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Research Article

In-situ Enzymatic Generation of Hydrogen Peroxide for Bleaching Purposes

Bleaching detergent formulations contain environmentally unfriendly bleaching agents (perborates and percarbonates), which cause aquatic eutrophication, although without these compounds detergents are much less efficient for the washing processes. In an effort to replace these compounds, in this study, hydrogen peroxide was generated as a bleaching compound by means of enzymatic reactions. Three different pathways were investigated. The first one was the H_2O_2 production from glucose by glucose oxidase. The second one was the production of H_2O_2 from carboxymethylcellulose (CMC) by the action of both cellulase, which promotes the hydrolysis of the polymeric chain, and glucose oxidase, which oxidizes the smaller fractions to produce H_2O_2. Finally, H_2O_2 was also obtained from ethanol, which is present in liquid detergents, by the action of the enzyme alcohol oxidase. In the search for maximal peroxide production, substrate concentration and enzymatic activities were optimized. The effect of H_2O_2 produced in the washing process was simulated by means of a process of cotton bleaching. Although enzymatic-reaction oxidations produced higher levels of hydrogen peroxide (up to 1 g/L after 8 h), higher improvement of cotton whiteness was achieved from CMC and from ethanol. The milder conditions of temperature and pH, biodegradability and less consumption of water and energy are advantageous for enzymes as good substitutes for H_2O_2 precursors and make them appropriate to be considered in detergent formulations. These enzymes could be combined with other oxidative enzymes, such as peroxidases, in order to lower the required temperature and use a pH close to the neutral value during the bleaching processes.

Keywords: Detergents, Hydrogen peroxide, Oxidative enzymes

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1 Introduction

The most extensively used laundry detergents are powdered formulations, due to their adequate washing capacity. Their efficiency is based on the involvement of different components. The surfactants contribute to the washing process by forming micelles and removing the particulate and oily stains. The presence of proteases helps to eliminate protein remains, such as blood, cacao, milk or starch. The sodium salt of carboxymethylcellulose (CMC) contributes to the elimination of the re-deposition of dust in the fabrics [1, 2]. The builders remove water hardness by ion exchange [3]. The bleaching agents (sodium perborate, sodium percarbonate) are mainly used in those detergents especially designed for bleaching and removing difficult stains, as they oxidize remains of coffee, tea, wine or fruit. Some of the traditionally applied builders were tripolyphosphates, which caused an excess of nutrients in lakes and reservoirs, stimulating the proliferation of aquatic plants and generating an oxygen default for the subaquatic fauna. On the other hand, some bleaching precursors, such as perborate, were found to be toxic for aquatic life, as they release boron to the waters. Despite the fact that the powdered formulations are being modified in order to decrease their environmental impact (by substituting phosphates with zeolites or other less toxic compounds), they are not yet environmentally friendly, as they still include builders and oxidant bleaching agents that contribute to aquatic eutrophication. Liquid compositions cannot include these compounds, as they are unstable in aque-

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ous media, thus reducing the environmental impact of detergents. Furthermore, liquid formulations are easily dosed, more comfortable due to their lower size and easier dilution in water. However, the absence of some of the components contained in powdered formulations diminishes their washing properties.

Some efforts were focused on the search for a solution to reach cleanliness in the laundry by maintaining clean water and the environment. Incorporating enzymes into detergent formulations poses numerous advantages: they are effective under mild conditions of temperature and pH, are 100 % biodegradable and allow a saving of water and energy. Enzyme-containing products comprise more than 80 % of the total laundry detergent market in the United States, Europe and Japan [4]. Proteases are by far the most commonly used detergent enzymes, but amylases, lipases and cellulases are often added [5]. Amylases degrade starch and other carbohydrates; lipases hydrolyze lipids and make them more soluble; cellulases are able to hydrolyze the glycosidic bonds of cellulose and remove microfibrils from the surface of cellulosic fabrics, enhancing color brightness [6]. Apart from these, other enzymatic reactions could be studied in order to improve the washing capacity of detergents and minimize their environmental impact. For instance, hydrogen peroxide producing oxidases are able to generate H_2O_2 in situ during the washing process under mild conditions of temperature and pH; thus they could be good substitutes for bleaching agents as H_2O_2 precursors in bleaching detergents.

H_2O_2-producing oxidases are electron-transfer oxidases which reduce molecular oxygen to hydrogen peroxide, by catalyzing a two-electron transfer [7]. These enzymes are flavoproteins which contain one very tightly but noncovalently bound flavine adenine dinucleotide (FAD) cofactor per monomer; this cofactor is first reduced to its hydrogenated form ($FADH_2$) and then re-oxidized to its native form by molecular oxygen, with the concomitant formation of H_2O_2 (see Fig. 1) [8, 9].

H_2O_2-producing oxidases were previously applied in textile technology in one of the main finishing stages of fabrics, i.e. the bleaching step after desizing and scouring of the fibers

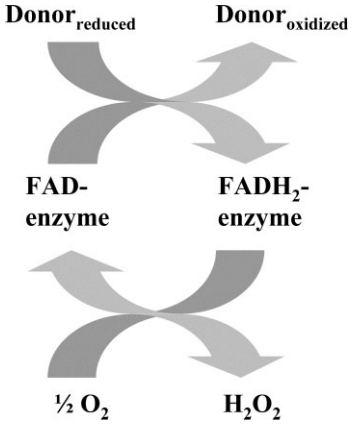

Figure 1. Catalytic cycle of H_2O_2-producing oxidases.

[10–14]. However, very few authors have studied their possible application in detergent formulations [15, 16]. Fig. 2 represents some systems for the enzymatic generation of H_2O_2 from different substrates that are usually present in detergent compositions or can be externally added: *(i)* H_2O_2 production from glucose by glucose oxidase; *(ii)* H_2O_2 production from CMC by the combination of two enzymatic reactions: the first one is based on the action of cellulases, which promote the hydrolysis of the polymeric chain; the smaller fractions can be later oxidized by glucose oxidase to produce H_2O_2; *(iii)* H_2O_2 production from ethanol, which is present in liquid detergents in concentrations up to 8 %, by the action of the alcohol oxidase enzyme [17, 18].

The first system involves the addition of glucose and glucose oxidase in the formulation. Glucose oxidase (β-*D*-glucose: oxygen 1-oxidoreductase (E.C. 1.1.3.4)) is an homodimer with a molecular mass of 130–320 kDa depending on the extent of glycosylation [19]. It catalyzes the oxidation of *D*-glucose to *D*-glucono-*δ*-lactone, which is further hydrolyzed to gluconic acid. Its substrate specificity was examined, and it was obtained that it also attacks other carbohydrates, although the reaction kinetics are slower [20]. This system was previously studied for the production of H_2O_2 in liquid detergents [15] and is analyzed here to lay the foundation for the second system.

The second system aims to exploit the presence of CMC and cellulases in the formulations to produce H_2O_2. Cellulases (E.C. 3.2.1.4) hydrolyze β-1,4-glycosidic bonds with a 6-*O* substituent on both adjacent glucose units of celluloses. Endoglucanases are a type of cellulases that cleave the cellulose chain internally, thus producing shorter polymer fragments and oligosaccharides [21]. CMC is a cellulose derivative with carboxymethyl groups bound to the hydroxyl groups of the glucose unit, and represents a good substrate for endoglucanases. Some of the oligosaccharides formed are able to act as a substrate of glucose oxidases, thus producing H_2O_2.

Finally, the aim of the third system is to make use of the percentage of ethanol present in liquid formulations (around 8 %) to produce H_2O_2. Alcohol oxidase (alcohol: oxygen oxidoreductase (E.C. 1.1.3.13)) is an oligomeric enzyme consisting of eight identical sub-units arranged in a quasi-cubic orientation [22]. Alcohol oxidase catalyzes the oxidation of primary low molecular weight alcohols into the corresponding aldehydes and H_2O_2, with a higher substrate affinity to ethanol [16].

The aim of this work is to establish an enzymatic system for the production of H_2O_2 as a bleaching agent in household laundries. The system is based on the addition of enzymes to the formulations; the selected enzymes are able to oxidize substrates that are present or can be added to detergents. This process provides two main advantages: the substitution of commonly applied bleaching agents by enzymes, thus preventing aquatic toxicity; and the generation of H_2O_2 in situ during the washing process, avoiding destabilization of components during storage. This work involves the analysis, optimization and comparison of different systems for the production of H_2O_2, as well as their application to a bleaching process.

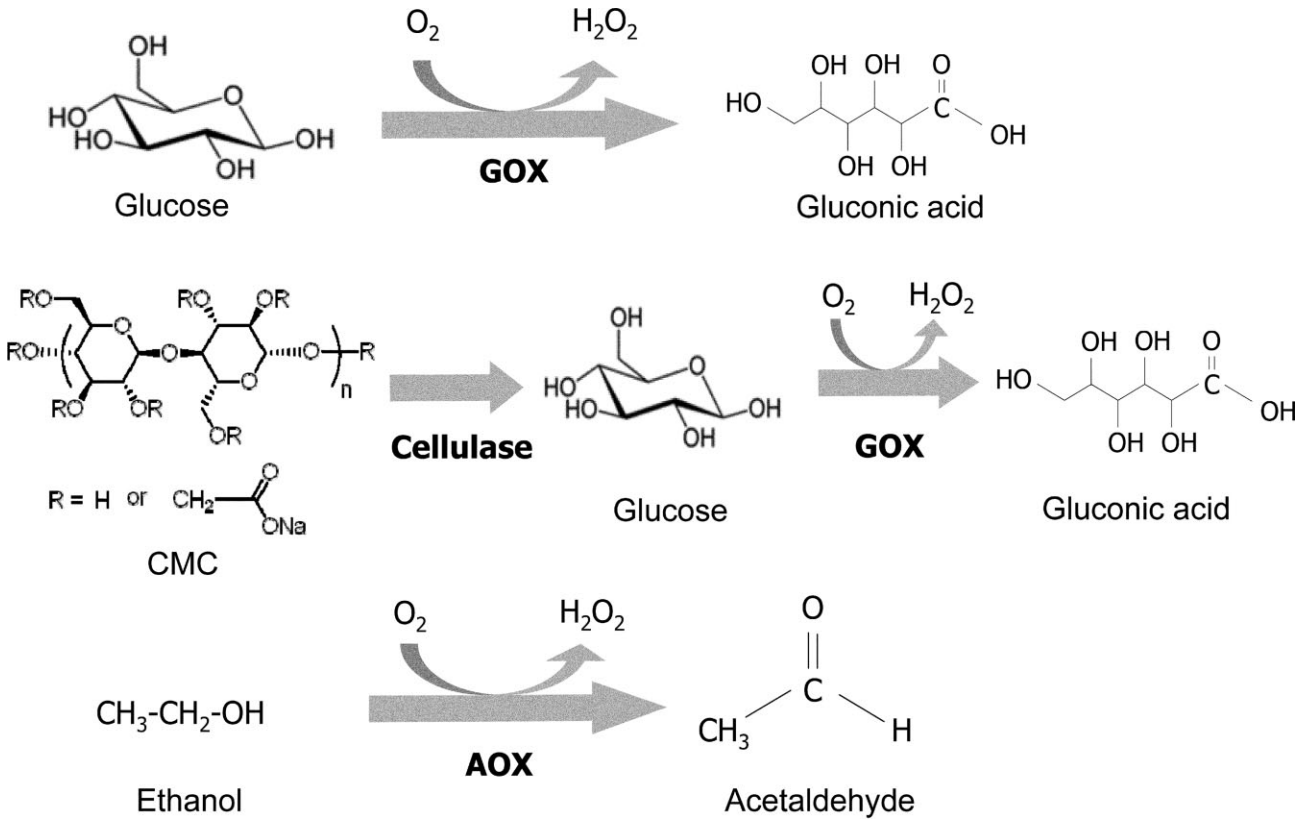

Figure 2. Enzymatic generation of H_2O_2 by three different systems. (GOX: glucose oxidase; AOX: alcohol oxidase).

2 Materials and Methods

2.1 Chemicals, Enzymes and Textile Substrates

Pure ethanol (99.0 %) was obtained from Panreac (Barcelona, Spain). The rest of the chemicals were purchased from the Sigma Chemical Company (Munich, Germany).

Glucose oxidase from *Aspergillus niger*, alcohol oxidase from *Pichia pastoris* and horseradish peroxidase (HRP) were obtained from the Sigma Chemical Company (Munich, Germany). Endoglucanase was kindly provided as a gift by Prof. G. Guebitz (Technical University of Graz, Austria).

The textile material used was 100 % cotton fabric (120 g/m^2).

2.2 H_2O_2 Production

The first system was studied by using glucose as a substrate and glucose oxidase as a catalyst. The 50 mL medium of the Erlenmeyer flasks consisted of: 4-12-20 g/L of glucose, 2-4-6 U/mL of glucose oxidase and 0.1 M of sodium acetate at pH 5.0. The assays were performed at 37 °C with an agitation speed of 100 rpm. Glucose oxidase activity, glucose consumption, H_2O_2 production and pH were followed for 8 h. Samples were taken each 2 h and analyzed in duplicate. Controls without enzyme were performed in parallel and no H_2O_2 production was observed.

The second system, i.e. the production of H_2O_2 from CMC, was studied in two steps. The reaction medium in the first step consisted of low or medium viscosity CMC with concentrations from 0.5 to 3 % [w/v]; 1-2-3 U/mL of endoglucanase and 0.1 M of sodium acetate at pH 5.0. The assays were performed at 37 °C and 100 rpm. The enzymatic activities and reducing-end sugars were followed at 2 h intervals during the 8-h experiment. Samples were analyzed in duplicate and controls without enzyme were simultaneously performed. In the second set of experiments, a double enzymatic reaction was performed. 50 mL-Erlenmeyer flasks with 3 % low viscosity CMC, 2-5-10 U/mL of endoglucanase, 4–6 U/mL of glucose oxidase and 0.1 M of sodium acetate at pH 5.0 were agitated at 100 rpm and 37 °C. Samples were taken each 2 h and enzymatic activities, reducing sugars and H_2O_2 production were measured.

In the third system of H_2O_2 production from ethanol, the medium consisted of 0.5-1-5-8 % of ethanol, 4 U/mL of alcohol oxidase and 0.1 M of potassium phosphate at pH 7.5. The assays were carried out at 25 °C for 8 h, and samples were taken every 2 h. The H_2O_2 production and alcohol oxidase activity were analyzed in duplicate. Controls without enzyme were performed in parallel and no production of H_2O_2 was obtained.

2.3 Measurements of Enzymatic Activity

Samples were purified first with Sephadex™ G-25 M PD10 columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) before activity measurements were carried out.

Glucose oxidase (GOX) activity was determined by a combined reaction with HRP. The H_2O_2 obtained during the oxidation of glucose by glucose oxidase activates the HRP, which catalyzes the oxidation of *o*-dianisidine. The reaction mixture contained a final concentration of 48 mM sodium acetate (pH 5.1), 0.16 mM *o*-dianisidine, 1.61 % [w/v] glucose, 1.94 U/mL HRP and up to 100 μL of supernatant in a total volume of 3.1 mL. Measurements were carried out at 35 °C at a wavelength of 500 nm in a Helios Gamma UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). The molar extinction coefficient of oxidized *o*-dianisidine was 7,500 $M^{-1}{\cdot}cm^{-1}$. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of *β-D*-glucose to *D*-gluconolactone and H_2O_2 per minute at pH 5.1 and 35 °C [23].

Carboxymethylcellulase (CMCase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.1 mL of the crude enzyme and 0.9 mL of low viscosity CMC solution (final concentration 2.4 %) in 50 mM sodium acetate buffer (pH 5.0) incubated at 37 °C for a period of 15 min. Reducing sugars were assayed by the dinitrosalicylic acid (DNS) method explained below. One unit of CMCase activity corresponded to 1 μmol of glucose equivalents released per minute under the assay conditions [24].

Alcohol oxidase (AOX) activity was determined by the oxidation of methanol to formaldehyde; the produced H_2O_2 catalyzes the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The reaction mixture contained a final concentration of 96 mM potassium phosphate (pH 7.5), 2 mM ABTS, 0.033 % [v/v] methanol, 0.83 U/mL HRP and up to 100 μL of supernatant in a total volume of 3.1 mL. Measurements were carried out at 25 °C at a wavelength of 405 nm in a Helios Gamma UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). The molar extinction coefficient of oxidized ABTS was 36,800 $M^{-1}{\cdot}cm^{-1}$. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of methanol to formaldehyde and H_2O_2 per minute at pH 7.5 and 25 °C [25, 26].

2.4 Measurement of Reducing Sugars

In order to determine the reducing sugars, 1 mL of sample was incubated with 3 mL of DNS reactive solution containing: 7.5 g/L DNS, 14 g/L NaOH, 216 g/L potassium sodium tartrate, 0.5 % [v/v] phenol and 6 g/L sodium metasulfite. The mixtures were boiled for 5 min and cooled at room temperature. Absorbance was measured at 640 nm in a Helios Gamma UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). The concentration of reducing sugars [g/L] was determined against glucose standards [27].

2.5 H_2O_2 Measurement

Hydrogen peroxide was assayed spectrophotometrically as previously described [28, 29]. Briefly, 200 μL samples of H_2O_2 were mixed to obtain a 1 mL mixture with final concentrations of 100 mM sodium phosphate buffer (pH 6.0), 0.282 mM ABTS and 1 U/mL of HRP. The colorimetric reaction was allowed to develop for 1 min, and the absorbance was followed at 420 nm in a Helios Gamma UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), versus a no-peroxide blank. The concentration of H_2O_2 (μM) was determined against H_2O_2 standards. Under these conditions, H_2O_2 concentration resulted to be linearly proportional to the maximum absorbance at 420 nm, and the proportional constant was obtained to be 85 $\mu M^{-1}{\cdot}cm^{-1}$.

2.6 Bleaching of Cotton Fabrics

The enzymatically desized cotton fabrics were first scoured with a solution of 10 g/L NaOH and 2 g/L Lutensol AT 25 at 98 °C for 1 h in an Ahiba Spectradye-Datacolor dyeing apparatus (Lawrenceville, NJ, USA) at a liquor to fabric ratio of 20:1 [30]. Afterwards, the fabrics were neutralized with 1 g/L acetic acid at 80 °C for 10 min and washed with tap water.

During bleaching experiments, the generation of H_2O_2 and bleaching of fabrics were forced to occur simultaneously. These experiments were performed in 50-mL Erlenmeyer flasks containing 20 mL of each medium and 1 g of fabric, and were carried out for 8 h under the conditions previously obtained as being optimal for the H_2O_2 production in each case. Both controls with conventional bleaching solution and hydrogen peroxide solution were performed in parallel at the same temperature of the enzymatic bleaching assays. Conventional bleaching solution contained as a percentage of fabric weight: silicate 3.5 %, soda ash 1 %, sodium hydroxide 1 % and 35 % of hydrogen peroxide at 4 % [11]. Hydrogen peroxide solution contained 35 % of 4 %-hydrogen peroxide (0.7 g/L). Other controls without enzyme and without substrate were performed in parallel in order to observe the possible interaction with the fabrics. After the treatment, the supernatants were measured for H_2O_2 production, and the fabric samples were washed with tap water before the whiteness measurement.

2.7 Fabric Whiteness of the Textile Material

The whiteness index of the bleached fabrics (Berger) was determined using a reflectance measuring Datacolor apparatus (Lawrenceville, NJ, USA) under the conditions of the standard illuminant D_{65} (LAV/Spec. Incl., d/8, D_{65}/10°). All the determinations in this study were performed in triplicate and the results represent mean values with less than 0.2 % of error.

3 Results

3.1 H_2O_2 Production from Glucose by Glucose Oxidase

Various experimental conditions, e.g. enzyme and substrate concentration, for the production of peroxide from glucose were tested. Although aeration was previously obtained to increase the production of H_2O_2 [10], an external addition of oxygen was not considered in these assays in order to simulate the conditions of detergent washing. The open flasks were shaken in a thermostatic bath.

Fig. 3 shows the profiles of H_2O_2 and glucose for 8 h at 37 °C, for experiments with an initial glucose oxidase activity of 6 U/mL. The H_2O_2 production rate decreased during the assay and, for low concentrations of glucose, the production stopped

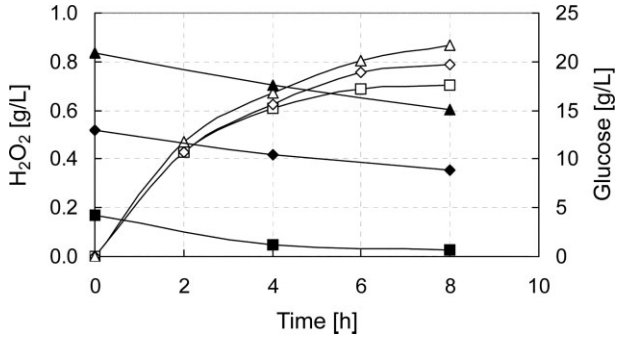

Figure 3. H_2O_2 production (open symbols) and glucose consumption (closed symbols) from 20 g/L (△), 12 g/L (◇) and 4 g/L (□) of glucose in the presence of 6 U/mL of glucose oxidase.

after 6 h. This plateau could probably be due to an inactivation of the enzyme or a decrease in the substrate concentration during the experiment. Regarding the inactivation of the enzyme, some measurements were performed during the experiments (data not shown) and it was found that approx. 1 U/mL is lost after 8 h. The inactivation of the enzyme could be due to the production of H_2O_2, which is an inhibitor of the enzymatic activity [31], or to the production of gluconic acid, which could decrease the pH and inhibit the protein [20]. The pH was followed along the assay and no decrease was detected, as the reaction medium was buffered with the appropriate solution; it can therefore be concluded that H_2O_2 is responsible for the inhibition of the enzyme. Although enzymatic activity is still remaining at the end of the experiment, it can be possible that low concentrations of enzyme are not able to maintain the reaction rate required for a constant production of H_2O_2. Concerning the substrate, glucose concentration was followed in the three assays, and it was observed that 3.59, 4.07 and 5.83 g/L of glucose were consumed for the 4, 12 and 20 g/L of initial glucose assays. Therefore, residual glucose is still remaining at the end of the experiment. However, considering a Michaelis-Menten behavior, the decrease in the reaction rate is probably due to a decrease in the substrate concentration, which is very low (around 0.4 g/L) at the end of the 4 g/L experiment.

Fig. 4 shows the effect of the combination of the two modified variables (glucose concentration and enzyme activity) on the H_2O_2 production. For low substrate concentrations, the increase of enzymatic activity from 4 to 6 U/mL did not involve high differences in peroxide production, as in this case, the reaction is limited by the substrate. However, the higher the initial glucose concentrations (12–20 g/L), the higher the differences in peroxide production between the experiments with 4 and 6 U/mL of glucose oxidase. From these data, the limits of operation can be established. Concerning glucose concentration, it should be higher than the K_m values (which depend on the activity and are usually around 20 g/L [20]), in order to obtain reaction rates close to the maximum. Regarding the enzymatic activity, when the concentration of glucose is low, 4 U/mL are enough to obtain a high peroxide production rate, although 6 U/mL help to increase the production of H_2O_2 when there is no substrate limitation. If this system is performed for the washing processes, a concentration of 20 g/L and 6 U/mL of enzyme produce a high quantity of H_2O_2; if the system is applied in combination with cellulase activity and CMC, the most appropriate concentration of glucose oxidase will depend on the concentration of the reducing sugars reached in the first reaction.

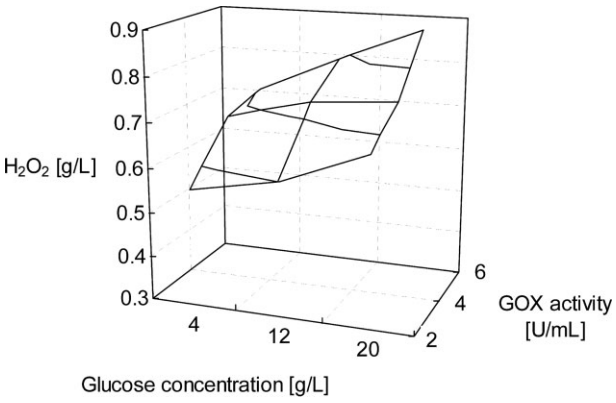

Figure 4. H_2O_2 production after 8 h in assays performed at different initial concentrations of glucose (4, 12 and 20 g/L) and different initial activities of glucose oxidase (2, 4 and 6 U/mL).

3.2 H_2O_2 Production from CMC by Cellulase and Glucose Oxidase

The optimization of H_2O_2 production in a combined reaction of cellulase and glucose oxidase was performed once the two enzymatic reactions were optimized separately.

The hydrolysis of CMC by cellulases was followed by measuring the reducing sugars, as they are the substrates for the second enzymatic reaction. Variables such as polymerization grade and concentration of CMC as well as enzymatic activity were modified. Some of these variables are highly dependent on the characteristics of CMC (see Tab. 1). There can be up to three substituents of carboxymethyl groups on each glucose unit, although the commercial CMC usually has less than 1.

The degree of polymerization (DP), i.e. the number of monomers per molecule of polymer, is responsible for the solubility of the CMC. The higher the DP, the higher the molecular weight and the lower the solubility of the polymer.

Assays were performed for 8 h, although after 4 h no further production of reducing sugars was observed. The plateau could be due to the inactivation of the enzyme or the total consumption of the CMC. Enzymatic activities were measured in order to prove that some enzymatic activity remained until the end of the experiments. Concerning substrate consumption, the percentage of reducing sugars with respect to those obtained if the polymer of CMC breaks to monomers was calculated to be around 20 % (see Tab. 2). Some authors [21] calculated the degree of polymerization of the oligosaccharides from CMC hydrolysis, which resulted to be between 1 and 15, the most abundant being those corresponding to nine monomers. Depending on the position of the substituents, sometimes the cellulase is not able to continue the hydrolysis, thus stopping the reaction before all the oligomers were broken to monomers.

Tab. 2 summarizes the results obtained after 4 h of enzymatic treatment of CMC at 37 °C. The influence of the type of CMC was determined comparing the reducing sugars obtained from low and medium viscosity CMC at the same initial concentration [w/v]. The degree of polymerization of low viscosity CMC is almost 3-fold lower than that of the medium viscosity CMC, but the total number of monomers in the two cases is the same, therefore the values of reducing sugars appeared to be the same. As low viscosity CMC has higher solubility, more concentrated solutions can be performed and a higher production of reducing sugars can be reached. Therefore, high and medium viscosity CMC can be disregarded. The enzymatic activity seemed not to affect the production of reducing sugars above 2 U/mL. For higher levels of enzymatic activities, the limitation of the reaction is probably due to the inability of the cellulase to perform the hydrolysis of the substituted oligomers.

Taking into account the optimizations of glucose oxidase and the cellulase reactions, the production of H_2O_2 from CMC was started considering 3 % of low viscosity CMC as a substrate. As 4 U/mL of glucose oxidase was enough to oxidize low concentrations of glucose, this activity was applied to analyze the effect of cellulase activity. Three values of cellulase activity (2, 5 and 10 U/mL) were considered, and similar profiles of H_2O_2 production were obtained (see Fig. 5). The reducing sugars reached a concentration which is the result of an equilibrium between their production from CMC hydrolysis and their consumption by oxidation with glucose oxidase. Considering this value and the formation of reducing sugars under the same conditions (see Tab. 2), the reducing sugars oxidized by glucose oxidase in the combined reaction result in 9 mM (1.6 g/L of glucose equivalents), which both stoichiometrically and experimentally corresponds to 0.30 g/L of H_2O_2. Among the possible substrates, glucose oxidase has more affinity towards glucose, although it is able to oxidize other low molec-

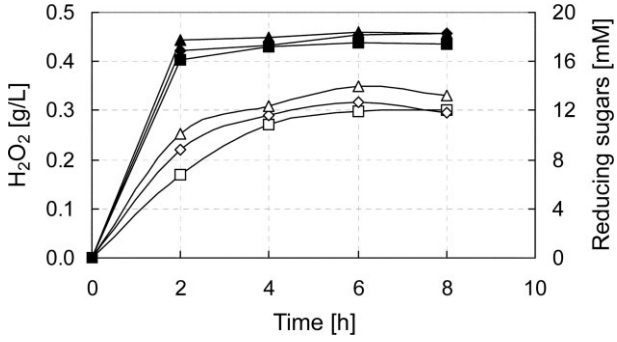

Figure 5. H_2O_2 production (open symbols) and consumption of reducing sugars (closed symbols) in the presence of 10 U/mL (△), 5 U/mL (◇) and 2 U/mL (□) of cellulase and 4 U/mL of glucose oxidase.

Table 1. Characteristics of CMC.

Viscosity	Degree of substitution	Degree of polymerization	Molecular weight [kDa]	Solubility [g/L]
Low	0.65–0.90	400	90	40
Medium	0.7	1,100	250	20
High	0.65–0.85	3,200	700	10

Table 2. Concentration of reducing sugars and percentage with respect to the maximum theoretical concentration for experiments performed at different types and concentrations of CMC and different cellulase activities, after 4 h at 37 °C.

CMC type	CMC [%]	Cellulase activity [U/mL]	Reducing sugars [mM]	Reducing sugars [%]
Medium μ	0.5	1	5.1	22.7
Medium μ	1	1	9.5	21.6
Low μ	1	1	9.5	21.6
Low μ	2	1	17.5	19.7
Low μ	3	1	23.1	17.4
Low μ	3	1	23.4	17.6
Low μ	3	2	27.3	20.5
Low μ	3	3	27.7	20.8

ular weight carbohydrates as mannose, galactose or xylose [20]. Therefore, 30 % of the reducing sugars formed from CMC correspond to glucose or other monomers easily oxidized by glucose oxidase.

Assays with 2 U/mL of cellulase and different glucose oxidase activities (4 and 6 U/mL) were also performed (data not shown). The peroxide production results verified those obtained in the first system, as at low concentrations of glucose the substrate limits the reaction rate. As a conclusion, 2 U/mL of cellulase and 4 U/mL of glucose oxidase were able to hydrolyze and oxidize 3 % CMC producing 0.30 g/L of H_2O_2.

3.3 H_2O_2 Production from Ethanol by Alcohol Oxidase

Various ethanol concentrations (0.5–8 %) for the production of peroxide by alcohol oxidase were tested (see Fig. 6). Although with low differences, after 2 h the production of H_2O_2 was proportional to the initial ethanol concentrations; however, after 4 h the peroxide production rate of the highest initial ethanol concentration assay started to decrease. The presence of high ethanol concentrations causes the inactivation of alcohol oxidase. Even so, concentrations above 0.9 g/L of H_2O_2 were obtained after 8 h of treatment.

3.4 Bleaching of Cotton

In order to evaluate the efficiency of the enzymatic systems for bleaching in detergents, a bleaching process was simulated. The possibility of bleaching duty clothes was rejected due to the difficulty in comparing the results to those from other authors.

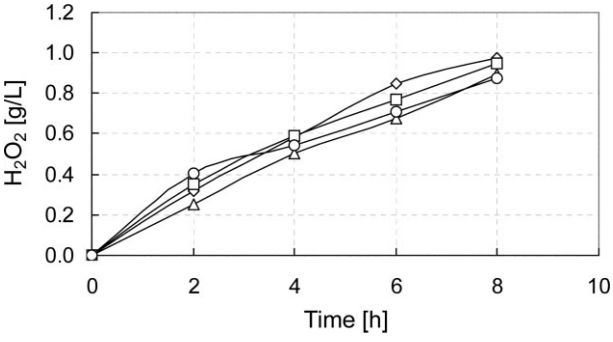

Figure 6. H_2O_2 production from 0.5 % (△), 1 % (◇), 5% (□) and 8 % (○) of ethanol in the presence of 4 U/mL of alcohol oxidase.

Therefore, the bleaching as a pretreatment step of fabrics like cotton was selected for being much more commonly studied. The capacity of the three enzymatic systems to bleach cotton fabrics was analyzed under the conditions resulting in the highest hydrogen peroxide production (see Tab. 3). The results of fabric whiteness were compared to those obtained with: *(i)* a conventional bleaching solution, composed by silicate, sodium carbonate, sodium hydroxide and hydrogen peroxide; and *(ii)* a hydrogen peroxide solution with the same concentration of that in the bleaching solution (0.7 g/L). These controls were performed at 37 °C and 25 °C, the same temperatures of the experiments with glucose oxidase and ethanol oxidase, respectively.

The control experiments achieved the highest whiteness improvement, the conventional bleaching solution being more efficient than the hydrogen peroxide by itself (see Fig. 7). The increase of temperature helped the bleaching systems to act. Furthermore, the pH has a strong effect, as the enzymatic system with alcohol oxidase performed at 25 °C and pH 7.5 was more efficient for bleaching than those enzymatic systems performed at 37 °C and pH 5.0. Moreover, the production of H_2O_2 from CMC was able to bleach the cotton more efficiently than its production from glucose, although the concentration of H_2O_2 achieved in the latter was higher than the level obtained from CMC. The whiteness of the controls both with glucose or glucose oxidase was not lower than the initial whiteness; therefore these compounds do not cause any interaction with the fabric which produces an increase of color. In a previous work [10], it had been observed that concentrations of glucose above 5 g/L resulted in a decrease of the bleaching capacity of the enzymatic system with respect to assays performed at lower concentrations. It was demonstrated that glucose has a stabilizing effect on the hydrogen peroxide, retarding the bleaching process. This hypothesis can also explain the data observed in Fig. 7. As the concentration of glucose in the experiments from CMC was much lower than 20 g/L, the impediment to the action of peroxide was diminished, resulting in a higher whiteness increase.

4 Discussion

This study tries to establish, not only the improvement of whiteness which is provided by three enzymatic systems, but also the feasibility of these systems to be incorporated in future detergent compositions. The starting point was the evaluation of the optimal conditions for the production of hydrogen peroxide. Reaching a concentration of 20 g/L of glucose after the

Table 3. Comparison of the three systems for the production of H_2O_2 and cotton bleaching.

System	Substrate	[Substrate]	Activity [U/mL]	*T* [°C]	pH	Treatment time [h]	H_2O_2 production [g/L]	Whiteness increase [Berger units]
(i) GOX	Glucose	20 g/L	6	37	5	8	0.87	0.8
(ii) Cell + GOX	Low μ CMC	3 %	2 (cell)+6 (GOX)	37	5	8	0.30	1.1
(iii) AOX	Ethanol	1 %	4	25	7.5	8	0.97	2.7

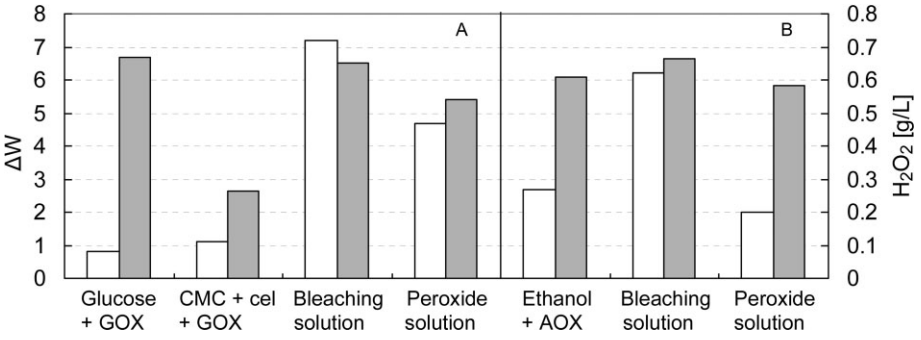

Figure 7. Cotton whiteness improvement (Berger whiteness) (white bars) and hydrogen peroxide production (gray bars) after 8 h of treatment with enzymatic systems, and controls with conventional bleaching solution and hydrogen peroxide solution. The differences in whiteness were calculated with respect to scoured fabric: (A) $T = 37\,°C$; (B) $T = 25\,°C$.

dilution of the detergent in the washing process means that a much higher concentration should be present in the detergent composition. This is also applicable to the other two systems, although in these cases lower concentrations of substrates are required. Regarding the enzymatic activity, low levels of enzyme are required in the three systems. Detergents usually have a high pH and work at high temperatures, although the reduction of these requirements is a constant objective. Therefore, working under neutral pH values and room temperatures should not be a problem, and in this regard the system based on the action of ethanol oxidase is more feasible, as this enzyme is more resistant to moderately basic medium.

Concerning the efficiency of each system to generate H_2O_2, the systems based on a direct oxidation reaction (from glucose and ethanol) achieved faster kinetics for hydrogen peroxide production and, consequently, higher levels of peroxide (1 g/L). To obtain peroxide from CMC, the complexity of two coupled reactions increased as some of the products of the first reaction were not substrates for the second one. Even so, H_2O_2 concentrations of 0.3 g/L were obtained from CMC. Other authors were also able to obtain concentrations of 1 g/L when working in aerated systems [11] or with higher enzymatic activities [12], which are conditions difficult to achieve in real washing processes. Even 3 g/L of peroxide were produced from 10 g/L of starch, by considering a two-step process consisting of starch degradation by amylases followed by glucose oxidation by glucose oxidase [13]. Nevertheless, the levels of peroxide reached from our systems are significant for bleaching purposes, as concentrations of 0.17 g/L are described to be sufficient [16].

The improvement in whiteness was not directly proportional to the level of H_2O_2 produced by the system. The peroxide actuation is highly influenced by the pH of the solution. Moreover, peroxide from glucose was not able to bleach the fabrics due to the stabilizing effect that glucose produces on hydrogen peroxide, thereby retarding the bleaching process. Strong conditions of pH (above 9) and temperature (above 90 °C) are usually applied for industrial bleaching processes. However, some studies tried to avoid these energy-consuming conditions by coupling the peroxide generation with peroxidase reactions where the enzyme is able to oxidize the compounds under much milder conditions [32, 33].

Although gluconic acid could have been able to reduce the pH of the medium, the use of a buffered solution avoided the modification of the pH, and did not affect both the bleaching yield and the enzymatic activity. Gluconic acid is a weak acid [19] acting as a chelator for bleaching (disturbing heavy metal ions), so the addition of peroxide stabilizing agents may be avoided [11, 13]. Neither inhibitory effects of hydrogen peroxide over glucose oxidase or ethanol oxidase were observed.

One more drawback for the application of these enzymatic systems in detergent compositions that must be overcome is the storage. The exposure of substrates and enzymes during the storage can result in premature hydrogen peroxide generation. In solid compositions, one solution can be the encapsulation of the enzyme to avoid contact with substrate until the moment arrives when the composition is dissolved in water [34]. In liquid formulations, the physical isolation of enzymes is more difficult. Some stabilization strategies are based on chemical additives, such as carboxylic acid salts and calcium chloride to protect against protease degradation [5]. Other authors have studied the inclusion of inhibitors, such as Ag^+ or Cu^{2+} ions, with an inhibitory effect that can be completely reversed by a further dilution of the system with water [15]. Another technique is the decrease of free water concentration [16].

5 Conclusions

The enzymatic generation of hydrogen peroxide using oxidases was demonstrated to be a very efficient solution for bleaching processes, since high levels of hydrogen peroxide and acceptable cotton whiteness improvement were achieved. Among the three systems, the one based on the hydrolysis and further oxidation of CMC allowed the use of the residual CMC and the cellulases from the detergent formulations, giving good bleaching results. On the other hand, the system based on the oxidation of ethanol by ethanol oxidase was able to produce the highest levels of peroxide and the highest improvement of whiteness, due to the ability to perform the bleaching process in a mildly basic medium (pH 7.5).

These enzymatic systems are able to produce hydrogen peroxide in situ during the washing process; they avoid the use of

bleaching agents, such as peroxoborate and carbonate peroxohydrate, which cause the eutrophication of lakes; they are effective under milder conditions, thus reducing energy and water consumption; therefore, they are more environmentally friendly, and allow a balance between the ecological impact and the bleaching efficiency to be to reached. Future work will be focused on the improvement of fabric whiteness under mild conditions, by combining the hydrogen peroxide production with peroxidases able to oxidize hardly recalcitrant compounds, or by using activators which allow the action of peroxide at low temperatures. Other aspects, such as bleaching times, application conditions, enzyme costs and storage stability will be studied as well, in order to improve the feasibility of the system to be applied in real detergent formulations.

6 References

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