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A THERMOMECHANICAL PROCESS FOR IMPROVING ENZYMATIC HYDROLYSIS OF BREWER'S SPENT GRAIN

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Abstract

Brewers' spent grain (BSG), the main low-value solid residue, is the major by-product of the brewing industry, representing around 85 % of the total by-product generated. This raw material is renewable, at low cost, largely available throughout the year and produced in large quantities. BSG was hydrothermally treated by instantaneous controlled pressure drop process (DIC) at five pressure levels (or five temperatures), with as objective to observe the effect of the processing pressure on enzymatic hydrolysis of BSG. Enzymatic hydrolysis was performed on the pretreated solids by celluclast (1.5L). The results showed that the hydrolysis yield of BSG treated at pressure levels of 2, 3, 4, 5 and 7 bar for a processing time of 15 min was strongly improved compared to hydrolysis yield of untreated BSG. The best hydrolysis yield was obtained, up to 100%, when the BSG was pretreated at more intensive conditions (5 and 7 bar for 15 min). The morphology of treated BSG samples was studied with scanning electronic microscopy (SEM). Untreated BSG exhibited rigid and highly ordered fibrils whereas the structure of pretreated BSG at 7 bar seems to have an important disruption.

Keywords: Brewers' spent grain (BSG); DIC hydrothermal treatment; Enzyme hydrolysis; SEM

1. Introduction

Nowadays, there is great challenge and political pressure to reduce the pollution arising from industrial activities. The majority of industries trying to adapt to this reality through modification of their processes so that their residues can be reused. Consequently, residues are generally not considered as waste but as a raw material for other purposes. Brewers' spent grain (BSG), the main low-value solid residue, is the major by-product of the brewing

industry, representing around 85 % of the total by-product generated (Fernández et al., 2008). According to Townsley (1979), cited by Mussatto et al. (2006), spent grain accounts, on average, for 31 % of the original malt weight, representing approximately 20 kg per 100 l of beer produced. The composition of BSG as described in the literature is variable, containing mainly hemicellulose in the form of arabinoxylans and cellulose. A hemicellulose and cellulose content varies between 19-40 % and 9-25% per dry matter, respectively (Xiros et al., 2008). BSG is available at low or no cost throughout the year, and is produced in large quantities by large breweries but also by the small ones.

Current technology for conversion of lignocellulose to bioethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by fermentation by microorganisms. Enzymatic hydrolysis of cellulose is a reaction carried out by cellulase enzymes, which are highly specific (Beguin, 1994). Cellulases are usually a mixture of several enzymes, among which at least three major groups are involved in the hydrolysis process of cellulose: (1) β -1-4-endoglucanase (EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber creating free chain ends; (2) β -1-4-exoglucanase or cellobiohydrolase (EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain ends; (3) β -glucosidase or cellobiase (EC 3.2.1.21.), which hydrolyzes cellobiose to produce glucose. Unfortunately, large amounts of enzymes are required and influence severely on the cost effectiveness of this technology. Because enzymatic hydrolysis is a heterogeneous reaction and requires direct physical contact between enzyme and substrate. These reactions can be affected by the physicochemical properties of the substrate such as crystallinity, degree of polymerization, surface area, and lignin and hemicelluloses contents (Chang and Holtzapple, 2000 ; Mansfield, et al., 1999). In lignocellulosic materials cellulose is physically associated with hemicellulose, and physically and chemically associated with lignin. The presence of these two fractions is reported to make the access of cellulase enzymes to cellulose difficult, reducing the efficiency of the hydrolysis.

Considerable research efforts have been made to improve conversion yields of lignocellulosic materials by the insertion of pretreatment step before to the enzymatic hydrolysis. The purposes of pretreatment is increasing of porosity as well as removing the lignin (Cara et al, 2008; Mosier et al, 2005). The purely physical processes of pretreatment can be summarized by an intense mechanical grinding in order to increase the accessible surfaces to enzymes, thus supporting the further hydrolysis and reducing the polymerization degree of cellulose. This kind of pretreatment can be interesting if the granulometry is very low, which limits its interest and its use due to the energy cost of grinding (< 2 mm), (Ogier et al., 1999). The steam explosion consists to expose the biomass up to high pressure (15 to 50 bar) and temperature (180 to 250 °C) in presence of steam during a determined time, up to 90 minutes (Sassner et al., 2008), followed by a rapid reduction in pressure, in order to breakdown the lignocellulosic structure. The treatment leads to a partial self-hydrolysis of hemicelluloses, depolymerisation of lignin and a destructuration of cellulose, largely dependent on the temperature of treatment. However, the yields of enzymatic hydrolysis are about 50% and some inhibiting compounds of the fermentation can appear following the pyrolysis of

cellulose. This treatment is often combined to acidification with H₂SO₄ or SO₂ (Silverstein et al., 2007; Rodriguez et al., 2007), to improve the yield of hydrolysis. Steam explosion with acid catalysis (H₂SO₄) exhibited better results than other techniques such as the Afex process (Ammonia Fiber EXplosion) developed in USA (Kim et al, 2008). Indeed, although AFEX process requires relatively low temperatures (between 50 and 90 °C), without formation of inhibitors, it remains not very competitive on woody substrates. Moreover, the high ammonia concentrations (1-2 kg/kg dm) require its recovery and recycling so that the process is economically interesting.

In order to obtain a higher yields of enzymatic hydrolysis than those described in the literature and limiting the production of inhibitors of alcoholic fermentation, a thermomechanical pretreatment termed "D.I.C." process (in french: Détente Instantanée Contrôlée) developed in our laboratory was performed on brewer's spent grain (BSG). This physical process is close to the steam explosion technology. The difference is that the DIC treatment comprises two additional steps. The first consist to the instauration of the initial vacuum before injection of the steam in processing vessel. This step, as demonstrated by Zarguili et al. (2006), allows to reduce the resistance of the air and thus to facilitate the diffusion of steam into the product. Consequently, the time necessary to reach the steam equilibrium temperature is reduced (Zarguili, 2006). The second step consists to the abrupt decompression which carries out towards the vacuum (50 mbar) instead of the atmospheric pressure like it's the case with the steam explosion treatment. The pressure drop is obtained by a very fast communication (Instantaneous) of the reactor with a vacuum tank that has a much larger volume. Due to the instantaneous character of this transformation as well as the adiabatic nature of the transition of steam inside the product, the self-vaporisation induces a fast cooling. The temperature is quickly stabilized at a balance temperature of the considered final pressure, limiting the reactions of degradations.

2. Materials and methods

All chemicals were purchased from Sigma Co. (USA). Deionised water was obtained via a Milli-Q system (Millipore, France).

2.1. Substrate preparation

Brewer's spent grains (BSG) were supplied by the Biotechnology Department of the University of La Rochelle (www.univ-lr.fr/science-infuse). BSG were obtained from a high gravity brewing using 100% malt (without addition of other cereal adjuncts). Brewing yield (ratio of extract on the grist) was 70%. As soon as obtained the untreated material was dried at 50 °C to 3% or 40% moisture content before thermomechanical treatment.

2.2. Enzymes

Celluclast 1.5L, the enzyme concentrate used for cellulose hydrolysis, was a commercial *Trichoderma reesei* cellulase preparation contains *endo*-glucanases, *exo*-glucanases, cellobiohydrolases and β -glucosidases. This preparation was a brownish liquid with a density of approx. 1.20 g/ml and contained 27 mg protein/ml. The cellulosic activity of concentrate

was 74 FPU/ml. One unit of FPU is defined as the enzyme amount, which releases 1 μ mol of glucose equivalents from Whatman n°1 filter paper in 1 min.

Fungamyl 800 L, a α -amylase from *Aspergillus oryzae* was used for starch liquefaction. The enzyme activity was 800 FAU/g (1 Fungal α -amylase FAU is the amount of enzyme which breaks down 5.26 g of starch per hour according Novozymes standard method for the determination of α -amylase). AMG 300 L, a glucoamylase from *Aspergillus niger* was used for saccharification. The enzyme activity was 300 AGU/ml. 1 Novo Amyloglucosidase Unit (AGU) is defined as the amount of enzyme which hydrolyzes 1 μ mol maltose per minute under standardized conditions according Novozymes. All the enzymes preparations were supplied by Novozymes (Bagsvaerd, Denmark).

2.3. Determination of the chemical composition of BSG.

Dilute acid and dilute alkali solution have been used to separated residual starch, hemicellulose, lignin and cellulose from BSG. In a first time, the BSG material was submitted to a reaction with 3% (v/v) H₂SO₄ solution at 120 °C for 1 h (Wen et al., 2004). In these conditions, only the starch and the hemicellulose were hydrolysed. The resulting solid material (cellulignin) was separated by centrifugation and the content was estimated by heating the weighed dry matter at 50 °C. The supernatant (starch and hemicellulose) was also dried and weighed. In a second time, the cellulignin fraction was treated with 2% (w/v) sodium hydroxide solution in a solid/liquid ratio of 1 g/20 g at 120 °C for 90 min, conditions for an efficient lignin hydrolysis (Mussatto and Roberto, 2005). The residual solid material (cellulose pulp) was separated by centrifugation, washed with water to remove the residual alkali, dried at 50 °C and weighed. The supernatant (lignin) content was estimated by heating the weighed dry matter.

2.4. Determination of residual starch composition by enzymatic hydrolysis

The dried BSG samples (50 mg) were mixed with 10 ml of citrate phosphate buffer pH 5 in a 10 ml tube. The mixture was treated with enzymes in two steps, liquefaction and saccharification. The first step, liquefaction, was performed at 50 °C with 32 U/l (10 g/l) of Fungamyl 800 L for 120 min. The second step, saccharification, was performed at 65 °C with 1.5 U/l (7 ml/l) of AMG 300 L for 60 min. During the starch hydrolysis, samples were withdrawn for analysis of reducing sugars content using 3,5-dinitrosalicylic acid method (DNS).

2.5. DIC hydrothermal treatment

The equipment and procedure of D.I.C. thermomechanical treatment (Fig.1) were largely described in previous studies (Rezzoug et al., 2000; Loisel et al., 2006; Zarguili et al., 2006). During the treatment, 40 g of BSG (16% dry basis) disposed in circular containers (diameter: 20 cm; height: 5 cm) were placed in the treatment reactor. An initial vacuum of 50 mbar was established. This initial vacuum allows reducing the resistance of air and then accelerating the heat transfer into the product. Consequently, the time necessary to reach the steam temperature equilibrium is reduced. Saturated steam was introduced into the vessel at fixed pressure and maintained for a predetermined time. In this study the processing pressure was fixed at 2 bar (122 °C), 3 bar (135 °C), 4 bar (143.6 °C), 5 bar (151.8 °C) and 7 bar (164.9°C).

The pressurisation is followed by an abrupt decompression towards vacuum (50 mbar). After the vacuum phase, atmospheric air is injected to return to atmospheric pressure for sample recovery.

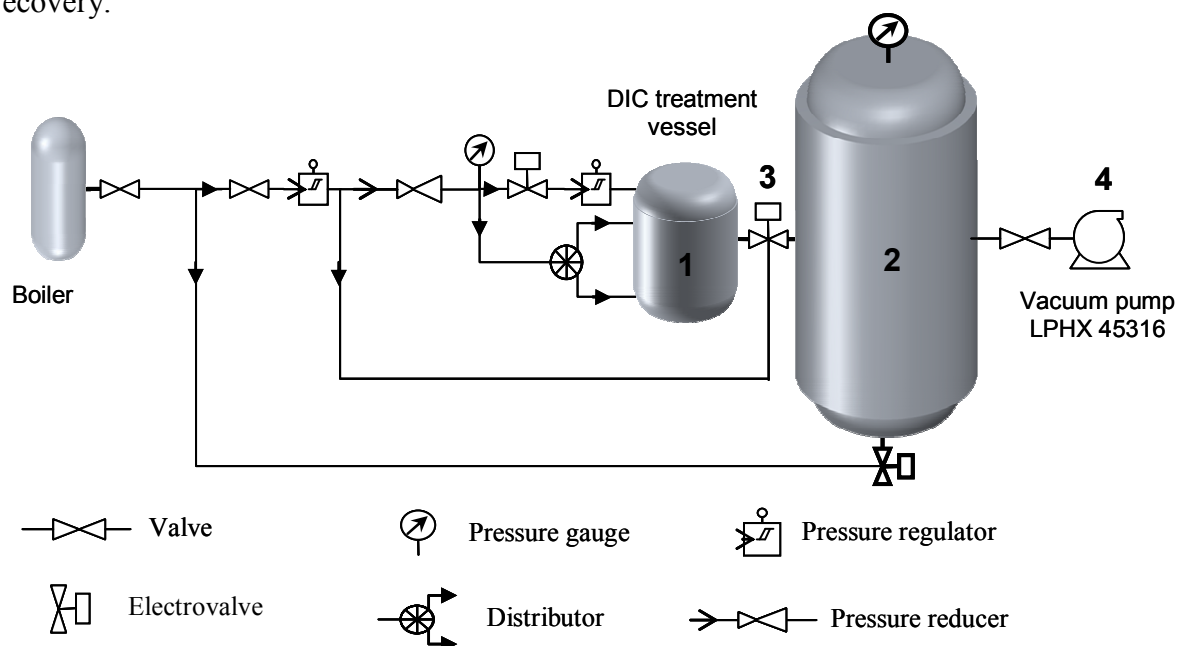


Figure 1. Experimental apparatus for pretreatment of BSG by Instantaneous Controlled Pressure Drop process. 1. treatment vessel; 2. Vacuum container; 3. Valve; 4. vacuum pump

2.6. Cellulose hydrolysis

Celluclast 1.5L (500 FPU/L) was added to 50 mM sodium citrate buffer (pH 5) and then mixed to the substrate (10 g/L). The experiments were carried out in 100 ml Erlenmeyer flasks containing 10 ml total reaction volume (the buffer–enzyme mixture). The flasks were sealed and incubated in a rotary shaker at 600 rpm at 50 °C during 20 h. To follow the hydrolysis, a flask was withdrawn at different times and the reaction mixture was immediately centrifuged at 4000 rpm for 10 min to remove solids. The liquid phase (hydrolyzate) was heated for 5 min on a boiling water bath to precipitate the protein and prevent further hydrolysis. The cellulose hydrolysis yield of samples was determined by 3,5-dinitrosalicylic acid method (DNS) method. As positive control of Celluclast 1.5L activity, the hydrolysis of pure microcrystalline cellulose (Avicel PH102) was carried in the same conditions. All reactions were carried in triplicate.

2.7. HPLC analysis

After acid hydrolysis of hemicellulose, glucose, xylose and arabinose concentrations in the hydrolysates were determined using a HPLC system from Agilent (1100 LC and differential refractometer, Waters model 410), with a UP6OH Uptisphere 6DIOL reversed-phase column (250 x 4 mm, 5 µm, Interchim) eluted with acetonitrile/water/acetic acid (82/18/0.1, v/v/v) at room temperature and at a flow rate of 1 ml/min.

2.8. Determination of reducing equivalents

During the cellulose hydrolysis samples were withdrawn for analysis of reducing sugars content using 3,5-dinitrosalicylic acid method (DNS). In all samples, the reaction was stopped by adding 1 volume of water and by heating at 100 °C for 5 min. After centrifugation of the solution at 3000 rpm for 10 min, 0.5 ml of diluted supernatant was transferred to a test tube and 0.5 ml of DNS reagent (10 g dinitrosalicylic acid + 200 ml NaOH 2M + 300 g potassium sodium tartrate + qsp 1l distilled water) was added. The tubes were allowed to stand for 10 min in boiling water and cooled to room temperature in ice water. The absorbance of the samples was measured at 550 nm using a spectrophotometer (Shimadzu mini UV 1240). Amount of reducing equivalents was calculated using a glucose standard curve. The cellulose hydrolysis yield of samples was calculated by:

$$\text{Cellulose hydrolyse (\%)} = \left(\frac{\text{Reducing sugar expressed as glucose in sample g/l}}{\text{weight of dried BSG in sample g/l}} \right) \times 100$$

2.9. Electronic microscopy

A Philips-FEI Quanta 200 ESEM/FEG Scanning Electron Microscope (Laboratoire commun d'Analyses, La Rochelle University) operated at 20 kV, with a detector of secondary electrons Everhardt-Thornley, was used to image the control sample and some treated BSG samples. To improve the conductivity of the sample and thus the quality of the SEM images, a high vacuum was achieved.

3. Results and discussion

3.1. Chemical composition of substrates

The chemical composition of the BSG samples and the sugar composition of hemicellulose are given in Table 1 and 2, respectively. The starch composition was determined by the glucose concentration obtained by acid hydrolysis and confirmed by enzymatic hydrolysis using a α -amylase from *Aspergillus oryzae* and a glucoamylase from *Aspergillus niger* (Fungamyl 800 L and AMG 300 L respectively). Arabinose and xylose are the products of hemicellulose degradation and are shown in Table 2, glucose is the product of starch and hemicellulose hydrolysis. Glucose, xylose and arabinose contents have been determined by HPLC analysis. These results shown that hemicellulose is composed of xylose/arabinose/glucose with a ratio 9/5/1.

Table 1. Chemical composition of BSG.

Component	Composition of BSG (g/100 g) ^d
Cellulose	24
Hemicellulose ^a	15
Residual starch ^b	23
Others ^c	37

^aDetermined by acid hydrolysis.

^bDetermined by acid and enzymatic hydrolysis.

^cOther components may include lignin, ash, protein, lipid.

^dValues correspondent to the mass recovered from each 100 g of the original BSG.

Table 2. Sugar concentration and corresponding yield after acid hydrolysis of 50 g/L of BSG.

Reducing sugar	Concentration (g/L)	Yield (g/100 g BSG)
Arabinose	2.5	5
Xylose	4.5	9
Glucose ^a	12 ^c	48

^aGlucose from hemicellulose and starch hydrolysis.

3.2. Enzymatic hydrolysis of DIC-pretreated BSG

The cellulose enzymatic hydrolysis of untreated BSG was compared to the hydrolysis of five BSG treated by DIC at several processing pressures (2, 3, 4, 5 and 7 bar) during 15 min. The effect of pressure level on the time course of BSG hydrolysis by Celluclast and on the maximum glucose yield obtained after 18 h are presented in Figure 2 and 3, respectively. In figure 2 we can observe that the hydrolysis yield of BSG treated by DIC at different pressures was strongly improved compared to those obtained from untreated BSG. The kinetics of pretreated BSG at pressure levels of 5 and 7 bar are very similar, more rapid and the glucose yield (Figure 3) obtained at these two conditions is higher than those obtained at low pressure (2, 3 or 4 bar) for a same processing time. In spite of low DIC conditions (pressure or temperature equal or smaller than 4 bar and 143.6 °C, respectively) the enzymatic hydrolysis of the treated BSG biomass was improved, by comparison with the conditions of steam explosion technology, where the treatment is carried out at higher pressures (15 to 50 bar) and temperatures (180 to 250 °C). (Sassner et al., 2008 ; Taherzadeh and Karimi, 2007, Negro et al., 2003). These results suggest that the DIC pretreatment produced physico-chemical modifications in the structure of BSG. The pressure drop towards vacuum induces a sudden vaporisation of water, an intense cooling and mechanical modifications of the structure (Rezzoug et al., 2000). The product initially wetted and relatively hot, has viscoelastic behaviour. The generated steam into BSG acts mechanically on the material which has not yet lost its thermorheologic characteristics and creates mechanical constraints which generate an alveolation. The size and distribution of this alveolation depend on the operating conditions and the characteristics of the material (Maache-Rezzoug and Allaf, 2005). The mechanical effect of the DIC pretreatment has probably damaged the structure of cellulosic biomass to make cellulose more accessible to the enzymes attack that converts the carbohydrate polymers into sugars. Mosier et al. (2005) argued that pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its sub-microscopic chemical composition so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. Figure 3 indicates that under the best conditions (7 bar corresponding to 163 °C and 15 min), starting from 100 g of BSG, 24 g of glucose can be obtained in 18 h, corresponding to a hydrolysis yield near to 100 % (Table 1). For a higher temperature (220 °C), Ruiz et al. (2007) reported that the steam explosion pre-treatment improves enzymatic hydrolysis yields by four times compared to un-pretreated sunflower stalks (from 18 to 72%).

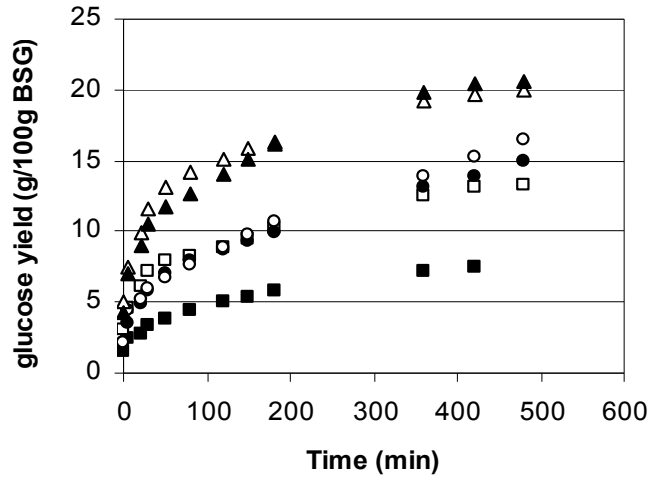


Figure 2. Kinetics of hydrolysis by Celluclast 1.5 L of BSG. Untreated BSG (dried at 50 °C at atmospheric pressure) (■); BSG DIC treated at 2 bar/15 min (□), 3 bar/15 min (●), 4 bar/15 min (○), 5 bar/15 min (△) and 7 bar/15 min (▲). Reactions were carried out with 10 g/L of BSG (HRE 40%) and 500 FPU/L of Celluclast 1.5L at 50°C in citrate phosphate buffer pH 5.

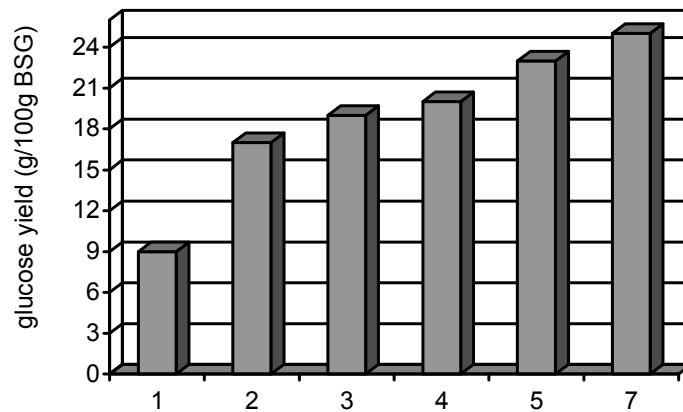


Figure 3. Effect of pressure on the final glucose yield for 18 h. Reactions were carried out with 10 g/L of BSG (HRE 40%) and 500 FPU/L of Celluclast 1.5 L at 50° C in citrate phosphate buffer pH 5. Untreated BSG (dried at 50°C and atmospheric pressure) (1), BSG DIC treated at 2 bar/15 min (2), 3 bar/15 min (3), 5 bar/15 min (5) and 7 bar/15 min (7).

3.3. Morphological structure of DIC treated samples

To verify effectiveness of DIC pretreatment in enhancing the hydrolysis yield scanning electronic microscopy was carried out to observe structural modifications and morphology of different BSG samples. We observe that the untreated BSG (dried at 50 °C and atmospheric pressure), exhibited rigid and highly ordered fibrils (Figure 4A). After a DIC treatment at 3 bar, the structure of the treated biomass is almost unchanged, but some cracks appear on the surface (Figure 4B). On the other hand, the material structure pretreated at 7 bar (Figure 4C) was strongly modified resulting in a structure very different from the two other. In this case the cellulose fibers were separated from the initial connected structure and fully exposed, thus

increasing the accessible surface area and the porosity. Consequently, these fibers were more susceptible to the enzymatic attack than those of the two other samples, justifying the better performance of the cellulose hydrolysis and the high glucose yield obtained at this pressure (Figure 3). Hu et al. (2008) reported that expansion at the end of pretreatment by steam explosion terminate the reaction and opens up the particulate structure of wood, improving the accessibility of cellulose fibrils to enzymes.

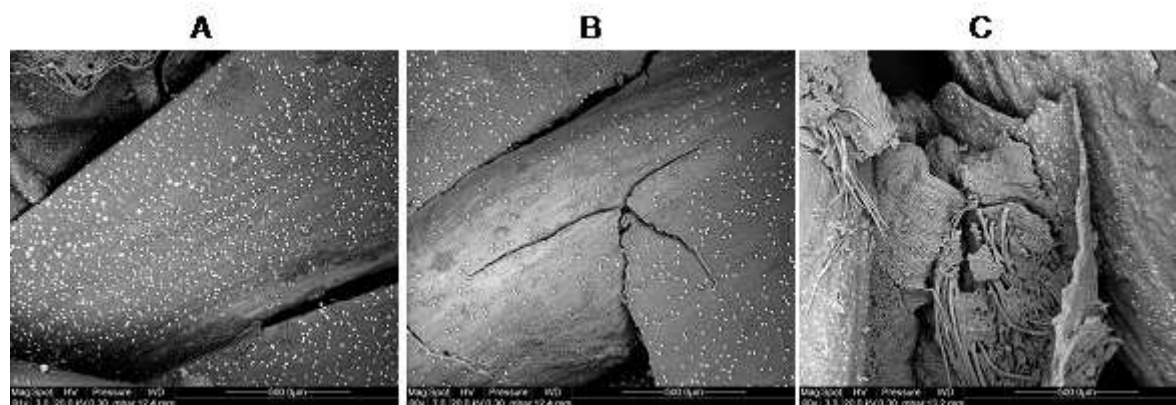


Figure 4. Scanning electronic microscopy (SEM) of untreated BSG (dried at 50°C and atmospheric pressure) (A), BSG treated by DIC at 3 bar (B) and BSG treated at 7 bar (C).

Conclusion

We can conclude that the proposed thermomechanical treatment improves enzymatic hydrolysis yields of BSG compared to that of untreated material. The pressure level has a great effect on hydrolysis efficiency. A significant increase of hydrolysis yields of BSG was observed under pretreatment conditions at low and intermediate pressure (from 2 to 4 bar) and strongest improvements were obtained with the more intensive conditions (5 and 7 bar). By considering the final glucose yield for 18 h, it increased from 9 g glucose/100 g BSG for untreated material to 24 g glucose/100 g BSG of pretreated BSG at 7 bar, corresponding to a hydrolysis yield close to 100 %. This improvement can be related with the SEM images which showed an important disruption of the structure of treated BSG at 7 bar compared to the structure of untreated sample.

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