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Effect of Ohmic Heating Pretreatment on Enzyme Production by Solid-State Fermentation of Brewer's Spent Grain

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Abstract

Solid-state fermentation (SSF) involves the growth of microorganisms on solid substrates, mimicking natural environments of many species. Due to sustainability concerns, transforming agro-industrial by-products into value-added products through SSF has been increasingly studied. Brewer's spent grain (BSG), the main by-product of beer production, mostly consists of barley grain husks, making BSG a great support for microorganism cultivation. Although autoclaving remains the standard sterilization and pretreatment method of substrates, electric field technologies and their attendant ohmic heating (OH) have great potential as an alternative technology. In the present work, pretreatment of BSG by OH was explored in SSF with *Aspergillus niger* to produce commercially valuable enzymes. OH favored the solubilization of phenolic compounds, total protein, and reducing sugars significantly higher than autoclaving. SSF of treated BSG led to the production of lignocellulosic enzymes, with xylanases being the most active, reaching 540 U/g, a 1.5-fold increase in activity compared to autoclaved BSG. Protease activity was also improved 1.6-fold by OH, resulting in 49 U/g. Our findings suggest that OH treatment is an effective alternative to autoclaving and that its integration with SSF is a sustainable strategy to enhance by-product valorization through enzyme production with many industrial applications, according to circular economy guidelines.

Keywords: *Aspergillus niger*; brewer's spent grain; enzyme production; ohmic heating; solid-state fermentation

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1. Introduction

The application of enzymes in industrial settings, such as in textiles, pharmaceuticals, bioenergy, and food sectors, allows for the development of sustainable processing and methods to enhance the quality of the final product. The use of energy and raw materials, along with the heightened awareness of environmental issues associated with the use and disposal of chemicals in landfills, water, or air emissions during chemical processing, are the main reasons for the employment of enzymes [1–4]. Today, the application of enzymes in industry is mainly limited by cost-effectiveness, and therefore, the development of sustainable and affordable methodologies for enzyme production are of the utmost interest.

Enzyme production is currently performed by fermentation mostly of the submerged type. Solid-state fermentation (SSF) is an ecofriendly fermentation process since it requires the near absence of free water in a solid substrate mixture containing enough moisture

to support microbial life. When performing SSF with filamentous fungi in particular, its hyphae state allows for colonization of the spaces between substrate particles to reach and obtain the available nutrients, as it happens in their natural environment, therefore promoting productivity [5]. This is especially true for filamentous fungi such as *Aspergillus niger*, which naturally secrete a wide range of enzymes as part of their natural metabolism to obtain the nutrients needed from the substrate that supports its growth. Because of this, the cultivation of filamentous fungi has been widely explored and implemented for production of enzymes [6]. The reduced power consumption and amount of water needed for fermentation, along with the use of agrifood by-products as substrates for microbial growth, make SSF with filamentous fungi a sustainable method for enzyme production.

The higher need for resources such as water, food, and energy as a result of global population growth makes the implementation of sustainable methods such as SSF of the utmost importance [7] since it allows an eco-friendly and sustainable production of value-added products. Additionally, the increase in global population will intensify environmental pollution and the generation of agro-industrial by-products. These residues are usually disposed of, or, in some cases, used as low-value animal feed, which is the case for brewer's spent grain (BSG).

BSG represents the most abundant by-product of the beer brewing process, with an annual production of 39 million tons [8]. Due to its low commercial value, the industry and scientific communities have been exploring its potential to new applications. Since it largely consists of barley grain husks, its composition contains mainly hemicellulose, cellulose, lignin, and protein, making it a great support for microorganism cultivation. Previous studies have shown that SSF with *A. niger* CECT 2088 is a suitable biotechnological process for producing lignocellulolytic enzymes, with BSG being the agro-industrial by-product that generated the highest production of lignocellulolytic enzymes, mainly xylanase [9].

Although BSG is considered a valuable fermentation substrate on its own, the application of pretreatments can further improve the extraction of valuable compounds from BSG. Conventional chemical-based extraction methods are usually implemented to aid in the disruption of cell membranes and contents, improving their bio-accessibility. However, "greener" approaches have recently been explored to reduce the impact on the environment and increase safety, feasibility, and cost effectiveness after scale-up [10]. One of the alternatives to chemical extractions is the application of electric field technologies that can promote rapid heating due to the controllable ohmic heating (OH) effect. OH and autoclaving are two methods that can be implemented as substrate pretreatment to aid in nutrient solubilization before fermentation. OH involves passing an alternate electric current through a semi-conductive matrix, generating heat internally, while autoclaving uses high-pressure steam to achieve sterilization. According to Joule's law, the degree of heating is proportional to the matrix's intrinsic electrical resistance. Therefore, this quick transformation of electric energy into thermal energy offers a benefit that could hasten membrane disruption and solute solubilization from cell membranes, as well as promote fast and uniform volumetric heating [11]. OH technologies have been widely implemented in the food industry, such as maintaining fruit juice quality, ensuring microbial safety with limited effects on nutritional changes. This technique is especially promising in this industry since it is both energy efficient and environmentally friendly when compared to traditional conventional heating methods [12]. However, the heat produced during OH depends on the electrical conductivity of the material being heated. As a result, OH is not effective for materials that include non-polar substances or have low moisture content. Thus, the heating rate will differ for each material and must be individually assessed.

Despite this limitation, electric field technologies and the consequent application of OH have previously been highlighted as valuable tools for extraction due to their

ability to induce electro-permeabilization of the cell membrane by altering transmembrane potential. This may result in electro permeabilization or other kinds of permeabilization effects and cause the release of intracellular compounds [13,14], thus affecting microbial metabolism and fermentation outcomes [15]. Although autoclaving remains the most common pretreatment method used in fermentation processes to enhance the breakdown of biomass and induce sterilization, it is here postulated that OH could serve as an alternative to autoclaving due to its ability to reach and maintain high temperatures throughout the substrate and consequently deconstruct the substrate structures. Moreover, with the ohmic treatment, lower temperatures are usually needed compared to conventional hydrothermal treatments [16].

In the present study, *Aspergillus niger* was grown in solid-state BSG to assess the production of industrially valuable enzymes, namely endo-1,4-β-glucanase, amylase, β-glucosidase, xylanase, pectinase, and protease. Additionally, the impacts of substrate pretreatment by OH and autoclaving were compared. The crude extract obtained from the SSFs was used to obtain a highly active, concentrated enzymatic cocktail.

Therefore, the production of industrially important enzymes from filamentous fungus *Aspergillus niger* utilizing pretreated BSG as a substrate allows for a low-cost, eco-friendly solution for textile processing, among other applications.

2. Materials and Methods

2.1. Microorganism

Aspergillus niger CECT 2088 was obtained from the Colección Española de Cultivos Tipo (CECT, Valencia, Spain) and stored at −80 °C in an aqueous solution of 1% (w/v) peptone and 30% (v/v) glycerol. The fungal strain was revived in potato dextrose agar medium (potato extract 4 g/L, dextrose 20 g/L, agar 15 g/L) for 7 days at 25 °C.

2.2. Raw Materials

Brewer's spent grain (BSG) was supplied by LETRA craft brewery (Vila Verde, Portugal). The material was dried (<10% moisture) [9] at 60 °C for 24 h and ground to a 10 mm particle size using a Retsch SM 300 cutting mill (Retsch GmbH, Haan, Germany). Residual moisture content was determined using a PMB202 moisture analyzer (Adam Equipment, Oxford, UK). Dried BSG was stored in hermetic bags at room temperature.

2.3. Substrate Pretreatment and Inoculation

Brewer's spent grain (BSG) was pretreated by autoclaving or OH after adjusting the initial moisture to 75% (w/w) with a solution of 0.03% w/w sodium chloride in distilled water. The salt was added to increase water electrical conductivity to a value of 1 mS/cm. For these experiments, approximately 10 g (dry weight) of substrate were used. Autoclaving (Panasonic labo autoclave MLS-3020U-PE, Nijverheidsweg, The Netherlands) was carried out at 121 °C for 15 min inside 250 mL Erlenmeyer flasks (the usual conditions used for sterilization). OH was performed in a cylindrical polytetrafluoroethylene reactor with two stainless steel electrodes, defining a total volume of 45 mL as described elsewhere [17]. Treatment conditions were set to a frequency of 20 kHz under a constant electric field of 11 V/cm, which allowed for conduction of thermal treatments at 145 °C for 20 min. These conditions guaranteed the necessary sterilization conditions, but also allowed for the study of the advantage of using ultra-high temperature (>140 °C) to promote electro and thermal disruption of the material to enhance its functionalization for further steps. The chosen temperatures and residence times are known to promote the thermal breakdown of structural components such as hemicelluloses [18,19]. During OH treatments, the temperature was measured with a type-K thermocouple (Omega Engineering, Inc., Stamford, CT, USA),

placed at the geometric center of the sample volume for temperature measurement, with a precision of ±1 °C. This thermocouple was linked to a USB-9161 data logger (National Instruments Corporation, Austin, TX, USA) for continuous data acquisition.

BSG subjected to OH was transferred to a 250 mL Erlenmeyer flask in sterile conditions and followed inoculation. The temperature was monitored for both pretreatments. The treated substrates were inoculated with five evenly distributed mycelium agar disks (1 cm diameter) of *Aspergillus niger* CECT 2088, previously grown in potato dextrose agar plates.

All SSFs were incubated at 27 °C for 7 days in the dark which, according to Guimarães et al. [9], is the fermentation period that results in peak enzymatic activity, predominantly from xylanase.

2.4. Extraction

For soluble compound monitoring, an extraction was performed by adding water to the fermented substrate at the previously optimized proportion of 1 g dry solid to 10 mL water, followed by agitation (150 rpm) and centrifugation (822× *g*) at 4 °C. The supernatant was filtered by vacuum with a 2.5 µm pore-size filter. All the extracted samples were kept frozen at −20 °C and thawed at the time of use.

2.5. Enzymatic Assays

The activity of endo-1,4-*β*-glucanase, xylanase, amylase, and pectinase were quantified by the dinitrosalicylic acid (DNS) method [20], *β*-glucosidase was determined using 4-nitrophenyl-β-D-glucopyranoside, and protease was determined using azocasein. The absorbance reading for all the above-mentioned colorimetric methods were performed on a 96-well plate using the ThermoScientific MultiSkan Sky plate reader (ThermoScientific, Reinach, Switzerland). One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 µmol/min of product under the conditions of the assay. Each enzymatic activity was expressed in units per gram of dry BSG (U/g).

2.5.1. Endo-1,4-*β*-Glucanase

The activity of endo-1,4-*β*-glucanase was measured using carboxymethylcellulose (CMC) as substrate as follows: 250 µL of 2% (w/v) carboxymethylcellulose in citrate buffer 0.05 M, pH 5, was incubated with the same volume of the extracted sample at 50 °C for 30 min. Released glucose, the only reducing sugar liberated from the CMC hydrolysis, was quantified by the addition of 500 µL of DNS reagent, and following the same procedure as described in analytical methods. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 µmol/min of glucose under the conditions of the assay.

2.5.2. Xylanase

The procedure to determine xylanase activity was similar to the one described for endo-1,4-*β*-glucanase activity but using xylan (Beechwood, Megazyme, main chain glycosidic linkage β-1,4 and *α*-1,2) 1% (w/v) as a substrate. Released xylose, the only reducing sugar released from xylan hydrolysis, was also quantified by the DNS method (as described for endo-1,4-*β*-glucanase activity). One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol/min of xylose under the assay conditions.

2.5.3. Amylase

Amylase activity was quantified using starch as a substrate. Briefly, 250 µL of 2% (w/v) starch substrate in sodium acetate buffer 0.05 M, pH 4.8, was incubated with 250 µL of the extracted sample at 40 °C for 30 min. The DNS method was used to quantify the released maltose (as described for endo-1,4-*β*-glucanase activity). One unit of enzymatic

activity was defined as the amount of enzymes required to release 1 µmol/min of maltose under the assay conditions.

2.5.4. Pectinase

Pectinase activity was quantified using 1% (w/v) pectin as a substrate, applying the same protocol as the one described for amylase. The DNS method was used to quantify the released D-galacturonic acid (as described for endo-1,4-*β*-glucanase activity). One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol/min of D-galacturonic acid under the assay conditions.

2.5.5. *β*-Glucosidase

The activity of *β*-glucosidase was determined by incubating 100 µL of the substrate, 4 mM 4-nitrophenyl-β-D-glucopyranoside, in 0.05 M citrate buffer, pH 5, with 100 µL of the extracted sample at 50 °C for 15 min. To stop the reaction, 0.6 mL of sodium carbonate 1 M was added, following the addition of 1.7 mL of distilled water. The mixture's absorbance was then at 400 nm. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 µmol/min of *p*-nitrophenol under the assay conditions.

2.5.6. Protease

Protease activity was determined by using azocasein as a substrate. An amount of 500 µL of 0.5% (w/w) azocasein in sodium acetate buffer 0.05 M, pH 4.8, was incubated with 500 µL of the extracted sample at 37 °C for 40 min in the dark. The reaction was then stopped by adding 1 mL of 10% trichloroacetic acid and centrifuged 0.6× *g* for 15 min. The supernatant was transferred to glass tubes and 1 mL 5 M potassium hydroxide and 1 mL water were added. The mixture's absorbance was then at 428 nm. One unit of enzyme activity was defined as the amount of enzyme that causes an increase of 0.01 absorbance comparatively to the blank (supernatant was replaced by sodium acetate buffer 0.05 M) per minute under assay conditions [21].

2.6. Analytical Methods

Reducing sugars in the extracts were quantified by the DNS method mixing 500 µL of extract with 500 µL of DNS and placing the mixture in a water bath at 100 °C for 5 min, followed by the addition of 1 mL of ultra-pure water before reading the absorbance at 540 nm.

Soluble protein quantification was performed using the Bradford method [22]. Briefly, 5 µL of sample were mixed with 200 µL of Coomassie blue reagent, mixed thoroughly and incubated for 10 min at room temperature in the dark. For less protein-rich samples, 150 µL of sample were mixed with 150 µL of Coomassie blue reagent instead. The final solutions were shaken for 30 s, and the absorbance was read at 595 nm and converted to concentration using bovine serum albumin as standard.

Total phenolic concentration was determined using the Folin–Ciocalteu method (Commission Regulation (EEC) No. 2676/90). For this, 10 µL of sample were mixed with 200 µL of sodium carbonate 15% (w/v) solution, 50 µL of Folin reagent and 740 µL of ultra-pure water and incubated at 50 °C for 10 min. Absorbance of the final incubated mixture was performed at 700 nm and converted to a concentration of caffeic acid.

Antioxidant activity was quantified by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by Dulf [23] and adapted by Estevão-Rodrigues et al. [24], and expressed in mg of Trolox equivalents per gram of dry substrate by absorbance reading at 517 nm.

The substrate used in this study has been previously characterized regarding its ashes, protein, total lipids, Klason lignin, cellulose, and hemicellulose by Guimarães et al. [9].

2.7. Scanning Electron Microscopy

Substrates were observed by scanning electron microscopy (SEM) before and after pretreatment using the Hitachi FlexSEM1000 (Hitachi, Tokyo, Japan). Additional details regarding image capture are described in the respective figures. No coating was applied in any of the observed samples.

2.8. Statistical Analysis

In general, three replicates were performed for all the analyses. Results were analyzed by *t*-test (two-sample assuming unequal variance) for phenolic compounds, reducing sugars, soluble protein, and antioxidant activity, or by one-way ANOVA for enzyme activity, applying the Tukey multiple comparisons test ($p < 0.05$).

3. Results and Discussion

3.1. BSG Pretretatment

BSG was pretreated by OH or autoclaving according to the conditions that led to different temperature profile exposures (Figure 1). OH conditions allow for not only the necessary sterilization conditions, but also for the use of ultra-high temperatures (>140 °C) that may promote electro and thermal disruption of BSG.

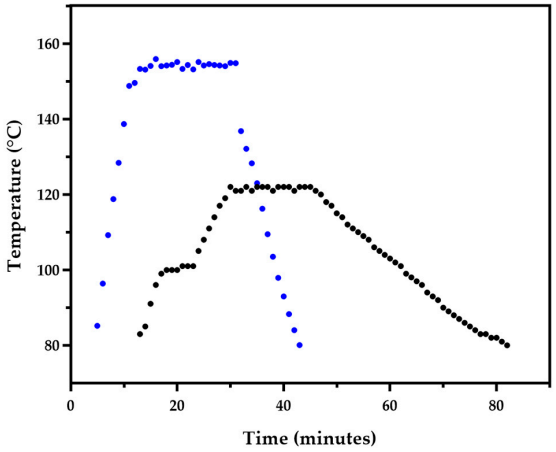


Figure 1. Thermic profile of OH (●) and autoclave (●) pretreatments.

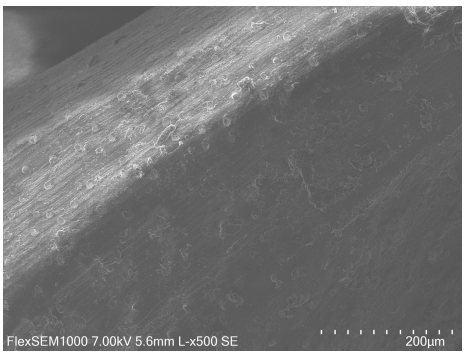
Moreover, OH allowed for the ability to reach a stable ultra-high temperature very fast, reaching 120 °C after 8 min from ambient temperature, and 140 °C after 10 min, while autoclaving took 29 min to attain 121 °C. Additionally, the rate of cooling down of OH is higher than the autoclave. Thus, OH represents an overall faster method for substrate pretreatment than the standard autoclaving; therefore, using OH as pretreatment at industrial settings may represent significant productivity improvement and power consumption savings. In this study, OH required an energy input of 0.21 kWh/kg ± 0.02 kWh/kg (calculated from voltage and current data recorded during the OH treatments) to reach the target temperature up to 150 °C, corresponding to an estimated energy efficiency ranging from 64% to 78% during the heating-up phase. This efficiency was determined by comparing the total electrical energy consumed with the theoretical thermal energy estimated using the classical heat transfer equation, $Q = m \cdot cp \cdot \Delta T$ (where Q is the energy absorbed or released in Joules, cp represents specific heat of substance in °C, m is the mass of substance in grams, and ΔT corresponds to the change in temperature in °C). Assuming a wet sample mass of 50 g and a specific heat capacity of 4184 J/(kg·K) (which reflects the high humidity of the sample), the theoretical energy required to raise the temperature to 140 °C was calculated to be approximately 27 kJ, which corresponds to 0.15 kWh/kg.

This level of energy efficiency is consistent with reported values for industrial autoclaves, which typically can range from 68% to 75% [25]. Notably, the energy efficiency reported in this study was achieved using a non-insulated laboratory-scale OH system, suggesting that higher efficiencies can be expected under industrial or continuous processing conditions with improved insulation and system design. However, the transition from laboratory-scale experiments to industrial applications requires thorough validation of both technological performance and economic viability. It is worth noting that several industrial-scale ohmic heating systems are already in operation for the continuous pasteurization and sterilization of commercial food products, which greatly facilitate the scale-up and industrial adoption of this technology [12].

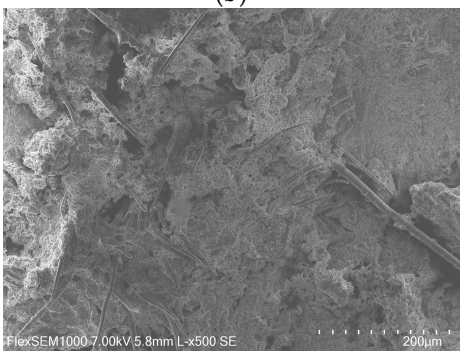
The substrate was observed with SEM in its three different states: original (no treatment) (Figure 2a), after autoclaving (Figure 2b), and after the application of OH (Figure 2c). In its original form, the vegetal tissues that constitute BSG form very organized, stable structures. After autoclaving, these structures seem to keep their integrity, showing visibly uniform texture. However, after the application of electric current in the previously mentioned conditions, the vegetal structures appear more disintegrated and show more exposed fibers.



(**a**)



(**b**)



(**c**)

Figure 2. Comparison of original BSG structure after pretreatment with OH and autoclaving. (**a**) BSG without pretreatment; (**b**) BSG pretreated by autoclaving; and (**c**) BSG pretreated by OH.

These observations go according to the previously mentioned studies that conclude that the application of electric fields can cause disruption of the cells that form the matrix or tissue. This disintegration of cellular structure, along with the heat generated internally, causes visible changes in the overall structure of BSG. Therefore, this sustains the hypothesis that OH could lead to a more effective release of valuable-added compounds present in BSG that could consequently improve the growth and metabolism of *Aspergillus niger*.

3.2. Substrate Characterization Before and After Fermentation

Phenolic compounds, antioxidant activity, reducing sugars, and soluble protein were quantified before and after pretreatment. Additionally, the same analyses were conducted after SSF with both pretreatments (Figure 3). SSF efficiency can be affected by the chemical composition of solid substrates, which can influence microbial growth and enzyme activity. For instance, high cellulose and hemicellulose concentrations may induce the production of lignocellulolytic enzymes, while the protein content can promote the synthesis of proteolytic enzymes [26].

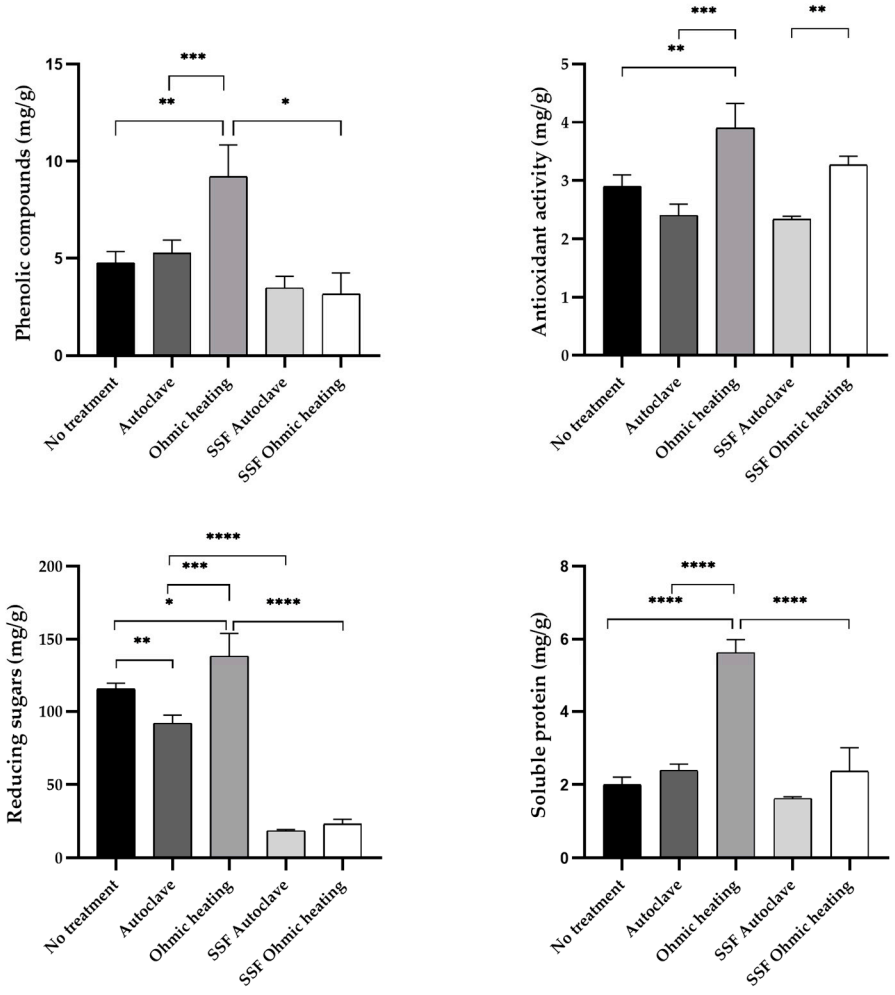


Figure 3. Characterization of aqueous extracts of untreated BSG after pretreatment and after SSF. All values are expressed as mg per g of dry BSG (mg/g). A *p*-value below 0.05 was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

Before fermentation, pretreatment of BSG by OH significantly increased phenolic compounds, antioxidant activity, reducing sugars, and soluble protein concentration in the aqueous extracts, compared to the extracts obtained in BSG without any treatment (Figure 3). In contrast, autoclaving the substrate did not cause a significant increase in any

of the monitored parameters. OH increased the concentration of phenolic compounds and antioxidant activity by 42.7% and 38.5%, respectively, compared to autoclaving. Regarding the liberation of reducing sugars, there is a slight decrease in autoclaved substrates, while OH increased their concentration, resulting in a difference of 33.6% between the two pretreatments. The increase in soluble protein content by OH is the most accentuated, resulting in more than double the concentration in the OH-treated BSG compared to the untreated substrate.

Overall, by comparing OH and autoclaving pretreatments, OH increased the release of soluble compounds in all cases, with significant differences to autoclaving for all analyses. Thus, the application of electric current and consequent induction of OH significantly improves the release of phenolic compounds, reducing sugars and soluble protein, and seems to be a better alternative to autoclaving for the release of these molecules.

For autoclaved substrates, there are no significant differences in either phenolic compound concentration, antioxidant activity, or soluble protein concentration, between the fermented and unfermented BSG (Figure 3). However, contrary to autoclaving, pretreating the substrate by OH leads to significant decrease in soluble proteins and phenolic compounds after fermentation, suggesting that the increased release of proteins and phenolics from BGS increased their bioavailability and could therefore benefit the growth of the fungus. In these conditions, these compounds could be taken up and metabolized by *A. niger* overtime or even liberated from the solid by the action of the enzymes secreted by *A. niger* [16–18]. For both pretreatments, a significant decrease in reducing sugar concentration is observed after fermentation, as expected, since the fungus could use the sugars solubilized by both pretreatment methods.

Additionally, it seems that OH improved the consumption of soluble proteins and phenolic compounds during fermentation since these compounds were at the highest concentrations before fermentation due to OH treatment. These results go accordingly with the previous observations by SEM (Figure 2), where the application of OH caused visible changes and apparent modification of the structure of BSG, suggesting increased solubilization of fiber compounds. This modification supports the hypothesis that OH causes the deconstruction of the matrix, inducing the solubilization of intracellular compounds. These aspects are especially relevant since differences in solubilization and increased bioavailability of these molecules could interfere with microbial growth and consequent enzymatic production from *A. niger.*

Crude extracts rich in phenolics have a wide range of industrial applications, such as in cosmetics, health [27], and, particularly, in the food industry, since they improve the quality and nutritional value of food [28]. In plant tissues such as BSG, phenolic compounds occur in free, esterified, and insoluble-bounded forms [29], which have low bioavailability. Therefore, there is a need to select suitable processes to increase the bioavailability of phenolics by facilitating their release [30]. As previously mentioned, pretreatment of the substrate by OH led to an increase in the release of these compounds, compared to the standard autoclaving (Figure 3). This reflects the potential application of OH not only for enhanced enzymatic production through SSF, but also as a tool for obtaining phenolic-rich extracts for other industrial applications.

It is currently unclear how phenolic compounds affect the growth and metabolism of microorganisms, and, in return, how different microorganisms affect total phenolic concentration during fermentation. In a study conducted by Ma et al. [31] on the fermentation of tea leaves by *A. niger*, it was hypothesized that *Aspergillus* plays an important role in the production of enzymes which catalyze hydrolysis, oxidization, conversion, and biodegradation of phenolic compounds, causing variations in phenolic compounds present in the substrate. Additionally, the total phenolic concentration seems to increase

or decrease depending on the microorganism strains [32]. In contrast to our results, Dulf et al. demonstrated that the total phenolic concentration increased by >21% for SSF with *A. niger* ATCC-6275 [33]. This further supports the hypothesis that different strains of *A. niger* could vary the outcome of phenolic compound degradation. Additional studies need to be conducted to infer the impact of different strains and substrate profiles on phenolic degradation and fermentation outcomes.

Regarding protein concentration, although complex nitrogen sources are usually used for enzymatic production, the requirement for a specific nitrogen supplement differs from organism to organism. Thus, the implementation of substrate pretreatment by OH could further enhance protein bioavailability in agricultural by-products, making them more suitable in industrial settings, such as in enzyme production, without need for further supplementation.

3.3. Enzymatic Activity After Fermentation

After SSF with pretreated BSG, the enzymatic activity of different types of lignocellulolytic enzymes (namely endo-1,4-β-glucanase, β-glucosidase, and xylanase), amylase, protease, and pectinase present in the aqueous extracts were quantified (Figure 4). Although the results show a slight but significant decrease in endo-1,4-β-glucanase and amylase activity, no significant differences were found in β-glucosidase and pectinase activities. Alternatively, a significant increase of 31% and 37% in xylanase and protease activities, respectively, was obtained in extracts of BSG treated by OH compared to autoclaved and fermented BSG. Xylanase was the enzyme with the highest values of activity, reaching around 540 U/g in SSF of OH-treated BSG. This increase in xylanase production may be related to the increase in hemicellulose exposure to the fungus in BSG treated by OH, as shown by the structural changes of the solid matrix (Figure 2). The application of OH also enhanced protease activity, resulting in the highest value of 49 U/g. Additionally, increased bioavailability of proteins, phenolic compounds, and reducing sugars induced by OH (Figure 3) at the beginning of fermentation, may contribute to faster fungal growth and metabolism at the initial time of fermentation and consequently improve enzyme secretion.

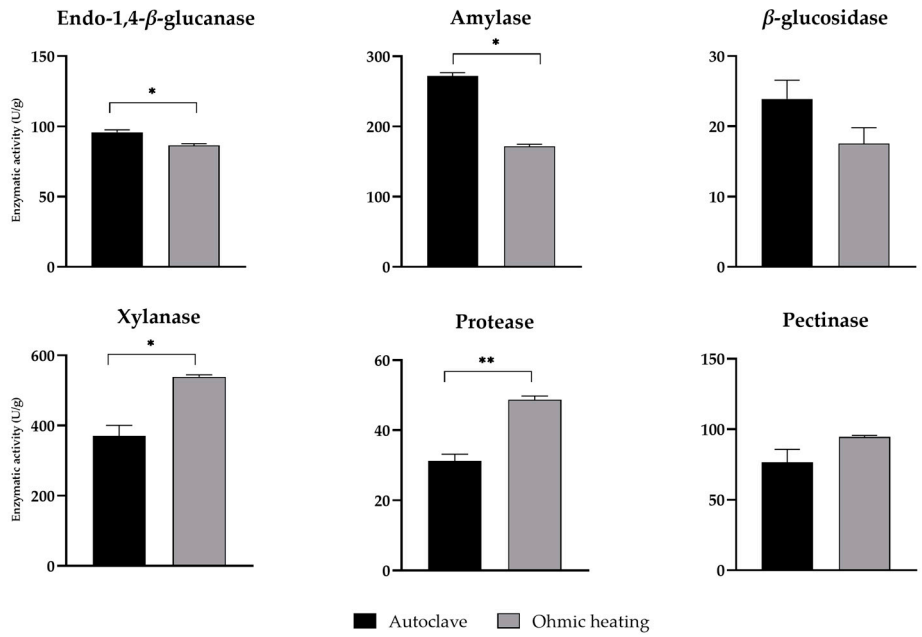


Figure 4. Endo-1,4-β-glucanase, amylase, β-glucosidase, xylanase, protease, and pectinase activities in crude extracts obtained from SSFs with BSG pretreated by autoclaving or OH. Enzymatic activity is expressed in units per gram of dry substrate (U/g). A p-value below 0.05 was considered statistically significant (* $p < 0.05$, ** $p < 0.01$).

Proteases are one of the largest groups of industrial enzymes, accounting for 60% of total global enzyme sales [34,35]. This group of enzymes has been applied in several industries, from detergents to leather processing, pharmacology, and others [36]. Alongside proteases, xylanases have great potential for industrial applications, such as in the textile, pulp, and paper industry, food processing, and lignocellulosic biomass saccharification, among others [36,37].

Xylanase is the most active enzyme produced by *A. niger* in various studies [6,35], thus being considered the most relevant enzyme in the enzymatic cocktail obtained. Hydrothermal treatments such as autoclave affect mostly the hemicellulose fraction of the lignocellulosic biomass [38]; thus, it is expected that xylanase is the most induced enzyme in hydrothermally treated biomass. The other enzymes act in other polysaccharides that are not so affected by hydrothermal treatment. Thus, the impact of OH on xylanase is related to the efficacy of pretreatment.

Due to the wide range of sectors in which enzymes are used, the market for these enzymes has grown significantly in recent years. With lignocellulolytic enzymes contributing to more than 20% of total revenue, the industrial enzyme market is projected to reach USD 8.7 billion in 2026 [39]. The market for enzymes is anticipated to rise further due to rising demand from sectors such as paper, leather, textiles, and biodiesel [40]. Given the rising market projections and demand, and the need to employ more sustainable approaches to enzyme production, the use of inexpensive substrates and minimization of water and energy consumption while simultaneously achieving high yields is imperative.

Therefore, the use of BSG is a sustainable and economic substrate for production of enzymes. For the case of xylanase and protease activity, this is further improved by the application of electrical fields as pretreatment. The ability to produce enzymes of high commercial interest such as xylanases and proteases using low-cost, eco-friendly substrates (such as BSG), and methods (SSF, for instance) is extremely important to meet the societal demands in an environmentally endangered planet. Further enhancing this enzymatic production with "greener" alternatives to the commonly used chemical approaches, such as OH, becomes a viable strategy for working around emerging issues.

4. Conclusions

While previous studies already highlighted the potential of BSG for lignocellulosic enzyme production through SSF, this study demonstrated the potential of using OH as a tool for further improving substrate composition, and consequently, enzymatic production. In contrast to conventional autoclaving, OH increased the deconstruction of the matrix's structures and enhanced the release and microbial up-take of substrate components. This is further reflected in enzymes production, where xylanase and protease were the most improved by OH pretreatment.

Our findings suggest that OH is an energy efficient and environmentally friendly strategy to enhance the production of enzymes of significant industrial interest, as it can reduce water consumption. Additionally, this method allows for potential valorization of agro-industrial by-products and thus leads to the reduction in their environmental impacts, contributing to a circular economy. To the best of our knowledge, the use of OH in the context of SSF and enzyme production is yet to be explored, especially regarding OH *versus* autoclaving, highlighting the novelty of this study and contributing towards alternative and sustainable practices.

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Abbreviations

The following abbreviations are used in this manuscript:

SSF	Solid-state fermentation
BSG	Brewer's spent grain
OH	Ohmic heating
CECT	Coleción Española de Cultivos Tipo
CMC	Carboxymethylcellulose
HPLC	High-performance liquid chromatography
DNS	Dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
SEM	Scanning electron microscopy

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