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Evaluation of different pretreatment strategies for protein extraction from brewer's spent grains

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ABSTRACT

Brewer´s spent grain (BSG) is an agro-industrial residue rich in proteins, which correspond to approximately 18–30% (w/w) of its composition in a dry weight basis. In the present study, different pretreatment strategies including alkaline, acid, enzymatic and hydrothermal, and their combinations, were evaluated and compared with the aim of extracting protein from BSG. From the different evaluated options, one-step dilute acid pretreatment (11,400 mg H_2SO_4/g BSG, 121 °C for 1 h) was able to extract 90% of total protein present in BSG. Sequential alkaline and dilute acid pretreatment increased the protein extraction to 95%. Hydrothermal pretreatment at 60 °C resulted in a protein extraction of approx. 64–66%, which was lower than those achieved by the chemical methods but still relevant as it was obtained under non-optimized process conditions. In addition, the selectivity of the process for protein recovery was higher for hydrothermal pretreatment than for the chemical processes. Hydrothermal pretreatment was then found as an interesting option for protein extraction from BSG, being also advantageous in terms of costs (requires low temperature and no addition of chemicals), and more environmentally friendly.

1. Introduction

A growing global population combined with other factors such as emerging economies in developing countries and urbanization have increased the demand for protein. Currently available resources for protein are mainly food crops such as wheat, soybean, and maize. However, the current traditional production capacities will not be able to meet the growing demand estimated for protein (Aiking, 2011). An alternative to the traditional resources is to recover protein from lignocellulosic biomass, such as agricultural wastes and by-products derived from industrial processes (Day, 2013). Extraction of protein from such biomass materials would provide more protein to meet the future demand of this food component, and it would lead to optimal exploitation and valorization of biomass. A challenge for processing these types of resources is to develop technologies that can extract protein from biomass with high yields and cost competitiveness.

Brewer's spent grain (BSG) is the most abundant side-stream from brewing industry, with an approximate generation of 20 kg (wet form) per 100 l of beer produced, which leads to an average annual global production of ca. 39 million tons (Lynch et al., 2016; Mussatto et al., 2006). BSG contains a high amount of protein in its composition (18–30% w/w), which vary depending on the raw material, adjuncts and brewing process used by the industry. Protein content in BSG is much higher when compared to many agricultural residues and by-products. Therefore, in the recent years, attention has been given in using BSG to obtain protein. So far, alkaline or enzymatic methods have been studied for this purpose (Niemi et al., 2013; Forssell et al., 2008).

In the present study, different pretreatment methods including alkaline, acid, enzymatic, hydrothermal, and their combinations, were evaluated and compared with the aim of extracting protein from BSG. Then, additional efforts were done to improve the extraction results when using hydrothermal pretreatment, as this is a more sustainable process option. Selectivity, i.e., the recovery of protein with a minimum effect in the other fractions of the feedstock (especially carbohydrates and lignin), was an important factor considered to select the most suitable strategy for protein recovery from BSG.

2. Experimental

2.1. Materials and chemicals

BSG used in this study was kindly provided by Carlsberg Research Lab (Copenhagen, Denmark). The material was produced from a standard pilsner brew using 30% barley as raw material. As soon as provided, BSG was oven dried at 60 °C until a moisture content of approx. 5%, being then stored at −20 °C before use. This material was called as

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"raw BSG".

Defatted BSG was obtained by mixing dried BSG with n-hexane (0.24 g BSG/ml) and keeping the mixture at 37 °C, 200 rpm, for 1 h in a shaking incubator. Then, the hexane phase was removed by centrifugation (4500 × *g*, 10 min, 25 °C). The defatting procedure was repeated several times until the n-hexane phase was colorless. At the end, the slurry with hexane was filtered through Whatman® grade 1 filter paper (GE Healthcare) and the recovered solid material was dried at room temperature until constant mass and weighted. The amount of lipids in BSG (wt %) was calculated by weight difference before and after the defatting process.

Protease alcalase® 2.4 l (P4860) produced by *Bacillus licheniformis* was purchased from Sigma-Aldrich and stored at 4 °C until use. The protease activity, determined according to the Sigma non-specific protease activity assay protocol (Cupp-Enyard, 2008), corresponded to 74.15 U/ml enzyme (One unit (U) is the amount of hydrolyzed casein able to produce 1.0 µmol of tyrosine per minute at pH 7.5 and 37 °C).

Paraformaldehyde 4%, glutaraldehyde 1% in phosphate buffer pH 7.0, and Embedding Technovit® 7100 Kit were purchased from Electron Microscopy Sciences. Light green SF yellowish (C.I. 42095), Lugol's solution (diluted iodine-potassium iodide solution), and calcofluor white stain for microbiology were purchased from Sigma-Aldrich.

2.2. Pretreatment strategies for protein extraction

2.2.1. Water-alkaline-acid sequential pretreatment

For the 3-steps sequential pretreatment, defatted BSG was initially mixed with MilliQ water (1:20 w/v) and incubated at 25 °C, 250 rpm for 1.5 h. Then, the remaining solid was recovered by centrifugation (5000 × *g*, 10 min) and the liquid phase was stored for analysis. The solid material after water pretreatment was submitted to a two sequential 1-h alkaline extraction using 110 mM NaOH (1:20 w/v) at 50 °C and 200 rpm. Both protein-rich extracts were collected by centrifugation (5000 × *g*, 10 min) and mixed. The resulting alkaline supernatant was stored for further analysis. The remaining solid was washed with water until neutral pH, oven-dried at 60 °C until constant mass, and weighted. Finally, the remaining solid material was submitted to dilute acid pretreatment using 1 M H_2SO_4 (16,150 mg H_2SO_4/g BSG) at 25 °C, 250 rpm for 1 h, followed by autoclaving at 121 °C for 1 h. The remaining solid was separated from the liquid by filtration using a Whatman® grade 542 filter paper (GE Healthcare), washed with MilliQ water until neutral pH, oven dried at 60 °C until constant mass, and weighted; while the liquid phase was collected and stored for further analysis. These assays were performed at least in quintuplicate.

2.2.2. Alkaline-acid sequential pretreatment

The 2-steps sequential pretreatment was performed under the same conditions used for the alkaline and acid steps described in 2.2.1. The only difference from the previous strategy was the absence of the initial water pretreatment step under room temperature. These assays were performed at least in quintuplicate.

2.2.3. One-step dilute acid pretreatment

Two different procedures were evaluated for the one-step dilute acid pretreatment. In the first procedure, BSG (raw and defatted) was mixed with the acid solution (120 mg H_2SO_4 solution/g total liquid solution) in a liquid to solid ratio of 10 g/g (1200 mg H_2SO_4/g BSG), and the mixture was autoclaved at 120 °C for 27 min (Mussatto and Roberto, 2006). Then, the content was filtered in Whatman® grade 542 (GE Healthcare) filter paper. The liquid fraction was stored for analysis, while the solid was washed until neutral pH (6.5), dried at 60 °C until constant mass and weighted. This pretreatment was performed in duplicate to each sample.

In the second procedure, BSG (raw and defatted) was mixed with the acid solution (40 mg H_2SO_4 solution/g total liquid solution) in a liquid to solid ratio of 285 g/g (11,400 mg H_2SO_4/g BSG), and the mixture was autoclaved at 121 °C for 1 h (Goldschmid, 1971). Then, the content was filtered, collected and analyzed as mentioned in the previous acid procedure. This pretreatment was performed in triplicate to each sample.

Table 1
Conditions used for hydrothermal pretreatment of raw brewer's spent grain.

Temperature (°C)	Reaction time (h)	Incubation condition
30, 45, 60	1, 2, 4, 6, 8, 12,16,20, 24	Shaker incubator - 250 rpm
75, 90	1, 2, 4, 6	Shaker incubator - 200 rpm
105, 120, 135	0.25, 0.5, 1, 2	Autoclave

2.2.4. Hydrothermal pretreatment

Raw BSG was submitted to hydrothermal pretreatment under different conditions of solid to liquid ratio (6.67, 5, 4 and 2.5% w/v), temperature (from 30 up to 135 °C), for 1 up to 24 h (Table 1). Defatted BSG was submitted to hydrothermal pretreatment using a solid to liquid ratio of 2.5% w/v, at 60 °C for 1 and 24 h.

After reaction, the samples were cooled down to room temperature in a cold water bath. Then, the samples were filtered in Miracloth (Merck®) as membrane. The liquid fraction was frozen and stored for further analysis, while the remaining solid was freeze-dried. The dried solid sample was weighted and stored at −20 °C for further analysis. The experiments were carried out at least in triplicate.

2.2.5. Alkaline pretreatment with ammonium carbonate

Raw BSG was submitted to alkaline pretreatment using 10 mM ammonium carbonate solution under different solid to liquid ratios (6.67, 5, 4 and 2.5% w/v), at 60 °C for up to 24 h. Pressure plus GL 45 bottles with polybutylene terephthalate (PBT) red screw caps (Duran®) were used for these assays. The bottles were filled with BSG and the alkali solution, kept at 60 °C and stirred at 250 rpm. After 2, 8 and 24 h of reaction, samples were withdrawn and cooled down to room temperature using a cold water bath. The supernatant was collected by vacuum filtration using Miracloth (Merck®) as membrane. The liquid fraction was stored at −20 °C until further analysis, while the remaining solid was washed with MilliQ water until reached a neutral pH, freeze-dried, weighted and stored at −20 °C for further analysis. The experiments were carried out in duplicate.

2.2.6. Enzymatic pretreatment

Enzymatic pretreatment of BSG was performed at pHs 6.25 and 8.0. For the experiments, raw BSG was mixed with the reaction medium (MilliQ water, pH 6.25, or 10 mM ammonium carbonate solution, pH 8.0) in order to obtain different solid to liquid ratios (6.67, 5, 4 and 2.5% w/v). Then, 100 µl of alcalase® 2.4 l per g raw BSG (equivalent to 7.415 U/g raw BSG) was added to each reaction medium. The reactions were performed in pressure plus GL 45 bottles with PBT red screw caps (Duran®), which were kept in a shaker incubator at 60 °C, 250 rpm, for up to 24 h. After 2, 8 and 24 h of pretreatment, the enzyme activity was stopped by heating up the medium to 95 °C for 10 min, and subsequent cooling it down to room temperature in a cold water bath. The liquid fraction and the remaining solid were collected and treated as described in Section 2.2.5. The experiments were carried out in duplicate.

2.3. Analysis for determination of BSG chemical composition and structure

2.3.1. β-Glucan, total starch and lipids

β-Glucan and starch contents were determined by using the mixed linkage beta-glucan assay kit and the total starch (amyloglucosidase/α-amylase method) assay kit, according to the manufacturer's (Megazyme International Ltd.) instructions. Total lipids were determined as described in 2.1 (defatting process).