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One-step process for producing prebiotic arabino-xylooligosaccharides from brewer's spent grain employing *Trichoderma* species

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ABSTRACT

Xylooligosaccharides (XOS) are prebiotic nutraceuticals that can be sourced from lignocellulosic biomass, such as agro-residues. This study reports for the first time an optimization study of XOS production from agro-residues by direct fermentation using two *Trichoderma* species. A total of 13 residues were evaluated as potential substrates for single-step production. The best results were found for *Trichoderma reesei* using brewers' spent grain (BSG) as substrate. Under optimal conditions (3 days, pH 7.0, 30 °C and 20 g/L of BSG), a production yield of 38.3 ± 1.8 mg/g (xylose equivalents/g of BSG) was achieved. The obtained oligosaccharides were identified as arabino-xylooligosacharides (AXOS) with degree of polymerization from 2 to 5. One-step fermentation proved to be a promising strategy for AXOS production from BSG, presenting a performance comparable with the use of commercial enzymes. This study provides new insights towards the bioprocess integration, enabling further developments of low-cost bioprocesses for the production of these valuable compounds.

1. Introduction

Due to the increasing of health consciousness and consumer awareness, the demand of prebiotic products as functional food ingredients has been growing in the recent years (Antov & Đorđević, 2017). According to the updated definition, a prebiotic can be defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Prebiotics are attractive compounds given their multidimensional beneficial effects on human and animal health, namely on gastrointestinal tract (*e.g.* pathogen exclusion, immune modulation), cardiometabolism (*e.g.* cholesterol lowering), mental health (*e.g.* energy and cognition) and bone (*e.g.* enhanced mineral absorption), among others (Gibson et al., 2017; Samanta et al., 2015).

Xylooligosaccharides (XOS) are one example of prebiotics that has gained attention due to their multidimensional benefic influences on human health and livestock, namely in preventing gastrointestinal disorders (Adebola, Corcoran, & Morgan, 2014). These compounds exhibit temperature and acidity stability in a higher range than inulin and FOS, making them a potential food ingredient (Courtin, Swennen, Verjans, & Delcour, 2009). Furthermore, XOS have acceptable organoleptic properties, not exhibiting toxicity or negative effects on human health (Aachary & Prapulla, 2009). They represent a remarkable niche amongst the prebiotics, especially because they are the only nutraceuticals that can be sourced from lignocellulosic biomass (Samanta et al., 2015).

There has been a great interest in the exploitation of lignocellulosic biomass, such as agro-residues, which are abundant and renewable resources (Samanta et al., 2015). Billions of tons of agro-residues are annually generated, making them cost-effective raw materials for the production of value-added compounds (Kumar & Satyanarayana, 2015). However, seasonality, variability in composition and spoilage risk during storage may limit the use of agro-residues (Batidzirai et al., 2016).

Chemically, XOS are oligomers with a ramified structure containing 2 to 7 xylose units linked through β-(1,4)-xylosidic bonds and decorated with a diversity of substituents, namely acetyl groups, uronic acids and arabinose units (Kumar & Satyanarayana, 2011). Xylan, the main constituent of hemicelluloses and the second most abundant carbohydrate in the lignocellulosic biomass (Nieto-Domínguez et al., 2017), is the precursor of XOS. Besides the origin of the biomass, the structural features of XOS depend also on the production process (Samanta et al., 2015). Different production methods are described, including chemical, auto-hydrolytic, enzymatic, or the combination of both (Kumar & Satyanarayana, 2015). Chemical or auto-hydrolytic methods present several problems, namely the contamination of the product by chemicals, the formation of undesired toxic by-products, such as hydroxymethylfurfural (HMF) and furfural, and the low control over the

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degree of polymerization (DP) (Chapla, Pandit, & Shah, 2012). Consequently, additional costs directly associated with the downstream process have to be considered (Bian et al., 2014). These methods generally require the use of specific and more robust equipment (Jain, Kumar, & Satyanarayana, 2015). On the other hand, the use of enzymes is a more environment-friendly approach, operating without application of noxious chemicals and being aligned with the perspective of a biodegradable process (Azelee et al., 2016). The most common method of XOS production is the combined one (Faryar et al., 2015), which includes the fractionation of the lignocellulosic material to obtain soluble xylan followed by its hydrolysis by xylanolytic enzymes (Chapla et al., 2012), such as endo-1,4-β-xylanases (EC 3.2.1.8) and endo-1,3-β-xylanases (EC3.2.1.32). The method presents high efficiency and specificity, and subsequently, lower amounts of xylose and other undesirable by-products are produced with this process (Akpinar, Ak, Kavas, Bakir, & Yilmaz, 2007). Besides, this two-step process for XOS production from lignocellulosic residues is neither cost effective nor easy to perform as it depends on both xylan extraction and enzymatic hydrolysis. Xylan is generally present as a xylan-lignin complex in the lignocellulosic biomass, thus it is necessary the previous pretreatment of the residues to increase the accessibility of the enzymes (Chapla et al., 2012). The low yields associated with this extraction step in addition to the cost of purchasing commercially available xylanases, may compromise the economic viability of the production process (Reddy & Krishnan, 2016).

One-step bioprocess to produce XOS would be an interesting approach for lignocellulosic biomass conversion. However, as far as we know, there are no studies specifically focused on the production of XOS by direct fermentation of lignocellulosic materials without the use of a previous conventional pretreatment. The successful use of this One-step strategy requires the application of suitable lignocellulosic materials and effective xylanase producers. This work describes, for the first time, an optimization study of XOS production from non-pretreated residues in a One-step using *Trichoderma* species, namely *T. reesei* and *T. viride*. In particular, *T. reesei* is considered a biosafety level 1 microbe, presenting several associated enzymatic products with GRAS status (Paloheimo, Haarmann, Mäkinen, & Vehmaanperä, 2016).

Direct fermentation is also compared with the XOS production using of a commercial enzyme preparation. In view of the above, this study will be useful for further development of a low-cost process for the production of XOS that adds a new dimension in developing a bioprocess under green environmental initiatives.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma (Germany). The commercial enzymatic preparation of endo-1,4-β-xylanase M3 from *Trichoderma longibrachiatum* and the standard XOS (xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6)) were purchased from Megazyme (Ireland).

The brewer's spent grains (BSG) were supplied by UNICER S.A. (Portugal). Coffee pellets and coffee silver skin were provided by Delta S.A. (Portugal). Chestnut shell resulting from a thermal peeling of the fruit was supplied by Alcino Nunes & Irmão Ltd. (Portugal). Raw chestnut shell was obtained from chestnut fruits collected in Trás-os-Montes (Portugal). Orange peel, vine pruning, passion fruit, corn cobs, garlic, onion were obtained by local harvest (Barcelos, Portugal). Banana and peanut were purchased at local supermarkets. The residues were dried overnight at 60 °C, triturated with a bench top blender and stored at room temperature until use. The relative humidity content of the raw materials was determined with a moisture analyzer MAC 50/1/NH apparatus, being < 5% (w/w).

2.2. Microorganism and culture conditions

Trichoderma reesei (MUM 9753) and *Trichoderma viride* (MUM 9754) were provided by Micoteca da Universidade do Minho (MUM) hosted at the Centre of Biological Engineering (CEB), University of Minho, Portugal. Stock cultures were stored at room temperature in suspensions of semi solid agar media 0.2% (w/v) (Oxoid), and then propagated at 25 °C on petri dishes of solid 4% (w/v) PDA (Biolife®).

2.3. Chemical characterization of BSG

BSG was chemically characterized according to NREL (National Renewable Energy Laboratory) protocols (NREL/TP-510-42618-42622-4218) for biomass compositional analysis. The methodology included acid hydrolysis with 72% (w/w) sulphuric acid of the extractive-free BSG, High Performance Liquid Chromatography (HPLC) quantification of sugars and degradation products present in the hydrolysate (Section 2.8) and gravimetric determination of acid insoluble lignin. The fraction of extractives was obtained using ethanol 80% (v/v) as solvent in a Soxtec 8000 FOSS apparatus. The hemicellulose and cellulose fractions were calculated from the HPLC data. The quantification of total proteins was performed using a Kjeltec 8400 Fosse Tecator equipment. The content of acid-insoluble lignin corresponds to the mass of the remaining solids after filtration without the structural ash content. The total amount of lignin was calculated as the sum of acid-insoluble and acid-soluble fractions.

2.4. Screening of different agro-residues for XOS production by direct fermentation

The ability of *T. viride* and *T. reesei* to use different residues as sole carbon source was investigated in 2% (v/v) of Vogel's 50x salts (Vogel, 1956) at pH 5.0. The 250 mL Erlenmeyer flasks were filled with 50 mL of medium and the dried residues were added at a final concentration of 100 g/L and 50 g/L. The solid–liquid mixture was sterilized at 121 °C during 15 min. The flasks were inoculated with a suspension of approximately 10^6 spores/mL of conidia in sterile solution containing 0.8% (w/v) NaCl and 0.05% (w/v) Tween 20. The inoculated flasks were incubated at 30 °C with orbital shaking (model Agitorb 200 IC; Norconcessus, Portugal) at 180 rpm during 10 days. Samples from the supernatant were collected, centrifuged and qualitatively analyzed by Thin Layer Chromatography (TLC) (Section 2.9).

2.5. Optimization of XOS production by direct fermentation of BSG with T. reesei

2.5.1. Effect of BSG amount, pH and temperature

The procedures described in Section 2.4 were adapted to BSG fermentations according to the variable under analysis. The effects of substrate concentration (5, 10, 20, 40, 60, 100 and 160 g/L), pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and temperature (20, 27, 30 and 37 °C) were studied during 6 days of fermentation. Samples from the supernatant were collected, centrifuged and further analyzed. A negative control, without inoculum, was performed in parallel for the optimal conditions selected, to evaluate the possible sugar extraction from BSG during the fermentation time.

2.5.2. Enzymatic activity

The enzyme activity was accessed using a soluble chromogenic substrate (Biely, Mislovičová, & Toman, 1985) according to Megazyme procedures for azo-xylan from birchwood. One unit of enzyme activity is defined as the amount of enzyme required to release one μmole of D-xylose reducing sugar equivalents from arabinoxylan, at pH 4.5 per minute at 40 °C.

2.5.3. Sugar analysis

The DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) was used to quantify the total reducing sugars, RS (mg), using xylose as standard for the calibration curve (Jeffries, Yang, & Davis, 1998). The sugar production yield, Y_{RS} (mg/g), was determined as the ratio between RS (mg) and the mass of BSG (g) used in the fermentation.

The XOS produced under optimal conditions were quantified by HPLC (Section 2.8) in terms of equivalent xylose (Xeq) after acid hydrolysis according to NREL protocols. Note that the supernatant was analyzed before and after acid hydrolysis to determine the amount of free xylose and also the xylose contained in the oligosaccharides. The XOS production yield expressed in terms of xylose equivalents, Y_{Xeq} (mg/g), was determined as the ratio between the xylose (mg) present in the XOS and the mass of BSG (g) used in the fermentation. The DP of the XOS was estimated by TLC (Section 2.9) and confirmed by HPLC (Section 2.8), after previous purification of the fermentative broth with activated charcoal (Section 2.7).

2.6. Production of XOS using commercial enzyme from T. longibrachiatum

A commercial enzymatic preparation of endo-1,4-β-xylanase M3 from *T. longibrachiatum* was used to hydrolyze 20 g/L of BSG in sodium acetate buffer (0.1 M) pH 4.5 at 40 °C. BSG was added to 50 mL of buffer and sterilized at 121 °C during 15 min. Then, the mixture was incubated with different concentrations of enzyme under orbital shaking (model Agitorb 200 IC; Norconcessus, Portugal) at 180 rpm during 24 h. A negative control, without enzyme, was performed in parallel for the optimal conditions selected, to evaluate the possible sugar extraction from BSG during the incubation time. The XOS produced were quantified in terms of equivalent xylose after acid hydrolysis determined by HPLC using the method for monosaccharides (Section 2.8). The enzyme activity was determined according to Section 2.5.2.

2.7. Partial purification of XOS produced by direct fermentation

To minimize the salt interference in the HPLC analysis for further estimation of the DP level, XOS produced by fermentation of BSG under optimal conditions were treated with activated charcoal. 10 mL of the supernatant were collected, centrifuged and loaded to 12.5*g* of activated charcoal. After the adsorption step the charcoal was washed with MiliQ water to remove the salts and other non-adsorbed components. The efficiency of salt removal was confirmed by electrical conductivity (WTW LF 538). Sugar desorption was performed using a mixture of butanol, acetic acid and water (2:1:1 v/v/v) as eluent. The collected eluent with sugars was evaporated to dryness and the samples were resuspended in water and further analyzed by HPLC (Section 2.8, method for oligosaccharides) to access the DP of the XOS produced.

2.8. Sugars analysis by HPLC

Two distinct HPLC methods were used, namely for monosaccharides and for oligosaccharides analysis. For monosaccharides (before and after acid hydrolysis), an HPLC (Knauer, Germany) fitted with Knauer-RI detector and an Aminex HPX 87H column (300 mm × 7.8; Biorad, USA) were used for quantification. 40 μL of sugar sample was eluted using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.7 mL/min and a temperature of 60 °C. Chromatographic grade sugars glucose, xylose, arabinose were used as standards for identification and quantification of sugars in the hydrolysates. Furfural and HMF concentrations were also determined by HPLC, using a Knauer-UV detector. For oligosaccharides (partial purified XOS (Section 2.7)), an HPLC (JASCO, Japan) fitted with ELSD detector (SEDERE, Sedex 85, France) and a Prevail Carbohydrate column (250 mm × 4.6 mm; Grace, USA) were used. A mixture of acetonitrile and water 68:32 (v/v) was used as mobile phase at a flow rate of 0.9 mL/min, temperature of 30 °C and 20 μL injection volume. Pure XOS (X2-X6) were used as standards for identification of the DP of the oligosaccharides produced.

2.9. Qualitative analysis of hydrolysis products using TLC

TLC silica gel plates (DCAlufolien Kieselgel 60, Merck) were used to analyze the composition of hydrolytic products obtained from the lignocellulosic residues. A volume of 3 μL of the hydrolytic products were spotted onto TLC plates and subjected to one ascending chromatography run using butanol, acetic acid and water (2:1:1 v/v/v) as mobile phase. The bands were detected by spraying with a staining solution containing 1% (w/v) diphenylamine and 1% (v/v) aniline in acetone, followed by heating at 120 °C during 10 min (Wu et al., 2013). Glucose at 2 g/L and a mixture of xylose and XOS (X2–X6, 2 g/L each) were used as standard. The retention factor, corresponding to the ratio between the distance traveled by the sample and the distance traveled by the solvent, was used to compare the different components of the spotted mixtures.

2.10. Confocal Laser Scanning Microscopy (CLSM) analysis of the cultivated BSG with T. reesei

For the CLSM analysis, the samples were prepared as follows. Firstly, the biomass attached to BSG was washed with distilled water for salts removal and fixed onto a slide using 4% (w/v) of paraformaldehyde, followed by ethanol addition at 50% (v/v). The samples were immersed with a 2.5 mg/ml Calcoflour White solution and incubated for 10 min in the absence of light. The excess of dye was removed with distilled water. The samples were analyzed using a Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000). Calcofluor was used for the detection of *T. reesei* (laser excitation line 405 nm and emissions filters BA 430–470) and auto-fluorescence (laser excitation line 488 nm and emissions filters BA 505–540) for BSG. Images were acquired with the program FV10-ASW 4.2 (Olympus).

3. Results and discussion

3.1. Screening of different agro-residues for XOS production by direct fermentation

A screening assay with 13 agro-residues (Section 2.1) was performed to evaluate their potential for XOS production by direct fermentation using *T. viride* and *T. reesei*. Prior inoculation, the residues in Vogel medium were sterilized by autoclave (weight losses < 5% (w/w)) which possibly contributed to enhance the access of the enzyme to the hemicellulose and also to the release some sugars. For each residue, two different concentrations were tested (50 and 100 g/L) and the fermentation process was monitored by TLC (*data not shown*) to obtain a qualitative analysis of the hydrolytic products composition. Table 1 presents the XOS potential of each residue directly fermented by *T. viride* and *T. reesei*. The number and intensity of the bands observed in TLC were used as a qualitative indication about the DP and concentration of the compounds, respectively, and these aspects were used as criteria to evaluate the potential of each residue. In addition, the fermentation time associated with the observation of the first TLC bands was also considered in the screening and it is indicated in Table 1. For all residues, the increase in substrate concentration did not affect the time of the first TLC bands that could be associated with oligosaccharides production. Only the intensity of the bands was affected, being more intense for the highest concentration tested.

Banana peel, passion fruit peel and orange peel did not reveal potential for XOS production. It is known that these three agro-residues are rich in pectin materials, therefore they are preferentially used to produce pectic oligosaccharides (Mamma, Kourtoglou, & Christakopoulos, 2008; Oberoi, Sandhu, & Vadlani, 2012; Yapo & Koffi, 2008).

Chestnut shell did not reveal relevant potential for XOS production

Table 1
Potential of several residues evaluated for XOS production using *Trichoderma viride* and *Trichoderma reesei*.

Residue	XOS potential[a]		Time (days)[b]	
	T. viride	*T. reesei*	*T. viride*	*T. reesei*
Banana peel, passion fruit peel, orange peel	−	−	N.O[c]	N.O[c]
Chestnut shell	+	+	5	4
Chestnut shell thermally treated	−	−	N.O[c]	N.O[c]
Corn cobs	−	−	N.O[c]	N.O[c]
Vine pruning	−	+	N.O.[c]	5
Garlic straw	+ +	+ +	4	3
Onion straw	+ +	+ +	4	3
Coffee silver skin	+ +	+ +	6	5
Coffee pellets	+	+	6	5
Peanut shell	−	+	N.O[c]	6
Brewers' spent grains	+ + +	+ + +	3	2

[a] Symbol scale evaluation in which (−) represents no potential and (+ + +) high potential.
[b] Time corresponding to the first observation of TLC bands that could be associated with oligosaccharides production.
[c] Not observed (N.O.).

possibly due to the constraint that lignin presents to the access of xylanases. This residue is mainly composed by lignin (~45% (w/w)) containing only ~8% (w/w) of xylan, expressed as xylose (Maurelli, Ionata, La Cara, & Morana, 2013). The xylan–lignin complex usually present in the lignocellulosic biomass makes it resistant to enzymatic hydrolysis (Chapla et al., 2012). Therefore, it was expected that thermally treated chestnut shell could be a more interesting residue since the high temperatures applied in the industrial peeling process could probably open the lignin chains. However, for both fungi, no TLC bands suggesting the production of oligosaccharides were observed.

XOS production by direct fermentation of corn cobs was not observed. However, the TLC revealed the presence of a single and strong band in the xylose region after 7 days (*T. viride*) and 3 days (*T. reesei*) of fermentation. The production of high amounts of xylose is undesired, since it is associated to low production of oligosaccharides and entails the need for a more complex downstream process (Bian et al., 2014).

Vine pruning and peanut shell demonstrated no potential for XOS production by direct fermentation with *T. viride* but revealed low potential when *T. reesei* was used. This observation suggests some differences in the enzymatic complexes produced by these microorganisms. Furthermore, peanut shell presented significant mixing problems due to its morphology. Therefore, mass transfer issues and aeration limitation could occur and negatively affect enzyme production, namely xylanase.

Coffee pellets also revealed low potential for XOS production by direct fermentation, probably due to the high amount of total lignin present in their composition (51.0% (w/w) determined according to the methodology described in Section 2.3). Garlic, onion straw and coffee silver skin showed higher potential for XOS production than coffee pellets and the first TLC bands were observed in 3 or 4 days, depending on the microorganism. However, coffee silver skin, composed by approximately 17% (w/w) of hemicellulose and 29% (w/w) of lignin (Ballesteros, Teixeira, & Mussatto, 2014), showed significant mixing problems, such as those discussed before for peanut shell.

Among all residues evaluated, BSG was found to be the most attractive for XOS production by direct fermentation. This residue provided a higher number of bands associated with XOS production and lower concentration of xylose in the fermentation medium (low intensity band in TLC). Additionally, the first bands were observed in 2 or 3 days of fermentation. BSG is the most abundant by-product of the brewing industry, being generated after the wort manufacture. Furthermore, BSG is a low cost residue, being continuously produced along the year (Mussatto, Dragone, & Roberto, 2006). It is estimated that 30 million tons of BSG are annually produced worldwide (Niemi et al., 2012). For all these reasons, BSG was selected as the most promising residue and it was used in further optimization studies.

3.1.1. Chemical characterization of BSG

BSG is mainly composed by the outer husk, pericarp and seed coat layers of the barley grain (Mussatto et al., 2006). The chemical composition of BSG used in this work was determined by acid hydrolysis (Section 2.3) and the results obtained are presented in the Supplementary information (Table S1). BSG contains 16.5% (w/w) of hemicelluloses which in turn are mainly composed by xylan, 10.3% (w/w), and arabinan, 5.1% (w/w), but also present a small amount of acetyl groups, 1.1% (w/w). Therefore, the hydrolysis of BSG hemicelluloses probably results on the production of arabino-xylooligosaccharides (AXOS). In fact, the use of hydrothermally treated BGS has been described for the production of AXOS (Gómez, Míguez, Veiga, Parajó, & Alonso, 2015). Furthermore, AXOS have also been reported to exert prebiotic effects in the colon of humans and animals through selective stimulation of beneficial intestinal microbiota (Broekaert et al., 2011).

3.1.2. Microorganism selection for AXOS production by direct fermentation

T. reesei was found to be a faster producer of AXOS by direct fermentation of BSG than *T. viride*. Fig. 1A and B shows the TLC of the hydrolytic products obtained for both fungi. The TLC bands suggesting AXOS production were observed first for *T. reesei*. This evidence was also observed for other residues presenting AXOS potential (Table 1). According to Fig. 1, both microorganisms are able to produce oligosaccharides with DP > 3. However, 3 bands can be observed for *T. reesei* (Fig. 1B), which possibly indicates oligosaccharides with DP from 3 up to 5, while for *T. viride* (Fig. 1A) only 2 clear bands are observed in the DP = 4 and DP = 5 region. The retention factor of TLC bands corresponding to BSG fermentation products may differ slightly from the XOS standard used, since the oligosaccharides produced in this work will probably have a different since the oligosaccharides produced in this work will probably have a different composition when compared to the standards. For instance, it has been reported the production of AXOS using BSG (Gómez et al., 2015). In addition, the TLC results suggest that *T. reesei* (Fig. 1B) consumes firstly the glucose released from BSG during the sterilization process, while *T. viride* (Fig. 1A) produces AXOS before the total consumption of glucose. This qualitative observation was confirmed by HPLC. Table S2 (Supplementary) shows the most abundant monosaccharides detected during BSG fermentation. Supernatants collected after 3 and 6 days showed that free glucose, xylose and arabinose are produced from BSG when *T. viride* is used, while *T. reesei* consumes those monosaccharides. The sugar production yields (Y_{RS}) obtained for both fungi are presented in Fig. 1C. A continuous increase of the Y_{RS} is observed for *T. viride*, while *T. reesei* presents maximum Y_{RS} at 3 days of fermentation. The higher Y_{RS} obtained for *T. viride* after 4 days of fermentation is possibly due to the production of monosaccharides (Table S2). An increased production of monosaccharides is undesirable as it requires a more complex downstream process. *T. viride* is described as a cellulose-degrading fungus, secreting large amounts of β-glucosidase which could be used to degrade the BSG cellulose (Li et al., 2016). Moreover, the ratio of β-glucosidase in its enzyme complex is higher than that in the enzyme complex from *T. reesei* (Wagner, Schwarzenauer, & Illmer, 2013). This fact can explain the differences obtained for Y_{RS} and monosaccharide concentration of each fungus. Based on the results obtained, *T. reesei* was selected as the most promising microorganism and it used for posterior optimization studies.

3.2. Optimization of the direct fermentation of BSG by T. reesei

3.2.1. Substrate concentration

The effect of the substrate concentration on AXOS production through direct fermentation by *T. reesei* was studied using different amounts of BSG in 2% (w/v) Vogel medium pH 5.0, 30 °C and 180 rpm

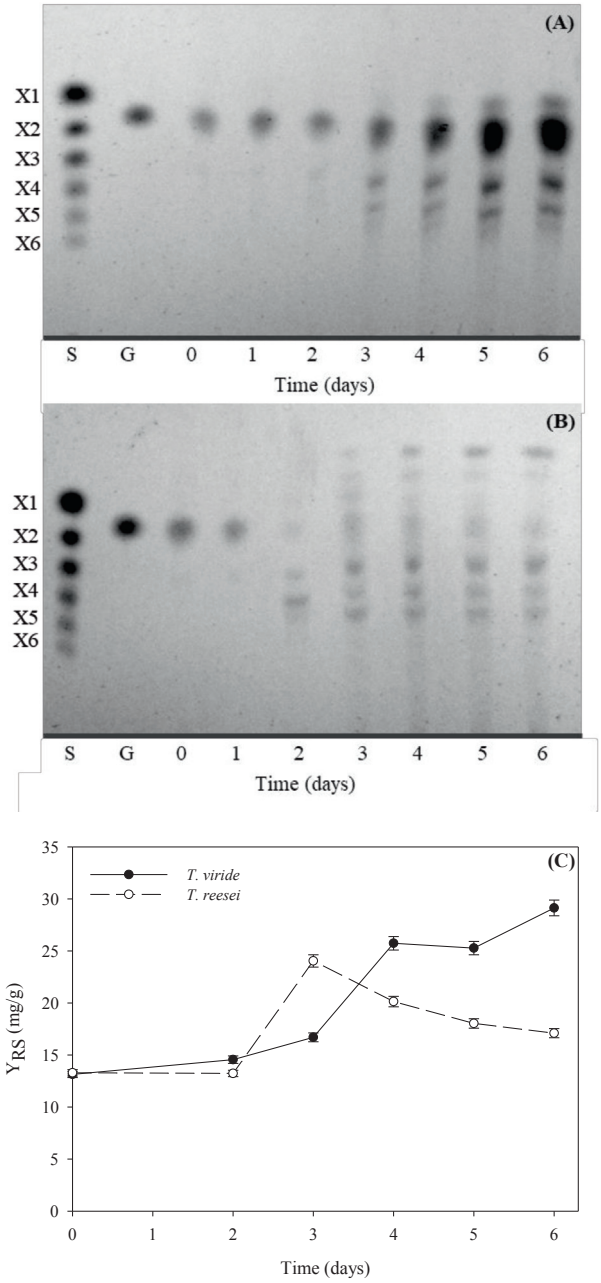

Fig. 1. TLC of the supernatants obtained from the direct fermentation of BSG by (A) *Trichoderma viride* and (B) *Trichoderma reesei* using 100 g/L of residue. Glucose (G, 2 gL/) and a mixture containing 2 g/L of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). (C) sugar production yield (Y_{RS}) obtained for *Trichoderma viride* and *Trichoderma reesei* using 10 g/L of BSG. The fermentation assays were performed at 30 °C and 180 rpm with BSG in 2% (w/v) Vogel media pH 5.0.

(Fig. 2A). In order to clearly illustrate the most significant differences, only the more relevant concentrations are represented in this figure. The maximum Y_{RS} was achieved after 3 days of fermentation for all BSG concentrations tested. Furthermore, the initial Y_{RS} values are similar for all substrate concentrations, except for 5 g/L. The difference observed for this concentration may be related with the sensitivity limit of the DNS method, which also explains the higher error associated to the Y_{RS} value at 0 days. The profile curves obtained for 5 and 10 g/L suggest a two-step metabolic behavior of *T. reesei*. First, the strain consumes sugars readily available in the medium and second, it degrades the residue to obtain more assimilative sugars. For concentrations of BSG up

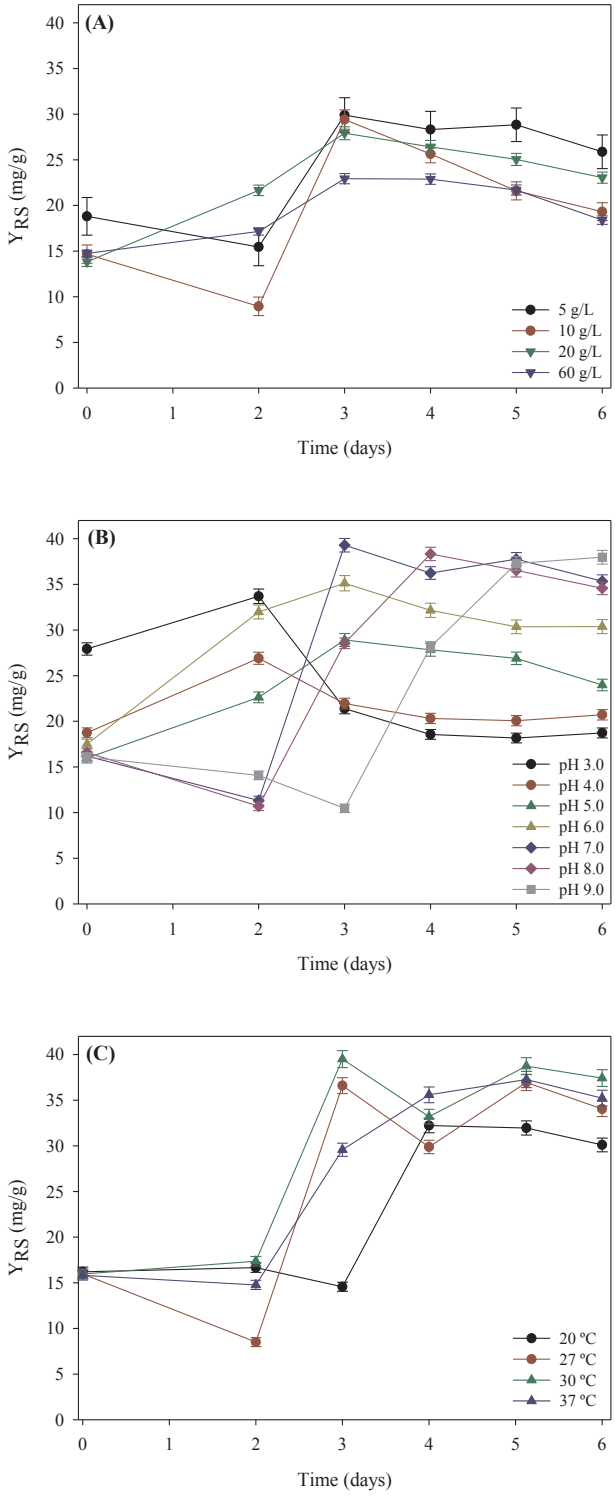

Fig. 2. Sugar production yield (Y_{RS}) obtained for *Trichoderma reesei* using: (A) different substrate concentration (5, 10, 20 and 60 g/L of BSG) at 30 °C, 180 rpm with BSG in 2% (w/v) Vogel media pH 5.0; (B) different pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) at 30 °C, 180 rpm and 20 g/L BSG in 2% (w/v) Vogel media; (C) different temperatures (20, 27, 30 and 37 °C) at 180 rpm and 20 g/L BSG in 2% (w/v) Vogel media pH 7.0.

to 20 g/L similar Y_{RS} values were obtained (29.9 ± 1.9, 29.4 ± 1.1 and 27.9 ± 0.7 mg/g for 5, 10 and 20 g/L of BSG, respectively). On the other hand, concentrations of BSG higher than 20 g/L had a negative impact on the Y_{RS}, reducing significantly its value (23.9 ± 0.6, 22.9 ± 0.6, 20.7 ± 0.5 and 19.4 ± 0.5 mg/g for 40, 60, 100 and

Table 2
Monosaccharide concentration and AXOS production yield, Y_{Xeq}, obtained by direct fermentation (20 g/L BSG, pH 7.0 and 30 °C) and using commercial enzyme preparation (20 g/L BSG, pH 4.5 and 40 °C).

Time (h)	Acid Hydrolysis[a]	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Y_{Xyl}[b] (mg/g)
Direct fermentation					
0	N	0.19 ± 0.01	N.O[c]	N.O[c]	N.O[c]
0	Y	0.55 ± 0.03	0.145 ± 0.009	0.060 ± 0.021	7.3 ± 0.5
72	N	N.O[b]	0.035 ± 0.012	0.033 ± 0.008	1.8 ± 0.6
72	Y	0.12 ± 0.01	0.800 ± 0.057	0.470 ± 0.053	40.0 ± 1.2
Commercial enzyme preparation					
0	N	0.08 ± 0.02	N.O[b]	0.08 ± 0.03	N.O[b]
0	Y	0.41 ± 0.08	N.O[b]	0.41 ± 0.06	N.O[b]
12	N	0.08 ± 0.03	0.06 ± 0.01	0.08 ± 0.02	3.2 ± 0.5
12	Y	0.72 ± 0.06	1.10 ± 0.08	0.72 ± 0.04	55.0 ± 1.9

[a] Without acid hydrolysis (N); with acid hydrolysis (Y).
[b] Ratio between the total amount of xylose (mg) and the amount of BSG (g).
[c] Not observed (N.O).

160 g/L of BSG, respectively). This evidence suggests possible inhibition by the substrate, but also mass transfer and aeration issues, possibly caused by an increase in the viscosity and density of the reaction mixture when higher concentrations of substrate are present (Akpinar et al., 2007). In this sense, and attending the sensitivity limit of both DNS and HPLC techniques, the optimal concentration of BSG selected was 20 g/L. The following optimization studies on the effect of pH and temperature were performed using this concentration. Additionally, xylanase production can also be affected by mixing problems associated with the use of high substrate concentrations since aeration limitation and agitation rate are important factor for the enzyme production by fungi (Lejeune & Baron, 1995). It is expected that variables which influence xylanase production may subsequently have an impact on XOS production. However, the optimal conditions for XOS production can be distinct from those for xylanase production, namely in terms of optimal time. For 20 g/L of BSG the maximal value of endo-xylanase activity (39.7 ± 4.1 U/mL) was achieved after 5 days of fermentation (Fig. S1, Supplementary). During the first 2 days the enzymatic activity remained at a basal level, corroborating the hypothesis of preferential consumption of the glucose released in the sterilization process. Furthermore, it is known that glucose can have a repression effect on xylanase production by *T. reesei* (Xiong, Turunen, Pastinen, Leisola, & von Weymarn, 2004).

3.2.2. Effect of pH and temperature

Extracellular pH and temperature are two of the several environmental factors affecting the growth of filamentous fungi and enzyme production (Häkkinen, Sivasiddarthan, Aro, Saloheimo, & Pakula, 2015). To study the pH effect on the Y_{RS}, 20 g/L of BSG were fermented by *T. reesei* at 30 °C using different pH from 3.0 up to 9.0 (Fig. 2B). The most suitable pH for the process was found to be pH 7.0, providing a Y_{RS} of 39.3 ± 0.7 mg/g after 3 days of fermentation. Interestingly, the curves corresponding to the neutral to alkaline pH values (7.0, 8.0 and 9.0) presented similar profiles. It is observed an initial decrease of the Y_{RS} probably due to the consumption of the sugars released by the sterilization process followed by a fast increase until reaching the maximum Y_{RS} value. Furthermore, the increase of the pH value led to an increase in the fermentation time associated to the maximum Y_{RS}. All the tested pH provided similar Y_{RS} at 0 days of fermentation, except pH 3.0. In this case, a higher value of Y_{RS} was obtained, suggesting that more sugars could be released from BSG during the sterilization process due to the acidification effect. The Y_{RS} variations observed for the several pH studied can probably be a consequence of the distinct enzymatic complexes produced by *T. reesei*. It is known that different types of enzymes, including different types of xylanases, can be produced by *T. reesei* depending on the pH of the medium (Häkkinen et al., 2015).

In order to study the effect of temperature on the Y_{RS}, 20 g/L of BSG were fermented by *T. reesei* at optimum pH (7.0) and using a temperature range from 20 °C up to 37 °C (Fig. 2C). The most suitable temperature found was 30 °C leading to a Y_{RS} of 39.5 ± 0.9 mg/g after 3 days of fermentation (Fig. 2C) temperature. At 37 °C it is observed a decrease of the Y_{RS} value possibly due to the negative effect of this temperature on *T. reesei* growth. The optimum growth temperature of *Trichoderma* fungi is in the range 20–28 °C (Chen, 2013). Moreover, temperature also has a significant effect on xylanase production. Irfan, Nadeem, and Syed (2014) observed a decrease on xylanase activity at temperatures > 30 °C for *T. viride* in solid-state fermentation. Maximal xylanase activity (59.4 ± 4.8 U/mL) for optimal pH (7.0) and temperature (30 °C) was observed after 5 days of fermentation (Fig. S2, Supplementary).

3.2.3. Quantification of AXOS produced under optimal conditions

The fermentation supernatant containing the AXOS produced under the optimal conditions (20 g/L of BSG, pH 7.0 and 30 °C) was quantified before and after acid hydrolysis to evaluate the fraction of free monosaccharides and the fraction of monosaccharides associated to the oligosaccharides composition. Table 2 shows the concentration of glucose, xylose and arabinose present in the supernatant obtained at 0 h and at the optimal time (72 h), with (Y) and without acid hydrolysis (N). Additionally, Y_{Xeq} (mg/g) in terms of equivalent of xylose per gram of BSG is also presented. Glucose was the only free sugar released from BSG during the sterilization process, as indicated by the TLC analysis (Fig. 1B). However, other oligosaccharides, such as gluco-oligosaccharides, were possibly also extracted from the residue. This fact may explain the increase of approximately 3-fold of the glucose amount after acid hydrolysis and also the presence of xylose and arabinose in lower amounts. The total consumption of glucose by the microorganism after 72 h of fermentation was confirmed. This is one of the main advantages of the direct fermentation approach using *T. reesei*, i.e. allowing the simplification of the downstream process, which generally represents up to 80% of the total entire production costs (Urmann, Graalfs, Joehnck, Jacob, & Frech, 2010). Moreover, the presence of a minor amount of free xylose (2.5%) and arabinose (2.4%) was found in the fermentation broth. The Y_{Xeq} obtained for the optimized process was 38.3 ± 1.8 mg/g. The TLC analysis (*data not shown*) suggested a DP between 2 and 5. This range of DP was also suggested by HPLC analysis (Section 2.8), after partial purification of the fermentation supernatant with activated charcoal (Section 2.7). The direct quantification by HPLC was not possible, since retention times of the produced AXOS were distinct from the ones presented by pure XOS standards.

In summary, AXOS composed mainly by xylose and arabinose were produced through direct fermentation of BSG with *T. reesei*.

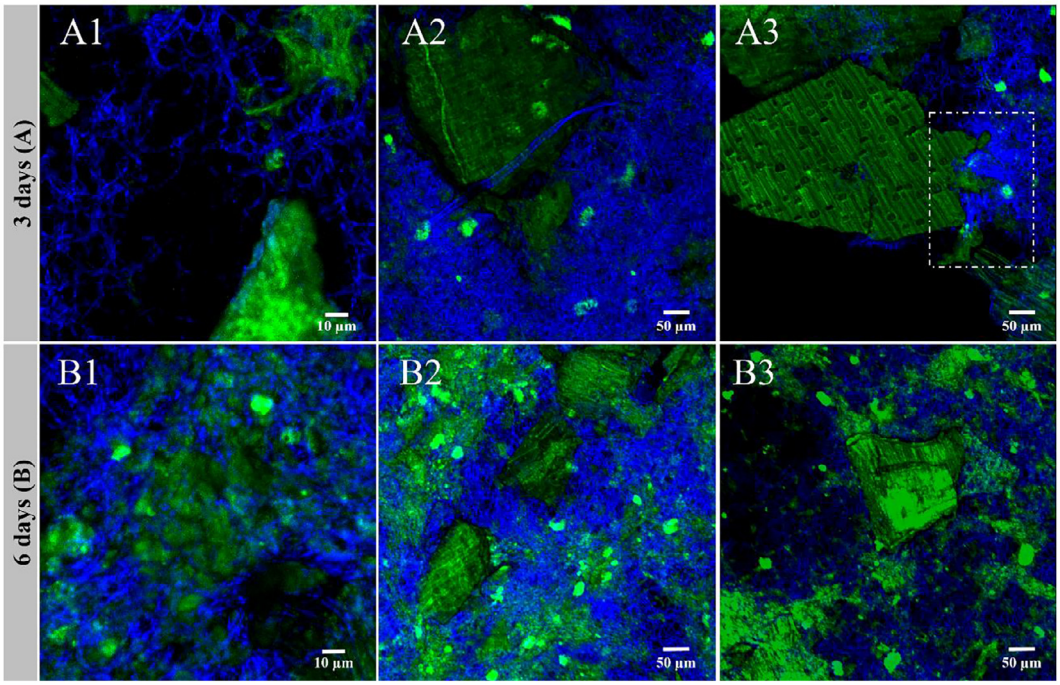

Fig. 3. Confocal Laser Scanning Microscopy images showing BSG (green channel) colonized by hyphae (blue channel) of *Trichoderma reesei* at (A) 3 days and (B) 6 days of fermentation under optimal operational conditions: 20 g/L of BSG, pH 7.0 and 30 °C. Dashed area shows the fungi adhesion to the intricate surface of BSG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.4. CLSM analysis of the cultivated BSG with T. reesei

BSG was directly fermented by *T. reesei* under optimal conditions and samples corresponding to 3 days (Fig. 3A1 to A3) and 6 days of fermentation (Fig. 3B1 to B3) were collected and prepared for Confocal Laser Scanning Microscopy (CLSM) analysis as described in Section 2.12. The labeling of the hyphae with the Calcofluor White allowed clear identification of the filamentous mycelia which greatly contrasted with the BSG auto fluorescence (Fig. 3). BSG without fungal cultivation was used as control to analyze the influence of the signal (*data not shown*). For all samples, the structural differences between the fungal mycelia and the BSG could be distinguished, through the particular pattern of the Calcofluor staining and the associated fluorescence signal. Fully resolved structures with high fluorescent signals compared to the background noise were observed and the fungal morphology was very clear. In general, it was perceptible the presence of a higher amount of BSG in the 3 days sample when compared with the 6 days sample. This observation confirms the residue degradation by *T. reesei* during the fermentation. However, cell wall damages in BSG were not visible. CLSM also showed the spatial distribution of the fungal networks on the surface of BSG revealing their intricate interaction with the residue, which probably enabled physical anchorage of the fungal mycelium and provided protection against its removal by mechanical forces such as shear stress resultant from the media agitation. In particular, this evidence is noticeably observed inside the dashed region on Fig. 3A3.

3.3. One-step strategy versus commercial enzyme

In order to compare the One-step fermentation with the use of commercial enzymes, a range between 0.02 U/mL and 6 U/mL of commercial xylanase from *T. longibrachiatum* was incubated with 20 g/L of BSG under the optimal conditions described in Section 2.6. BSG in sodium acetate buffer was sterilized before incubation with the enzyme The Y_{RS} profiles obtained by enzymatic hydrolysis were significantly different from those obtained for direct fermentation (Fig. S3, Supplementary). These differences are mainly associated with the fungal metabolism behavior, since, as expected, the consumption of the initial sugars present in the media was not observed with the enzymatic hydrolysis. In general, after 12 h of reaction the maximum Y_{RS} value seems to be achieved. However, the increase of the enzyme concentration led to the reduction of the time needed to reach the maximum Y_{RS}. In addition, the increase of the enzyme concentration up to 2 U/mL also resulted in the increase of the maximum yield obtained. However, for 2 U/mL and 6 U/mL the maximum Y_{RS} obtained (59.0 ± 1.9 mg/g and 61.3 ± 1.9 mg/g, respectively) was similar, which suggests that the substrate starts to be the limiting variable for concentrations higher than 2 U/mL. Therefore, 2 U/mL and 12 h were found to be the optimal enzyme concentration and time for BSG hydrolysis by commercial xylase from *T. longibrachiatum*. The Y_{Xeq} value at 12 h of reaction was determined before and after acid hydrolysis to evaluate the fraction of free monosaccharides. Table 2 shows the concentration of glucose, xylose and arabinose, and also the Y_{Xeq} obtained for the reaction mixture supernatant at 0 h and 12 h. Similarly to direct fermentation, AXOS mainly composed by xylose and arabinose were produced by enzymatic hydrolysis of BSG. The Y_{Xeq} achieved at 12 h (52.0 ± 2.4 mg/g), is approximately 1.36 times higher than that obtained by direct fermentation (38.3 ± 1.8 mg/g). The amount of free xylose (2.4%) is also similar to that obtained by direct fermentation. However, higher amount of arabinose 3.1% and glucose 3.1% is present in the reaction mixture after 12 h of enzymatic hydrolysis. These fractions were calculated with respect to the total sugars production after acid hydrolysis. Similarly to direct fermentation, AXOS with DP between 2 and 5 were produced using the commercial enzyme.

It should be noted that two negative controls were performed in parallel, without inoculum or enzyme addition, in order to ensure that the AXOS production was exclusively due to the microorganism metabolism or enzymatic hydrolysis, respectively. Both negative controls presented relatively stable values of total reducing sugars along time, which may exclude the possible interference of the incubation process itself on AXOS production (data not shown).

Taking into account the cost of purchasing commercial enzymes, the proximity of the Y_{Xeq} values, the equivalence of DP obtained, as well as

the amount of free monosaccharides produced, it seems that the direct fermentation of BSG can be a promising production approach for XOS production. Table S3 (Supplementary) shows the yields of XOS produced (mg) per gram of xylan, $Y_{XOS/xylan}$ (mg/g), reported in the literature for enzymatic hydrolysis processes. The Y_{Xeq} (mg/g) obtained in this study by direct fermentation (38.3 ± 1.8 mg/g) and by enzymatic hydrolysis (52.0 ± 2.4 mg/g), were converted to $Y_{XOS/xylan}$ (mg/g), considering the BSG composition in xylan (Section 3.1.1) and the stoichiometric correction factor to account for the molecular weight gain during acid hydrolysis as described in NREL protocols.

The highest yield present in Table S3 (Supplementary) was reported by Reddy and Krishnan (2016) using a β-xylosidase-free xylanase from *B. subtilis* to hydrolyze pretreated sugarcane bagasse during 30 h. This same residue was used by Bragatto, Segato, and Squina (2013) and Bian et al. (2013) presenting lower yields but shorter production times (8h and 12 h, respectively). The yield obtained in the current work for the commercial xylanase was higher than those reported in Table S3 (Supplementary), except for the yield achieved by Reddy and Krishnan (2016). On the other hand, the yield associated to direct fermentation was lower than the those presented by Bragatto et al. (2013), Faryar et al. (2015) and Reddy and Krishnan (2016), but similar to the yield reported by Azelee et al. (2016). Depending on the substrate and the specificities of the production process, the production yield seems to vary widely, between 100 mg/g and 600 mg/g. Interestingly, several authors reported similar yield values, in a range of 90–114 mg/g (Samanta et al., 2012, 2014; Seesuriyachan, Kawee-ai, & Chaiyaso, 2017), although using different residues and different xylanases sources. Despite these facts, it is important to highlight that the $Y_{XOS/xylan}$ do not represent the overall production process yield, since the contribution associated to the residues pretreatment and/or xylan extraction is not considered in Table S3 (Supplementary), and it will significantly decrease the yield of the production process. For instance, Faryar et al. (2015) reported one of the highest Yxos/xylan values, however if the yield of xylan extraction is considered, the overall process yield will be 20.3 mg of XOS per gram of wheat straw, which is lower than the yield reported in this work for direct fermentation (38.3 ± 1.8 mg/g) and enzymatic hydrolysis (52.0 ± 2.4 mg/g).

Direct fermentation presents a higher production time, however it should be noted that the time reported in Table S3 (Supplementary) for the references from the literature does not include the duration time of the pretreatment, neither the time associated to the enzyme production when it is not purchased. For instance, Bian et al. (2013) used crude xylanase extract to hydrolyze pretreated sugarcane bagasse during 12 h, however 7 days of cultivation were needed to obtain the xylanase crude extract.

In summary, given all the reasons above, the proposed approach in this is a promising strategy for the production of XOS using sterilized raw BSG (without conventional pretreatment), either by direct fermentation of *T. reesei* or by the use of a commercial xylanase.

4. Conclusions

The current study clearly highlights the potential of using an agro-industrial reside, BSG, and direct fermentation by *T. reesei* to produce AXOS, a prebiotic functional food ingredient. Comparing the use of direct fermentation with the application of commercial enzymes, it was concluded that direct fermentation is an attractive and advantageous approach to hydrolyze BSG and produce AXOS. Further optimization of the bioprocess operation mode to deal with substrate inhibition and its scale-up are foreseen. Additionally, the AXOS produced should be evaluated regarding their chemical structure and prebiotic activity.

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Author declaration

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.080.

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