BioEnergy Research (2019) 12:583–592
https://doi.org/10.1007/s12155-019-09991-8

Production of Biomass-Degrading Enzymes by *Trichoderma reesei* Using Liquid Hot Water-Pretreated Corncob in Different Conditions of Oxygen Transfer

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Published online: 8 June 2019

Abstract
Enzymatic hydrolysis accounts for 20% of the total cost in the conversion process of lignocellulosic biomass into bioethanol. Therefore, production of biomass-degrading enzymes by using lignocellulosic residue as a fermentation substrate may be an alternative to decrease the production costs. In this study, corncob (CC) has been pretreated by liquid hot water (LHW) at 200 °C for 30 min and used as inducer source for production of biomass-degrading enzymes by *Trichoderma reesei* MUM 97.53. The pretreatment was used to increase the cellulose content and the accessibility to lignocellulosic material. Although the filamentous fungus secreted a broad range of cellulolytic and hemicellulolytic enzymes when grown on untreated CC, higher enzyme productions were obtained when cultured on LHW-pretreated CC in a 2-L stirred tank bioreactor (STB). Besides, the effects of aeration (2 and 4 vvm) and agitation (150 and 250 rpm) rates on enzyme production were studied by submerged fermentation in a batch STB and correlated with the volumetric oxygen transfer coefficient (k_La). Maximal cellulase, xylanase, and β-xylosidase productions were found at 150 rpm and 4 vvm, while the highest β-glucosidase levels were obtained at 150 rpm and 2 vvm, that corresponded to k_La values of 32.50 h^{-1} and 16.41 h^{-1}, respectively. At higher agitation, a lower enzymatic production was observed probably due to the high shear stress in the fungal hyphae.

Keywords Cellulases · Xylanases · *Trichoderma* · Autohydrolysis · Bioreactor · k_La

Introduction

Biomass-degrading enzymes are one of the greatest challenges in the use of the biochemical route for lignocellulosic biomass conversion into add-value bioproducts. This is mainly due to the high production cost of the enzymes, the low yields, and the considerable quantities of enzymes that are required during the process. For example, in the bioethanol production, the enzyme cost represents about 15–28% of the bioethanol selling price, or 20% of the overall bioethanol production costs [1]. Additionally, it has been reported that the biggest cost in the cellulase production is in the raw materials and consumables and that this cost varies according to the production approach, being the integrated method, where the cellulose is used as raw material to produce the cellulase, the most cost-effective compared to others that use glucose as raw material [2].

Thus, recent studies have focused on the improvement of the fermentation processes for microbial enzyme production, as well as in the development of hyper-producing microbial strains, in order to reduce the costs and make the process economically viable [3–5]. Other possibility is the use of lignocellulosic biomass as a inducer source for the production of enzymes. In fact, lignocellulosic biomass has been considered a promising material for production of these enzymes because of its abundance, low cost and wide availability [6]. Besides, it has been shown that better performances are obtained when an enzyme complex is prepared from the same lignocellulosic material that is meant to be hydrolyzed by that complex [7–9].

Various types of lignocellulosic biomass, such as hardwood, bagasse, and straws, have been studied as inducer sources or fermentation substrate to improve the lignocellulosic enzyme production [10, 11]. One of the ways to improve the production is to use pretreated biomass which can lead to an improvement in production and yield since the pretreatment breaks the lignocellulosic structure and makes cellulose

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and hemicellulose more accessible for the fermenting microorganisms [7, 8]. Hydrothermal pretreatment, such as liquid hot water (LHW), has been considered a suitable and cost-effective pretreatment for biomass fractionation. It depolimerizes the hemicellulose and a small portion of lignin into the hydrolysates, while it recovers a more accessible cellulose-lignin fraction. Additionally, this pretreatment presents some advantages compared to other pretreatment technologies, like simple and economical operation, limited equipment corrosion problems, reduced polysaccharide losses, and inhibitor formation; does not require the addition and recovery of chemicals different from water; and is considered an environmentally friendly process [12]. Many researchers have used lignocellulosic hydrolysates and pretreated solids for enzyme production and have showed that these pretreated materials improve enzyme production [11–16].

Cellulases and hemicellulases are produced by a wide variety of microorganisms, such as bacteria and fungi. However, filamentous fungi are known by their ability to secrete large amounts of protein, among which, enzymes. Most commercial cellulases and hemicellulases are produced by submerged cultivation using filamentous fungi of the genera *Trichoderma* and *Aspergillus* [17]. These aerobic microorganisms require good oxygen transfer, which is related to the aeration and agitation rates, to achieve good growth and subsequent enzyme production. However, the shear stress caused by the stirred tank bioreactor (STB) turbine, as a result of agitation intensity, on mycelia causes a negative impact on growth and enzyme production [18, 19]. This calls for the optimization of the fermentation conditions. Other authors have studied the interactive influence of aeration and agitation rates on the volumetric oxygen transfer coefficient (k_La) to improve the production of several bioproducts, together with the negative impact of shear forces on microorganism and bioproduct production [20–24].

The current study describes the effects of aeration and agitation rates in a lab-scale stirred tank bioreactor (STB) on the production of biomass-degrading enzymes, namely cellulase, β-glucosidase, xylanase, and β-xylosidase, by a selected strain of *Trichodema reesei* MUM 97.53 cultivated under batch fermentation, using corncob pretreated by liquid hot water (LHW) at 200 °C for 30 min as substrate.

Materials and Methods

Material

Corncob (CC) was kindly supplied by a local farmer (Caíde de Rei, Lousada, Northern Portugal). The material was dried at 40 °C for 12 h. After that, it was cut into small chips (1–3 cm), milled and sieved on particle sizes of 1 mm, and stored at room temperature.

Chemical Composition

The chemical composition of untreated and pretreated CC was determined according to the standard Laboratory Analytical Procedures (LAPs) for biomass analysis provided by the US National Renewable Energy Laboratory (NREL) [25]. The analyzed components were glucan, xylan, arabinan, acetyl gropus, lignin, and ash.

Glucose, xylose, arabionose, and acetic acid were analyzed by high-performance liquid chromatography (HPLC) in a Jasco chromatograph + sampler (JASCO Intelligent Sampler AS 2057 Plus) with a refractive index (RI) detector, using a Metacarb 87H column (300 × 7.8 mm, Varian, USA) preheated at 60 °C. The mobile phase (0.005 mol L^{-1} H_2SO_4 in Milli-Q water) was filtered through 0.2 μm Millipore® nylon filter and degassed. The flow rate was 0.7 mL min^{-1}. All measurements were made in duplicate.

Liquid Hot Water Pretreatment

LHW pretreatment was carried out in a 160-mL stainless steel cylinder reactor (4.0 × 12.4 cm), with working volume of 50 mL. Milled CC samples and water were mixed in order to obtain 10% (*w*/*v*) solid loading. The reactor was submerged in an oil bath with an open heating circulator (Julabo Labortechnik GmbH, Seelbath, Germany) with PID temperature control at 200 °C for 30 min (log(R_0) of 4.42). After that, the reactor was immediately cooled in an ice bath to quench the reaction. The insoluble solids were separated from the liquid fraction by vacuum filtration (filter paper) and used as substrate for fermentation.

Microbial Strain

Tricoderma reesei MUM 97.53 was kindly provided by MUM (Micoteca da Universidade do Minho, Portugal), that is a member of the European Culture Collections Organization (ECCO) and the World Federation for Culture Collections (WFCC) and is also registered in the WDCM (World Data Centre of Microorganisms) with the number 816. Fungus stock cultures were propagated on PDA medium plates at 30 °C for 1 week. Thereafter, the plates were stored at 4 °C until use.

Medium and Inoculum Preparation

The microorganism was grown in 500-mL Erlenmeyer flasks, containing 125-mL Mandels medium [26], and 1% (*w*/*v*) unwashed slurry of CC solids recovered after LHW pretreatment, as inducer source of biomass-degrading enzymes. After autoclaving, the flasks were inoculated with a spore suspension, containing 2.5×10^{10} spores mL^{-1}, determined in a Neubauer counting chamber, and incubated on a rotatory

shaker at 30 °C, 100 rpm, for 72 h. After that, this culture was used to determine the studied enzyme activities and as pre-growth culture to start the bioreactor fermentation. Untreated CC was also used in shake flask fermentation at the same conditions described above.

Bioreactor Configuration and Operating Conditions

Enzyme production was carried out in a lab scale 2 L STB (Bioengineering AG CH-8636, Wald, Switzerland) with 1.25 L working volume, equipped with two 6-bladed Rushton turbines and automatic monitoring and control facilities for temperature, pH, aeration, and agitation rates.

Batch fermentations in the STB were initiated with an inoculum of 10% (*v*/*v*) of the bioreactor volume of a *T. reesei* MUM 97.53 culture that was pre-grown at 30 °C for 72 h. STB containing Mandels medium, pH 5.4 ± 0.2, and 1% (*w*/*v*) unwashed slurry of CC solids recovered after LHW pretreatment was operated to optimize aeration and agitation rates for the production of biomass-degrading enzymes. Two levels of airflow rates (2 and 4 vvm) were studied, and at each airflow rate, two different agitation rates (150 and 250 rpm) were tested; the fermentation was performed at 30 °C for 10 days (Fig. 1).

Dissolved oxygen (DO) and pH probes (Mettler-Toledo, Columbus, Ohio, EUA) were used to monitor the DO and the pH, respectively. One milliliter of antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) was used at the beginning of fermentation. Estimation of enzyme production was carried out at every 24 h interval.

Enzymatic Assays

Cellulase and xylanase activities were determined by measuring the released reducing sugars by the 3,5-dinitrosalicylic acid (DNS) method [27], using glucose or xylose, respectively, as standard. The cellulase (endo- and exo-glucanase) assay was performed at 55 °C for 30 min, using Whatman No. 1 filter paper as substrate (10 mm × 30 mm) in 0.05 M sodium citrate buffer, pH 4.8, according to [28], and expressed as Filter Paper Unit per milliliter (FPU/mL). The xylanase assay was performed at 60 °C for 20 min, using 1% (*w*/*v*) birchwood xylan (Sigma-Aldrich, St. Louis, MO, USA) in citrate-phosphate buffer, pH 6.0, as substrate, according to [19], and expressed as International Unit per milliliter (IU/mL).

The β-glucosidase assay was performed at 50 °C for 10 min, by monitoring the hydrolysis of 5 mmol L^{-1} *ρ*-nitrophenol-β-D-glucopyranoside (PNP-glu) in 0.05 M sodium citrate buffer, pH 4.8, while the β-xylosidase assay was

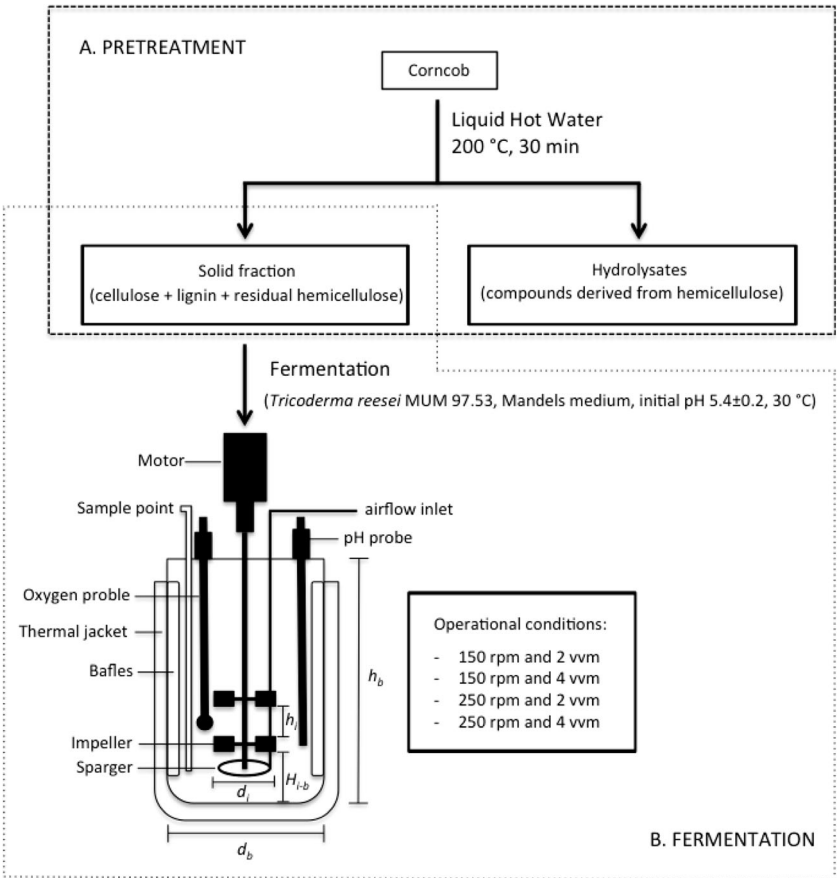

Fig. 1 The overall scheme of the fermentation process. A. Pretreatment step. B. Fermentation step in a 2 L STB. Diagram of stirred tank: d_b, bioreactor diameter (9.5 cm); h_b, bioreactor height (30 cm); d_i, impeller diameter (4 cm); h_i, distance between impellers (5.5 cm); $h_{i\text{-}b}$, distance between the impeller and the bottom of the reactor (5 cm)

performed at 70 °C for 15 min, using 5 mmol L^{-1} ρ-nitrophenol-β-D-xylopyranoside (PNP-xyl) in citrate-phosphate buffer, pH 4.5, as substrate [19]. The released ρ-nitrophenolate of both assays was estimated with 1 mol L^{-1} sodium carbonate, using ρ-nitrophenol as standard. One international unit (IU) of enzymatic activity was defined as the amount of enzyme that releases 1 μmol of product per minute under the assay conditions. All assays were performed in duplicate. The values presented correspond to mean values of replicate experiments.

k_La Measurement

The volumetric oxygen transfer coefficient (k_La) was measured at 30 °C in cell-free medium by the dynamic gassing-out method [29]. This method was performed by sparging nitrogen until the dissolved oxygen (DO) concentration falls close to zero and then monitoring the DO concentration after the start of the humidified air injection into the bioreactor. At this moment, the oxygen transfer process to the medium begins and continues until DO in the liquid reaches the saturation. DO concentration values were measured on-line using an O_2 electrode (CellOx 325, WTW) and recorded through a data acquisition board. Two measurements were done for each condition. The influence of oxygen electrode on k_La was considered negligible due to the low probe response time (6 s), which was significantly lower than $1/k_La$, corresponding to an experimental error lower than 6%.

The variation on DO concentration with time, t, was recorded, and k_La values were calculated according to the equation:

$$\ln(C^*-C) = \ln(C^*-C0)-k_La.t \quad (1)$$

where C^* and C are, respectively, the saturation concentration of oxygen and oxygen concentration in the liquid. Assuming the liquid phase as homogeneous and being $C0$ the concentration of oxygen at $t = 0$, the k_La was determined by plotting $\ln(C^* - C)$ against time (t) using MATLAB (MathWorks, Natick, MA, USA), version 7.2.0.232 (R2006a).

Results and Discussion

Influence of the Biomass Pretreatment in Enzyme Production

CC was pretreated by LHW at 200 °C for 30 min, and the recovered unwashed slurry of CC solids was used as fermentation substrate for the production of cellulases and xylanases in shake flasks. This pretreatment condition was selected based on a previous work that investigated several conditions of LHW pretreatment [30]. Untreated CC contained approx. 38.0% cellulose and 31.5% hemicellulose. Once the LHW pretreatment removes mainly the hemicellulose from lignocellulosic biomass, the cellulose content of pretreated CC increased to 60.5% and the hemicellulose content was reduced to 13.4%, of which 10.7% is xylan (Table 1). Liquid fraction contained 22 g/L of xylooligossacarides [30]. Part of the hemicellulose contained in the solid fraction may be free hemicellulose that was removed in the pretreatment process but remained impregned in the solids after the drying process, since the solids were not washed after the pretreatment. Unwashed solids were used as substrate in the fermentation since this hemicellulose could be advantageous for the xylanase production.

Figure 2 presents the enzyme production obtained by *Trichoderma reesei* MUM 97.53 after 72 h of fermentation, using untreated and pretreated CC. The cellulase (FP activity) and β-glucosidase productions were, respectively, 18.4% and 47.9% higher on pretreated CC than on untreated CC. This influence is explained by the effect of the hydrothermal pretreatment on CC, which provokes the disruption of the structure of the lignocellulosic matrix, and thus increases the availability of the lignocellulosic components and the accessibility of the microorganisms to it, after its fractionation by the pretreatment. Xylanase and β-xylosidase productions also increased when pretreated CC was used, achieving rates of 28.2% and 5.1%, respectively, even with the removal of part of the hemicellulose from the solid fraction after the pretreatment. This may be associated with improved accessibility of the microorganism to the hemicellulose fraction due to disruption of the lignocellulosic matrix.

Cunha et al. [31] reported 64% higher endoglucanase production in steam-explosion pretreated sugarcane bagasse (SCB) as substrate than in untreated SCB, in sequential fermentation (SF). Those authors used a pre-culture initiated as solid state fermentation (SSF) under static conditions for 24 h, which was continued by sequential submerged fermentation (SmF) in an orbital shaker, with addition of an enriched nutrient medium for 48 h, and finally used to initiate the enzyme production in the STB.

In the current work, 3.5 FPU mL^{-1} of cellulase and 3.8 IU mL^{-1} of β-glucosidase were achieved after 72 h with the

Table 1 Composition of untreated and pretreated corncob by liquid hot water at 200 °C for 30 min

Components	Corncob (%)	Pretreated corncob (%)
Cellulose	37.95 ± 1.81	60.55 ± 3.84
Hemicellulose	31.52 ± 2.09	13.40 ± 1.77
Lignin	19.09 ± 0.21	20.56 ± 0.08
Ash	0.77 ± 0.07	0.34 ± 0.02
Moisture	6.86	5.06

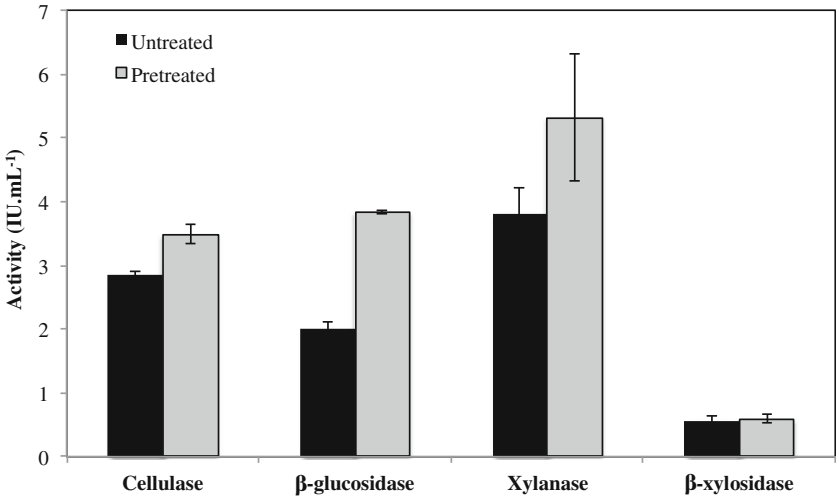

Fig. 2 Production of cellulase, β-glucosidase, xylanase, and β-xylosidase in untreated corncob (black column) and LHW-pretreated corncob (gray column) in shake flasks. The microorganism was cultivated at 30 °C, 100 rpm during 72 h

LHW-pretreated biomass on shake flasks. These production values are higher than those obtained by Gottschalk et al. [32] that achieved a cellulase production of 1.7 FPU mL^{-1} and 0.34 IU mL^{-1} of β-glucosidase on shake flasks culture of *T. reesei* using 3% (*w*/*v*) lactose. However, the same authors also observed a lower FP activity (0.42 FPU mL^{-1}) and an improved β-glucosidase activity (45.6 IU mL^{-1}) on shake flask culture of *Aspergillus awamori* using 3% (*w*/*v*) wheat bran. These differences may be related with the inducer source, as well as with the fungal species.

In relation to xylanase production, a value of 5.3 IU mL^{-1} was achieved using pretreated CC. This production was lower than xylanase production reported in a previous work by Michelin et al. [16], where 14 IU mL^{-1} and 13.2 IU mL^{-1} were obtained in cultures of *A. terricola* and *A. ochraceus*, respectively, with a mixture of untreated CC and CC hydrolysates from LHW pretreatment. Gottschalk et al. [32] also studied the xylanase production by *T. reesei* and *A. awamori* cultures, and 12.6 and 79.1 IU mL^{-1}, respectively, were obtained.

These results could be related with the fungal specie, since *T. reesei* is known to be an efficient fungus for production of cellulase. However, it has been described that β-glucosidase enzyme is produced in very small quantities by this microorganism [33]. On the other hand, xylanase production has been mainly studied in the *Aspergillus* species [15, 16, 19, 34–36].

The pretreatment, as well as the fungal specie, appears to have influenced enzyme production, and the higher enzyme levels obtained with the LHW-pretreated material were probably due to the increased accessibility of the microorganism to the lignocellulosic components due to the disruption of the lignocellulosic matrix caused by the pretreatment. Additionally, the absence or low content of inhibitory by-products, characteristic of LHW pretreatment [37], associated with this improved accessibility may have favored the increase on enzyme production.

Influence of Aeration and Agitation Rates in the Enzymatic Production

In this work, batch fermentation was run on STB with the *T. reesei* MUM 97.53 fungus, and the influence of aeration and agitation rates on biomass-degrading enzyme production, by using LHW-pretreated CC, as fermentation substrate, were analyzed. Two different airflow rates, namely 2 and 4 vvm, were analyzed, and at each airflow rate, two agitation rates, i.e., 150 and 250 rpm, were tested.

Figure 3 shows the enzymatic production (cellulase, β-glucosidase, xylanase, β-xylosidase), and dissolved oxygen (DO) and pH behaviors during 10 days fermentation. The highest enzymatic levels were observed in the condition of lower agitation, being the best production achieved at 150 rpm and 4 vvm for cellulase (8.4 FPU ml^{-1} after 5 days fermentation; Fig. 3a), xylanase (11.1 IU ml^{-1} after 3 days fermentation; Fig. 3c), and β-xylosidase (2.1 IU ml^{-1} after 2 days fermentation; Fig. 3d), while for the β-glucosidase activity (5.8 IU ml^{-1} after 10 days fermentation; Fig. 3b), the best production was achieved at 150 rpm and 2 vvm condition. In addition, β-glucosidase activities continued to show an upward trend after 10 days fermentation (Fig. 3b), suggesting that maximum values had not been reached. This late production can be associated with the complexity of the substrate, since β-glucosidase production occurs after the production of the endoglucanase, that cleaves the cellulolytic polymers randomly to produce new chain ends including cellobiose, that is a substrate for β-glucosidase [38]; this may explain that the reason for the β-glucosidase is still increasing after 10 days of fermentation. Similar results were observed by Reis et al. [39] and Li et al. [40] regarding this late β-glucosidase production.

Patel et al. [41] studied the growth of *T. reesei* RUT-C30 in different bioreactors and found that lower agitation rate resulted in higher FP activity, although higher agitation frequencies

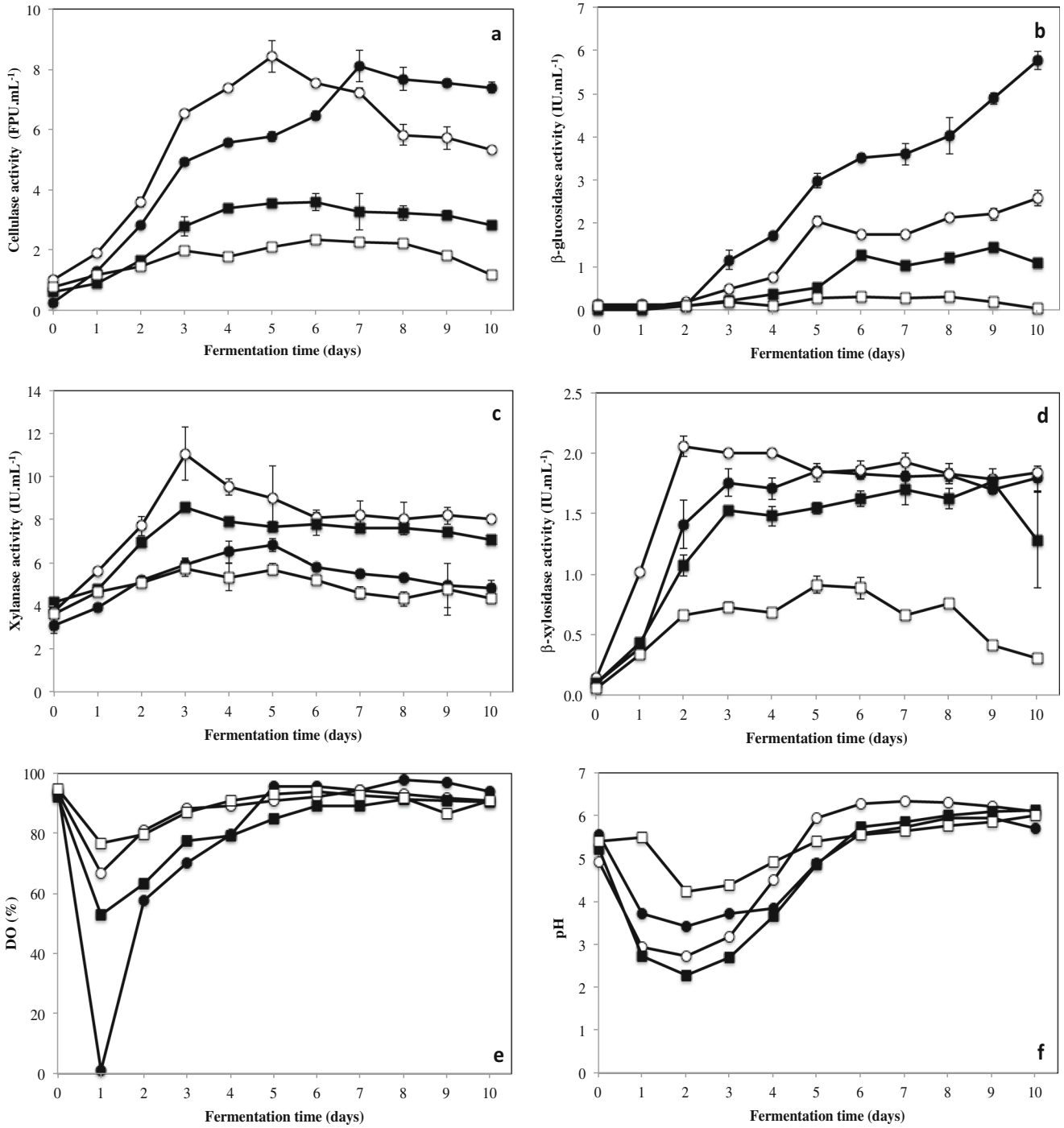

Fig. 3 Time course for cellulase activity (**a**), β-glucosidase activity (**b**), xylanase activity (**c**), β-xylosidase activity (**d**), dissolved oxygen (**e**), and pH (**f**) in the stirred tank bioreactor at 150 rpm and 2 vvm (black circle); 150 rpm and 4 vvm (white circle); 250 rpm and 2 vvm (black square); and 250 rpm and 4 vvm (white square). The microorganism was cultivated at 30 °C during 10 days

increased the biomass, due to a better oxygen supply. The agitation rate and the airflow in cultures of filamentous fungi strongly influence the growth and production of extracellular enzymes [42]. In the current work, it was possible to improve enzyme production from 50 to 95%, as in the case of β-glucosidase, by comparing the best conditions for each enzyme production with the worst studied conditions (250 rpm, 4 vvm).

The lower enzyme production observed at 250 rpm when compared to 150 rpm can be related to the shearing effect of the STB turbines. In practice, it has been often reported that the shear stress imposed on mycelial microorganisms at vigorous agitations could lead to morphological and physiological changes, leading to a decrease in enzyme productivity [19, 20, 43]. In addition, the highest enzymatic activity (cellulase, xylanase, and

Table 2 Production of biomass-degrading enzymes by filamentous fungi in bath fermentation in lab- and pilot-scale STB

Strain	Substrate	Fermentation conditions			Enzyme activities				References
		T (°C)	pH	Scale (L)	Cellulase (FPU/mL)	BGL (IU/mL)	Xylanase (IU/mL)	BXL (IU/mL)	
Trichoderma reesei M.U.M. 97.53	LHW-pretreated CC	30	5.0	2	8.4	5.8	11.1	2.1	This work
Aspergillus terreus	Delignified OPEFB fiber	29	5.5	2	2.3	16.0	n.d.	n.d.	Shahriarinour et al. [49]
Penicillium oxalicum	Wheat bran plus avicel	28	5.0	7	1.3	4.0	n.d.	n.d.	Saini et al. [50]
Trichoderma harzianum P49P11	DSB plus sucrose	29	5.0	3	1.2	17.3	80.0	n.d.	Delabona et al. [51]
Penicillium echinulatum S1M29	Cellulose (Celuflok E®)[a]	28	6.0	2	5.9	5.8	~155.0	n.d.	Reis et al. [39]
Trichoderma reesei Rut C30 ATCC 56765	MC plus corn steep liquor	26	5.0	5	8.0	n.d.	n.d.	n.d.	Ma et al. [52]
Aspergillus terricola	Wheat bran	30	6.0	2	n.d.	n.d.	7.5	< 0.1	Michelin et al. [53]
Aspergillus niger van Tieghem	Corncob	30	6.0	8	n.d.	n.d.	1.8	0.4	Michelin et al. [19]
Trichoderma reesei Aspergillus niger	Cellulose-yeast extract and lactose	30	4.8	3	7.1	n.d.	n.d.	n.d.	Ahamed and Vermette [54]
Aspergillus niger	Wheat bran	30	5.6	5	n.d.	9.3	n.d.	n.d.	Abdella et al. [55]
Penicillium echinulatum S1M29	Cellulose plus glycerol	28		5	0.75	1.15	40	n.d.	Schneider et al. [56]

BGL beta-glucosidase activity, *BXL* beta-xylosidase activity, *LHW-pretreated CC* corncob pretreated by liquid hot water, *OPEFB* oil palm empty fruit bunch, *DSB* sugarcane bagasse pretreated by steam followed by delignification with NaOH, *MC* microcrystalline cellulose, *n.d.* not determined

[a] Medium also containing sucrose, soybean meal, wheat bran in lower concentrations

β-xylosidase) at 150 rpm and 4 vvm when compared to 150 rpm and 2 vvm can be related with the DO deprivation in the last condition. At lower aeration, the fermenter may suffer O_2 depletion due to poor mixing of its content and thus the movement of fungal biomass may be hampered [44]. It is important highlight that the highest β-glucosidase levels were achieved in the end of the fermentation, when the DO concentration was higher than 90% of saturation.

Figure 3e shows that DO concentration presented a similar behavior for all conditions studied, i.e., DO concentration was maintained above 50% of saturation, with exception of the conditions of 150 rpm and 2 vvm, where DO concentration fell down to zero after 1 day of fermentation. This drop in DO concentration at the beginning of fermentation can be attributed to an increase in oxygen consumption in the exponential phase of the fungal growth due to the fast increase in cell concentration in the first hours of fermentation [19, 42].

It is also known that the microbial physiology of filamentous fungal cells is significantly influenced by the DO concentration in suspended cultures, and it has been suggested that the critical DO concentration for fungal cells in culture should be greater than around 20% of the saturation DO value [45, 46]. In this study, no effort was made to maintain the DO above 20% in order to investigate the effect of DO on enzyme production by the fungus *T. reesei* MUM 97.53. The results suggested that, as already reported by Michelin et al. [19] and Ghoshal et al. [44], the oxygen transfer rate from the gas phase to the liquid phase was lower than the oxygen uptake rate of the microorganism in the liquid phase, i.e., the combination of the studied agitation (150 rpm) and aeration (2 vvm) rates probably was not enough to maintain the oxygen supply at sufficient levels to cover the demand of the microorganism during the exponential growth phase. On the other hand, Reis et al. [39] varied the stirring and air flow rates in order to maintain a satisfactory concentration of dissolved oxygen in the culture media (oxygen level above 30% of air saturation) and avoid depletion of oxygen on culture medium.

As observed for DO concentration, the pH of the medium decreased in the beginning of the fermentation (Fig. 3f) but increased again after that. Fontana et al. [42] observed a minimum pH of 2.7 in a STB, reaching 5.10 at the end of the fermentation process by *Aspergillus oryzae*. This drop in pH

Table 3 k_La values for the conditions used in the STR

Run	Agitation speed (rpm)	Airflow rate (vvm)	k_La (h^{-1})
1	150	2	16.41 ± 1.46
2	150	4	32.50 ± 3.49
3	250	2	18.47 ± 2.33
4	250	4	31.21 ± 0.38

The values presented correspond to mean values of two replicate experiment

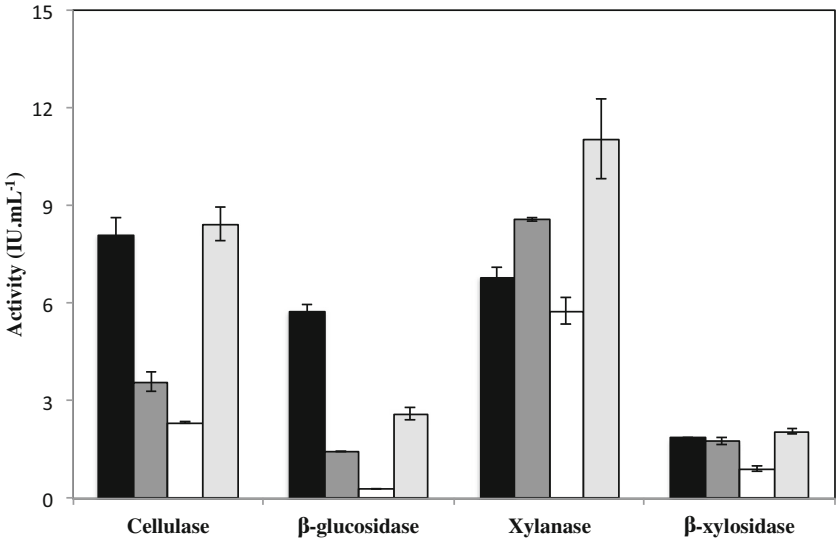

Fig. 4 Production of cellulase, β-glucosidase, xylanase, and β-xylosidase in the stirred tank bioreactor at a k_La of 16.41 h^{-1} (black column), 18.47 h^{-1} (dark gray column), 31.21 (white column), and 32.50 h^{-1} (light gray column). The enzymatic activities correspond to the maximum activity of each enzyme

could be related to the production of acid metabolic by-products, as well as with the acetic acid released from hemicellulose during the degradation of the lignocellulosic substrate by the produced xylanases. Similar trend in pH decrease was also reported by Khanahmadi et al. [47] and Abdella et al. [48].

Table 2 presents the production of cellulases and xylanases by a few prominent microorganisms in laboratory and pilot-scale bioreactors under batch submerged fermentation. The current work presented good levels of cellulase activities; however, better xylanase levels were reported by other authors (see Table 2). This can be related to the removal of a great part of the hemicellulose from lignocellulosic biomass, as well as to the fungus species used in the works.

Relation of the Volumetric Oxygen Transfer Coefficient (k_La) with Enzyme Production

k_La is the most significant parameter to measure transfer phenomena in a bioreactor, including oxygen transfer. Those are affected by many factors, including agitation speed and/or airflow rate [19]. Generally, the increase of k_La values is achieved by increasing agitation or airflow values; however, this practice is limited to a certain extent due to high shear rate, excessive foaming, and increased process costs. Thus, increasing these variables beyond a critical value decreases both mass transfer efficiency as well as microbial activity [23].

The influence of aeration and agitation rates on k_La was evaluated under the studied conditions. Results presented on Table 3 show that aeration rate was the most important variable to improve oxygen transfer, while k_La was less sensitive to the increment of the agitation speed. Mixing is very crucial for maximum productivity in microbial fermentation, and it could be achieved through agitation and aeration. But, agitation at higher stirring speeds may cause vortex formation, as well as free cell disruption in the reactor by forces, which may result in poor mass transfer (oxygen/substrate). Therefore, it is important to provide optimum combination of agitation and aeration in free cell batch bioreactor process [43]. Mass transfer achieves a critical value at 250 rpm and 4 vvm, where no improvement in mass transfer was observed with increasing agitation. This condition corresponded to the lowest enzymatic activities (Fig. 4). This drop in enzyme activity can be related to the negative impact of shear stress caused by the STB mixing turbine on mycelia [19].

The highest cellulase, xylanase, and β-xylosidase activities were observed for a k_La value of 32.50 h^{-1}, while the highest β-glucosidase activity was verified at 16.41 h^{-1}. These k_La values were obtained for the lower agitation speed (150 rpm), showing the high sensitivity of the fungus to the shear stress (Fig. 4).

Zhou et al. [24] studied the effects of agitation and aeration on the production of a glycoprotein based on k_La and verified that k_La values increased as agitation speed and aeration rate increased in the bioreactor. At agitation speeds of 150 to 300 rpm and an aeration rate of 1 vvm, they reported similar k_La values (14.53–32.82 h^{-1}) to this work. However, although k_La values increased with agitation and aeration rates, they verified that too high k_La had a negative effect on glycoprotein production due to the high shear force caused by high agitation rate, which could destroy the structure of cell and mycelium, and affect the biosynthesis of glycoprotein. The harmful effect of the shear forces due to the higher agitation rate has been reported to cause a reduced enzyme production in some filamentous fungi by other authors [19, 20, 43, 47].

Conclusions

The findings of this work demonstrate the potential of liquid hot water pretreatment to improve the production of biomass-degrading enzymes using pretreated biomass as fermentation

substrate, as well as the influence of the aeration rate and agitation speed on k_La and enzyme production. Results suggest that variables such as aeration and agitation are the key when defining a strategy to optimize the production of fungal enzymes, once aeration supplies the necessary oxygen for cell growth and agitation could assure an efficient mixing of oxygen, heat, and nutrients, as well as disperse the air into smaller bubbles to improve the gas-liquid contact area. Besides, k_La could be improved by increasing aeration and/or agitation but with technical and physiological limitations, since the very high agitation speed may lead to the reduction of enzymatic activity due to shear stress.

Acknowledgments The authors thank Professor Dr. Nelson Lima from MUM (Micoteca da Universidade do Minho, Portugal) by the fungal strain.

Funding Information Michele Michelin is a recipient of a FCT fellowship (SFRH/BPD/100786/2014). This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte.

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