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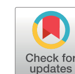
Bioprocessing of brewers' spent grain for production of xylanopectinolytic enzymes by *Mucor* sp.



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ABSTRACT

The potential of microwave and ultrasound was evaluated for the pretreatment of brewer's spent grain (BSG). Under optimal conditions of microwave and ultrasound pretreatments, reducing sugar yields per 1 g of pretreated BSG were 64.4 ± 7 mg and 39.9 ± 6 mg, respectively. Subsequently, the pretreated BSG was evaluated as a substrate for production of Xylanopectinolytic enzymes using fungi isolated from spoiled fruits. Out of twenty-nine (29) isolates recovered, *Mucor* sp. (AB1) isolated from Bramley apple (*Malus domestica*) produced xylanopectinolytic enzymes with higher specific activity, and was selected for further studies. The highest enzyme activity (137 U/g, and 67 U/g BSG, for pectinase and xylanase, respectively) was achieved in a medium that contained 15 g of BSG, at pH 6, temperature of 30 °C, supplemented with 1% xylan or pectin for inducing the production of xylanase or pectinase, respectively. The partially purified xylanopectinolytic enzymes were optimally active at 60 °C and pH 5.

1. Introduction

Globally, beer is the primary consumed alcoholic beverage, and the fifth most consumed beverage overall. Brewer's spent grain (BSG) is the solid barley waste produced during mashing of malted barley, and is separated from the wort in beer brewing. The average global by-production of BSG is 39 million tonnes annually, with approximately 3.4 million tonnes of BSG produced annually in Europe alone (Lynch et al., 2016). However, the BSG has a high moisture content (up to 80%) and polysaccharides (40–50% on dry weight basis) which make it susceptible to microbial growth and spoilage after few days of storage. Thus, it is used instantly as an animal feed or is disposed in landfill sites.

BSG holds potential as a low-cost feedstock option that is rich in sugars for biorefinery applications (Hassan et al., 2019a). However, achieving enzymatic hydrolysis of sugars in BSG requires energy-intensive pretreatment process to fractionate the lignocelluosic complex matrix and improve digestibility (Hassan et al., 2019b). Interestingly, both ultrasound and microwave have potential as energy efficient green technologies for pretreatment of lignocelluosic biomass prior to further conversions (Hassan et al., 2018).

Microwave-assisted pretreatment of lignocellulose has been reported to be a time and energy efficient process, that does not lead to generation of inhibitors or superficial overheating of biomass (Aguilar-Reynosa et al., 2017). The efficiency of microwave-assisted pretreatment of lignocellulose may be attributed to the energy absorbance properties of such carbon materials, and the heating properties of the microwave electromagnetic fields. Ultrasound technology has also been investigated for pretreatment of lignocellulose, with advantages of reduction in duration of treatment and chemicals required. Cavitation bubbles are formed during the ultrasound-assisted pretreatment that leads to fractionation of lignocellulose, and thus, increase in the accessibility to hydrolytic enzymes. Therefore, this study aims to compare the effectiveness of microwave and ultrasound-assisted pretreatments of BSG for further use as a microbial growth substrate. Microwave-assisted pretreatment of BSG was carried out at varying power levels (100, 250, 440, 600, and 1000 W) for 30, 60, and 90 s. Additionally, BSG was pretreated using different ultrasound frequencies (25, 35, 45, 130, and 950 kHz) for 20, 40, and 60 min. The pretreated BSG was then incorporated into fermentation media used for production of microbial enzymes.

This study focused on production of xylanopectinolytic enzymes by fungi, as fungi represents the main producers of the most industrial lignocellulose-degrading enzymes. Hence, the pretreated BSG samples were subjected to fermentation by fungi isolated from spoiled fruits. Although no studies have been carried out on fermentation of BSG for production of pectinase, few researchers such as Knob et al. (2013) has

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investigated the production of xylanase by fermentation of BSG. Therefore, this study aims to investigate the effectiveness of pretreated BSG as a growth substrate in the production of xylanopectinolytic enzymes by fungi.

2. Materials and methods

2.1. Materials

Brewer's spent grain (BSG) was generously donated by a local brewery in Dublin, Ireland. Once received, BSG was dried at 60 °C for 48 h, then milled, and sorted using a 350-μm sieve. It was then maintained at ambient temperature in a dry place for further experiments. All chemicals required for experimentation were purchased from Sigma Aldrich (Ireland), and were of analytical grade. Beechwood xylan and pectin from citrus peel were used to assay xylanase and pectinase activities, respectively. The cellulase from *Trichoderma reesei* (aqueous solution, ≥700 units/g) showed 77 FPU/ml when assessed by a protocol developed by National Renewable Energy Laboratory (Adney and Baker, 1996). Hemicellulase was from *Aspergillus niger* (powder, 0.3–3.0 unit/mg solid). A hemicellulase stock solution at a concentration of 10 g/l was prepared by dissolving hemicellulose powder in sodium acetate buffer (pH 4.8, 500 mM), and then the protocol developed by Rickard and Laughlin (1980) was used to assay activity (72 U/ ml).

2.2. Methods

2.2.1. Pretreatment and characterization of substrate (BSG)

2.2.1.1. Microwave pretreatment. A domestic microwave oven with a frequency setting at 50 Hz and adjustable power outputs was used. Fixed solid loading experiments were carried out by immersing five grams of biomass in 50 ml of deionized water in a 200-mL flask. The flask was placed at the center of a rotating circular plate in the microwave oven. Pretreatments were carried out at 100, 250, 400 and 600 W, for 30, 60, and 90 s. The mixture was then centrifuged, oven-dried, and the biomass stored for further analysis.

2.2.1.2. Ultrasound pretreatment. Three different ultrasound baths were used: (a) a multi-frequency ultrasonic bath with selectable frequencies of 25 and 45 kHz (Elma Schmidbauer, Transonic TI-H-10, output 550 W), (b) a multi-frequency ultrasonic bath with selectable frequencies of 35 and 130 kHz (Fisher Bio-block Scientific, Transonic TI-H-10, output 750 W), and (c) an ultrasonic tank with a frequency of 950 kHz (Kaijo, Quava Mini Ultrasonic System, output 100 W). Fixed solid loading experiments were carried out by immersing five grams of biomass in 50 ml of deionized water in a 200-mL flask. Pretreatments were carried out at 25, 35, 45,130 and 950 kHz for 20, 40, and 60 min. The mixture then centrifuged, oven-dried and the biomass stored for further analysis.

2.2.1.3. Total reducing sugar analysis. Prior to the sugar analysis, enzymatic hydrolysis was carried out to facilitate the release of sugars from the pretreated samples as follows: a total of 10 ml mixture of 1 g of BSG, 158.76 µl of cellulose (77 FPU/ml), and 153.3 µl hemicellulase (72 U/ ml) in distilled water at pH 5.4 were mixed and maintained at 50 °C for 5 days (Ravindran et al., 2018). Afterwards, the hydrolysate liquors were collected, and the reducing sugar concentration was estimated by the dinitrosalicylic acid (DNS) method (Miller, 1959). Analysis of variance (ANOVA) and multiple comparisons (Fischer's least significant difference test) were applied to determine any significant differences using STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA). Values of $P < 0.05$ were considered as significant. Pretreatment conditions that resulted in the highest total reducing sugars were used for pretreating BSG prior to being used as substrate for production of enzymes.

2.2.1.4. FTIR analysis. FTIR analysis was carried out to investigate any chemical and structural changes in the BSG by assessing possible variations in functional groups after pretreatments, compared with native BSG. A Perkin Elmer Spectrum GX FTIR (UATR) Microscope (USA) was used, and the FTIR spectra were recorded from 4000 to 400 cm^{-1}, with 32 scans at a resolution of 0.3 cm^{-1} in transmission mode (Raghavi et al., 2016). The observed FTIR peaks at different wave numbers (cm^{-1}) were assigned to the corresponding functional groups of lignocellulose according to data from the literature.

2.2.2. Isolation and screening of xylanopectinolytic enzyme-producing fungi

2.2.2.1. Isolation of fruit-rotting fungi. Samples of nineteen different spoiled fruits (Pome, Stone, and Soft fruits) were randomly collected in January and February 2019 from a commercial pack-house facility in Dublin, Ireland. The type of fruits collected was dependent upon availability; fruits (and varieties) namely: apple (Bramley, and Gala), blackberry (Tupi), blueberry (Emerald, and Duke), grape (Crimson, Melody, Tawny, Sugraone, and Sugarthirteen, Starlight), raspberry (Kweli, Imara, and Framboises), strawberry (Calinda, Rociera, Sensation, and Marquis), and cherry (Regina). The fruits were collected in separate sterile polythene bags and transferred to the laboratory for the study. Suspected fungal growth visible on the fruits was aseptically transferred to potato dextrose agar (PDA) plates and incubated at 25 °C until fungal growth developed on the medium surface. Isolates were serially sub-cultured until pure and transferred to PDA slants for storage at 5 °C. Isolates were identified to genus level based on macroscopic appearance and microscopic characteristics under 40× objective lens of a light microscope (after staining with a lactophenol cotton blue dye), and compared with a standard mycological atlas (Watanabe, 2010).

2.2.2.2. Qualitative screening of xylanopectinolytic enzyme-producing fungi. The presence of xylanases and pectinases was detected as described earlier (Félix et al., 2016) differential, substrate-containing media. Briefly, the various substrates [0.5% (*w/v*) xylan, and 0.5% (w/v) pectin, respectively] were independently added to a medium containing 0.5% (w/v) malt extract and 1.5% (w/v) agar. Each isolate was inoculated individually on enzyme assay plates and incubated at 25 °C for 7 days. The activities were detected by the formation of a halo around the mycelium after flooding the plates with Congo red (1.0 mg/ml) for 15 min, discarding the solution, and washing cultures with 1.0 M NaCl. The appearance of hydrolysis zones (halo) around the fungal growth was observed against the red background and considered as positive result. All fungal isolates that showed a positive result were further assessed for their enzymatic activities via a quantitative assay.

2.2.2.3. Quantitative determination of xylanopectinolytic enzymes produced by Fungi. Following qualitative screening, the fungal isolates were further screened for yield potential by quantitative determination in submerged fermentation using basal liquid media (Mandels and Weber, 1969) supplemented with 1.0% (*w/v*) of either xylan or pectin for determination of xylanase and pectinase, respectively (Deep et al., 2014). For each experiment, 50 ml of basal liquid media was poured in 250 ml Erlenmeyer conical flasks and then was autoclaved at 15 psi and 121 °C for 20 min. After cooling, each flask was inoculated with a PDA agar plug of growth (0.5 cm) which had previously been cultivated for 7-days. Flasks were incubated in a rotary incubator (150 rpm) at 30 °C for seven days. After incubation, the supernatants were collected separately by filtration using Whatman filter paper (No.1) followed by centrifugation at 10,000 rpm at 4 °C for 10 min. The supernatants were used for protein estimation and enzyme activity assay as crude enzyme.

Table 1
Effect of microwave power on sugar yield from BSG.

Time (s)	Reducing Sugar Yield (mg/g of biomass) at different microwave power (W)									
	100		250		440		600		1000	
	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
30	24.4	0.2[a]	27.8	8.5[ab]	37.2	1.2[bc]	42.1	3.7[cd]	50.9	0.7[d]
60	37.9	1.2[a]	41.4	8.2[b]	46.4	1.0[bc]	64.4	6.3[bc]	51.2	1.6[c]
90	38.2	0.2[a]	41.3	0.7[b]	46.3	4.7[bc]	64.4	7.0[bc]	51.1	2.1[c]

Where reducing sugar yields for native BSG = 24.5 ± 0.3 mg/g of biomass.
Means not sharing the same letter are significantly different (LSD) at $P < 0.05$ probability level for respective microwave power levels.

2.2.3. Production of xylanopectinolytic enzymes using BSG under different conditions

The best xylan and pectin degrader among all the fungi tested was selected for solid-state fermentation (SSF) using BSG as a fermentation medium. Pretreated BSG was used as the main carbon source (5.0 g), moisturized (77.5%) with basal medium (Mandels and Weber, 1969) as described by Gautam et al. (2018) and supplemented with 1 0.0% xylan or pectin for inducing the production of xylanase or pectinase, respectively. The approach of "one-factor-at-one-time" was employed for optimization of different cultural parameters for enzyme production, where during each run a single factor was varied and other factors were kept constant. The factors studied were BSG concentration (5.0, 10, 15, 20 and 25% *w/v*), pH (4.0, 5.0, 6.0, 7.0 and 8.0), and temperature (20, 25, 30 and 37 °C). The optimized culture conditions derived from these experiments were then used for production of enzyme.

Following media sterilization by autoclaving at 121 °C for 30 min, five 7-mm-diameter agar plugs of actively growing fungi were used as inoculum. Flasks were incubated at 30 °C (except for temperature studies) for 7 days, and then 50 ml of distilled water was added to each flask. Substrate in flasks was crushed with glass rod, and then shaking was applied at 150 rpm for 60 min in an incubator shaker. Following shaking, the flasks were harvested by squeezing the BSG, and the supernatant was further centrifuged at 10,000 rpm at 5.0 °C for 15 min. The cell-free supernatants were treated as crude enzyme and were used for protein estimation and enzyme activity assay.

2.2.4. Protein and enzyme assays

2.2.4.1. Assay of total protein. Total protein concentration was determined by the Bradford assay using the absorbance values of the dye at 595 nm using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.2.4.2. Assay of enzyme activity. Xylanase, and pectinase activities were assayed by determining the liberated reducing sugars (xylose and D-galacturonic acid, respectively) resulted from the enzymatic hydrolysis of the corresponding substrates (xylan, and pectin, respectively) using 3,5-dinitrosalicylic acid reagent (Miller, 1959). The supernatant from the culture broth was served as the source of the enzyme), a standard curve was graphed for each sugar (xylose, and D-galacturonic acid), and the absorbance of the solution was measured at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that produces 1.0 μmol of reducing sugar per ml per minute ($\mu mol\,min^{-1}$) under the assay conditions.

2.2.4.3. Partial purification and concentration of pectinases and xylanases. Crude enzymes were centrifuged at 12,000 *g* for 15 min. The resulting cell-free supernatants were fractionated by ammonium sulfate between 50 and 80% saturation to find the optimum saturation. Extracts were further purified by centrifugal ultrafiltration using a 10-kDa MW cut-off membrane.

2.2.4.4. Determination of pH and temperature profiles. In order to determinate the optimal pH for enzyme activity, the xylanase and pectinase were studied over a pH range of 2.0–11.0 at 50 °C with 1.0% xylan and 1.0% pectin, respectively. To determinate the optimal temperature for activity, the enzymes were tested at different temperatures between 20 and 80 °C, and at the optimum pH of each enzyme.

2.2.5. Statistical data analysis

The analysis of variance (AVOVA) was performed through STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA). The calculations of the Fisher's least significant difference (LSD) were applied to the multiple comparisons. Treatments were carried out in triplicates and average values were reported.

3. Results

3.1. Pretreatment of BSG for recovery of fermentable sugars

3.1.1. Microwave pretreatment

Microwave heating can replace the conventional heating in disruption of the complex structures of lignocelluloisic biomass and achieve higher production, minimal energy loss, and lower cost. Thus, Microwave-assisted pretreatments were carried out at 100, 250, 400, 600 and 100 W, for 30, 60, and 90 s. Table 1 shows the effect of microwave power on sugar yield from pretreated BSG. The maximum sugar yield (64.4 ± 7.0 mg/g of BSG) was obtained at 600 w after 90 s, as compared to 24.5 ± 0.3 mg of reducing sugar obtained from every 1 g of native BSG before pretreatments.

Analysis of variance and Fischer's Least Significant Difference (LSD) were performed to determine whether the changes in fermentable sugars derived from BSG brought about by microwave-assisted pretreatments at different power levels were significantly different to each other (Table 1). ANOVA revealed that all the microwave-assisted pretreatments were significantly different ($P < 0.05$) at different power levels. However, not all the microwave-assisted pretreatments were significantly different when comparing fermentable sugars derived from BSG pretreated by microwave at different time intervals but constant power level. For example, the microwave-assisted pretreatments for BSG at 100 W for 30, 60, and 90s were statistically similar as far as fermentable sugars were concerned. The same was observed for the microwave-assisted pretreatments for BSG at 440 W for 30, 60, and 90s.

Nevertheless, the results from microwave-assisted pretreatments for BSG at higher power (600 or 1000 W) for short time (30s) were significantly different when compared to their counterparts for longer time (60, and 90s). The same was observed for the microwave-assisted pretreatments for BSG at 250 W. Overall, the statistical analysis suggested that microwave power has significant impact on pretreatment process as compared to pretreatment time in most cases. Increasing microwave power had positive effect on fermentable sugars yield; pretreatment time can be reduced by increasing microwave power. However, for longer pretreatments (60, 90s) the highest fermentable

sugar yield was produced at 600 W power level, and increasing microwave power level to 1000 W had a negative effect on fermentable sugar yield.

This is in agreement with results obtained by Binod et al. (2012) who evaluated the microwave-assisted pretreatments of sugarcane bagasse at different power levels (100, 180, 300, 450, 600 and 850 W). The authors reported that the highest sugar yield was obtained from sugarcane bagasse at 600 W microwave power using either microwave-alkali (MAL) pretreatment or microwave-alkali followed by acid (MAA) pretreatment. Furthermore, Mithra et al. (2017) recommended the same microwave power (600 W) after optimization of microwave-assisted dilute acid pretreatment for peels of root crops and vegetables. The authors found that microwave power had the greatest influence in fermentable sugar yield as compared to other factors studied, such as dilute sulphuric acid concentration, and irradiation time. Similar results that found 600 W microwave power to be optimal were reported by Kuittinen et al. (2016) who studied the effect of two microwve power levels (600 and 1200 W) on pretreatment of Norway spruce under high pressure- temperature. Recently, Tiwari et al. (2019) investigated the application of microwave-alkali pretreatment at different microwave power levels (100 to 600 W) and times (1 to 6 min) on banana peel waste to achieve optimal fermentable sugars. Interestingly, the study found that the maximum reducing sugar (0.561 g/g of biomass) was obtained after microwave-assisted alkali pretreatment at 600 W for 2 min. It is worth notice that most studies integrate chemical peatreatment with microwave pretreatment to improve process efficiency, in spite of disadvantages of chemical pretreatments that encouraged the investigating of new clean technologies like microwave. In this study, Microwave investigated as sole pretreatment technology for lignocellulose to achieve energy-efficient and chemicals-free process.

Microwave electromagnetic fields target the polar part of the biomass selectively, leading to a rise in temperature in the internal surface of lignocellulose. Increasing temperature also affects the degree of lignocellulose polymerization and crystallization, so that biomass becomes more accessible to hydrolytic enzymes. However, excessive heating (resulting from high microwave power or long exposure time) can lead to formation of "hot spots", degradation of useful components like glucose and the formation of unwanted components such as furfural, formaldehyde, acetaldehyde, and dihydroxyacetone. Thereby, it is essential to find a proper microwave power and exposure time to achieve efficient pretreatment.

3.1.2. Ultrasound pretreatment

Ultrasound pretreatments were carried out at varying frequencies (and ultrasound power): 25 (550 W), 35 (750 W), 45 (550 W), 130 (750 W) and 950 kHz (100 W) for 20, 40, and 60 min. Table 2 shows the effect of ultrasound frequency on sugar yield from pretreated BSG. The maximum sugar yield (39.9 ± 6.1 mg/g of BSG) was obtained at low ultrasound frequency (25 kHz, 550 W) after 60 min. (compared to 24.5 ± 0.3 mg of reduced sugar obtained from every 1 g of native BSG before pretreatments).

Analysis of variance and Fischer's Least Significant Difference (LSD) were performed to determine whether the changes in fermentable sugars derived from BSG brought about by ultrasound-assisted pretreatments at different power levels were significantly different to each other. ANOVA revealed that all the ultrasound-assisted pretreatments were significantly different ($P < 0.05$) at different frequencies of each power levels. Moreover, most of the ultrasound-assisted pretreatments were significantly different ($P < 0.05$) at different frequencies and different power levels (Table 2).

However, some pretreatments at constant ultrasound frequency were insignificantly different at different time intervals. For example, neither ultrasound-assisted pretreatments at 25 KHz (550 W) nor 35 KHz (750 W) were significantly different at different pretreatment times. The same was observed for the ultrasound-assisted pretreatments after 40 and 60 min at an ultrasound frequency of 45 KHz (550 W), and after 20 and 40 min at ultrasound frequencies of 130 KHz (750 W) and 950 KHz (100 W). Overall, the results suggested that ultrasound pretreatment at a frequency as low as 25 KHz is always yield higher fermentable sugars from pretreatments at higher frequencies (regardless of the ultrasound power levels) at a given pretreatment time. Additionally, pretreatment time can be reduced by decreasing the ultrasound frequency. The literature is dominated by pretreatments at low-ultrasound frequencies (< 50 kHz) because of the short acoustic cycle which allow the growth, radial motion and collapse of bubbles. However, considering the longer required pretreatment time and lower reducing sugar yield of ultrasound pretreatment, as compared to microwave pretreatment, the latter method was selected for all subsequent studies.

Ultrasound used for pretreatment of lignocellulosic biomass to disrupt the complex structures of lignocellulosic biomass due to the cavitation effect. Cavitation bubbles are produced in a liquid medium as a result of ultrasound waves, and collapse to produce a strong mechanical effect on lignocellulosic solid biomass, as well as producing free radicals such as H• and HO• from the liquid medium. Moreover, the "hot spots" that develop on the surface of the solid biomass (as a result of the heat generated during the collapse of the cavitation bubbles) can introduce chemical changes in the lignocellulosic complex structure. It has previously been reported that longer ultrasound pretreatment times may lead to greater sugar recovery (Rehman et al., 2014). However, exposure to long times of ultrasound pretreatment may cause adverse effects due to collision and aggregation between the particles, and also increase energy consumption during the process. Thereby, it is essential to find a proper ultrasound power and frequency that may achieve an efficient process within shortest exposure time.

3.1.3. FTIR characterization of pretreated BSG

The chemical changes in the functional groups of the BSG were studied based on FTIR analysis (Fig. 1). The microwave pretreated BSG displayed significant decreases in band intensities at characteristic peaks for cellulose, hemicellulose and lignin (Table 3) as compared to ultrasound-pretreated and native BSG.

Although all BSG samples (pretreated and native alike) exhibited a

Table 2
Effect of ultrasound frequency on sugar yield from BSG.

Time (min)		Reducing Sugar Yield (mg/ g of biomass)									
	US Power	550				750				100	
	US Frequency	25		45		35		130		950	
		Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
20		32.1	7.0[a]	24.5	0.3[b]	25.2	3.5[ab]	24.5	0.7[b]	24.4	0.5[b]
40		33.6	0.0[a]	25.0	4.9[ab]	28.4	1.7[ab]	24.6	0.5[b]	24.6	0.2[b]
60		39.9	6.1[a]	31.8	1.6[ab]	31.8	1.6[ab]	27.9	3.8[bc]	25.0	1.7[bc]

Where reducing sugar yields for native BSG = 24.5 ± 0.3 mg/g of biomass.
Means not sharing the same letter are significantly different (LSD) at $P < 0.05$ probability level for respective ultrasound frequency and power level.

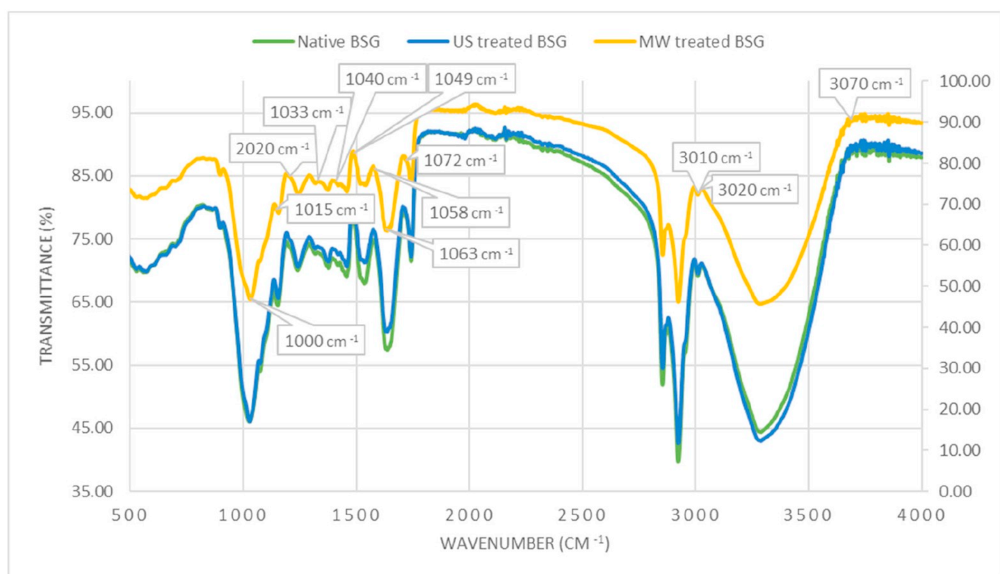

Fig. 1. FTIR spectra of native, microwave pretreated, and ultrasound pretreated BSG.

band at 1033 cm^{-1} which is characteristic of cellulose content in BSG (Silbir et al., 2019) in their respective spectra, microwave pretreated BSG showed the highest transmittance (i.e., the lowest absorption intensity). Similar to the peak at 1033 cm^{-1}, microwave pretreated BSG shows the highest transmittance at different bands that represent different functional groups. These results are related to the fact that microwave pretreatment was more efficient in changing the chemical composition of BSG. Similar results were also reported by other researchers (Silbir et al., 2019) who used FTIR to identify the functional groups present in BSG samples. Moreover, the results were consistent with reported data in the literature (Hou et al., 2019) for other lignocellulosic biomass pretreated with microwave.

3.2. Isolation and screening of xylanopectinolytic enzyme-producing fungi

3.2.1. Isolation xylanopectinolytic enzyme-producing fungi

Twenty-nine fungal isolates were obtained from spoiled-fruit samples of 19 different varieties (Table 4). The isolates were distributed in three genera, namely: *Mucor* spp. (19 isolates), *Pencillium* spp. (6 isolates), and *Aspergillus* spp. (4 isolates). All isolates were selected for subsequent pectinase and xylanase production screening. The low pH of fruit gives fungi a competitive advantage over bacteria. Moreover, fungi can spread on commodities in cold-storage (Moss, 2008). Some fungi have the ability to produce pectinolytic enzymes to soften the plant tissue causing rot, and others infect through wounds caused during handling and packing of the fruit. *Aspergillus* spp., *Pencillium* spp., and *Mucor* spp. are among the commonly encountered species causing spoilage of fruit (Moss, 2008).

3.2.2. Screening of xylanopectinolytic enzyme-producing fungi

In the current study, three candidates were obtained from qualitative screening as pectinase producers (*Mucor* sp. AB1, *Penicillium* sp. GS1 and GS2) and three candidates as xylanase producers (*Penicillium* sp. GS1, *Mucor* sp. AB1 and RI2,). Among these, *Mucor* sp. coded AB1 displayed the highest extracellular pectinase and xylanase activity after the quantitative determinations (Table 5) and was, therefore, retained for all subsequent studies. In previous study, *Mucor* sp. has been reported as higher polygalacturonase producer than *Aspergillus* sp. (Thakur and Gupta, 2012) when authors compared the activity of polygalacturonase produced by *Aspergillus* sp., *Byssochlamys* sp., and *Mucor* sp. However, the literature is dominated by studies that employ *Aspergillus* sp. for production of xylanopectolytic enzymes.

There are relatively few publications on production of xylanase and pectinase by fungi that belong to the species of the genus *Mucor*. Daling et al. (2008) reported in their patent the production of xylanases by *Mucor rouxii* under aerobic fermentation in media containing xylan as inducer. Behnam et al. (2016) reported the production of xylanases by *Mucor indicus* (43.1 U/g of dry substrate) and *Mucor hiemalis* (43.8 U/g of dry substrate) on wheat bran as fermentation medium; these authors found that the optimum fermentation conditions were a temperature of 40 and 43.4 °C, moisture percent of 49.8 and 54.2%, and incubation time of 51.3 and 53.2 h for *Mucor indicus*, and *Mucor hiemalis,*

Table 3
FTIR peak assignments for lignocellulose.

Wave number (cm^{-1})	Assignment	References
1000	C–O stretching, C–C stretching of cellulose	(Szymanska-Chargot and Zdunek, 2013)
1015	C–O stretching, C–C stretching of pectin	(Szymanska-Chargot and Zdunek, 2013)
1020	C–C, C–OH, C–H ring and side group vibrations	(Fan et al., 2012)
1033	C–O stretching, C–C stretching of cellulose	(Szymanska-Chargot and Zdunek, 2013)
1040	C–O stretching, C–C stretching of xyloglucan	(Szymanska-Chargot and Zdunek, 2013)
1049	C–OH bending	(Adapa et al., 2011)
1058	C=O stretching of hemicellulose and lignin	(Wang et al., 2018)
1063	C–O–C asymmetrical stretching of cellulose, hemicellulose	(Sills and Gossett, 2012)
1072	C–O stretching, C–C stretching of xyloglucan	(Szymanska-Chargot and Zdunek, 2013)
3010	O–H stretching vibration	(Abidi et al., 2011)
3020	O–H stretching vibration	(Abidi et al., 2011)
3070	O–H stretching vibration	(Abidi et al., 2011)

Table 4
List of fruit-spoilage fungi isolated from infected fruits.

Code	Fungal isolate	Fruit	Fruit variety
AB1	*Mucor* sp.	Apple	Bramely
AB2	*Mucor* sp.	Apple	Bramely
AG1	*Penicillium* sp.	Apple	Gala
GC1	*Mucor* sp.	Grape	Crimson
GM1	*Aspergillus* sp.	Grape	Melody
GM2	*Mucor* sp.	Grape	Melody
GM3	*Penicillium* sp.	Grape	Melody
GT1	*Aspergillus* sp.	Grape	Tawny
GSO1	*Mucor* sp.	Grape	Sugraone
GS1	*Penicillium* sp.	Grape	Starlight
GS2	*Penicillium* sp.	Grape	Starlight
GS3	*Mucor* sp.	Grape	Starlight
GST1	*Aspergillus* sp.	Grape	Sugarthirteen
BT1	*Mucor* sp.	Blackberry	Tupi
BE1	*Mucor* sp.	Blueberry	Emerald
BD1	*Mucor* sp.	Blueberry	Duke
BD2	*Mucor* sp.	Blueberry	Duke
RI1	*Penicillium* sp.	Raspberry	Imara
RI2	*Mucor* sp.	Raspberry	Imara
RK1	*Mucor* sp.	Raspberry	Kweli
RK2	*Penicillium* sp.	Raspberry	Kweli
RF1	*Mucor* sp.	Raspberry	Framboises
SC1	*Mucor* sp.	Strawberry	Calinda
SC2	*Mucor* sp.	Strawberry	Calinda
SR1	*Mucor* sp.	Strawberry	Rociera
SR2	*Aspergillus* sp.	Strawberry	Rociera
SS1	*Mucor* sp.	Strawberry	Sensation
SM1	*Mucor* sp.	Strawberry	Marquis
CR1	*Mucor* sp.	Cherry	Regina

Table 5
Secondary evaluation of xylanases and pectinases production by fruit-spoilage fungi.

Code	Fungal isolate	Xylan-supplemented medium		Pectin-supplemented medium	
		Total cell-free protein (mg/ml)	Xylanase activity (u/ml)	Total cell-free protein (mg/ml)	Pectinase activity (u/ml)
AB1	*Mucor* sp.	28.1 ± 0.7	9.8 ± 0.2	14.5 ± 0.1	14.4 ± 0.6
GS1	*Penicillium* sp.	23.3 ± 0.4	7.4 ± 0.1	11.0 ± 0.1	7.7 ± 0.8
GS2	*Penicillium* sp.	–	–	10.4 ± 0.3	0.4 ± 0.0
RI2	*Mucor* sp.	21.4 ± 0.6	7.2 ± 0.1	–	–

respectively.

The pectinolytic enzyme (polygalacturonase) was found to be optimally produced by *Mucor circinelloides* ITCC 6025 after 48 h of fermentation at 30 °C and pH 4.0 with pectin methyl ester (1% *w/v*) as carbon source (Thakur et al., 2010). Moreover, Sharma et al. (2013) reported the production of polygalacturonase by the same isolate of *Mucor circinelloides* ITCC 6025 on medium containing pectin (1% w/v) as sole carbon source after fermentation for 48 h at 30 °C and pH 4.5.

3.3. Production of xylanopectinolytic enzymes using BSG

3.3.1. Optimization of production

Microwave pretreated BSG at 600 W for 90 s was used for solid-state fermentation by *Mucor* sp. AB1. Various process parameters influencing pectinases and xylanases production viz., substrate loading (5, 10, 15, 20, and 25 g), incubation temperature (20, 25, 30, and 37°), and pH (Aguilar-Reynosa et al., 2017; Ahmad et al., 2009; Ahmed et al., 2016; Ahmed et al., 2012; Behnam et al., 2016) were studied.

Fig. 2 shows the effect of substrate loading, incubation temperature, and pH on pectinase and xylanase production by *Mucor* sp. AB1. Enzyme activities of 137 U/g, and 67 U/g BSG, for pectinases and xylanases, respectively weres achieved in medium that contained 15 g of BSG, at pH 6.0, and at a temperature of 30 °C. Temperature and pH play an important role in the synthesis of microbial enzymes. The optimum temperature for production of pectinase and xylanase by *Mucor* sp. AB1 was 30^{0}C. Temperature beyond 30 °C led to a decrease in enzyme specific activity. Most of fungi are mesophiles with optimum growth temperatures between 25 and 30 °C (Dix and Webster, 1995).

The same optimum temperature was reported by Thakur et al. (2010) for production of Polygalacturonase by *Mucor circinelloides* ITCC 6025, and was also reported by Ahmad et al., (2009) for production of Xylanase by *Aspergillus niger*. Similar temperatures were reported for optimal production of xylanase, ranging from 28^{0}C by *Aspergillus niger* (Bhushan et al., 2012) to 32.5^{0}C by *Fusarium* sp. BVKT R2 (Ramanjaneyulu and Rajasekhar Reddy, 2016). Furthermore, increasing pH from 4.0 to 6.0 increased the production of pectinase and xylanase. However, enzyme specific activity then decreased until pH 8.0. This can be attributed to the fact that most fungi grow optimally at a low pH between 5.0 and 6.0. In earlier studies, Bhushan et al. (2012) reported similar results while, according to Ramanjaneyulu and Rajasekhar Reddy (2016) and Terrone et al. (2018) a pH value of 5.0 was an optimum.

The optimum biomass loading for production of pectinases and xylanases by *Mucor* sp. was 15 g per 250-ml conical flask, with any further increases suppressing enzyme activity. In addition to the availability of more nutrients, more inducers are available to the fungus at the optimum substrate concentration that in turn enhance the production of enzymes (Ahmed et al., 2016). In an earlier study, Kavya and Padmavathi (2009) found that maintaining the substrate concentration (wheat bran) at 10 g/250 ml conical flask yielded the maximum production of xylanase by *Aspergillus niger*. However, the optimum substrate concentration for enzyme production varies widely with varying substrate type, and also the water absorbed onto the substrate surface (Pandey, 1992).

3.3.2. Partial purification and concentration

Following removal of biomass by centrifugation, pectinase and xylanase produced by *Mucor* sp. AB1 were purified by ammonium sulphate precipitation. An ammonium sulphate fraction at 80% saturation resulted in a 95% and 76% yield for pectinases and xylanases respectively. The ammonium sulfate enriched fraction of pectinases and xylanases were further concentrated to 14-fold and 12-fold respectively by ultrafiltration (Table 6).

3.3.3. Effect of temperature and pH

Fig. 3 depicts the effect of different temperatures and pH on the enzyme activity of the enriched pectinase and xylanase from *Mucor* sp. AB1. The pectinase activity was steadily increased with an increase in the temperature up to 50 °C and pH up to 5 with no significant increase at 60 °C and pH 6 before declining at 70 °C and pH 7 or higher. While, the xylanases activity was steadily increased with an increase in the temperature up to 60 °C and pH up to 5.0 before declining at 70 °C and pH 6 or higher. This temperature-optima at 60 °C is similar to that reported for xylanase obtained from *Penicillium glabrum* (Knob et al., 2013) and *Trichoderma harzianum* (Ahmed et al., 2012), as well as pectinase obtained from *Aspergillus fumigatus* (Okonji et al., 2019). However, slightly higher optimal temperatures have been reported for other xylanases, such as 70 °C for the purified xylanase from *Rhizomucor pusillus* (Robledo et al., 2016) and *Chaetomium thermophilum* (Ahmed et al., 2012). Moreover, slightly lower optimal temperatures have been reported for other pectinases, such as 55 °C for the purified polygalacturonase from *Rhizomucor pusillus* (Siddiqui et al., 2012) and 50 °C for the purified polygalacturonase from *Aspergillus niger* MCAS2 (Khatri et al., 2015).

These results are in accord with that reported by Gautam et al. (2018) that maximum activity of the xylanase from *Chizophyllum commune* ARC-11 was at pH 5. Similar results have been reported for polygalacturonase from *Rhizomucor pusillus* (Siddiqui et al., 2012), as

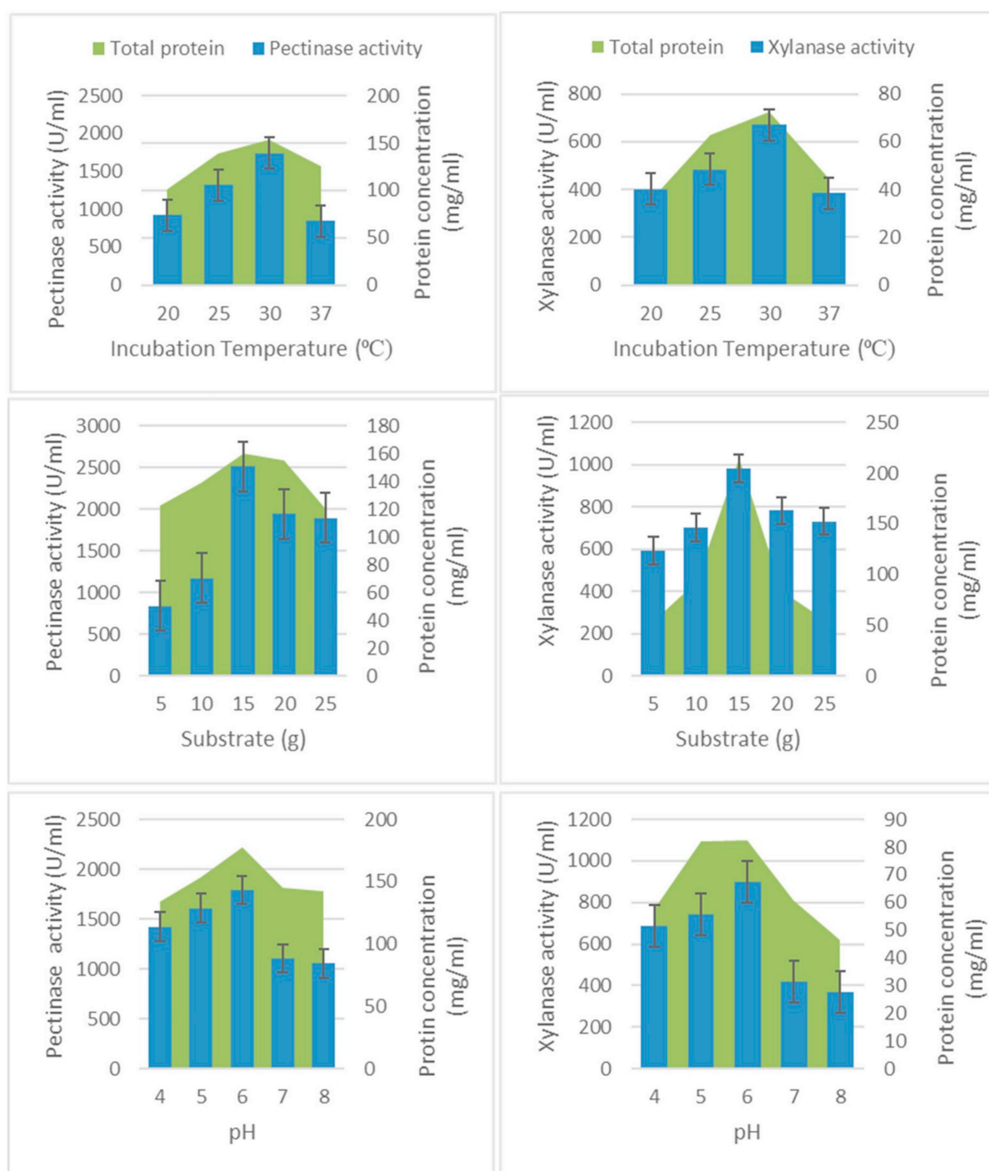

Fig. 2. Effect of temperature, substrate, and pH on pectinases and xylanases production by *Mucor* sp. (AB1).

well as xylanases by *Fusarium* sp. BVKT R2, and *Trichoderma harzianum* (Khatri et al., 2015). However, slightly higher optimal pH values have been reported for other pectinases and xylanases, such as pH 6.0 for pectinase from *Neurospora crassa* (Polizeli et al., 1991), or xylanase from *Chaetomium thermophilum* (Ahmed et al., 2012) and *Rhizomucor pusillus* SOC-4A (Robledo et al., 2016). Moreover, pH values of up to 6.5 have been reported as the optimum pH for xylanase (Terrone et al., 2018), and pectinase (Rasheedha Banu et al., 2010) from *Penicillium chrysogenum*. On the other hand, lower optimal pH has been reported for other xylanases, such as pH 3.0 for the xylanase from *Penicillium glabrum* (Knob et al., 2013).

4. Conclusion

Microwave technology is more effective in pretreatment of BSG for fermentable sugar release as compared to ultrasound. Optimal conditions for microwave-assisted pretreatment of BSG were found to be 600 W for 90s. When the pretreated BSG was used for xylano-pectolytic enzyme production by *Mucor* sp. under optimized conditions (15 g biomass/250 ml conical flask, pH 6.0, and temperature of 30 °C), the

Table 6
Summary of purification of pectinases and xylanases.

Purification step	Total protein (mg)		Total activity (U)		Specific activity (U/mg)		Yield (%)		Purification (fold)	
	PE	XY	PE	XY	PE	XY	PE	XY	PE	XY
Crude	289.51	214.14	2057.72	996.25	4.65	4.65	100	100	1	1
$(NH_4)_2SO_4$	230.07	136.83	1943.62	760.21	8.45	5.56	94.46	76.31	1.19	1.18
Ultrafiltration	15.62	12.51	1496.48	687.15	95.82	54.93	72.73	68.97	13.48	11.81

Where: PE = Pectinases, and XY = Xylanases.

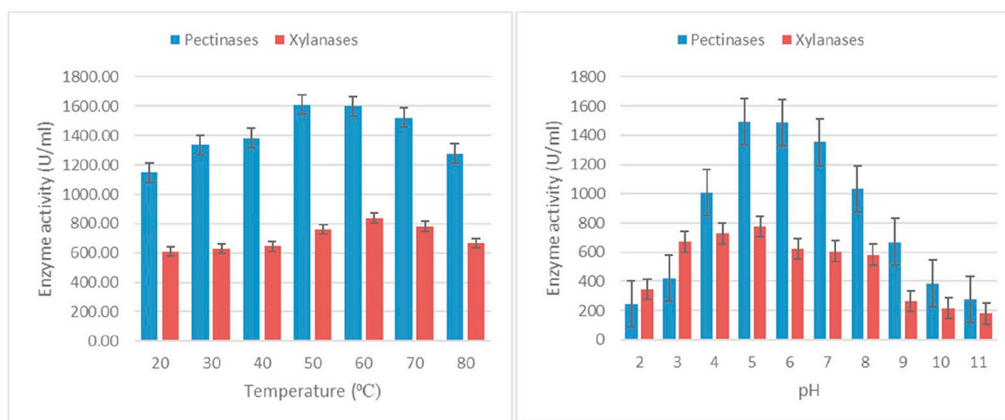

Fig. 3. Effect of temperature, and pH on pectinases and xylanases activity.

enzyme activity was 137 U/g, and 67 U/g BSG, for pectinases and xylanases, respectively. The temperature and pH profiles (60 °C and pH 5) of the Xylanopectinolytic enzymes obtained in this study make the enzymes advantageous in various industrial processes.

Author contributions

Shady S. Hassan, Conceptualization; Shady S. Hassan, Amit K. Jaiswal, Data curation; Shady S. Hassan, Formal analysis; Shady S. Hassan, Gwilym A. Williams, and Amit K. Jaiswal, Funding acquisition; Shady S. Hassan, Investigation; Shady S. Hassan, Brijesh K. Tiwari, Gwilym A. Williams, and Amit K. Jaiswal, Methodology; Gwilym A. Williams, and Amit K. Jaiswal, Project administration; Brijesh K. Tiwari, Gwilym A. Williams, and Amit K. Jaiswal, Resources; Shady S. Hassan, Amit K. Jaiswal, Software; Brijesh K. Tiwari, Gwilym A. Williams, and Amit K. Jaiswal, Supervision; Brijesh K. Tiwari, Gwilym A. Williams, and Amit K. Jaiswal, Validation; Shady S. Hassan, Visualization; Shady S. Hassan, Original draft; Shady S. Hassan, Brijesh K. Tiwari, Gwilym A. Williams, and Amit K. Jaiswal, Review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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