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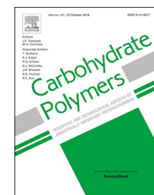

Single-step production of arabino-xylooligosaccharides by recombinant *Bacillus subtilis* 3610 cultivated in brewers' spent grain

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ABSTRACT

Brewers' spent grain (BSG) is an inexpensive and abundant brewery by-product that can be used to produce prebiotic arabino-xylooligosaccharides (AXOS). In this study, *Bacillus subtilis* 3610 was used, for the first time, to produce AXOS through direct fermentation of BSG. Additionally, the microorganism was genetically modified to improve the AXOS production. The xylanase gene *xyn2* from *Trichoderma reesei* coupled with a secretion tag endogenous to *B. subtilis* was cloned in pDR111 and integrated into its chromosome. After optimization by experimental design, AXOS with a degree of polymerization ranging from 2 to 6 were obtained. The maximum production yield expressed in xylose equivalents per amount of BSG (54.2 ± 1.1 mg/g) represents an increase of 33% comparing to the wild type. When compared with the enzymatic hydrolysis process, single-step fermentation with *B. subtilis* proved to be a very promising low-cost strategy for the simultaneous production of AXOS and valorization of BSG.

1. Introduction

The prebiotics global market is expected to be driven by an increasing demand of functional food ingredients, as consumers pay more attention to their health and well-being (Antov & Đorđević, 2017). Xylooligosaccharides (XOS) are one type of prebiotics that has been the focus of several studies given their different beneficial effects on several physiological functions of human and animal health (Aachary, Gobinath, Srinivasan, & Prapulla, 2015; Gibson et al., 2017). XOS are also considered highly price competitive due to its minimal recommended daily intake (2.1 g) compared to other prebiotics (Frost & Sullivan, 2015). Additionally, they present acceptable organoleptic properties, temperature and acidic stability, hence making them potential food ingredients (Courtin, Swennen, Verjans, & Delcour, 2009).

XOS are oligomers built from xylose residues linked through (β1,4)-linkages (Kumar & Satyanarayana, 2011). The main chain of xylose can be branched with several side substituents, namely acetyl groups, arabinose, glucuronic acids, and galactose residues (Coelho, Rocha, Moreira, Domingues, & Coimbra, 2016).

These compounds can be sourced from lignocellulosic biomass, such as agro-residues, which are an abundant and renewable resource (Samanta et al., 2012). They are obtained though the hydrolysis of xylan, the main constituent of hemicelluloses (Bian et al., 2013). XOS can be produced chemically, through auto-hydrolysis, enzymatic hydrolysis or a combination thereof (Carvalho, Neto, Silva, & Pastore, 2013). While the chemical or auto-hydrolytic processes originate undesired by-products (Yang, Xu, Wang, & Yang, 2005), including toxic compounds such as hydroxymethylfurfural (HMF) and furfural, the use of enzymes presents high efficiency and specificity, allowing a higher control over the degree of polymerization (DP) and lower costs associated with the downstream processes (Bian et al., 2014). Moreover, the enzymatic process does not require the use of noxious chemicals, being a more environment-friendly approach (Antov & Đorđević, 2017). Since xylan is generally present as a xylan-lignin complex in the lignocellulosic biomass (Samanta et al., 2012), XOS are mainly produced by a combination of methods (Carvalho et al., 2013), including a first step of fractionation of the lignocellulosic material to obtain soluble xylan followed by its hydrolysis by xylanolytic enzymes (Rico, Gullón,

Abbreviations: BSG, brewers' spent grain; AXOS, arabino-xylooligosaccharides; XOS, xylooligosaccharides; DP, degree of polymerization; Y_{RS}, sugar production yield; Xeq, equivalent xylose; Y_{Xeq}, production yield expressed in terms of xylose equivalents; Xyl, xylose; Xyl_{subst}, substituted xylose residues; Y_{Xyl}, free xylose yield; Y_M, monosaccharides production yield

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Alonso, Parajó, & Yáñez, 2018). The low yields associated to the xylan extraction step in addition to the cost of producing or purchasing commercially available xylanases, may compromise the economic viability of the production process (Reddy & Krishnan, 2016).

Brewers' spent grain (BSG) represents the most abundant lignocellulosic by-product of the brewing industry (Steiner, Procopio, & Becker, 2015) and it has been used as raw material for the production of arabino-xylooligosaccharides (AXOS) (Coelho, Rocha, Saraiva, & Coimbra, 2014). We previously found that a single step bioprocess to produce AXOS through the direct fermentation of BSG by *Trichoderma* strains (Amorim, Silvério & Rodrigues, 2018). Despite the undeniable advance of using an integrated process for XOS production, a considerably high production time (3 days) was reported. The development of a single-step process using bacterial strains as biocatalysts can be a suitable alternative to decrease the fermentation time and improve the XOS production. *B. subtilis* is a major industrial microorganism with high potential for XOS production (Subramaniyan & Prema, 2002).

This work describes, for the first time, the optimization of AXOS production from non-pretreated BSG in a single step using the wild type *Bacillus subtilis* 3610. Moreover, to further improve the production yield, a mutant *B. subtilis* 3610 strain expressing the xylanase gene from *Trichoderma reesei* xyn 2 was constructed.

2. Experimental

2.1. Materials

All chemicals, media and media components were of analytical grade obtained from Sigma-Aldrich Chemical Ltd., unless specified otherwise. The brewer's spent grains (BSG) were kindly supplied by Super Bock Group (Portugal). This residue contains 22.3 ± 0.6% (w/w) of insoluble lignin, 4.7 ± 0.3% (w/w) of acid soluble lignin, 16.9 ± 0.4% (w/w) of extractives (which include non-structural sugars, inorganic material, nitrogenous material, among others), 19.2 ± 0.5% (w/w) of proteins, 17.5 ± 0.3% (w/w) of cellulose, 4.54 ± 0.04% (w/w) of ashes and 16.5 ± 0.7% (w/w) of arabinoxylan with 10.3 ± 0.2% (w/w) of xylose (Amorim et al., 2018). This chemical composition is in accordance to Vieira et al. (2014), who used BSG obtained from the same brewery.

2.2. Bacterial strains, plasmids and standard media for cloning

The TG1 chemically competent *Escherichia coli* cells were used as host for the recombinant plasmid propagation. *B. subtilis* 3610 used for sub-cloning and expression, and the integration vector pDR111 were provided by Prof. Alan Grossman, MIT. This plasmid contained a ribosome binding site sequence specific for *Bacillus*, a lac operator/bacteriophage SPO1 promoter (Yansura & Henner, 1984), a starch utilization loci (*amy*E) for double cross-over into the *B. subtilis* chromosome, as well as ampicillin and spectinomycin selection markers (Britton et al., 2002).

Positive *E. coli* clones were selected in Luria-Bertani (LB) agar medium (Difco, New Jersey, USA) containing ampicillin 100 µg/mL and were further sequenced (GENEWIZ Inc., New Jersey, USA) to confirm the absence of mutations. *Bacillus* transformants were firstly selected in LB agar medium with spectinomycin 100 µg/mL and then, screened for integration on a starch plate with wescodyne to better visualize clear halos around the colonies. An *amy*E + control was prepared in parallel.

2.3. Cloning the xylanase gene from T. reesei in B. subtilis

DNA was manipulated using standard procedures. GenCatch™ kit from Epoch Life Science (USA) was used for DNA gel extraction and ZymoPURE™ plasmid miniprep kit from Zymo Research (USA) was used for plasmid DNA extraction.

Two *Bacillus* clones were built, namely clone 1 containing the xylanase gene *xyn2* from *T. reesei* (Genbank accession number X69574.1) and clone 2 containing a secretion tag endogenous to *B. subtilis* (UniprotKB A0A0S2II12|) coupled to xylanase *xyn2* N-terminus site. The Tag-*xyn2* DNA fragment was synthetized by Integrated DNA Technologies Inc., Coralville (USA). The DNA fragments were then ligated into the pDR111 vector by Gibson Assembly (Gibson et al., 2009) using the primers presented in Appendix A, Table A1 (in Supplementary material). The primers were designed to allow a ligation that excluded the *lacI* repressor sequence from the pDR111 vector. In the absence of *lacI* repressor the heterologous expression becomes constitutive (Yansura & Henner, 1984).

E. coli and *B. subtilis* were transformed by heat shock and natural competence, respectively. The positive colonies were confirmed by colony PCR.

2.4. Fermentation pre-inoculum

One colony of *B. subtilis* grown on LB agar plate overnight at 37 °C was picked and transferred to 2 mL of LB medium. Spectinomycin (100 µg/ml) was added when required for clones selection. Then, the cells were cultured at 37 °C and 250 rpm during approximately 2 h until reaching an OD at 600 nm (OD_{600}) around 1.0. This starter culture was further diluted to an initial OD_{600} around 0.020 into fermentation media.

2.5. Fermentation of BSG with B. subtilis wild type: medium selection, sterilization effect and optimization of pH, temperature and BSG concentration

The 250 mL flasks were filled with 25 mL of different media, namely LB (Bertani, 1951), in 2% (v/v) of Vogel's 50x salts (Vogel, 1956), M9 (Miller, 1972), and $S7_{50}$ (Jaacks, Healy, Losick, & Grossman, 1989) at pH 7.0 containing 20 g/L of BSG (Section 3.1.1). The solid-liquid mixture was sterilized at 121 °C during 15 min. The cells (Section 2.4) were cultured at 37 °C and 150 rpm during 36 h.

After selecting the Vogel medium, the sterilization effect on the fermentation process was evaluated comparing, non-sterile BSG and BSG sterilized by both UV and autoclave. For non-sterile condition, BSG (20 g/L) was added to Vogel medium previously sterilized by filtration (0.2 µm membrane). For UV sterilization, BSG was treated with an UV lamp during 60 min at a distance of 30 cm and further added to filter-sterilized Vogel medium (20 g/L BSG). These conditions were compared with BSG-Vogel mixture autoclave-sterilized (121 °C, 1 atm, 15 min). All the media were inoculated with *B. subtilis* wild type (Section 2.4). In parallel, 3 controls were prepared under the same tested conditions but without inoculum.

A preliminary assessment of the individual effects of substrate concentration (10, 20, 40 and 60 g/L BSG), initial pH (5.0, 6.0, 7.0 and 8.0) and temperature (30, 35, 37, 40, 45, 50 and 60 °C) was performed during 32 h using *B. subtilis* wild type. The optimum condition selected was then used to compare the performance of wild type with clone 1 and 2.

2.6. Optimization of XOS production by single-step fermentation of BSG with B. subtilis clone 2

A Box–Behnken experimental design was used to study the effects of substrate concentration, pH and temperature during 32 h of fermentation of BSG by the *B. subtilis* clone 2. The STATISTICA 7 software was used for the generation, analysis and optimization of the experimental design. The sugar production yield, Y_{RS} (mg/g), determined as the ratio between total reducing sugars (mg) and the mass of BSG (g) used in the fermentation, was defined as the response variable. The experimental design contained three blocks and a central point with three replicates (a total of 15 experiments). The ranges used for each variable are provided in Table 1.

Table 1
Experimental design performed to evaluate the effect of BSG concentration, pH and temperature (T) on the sugar production yield (Y_{RS12h}) at 12 h obtained for the fermentation of BSG by *Bacillus subtilis* clone 2.

Run	Independent variables			Dependent variable
	BSG (g/L) X1	pH X 2	T (°C) X3	Y_{RS12h} (mg/g) Y1
1	15	6.5	45	29.35
2	25	6.5	45	27.89
3	15	7.5	45	28.63
4	25	7.5	45	26.77
5	15	7.0	43	27.90
6	25	7.0	43	28.36
7	15	7.0	47	20.92
8	25	7.0	47	27.38
9	20	6.5	43	23.64
10	20	7.5	43	22.11
11	20	6.5	47	20.94
12	20	7.5	47	25.56
13	20	7.0	45	35.01
14	20	7.0	45	34.98
15	20	7.0	45	35.34

2.7. Analytical methods

For all the fermentation assays, samples from the supernatant were collected, centrifuged and further analyzed.

2.7.1. Reducing sugars analysis and xylanase activity

The DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) was used to quantify the total reducing sugars. The microorganisms xylanase activity was accessed using a soluble chromogenic substrate according to the supplier (Megazyme) guidelines for azo-xylan from birchwood.

2.7.2. AXOS quantification by acid hydrolysis

The AXOS produced under optimum conditions were quantified by HPLC in terms of equivalent xylose (Xeq) after acid hydrolysis with H_2SO_4 at a final concentration of 4% (v/v) according to (National Renewable Energy Laboratory) protocols (NREL/TP-510-42618-42622-4218). Note that the fermentation supernatant was analyzed by HPLC before and after acid hydrolysis to determine the amount of free xylose and also the xylose contained in the oligosaccharides. For that purpose, an HPLC (Knauer, Germany) fitted with Knauer-RI detector and an Aminex HPX 87H column (300 mm x 7.8; Biorad, USA) were used for quantification. The 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.

The XOS production yield, Y_{Xeq} (mg/g), expressed in terms of Xeq was determined as the ratio between the xylose (mg) present in the AXOS and the mass of BSG (g) used in the fermentation.

2.7.3. Qualitative analysis of AXOS using TLC

TLC silica gel plates (DCAlufolien Kieselgel 60, Merck) were used for qualitative analysis of the AXOS composition obtained from the BSG. The supernatant samples 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). A mixture of xylose and XOS (X2–X5, 2 g/L each) was used as standard.

2.7.4. AXOS purification

AXOS produced by fermentation of BSG under optimal conditions were treated with activated charcoal. 20 mL of the supernatant were collected, centrifuged and loaded to 13 g of activated charcoal. After the adsorption step the charcoal was conveniently washed with MiliQ water to remove the salts and other non-adsorbed components. 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. The oligosaccharides partially purified by activated charcoal were separated by HPLC (JASCO, Japan) fitted with ELSD detector (SEDERE, Sedex 85, France) and a Prevail Carbohydrate column (250 mm × 4.6 mm; Grace, USA) was 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. AXOS with different DP were individually collected and further analyzed by GC–MS (Section 2.7.5)

2.7.5. AXOS glycosidic linkage analysis

The AXOS were methylated using CH_3I, acid hydrolysed, reduced, and acetylated. The partially methylated alditol acetates were analyzed by GC–MS (Coelho et al., 2016). The AXOS DP was obtained by the calculation of the relative amount of total xylose divided by the amount of terminally linked xylose. This mode of estimation is based on the principle that xylose (Xyl) does not occur as AX branching residues. The branching degree was calculated as the ratio between the branching points in substituted Xyl residues (Xyl_{subst}) and the total amount of Xyl (Xyl_{total}): [Xyl_{subst}/Xyl_{total}], where Xyl_{subst} is the sum of the amount of monosubstituted residues (2,4- Xyl + 3,4-Xyl) + twice the amount of disubstituted residues (2,3,4-Xyl).

3. Results and discussion

3.1. Optimization of the production of AXOS by single-step fermentation of BSG using the B. subtilis wild type

3.1.1. Medium selection for AXOS production

Different culture media were tested for the production of AXOS by single-step fermentation of BSG with *B. subtilis* 3610 wild type, namely a complex medium (LB) and three minimal media (Vogel, M_9 and $S7_{50}$) (Fig. 1). Envisioning an economically sustainable process, the use of minimal media is preferable over complex ones. However, LB fermentations allow comparing the performance of minimal media with a scenario in which the expectable cell growth would be promoted in a higher extent.

For all minimal media, the Y_{RS} values at 0 h correspond to the reducing sugars released from BSG during the sterilization process (Fig. 1). These sugars were determined using the DNS method, which

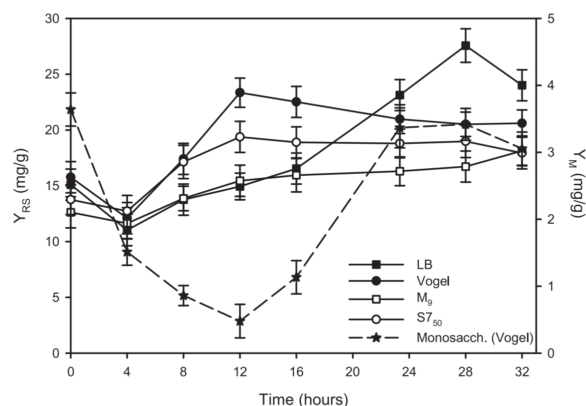

Fig. 1. Sugar production yield (Y_{RS}) obtained for the single-step fermentation of BSG by *Bacillus subtilis* 3610 using different culture media (LB, Vogel, M_9 and $S7_{50}$) at pH 7.0, 37 °C and 150 rpm, and monosaccharides production yield (Y_M) obtained for Vogel medium. Results represent the average of three independent assays ± standard deviation.

does not allow to discriminate the sugars present in the mixture (Section 2.7). Therefore, HPLC analysis was used as a complementary method to identify and quantify the free monosaccharides present in the culture media. It was found that xylose is the main free sugar released in all the minimal media at 0 h (74.2, 80.1 and 72.8 mg/L for M_9, $S7_{50}$ and Vogel, respectively), but also some glucose and arabinose can be found.

Vogel medium provided the highest maximum value of Y_{RS} (Y_{RSmax}) in the lowest period of time. Also, for this medium it is particularly notorious a two-step metabolic behavior of *B. subtillis* wild type, including an initial decrease of the Y_{RS} (0–4 h), which may probably be caused by the consumption of released xylose followed by an increase of the Y_{RS} values until the maximum value is reached at 12 h. Interestingly, at 12 h the production yield of monosaccharides (Y_M) is significantly low (0.48 ± 0.25 mg/g) (Fig. 1), suggesting that the Y_{RS} value mainly comprise the oligosaccharides, namely AXOS. Thus, it seems that there is an optimum time point around 12 h of fermentation in which complex sugars are accumulated and it is framed, firstly by the consumption of the free sugars in the media (0 h–4 h) and secondly by the degradation of the produced oligosaccharides (16 h–32 h), as suggested by the increase of free monosaccharides in this same period (Fig. 1).

The LB medium presented the highest Y_{RSmax} (27.6 ± 1.5 mg/g) at 28 h. This medium contains higher amounts of assimilative sugars when compared to the minimal media, which could possible lead to a higher cell concentration and subsequently to the highest Y_{RSmax}, however requiring more time to consume all the initial available sugars.

Bearing in mind the process sustainability and based on the Y_{RSmax} values, the Vogel medium was selected for further studies.

3.1.2. Sterilization effect on AXOS production by single-step fermentation

All media studied in the previous section (Section 3.1.1) were prepared by adding BSG to the culture medium followed by a sterilization at 121 °C, 1 atm during 15 min. This sterilization may bear some similarity to a thermal pretreatment of lignocellulosic biomass, namely liquid hot water and steam explosion. However, it operates at milder conditions compared to these pretreatments, which generally require 160–240 °C and 1–30 atm for few seconds to few minutes (Behera, Arora, Nandhagopal, & Kumar, 2014).

To better understand the role of the sterilization on the production of AXOS from BSG by *B. subtilis* 3610, 3 different conditions were studied, namely the use of BSG non-sterile, and BSG sterilized by both UV and autoclave. In parallel, 3 controls were performed under the same conditions but without inoculum (Fig. 2).

As expected, the sterilization by autoclave led to the release of sugars into the medium, which possibly stimulated the biomass growth, resulting in the highest Y_{max} (23.4 ± 1.3 mg/g) achieved at the lowest time observed (12 h). It was found that even without this initial amount of sugars in the medium, bacteria can grow using BSG as the only carbon source (BSG sterilized by UV) although taking more time to achieve the highest yield (28 h). Interestingly, the difference between Y_{RSmax} and Y_{RSmin} is comparable for BSG sterilized by UV (17.5 mg/g) and by autoclave (11.3 mg/g), thus suggesting that the microorganism metabolism itself can perform similarly using both processes.

For non-sterile control, it was possible to observe a slight increase of Y_{RS} at 24 h, followed by a decrease at 32 h, which may be due to the activity of the original microflora present in BSG. Therefore, it seems clear that a sterilization process will be essential and cannot be excluded. On the other hand, the profile curve of non-sterile control and UV control suggest that the incubation process itself contribute to the sugars release into the medium (0–4 h). Nonetheless, it should be noted that the sugar concentrations associated to the lowest Y_{RS} values are close to the lower sensitivity limit of the DNS method.

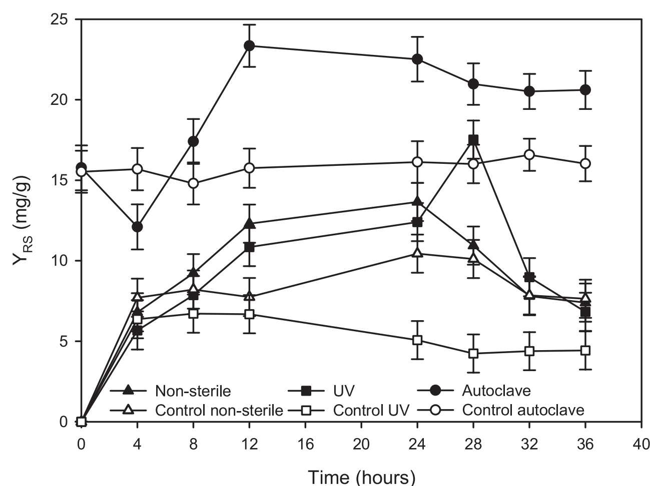

Fig. 2. Sugar production yield (Y_{RS}) obtained from the single-step fermentation of BSG by *Bacillus subtilis* 3610 in Vogel medium at pH 7.0, 37 °C and 150 rpm, using different methods for the BSG sterilization: non-sterile, UV and autoclave. The corresponding controls were performed under the same conditions but without inoculum. Results represent the average of three independent assays ± standard deviation.

3.1.3. Optimization of the BSG concentration, pH and temperature for the production of AXOS

The concentration of BSG, pH and temperature were separately studied to evaluate their individual effect on the production of AXOS by single-step fermentation of BSG using the *B. subtilis* 3610 wild type (Fig. 3).

The Y_{RSmax} was achieved after 12 h of fermentation for all BSG concentrations tested (Fig. 3A). Moreover, except for 60 °C (Fig. 3C), all the profile curves obtained suggest a two-step metabolic behavior of *B. subtilis* as previously described (Section 3.1.1). When 10 g/L and 20 g/L of BSG were used, similar Y_{RSmax} values were obtained (21.4 ± 1.5 and 22.1 ± 1.3 mg/g, respectively). However, for 10 g/L BSG the concentrations of sugars measured are close to the low sensitivity limit of the DNS method. On the other hand, concentrations of BSG higher than 20 g/L had a negative impact on the Y_{RSmax}, reducing significantly its value (19.5 ± 1.2 and 15.8 ± 0.9 mg/g for 40 and 60 g/L of BSG, respectively). This effect could possibly be caused by different phenomena. On one hand, due to substrate inhibition and on the other hand, mass transfer and aeration issues (Aachary & Prapulla, 2009; Helianti et al., 2016) as consequence of the increase of viscosity and density of the reaction mixture (Figueiredo, Carvalho, Brienzo, Campioni, & Oliva-Neto, 2017; Moteshafi, Hashemi, Mousavi, & Mousivand, 2016).

In this sense, and taking into account the low sensitivity limit of both DNS and HPLC methods, 20 g/L was selected as the optimum concentration of BSG to perform the following studies on the effect of pH and temperature.

The most appropriate pH was found to be pH 7.0, leading to a Y_{RSmax} of 23.2 ± 0.7 mg/g after 12 h of fermentation (Fig. 3B). Interestingly, the curves corresponding to the neutral to alkaline pH values (7.0 and 8.0) presented similar profiles. It has been reported that alkaline conditions can improve the efficiency of the enzymatic reactivity towards the carbohydrates present in lignocellulosic biomass (Kim & Holtzapple, 2005).

However, the optimum pH for this fermentation process would entail a compromise between the pH conditions more favorable for sugars extraction (Amorim et al., 2018) and the ones more suitable for bacteria growth and xylanase production. Other authors also reported an optimum pH around 7.0 for *B. subtilis* xylanase production, using different substrates (Ho & Heng, 2015; Kallel et al., 2014). Furthermore, the significant differences between the curve profiles, particularly for

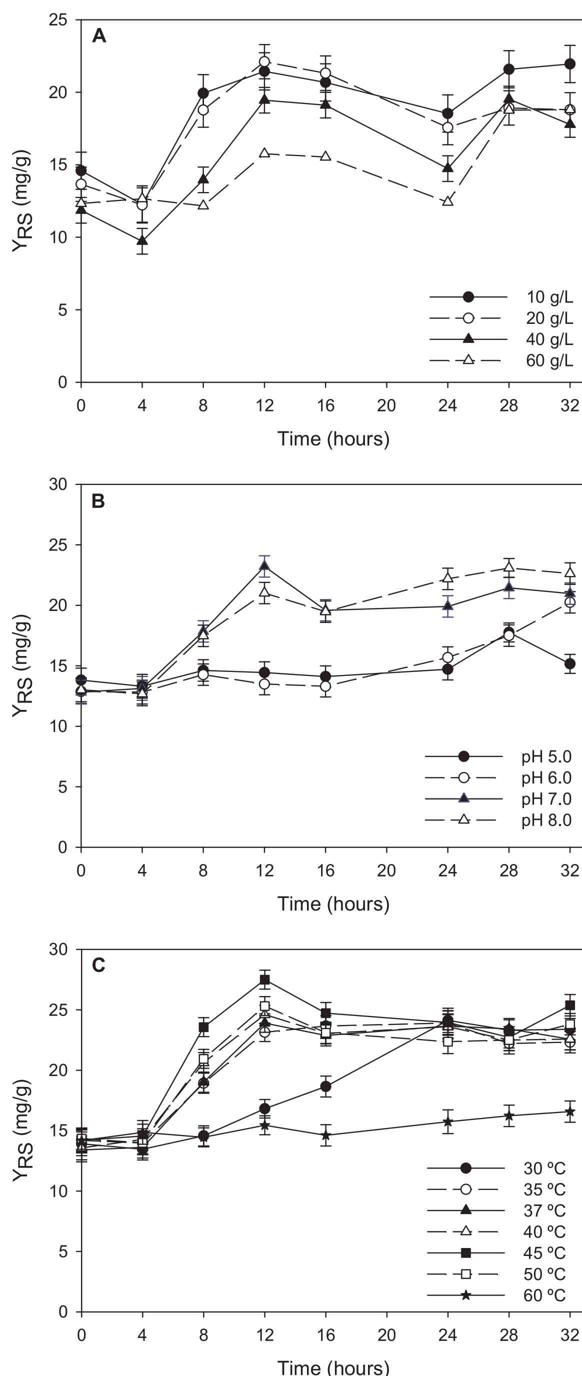

Fig. 3. Sugar production yield (Y_{RS}) obtained for *Bacillus subtilis* 3610 wild type in Vogel media at 150 rpm using: (A) different substrate concentration (10, 20, 40 and 60 g/L of BSG) at 37 °C and pH 7.0; (B) different pH (5.0, 6.0, 7.0 and 8.0) at 37 °C and with 20 g/L of BSG; (C) different temperature (30, 35, 37, 40, 45, 50 and 60 °C) at pH 7.0 and with 20 g/L of BSG. Results represent the average of three independent assays ± standard deviation.

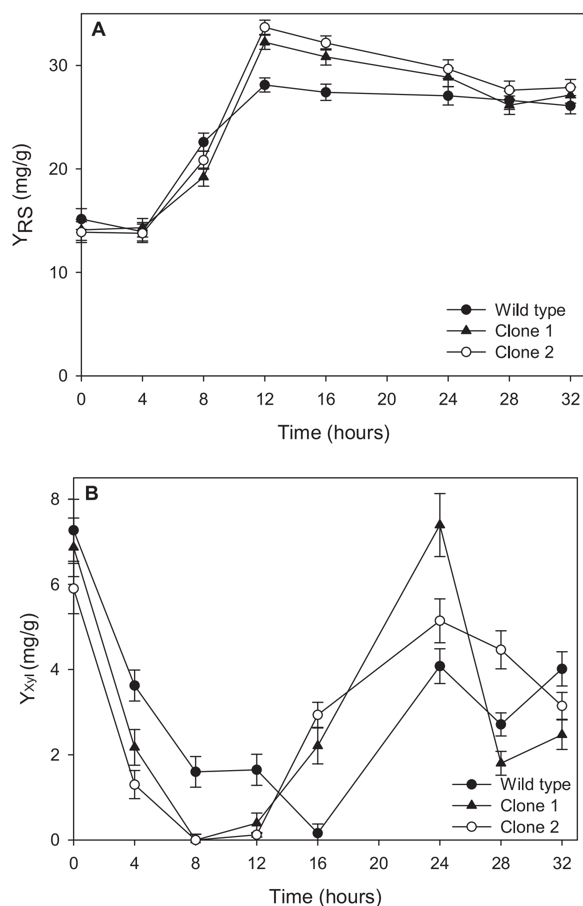

Fig. 4. Sugar production yield (Y_{RS}) (A) and Free xylose yield (Y_{Xyl}) (B) obtained for *Bacillus subtilis* 3610 wild type (wt), *Bacillus subtilis* clone 1 (transformant containing the xylanase gene *xyn2* from *Trichoderma reesei*) and *Bacillus subtilis* clone 2 (transformant containing the xylanase gene *xyn2* from *Trichoderma reesei* coupled with a secretion tag endogenous to *Bacillus subtilis*) using 20 g/L of BSG in Vogel media at pH 7.0, 45 °C and 150 rpm. Results represent the average of three independent assays ± standard deviation.

pH 6.0 and 7.0, reveals a high sensitivity of the process to this variable. Some of these variations can probably be a consequence of the distinct enzymatic complexes produced by *B. subtilis* (Banka, Guralp, & Gulari, 2014). The most suitable temperature was found to be 45 °C, leading to a Y_{RSmax} of 27.5 ± 0.8 mg/g after 12 h of fermentation (Fig. 3). It seems that at this temperature an optimum synergy is reached between the positive effect of high temperatures in the release of sugars and in the increase of the hydrolysis reaction rates (Heinen et al., 2017), and the optimum growth temperature for *B. subtilis*, which is reported to be between 30 and 37 °C (Korsten & Cook, 1996), and optimum temperature for xylanase production, around 35–37 °C (Helianti et al., 2016; Irfan, Asghar, Nadeem, Nelofer, & Syed, 2018). For temperatures higher than 50 °C, a decrease of the Y_{RS} value was observed possibly due its negative effect on *B. subtilis* growth (Irfan et al., 2018).

In summary, the best conditions for *B. subtilis* wild type fermentation were 20 g/L of BSG, 45 °C and pH 7.0, leading to a Y_{RSmax} of 27.5 ± 0.8 mg/g at 12 h.

3.2. Evaluation of the transformants performance for the production of AXOS

The performance of the *B. subtilis* wild type as AXOS producer by single-step fermentation of BSG was compared with the clone 1 and clone 2 ones (Fig. 4) under the optimum process conditions previously established (Section 3.1).

From Fig. 4A, it was found that for all tested strains the Y_{RSmax} was attained after 12 h of fermentation. Moreover, all the production curve profiles are comparable with each other, suggesting that the expression of the heterologous xylanase did not entail a metabolic burden to the cells. However, both clones achieved a higher Y_{RS} value than the wild type (30.8 ± 0.8 mg/g for clone 1, 33.7 ± 0.7 mg/g for clone 2 and 28.1 ± 0.8 mg/g for the wild type), with clone 2 showing the highest improvement (20%). The similar Y_{RSmax} values achieved for both clone 1 and clone 2, suggest that *B. subtilis* was able to secret the heterologous

xylanase even in the absence of the secretion tag. In fact, this microorganism is recognized for its high protein-secreting capability (Van Zyl, den Haan, & la Grange, 2013).

The better performance of the mutant strains was also confirmed through analysis of the xylanase activity. For all strains, the maximum enzymatic activity was reached at 24 h. Clone 1 and clone 2 presented a 28% and 39% increase in the enzymatic activity, respectively, when compared to the wild type (data not shown). Besides leading to the highest Y_{RSmax} and xylanase activity, clone 2 also presented the lowest amount of free xylose per amount of BSG (Y_{Xyl}) at 12 h (0.12 ± 0.05 mg/g) (Fig. 4B), therefore it was selected as the most interesting strain for the following studies.

3.3. Optimization of AXOS production by single-step fermentation of BSG with B. subtilis clone 2

The sugars production yield was optimized for the *B. subtilis* clone 2 by a Box-Behnken (BB) design. The evaluation of the pH, temperature and BSG effects on the production of AXOS by *B. subtilis* wild type (Section 3.1) allowed to delineate a solution space which was taken as a basis to define the range limits of the same variables under study now for *B. subtilis* clone 2 (Table 1).

Samples were collected along time for the quantification of reducing sugars by DNS and the Y_{RS} at 12 h was selected as the dependent variable (Table 1). The $Y_{RS\ 12h}$ varied in the range of 20.92–35.34 mg/g. The experimental design allowed the mathematical modelling of Y_{RS12h} (mg/g) depending on the normalized variables, *pH*, temperature, *T* (°C) and amount of *BSG* (g/L), as a second degree polynomial regression as expressed in Eq. (1):

$$Y_{RS12h} = a_0 + a_1 \cdot BSG + a_2 \cdot BSG^2 + a_3 \cdot pH + a_4 \cdot pH^2 + a_5 \cdot T + a_6 \cdot T^2 \quad (1)$$

where a_0 and $a_{1\ to\ 6}$ are the regression coefficients calculated from experimental data by multiple regression using the least-squares method and their significance was determined by p-value and t-value (Table 2).

The optimum solution calculated through this model was 20.6 g/L BSG, pH 7.0 and 44.9 °C. Interestingly, this solution corresponds approximately to the conditions settled for the central point of the design (experiments number 14–16), which in turn correspond to the optimum solution previously found for the wild type (Section 3.1.3) by varying each variable individually.

The relation between the independent variables and maximum yield was well described by the empirical model (R^2 = 0.85921 and predicted-R^2 = 0.75362). Analyzing the results from Table 2, temperature was found to be the most influent variable on yield related in a quadratic way, with p-value = 0.0004 (representing > 99.99% of the significance level) and t-value = 5.751 followed by pH with p-value = 0.0034 (> 99%) and t-value = 4.101.

In order to better analyze and discuss the data, a response surface is presented in Fig. 5.

The response surface corresponds to the variation of the maximum yield with the independent variables pH and temperature fixing the variable BSG at the optimum solution given by the quadratic model (20.6 g/L). A region of optimal solution corresponding to temperatures around 45 °C and pH 7.0 is easily identifiable.

Table 2
Effect of BSG concentration, pH and temperature on the sugar production yield Y_{RS} at 12 h of fermentation.

	Coefficient	Standard error	t-value	p-value
a_0	25.79	0.678	38.023	0.0000
a_1	0.90	0.831	0.543	0.6022
a_2	1.94	0.611	1.583	0.1520
a_3	0.31	0.831	0.188	0.8553
a_4	5.01	0.611	4.101	0.0034
a_5	−1.80	0.831	−1.083	0.3105
a_6	7.03	0.611	5.751	0.0004

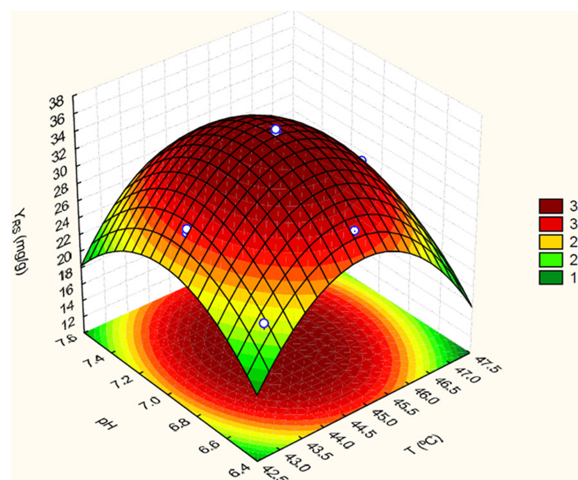

Fig. 5. Response surface of calculated sugar production yield (Y_{RS}) *Bacillus subtilis* clone 2 (transformant containing the xylanase gene *xyn2* from *Trichoderma reesei* coupled with a secretion tag endogenous to *Bacillus subtilis*) at 12 h of fermentation, calculated dependence of Y_{RS} on temperature and pH.

3.4. Quantification and chemical characterization of AXOS produced by B. subtilis clone 2

The AXOS produced under optimum conditions (20 g/L of BSG, pH 7.0 and 45 °C) were quantified by HPLC before and after acid hydrolysis to evaluate the fraction of free monosaccharides present in the fermentation supernatant. Table 3 shows the concentration of glucose, xylose and arabinose present in the supernatant obtained at 0 days and at the optimum time (12 h). Additionally, Y_{Xeq} (mg/g) in terms of equivalent of xylose per gram of BSG is also presented for monosaccharides and oligosaccharides.

Xylose and arabinose were the only monosaccharides released from BSG during the sterilization process by autoclave. Small amounts of oligosaccharides may also be extracted from the residue. This fact can explain the increase of approximately 2- and 4-fold of the xylose and arabinose amount after acid hydrolysis. The presence of glucose may be due to the degradation of the remaining starch present in BSG, which is confirmed by the characteristic linkages present of (1→4)-linked glucose residues, together with terminally-linked and (1→4,6)-linked glucose residues (data not shown). After 12 h of fermentation, both wild type and clone 2 presented a low amount of monosaccharides. The preferential consumption of these sugars is one of the main advantages of using a single-step fermentation approach, *i.e.* it greatly simplifies the downstream processes, which generally represent up to 80% of the total production costs (Urmann, Graalfs, Joehnck, Jacob & Frech, 2010). Discounting the amount of free xylose, the Y_{Xeq} obtained for clone 2 was 54.2 ± 1.1 mg/g. The microorganism improvement by genetic engineering resulted in an increase of 33% in the Y_{Xeq}, when compared to the wild type. The TLC analysis (Appendix A, Fig. A1 in Supplementary material) suggested the presence of 2- and higher DP XOS.

The glyosidic linkages present in AXOS composition were determined by GC–MS (Section 2.7). Methylation analysis showed the existence of AXOS composed of (1→4)-linked-xylopyranosyl residues to which arabinofuranose units are linked as side chains (data not shown). Furthermore, (1→5)-linked-arabinofuranosyl residues were also detected, which may be indicative of ferulic acid esterified in position *O*-5 of the arabinose, as reported in cereals such as BSG (Coelho et al., 2014, 2016; Mandalari et al., 2005). The diversified number of arabinose substitution patterns observed in AXOS should explain the different

Table 3
Monosaccharide concentration and AXOS production yield, Y_{Xeq}, obtained by *Bacillus subtilis* wild type and clone 2 (20 g/L BSG, pH 7.0 and 45 °C).

		Time (h)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Y_{Xeq} (mg/g)
Wild type	M[a]	0	N.O.[b]	0.059 ± 0.017	0.017 ± 0.009	2.95 ± 0.87
	O[a]	0	0.473 ± 0.032	0.056 ± 0.018	0.051 ± 0.012	2.80 ± 0.91
	M	12	N.O.[b]	0.015 ± 0.002	0.006 ± 0.001	0.76 ± 0.08
	O	12	0.122 ± 0.025	0.814 ± 0.033	0.362 ± 0.023	40.67 ± 1.65
Clone 2	M	0	N.O.[b]	0.055 ± 0.015	0.015 ± 0.007	2.76 ± 0.77
	O	0	0.491 ± 0.040	0.062 ± 0.016	0.055 ± 0.013	3.06 ± 0.82
	M	12	N.O.[b]	0.013 ± 0.001	0.012 ± 0.008	0.67 ± 0.05
	O	12	0.307 ± 0.021	1.085 ± 0.021	0.420 ± 0.017	54.24 ± 1.10

[a] Monosaccharides (M); oligosaccharides (O).
[b] Not observed (N.O).

Table 4
Glycosidic linkage composition (mol%).

Glycosidic linkage	Arabino-oligosaccharides (AXOS)[a]		
	A	B	C
t-Ara*f*	2.0	7.7	8.9
3-Ara*f*		0.5	
5-Ara*f*	1.2	1.8	2.5
3,5-Ara*f*		0.5	tr[b]
Total	3.1	10.5	11.3
t-Xyl*p*	17.3	8.8	8.4
3-Xyl*p*		0.3	0.3
4-Xyl*p*	54.1	36.7	36.3
2,4-Xyl*p*	0.6	1.5	2.0
3,4-Xyl*p*	0.2	3.0	1.2
2,3,4-Xyl*p*		1.9	1.4
Total	72.2	52.2	49.6
t-Glc*p*	14.9	8.8	13.8
3-Glc*p*		1.0	1.2
4-Glc*p*	4.3	23.7	21.1
6-Glc*p*			0.7
3,4-Glc*p*		0.4	0.2
4,6-Glc*p*		0.4	1.1
Total	19.2	34.3	38.0
t-Gal*p*	4.9	1.4	
6-Gal*p*		0.7	
Total	4.9	2.1	
4-Man*p*	0.6	0.9	1.0
Total	0.6	0.9	1.0
AXOS average DP[c]	4	6	6
% Branching	1.0	15.7	12.0

[a] AXOS fractions individually collected by HPLC.
[b] Trace amount (tr).
[c] Degree of polymerization (DP).

retention times obtained by HPLC when compared with those of pure unbranched XOS standards. Moreover, the GC–MS analysis of the partially methylated alditol acetates derived from AXOS showed an average polymerization degree, calculated as the ratio of total xylose residues/terminally-linked xylose residues of 4 and 6, assuming that the xylose residues are only present in the backbone and absent as substituents. The ratio of branched to total xylose residues showed that the AXOS with an average DP 4 presented a very low degree of branching (1%) whereas the average DP 6 populations presented 16% and 12% of branching. These populations showed different substitution patterns of the xylan backbone, where 3,4-linked Xyl*p* is the main representative branching in the DP6 16% branched fraction (proportion of 3,4-Xyl*p*, 2,4-Xyl*p*, and 2,3,4-Xyl*p* of 3.0, 1.5, and 1.9 mol%), and 2,4-linked Xyl*p* is the main representative branching in the DP6 12% branched fraction (proportion of 3,4-Xyl*p*, 2,4-Xyl*p*, and 2,3,4-Xyl*p* of 1.2, 2.0, and 1.4 mol%, respectively). The higher branching is also in accordance with the higher proportion of 2,3,4-Xyl*p*, *i.e.* the disubstituted xylose residue (Table 4).

In summary, AXOS composed mainly by xylose and arabinose were produced in a higher extent by single-step fermentation of BSG using *B. subtillis* clone 2 as compared to the wild type. Furthermore, some oligomers of maltodextrins and (β1→3)-glucans are also present in the AXOS-rich fractions, comprising 22–42 mol% of glucose residues. However, the prebiotic effect of maltodextrin is reported in the literature (Yeo & Liong, 2010).

Table 4 shows the yields of XOS produced (mg) per gram of xylan, $Y_{XOS/xylan}$ (mg/g) that have been reported in the literature for enzymatic hydrolysis processes. The Y_{Xeq} (mg/g) obtained in this study using *B. subtilis* clone 2 (54.2 ± 1.1 mg/g) was converted to $Y_{XOS/xylan}$ (mg/g), considering the BSG composition in xylan (Section 2.1) and the stoichiometric correction factor to account for the molecular weight gain during acid hydrolysis as described in NREL protocols.

Reddy and Krishnan (2016) reported the highest yield presented in Table 4, using a β-xylosidase-free xylanase from *B. subtilis* to hydrolyze pretreated sugarcane bagasse during 30 h. Bian et al. (2013) and Bragatto, Segato, and Squina (2013) used the same residue, achieving lower yields but significant shorter production times (8 h and 12 h, respectively), which in turn translates into a higher productivity. Romero-Fernández et al. (2018) reported the lowest production time (5 h), however using commercial wheat arabinoxylan.

The yield obtained in the current work for the *B. subtilis* clone 2 was similar to that reported by Amorim et al. (2018) using direct hydrolysis of BSG by a commercial xylanase from *Trichoderma longibrachiatum*. On the other hand, the yield herein associated to single-step fermentation was lower than the those presented by Rajagopalan, Shanmugavelu, and Yang (2017) and Reddy and Krishnan (2016), but similar to the one reported by Bragatto et al. (2013).

The production yield seems to depend greatly on the substrate and the production process, varying approximately between 100 mg/g and 700 mg/g. Nonetheless, although using different residues and xylanases sources, several authors reported comparable yield values, in a range of 110–114 mg/g (Akpinar, Erdogan, Bakir, & Yilmaz, 2010; Samanta et al., 2012; Seesuriyachan, Kawee-ai, & Chaiyaso, 2017), which may indicate a certain limitation of the production processes, and therefore revealing a need for development of different production approaches.

Additionally, it is important to highlight that the $Y_{XOS/xylan}$ values presented in Table 5 do not reflect the overall production process yield, except the ones reported in this study and by Amorim et al. (2018). Generally, the yields associated to the residues pretreatment and/or xylan extraction, as well as the enzyme production (when it is not purchased) are not being considered, otherwise they would significantly decrease the reported yields of the production process. For instance, Rajagopalan et al. (2017) reported one of the highest Yxos/xylan values, however if the yield of xylan extraction from mahogany (77.2%) and mango sawdust (62.6%) were considered, the yields values would be closer to the ones that we herein report, namely 441.6 and 315.5 mg/g, respectively. In addition, the overall process yield and production time, would also contemplate the yield associated to the

Table 5
XOS production using enzymatic hydrolysis and direct fermentation.

Substrate	Biocatalyst	Time (h)	$Y_{XOS/xylan}$[a] (mg/g)	Reference
Tobacco stalk xylan	Commercial *A. niger* xylanase	24	114.0	Akpinar et al. (2010)
Natural grass xylan	Commercial xylanase *T. viridae*	10.11	110.0[b]	Samanta et al. (2012)
Delignified sugarcane bagasse	Recombinant *B. subtilis* xylanase	8	416.6[c]	Bragatto et al. (2013)
Pretreated sugarcane bagasse	Crude *P. stipitis* xylanase	12	318.0	Bian et al. (2013)
Garlic straw xylan	*B. mojavensis* UEB-FK xylanse	8	272.6-307.4	Kallel et al. (2014)
Pretreated sugarcane bagasse	β-xylosidase-free xylanase of *B. subtilis* KCX006	30	670.0[c]	Reddy and Krishnan (2016)
Mahogany and mango xylan	*Clostridium* sp. BOH3 xylanase	12	572 and 504[c]	Rajagopalan et al. (2017)
Pretreated corn cob	*Streptomyces thermovulgaris* TISTR1984 xylanase	18	106.6[c]	Seesuriyachan et al. (2017)
Commercial wheat arabinoxylan	Immobilized *Streptomyces halstedii* JM8 *Xys1Δ xylanase*	5	312.5[c]	Romero-Fernández et al. (2018)
Raw BSG	Commercial xylanase *T. longibrachiatum*	12	444.3	Amorim et al. (2018)
	T. reesei Direct Fermentation	72	326.2	
	B. subtilis clone 2 Direct Fermentation	12	463.41	This work

[a] Yields are represented in terms of amount XOS per amount of xylan, $Y_{XOS/xylan}$ (mg/g).
[b] Highest yield observed in terms of maximization of xylobiose production.
[c] Calculated from text information and converted to appropriate units.

production and purification of the *Clostridium* sp. BOH3 xylanase and the time required for its production (2 days). Contrariwise, single-step fermentation yields represent the overall production process and its respective production time. For instance, in the work by Bian et al. (2013), 12 h was the time reported for the hydrolysis of pretreated sugarcane bagasse with crude xylanase, however additional 7 days were required to obtain this crude extract. Hence, comparisons between different studies are not straightforward and must be carefully drawn.

In summary, based on the above discussion, the single step approach used in this study is an advantageous strategy for the production of AXOS from BSG.

4. Conclusions

The current study clearly highlights the potential of using BSG, an agro-industrial byproduct, and single-step fermentation by *B. subtilis* to produce AXOS together with maltodextrins, which are prebiotic ingredients. Genetic engineering allowed to optimize this microorganism by cloning the *T. reesei* xylanase gene into the *B. subtilis* chromosome, which led to a relevant increase of the process yield (33%). Comparing the enzymatic hydrolysis process with the single-step fermentation approach, it was concluded that the last is a more attractive and advantageous approach to hydrolyze BSG and produce AXOS. Further optimization of the bioprocess operation mode to deal with possible substrate inhibition and its scale-up are foreseen.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.07.017.

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