Research Article

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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 to their commercial deployment. This work aims at investigating the *Moesziomyces aphidis* yeast cultivation conditions that deliver maximal xylanase titres, yields and productivities using low-cost nitrogen (N) and carbon (C) sources.

Results: $NaNO_3$ and KNO_3 supplementation improved xylanase production 2.9- and 2.7-fold (against 67.2 U mL^{-1}), respectively, using xylan as C source. Interestingly, the use of KNO_3, instead of $NaNO_3$, results 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 N sources. A 4.9-fold increase in xylanase production, with high specific activity, is 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^{-1}) by yeasts, using a media formulation containing dilute-acid pre-treated brewery spent grains (BSG), as C source and inducer, supplemented with KNO_3 and CSL. Replacement of dilute-acid pre-treatmed BSG by untreated BSG had low impact on xylanase production, of only 6%.

Conclusion: Efficient production of *M. aphidis* xylanolytic enzymes, using low-cost N and C 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 such as brewing (using BSG as substrate for enzyme production), pulp and paper (benefiting from the cellulase-free xylanase activity) or lignocellulosic ethanol (for cellulase supplementation).

Supporting information may be found in the online version of this article.

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

INTRODUCTION

Xylanases have attracted considerable attention as a consequence of their application in many industrial processes such as enzymatic bleaching of paper pulp, juice and beer clarification, extraction of plant oils, texture improvement in commercial bakery goods, 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]

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Extensive research on microbial cellulase and xylanase production has been performed with filamentous fungi (e.g. *Aspergillus* spp., *Trichoderma* spp., *Penicillium* spp.) and bacteria (*Bacillus* spp., *Streptomyces* spp.), which are efficient enzyme producers,[1] yet a limited number of studies on this topic has been performed with yeasts. Yeast fermentation combines the potential benefits of the homogeneous scalable submersed fermentation in stirrer tank bioreactors, possible in bacterial cultures, with the higher robustness characteristic of fungal cultures. In addition, yeast fermentation allows the presence of inhibitory compounds driven from complex carbon (C) and nitrogen (N) sources obtained from agro-industrial residues. Among yeasts, those belonging to the genera *Cryptococcus*, *Scheffersomyces*, *Candida*, *Trichosporon*, *Dekkera*, *Hanseniaspora*, *Metschnikowia*, *Rhodotorula*, *Sugiyamaella* and *Wickerhamomyces* have been 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, previously were found to 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 the 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]

In order to enter the xylanase market, estimated at an annual value of US$200 million,[13] cost-effective xylanase production processes using low-cost substrates is mandatory. Xylan is a known inducer of the regulatory mechanisms for xylanase production. Therefore, the use of complex and inexpensive substrates rich in xylan, it is particulary relevant in bioprocesses with natural xylanase-producers. Xylan is found naturally 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 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 also is often required, particularly in relation to the N source. The optimization of the culture media with the combined use of low-cost C and N sources significantly contributes to minimizing production costs. Moreover, the production costs can be reduced yet further by streamlining the bioprocess, namely by minimizing or eliminating lignocellulose pretreatment.

This work aims at improving the production of xylanases from lignocellulosic materials by *M. aphidis* PYCC 5535^T, with focus on the optimization of N supplementation. Brewery spent grain (BSG) is an abundant residue from the brewing industry with low, zero or negative value, and we previously established 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 byproducts or residues 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 use of these residues for on-site enzyme production contributes to circular economy concepts. In the case of xylanases, potential end-users include industries such as 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 cellulase 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^{-1}; malt extract 3 g L^{-1}; peptone 5 g L^{-1}; glucose 10 g L^{-1}; agar 20 g L^{-1}). 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^{-1}), $NaNO_3$ (3 g L^{-1}), $MgSO_4$ (0.3 g L^{-1}), KH_2PO_4 (0.3 g L^{-1}) and yeast extract (1 g L^{-1}).

Raw material and cultivation conditions

The BSG, kindly provided by Sociedade Central de Cervejas (Vialonga, Portugal), was ground with a knife mill to particles <1.5 mm and homogenized in a defined lot. BSG then was 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 mol L^{-1} NaOH. This pretreated slurry material subsequently was used for *M. aphidis* PYCC 5535^T cultivation.

D-Xylose, commercial grade beechwood xylan (Sigma-Aldrich, St Louis, MO, USA), pretreated BSG and untreated BSG were directly tested as C source for cultivation of *M. aphidis* PYCC 5535^T with supplementation of $NaNO_3$ (3 or 5 g L^{-1}), KNO_3 (3 g L^{-1}), NH_4NO_3 (3 g L^{-1}), NH_4Cl (3 g L^{-1}), $(NH_4)_2SO_4$ (3 g L^{-1}), peptone (3 g L^{-1}), yeast extract (3 or 10 g L^{-1}), urea (3 g L^{-1}) or corn steep liquor (CSL) (10 or 20 g L^{-1}) in the culture media. The standard cultivation medium comprised $MgSO_4$ (0.3 g L^{-1}), KH_2PO_4 (0.3 g L^{-1}), xylan (40 g L^{-1}) and yeast extract (1 g L^{-1}), and 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 mmol L^{-1} potassium phthalate buffer and incubated at 50 °C for 30 min. Subsequently, 0.6 mL 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 reducing sugar D-xylose equivalent min^{-1}.

β-Xylosidase was determined as described previously.[18] The supernatant culture sample was appropriately diluted in a reaction mixture (0.3 mL), containing 5 mmmol L^{-1} 4-nitrophenyl-β-D-xylopyranoside (pNPX; Sigma Aldrich) in 50 mmmol L^{-1} potassium phthalate buffer pH 5.5. After incubation at 50 °C for 30 min, 0.15 mL of 1 mol L^{-1} 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 min^{-1}.

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

Characterization of xylanase crude extracts

Extracellular xylanolytic crude extracts obtained from 7-day 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 ANOVA and *P*-values of the differences between groups are corrected for simultaneous hypothesis testing according to Tukey's honestly significant difference (HSD) 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 C source and different inorganic- (nitrate- and/or ammonium-) or organic-based N sources (at 3 g L^{-1}) were first assessed for cell biomass, extracellular protein and xylanase activity improved production (Fig. 1, Supporting Information, Fig. S1). 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^{-1}) in relation to the standard condition improved the xylanase volumetric activity by 2.9-fold (to 194 U mL^{-1}) and the total extracellular protein by 3.6-fold. A higher $NaNO_3$ supplementation load (at 5 g L^{-1}) further increased the xylanase volumetric activity to 314 U mL^{-1} (with similar biomass and protein content to the condition with $NaNO_3$ 3 g L^{-1}) (data not shown). The highest xylanase specific activities were achieved with KNO_3 (61.6 U mg_{prot}^{-1}) and peptone (40.3 U mg_{prot}^{-1}), as a consequence of the 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^{-1} (Fig. 2). The extracellular β-xylosidase activity obtained using standard media (29 mU mL^{-1}). $NaNO_3$, KNO_3 or peptone supplementation significantly increased β-xylosidase volumetric activity (5- to 8-fold), with the highest value (207 mU mL^{-1}) achieved when *M. aphidis* PYCC 5535^T was cultivated in xylan medium with KNO_3 supplementation.

The xylanase:β-xylosidase activity ratio in the extracellular crude extracts were calculated, ranging from 863 with KNO_3 to 2586 with peptone supplementation.

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

In order to assess the effect of different N sources on xylanase activity, three N sources were selected for further studies: two inorganic ($NaNO_3$ or KNO_3) and one organic (peptone), all at 3 g L^{-1}. Those were supplemented individually into medium containing xylan or D-xylose as C source at 40 g L^{-1} (Table 1). Among the N sources tested, peptone led to the highest xylanase volumetric activity both in the presence of xylan (371 U mL^{-1}) and of D-xylose (294 U mL^{-1}) (Table 1). In relation to the influence of inorganic N sources on xylanase volumetric activity, $NaNO_3$ was preferred in the presence of xylan (194 U mL^{-1}) but KNO_3 was

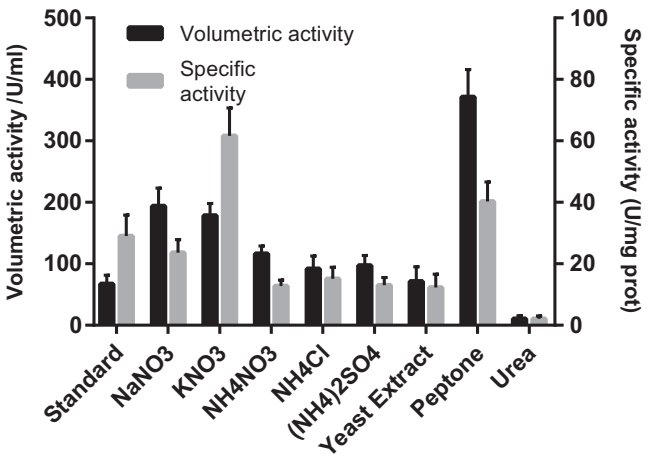

Figure 1. Xylanase volumetric and specific activities in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27 °C) in xylan (at 40 g L^{-1}) supplemented with the listed N sources (at 3 g L^{-1}).

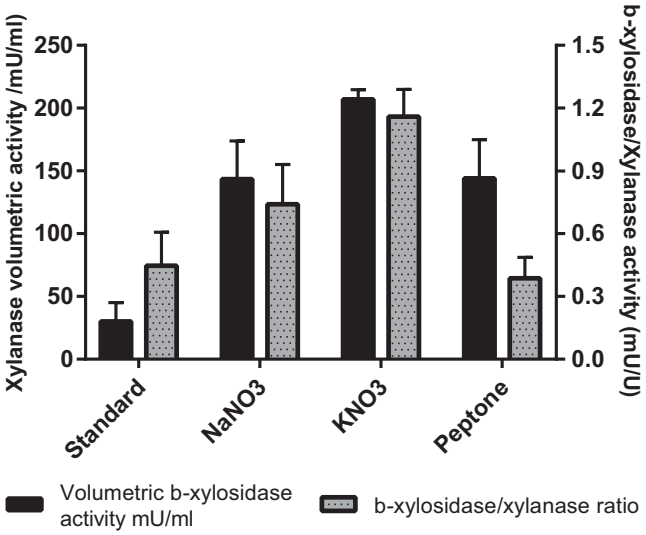

Figure 2. Volumetric β-xylosidase activity and β-xylosidase:Xylanase activity ratio assessed in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27 °C) in xylan (40 g L^{-1}) supplemented with the listed N sources (at 3 g L^{-1}).

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^{-1}, as C source) supplemented with the listed N sources (at 3 g L^{-1})

		Volumetric activity (U mL^{-1})	Specific activity (U $mg_{protein}^{-1}$)
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

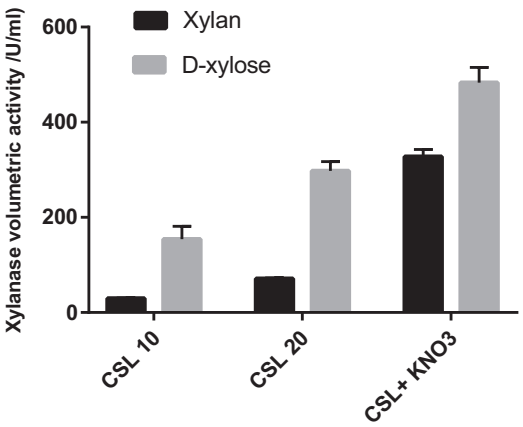

Figure 3. Xylanase volumetric activity of extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27 °C) on xylan (black) or D-xylose (light gray) (at 40 g L^{-1}) supplemented with CSL at 10 g L^{-1} (CSL 10), CSL at 20 g L^{-1} (CSL 20), or CSL at 10 g L^{-1} and KNO_3 at 3 g L^{-1} (CSL + KNO_3).

preferred in the presence of D-xylose (220 U mL^{-1}). However, the specific activity was 2.6- to 3.0-fold higher with KNO_3 than with $NaNO_3$.

The higher xylanase volumetric activity obtained with the organic N source (peptone) may not contribute positively to the economy of the process because peptone is more expensive than inorganic N sources such as $NaNO_3$ and KNO_3. Therefore, an alternative relatively low-cost organic N source, CSL, was assessed. The use of 10 g L^{-1} CSL resulted in high biomass yield and very low total extracellular protein content (data not shown) at values of 2–4 g L^{-1}, which are in the range of that 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 and 30 U mL^{-1}, respectively (Fig. 3). This trend is similar to the one obtained when yeast extract, a much more expensive N source, was used (at 10 g L^{-1}) in supplementation of D-xylose or xylan medium, which resulted in xylanase volumetric activities of 529 and 115 U mL^{-1}, respectively (data not shown). Interestingly, doubling the CSL supplementation (to 20 g L^{-1}) caused the xylanase volumetric activity to increase around 2-fold, to 297.7 U mL^{-1} in D-xylose medium and 71.2 U mL^{-1} in xylan medium (Fig. 3).

The combination of organic and inorganic N sources [CSL (10 g L^{-1}) and KNO_3 (3 g L^{-1})] was evaluated, using D-xylose or xylan as C source. Under these conditions, the xylanase volumetric activity increased 3-fold (≤483.4 U mL^{-1}) in D-xylose medium and 10-fold (≤327.5 U mL^{-1}) in xylan medium, when compared to the sole use of CSL supplementation (at 10 g L^{-1}) as N source (Fig. 3). The combination of CSL and KNO_3 triggered a 2-fold improvement of the xylanase volumetric activity relative to KNO_3 (3 g L^{-1}) only as N source, (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}^{-1} in D-xylose medium and 87 U mg_{prot}^{-1} 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

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

The N supplementation conditions that promote higher xylanase production in the previous section were applied, but using pretreated BSG as C source, instead of D-xylose or xylan. Therefore, BSG was first subjected to a dilute-acid pretreatment and assessed at 11% (w/v) (equivalent to 15 g L^{-1} xylan) for xylanase production with or without supplementation of CSL (at 10 g L^{-1}) and KNO_3 (at 3 g L^{-1}). The CSL/KNO_3 supplementation improved both the volumetric and the specific activity, up to 865 ± 10 U mL^{-1} and 116 ± 6 U mg_{prot}^{-1}, respectively, compared to the xylanase activity obtained without N supplementation (517 ± 6 U mL^{-1}, 57 ± 6 U mg_{prot}^{-1}) (Fig. 4). A volumetric xylanase productivity of 5.2 U mL^{-1} h^{-1} was achieved (with N 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 option of avoiding the use of BSG pretreatment were assessed. When BSG load was reduced to 7% (w/v) (equiv. 10 g L^{-1} xylan), but CSL/KNO_3 supplementation was maintained, xylanase volumetric and specific activities of 438 ± 62 U mL^{-1} and 84 ± 12 U mg_{prot}^{-1}, respectively, were obtained. This means a yield of 43.8 U mg^{-1} xylan, which is lower than the 57.3 U mg^{-1} xylan obtained with 11% (w/v) of BSG and CSL/KNO_3 supplementation, but still quite competitive and higher than that obtained with 11% (w/v) BSG without supplementation (34.2 U mg^{-1} xylan) (Table S1). The direct use of (untreated) BSG without N 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^{-1} and 47 ± 7 U mg_{prot}^{-1}, respectively, which corresponded to a yield of 41.3 U mg^{-1} xylan. Remarkably, the xylanase volumetric activity obtained with untreated BSG was as high as 94% of that obtained with pretreated BSG (Fig. 4) and slightly higher than the one obtained when using xylan as substrate (at 40 g L^{-1})

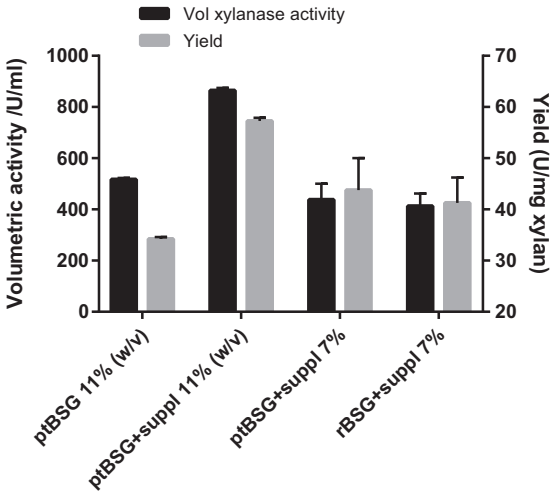

Figure 4. Xylanase volumetric activity (U mL^{-1}) and yield (U mg^{-1} xylan) in extracellular extracts of *M. aphidis* PYCC 5535^T obtained after 7 days of cultivation (150 rpm, 27 °C) in BSG (11% or 7.1% w/v, eqiv. 15 or 10 g L^{-1} xylan, respectively), with or without pretreatment (ptBSG or rBSG, respectively), with or without supplementation (suppl) of CSL (at 10 g L^{-1}) and KNO_3 (at 3 g L^{-1}).

with the same CSL/KNO_3 supplementation (see previous section, Fig. 3). The utilization of untreated BSG with CSL/KNO_3 supplementation resulted in the highest β-xylosidase activity determined in this work, 462 mU mL^{-1}, with a β-xylosidase:xylanase activity ratio of 1.21 (mU U^{-1}), whereas the use of pretreated BSG with CSL/KNO_3 supplementation generated a β-xylosidase activity of 381 mU mL^{-1}, with a respective β-xylosidase:xylanase activity ratio of 0.44 (mU U^{-1}).

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 (Fig. S2). The optimum pH and temperature for xylanase activity were 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 a potential N limitation during enzyme production from D-xylose and xylan media, as those culture media protocols were designed primarily for biosurfactant production.[16] However, the current study reveals that the supplementation of D-xylose or xylan medium with nitrate-based and/or organic-based N sources improved the production of xylanolytic enzymes (see Fig. 1 and Table 1). Peptone generated higher (≤2.6-fold) xylanase volumetric activities than the nitrate-based counterparts, but the higher cost of this organic N source (≈US $10 000 ton^{-1}, crude industrial grades)[19] compare to the nitrate-based ones (US$400–600 ton^{-1}), most probably balances the preference to the latter, with a cost per enzyme unit of US$0.006–0.010 per xylanase pmillion U. Therefore, the higher specific activity obtained using peptone and KNO_3 (35–62 U mg_{prot}^{-1}) (see Table 1), and the high cost of peptone, prompted the investigation of the use of CSL as an alternative cheaper (≤US$400 ton^{-1}) organic N source.[20] CSL is a by-product of corn processing with significant content of macronutrients needed for microbial conversion processes, including relatively low content of carbohydrates and inorganic N and high content of organic N.[21,22]

The combination of CSL (at 10 g L^{-1}) with KNO_3 (at 3 g L^{-1}) 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 N sources alone (see Fig. 3). Using D-xylose or xylan medium with CSL/KNO_3 supplementation, the xylanase volumetric activities were >320 U mL^{-1}, reaching the highest specific activities achieved in this study, higher than 85 U mg_{prot}^{-1}. Although the estimated N source cost per enzyme unit (US$0.012–0.018 per xylanase pmillion U) increased in comparison with, for example, 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 C source agro-industrial residue from the brewing industry, 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^{-1} 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^{-1} and 57 U mg_{prot}^{-1} to 865 ± 10 U mL^{-1} and 116 ± 6 U mg_{prot}^{-1}, the highest values obtained in this study (see Fig. 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 were 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 of C and N are a direct contribution to the reduction of the costs of raw materials, which accounts for 30–40% 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 of lignocellulose materials in a biorefinery context.[24,25]

The influence of substrate concentration also was assessed by processing pretreated BSG at 7% (10 g L^{-1} xylan equivalent) against the 11% (15 g L^{-1} xylan equivalent) mentioned above, both with CSL/KNO_3 supplementation. The reduction in the substrate concentration led to a small decrease of xylanase specific activities from 57.3 to 43.8 U mg^{-1} xylan, respectively. Those values are still relatively high when compared to other enzyme producers and conditions.[24,26,27]

Because BSG is an agro-industrial residue, it is a somewhat processed lignocellulosic material that thereby avoids the need for

pretreatment before the enzyme production process, which should result in beneficial savings in energy and chemicals use. Hence, eliminating the dilute-acid pretreatment with HCl and using directly untreated BSG at 7% (10 g L^{-1} xylan equivalent) with CSL/KNO_3 supplementation for enzyme production was investigated. Under such conditions, xylanase yield further decreased to 41.3 U mg^{-1} xylan yet this was still as high as 94% of the xylanase volumetric activity obtained with pretreated BSG (see Fig. 4). Therefore, the elimination of the BSG pretreatment step in xylanase production can contribute to the economy of the process, because most pretreatments require high energy consumption, chemicals and longer reaction times.[28] Circumventing the need for pretreatment not only reduces production costs, but also implies reduction on toxic byproducts typically formed during secondary reactions in the BSG pretreatment. In this regard it is important to notice that although the requirement for balanced β-xylosidase and xylanase activities depend on the application purposes, notably the highest β-xylosidase activity actually was obtained by using untreated BSG medium supplemented with CSL and KNO_3 (462 mU mL^{-1}). In fact, KNO_3 had already stood out as the best N source for β-xylosidase production in xylan medium (see Fig. 2).

This study underscores the versatility of *M. aphidis* PYCC 5535T with sucessfully production of xylanolytic enzymes with: (i) the use of different potential cost-effective N sources, including $NaNO_3$, KNO_3, peptone and CSL, in the range of US$0.006–0.018 per xylanase million U; (ii) a wide range of concentration of C source (xylan equivalent) in the lignocellulosic material; and, for specific residues, (iii) circurventing the need for 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 N source. Also, the requirement of CSL in the supplementation of BSG, already rich in organic N, could be evaluated. These considerations in relation to pretreatment, C source concentration and N supplementation also could be extended to other lignocellulosic materials for on-site enzyme production, for example in lignocellulosic ethanol processes.

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ABBREVIATIONS

BSG	Brewery spent grains
CSL	Corn steep liquor
MEL	mannosylerythritol lipids

REFERENCES

1 Motta FL, Andrade CP and Santana HA, A review of xylanase production by the fermentation of xylan: classification, characterization and applications, in *Sustainable Degradation of Lignocellulosic Biomass—Techniques, Applications and Commercialization*, ed. by Chandel A and Silva SS. InTech, London, pp. 251–276 (2013).
2 Paës G, Berrin JG and Beaugrand J, GH11 xylanases, structure/function/properties relationships and applications. *Biotechnol Adv* **30**: 564–592 (2012).
3 Lara AL, Santos RO, Cadete RM, Ferreira C, Marques S, Gírio F *et al.*, Identification and characterisation of xylanolytic yeasts isolated from decaying wood and sugarcane bagasse in Brazil. *Antonie van Leenwenhoek* **105**:1107–1119 (2014).
4 Carrasco M, Rozas JM, Barahona S, Alcaíno J, Cifuentes V and Baeza M, Diversity and extracellular enzymatic activities of yeasts isolated from King George Island. *BMC Microbiol* **12**:1–9 (2012).
5 Fall R, Phelps P and Spindler D, Bioconversion of xylan to tryglycerides by oil-rich yeasts. *Appl Environ Microbiol* **47**:1130–1134 (1984).
6 Leathers TD, Detroy RW and Bothast RJ, Induction and glucose repression of xylanase from a color variant strain of *Aureobasidium pullulans*. *Biotechnol Lett* **8**:867–872 (1986).
7 Morais C, Cadete RM, Uetanabaro AP, Rosa LH, Lachance M and Rosa CA, D-xylose-fermenting and xylanase-producing yeasts species from rotting wood of two Atlantic rainforest habitats in Brazil. *Fungal Genet Biol* **60**:19–28 (2013).
8 Stevens BJH and Payne J, Cellulase and xylanase production by yeasts of the genus Trichosporon. *J Gen Microbiol* **100**:381–393 (1977).
9 Romero A, Mateo J and Maicas S, Characterization of an ethanol-tolerant 1,4-β-xylosidase produced by *Pichia membranifaciens*. *J Gen Microbiol* **100**:381–393 (2012).
10 Faria NT, Marques S, Fonseca C and Ferreira FC, Direct xylan conversion into glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma antarctica* PYCC 5048^T. *Enzyme Microb Technol* **71**:58–65 (2015).
11 Ho HL and Hood JS, Optimisation of medium formulation and growth conditions for xylanase production by *Aspergillus brasiliensis* in submerged fermentation (SmF). *J Biodivers Biopros Dev* **1**:1–13 (2014).
12 Mullai P, Fathima NS and Rene E, Statistical analysis of main and interaction effects to optimize xylanase production under submerged cultivation conditions. *J Agric Sci* **2**:144–153 (2010).
13 Amaro-Reyes A, García-Almendárez BE, Amaya-Llano S, Castaño-Tostado E, Guevara-Gonzalez RG, Loera O *et al.*, Homologue expression of a fungal endo-1, 4-β-D- xylanase using submerged and solid substrate fermentations. *J Biotechnol* **10**:1760–1767 (2011).
14 Awasthi MK, Sarsaiya S, Patel A, Juneja A, Singh RP, Yan B *et al.*, Refining biomass residues for sustainable energy and bio-products: an assessment of technology, its importance, and strategic applications in circular bio-economy. *Renewable Sustainable Energy Rev* **127**: 109876 (2020).
15 Farinas CS, Solid state fermentation for the on site production of cellulolytic enzymes and their use in the saccharification of lignocellulosic biomass, in *Current Developments in Biotechnology and Bioengineering*, ed. by Pandey A, Larroche C and Soccol C. Elsevier Science Publishers, Amstrdam, pp. 169–179 (2018).
16 Faria NT, Marques S, Ferreira FC and Fonseca C, Production of xylanolytic enzymes by *Moesziomyces* spp. using xylose, xylan and brewery's spent grain as substrates. *New Biotechnol* **49**:137–143 (2019).
17 Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**:426–428 (1959).
18 Berghem LER and Pettersson LG, The mechanism of enzymatic cellulose degradation. *Eur J Biochem* **37**:21–30 (1973).
19 Mao R, Teng D, Wang X, Zhang Y, Jiao J, Cao X *et al.*, Optimization of expression conditions for a novel NZ2114-derived antimicrobial peptide-MP1102 under the control of the GAP promoter in Pichia pastoris X-33. *BMC Microbiol* **15**:57 (2015). https://doi.org/10.1186/s12866-015-0389-5.
20 Maddipati P, Atiyeh HK, Bellmer DD and Huhnke RL, Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract. *Bioresour Technol* **102**: 6494–6501 (2011). https://doi.org/10.1016/j.biortech.2011.03.047.
21 Nascimento RP, Junior NA, Pereira N Jr, Bon EPS and Coelho RRR, Brewer's spent grain and corn steep liquor as substrates for cellulolytic enzymes production by *Streptomyces malaysiensis*. *Lett Appl Microbiol* **48**:529–535 (2009).

22 Edwinoliver NG, Thirunavukarasu K, Purushothaman S, Rose C, Gowthaman MK and Kamini NR, Corn steep liquor as a nutrition adjunct for the production of *Aspergillus niger* lipase and hydrolysis of oils thereof. *J Agric Food Chem* **57**:10658–10663 (2009).
23 Ximenes EA, Dien BS, Ladisch MR, Mosier N and Cotta MA, Enzyme production by industrially relevant fungi cultured on coproduct from corn dry grind ethanol plants. *Appl Biochem Biotechnol* **137**:171–183 (2007).
24 Saini S and Sharma KK, Fungal lignocellulolytic enzymes and lignocellulose: a critical review on their contribution to multiproduct biorefinery and global biofuel research. *Int J Biol Macromol* **193**: 2304–2319 (2021). https://doi.org/10.1016/j.ijbiomac.2021.11.063.
25 Moran-Aguilar MG, Costa-Trigo I, Calderón-Santoyo M, Domínguez JM and Aguilar-Uscanga MG, Production of cellulases and xylanases in solid-state fermentation by different strains of *Aspergillus niger* using sugarcane bagasse and brewery spent grain. *Biochem Eng J* **172**: 108060 (2021). https://doi.org/10.1016/j.bej.2021.108060.
26 Otero DM, Braga AC and Kalil SJ, Diversification of nitrogen sources as a tool to improve endo-xylanase enzyme activity produced by *Cryptococcus laurentii*. *Biocatal Agric Biotechnol* **32**:101941 (2021). https://doi.org/10.1016/j.bcab.2021.101941.
27 Dutta PD, Neog B and Goswami T, Xylanase enzyme production from *Bacillus australimaris* P5 for prebleaching of bamboo (Bambusa tulda) pulp. *Mater Chem Phys* **243**:122227 (2020). https://doi.org/10.1016/j.matchemphys.2019.122227.
28 Kuo P and Yu J, Process simulation and techno-economic analysis for production of industrial sugars from lignocellulosic biomass. *Ind Crops Prod* **155**:112783 (2020). https://doi.org/10.1016/j.indcrop.2020.112783.

