50 °C. However, there is a range of relative activity over 80 % between 40 and 70 °C.

3.2.3. pH stability of N-acetylglucosamine oxidase (NagOX) and Cellobiose oxidase (COX)

Fig. 3A illustrates the residual activity of NagOX at varying pH levels over time. Regardless of the optimal pH value for NagOX (pH 9.0), neutral or slightly acidic conditions result in higher enzyme stability: at pH 6.0 NagOX exhibits a half-lifetime of 429 h, which is 14-fold higher than at pH 9.0. In contrast, the time course inactivation of COX (Fig. 3B) reveals superior stability across the entire range of pH compared to NagOX. Notably, COX presents better stability under mildly acidic

Table 2
Substrate screening of Cellobiose oxidase from Microdochium nivale (COX) and,N-acetylglucosamine oxidase L251R from Ralstonia solanacearum (NagOX) on 50 mM Britton-Robinson buffer. Cellobiose 20 mM for COX and Glucose 50 mM for NagOX, were set as 100 % of relative activity.

Carbohydrate	COX (%)	NagOX (%)
D-(+)-Glucose	27.6	100.0
D-(+)-Cellobiose	100.0	3.0
D-(+)-Galactose	12.1	76.2
D-(+)-Mannose	0.0	4.4
D-(+)-Fructose	0.0	0.0
D-(+)-Xylose	24.8	0.0
D-(+)-Maltose	41.4	2.8
D-(+)-Saccharose	20.6	0.0
Maltotriose	15.1	0.0
Pectin	0.0	0.0
Amilopectin	0.0	0.0
Dextrin	0.0	0.0

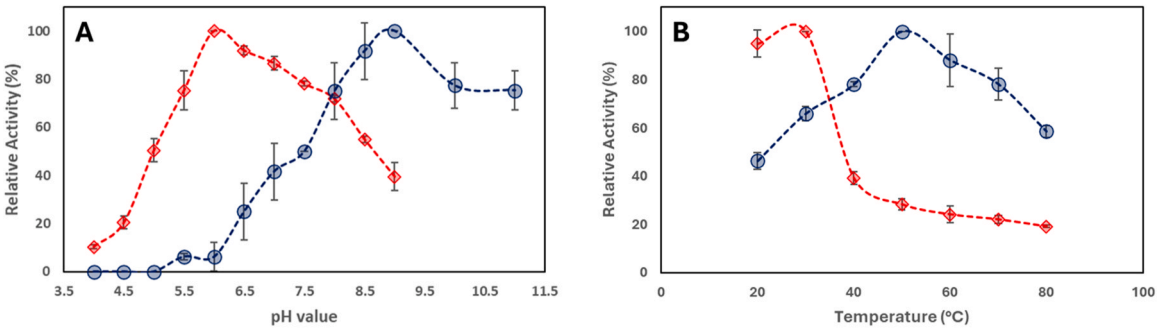

Fig. 2. pH (A) and Temperature (B) profile of COX (◊) and N-acetylglucosamine oxidase (○). The study was performed on 50 mM Britton-Robinson buffer using 20 mM of cellobiose and glucose 50 mM as a substrate respectively for each enzyme.

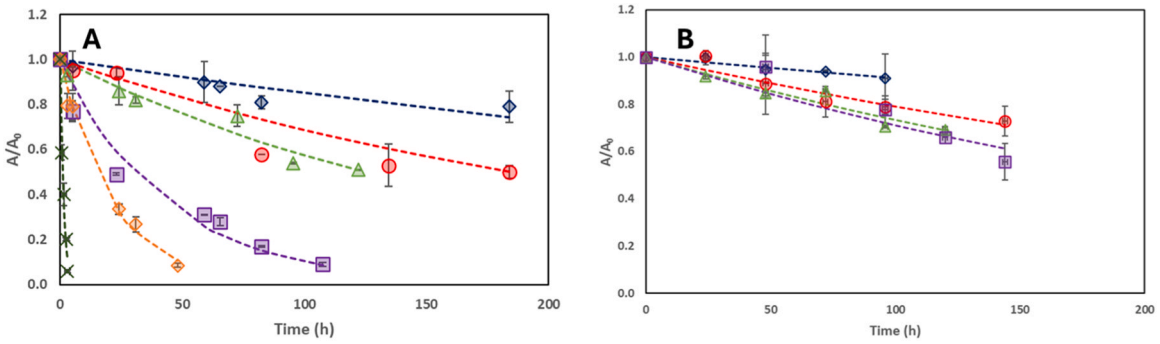

Fig. 3. Time course inactivation of (A) NagOX L251R at 50 °C and (B) COX at 30 °C at pH 6.0 (◊), 7.0 (○), 8.0 (△), 9.0 (□), 10.0 (⬨) and 11.0 (✗).

conditions, with a half-lifetime of 743 h, which is 3.6-fold higher than at pH 9.0. Moreover, the optimal conditions for activity align with the maximum stability conditions for COX.

The concept of catalytic potential defines the overall activity that a catalyst can exhibit under specific conditions of pH and temperature, considering both the specific activity and stability. In mathematical terms, it represents the integral of the function that describes the enzyme inactivation (Eq. 2). This parameter provides a more

comprehensive understanding of the optimal conditions for the reaction, where maximum activity can be effectively harnessed.

Considering the enzyme inactivation kinetics at varying pH values, the half-life time attained under these conditions, and the enzyme activity shown at these specific pH values, the catalytic potential was determined (Table 3). See supplementary material for time course inactivation at specific activity.

For NagOX, optimal catalytic activity was observed at pH 8.0. While NagOX demonstrated significant oxidative activity at alkaline pH, including peak activity, rapid enzyme inactivation under these conditions necessitated a reduction in reaction pH to maximize biocatalyst utilization.

On the other hand, the maximum catalytic potential for COX was attained at pH 6.0, where the stability and activity reached their peak values. Consequently, subsequent studies related to the production of hydrogen peroxide were conducted under these specified conditions, pH 8.0 and 50 °C for NagOX and pH 6.0 and 30 °C for COX.

Given the characteristics of the carbohydrate-rich effluent from the pre-wash stage and the information collected from the exhaustive characterization of both N-acetylglucosamine oxidase (NagOX) and Cellobiose oxidase (COX), two enzymatic strategies were developed for a bioprocess for hydrogen peroxide production (Fig. 4).

Considering the high promiscuity of COX for different carbohydrates presents in the effluents, hydrogen peroxide production will be conducted at pH 6.0 and 30 °C, with the enzyme directly applied to the wastewaters. Given the carbohydrate profile of the selected wastewater and the promiscuity of COX, the maximum concentration of hydrogen peroxide is anticipated to reach 13.8 mM.

In contrast, due to the pH and temperature profiles as well as operational stability, NagOX will be employed at pH 8.0 and 50 °C. However, an additional step must be assessed due to NagOx's specificity towards glucose as the primary substrate. To achieve this, a preliminary treatment using glucoamylases is necessary to obtain higher glucose concentrations. Accordingly, the theoretical maximum concentration of hydrogen peroxide is expected to range between 40 and 45 mM.

3.3. Production of hydrogen peroxide using real wastewaters from cotton industry

Once the wastewater from the pre-wash stage was identified as a suitable substrate (in terms of availability during the process and composition), and the optimal conditions for both carbohydrate oxidases were determined, of hydrogen peroxide production was studied in a batch mode.

3.3.1. Analysis of effectiveness of carbohydrate oxidases

To assess the effectiveness of both NagOX and COX in producing hydrogen peroxide within real wastewater, a range of different enzymatic activity loads were studied. This analysis provides crucial insights into the capability of both strategies in terms of productivity and the final concentration of hydrogen peroxide, as well as establishing the basis for optimizing the enzymatic system.

The time course of H_2O_2 production was evaluated for both biocatalysts. The results demonstrated significant discrepancies in the performance of COX and NagOX. In the case of COX (Fig. 5A), as previously mentioned, it is added directly to the wastewater due to the recognition of the different carbohydrates. It can be observed that there is a direct correlation between the amount of oxidative activity offered and the increasing production of hydrogen peroxide during the reaction. Moreover, considering the final concentration of H_2O_2 reached (12.2 ± 0.20 mM), at 0.7 AU/mL, 1.3 AU/mL and 10 AU/mL, it is comparable to the maximum hydrogen peroxide that can be achieved (13.8 mM), confirming that the application of COX can oxidize almost all the total carbohydrates in the wastewaters over a period of 5 h.

Conversely, as previously stated, the use of NagOX (Fig. 5B) in comparison with COX, requires a wastewater pretreatment with glucoamylases, as NagOX is primarily substrate-specific for glucose. Unlike COX, the offered oxidative activity demonstrated a limited effect in the initial stage of the reaction. The concentration of hydrogen peroxide increases during the first hour, after which it either increases slowly or stops increasing altogether. This phenomenon can be attributed to two primary causes: Firstly, hydrogen peroxide is unstable in alkaline conditions, decomposing into radical species (-OH), which exhibit a bleaching effect [46,54]. In addition, hydrogen peroxide can also act as an enzymatic inactivating agent, modifying methionine residues and affecting the enzyme stability [19]. This negative effect is more pronounced with NagOX than COX, as the reaction with NagOX is carried out at pH 8.0, while for COX the pH is set at 6.0. Also, the decrease in pH value during the reaction due to the formation of gluconic acid as secondary product of glucose oxidation may affect the activity of NagOX, as its pH profile indicates that the activity is low at acidic pH values. Meanwhile, the initial effect is more pronounced at the beginning of the reaction, while the second effect becomes more evident as the activity offered increases.

It is noteworthy that the initial glucose concentration in the wastewaters after being pretreated with glucoamylase is 40–45 mM. This indicates that the maximum hydrogen peroxide concentration reached of 10.9 ± 0.13 mM (10 AU/mL), correspond to a yield of reaction of nearly 25 %. Studies employing a saccharification process prior to glucose oxidation have reported lower glucose concentrations, highlighting the variability of the wastewaters. Moreover, these studies reveal that the application of glucose oxidase results in a final hydrogen peroxide concentration of 22 mM after the application of glucose oxidase, particularly in acidic conditions, which allows for a more stable hydrogen peroxide environment [37].

3.3.2. Operation mode: pH control system

Considering the fluctuation of pH levels throughout the reaction and the effect on the relative activity, both enzymes were evaluated in a pH-controlled system. To assess the effectiveness of a pH-controlled system in both strategies, an oxidative activity of 1 AU/mL was chosen, ensuring sufficient sensitivity to observe differences between both systems (Fig. 6).

The time course reaction for NagOX (Fig. 6A), demonstrates that the implementation of a pH-controlled system led to significantly enhanced results compared to a system without control. Maintaining the pH of the reaction at 8.0 allows the enzyme to achieve its maximum rate. Consequently, in the case of maintaining the pH 8.0, NagOX reached the maximum H_2O_2 concentration (26.5 mM) after 19 h, while the reaction without control reached the same concentration after 24 h. The pH variation observed during both reactions (Fig. 6B) supports the hypothesis that the optimum pH of NagOX plays a critical role in its implementation, reinforcing the idea that pH-controlled should be applied.

Regarding COX, the implementation of a pH-controlled system has no significant impact on COX performance (Fig. 6C). The two systems followed a resembling evolution during the reaction, reaching a similar concentration of hydrogen peroxide after 24 h of reaction. Based on the same analysis of NagOX and considering the pH variation during both

Table 3
Half lifetime ($t_{1/2}$) and catalytic potential (CP) of NagOX and COX at different pH values.

	NagOX		COX	
pH value	**$t_{1/2}$ (h)**	**CP (AU)**	**$t_{1/2}$ (h)**	**CP (AU)**
6.0	428.9	729.0	743.0	**10974.4**
7.0	185.5	2246.4	295.5	4026.6
8.0	125.5	**2725.8**	222.2	2442.3
9.0	30.2	832.4	204.2	1489.5
10.0	14.8	352.9	-	-
11.0	0.9	22.4	-	-

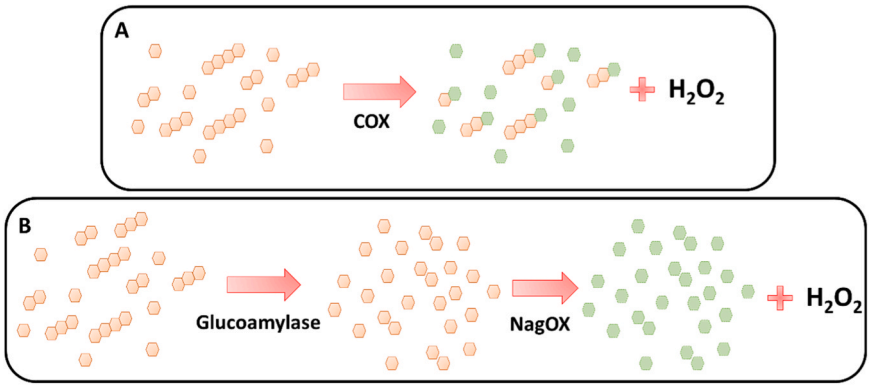

Fig. 4. Enzymatic strategies for hydrogen peroxide production using pre-washer from cotton industrial processing effluent as feedstock. (A) Utilization of cellobiose oxidase (COX) directly in the effluents. (B) Utilization of N-acetylglucosamine oxidase (NagOX) prior saccharification of effluents using glucoamylases.

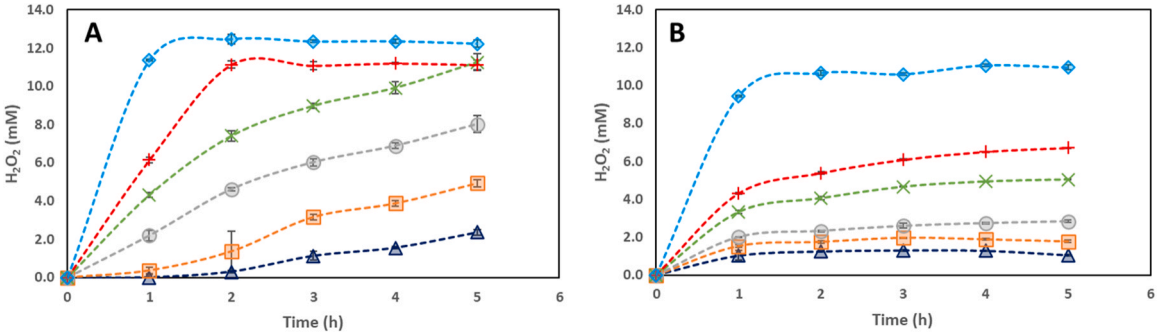

Fig. 5. Time course reaction of hydrogen peroxide production on pre-washer wastewater using COX (A) at 30 °C and pH 6.0 and NagOX (B) at 50 °C at pH 8.0 at different amount of catalytic activity. 0.07 AU/mL (△) 0.17 AU/mL (□) 0.3 AU/mL (○) 0.7 AU/mL (✕) 1.3 AU/mL (+) 10 AU/mL (◇).

reactions (Fig. 6D), it can be concluded that the hydrogen peroxide production by COX could be operated without a pH-controlled system.

3.3.3. *Feedstock flexibility*

The fluctuation in the parameters of effluents was a subject of concern in relation to the flexibility of the system. This is a common challenge in process development when wastes are used as feedstock. The treatment of different types of cotton fabric (e.g., fabric size and level of thread) employs different quantities of chemicals in the sizing stage. Consequently, the degradation of the starch could result in a different composition in terms of carbohydrate profile and total carbohydrate content, as well as a change in color. Based on the aforementioned information, the production of hydrogen peroxide was carried out using two different batches of pre-washing wastewater to evaluate feedstock flexibility.

To facilitate the comparison between both enzymes and operational modes, in relation to the type of wastewater, a summary of the final concentration of hydrogen peroxide and productivity is provided in Table 4.

The analysis of the initial wastewater sample revealed significantly higher concentrations of hydrogen peroxide and productivity when NagOX was employed in comparison to COX. However, the analysis of the second batch of wastewater revealed a decline in the concentration of hydrogen peroxide when NagOX was applied (Fig. 7A), whereas COX was able to maintain the concentration of H_2O_2, by achieving the same level of productivity (Fig. 7.B). It is noteworthy that the second wastewater batch exhibited elevated values of COD and glucose equivalent concentration (3-fold of COD and a 5-fold glucose equivalent) (Table S.2). Furthermore, the liquor presented a darker color. These observations may indicate the presence of higher concentrations of other chemicals involved in the process. Considering these assumptions, COX may be more robust in the presence of fluctuations in the wastewater parameters as well as the pH variations during reaction, whereas NagOX performance is highly dependent on the pH and composition of the wastewaters.

A comparison of productivity results from this study compared with those reported in literature using wastewaters from the cotton processing, NagOX and COX in these conditions produce 10–20 times less H_2O_2.Related to the above, the most notable distinction between this study and those previously published is the previous studies used at least 10-fold more enzyme activity per mL of media [5,12,38]. Furthermore, these studies employed commercial preparations of glucose oxidase (GOX) enzymes, which are primarily designed for use in the food and beverage industry rather than the textile industry. Such commercial GOX preparations can exhibit a high catalase activity, which can result in the consumption of hydrogen peroxide [61]. The presence of catalases represents a significant drawback in the practical application of the system.

Physicochemical parameters, including chemical oxygen demand (COD) and pH, were measured following hydrogen peroxide production. While COD levels remained largely unchanged, indicating the high oxidation potential of the acids generated from carbohydrate oxidation, pH values were found to vary between 5.5 and 8.0 depending on the specific production strategy and modality employed.

3.4. *Proof of concept*

To evaluate the practical application of enzymatically produced

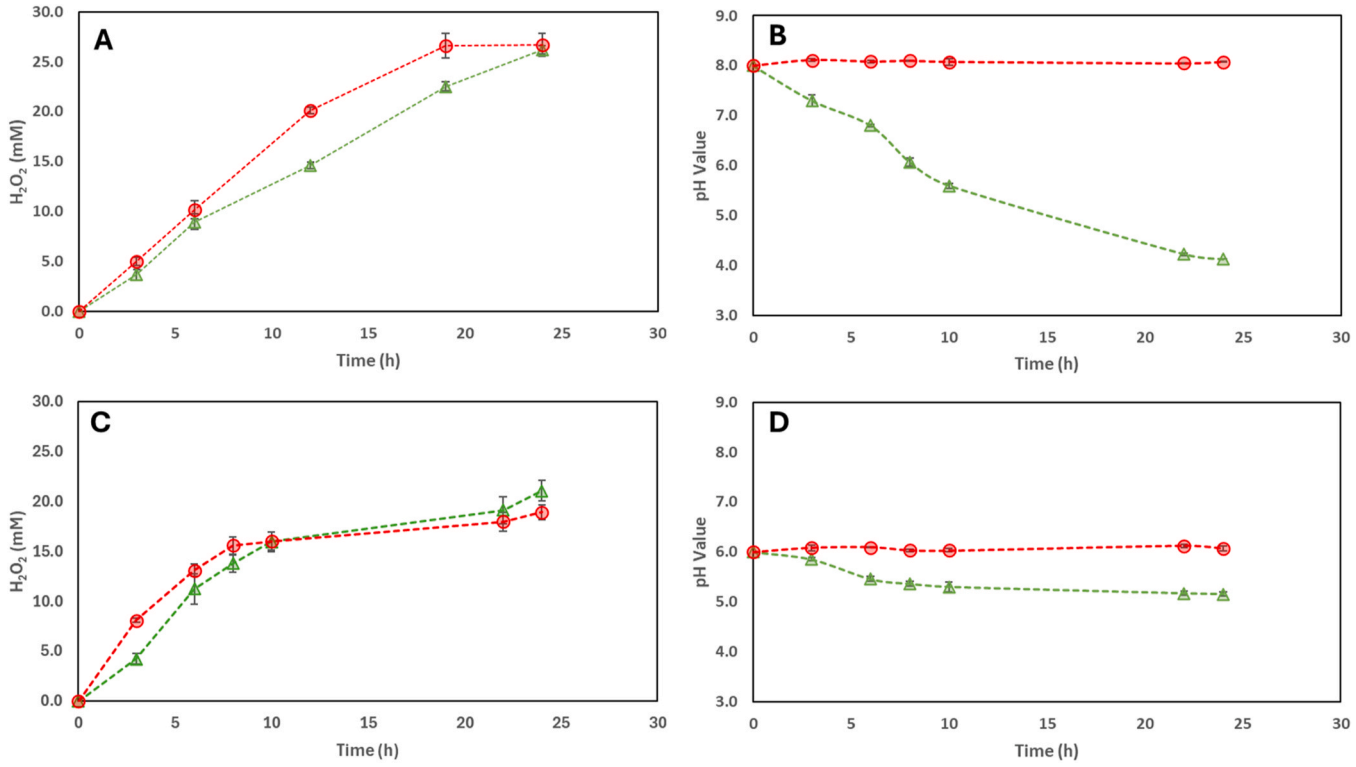

Fig. 6. Time course reaction of hydrogen peroxide using **(A)** N-acetylglucosamine oxidase (NagOX) at pH 8.0 and 50 °C, and **(B)** the pH fluctuation during reaction, **(C)** Cellobiose oxidase (COX) at pH 6.0 and 30 °C, and **(D)** the pH fluctuation during reaction. Reaction performed with no pH control (Δ), with pH control (o).

Table 4
Summary of hydrogen peroxide concentration and productivity using 1 AU/mL of NagOX and 1 AU/mL of COX on two different batches of wastewater from cotton processing. NagOX was employed at pH 8.0, 50 °C and with a pH-control system. COX was employed at pH 6.0, 30 °C and without a pH-control system.

	NagOX		COX	
Batch n°	**H_2O_2 produced [mM]**	**Productivity ($mM \cdot L^{-1} h^{-1}$)**	**H_2O_2 produced [mM]**	**Productivity ($mM \cdot L^{-1} h^{-1}$)**
1	26.7 ± 1.16	1.40 ± 0.07	21.07 ± 1.05	0.88 ± 0.04
2	17.41 ± 0.59	0.88 ± 0.00	21.71 ± 0.18	0.90 ± 0.01

hydrogen peroxide from wastewater, bio-bleaching experiments were conducted on cotton fabric. The whiteness index (WI%), measured in Berger Degrees, was assessed. The results of the cotton fabric samples were compared to those obtained using distilled water and a hydrogen peroxide activator, TAED.

As shown in Table 5, the color of the wastewater significantly impacted the final whiteness index of the cotton fabric compared to controls using distilled water. The addition of TAED as a hydrogen peroxide activator markedly enhanced bleaching, resulting in a nearly 3-point increase in whiteness index compared to distilled water alone. Furthermore, decoloring the wastewater with active charcoal at a 1:20 ratio significantly reduced its darkening effect. After 30 min of incubation, the color was significantly removed with no negative impact in the hydrogen peroxide concentration, improving the whiteness index by almost 10 points under identical conditions. These results demonstrate promising potential for the industrial implementation of a continuous valorization strategy for cotton textile wastewater. Visual representation of wastewater before and after being decolored as well as the effect on the fabric in a bleaching process are presented in the supplementary material.

Considering that hydrogen peroxide was produced at 1 AU/mL, higher oxidative activity, potentially achieved by increasing enzyme activity, could significantly increase the final hydrogen peroxide concentration. This approach could potentially reduce reliance on commercial hydrogen peroxide in the bleaching stage [55].

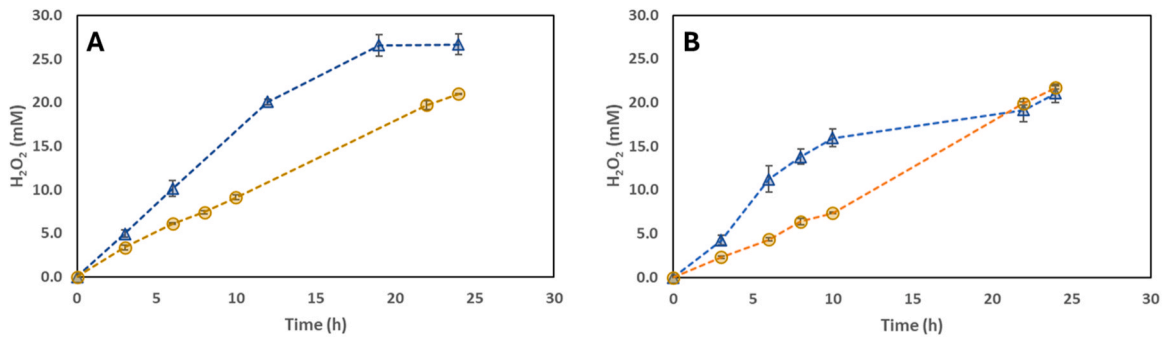

Fig. 7. Time course reaction of hydrogen peroxide production on pre-washer wastewater using 1 AU/mL of **(A)** NagOX at 50 °C and pH 8.0 with a pH-control system, and **(B)** COX at 30 °C at pH 6.0 without a pH-control system. Reaction performed on batch one (Δ) and batch two (o) of wastewater from cotton processing.

Table 5
Berger degree (%WI) of cotton fabric samples after 1 h of bleaching process at different conditions. (B) Distilled water pH 8.0, 90ºC, 20 mM commercial H_2O_2, (C) wastewater pH 8.0, 90ºC, 20 mM enzymatically produced H_2O_2, (D) wastewater pH 8.0, 90ºC, 20 mM enzymatically produced H_2O_2 and 5 g/L TAED as hydrogen peroxide activator, (E) decolored wastewater pH 8.0, 90ºC 20 mM enzymatically produced H_2O_2 and 5 g/L TAED as hydrogen peroxide activator.

Condition	Berger Degree (WI%)
A (Unbleached)	16.5 ± 0.10
B	23.9 ± 0.11
C	18.4 ± 0.08
D	26.1 ± 0.75
E	34.9 ± 0.09

4. Conclusions

A characterization of effluents from different stages of the cotton textile industry was performed, and the carbohydrate profile, as well as a total sugar content was determined. The wastewater from the pre-wash step of the process was identified as the optimal substrate to produce hydrogen peroxide for bio-bleaching purposes. Furthermore, a comprehensive characterization of N-acetylglucosamine oxidase (NagOX) and Cellobiose oxidase (COX) was conducted, and based on the findings, two distinct strategies were proposed. Both strategies were successfully implemented in real wastewater, resulting in the production of significant quantities of hydrogen peroxide. In the case of NagOX, the process was implemented with a pH-control system, given that the enzyme presents an alkali profile and is more sensitive to a variation of the pH in the media. Moreover, COX demonstrated great robustness and flexibility, thereby enabling the strategy to function without the need for pH control. Additionally, different batches of real industrial wastewater were tested, aiming to evaluate the robustness and flexibility of the process. Regarding that COD levels remained relatively stable, likely a consequence of the propensity of glucose oxidation intermediates to undergo continued oxidation.

The results indicate that both strategies could be employed, even in the presence of natural fluctuations in the substrate. In conclusion, both carbohydrate oxidases are promising biocatalysts for the reuse of wastewater to produce hydrogen peroxide, which has the potential to mitigate the environmental impact of the cotton industry.

In terms of the whiteness index in cotton fabric after being treated in a bleaching process using wastewater, the results showed that the color of the effluent represents a problem. However, the utilization of a active charcoal to remove the dark color in addition of TAED as a hydrogen peroxide activator could be employed as a strategy to mitigate this disadvantage, allowing to reduce the water and energy necessary to bleach cotton fabrics.

CRediT authorship contribution statement

Romero Oscar: Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Guillén Marina:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Álvaro Gregorio:** Supervision, Resources, Funding acquisition. **Fredes Yerko:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL tool in order to improve the grammar and English of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper: Oscar Romero reports financial support was provided by European Union's Horizon 2020. Oscar Romero reports financial support was provided by Generalitat de Catalunya 2021 SGR 00143. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.115902.

Data availability

The data are available in CORA data repository: https://doi.org/10.34810/data1534

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