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Sugars from pine bark by enzymatic hydrolysis Effect of sodium chlorite treatments *

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Summary. For the utilization of polysaccharides from *Pinus pinaster* bark, both untreated and NaOH-treated samples were delignified to different stages using $NaClO_2$ and submitted to enzymatic hydrolysis with *Trichoderma reesei* cellulase and *Aspergillus niger* beta-glucosidase. In the best conditions, samples treated with 1% NaOH for 15 min and $NaClO_2$ for 7 hours the conversion of polysaccharides into sugars accounts for the 75% of the potential yield, and the glucose obtained amounts to 87% of the theoretical value.

Introduction

Galicia, the northwest country of Spain, produces 40,000 TM/year of *Pinus pinaster* bark (oven-dry basis), a renewable lignocellulosic material which is wasted in great proportion. Both polysaccharides and polyphenols contained in bark are potentially useful for the manufacture of marketable chemicals (Parajó et al. 1983).

In this paper, enzymatic hydrolysis is carried out in order to recover the polysaccharides existing in a lignocellulosic waste. The sugars thus obtained could provide a fermentation medium to be used for production of many chemicals.

When pine bark is considered as substrate for the enzymatic hydrolysis, some problems must be considered:

The complexity of its chemical nature: pine bark contains chemical fractions with very different composition and properties.

The kind and proportion of its phenolic compounds. These compounds include water-soluble polyphenols (condensed tannins), alkali-soluble polyphenols (with polyflavonoid structure) and alkali-insoluble polyphenols (analyzed as Klason lignin).

For these reasons the pine bark is a poor source of carbohydrates, and it is necessary to apply pretreatments in order to improve the yields on enzymatic hydrolysis and render the process economically feasible.

Many types of pretreatments have been proposed to enhance the rate and yield of sugars by means of enzymatic hydrolysis of lignocellulosics (Cowling et al. 1975; Fan et al. 1982). The optimum solution depends on the substrate involved. In some

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cases, a mechanical treatment (which reduces the cristallinity of cellulose and raises the specific surface) improves the results (Mandels et al. 1974; Gharpuray et al. 1983). However on other occasions treatments with chemicals are necessary to modify the structure of the substrate and disrupt the physico-chemical associations between polysaccharides and phenolic fraction (Thonart et al. 1979).

Among the chemicals that have been used for the pretreatment of lignocellulosics we have focused our attention on NaOH, which dissolves partially the phenolic fraction, causes swelling and increases the available surface area (Shimizu 1981; Gharpuray et al. 1983; Grohman et al. 1984) and $NaClO_2$, frequently used as delignifying agent.

The bark used in the experiments was analyzed to determine the type and amounts of its chemical fractions. Both untreated and alkali-treated bark samples were submitted to chlorite treatments, and the variations in chemical composition caused by this treatment were studied. The materials obtained with an increased carbohydrates content were used as substrates for enzymatic hydrolysis.

Material and methods

The *Pinus pinaster* bark samples were collected in a particleboard plant located in Santiago de Compostela (Spain). The samples utilized came directly from the debarking of trees. The bark was air-dried and milled using a Wiley mill fitted with 1 mm screen. The particle size distribution of samples is shown in Table 1, where it can be seen that the smaller the particles are the higher is their weigth percent.

The milled bark samples were properly homogenized to ensure the identity of composition in every part of the lot. Using this material, the following analytical determinations were performed:

1. Moisture content (Browning 1965)
2. Ash (Browning 1965)
3. Extracts, using the sequential extractions proposed by Labosky (Labosky 1979)
4. Aromatic content (Labosky 1979)
5. Suberin (Meara 1955)
6. Klason lignin, measured as the insoluble residue after 1% NaOH and 72% sulfuric acid treatments (Chang 1965).

Formaldehyde-condensable polyphenols (Stiasny polyphenols) extracted by alkaline solutions, were determined using both a gravimetric method (Wissing 1955)

Table 1. Particle size distribution of bark samples

Particle size, mm	Weight percent
1.0 –0.5	37.6
0.5 –0.25	22.3
0.25 –0.12	16.7
0.12 –0.071	10.5
0.071–0	12.9

and the spectrophotometric procedure already described by the authors (Vázquez et al. 1987 b).

Polysaccharide content, measured from the sugars obtained by a quantitative acid hydrolysis performed under previously reported conditions (Browing et al. 1957). The overall concentration of reducing sugars was measured, using a spectrophotometric method (Somogyi 1952), by comparison between the reducing power of hydrolyzates and pure glucose solutions. The results are reported as "glucose equivalent". The hydrolyzates were concentrated, centrifuged, filtered through 0.45 μm membranes and analyzed by HPLC using a Bio-Rad HPX87H column and water as eluant.

The alkaline extractions previous to delignification treatments were performed in a batch reactor at 95 °C for 15 min under the conditions previously selected Vázquez et al. 1986). The raffinates were washed with water during 15 min at 60 °C and air-dried. The solid/liquid ratio was 1/10 in alkaline extractions and washings.

Both untreated and alkali-treated bark samples (25 g of dry solid), were submitted to delignification (using sodium chlorite and acetic acid) in Erlenmeyer flasks maintained at 70 °C, with magnetic stirring. The solid/liquid ratio used was 5/100. The time taken for oxidizing treatments varied from 0.5 to 8 hours. The initial concentrations of sodium chlorite and acetic acid were 1.5 and 0.5% (w/w) respectively. Each hour, fresh chlorite and acetic acid were added in equal amounts to those initially used.

The samples obtained at different times were washed with water at 60 °C during 15 min (solid/liquid ratio = 5/100) and air-dried. Each sample was submitted to both acid and enzymatic hydrolysis. The extracts from acid hydrolysis were analyzed for sugars by the spectrophotometric and HPLC methods mentioned above. The acid-insoluble residue, which does not entirely represent the Klason lignin contained in pine barks, was also measured to study its influence on the enzymatic saccharification.

The enzymatic hydrolyses were performed using *Trichoderma reesei* cellulases (Celluclast, Novo), an enzymatic complex that exhibits low beta-glucosidase activity and it is strongly inhibited by cellobiose (Mandels, 1963; Ghose 1969, 1971; Mangat 1978; Lee et al. 1982). Therefore, the hydrolysis medium was enriched with beta-glucosidase from *Aspergillus niger* (Novozym, Novo). The operational conditions were: Temperature: 48.5 °C, pH: 4.85 (0.05 N citrate buffer), Solid/liquid ratio: 4/100, Cellulase activity (according to Mandels et al. 1976): 0.3 FPU/ml and Beta-glucosidase activity (according to Paquot et al. 1982): 4.0 UI/ml. Preservative: 0.01% formaldehyde.

Experiments were performed in 100 ml Erlenmeyer flasks with magnetic stirring (working volume 70 ml).

Samples (1 ml) were obtained from hydrolysis media after 1, 2, 4, 6 and 24 hours. The samples were centrifuged, filtered through 0.45 μm membranes and analyzed by both the spectrophotometric and the HPLC method described above. In the determination of reducing sugars from bark, the DNS method (Miller 1959) showed interferences, but the Somogyi-Nelson procedure provided correct analytical results.

Results and discussion

The results obtained in the analysis of bark are summarized in Table 2. The fraction soluble in apolar organic solvents, mostly waxes, amounts to 2.4 weight percent of samples. The fraction dissolved by polar organic solvents consists mainly of polyflavonoids with phloroglucinol like A rings (Vázquez et al. 1987 a). The fractions soluble in water or 1% NaOH have spectral and chromatographic properties that demonstrate their closely interrelationship with the compounds extracted using polar organic solvents (Vázquez et al. 1987 a). The polyphenols solubilized by neutral and alkaline extractions also belong to the flavonoid family, and the differences in their solubility seem to be due to variations in their degree of polymerization and accessibility rather than to differences in their chemical constitution (Hemingway et al. 1976).

The aromatic content provides an index of all the polyphenols present in bark, and the Klason lignin measures the NaOH insoluble polyphenols. The two kinds of compounds differ notably in chemical composition (Browning 1965). The glucose equivalent gave a value higher than the overall amount of monosaccharides. The difference between these values is due to the existence of uronic acids in bark hydrolyzates, which have reducing power but are not included in HPLC data. The column utilized in HPLC analysis resolves glucose and arabinose. Xylose, mannose and galactose (sugars also present in *Pinus pinaster* bark hydrolyzates) are eluted in a single peak.

When the bark samples are extracted with NaOH under the conditions mentioned above, 29.6% of bark is dissolved. During this treatment the polysaccharide

Table 2. Chemical composition of the bark lot (results expressed as weight percent of oven dried samples)

Fraction	Amount
Ash	1.0%
Extracts	
Hexane	1.6%
Benzene	0.8%
Ether	1.0%
Ethanol	11.6%
Water	3.0%
1% NaOH	20.6%
Aromatic content	60.0%
Suberin	0.9%
Klason lignin	34.2
Products of acid hydrolysis	
Glucose equivalent[a]	29.3
Glucose[b]	15.8
Other sugars[b]	11.9

[a] From spectrophotometric determination
[b] From HPLC data. Other sugars are mannose, xylose, arabinose and galactose (Vázquez et al. 1987 a)

losses account for 8.9% of the initial polysaccharides present in bark. The losses are due to the removal of hemicelluloses, and the cellulose does not vary. Roughly 85% of the material dissolved on alkaline extraction consists of formaldehyde-condensable polyphenols, which have been identified as active agents for the production of adhesives (Yazaki et al. 1977). The NaOH extraction of bark has important effects: it provides significant amounts of extracts with potential use, causing limited carbohydrate losses from the solid phase and increases the polysaccharide content of raffinates up to 37.9% (where 6/10 of this content corresponds to cellulose). Other reported effects caused by alkaline extractions are swelling, increase of the surface area and decoupling of lignin-carbohydrate associations (Shimizu 1981; Fan et al. 1982; Gharpuray et al. 1983a). These factors favor the enzymatic hydrolysis of polysaccharides, but the advantage obtained when raffinates from caustic extractions of bark are hydrolyzed is very small (see below).

To improve the yields on hydrolysis it is necessary to perform other treatments which facilitate the enzymatic attack and alter the chemical structure of the phenolic fraction, avoiding the inactivation of enzymes by these compounds. Our previous work (unpublished) showed that oxidizing agents are the most effective chemicals to enhance the enzymatic hydrolysis of bark. Among the delignifying agents cited in literature (Toyama 1972; Dunlap et al. 1976; Millett 1976; Gould 1984, 1985) we have chosen sodium chlorite. In acid medium the chlorite generates ClO_2 that transforms the lignin in soluble compounds without significant losses of carbohydrates (Dunlap et al. 1976; Blanch et al. 1983). This procedure has been used as an analytical method for holocellulose determination (Timell 1961) and for physico-chemical studies of pure delignification (Sudo et al. 1976). In addition, the chlorite treatments cause changes in the crystallinity of the cellulose (Blanch et al. 1983) that could promote the hydrolysis process.

Milled bark samples (without chemical pretreatment) and alkali-pretreated barks were submitted to delignification. The effects obtained are shown in Fig. 1. Both weight loss and polysaccharide content of the samples increase with the duration of the chlorite treatments. In alkali-pretreated samples, the delignification is mainly accomplished in 4 hours (samples with 70% of polysaccharides content and 3.5% acid insoluble residue were obtained). When untreated bark samples were submitted to oxidation, the effects caused were less: the product obtained after 8 hours of delignification contains 69.4% of carbohydrates and 4.8% of acid-insoluble residue.

From the above it can be seen that the overall fraction corresponding to polysaccharides and acid-insoluble residue of samples account for far less than 100 weight percent of the samples. This is due to the generation of acid-soluble lignin during delignification. This behaviour was previously found with woods (Sudo 1976), but the reported results are roughly one half of those determined in our case. The presence of non-sugar compounds in the acid hydrolyzates from delignified bark samples was confirmed in the HPLC chromatograms. The rapid formation of acid-soluble lignin seems to be related to the small particle size used in this work.

During the delignification of untreated bark samples, up to 11% of the polysaccharides is lost. The hemicellulose fraction is selectively removed, but the amount

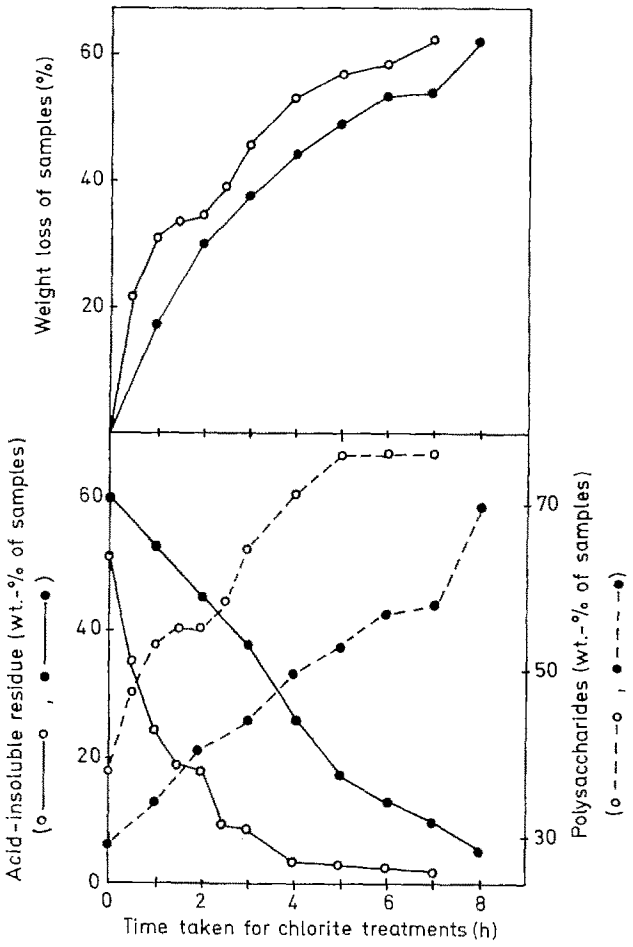

Fig. 1. Effect of delignification. ● Bark not pretreated, ○ Alkali pretreated bark

of cellulose is not altered. Therefore, the cellulose/polysaccharides ratio increases during chlorite treatments to reach the value 0.6.

When alkali-treated bark samples are submitted to delignification the carbohydrate losses rise with time to 16% of the initial polysaccharides. The hemicelluloses are preferentially removed, and cellulose is also affected (4 of each 10 grams of dissolved carbohydrates correspond to cellulose). The solid obtained after 7 hours of treatment has a cellulose/polysaccharides ratio of 0.64.

The samples obtained at low degrees of delignification form a heterogeneous material: the biggest particles were dark, revealing a low degree of delignification. In contrast, the smallest particles were readily bleached. The samples delignified during more than four hours were white in colour. In these cases, the swelling produced by chlorite treatments provoked the loss of particle structure: a gel-like material is obtained, which becomes a hard solid after drying. These samples were newly milled before acid and enzymatic hydrolysis.

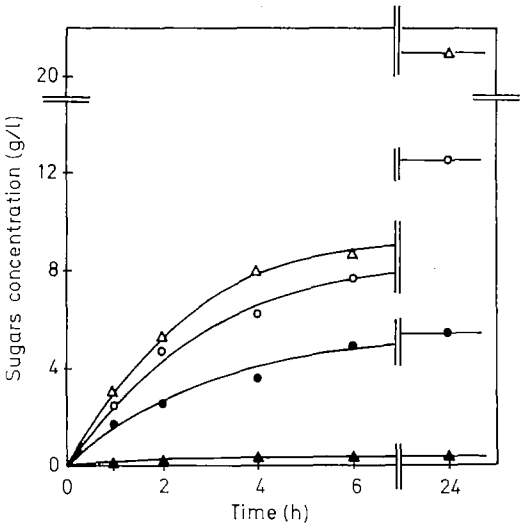

Fig. 2. Sugars produced in the enzymatic hydrolysis of untreated or $NaClO_2$ treated bark samples. ▲ Untreated bark, bark treated with chlorite ● for 4 h; ○ for 6 h; △ for 8 h

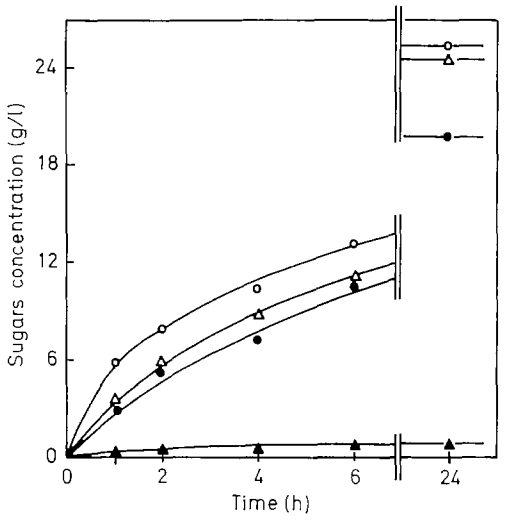

Fig. 3. Sugars produced in the enzymatic hydrolysis of NaOH treated or NaOH-$NaClO_2$ treated bark samples. ▲ Alkali-treated bar. Bark treated with NaOH (15 min.) and chlorite ● (3 h); △ (5 h); ○ (7 h)

Figures 2 and 3 show the kinetics of hydrolysis of some representative samples obtained at different stages of delignification. Both rates and yields of enzymatic hydrolysis strongly depend on the extent of delignification. During the first hour of hydrolysis, the glucose amounts to 76–100% of the sugars produced. After 24 hours, the glucose accounts for 72–90% of the overall sugars. When using samples with high degrees of delignification, the glucose/sugars ratio is 0.75–0.80.

Figure 4 shows the overall sugars and glucose produced after 24 hours of enzymatic hydrolysis from samples delignified at different levels. Figure 5 shows the hydrolysis percentages reached for the different substrates after 24 hours. The results indicate a gradual increase in yield when the time of delignification increases. Using alkali and chlorite treated samples, only minor advantages are ob-

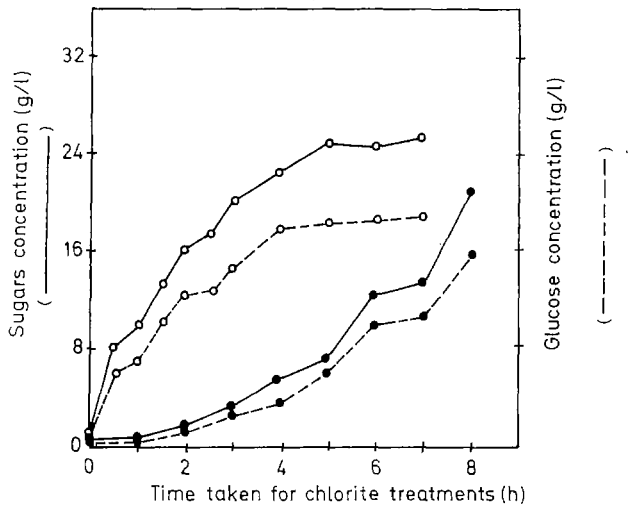

Fig. 4. Results obtained after 24 hours of enzymatic hydrolysis using delignified bark samples. ● Untreated or $NaClO_2$ treated bark samples; ○ NaOH or NaOH–$NaClO_2$ treated bark samples

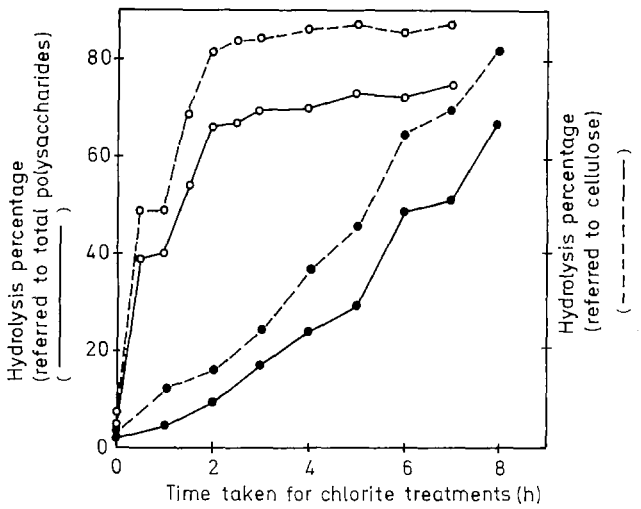

Fig. 5. Yields in sugars or glucose obtained after 24 hours of enzymatic hydrolysis. ● Untreated or $NaClO_2$ treated barks; ○ NaOH or NaOH–$NaClO_2$ treated barks

tained when the delignification was prolonged for more than 4 hours. In contrast, the barks pretreated with chlorite reach significant yields only after 7 hours of treatment.

Figure 6 allows an easy visualization of the influence of delignification on the hydrolysis yield. It can be seen that a strong interrelationship between the amount of acid-insoluble residue and the sugars produced on hydrolysis exists. Considering samples of the same proportion in acid-insoluble residue, we find that the barks

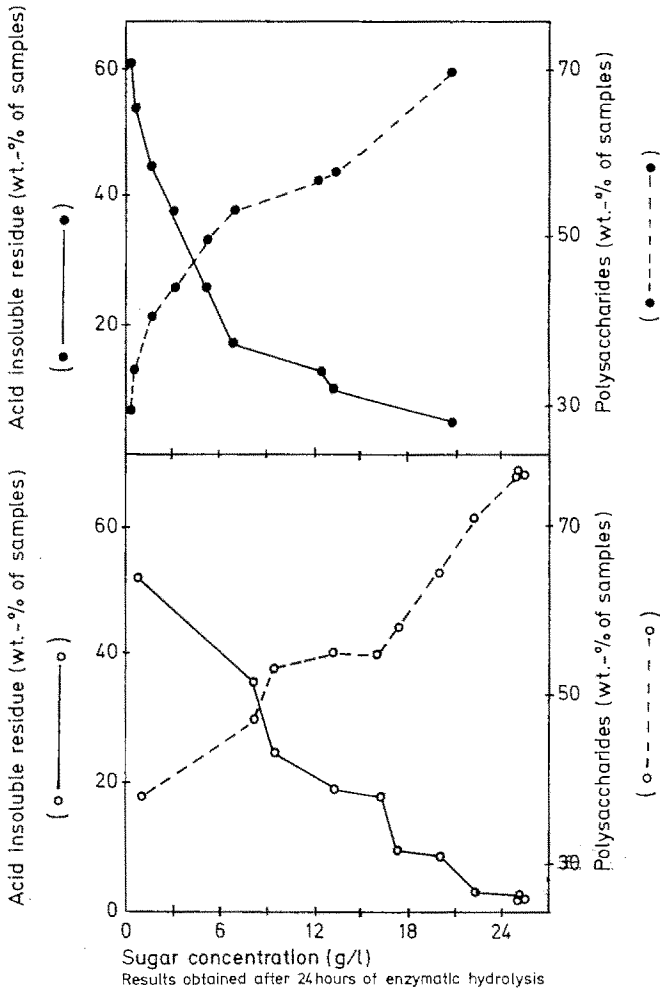

Fig. 6. Relationship between sugar concentration after 24 h of enzymatic hydrolysis, acid-insoluble residue and polysaccharides. ● Untreated or chlorite-treated barks; ○ NaOH or NaOH–$NaClO_2$ treated barks

treated with NaOH and $NaClO_2$ provide better results than the $NaClO_2$ treated barks. Thus, to reach 15 g of sugars/l it is necessary to decrease the acid-insoluble residue of chlorite treated barks to below 9% (value reached after 7 hours of delignification). In contrast, when NaOH pretreated barks are hydrolyzed, the same sugar concentration is obtained using samples with 18% acid-insoluble residue (which is reached after 2 hours of delignification). Therefore, to get 15 g of sugars/l, the 85% or 65% of the acid-insoluble residue can be eliminated using $NaClO_2$ or NaOH–$NaClO_2$ treated barks, respectively. These values are far above the 30–60% of delignification previously reported as the necessary pourcentage to enhance significantly the enzymatic hydrolysis yields from different substrates (Sudo et al. 1976; Gharpuray et al. 1983 a).

The differences between the susceptibility of $NaClO_2$ and NaOH–$NaClO_2$ treated bark towards enzymatic hydrolysis decreases when the degree of delignification increases. Thus, the hydrolysis yields of NaOH-chlorite treated barks containing 20% of acid-insoluble residue is twice as high as those attained using $NaClO_2$ treated barks containing the same amount of acid-insoluble residue, but when the acid-insoluble residue contained in both types of samples is 5%, the yields of hydrolysis differ only by a factor of 1.1.

When strongly delignified bark samples are hydrolyzed, high yields of hydrolysis are obtained. The sample treated with sodium chlorite for 8 hours produces 20.9 g of sugars/l and 15.7 g of glucose/l after 24 hours of enzymatic hydrolysis. These results account respectively for 67 and 83% of their potential values. The results improve when using NaOH–$NaClO_2$ treated barks, Thus, the sample obtained after 7 hours of delignification gave 25.5 g of sugars/l and 18.9 g of glucose/l. These values correspond to 75 and 87% of the potential yields, respectively.

Conclusions

Pine bark is a very resistent substrat towards enzymatic hydrolysis: in the operational conditions used in this work, only 0.41 g of sugars/l were obtained after 24 hours, i.e., 3.1% of the theoretical yield.

When the bark is submitted to treatment with NaOH, an important part of the solid is dissolved, but the carbohydrate losses are not important. The proportion of samples corresponding to polysaccharides rises to 37.9%, but the saccharification process is not significantly improved (only 0.86 g of sugars/l are obtained after 24 hours).

In order to reach a significant yield of hydrolysis, a delignification step was performed. Both untreated and alkali treated barks were delignified with sodium chlorite. Solids containing up to 75% carbohydrates were obtained without important polysaccharide losses. In samples with a high degree of delignification, the cellulose accounts for the 60–64% of overall carbohydrates.

In the enzymatic hydrolysis the glucose is the main product (it amounts to 72–100% of total sugars). To reach 15 g of sugars/l, the delignification must be prolonged during 7 or 2 hours for $NaClO_2$ or NaOH–$NaClO_2$ treated barks, respectively. Using the most delignified sample (treated with 1% NaOH for 15 min and $NaClO_2$ for 7 hours), 75% of polysaccharides were converted into sugars, and 87% of cellulose was transformed into glucose.

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