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High cellulase-free xylanases production by *Moesziomyces aphidis* using low-cost carbon and nitrogen sources

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Abstract

BACKGROUND: Enzymes involved in xylan hydrolysis have several industrial applications. Selection of efficient microbial hosts and scalable bioreaction operations can lower enzyme production costs and contribute for their commercial deployment. This work aims to investigate *Moesziomyces aphidis* yeast cultivation conditions contributing for maximal xylanase titres, yields and productivities using low-cost nitrogen and carbon sources.

RESULTS: $NaNO_3$ and KNO_3 supplementation improves xylanase production 2.9 and 2.7-fold (against 67.2 U/ml), respectively, using xylan as carbon source. Interestingly, the

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use of KNO_3, instead of $NaNO_3$, resulted in 2 to 3-fold higher specific activity, highlighting the potassium ion role. In addition, this study investigates synergetic effects on using ionic and organic nitrogen sources. A 4.9-fold increase on xylanase production, with a high specific activity, was attained combining KNO_3 and corn steep liquor (CSL). Exploring the previous findings, this study reports one of the highest extracellular xylanase production titres (864.7 U/ml) by yeasts, using a media formulation containing dilute-acid pre-treated brewery spent grains (BSG), as carbon source and inducer, supplemented with KNO_3 and CSL. Replacement of dilute-acid pre-treatment BSG by untreated BSG had low impacts xylanase production, of only 6%.

CONCLUSION: Efficient production of *M. aphidis* xylanolytic enzymes, using low-cost non treated nitrogen and carbon sources, is attractive for deployment of on-site enzyme production targeting different biotechnological applications under circular economy and biorefinery concepts. Potential xylanases end-users include industries like brewing (using BSG as substrate for enzyme production), pulp and paper (benefiting from the cellulase-free xylanase activity) or lignocellulosic ethanol (for cellulases supplementation).

Keywords

Xylanases; *Moesziomyces aphidis*; brewery's spent grain; enzymatic hydrolysis

ABBREVIATIONS

MEL – mannosylerythritol lipids

CSL – Corn steep liquor

BSG – Brewery spent grains

INTRODUCTION

Xylanases have attracted considerable attention due to their application in many industrial processes such as enzymatic bleaching of paper pulp, juice and beer clarification, extraction of plant oils, texture improvement in bakery, bioconversion of agricultural waste, bioscouring in textiles and improvement of animal feed digestibility.[1] This class of molecules acts directly in the breakdown of a constituent of lignocellulosic biomass, xylan, which is the second most common polysaccharide found in nature, a complex heteropolysaccharide comprising a backbone of D-xylose residues linked by β-1,4-glycosidic bonds, which may be substituted with side chain branches containing α-L-arabinofuranosyl, 4-O-methyl-D-glucuronosyl, acetyl, feruloyl and p-coumaroyl units. The enzymatic hydrolysis of xylan requires the action of several enzymes, being the most relevant the endo-β-1,4-xylanase (EC 3.2.1.8), that cleaves glycosidic bonds to produce xylooligosaccharides, and β-1,4-xylosidase (EC 3.2.1.37), responsible for the final breakdown of xylooligosaccharides into D-xylose.[2]

While extensive research on microbial cellulase and xylanase production has been performed with filamentous fungi (*Aspergillus spp.*, *Trichoderma spp.*, *Penicillium spp.*, etc.) and bacteria (*Bacillus spp.*, *Streptomyces spp.*), which are efficient enzyme producers,[1] a limited number of studies on this topic has been performed with yeasts. Yeasts fermentations combine the potential benefits of allowing homogeneous scalable submersed fermentations on stirrer tank bioreactors, possible in bacterial cultures, with higher robustness, characteristically of fungi cultures. In addition, yeast fermentation allows the presence of inhibitory compounds driven from complex carbon and nitrogen sources obtained from agro-industrial residues. Among yeasts, those belonging to the genera *Cryptococcus*, *Scheffersomyces*, *Candida*, *Trichosporon*, *Dekkera*,

Hanseniaspora, *Metschnikowia*, *Rhodotorula*, *Sugiyamaella* and *Wickerhamomyces* were described as cellulase and/or xylanase producers.[3-9] However, and contrary to the trend of relatively low xylanase activity found in yeasts, *Moesziomyces*/*Pseudozyma* spp., anamorphic basidiomycetous yeasts belonging to the Ustilagomycetes, were previously described by grow and produce xylanases directly from xylan.[10] From the strains assessed, *M. aphidis* PYCC 5535^{T} revealed superior xylanase production, with xylanase volumetric activities 60-fold higher than those reported in literature for other yeasts, and at a level that can be compared to those reported for filamentous fungi, known as efficient enzyme producers.[11,12]

To enter the xylanase market, estimated at an annual value of 200 million dollars,[13] cost-effective xylanases production processes using low-cost substrates is mandatory. The use of complex and inexpensive xylan-containing substrates for bioprocesses with natural xylanase-producers is particularly relevant due to typical carbon source inducing regulation mechanisms. Xylan is naturally found in lignocellulosic biomass, including agricultural, forestry and municipal solid residues. The use of these residues as feedstock for biorefineries is regarded as a favorable option for the sustainable development, particularly under circular economy approaches.[14] In fact, many lignocellulose bioconversion processes make use of hydrolases (including xylanases) that can be produced on-site using the same substrate, with potential advantages on the development of more efficient feedstock-specific enzymes and avoiding downstream (e.g. concentration and stabilization) and transportation costs.[15]

Supplementation of the culture media for enzyme production is also often required, particularly in relation to the nitrogen source. The optimization of the culture media with

the combined use of low-cost carbon and nitrogen sources significantly contributes to minimize production costs. Moreover, the production costs can be further reduced by streamlining the bioprocess, namely by minimizing or eliminating lignocellulose pretreatment.

This work aims to improve the production of xylanases from lignocellulosic materials by *M. aphidis* PYCC 5535^T, with focus on the optimization of nitrogen supplementation. Brewery spent grain (BSG) is an abundant residue from the brewing industry with low, zero or negative value, and we did previously establish its use as a reference lignocellulosic substrate able to induce high levels of xylanase production.[16] The current study investigates the influence of BSG concentration, BSG pretreatment and additional inorganic and organic supplements on optimal xylanase production. The utilization of agro-industrial wastes represents an interesting source of proteins, carbohydrates, lipids and other essential minerals that may be exploited for the bioproduction of value-added products, such as enzymes. The on-site possible enzyme production from these residues can follow circular economy and biorefinery concepts. In the case of xylanases, potential end-users include industries like brewing (using BSG as substrate for enzyme production and xylanase utilization in the brewery production process), pulp and paper (benefiting from the cellulase-free xylanase activity) or lignocellulosic ethanol (for cellulases supplementation).

MATERIALS AND METHODS

Yeast strain and maintenance

Moesziomyces (Pseudozyma) aphidis PYCC 5535^T (CBS 6821) was obtained from the Portuguese Yeast Culture Collection (PYCC), UCIBIO/Requimte, FCT/UNL, Portugal.

Yeasts were cultivated for 3 days at 25°C on Yeast Malt Agar (YM–agar) medium (yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L; agar, 20 g/L). Stock cultures were prepared by propagation of yeast cells in liquid medium as described below for the inoculum and storage (in 20% v/v glycerol aliquots) at -70°C for later use. Inoculum was prepared by incubation of stock cultures of *M. aphidis* PYCC 5535^T at 28°C, 140 rpm, for 48 h, in liquid medium containing glucose (40 g/L), $NaNO_3$ (3 g/L), $MgSO_4$ (0.3 g/L), KH_2PO_4 (0.3 g/L) and yeast extract (1 g/L).

Raw material and cultivation conditions

Brewery spent grain (BSG), kindly provided by Sociedade Central de Cervejas (Vialonga, Portugal), was grounded with a knife mill to particles smaller than 1.5 mm and homogenized in a defined lot. BSG was then stored in plastic containers at room temperature. BSG dry matter content was 95% (w/w), with polysaccharides accounting for 36.2 g/100 $g_{dry\ solids}$, of which glucan at 22.1/100 $g_{dry\ solids}$ and xylan at 14.1 g/100 $g_{dry\ solids}$. BSG was pretreated at 121°C for 15 min with 0.16 N HCl in a liquid-to-solid ratio of 9 (w/w) using an autoclave. The pH was adjusted to 5.5 using 4 M NaOH. This pretreated slurry material was subsequently used for *M. aphidis* PYCC 5535^T cultivations. D-Xylose, commercial grade beechwood xylan (Sigma-Aldrich, USA), pretreated BSG and untreated BSG were directly tested as carbon source for cultivation of *M. aphidis* PYCC 5535^T with supplementation of $NaNO_3$ (3 or 5 g/L), KNO_3 (3 g/L), NH_4NO_3 (3 g/L), NH_4Cl (3 g/L), $(NH_4)_2SO_4$ (3 g/L), peptone (3 g/L), yeast extract (3 or 10 g/L), urea (3 g/L), or corn steep liquor (CSL) (10 or 20 g/L) in the culture media. The standard cultivation medium was comprised by $MgSO_4$ (0.3 g/L), KH_2PO_4 (0.3 g/L), xylan (40 g/L) and yeast extract (1 g/L) and it was inoculated with 10% (v/v) of inoculum culture and incubated at 28°C, 140 rpm, for 7 days.

All experiments were carried out at least in duplicate.

Enzyme activity assays

Xylanase activity was assessed through the release of reducing sugar from xylan measured by the 3,5-dinitosalicylic acid (DNS) method described by Miller.[17] The supernatant culture sample was appropriately diluted with 0.1 mL of 1% (w/v) beechwood xylan solution in 50 mM potassium phthalate buffer and incubated at 50°C for 30 min. Subsequently, 0.6 mL of DNS reagent was added, stopping the reaction, and the solution was boiled for 5 min and cooled to room temperature. Reducing sugars were estimated using a D-xylose calibration curve, with absorbance of samples and standards measured at 550 nm. Each reaction and its control (without incubation) were run in quadruplicate. One unit (U) of xylanase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar D-xylose equivalent per minute.

β-Xylosidase was determined as previously described.[18] The supernatant culture sample was appropriately diluted in a reaction mixture (0.3 mL), containing 5 mM 4-nitrophenyl-β-D-xylopyranoside (pNPX, Sigma, USA) in 50 mM potassium phthalate buffer pH 5.5. After incubation at 50°C for 30 min, 0.15 mL of 1 M Na_2CO_3 was added to stop the reaction. The p-nitrophenol absorbance (pNP) was measured at 405 nm. One unit (U) of β-xylosidase activity was defined as the amount of enzyme releasing 1 µmol pNP per minute.

Extracellular protein content was assessed using Pierce™ BCA protein assay kit (Thermo Scientific, USA) on 1 mL of culture broth supernatant recovered by centrifugation (10 min at 13,000 rpm).

Characterization of xylanase crude extracts

Extracellular xylanolytic crude extracts obtained from 7-days culture, at 28°C, of *M. aphidis* PYCC 5535^T, were used to assess:

(i) extracellular xylanase activity for pH ranging from 3.0 to 8.0 at 50°C and for temperature ranging from 20°C to 72°C, at pH 5.

(ii) xylanase stability by assaying the residual activity along 48-h incubation at pH 5 and 50°C.

Statistical analysis

Statistics were performed by analysis of variance (ANOVA) and p-values of the differences between groups are corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p<0.05$.

RESULTS

Screening and selection of nitrogen sources for the production of xylanolytic enzymes

The use of xylan as carbon source and different inorganic- (nitrate- and/or ammonium-) or organic-based nitrogen sources (at 3 g/L) were first assessed for cell biomass, extracellular protein and xylanase activity improved production (Figure 1, Figure A1). Apart from urea, all nitrogen sources tested have promoted abundant cell growth and higher xylanase volumetric activity than the standard condition. Nitrate-based nitrogen

sources ($NaNO_3$ and KNO_3) and peptone contributed to the higher xylanase volumetric activity measured. The 10-fold higher $NaNO_3$ concentration (3 g/L) in relation to the standard condition improved the xylanase volumetric activity in 2.9-fold (to 194 U/mL) and the total extracellular protein in 3.6-fold. A higher $NaNO_3$ supplementation load (at 5 g/L) further increased the xylanase volumetric activity to 314 U/mL (with similar biomass and protein content to the condition with $NaNO_3$ 3 g/L) (data not shown). The highest xylanase specific activities were achieved with KNO_3 (61.6 U/mg_{prot}) and peptone (40.3 U/mg_{prot}), due to relatively low extracellular protein production and relatively high xylanase volumetric activity, respectively.

The extracellular β-xylosidase volumetric activities in the extracellular extracts were also assessed for the conditions showing higher xylanase activity, i.e. the standard, $NaNO_3$, KNO_3 and peptone supplemented at 3 g/L (Figure 2). The extracellular β-xylosidase activity obtained using standard media (29 mU/mL). $NaNO_3$, KNO_3 or peptone supplementation significantly increased β-xylosidase volumetric activity (5- to 8-fold), with the highest value (207 mU/mL) achieved when *M. aphidis* PYCC 5535^T was cultivated in xylan medium with KNO_3 supplementation.

The ratio xylanase activity/β-xylosidase activity in the extracellular crude extracts were calculated, ranging from 863 to 2,586 with KNO_3 and peptone supplementation respectively.

Combination of carbon and nitrogen sources for cost-effective xylanase production

Considering the results of the effect of the different nitrogen sources firstly assessed on the xylanase activity, three nitrogen sources were selected for further studies, two inorganic ($NaNO_3$ or KNO_3) and one organic (peptone), all at 3 g/L. Those were individually supplemented into medium containing xylan or D-xylose as carbon source at 40 g/L (Table 1). Among the nitrogen sources tested, peptone led to the highest xylanase volumetric activity both in the presence of xylan (371 U/mL) and of D-xylose (294 U/mL) (Table 1). In relation to the influence of inorganic nitrogen sources in xylanase volumetric activity, $NaNO_3$ is preferred in the presence of xylan (194 U/mL) but KNO_3 is preferred in the presence of D-xylose (220 U/mL). Still, the specific activity is 2.6- to 3.0-fold higher with KNO_3 than with $NaNO_3$.

The higher xylanase volumetric activity obtained with the organic nitrogen source (peptone) may not contribute positively to the process economy as peptone is more expensive than inorganic nitrogen sources like $NaNO_3$ and KNO_3. Therefore, an alternative relatively low-cost organic nitrogen source was assessed: corn steep liquor (CSL). The use of CSL (at 10 g/L) resulted in high biomass yield and very low total extracellular protein content (data not shown) at values of 2-4 g/L, which are on the range of the one obtained with KNO_3 supplementation. Significantly higher xylanase volumetric activity was observed in D-xylose medium (5.3-fold) than in xylan medium, at values of 154 U/mL and 30 U/mL, respectively (Figure 3). This trend is similar to the one obtained when yeast extract, a much more expensive nitrogen source, was used (at 10 g/L) in supplementation of D-xylose or xylan medium, which resulted in xylanase volumetric activity of 529 U/mL and 115 U/mL, respectively (data not shown). Interestingly, when doubling the CSL supplementation (to 20 g/L), the xylanase

volumetric activity increased around 2-fold, to 297.7 U/mL in D-xylose medium and 71.2 U/mL in xylan medium (Figure 3).

The combination of organic and inorganic nitrogen sources, CSL (10 g/L) and KNO_3 (3 g/L) was evaluated, using D-xylose or xylan as carbon source. Under these conditions, the xylanase volumetric activity increased 3-fold (up to 483.4 U/mL) in D-xylose medium and 10-fold (up to 327.5 U/mL) in xylan medium, when compared to the use of sole use of CSL supplementation (at 10 g/L) as nitrogen source (Figure 3). When compared to the sole use of KNO_3 (3 g/L) as nitrogen source, the combination of CSL and KNO_3 triggered a 2-fold improvement of the xylanase volumetric activity (see Table 1). These results revealed a synergistic effect on the combination of CSL and KNO_3, not only in relation to the volumetric activity but also to the specific activity. In fact, the highest specific activity was achieved under this condition, 149 U/mg_{prot} in D-xylose medium and 87 U/mg_{prot} in xylan. This feature is particularly relevant for downstream and application processes.

The use of BSG for xylanase production – influence of nitrogen source supplementation substrate concentration and pretreatment

While D-xylose and xylan allowed a comprehensive study of the impact of nitrogen supplementation on xylanase production, the development of cost-effective industrial processes benefit from the use of inexpensive carbon sources.

Considering the nitrogen supplementation conditions that promote higher xylanase production in the previous section were applied, but using pretreated BSG as carbon source, instead of D-xylose or xylan. Therefore, BSG was first subjected to a dilute-acid

pretreatment and assessed at 11% (w/v) (eq. 15 g/L xylan) for xylanase production with or without supplementation of CSL (at 10 g/L) and KNO_3 (at 3 g/L). The CSL/KNO_3 supplementation improved both the volumetric and the specific activity, up to 865±10 U/mL and 116±6 U/mg_{prot}, respectively, if compared to the xylanase activity obtained without nitrogen supplementation (517±6 U/mL, 57±6 U/mg_{prot}) (Figure 4). A volumetric xylanase productivity of 5.2 U/mL/h was achieved (with nitrogen supplementation).

In order to further evaluate the robustness and flexibility of the approach envisaged to promote cost-effective xylanase production, the substrate concentration and the possibility to avoid the use of BSG pretreatment were assessed. When BSG load was reduced to 7% (w/v) (eq. 10 g/L xylan), but CSL/KNO_3 supplementation maintained, a xylanase volumetric and specific activities of 438±62 U/mL and 84±12 U/mg_{prot}, respectively, was obtained. This means a yield of 43.8 U/mg of xylan, which is lower than the 57.3 U/mg of xylan obtained with 11% (w/v) of BSG and CSL/KNO_3 supplementation, but still quite competitive and higher than the one obtained with 11% (w/v) of BSG without supplementation (34.2 U/mg of xylan) (Table A1). The direct use of (untreated) BSG without nitrogen supplementation led to residual growth and xylanase production. However, robust growth and xylanase production was attained using untreated BSG, at 7% (w/v) with CSL/KNO_3 supplementation; leading to volumetric and specific activities of 413±50 U/mL and 47±7 U/mg_{prot}, respectively, which corresponding to a yield of 41.3 U/mg of xylan. Remarkably, the xylanase volumetric activity obtained with untreated BSG is as high as 94% of that obtained with pretreated BSG (Figure 4) and slightly higher than the one obtained when using xylan as substrate (at 40 g/L) with the same CSL/KNO_3 supplementation (see previous section, Figure 3). Interestingly, the utilization of untreated BSG with CSL/KNO_3 supplementation resulted in the highest β-

xylosidase activity determined in this work, 462 mU/mL, with a ratio of β-xylosidase/xylanase activity of 1.21 (mU/U), while the use of pretreated BSG with CSL/KNO_3 supplementation generated a β-xylosidase activity of 381 mU/mL, with the respective ratio of β-xylosidase/xylanase activity of 0.44 (mU/U).

Characterization of extracellular enzymatic extracts

Crude extracellular extracts from *M. aphidis* PYCC 5535^T with the highest xylanase activity (from pretreated BSG, 11% w/v, with CSL/KNO_3 supplementation, after 4 days at 28°C) were assessed for xylanase activity profile in relation to pH and temperature as well as thermal stability (at optimum pH and temperature) in comparison to a commercial xylanase (Figure A2). The optimum pH and temperature for xylanase activity was estimated at 5.0 and 50°C, respectively. Also, the stability of the xylanolytic crude extract was compared with a commercial xylanase and the obtained xylanase extracts performed slightly better than the commercial xylanase, with 90% of the initial activity after 6-h incubation, and 40% after 12 h.

DISCUSSION

The commercialization of xylanolytic enzymes requires sustainable production processes, preferentially using low-cost substrates and minimal chemical, energy and water inputs, while generating high titers (volumetric activities), yields and productivities, achieving high purities (specific activities) and requiring minimal upstream and downstream processing. Recently, *M. aphidis* PYCC 5535^T, known to produce biosurfactants, also emerged as an excellent producer of cellulase free xylanolytic enzymes from D-xylose, xylan and BSG.[10,16] However, the superior production from BSG, a substrate rich in

protein, pointed out for a potential nitrogen limitation during enzyme production from D-xylose and xylan media, as those culture media protocols were primarily designed for biosurfactant production.[16] Still, the current study reveals that the supplementation of D-xylose or xylan medium with nitrate-based and/or organic-based nitrogen sources improve the production of xylanolytic enzymes (see Figure 1 and Table 1). Peptone generate higher (up to 2.6-fold) xylanase volumetric activities than the nitrate-based counterparts, but the higher cost of this organic nitrogen source (around 10,000 USD/ton, crude industrial grades)[19] compare to the nitrate-based ones (400-600 USD/ton), most probably balance the preference to the later, with a cost per enzyme unit of 0.6-1.0 USD cents per xylanase MU. Therefore, the higher specific activity obtained using peptone and KNO_3 (35-62 U/mg_{prot}) (see Table 1), and the high cost of peptone, prompted the investigation of the use of corn steep liquor (CSL), as alternative cheaper (up to 400 USD/ton) organic nitrogen source.[20] CSL is a sub-product of corn processing with significant content on macronutrients needed for microbial conversion processes, including relatively low content in carbohydrates and inorganic nitrogen and high content in organic nitrogen.[21,22]

The combination of CSL (at 10 g/L) with KNO_3 (at 3 g/L) revealed a synergic effect on xylanase production, meaning that the volumetric activities obtained were higher (1.3- to 1.6-fold) than the sum of the ones obtained with each of the nitrogen sources alone (see Figure 3). Using D-xylose or xylan medium with CSL/KNO_3 supplementation, the xylanase volumetric activities were higher than 320 U/mL, reaching the highest specific activities achieved in this study, higher than 85 U/mg_{prot}. Although the estimated nitrogen source cost per enzyme unit (1.2-1.8 USD cents per xylanase MU) increased in comparison with e.g. the use of KNO_3 alone, the higher volumetric and specific activities

obtained with the CSL/KNO_3 may pay-off when accounting for downstream processing costs.

BSG, a low-cost carbon source obtained, as an agro-industrial residue, from the brewing industry and already known as an excellent inducer of xylanase production with *M. aphidis* PYCC 5535^T,[15] was used as reference lignocellulosic substrate. The CSL/KNO_3 supplementation of pretreated BSG (at 11% w/v, 15 g/L xylan equivalent) was evaluated to further improve xylanase production. The CSL/KNO_3 supplementation improved both volumetric and specific activities from 517±6 U/mL and 57 U/mg_{prot} to 865±10 U/mL and 116±6 U/mg_{prot}, the highest values obtained in this study (see Figure 4), with expected beneficial impact on recovery, concentration and enrichment/purification enzyme downstream processing stages. The results on xylanase volumetric activity produced by *M. aphidis* PYCC 5535^T in BSG media supplemented with CSL/KNO_3 are at the same level, or higher, than those reported for industrial xylanase producers, such as the filamentous fungi *Trichoderma reesei* and *Aspergillus awamori*, in lignocellulosic substrates.[23]

The application of such strategies that use cheap renewable sources carbon and nitrogen are a direct contribution to the reduction of the raw materials cost, which accounts for 30 to 40% of the total enzyme production cost. This can, consequently, positively impact on cost reductions of the saccharifying enzymes, overcoming a major barrier for industrial scale-up and commercial use lignocellulose materials in a biorefinery context .[24,25]

The influence of substrate concentration was also assessed by processing pretreated BSG at 7% (10 g/L xylan equivalent) against the 11% (15 g/L xylan equivalent) above mentioned, both with CSL/KNO_3 supplementation. The reduction on the substrate concentration lead to a small decreased of xylanase specific activities from 57.3 to 43.8

U/mg xylan, respectively. Those values are still relatively high when compared to other enzyme producers and conditions.[24,26,27]

Since BSG is an agro-industrial residue, and therefore a somewhat processed lignocellulosic material, avoiding the need for pretreatment prior to the enzyme production process, would result on beneficial energy and chemicals savings. Hence, eliminating the dilute-acid pretreatment with HCl and using directly untreated BSG at 7% (10 g/L xylan equivalent) with CSL/KNO_3 supplementation for enzyme production was investigated. On such conditions, xylanase yield further decreasing to 41.3 U/mg xylan, but still as high as 94% of the xylanase volumetric activity obtained with pretreated BSG (see Figure 4). Therefore, the elimination of the pretreatment step on xylanases production using BSG can be beneficial, since most pretreatments require high energy consumption, chemicals, and longer reaction times.[28] Circumventing the need of pretreatment not only reduces to lower production costs, but also implies reduction toxic by-products, as those are typically formed through secondary reaction over the BSG pretreatment. In this regard it is important to notice that, while the requirement for balanced β-xylosidase and xylanase activities depend on the application purposes, notably the highest β-xylosidase activity was actually obtained by using untreated BSG medium supplemented with CSL and KNO_3 (462 mU/mL). In fact, KNO_3 had already stood out as the best nitrogen source for β-xylosidase production in xylan medium (see Figure 2).

This study provides versatility in the production of xylanolytic enzymes from *M. aphidis* PYCC 5535^T with respect to: i) potential cost-effective nitrogen sources, including $NaNO_3$, KNO_3, peptone and CSL, in the range of 0.6-1.8 USD cents per xylanase MU;

ii) concentration of carbon source (xylan equivalent) in the lignocellulosic material; iii) the requirement of pretreatment when using an agro-industrial residue, namely BSG.

The optimization of the production process can take place depending on the application purpose and on an integrated techno-economic and environmental assessment. For example, BSG pretreatment can be improved by attempting pretreatment with HNO_3 instead of HCl, followed by neutralization with KOH, to generate KNO_3 to be used as nitrogen source. Also, the requirement of CSL in the supplementation of BSG, already rich in organic nitrogen, could be evaluated. These considerations in relation to pretreatment, carbon source concentration and nitrogen supplementation could also be extended to other lignocellulosic materials for on-site enzyme production, e.g. in lignocellulosic ethanol processes.

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TABLE CAPTIONS

Table 1 - Xylanase volumetric and specific activities in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in D-xylose or xylan (at 40 g/L, as carbon source) supplemented with the listed nitrogen sources (at 3 g/L).

Table A1 - Xylanase volumetric activity, protein level and specific activity of extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in pretreated (pt) or non-pretreated (r) BSG at 11% (w/v) or 7.1% (w/v), with or without supplementation with CSL (10 g/L) and KNO_3 (3 g/L).

FIGURE CAPTIONS

Figure 1 – Xylanase volumetric and specific activities in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in xylan (at 40 g/L) supplemented with the listed nitrogen sources (at 3 g/L).

Figure 2 – Volumetric β-xylosidase activity and β-xylosidase/ Xylanase activity ratio assessed in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in xylan (40 g/L) supplemented with the listed nitrogen sources (at 3 g/L).

Figure 3 - Xylanase volumetric activity of extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) on xylan (black) or D-xylose (light grey) (at 40 g/L) supplemented with CSL at 10 g/L (CSL 10), CSL at 20 g/L (CSL 20), or CSL at 10 g/L and KNO_3 at 3 g/L (CSL + KNO_3).

Figure 4 - Xylanase volumetric activity (U/mL) and yield (U/mg xylan) in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in BSG (11% or 7.1% w/v, eq. 15 or 10 g/L of xylan respectively), with or without pretreatment (ptBSG or rBSG, respectively), with or without supplementation (suppl) of CSL (at 10 g/L) and KNO_3 (at 3 g/L)

Figure A1 - Maximum biomass (OD640nm) and protein concentration (g/l) achieved after 7 days-cultivation (150 rpm, 27°C) of *M. aphidis* PYCC 5535^T on xylan (40 g/L) and and standard media ($MgSO_4$ (0.3 g/L), KH_2PO_4 (0.3 g/L), xylan (40 g/L) and yeast extract (1 g/L)) compared with conditions further supplemented with the listed nitrogen sources (3 g/l, except 10 g/l of yeast extract). Typical concentrations profiles are presented. Coefficients of variation for biomass and protein concentrations obtained for these assays usually range between 3-10% and 5-15%, respectively.

Figure A2 - Biochemical characterization and thermal stability. Effect of temperature (A) and pH (B) on xylanase activity in extracellular crude extracts obtained by growing *M. aphidis* PYCC 5535^T (MAX-B) on 11% (w/v) BSG. Xylanase activity was determined using citrate buffer (pH 3.5-6) and phosphate buffer (pH 6-8), at 50 ºC for pH profile, and temperature profile was obtained at 5.5. Thermal stability (C), i.e. effect of pre-incubating (for the given period) at the temperature of 50°C on the residual enzymatic activities assessed for MAX-B (filled triangles) and commercial xylanase (open triangles).

Table 1 - Xylanase volumetric and specific activities in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in D-xylose or xylan (at 40 g/L, as carbon source) supplemented with the listed nitrogen sources (at 3 g/L).

		Volumetric activity (U/ml)	**Specific activity** ($U/mg_{protein}$)
D-xylose	**$NaNO_3$**	114.8 ± 15.8	16.9 ± 2.9
	KNO_3	219.6 ± 10.6	50.8 ± 5.6
	Peptone	294.0 ± 25.6	35.6 ± 4.7
Xylan	**$NaNO_3$**	193.9 ± 29.4	23.6 ± 4.3
	KNO_3	178.7 ± 19.6	61.6 ± 9.1
	Peptone	371.3 ± 44.7	40.3 ± 6.3

Table A1 - Xylanase volumetric activity, protein level and specific activity of extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27°C) in pretreated (pt) or non-pretreated (npt) BSG at 11% (w/v) or 7.1% (w/v), with or without supplementation with CSL (10 g/L) and KNO_3 (3 g/L).

Condition	Volumetric activity (U/ml)	Total Protein (mg/ml)	Specific activity (U/mg protein)	Activity per mg xylan (U/mg xylan)
ptBSG 11% (w/v)	516.8 ± 6.0	9.1 ± 0.9	56.7 ± 5.8	34.2 ± 0.4
ptBSG (11% w/v) with supplementation	864.7 ± 9.5	7.5 ± 0.4	115.8 ± 6.2	57.3 ± 0.6
ptBSG (7% w/v) with supplementation	437.6 ± 62.4	5.2 ± 0.1	84.0 ± 12.1	43.8 ± 6.2
rBSG (7% w/v) with supplementation without pretreatment	412.8 ± 49.6	8.8 ± 0.7	47.1 ± 6.7	41.3 ± 5.0