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Effects of the Addition of Poly(ethylene Glycol) and Non-ionic Surfactants on Pretreatment, Enzymatic Hydrolysis, and Ethanol Fermentation

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Abstract
The consolidation of cellulosic ethanol on the market is fundamental to mitigate the consumption of fuels from fossil sources and to reduce the impact caused by the large generation of agro-industrial waste. In order to achieve this objective, some challenges of cellulosic ethanol technology must be overcome, including the improvement of the cellulosic ethanol production steps. Several studies propose the use of poly(ethylene glycol) (PEG) and non-ionic surfactants (such as Tween 80, Tween 20, and Triton X-100) as a way to increase cellulosic ethanol titers. The benefits attributed to the PEG and non-ionic surfactants go beyond the increase of the concentration of free cellulases during enzymatic hydrolysis. Successful cases of pretreatments of lignocellulosic biomasses assisted by PEG and surfactants and the detoxification of inhibitor-rich hydrolysates with PEG reveal the existence of a plethora of positive mechanisms. Therefore, the present review article is focused on the benefits and mechanisms involved in the addition of PEG and non-ionic surfactants in the pretreatment, enzymatic hydrolysis, and ethanol fermentation steps. Interactions between additives and lignin as well as schemes based on high PEG concentrations were also discussed in detail.

Keywords PEG · Tween · Biomass · Hydrolysis · Detoxification · Fermentation

Introduction

The crises in the oil industry and population growth put pressure on governments to consolidate the use of renewable energy. Ethanol is a biofuel derived from a microbiological route that can reduce the burning of fossil fuels in automobiles, an important source of greenhouse gases (~29% of the total) [1, 2]. Despite having an energy density 33% lower than gasoline, gasoline–ethanol blends have better octane rating and better combustion efficiency than isolated gasoline. It is noteworthy that the extensive use of ethanol as a liquid fuel occurs mainly in the USA and Brazil. Both countries also head the global ethanol market, which is supplied mainly by corn and sugar cane crops. The starch obtained from corn kernels and the sucrose obtained from sugarcane are easily converted into fermentable sugars and correspond to the main raw materials of the first-generation ethanol. Although ethanol based on starch or sucrose is treated as a renewable fuel, its production chain requires the practice of monocultures and reduces the planting area available for food production [3]. The competition between fuel and food is even more significant when assessing the consumption of water, labor, and capital invested in plantations and their processing [4]. In this sense, several technologies have become popular to produce ethanol from plant residues, more specifically lignocellulosic biomasses from industrial, forestry, and municipal wastes [5]. This type of ethanol is commonly called second-generation ethanol or cellulosic ethanol.

The lignocellulosic biomass is considered the most promising raw material for the ethanol production because of its carbohydrate content, low cost, and the fact that it does not compete with the food production [6]. The consumption of

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cellulosic ethanol also presents advantages over first-generation ethanol in the carbon dioxide emission since most of the gas is reabsorbed in the cycle, being closer to the carbon neutral condition [7]. However, the ethanol production from lignocellulosic biomass is not a simple task. The conversion of lignocellulosic biomass into ethanol by enzymatic route starts with the pretreatment step, a process that aims to disorganize the lignocellulosic matrix and facilitate access to cellulose and hemicellulose. Thereafter, in the enzymatic hydrolysis step, an enzyme cocktail is used to cleave the polysaccharide chains into monosaccharides. Finally, these sugars are metabolized by microorganisms in an anaerobic environment, and the ethanol generated must be directed to the purification steps [8].

Although there are already companies capable of producing large volumes of cellulosic ethanol (e.g., Iopen, POET, DuPont, and Abengoa), the largest share of this market is currently dominated by producers that use starchy materials or molasses as a raw material. The cellulosic ethanol production still faces some challenges that undermine the competitiveness of this biofuel, such as the low ethanol yield per mass of substrate. The low efficiency of delignification in pretreatments (mainly hydrothermal and acids), the decline in the catalytic activity of cellulases during enzymatic hydrolysis, and the inhibitory substances generated by the thermal degradation of biomass are recurrent problems in the cellulosic ethanol schemes. To solve these problems, studies have recommended the use of additives, such as poly(ethylene glycol) (PEG) and non-ionic surfactants. Authors such as Qing et al. [9] and Sindhu et al. [10] reported that the addition of PEG in the pretreatment increased the delignification of the pretreated biomass and, consequently, increased the ethanol titers. Furthermore, Liu et al. [11] proposed the addition of high PEG concentrations only in the stages of simultaneous saccharification and fermentation (SSF). Shortly, there is a range of options that can help the researcher in his decision-making, and the recent literature has already compiled studies of cellulosic ethanol production assisted by PEG and non-ionic surfactants. The papers of Eckard et al. [12] and Zheng et al. [13] show excellence in the description of the benefits associated with the use of additives during enzymatic hydrolysis; however, some effects of the additives were not taken into account, e.g., the ability to detoxify environments rich in inhibitors. In addition, new reports and understandings about the phenomena associated with PEG and non-ionic surfactants emerged after the publication of these reviews.

Thus, this review provides a more up-to-date overview of the benefits and mechanisms of action of the additives during the course of the cellulosic ethanol scheme. The properties of PEG and derivatives and their applications in research fields are discussed in the second section. The effects of PEG and non-ionic surfactants on pretreatment, enzymatic hydrolysis, and ethanol fermentation are discussed in the third, fourth, and fifth sections, respectively.

Poly(ethylene Glycol) and Non-ionic Surfactants

Poly(ethylene glycol) (PEG) is a polyether generated from ethylene glycol via ring-opening polymerization (Fig. 1). Using an alkaline initiator, the reaction involves the nucleophilic attack of a methylene group from the ethylene glycol to open the epoxide ring to form propagation species until the polymer reaches the desired chain size [14]. Then, the polymer mixture is separated by distillation or size exclusion chromatography, and the obtained products must have a specific chain size [15]. When the polymer has a molecular mass greater than 30 kDa, other nomenclatures, such as poly(ethylene oxide) (PEO) and poly(oxyethylene) (POE), can also be used [16].

The melting point of PEG is a function of the polymer chain size; PEGs with a molecular mass of up to 700 Da are presented in the liquid state at room temperature, while PEGs with molecular mass greater than 700 Da are presented in the solid state. Despite the molecular mass, PEG has high solubility in polar solvents, such as water. For example, PEG 400 is fully miscible with water, whereas a PEG 2000 solution can reach a concentration greater than 60% (w/w) at 20 °C [17]. According to Hammouda [18], the distance between the PEG units is precise and matches exactly with the water molecules, which explains the high solubility of the polymer when compared to other aliphatic polyethers. PEG offers excellent tribological properties and can be used as hydraulic fluid and gear and compressor lubricants [19]. Due to its biocompatibility, PEG is considered an ideal polymer for pharmaceutical, cosmetic, and medical applications [16]. Enzymes modified by the PEG grafting may show better catalytic activities and stability in organic solvents [20, 21]. In bioactive transport studies, the covering of drugs or bioactive vehicles is generally performed by covalent binding with PEG (PEGylation) since the polymer is inert and therefore mitigates the recognition of the immune system [22].

PEG is a recognized precursor to non-ionic surfactants. PEG-based non-ionic surfactants are composed of one or more PEG chains in the hydrophilic portion, while alkylated phenol, fatty acids, or long-chain alcohols make up the hydrophobic portion [23]. Consequently, the properties of these surfactants are controlled by the PEG chain size and the hydrophobic group present in the structure, in other words, by the hydrophilic–lipophilic balance (HLB) [24]. PEG-based non-ionic surfactants generally have HLB values above 13.0, so they are widely used as cleaning agents and detergents [25]. Triton X-100 (HLB = 13.5), Tween 20

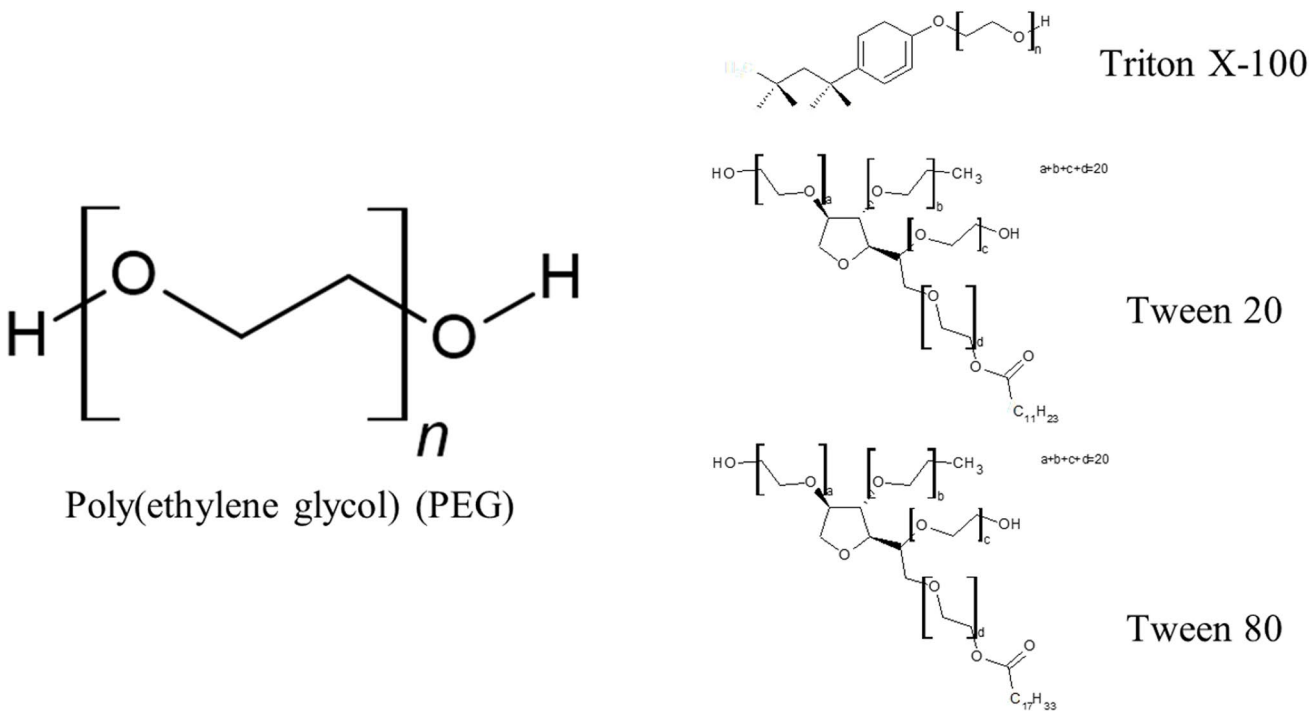

Fig. 1 Molecular structure of poly(ethylene glycol) (PEG) and some non-ionic surfactants

(polysorbate 20; HLB = 16.7), and Tween 80 (polysorbate 80; HLB = 15.0) are some of the most well-known PEG-based non-ionic surfactants. The chemical structure of these surfactants is shown in Fig. 1. Inspired by green chemistry, other PEG-based surfactants have been prepared using natural ingredients such as tannin acids [26], lignins [27], and rosins [28]. PEG is also the base material for the synthesis of copolymers with attractive properties, being generally inserted to modulate the hydrophilicity of hydrophobic polymers [29]. As well as surfactants, several PEG copolymers are capable of self-assembling in micelles, polymersomes, and capsules [30]. In particular, ethylene oxide–propylene oxide triblock copolymers (EOPO triblock copolymers), a type of PEG-based copolymer, can be successfully used as a drug and gene carrier in therapies [31, 32].

The first studies involving the use of PEG and non-ionic surfactants in the conversion of cellulose-rich materials into ethanol date back to the 1980s. Hahn-Hägerdal et al. [33] and Tjerneld et al. [34] proposed the use of PEG/dextran systems as an environment for enzymatic hydrolysis of cellulose and the subsequent recovery of residual cellulases. Hahn-Hägerdal et al. [35, 36] investigated the role of PEG as a modifier of osmoregularity in ethanol fermentations. Castanon and Wilke [37] used 0.1% (w/v) Tween 80 to increase the enzymatic digestibility of newspaper by up to 33%. Because of their practicality, PEG and its derivatives have been widely used as additives for enzymatic hydrolysis of lignocellulosic biomasses, and new articles appear until today. In addition, other lines of research have proposed PEG-assisted pretreatments as a way to improve ethanol titers [9, 38]. However, it is important to note that the PEG mechanisms for each of these situations are quite distinct, and therefore, they must be treated separately. The limitations, mechanisms, and literature associated with the use of PEG and its derivatives in the pretreatment, enzymatic hydrolysis, and ethanol fermentation are addressed in greater depth in the following topics.

Impact of PEG and Non-ionic Surfactants on Lignocellulosic Pretreatments

PEG and its derivatives can act as biomass modifiers in different pretreatments; however, most applications are linked to the acid and hydrothermal pretreatments, mainly to mitigate the negative effects of lignin and pseudo-lignin droplets.

Both acid and hydrothermal pretreatments are effective in reducing the recalcitrance of the lignocellulosic biomass. In these pretreatments, either by adding a catalyst (for acid pretreatment) or by using high temperatures, hydronium ions are available in the environment and promote the depolymerization of hemicellulose [39]. As a result, the pretreated biomass has higher enzymatic digestibility than the untreated biomass. This behavior is observed for a wide range of severity values, but it is often discussed that drastic pretreatments (temperatures above 180 °C and/or high retention

times) can limit enzymatic digestibility [40, 41]. Selig et al. [42] suggest that when the glass transition temperature of the lignin is reached (~ 120–200 °C), the biopolymer melts and migrates to the biomass surface or into the liquid due to the effects of capillarity and hydrophobic interactions. It is noteworthy that lignin is chemically incompatible with water and other polar solvents; therefore, it coalesces in these environments, and lignin droplets (~ 0.2–70.0 μm) are formed. After the interruption of the pretreatment and reduction of the ambient temperature, the lignin droplets harden and can be deposited on the surface of the pretreated biomass. A summary of this phenomenon is shown in Fig. 2.

Images of the lignin droplets can be recorded by scanning electron microscopy (SEM), just as the ones obtained by Selig et al. [42], Donohoe et al. [43], Hansen et al. [44], and Lin et al. [45]. It is important to mention that chemical changes in lignin also occur simultaneously with the formation of lignin droplets. Under acidic conditions, native lignin can be dehydrated to carbocations, which are highly susceptible to nucleophilic attacks, mainly by lignin fractions [46]. Therefore, lignin droplets show a high condensation degree (higher content of C–C bonds) to the detriment of aryl-ether bonds (e.g., β-O-4) when compared to native lignin [46].

Sugars released from biomass during acidic and hydrothermal pretreatments can be dehydrated to furan aldehydes: hydroxymethylfurfural (HMF) from glucose and furfural from xylose. Under this condition, furfural and HMF can be progressively transformed into levulinic acid and formic acid, and then aromatic structures are also generated, which are called pseudo-lignin [40, 47]. According to Shinde et al. [48], the intermediates 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenotriol are generated, respectively, from furfural and HMF, and they are essential for the formation of pseudo-lignin. Pseudo-lignin has a higher content of aliphatic groups than lignin; however, it is not possible to distinguish them in the analysis of biomass composition [49]. This explains the fact that acid pretreated biomass may have a higher Klason lignin content than untreated biomass [48]. In addition, pseudo-lignin is also distributed during pretreatments as droplets, and it is not possible to establish apparent differences with the lignin droplets.

Although acid and hydrothermal pretreatments favor the porosity of the pretreated material [50], it is often reported that the droplets of lignin and pseudo-lignin inhibit enzymatic hydrolysis [9]. According to Selig et al. [42], besides behaving as a physical barrier to enzymatic access, lignin droplets also increase the surface area for non-productive adsorption of cellulases. Kumar et al. [40] evaluated the formation of pseudo-lignin from crystalline cellulose during pretreatments with diluted acids. The authors observed that the formation of pseudo-lignin is a direct function of temperature and that it is favored in the presence of free sugars. They also reported that enzymatic hydrolysis of cellulose pretreated with 2% (w/w) sulfuric acid and 180 °C for 40 min resulted in a cellulose conversion of only 66%, while untreated cellulose achieved conversion equal to

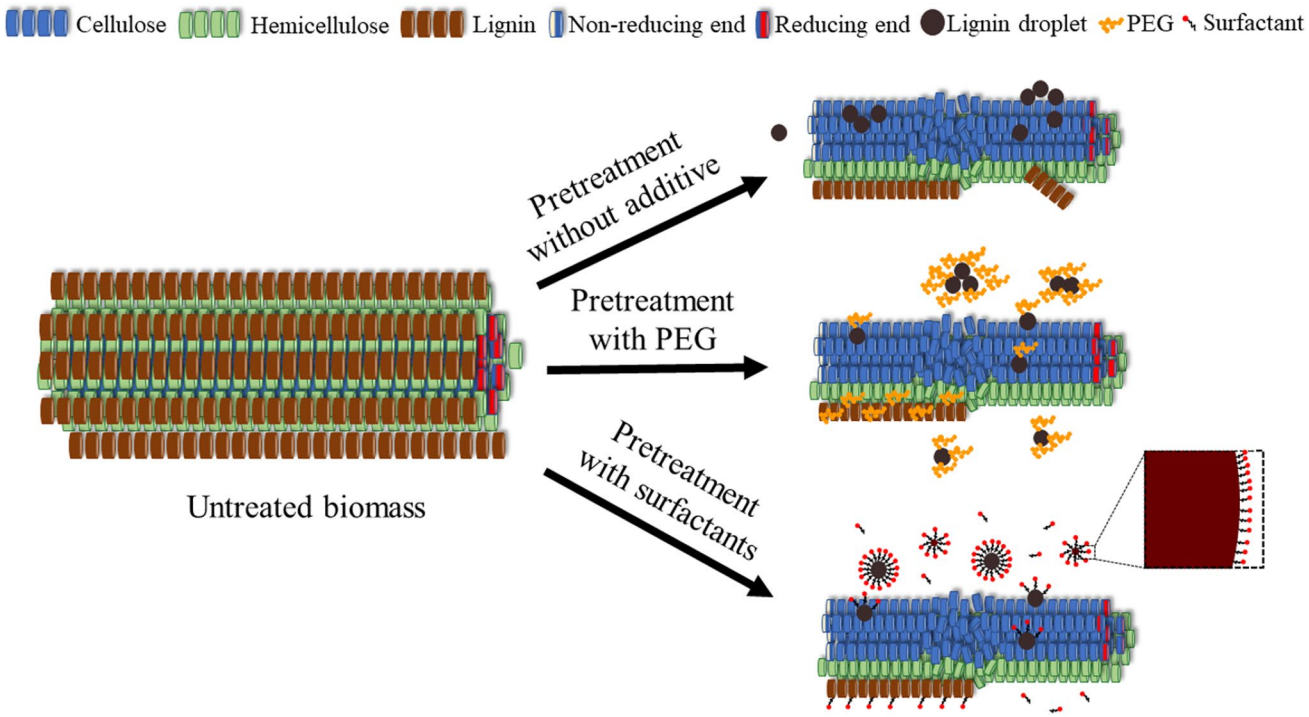

Fig. 2 Effect of poly(ethylene glycol) (PEG) and surfactants on the deposition of lignin droplets during pretreatment of lignocellulosic biomass

94.5%. Sipponen et al. [51] observed that the cellulosic conversion of hydrothermal pretreated wheat straw is inversely proportional to the surface area of the lignin. Pielhop et al. [41] reported that the enzymatic digestibility of pretreated spruce wood without droplets of lignin and pseudo-lignin was 64% higher than the control sample. Hu et al. [52], He et al. [39], and Schmatz et al. [53] reported that hydrophobic interactions between lignin droplets and cellulases reduce cellulolytic conversion.

To avoid the aforementioned adverse effects, extraction steps can be applied to the pretreated biomass. Alkaline reagents and organic solvents can remove droplets of lignin and pseudo-lignin and promote cellulose swelling [54]. Notorious results of extraction after pretreatment are shown in Sipponen et al. [51], Huang et al. [54], and Wu et al. [55]. Despite this, it increases the time required for conditioning the biomass or requires the use of an appropriate reactor. Considering the disadvantages of extraction after pretreatment, studies have proposed that the removal of lignin and pseudo-lignin should be carried out simultaneously with the course of the pretreatment. A possible way is through the addition of 2-naphthol and 2-naphthol-7-sulfonate, effective carbocation sequestrators, to suppress the formation of droplets of lignin and pseudo-lignin [56]. However, carbocation scavengers are generally toxic compounds for enzymes and microorganisms, so the washing of pretreated biomass must be exhaustive. To overcome the adversities of lignin and pseudo-lignin, other studies have proposed the use of small dosages of PEG and its derivatives in the pretreatment step.

Kurakake et al. [38] investigated for the first time the use of non-ionic surfactants in a hydrothermal pretreatment. In this case, Tween 20 was used as an additive for the autohydrolysis of hardwood and sugarcane bagasse under temperatures of 170, 180, and 190 °C. Regardless of the temperature used, biomasses pretreated with Tween 20 solutions had a Klason lignin content approximately 10% lower than biomasses pretreated with water. As a result, the sugar release was also superior in biomasses pretreated with Tween 20. Although the authors did not have an understanding of the formation of the droplets, they have already suggested that the surfactant may act as an extracting agent of hydrophobic products. Lignin and other hydrophobic products resulting from polysaccharide dehydration can migrate into micelles and remain in the liquid phase. The same understanding was given by Kim et al. [57] during pretreatment of recycle newspaper. In 2010, Qing et al. further evaluated the impact of PEG and surfactants on the pretreatment of lignocellulosic materials [9]. They investigated the impact of Tween 80 and PEG 4000 on the delignification of acid pretreated corn straw. The presence of Tween 80 and PEG 4000 during the acid pretreatment increased the delignification of corn stover to 17% and 10%, respectively; while the additive-free experiment achieved only 8% delignification. These evidences were confirmed after SEM analyses and wettability tests, which showed that samples pretreated with additives had a smaller amount of lignin droplets and greater hydrophilicity. Therefore, the authors suggest that as a mechanism of action the stabilization of lignin fragments in the liquid phase, as seen in Fig. 2. Due to their functional groups, the additive molecules can adsorb on the surface of lignin and pseudo-lignin droplets by hydrophobic interactions or hydrogen bonds. For example, PEG has several ether oxygens and two hydroxyl groups capable of establishing multiple hydrogen bonds and is a recognized agent to solubilize lignin (up to a concentration of 2%; w/w) [58]. When surfactants are added in concentrations higher than the critical micelle concentration (CMC), the generated micelles can capture hydrophobic substances, especially for pseudo-lignin precursor substances. As a result, lignin redeposition is hampered, which generally leads to a reduction in the lignin content of the pretreated material and, consequently, better enzymatic digestion results [9].

Disregarding the economic feasibility of the processes, it is important to highlight that surfactants are always reported as potential additives for pretreatments, and the mechanism proposed by Qing et al. [9] often cited. Recent studies indicate that the addition of surfactants can contribute to the removal of lignin as well as improve enzymatic digestibility involving other types of pretreatments. These pretreatments include extrusion [59], microwave [60], alkaline [61–63], organosolv [64], and ionic liquids [65–67].

Although most of the literature suggests positive effects of surfactants on pretreatments, it is important to explore studies that showed different results. Qi et al. [68] suggested the addition of Tween 20 in acid pretreatments of wheat straw as a way to increase sugar recovery after enzymatic hydrolysis, and this expectation was indeed fulfilled. For example, wheat straw pretreated in the presence of 2% (w/v) sulfuric acid and 1% (w/v) Tween 20 achieved a glucose yield equal to 73.3% after 72 h of enzymatic hydrolysis, while the wheat straw pretreated with only 2% (w/v) sulfuric acid obtained 62.2% yield under the same hydrolysis conditions. Such behavior was also observed for other dosages of acid and surfactant. Despite this, the authors were unable to elucidate any effects of Tween 20 on the chemical composition of pretreated materials. As expected, the insoluble lignin content increased with increasing acid dosage, but materials pretreated with or without Tween 20 had identical lignin contents. Although the authors did not highlight in the text, it is easy to interpret that the surfactant was unable to limit the formation of pseudo-lignin in those pretreatment conditions and that the improvement in enzyme digestibility was only due to the blocking of adsorption sites by attaching surfactants onto lignin (these aspects will be detailed in fourth section). This is the same conclusion as the study by Tong et al. [69] on poplar wood steam explosion

pretreatments assisted by JFC-M, an industrial surfactant. In 2014, Hu et al. investigated the effects of non-ionic surfactants on acid pretreatment of poplar holocellulose [70]. Using a temperature of 180 °C and 1% (w/w) sulfuric acid, the authors reported that the addition of 5% (w/w) Tween 80 in the acid pretreatment did not suppress the formation of pseudo-lignin; otherwise, there was an increase in the insoluble lignin content at the cost of the decrease in the cellulose content. The pretreated materials assisted by Tween 80 and control (surfactant-free condition) had 52.1% and 42.0% (w/w) of insoluble lignin, respectively. In infrared spectroscopy analyses, holocellulose pretreated with Tween 80 exhibited peaks of C = O streching (~ 1,705 cm^{-1}) and aromatic C = C stretching (~ 1,615 cm^{-1}), which are characteristic of pseudo-lignin [70].

Alternatively, it is possible to use PEG in other pretreatment strategies. Lai et al. [71] proposed the in situ modification of corn straw by PEG grafting to improve the results of enzymatic digestibility. It was observed that the alkaline pretreatment with PEG grafting did not increase the delignification values. In spite of this, the samples pretreated with PEG grafting showed less cellulase adsorption capacity and greater cellulosic conversion than other samples. The authors suggest that the addition of PEG chains in lignin structure reduces hydrophobic interactions between lignin and cellulases. The other hypothesis raised was that phenolic hydroxyl groups are reduced with the PEG grafting, and therefore, possible hydrogen bonds between the functional group in lignin and the enzymes are eliminated. Gong et al. [72] investigated the impact of PEG on pelletization and pretreatment of wheat straw and pine wood. Pelletization is a necessary procedure to increase the flowability of lignocellulosic biomass, which is essential for these materials to be considered as commodities; however, the short ring die lifespan is a limiting factor. Gong et al. observed that PEG 6000 behaved like a lubricant during pelletizing and it reduced the maximum ejection friction by 34% for wheat straw and 29% for pine wood. The PEG 6000-assisted pretreated biomasses also showed a sugar yield 256% higher than the control experiment (without the polymer).

Impact of PEG and Non-ionic Surfactants on Enzymatic Hydrolysis of Lignocellulosic Biomass

The enzymatic hydrolysis is a critical step for the success of cellulosic ethanol production, and therefore, there is a great effort by research groups to solve its bottlenecks. In fact, pretreatments are able to mitigate the consumption of enzymes and increase the productivity of the global process [73]. However, pretreatments that perform the precise fractioning of the lignocellulosic biomass are expensive, and they are not attractive for the industrial scale. In addition, the performance of conventional pretreatments, such as acid, alkaline, alkaline-oxidative, and organosolv pretreatments, is sometimes not enough to satisfactorily increase enzymatic digestibility. As previously mentioned, acid pretreatments can increase both the lignin exposure and the non-productive adsorption capacity of cellulases (due to the formation of lignin and pseudo-lignin droplets), so that low enzymatic digestibility is achieved. This behavior is more significant when biomasses with a high lignin content are used, as reported by Huang et al. [54] and Mariano et al. [74]. As an alternative, the addition of non-ionic surfactants has been recommended for decades to improve enzymatic hydrolysis of lignocellulosic biomass. In addition to their ability to change properties of biphasic systems, non-ionic surfactants can interact directly with enzymes or act on their inhibitors [12]. The main positive mechanisms of non-ionic surfactants include (i) the increase in the catalytic activity of cellulases, (ii) the increase in enzyme stability and prevention of enzyme denaturation, (iii) improving cellulose accessibility by disrupting lignocellulosic biomass, and (iv) the reduction of non-productive adsorption of cellulases [12, 75].

The beneficial effects of the presence of non-ionic surfactants on the catalytic activity of cellulases have already been observed in the recent literature. Non-ionic surfactants can interact directly with enzymes via hydrophobic interaction, resulting in more efficient conformations for a given environmental condition [76]. Eckard et al. [77] observed that non-ionic surfactants affect the α-helix and β-pleated sheet portions of cellulases in infrared spectrophotometry analysis. The generated micelles can also increase the solubility of the enzymes since they prevent protein aggregation [78]. Zhou et al. [79] reported that the relative activity of β-glucosidases increased to 115% with the addition of 5 g/L Tween 80, while the control experiment reached only 85% after 5 h of incubation. Shi et al. [80] observed that the addition of Tween 80 increased the activity of endoglucanases by up to 110% after 1 h.

Lignocellulosic-degrading enzymes, like all enzymes, are sensitive to the operational conditions of biotechnological processes. The catalytic activity of cellulases is a function of temperature in the range between 10 and 50 °C. However, above the optimum temperature for catalysis (~ 50 °C), the native conformation of cellulases is disrupted, which leads to a decrease in catalytic activity [75, 81]. Evidence of the benefits of non-ionic surfactants on thermal deactivation of enzymes has been reported in the literature. Eriksson et al. [75] showed that the addition of Tween 20 increased the thermal stability of cellulases from *Trichoderma reesei*. The authors noted that the enzyme deactivation temperature increased by 2 °C with the addition of the surfactant after fluorescence analysis. Although they did not work with cellulases, Lee et al. [82] reported that EOPO, a PEG-based

copolymer, limited the aggregation of denatured lysozyme by heating and recovered its catalytic activity.

The deactivation of cellulases by mechanical stress or contact at the air–liquid interface is also another concern during enzymatic hydrolysis. Molecular oxygen is one of the sources of cellulose deactivation by oxidation, mainly exoglucanases [83, 84]. In 1982, Reese [85] observed that the catalytic activity of *Trichoderma reesei* cellulases decreased by 20% after 5 h when the enzyme solution was stirred. Bhagia et al. [86] attributed the enzymatic deactivation at the air–liquid interface as the main cause for incomplete cellulose hydrolysis under conditions with low dosage of cellulases (5 mg of accelerase per 1 g of substrate). Okino et al. [87] observed that the addition of Tween 80 reduced the negative effect of agitation on the cellobiohydrolase II (CBH II) activity at 30 °C. The authors suggested that the surfactant acts by reducing the surface hydrophobicity of CBH II and preventing the enzyme denaturation. A representation of the effect of surfactant on the deactivation of enzymes at the air–liquid interface is shown in Fig. 3. Similar results were obtained by Yang et al. [88]. They observed that the addition of Tween 80 reduced the loss of activity of avicelase and carboxymethyl cellulase (CMCase) in different agitation conditions.

Reactive oxygen species generated by lytic polysaccharide monooxygenases can also cause cellulases to be inactivated [89]. One possibility to minimize enzymatic deactivation by oxidation with air is to avoid agitation. This procedure is executable on a laboratory scale; however, on an industrial scale, serious problems of temperature gradients would arise [90]. When surfactants are added to the enzyme solution, they occupy the surface sites of the liquid phase and reduce the contact of the enzymes with air [90]. Bhagia et al. [86] reported that the addition of 5 mg of Tween 80 increased the cellulosic conversion from 49 to 71% after 5 days of enzymatic hydrolysis of microcrystalline cellulose. Bhagia et al. [90] observed that the addition of 5 mg of Tween 20 increased the cellulosic conversion of filter paper from 74 to 87% and the cellulosic conversion of cotton linters from 39 to 49% after 11 days of enzymatic hydrolysis. Tejirian and Xu [91] described that phenolic compounds can inhibit enzymatic hydrolysis since they can form complexes with cellulases or adsorb onto substrates. To solve this problem, PEG 4000 was added in an aqueous medium. According to the authors, PEG 4000 can disrupt the enzyme–phenolic compound complex and increase the enzyme activity.

The improvement in the cellulose accessibility is often attributed to the addition of PEG and non-ionic surfactants. Obviously, it is not easy to dissociate this mechanism with the reduction of non-productive adsorption of cellulases; however, here the focus will be on physical changes promoted by surfactants. For example, Helle et al. [92] and Kaar and Holtzapple [93] suggested that surfactants can modify biomass structures and, therefore, increase the accessible surface area of cellulose. This was most evident in the work of Mo et al. [94]. They observed that PEG 8000 increased the enzymatic hydrolysis of hornified newsprint waste by 42%. Non-ionic surfactants can also increase biomass swelling and weaken bonds between the biomass components, as suggested by Seo et al. [95]. In experiments using SEM, the authors observed that the addition of Tween 20 caused the collapse of cell walls and led to the generation of more pores (~ 10–50 nm). Also, surfactants promote the reduction of surface tension, which makes it possible to reduce viscosity and, consequently, can improve the mass transfer of the enzymatic process [96].

The reduction of non-productive adsorption onto lignin is fundamental for the good performance of enzymatic hydrolysis, as shown in Fig. 4. In an aqueous environment free of these additives, cellulases bind to lignin molecules, which reduce the catalytic activity available during hydrolysis either by limiting the mobility of the enzymes or deactivation by conformational changes in the enzyme [97]. Carbohydrate binding modules (CBM) serve to approximate the catalytic domains of cellulose chains but are often associated with non-productive adsorption of cellulases due to hydrophobic interactions with lignin [98]. Electrostatic interactions and hydrogen bonds are also important in the

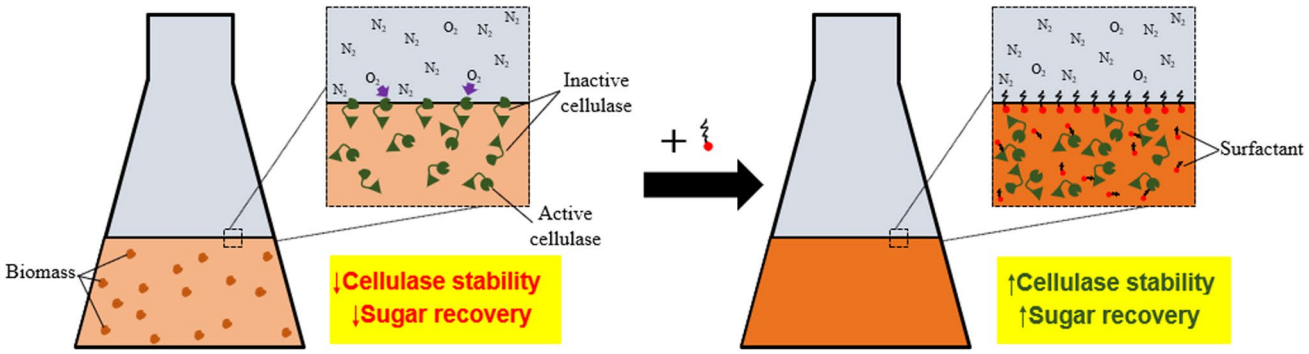

Fig. 3 Effect of additives (PEG and surfactants) on the cellulase deactivation at the air–liquid interface

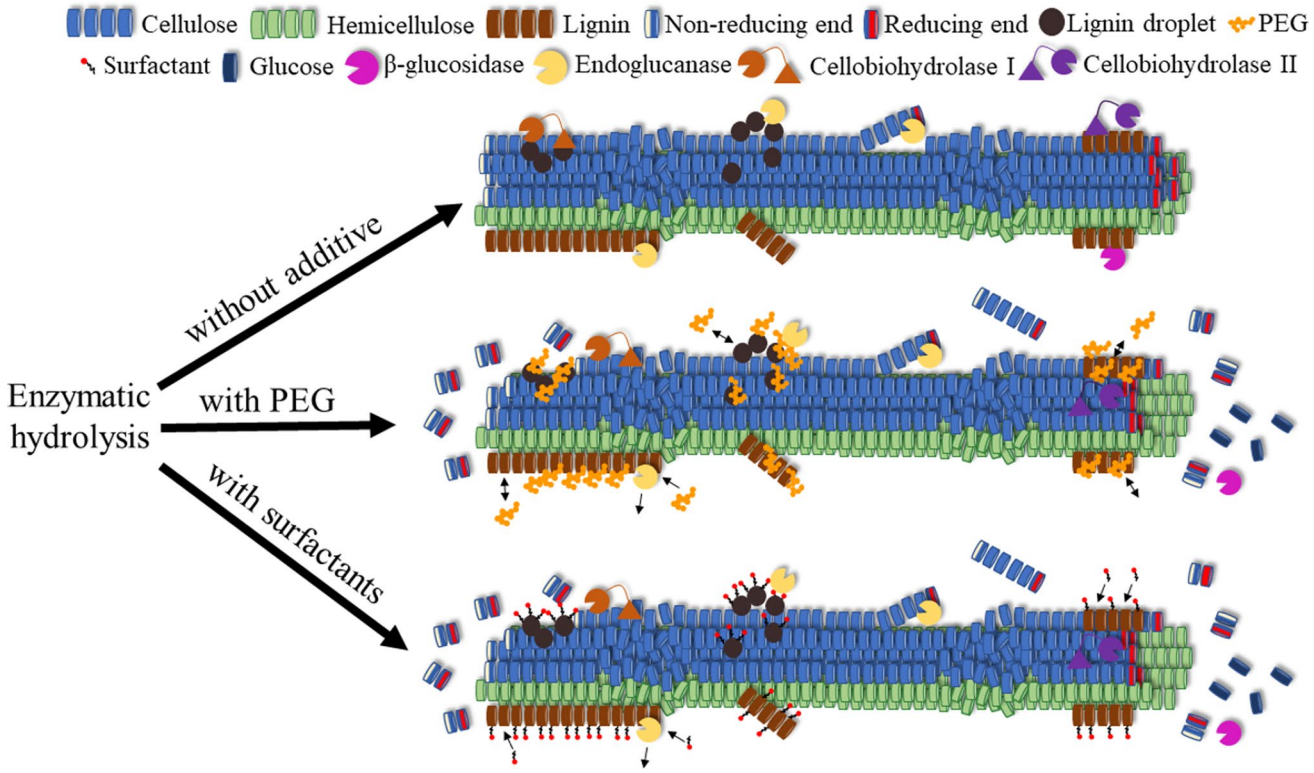

Fig. 4 Effect of polyethylene glycol (PEG) and surfactants on non-productive adsorption of enzymes during enzymatic hydrolysis of lignocellulosic biomass

non-productive adsorption of cellulases. Both cellulase and lignin have ionizable functional groups, with emphasis on the carboxyl, amino, and phosphate groups in the cellulases, while carboxyl, phenolic hydroxyl, and hydroxyl are abundant in the lignin structure. Considering that the optimal pH for cellulases is between 4.8 and 5.0, lignins have a negative net charge and can interact with cellulases whose isoelectric points are at higher values [98]. In a non-ionizable state, phenolic hydroxyl groups of lignin molecules are responsible for establishing hydrogen bonds between cellulases and lignin. In fact, the extent of non-productive adsorption is often associated with the content of phenolic hydroxyl groups in lignins [71]. In order to overcome this problem, additives are added to the system to block non-productive adsorption sites, especially PEG and non-ionic surfactants. When non-ionic surfactants are used as an additive, the mechanism is based on the adhesion of the additive to lignin by its tail, while the hydrophilic group is displaced towards the liquid phase. Hydrophobic interactions, dispersion interactions, and polar interactions (hydrogen bonds and dipole-involving) are involved in the adhesion of surfactants to lignin [99]. As a consequence, the enzymatic activity of cellulases is preserved, and the enzymes remain able to act on cellulose [75, 100]. Due to the absence of a hydrophobic tail, it is suggested that hydrogen bonding is the dominant driving force when PEG is used as an additive [99]. It is important to emphasize that these are the most frequent indications of intermolecular forces in the interactions between cellulases and lignin and additives and lignin, but the reader should be aware that their magnitude is strongly dependent on the characters in the system. Recently, numerous techniques have been developed to help understand cellulase–lignin and surfactant–lignin interactions, such as nuclear magnetic resonance, quartz crystal microbalance with dissipation (QCM-D), surface plasmon resonance (SPR), and atomic force microscopy, among others.

A vast number of reports are present in the literature about the benefits of PEG and non-ionic surfactants on the non-productive adsorption of cellulases. Börjesson et al. [101] reported that the addition of 2.5 g/L PEG 4000 reduced the adsorption of cellulases by 30% and 62.3% for exoglucanases and endoglucanases, respectively. Zhu et al. [102] reported that non-ionic surfactants are effective for recovering cellulases adsorbed onto acid pretreated corn straw. They also observed that high concentrations of ethylene glycol (the basic unit of PEG) can recover 76% cellulases. Seo et al. [95] reported the benefits of adding Tween 20 in the enzymatic hydrolysis of lignocellulosic biomass, noting a correlation with residual lignin content. The authors noted that the addition of this surfactant increased cellulosic

conversion by 9–21% with lignin-rich samples, while cellulosic conversion increased only 1.0–8.5% with lignin-poor samples. In the same way, Chen et al. [100] observed that the addition of Tween 20 can reduce incubation time and promote cellulase saving in the hydrolysis of wheat straw, but it can reduce by half the enzymatic digestibility of the filter paper. Nogueira et al. [103] reported that EOPO 5800 performed better than PEG 4000 to preserve cellulolytic activity in adsorption experiments using crystalline cellulose or a cellulose–lignin mixture.

The timing of the surfactant addition is also relevant in the enzymatic hydrolysis experiments. The simultaneous addition of PEG 4000 and substrate resulted in reduced losses of free cellulases than the late addition of the surfactant, as observed by Li et al. [104]. The study also pointed out that the adsorbed enzymes can be partially recovered with the late addition of PEG 4000 and that the irreversible adsorption of the enzymes (i.e., which could not be undone with the late addition of PEG 4000) occurred especially with lignin-rich samples. This behavior is in line with QCM-D analyses. According to Jiang et al. [99], the adsorption of PEG onto lignin is almost completely reversible, suggesting that simultaneous addition is the best choice of operation. In the case of using surfactants as additives, dispersion interactions ensure that a portion of the additives remains irreversibly adsorbed even after rinsing the lignocellulosic substrate. Thus, the addition of the surfactant can be carried out well before the addition of cellulases, especially in the pretreatment step.

Impact of PEG and Non-ionic Surfactants on Ethanol Fermentation (Considering the Hemicellulose Hydrolysates)

Surfactant-based cell disruption methods have become popular over the years. Surfactants can solubilize lipids and proteins and create pores along the cell membrane, which destabilizes the transit of compounds inside and outside the cell and eventually leads to the total cell lysis. The success of the cell disruption is linked to the operating conditions used. For example, sodium dodecyl sulfate, an anionic surfactant, is able to easily break cells in a few seconds and causes protein denaturation [105]. It is noteworthy that this phenomenon has an important analytical function, but it is not desirable for the cellulosic ethanol scheme since the cells must remain unscathed during fermentation. In addition, depending on the systems used (type and concentration of surfactant and type of strain), other phenomena associated with the use of surfactants may appear, and not all of them necessarily have negative effects for ethanol fermentation.

Cells adapt quickly to environmental stresses to ensure their survival, e.g., in response to reduced water activity. The presence of PEG and derivatives generates the initial loss of intracellular water and then promotes the accumulation of substances to balance the osmotic shock of the cytoplasm, also called osmoregulators [106, 107]. Glycerol is commonly synthesized by *S. cerevisiae* to counteract hyperosmotic stress, which is also a by-product of anaerobic glucose metabolism [108]. Because of this fact, studies have been carried out to evaluate the involvement of osmotic regulation in ethanol fermentation. Yeasts grown in a low water activity environment showed higher activity of enzymes involved in the conversion of glucose into ethanol, such as phosphofructokinase [109] and alcohol dehydrogenase [110].

Considering the potential of non-ionic surfactants in the enzymatic hydrolysis of biomass, Hahn-Hägerdal et al. [36] investigated the role of PEG on the ethanol production. It was observed that the ethanol yield obtained by *Candida tropicalis* increased by 25% when xylose was used as a carbon source and 21% (w/v) PEG was used as an additive. The result was attributed to the fact that PEG can act as an extracting agent and also suppresses the formation of xylitol. Lee et al. [111] evaluated the impact of surfactants on ethanol fermentation. The authors investigated the use of Tween 20, Tween 80, and Triton X-100 in concentrations of 0.1% and 1.0% (w/v) in simulated and real hydrolysates (from pretreated wood by steam explosion) on the ethanol production by *S. cerevisiae* HI-7. They observed that Tween 20 and Tween 80 improved the ethanol production from simulated hydrolysate without significantly affecting cell growth. On the other hand, only the experiment with 1% (w/v) Tween 80 showed a notorious fermentation result using real hydrolysate, reaching 25 g/L ethanol while the control experiment obtained 23 g/L ethanol. This behavior was attributed to the fact that surfactants can improve the mass transfer of glucose (substrate). In 2003, Alkasrawi et al. [112] reported that the addition of 2.5 g/L Tween 20 increased the final ethanol production by 11.8% from the liquid fraction of the softwood pretreatment. Wei et al. [113] reported that the addition of 0.05–0.40% (w/v) Tween 80 increased the ethanol yield from 70 to 85% in the conversion of softwood hemicellulose. Nasirpour et al. [114] investigated the effects of PEG 4000 on the ethanol fermentation of *Zymomonas mobilis* and the cell membrane characteristics of the strain. The cell growth of *Z. mobilis* was not affected by the use of 1% (w/v) PEG 4000, but fermentation with 3% and 5% (w/v) PEG 4000 reduced the optical density by 10% and 50%, respectively. After incubation with the polymer, the hydrophobicity and redox potential of the cell membrane increased as a function of the concentration of PEG 4000. Thus, it was inferred that PEG changes the amount of lipopolysaccharides in the strain and, therefore, increases the cell membrane permeability. This mechanism was attributed to the fermentability gain in the presence of 1% (w/v) PEG 4000 since ethanol can migrate more easily to the external environment, and

negative effects, such as inhibition of glucose transport and metabolism, were minimized.

The addition of non-ionic surfactants has been shown to be beneficial in systems with fermentation inhibitors for removing them or blocking their negative effects. Tu et al. [115] reported that the use of 0.2% (w/v) Tween 80 increased the ethanol yield of HMF-rich hydrolysate by 58% after 12 h of cultivation. Dhamole et al. [116] investigated the potential of thermosensitive copolymers (EOPO 2360 and EOPO 2500) to increase the fermentability of corn straw hydrolysates. The micelles formed by the copolymers were able to remove more than 90% of lignin-derived phenolic compounds (p-coumarylic acid, ferulic acid, vanillin, and syringaldehyde), furfural (~25%), acetic acid (~20%), and HMF (~10%). The hydrolysate with 1% (w/v) EOPO achieved productivity 30% higher than the control experiment, while the treatment with 5% (w/v) EOPO achieved a productivity twofold higher. Lee et al. [117] proposed the direct addition of Tween 80 to solve inhibition problems in acetone–butanol–ethanol fermentations by *Clostridium tyrobutyricum*. The presence of 1 g/L Tween 80 improved the fermentation performance with p-coumarylic acid and ferulic acid. Using rice straw hydrolysate as a substrate, the butyric acid production increased more than 80 times with 8.7 g/L Tween 80. One hypothesis assumed to justify this result was that the surfactant micelles can sequester the inhibitory compounds, so that negative effects are blocked. This strategy was titled as in situ detoxification since the inhibitor blocking occurred simultaneously with the fermentation. Mithra et al. [118] observed that Tween 20 and the combination between Tween 20 and PEG 4000 were effective in removing 50% phenolic compounds from hydrolysates from agro-industrial residues without compromising the content of fermentable sugars. Guan et al. [119] also reported benefits of in situ detoxification by Tween 80 in SHF and SSF of switchgrass.

Liu et al. [120] proposed the use of high PEG concentrations in order to boost the fermentation performance. These authors observed that the PEG concentration strongly affects the ethanol production in cultivation of *S. cerevisiae* using only glucose as a nutrient. Under the concentration of PEG equal to 250 g/L, ethanol production reached a maximum value of 175 g/L, while the control experiment obtained only 159 g/L. Cell viability was assessed throughout the fermentation process, and it was revealed that PEG can act as a yeast vitalizing agent. Cell viability in the PEG 400 experiment was twice the cell viability observed in the PEG-free experiment after 60 h of cultivation. This hypothesis was confirmed in cultivations with recycled cells, in which cells obtained from cultivations with PEG are still able to produce ethanol after 4 cycles, but cells recycled without PEG were completely inhibited in the same condition. In another approach, PEG was successfully recycled over 4 cycles by liquid–liquid extraction, and there was no impact on glucose consumption and ethanol production [120]. The same authors evaluated the effects of PEG in simulated fermentations with typical fermentation inhibitors (phenolic compounds and furan aldehydes) and SSF from acid pretreated pine in a study carried out in 2016. Phenol was chosen as a model phenolic compound, and it showed strong inhibition on the fermentation performance, reaching an ethanol production of only 111 g/L (30% reduction). However, when PEG 1000 was added to the system, the inhibition effect was blocked by PEG, and the ethanol production reached the same level as the control experiment (without phenol and PEG). It was observed that better ethanol production values accompanied the increase in the molecular mass of the polymer. In light of the results, the authors tried to name the approach as PEG detoxification or in situ detoxification by adding PEG. In nuclear magnetic resonance analysis, the authors suggest that the oxygen of the PEG ether groups interact with the hydroxyl groups of the inhibitors, limiting contact with the cell membrane, as can be seen in Fig. 5. The approach was effective for a wide range concentration of fermentation inhibitors (0–3 g/L) and concentration of inoculum ($0.4–1.6 \times 10^8$ cell per mL). In SSF, the addition of PEG 1000 also increased ethanol production by 242% (24 g/L ethanol) when compared to SSF without PEG 1000, which demonstrated that the polymer can be an alternative to conventional detoxification methods [11].

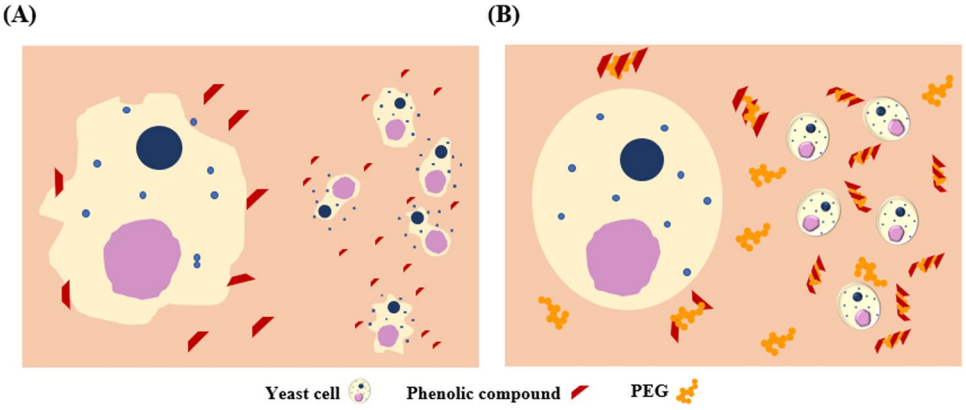

Fig. 5 Cell lysis caused by phenolic compounds (**A**) and blocking the effects of inhibition by the presence of polyethylene glycol (PEG) (**B**)

The effects of high PEG concentrations on cellulosic ethanol production were investigated by Nogueira et al. [121]. The authors proposed to test the effectiveness of the strategy of adding high PEG concentrations in real systems, in which green coconut fiber (GCF) was chosen as the main raw material. First, the studies were conducted with the addition of high concentrations of PEG 400 to increase the fermentability of pretreated GCF and pretreatment liquid fractions. SSF experiments with 200 g/L PEG 400 increased ethanol production by 43% using hydrothermal pretreated GCF; however, the addition of the polymer did not affect the ethanol titers obtained from the pretreated alkaline GCF. The addition of PEG 400 and 50% (v/v) liquid fraction of hydrothermal pretreatment in the SSF increased the ethanol yield from 39 (control experiment; without liquid fraction) to 87%. More recently, the authors investigated the mechanisms that promote the cellulosic ethanol production [103]. In addition to the detoxification effect, the authors were concerned about evaluating whether the high polymer concentrations would affect enzymatic digestibility. In this situation, the authors tested the non-ionic surfactants Tween 80 and Triton X-100 and the polymers PEG 4000 and EOPO 5800. The use of both polymers made it possible to maintain the cellular viability of *S. cerevisiae* in a stressful environment (with 2 g/L phenol) and mitigated the non-productive adsorption of cellulases in GCF (either untreated or pretreated). The SSF experiments with hydrothermal pretreated or diluted acid pretreated GCF showed an increase in ethanol production when the pretreatment liquid fraction and polymers were added. Unusual results were observed in the SSF experiments involving untreated GCF and PEG 4000, which reached 9.7 g/L ethanol and an ethanol yield equal to 89.8%. This behavior was attributed not only to the mitigation of the non-productive adsorption of cellulases, but also to the structural changes in biomass caused by PEG. Another paper was prepared by the same research group in order to explore the use of high PEG concentrations to dispense with the application of chemical and physical–chemical pretreatments. In Nogueira et al. [122], the authors proposed to increase the solid loading in the batch SSF and fed-batch SSF of GCF to the values of 20% and 30% (w/v) solids using 150 g/L PEG 1500, respectively. The maximum concentration of ethanol equal to 35.1 g/L was reached in the fed-batch SSF strategy, which corresponds to 66.8% after 48 h of operation.

Insights and Future Perspectives

In fact, PEG and non-ionic surfactants have been applied for decades as additives in cellulosic ethanol studies, and their benefits have been proven several times. However, the use of additives should not be considered unrestricted. Readers should keep in mind that the present paper only made a more up-to-date compilation of studies that pointed out advantages of using additives and their respective mechanisms. Due to the structural complexity of lignocellulosic biomass, it is unwise to create expectations when using a given dosage of PEG and surfactants on the cellulosic ethanol production using another biomass as a raw material. As mentioned in the third section, studies have already reported that the use of surfactants does not limit the formation of pseudo-lignin, but perhaps they will favor it in some situations. Therefore, if on the one hand the surfactant-assisted pretreatment already blocks the non-productive adsorption of cellulases onto lignin, on the other hand, it can limit the generation of sugars due to the cellulose loss. Zhou et al. [79] reported that non-ionic surfactants did not show a consistent improvement on the enzymatic digestibility of crystalline cellulose. In addition, PEG is a polymer whose manufacture is still largely dependent on petroleum, being incompatible with the concept of cellulosic ethanol as a renewable fuel.

Data on techno-economic evaluation involving surfactant-assisted strategies can already be collected in the literature, but they are still scarce. Both studies by Tu and Saddler [123] and Kadhum et al. [124] focused only on the feasibility of using non-ionic surfactants as an additive to enzymatic hydrolysis. To the knowledge of the authors, no study of this style has been carried out in strategies with PEG-assisted pretreatment. Techno-economic assessments would be welcome for PEG detoxification (in situ detoxification with the addition of PEG). Although these conditions make it possible to dispense with the need for chemical pretreatment, according to studies by Nogueira et al. [103, 122], operations with PEG concentrations of up to 200 g/L should require the effective recycling of the polymer by several batches. Some important points should be investigated in greater depth in future studies:

- Evaluate the best time to add PEG or non-ionic surfactants in the cellulosic ethanol production.
- Perform the techno-economic evaluation of cellulosic ethanol production with the addition of PEG and other surfactants in the pretreatment, enzymatic hydrolysis, and fermentation steps.
- Perform life cycle analysis on the cellulosic ethanol production schemes with addition of PEG or surfactants.

Conclusions

The present study brings to light a more complete view of the mechanisms and effects of PEG and non-ionic surfactants on cellulosic ethanol production. Blocking non-productive adsorption sites on lignin is undoubtedly crucial for the enzymatic digestion of lignin-rich materials, but other

mechanisms, such as minimizing enzyme deactivation at the air–liquid interface and additive–enzyme interactions, may be more important to explain the behavior of systems with cellulose or low lignin content materials as substrate. Pretreatments assisted with additives can improve cellulose enrichment and hydrophilicity in the pretreated material, especially using non-ionic surfactants. In the fermentation stage, PEG and non-ionic surfactants can act as yeast vitalizers and detoxifying agents against lignin-derived phenolics, which allows for greater conversions of sugars into ethanol. In summary, the insertion of additives at any stage of the cellulosic ethanol scheme is indeed a valid practice to increase cellulosic ethanol titers, as long as it does not compromise the economic viability of the process. Thus, it is expected that the information contained in this study can collaborate with researchers and enthusiasts regarding the efficient use of these additives in the context of cellulosic ethanol, so that this technology becomes increasingly robust.

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Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest The authors declare no competing interests.

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